

CULTURE DEPENDENT ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF ENDOPHYTES

Abstract

Plants have been exploited for medicinal purposes since time immemorial and today, man is faced with an emerging, immediate need to find alternate sources for the development of novel drugs due to both the overexploitation of plant wealth and the development of antibiotic resistance in microbes. Dawn of novel viruses such as SARS-CoV2, bringing the all-powerful human world to a standstill at times, are also only adding fuel to this burning global concern. Endophytes are those groups of organisms that inhabit plants in an apparent symbiotic relationship and are reported to have a direct or indirect role in the production of various secondary metabolites. These secondary metabolites show many similarities to those produced in plants and are proven to have a significant role in the plant's pharmacognostic, stress-tolerant and growth-promoting properties. The metabolites produced by endophytes can be used to develop drugs through more eco-friendly means, wherein the exploitation of the plant wealth will be minimal and drug development will be much quicker. Numerous reports have been put forward suggesting the capability of endophytes as a potential crop improvement tool. The frequent use of chemical pesticides and fertilizers has had a marked impact on soil health which calls for biocontrol agents such as endophytes to tackle the problem. Endophytes can thus be potential candidates for novel innovations leading to agricultural and medicinal field improvements. The isolation, identification and characterization of endophytes are of paramount importance in the current scenario.

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I. INTRODUCTION

De Bary, being the one who coined the term ‘endophyte’ in 1866, described endophytes as any organism colonizing within the plant tissues (Arnold, 2008). According to the modern-day concept, endophytes are only those organisms that reside within apparently healthy plant tissues without causing the formation of any symptoms in the host (Petrini, 1991; Afzal et. al., 2019). Archea, bacteria, fungi or viruses can constitute the endophytic microflora (Bacon et. al., 2000). Endophytic presence and its significant role in secondary metabolite production have been reported from almost all plants studied to date (Rigobelo and Baron, 2021). Natural bioactive compounds isolated from endophytes have played a significant role in the discovery of resources used for the betterment of medical, agricultural and food industries. Disease-causing bacteria becoming more resistant to antibiotics is a major area of concern that demands the development of alternate sources of antimicrobials (Dias et al., 2012). Adverse use of chemical fertilizers has also taken a huge toll on soil composition and fertility which demands the inevitable requirement of novel methods for crop improvement such as the use of plant growth-promoting bacteria (Lopes et al., 2018). Endophytes are reported to be involved in plant growth promotion by producing plant hormones such as auxin and cytokinin or by their action against infectious pathogens through the production of siderophores or other antimicrobials (Anu, 2012). Reports on the ability of endophytes to help plants adapt to stressed conditions also throw light on their potential to help plants adapt to climate change, which is a matter of global concern today (Suryanarayanan and Shaanker, 2021). These facts make the study of endophytes all the more important.

Two significant methods to study the plant microflora are the culture-dependent and culture-independent methods. In culture-dependent methods appropriate nutrient media are used for microbial culture and proper incubation under suitable conditions is provided. In culture-independent methods, also known as metagenomics, the DNA of the microbes are directly subjected to sequencing (Chimwamurombe et al. 2016; Fadiji and Babalola 2020). Identification of culturable endophytes is most commonly carried out by sequencing their conserved sequences. 16S rRNA, 16D rDNA or 23S rDNA sequences are commonly used for bacteria and ‘internal transcribed spacer’ (ITS) is used for fungi. These sequences are then compared with databases such as GenBank (Mousa et. al., 2015; Deutsch et. al., 2021). Endophytic characterization can be based on plant growth promoting activities such as phosphate solubilization, IAA production, siderophore production, ammonia and organic acid production and HCN production (Bhutani et al., 2021).

II. ISOLATION OF ENDOPHYTES

1. Collection of Plants

Healthy plants or plant parts that do not show any apparent symptoms of disease or damage have to be selected (Gupta et al., 2022). Endophytes colonize the plant roots, leaves, and stem and are also seen in relatively lesser numbers in flowers, fruits and seeds (Lodewyckx et al., 2002). Plant organs are colonized by different endophytes based on the chemical composition of the particular organ (Bulgarelli et al., 2013). The most colonized parts are often roots, as it is considered as the most important path of entry of microbes from the rhizosphere to the plant through root hairs (Maela, 2019). Whole plant, leaf, root, fruit or

seed can be used for endophyte isolation (Xia et al., 2015; Soni et al., 2021; Sangwan et al., 2021).

2. Surface Sterilization

Surface sterilization is a key process in endophyte isolation. The diversity of endophytes can be significantly affected by even the concentration and time of exposure of the surface sterilant (Yu et al., 2022). Serial washing was first introduced to remove bacteria and spores from plant parts such as roots. Sterile distilled water with glass beads was used to vigorously shake roots which removed non-endophytic bacteria (Harley and Waid, 1955; Rovira et al., 1974). Galaaen and Venn (1979) isolated endophytic fungi from the roots of spruce using serial washings. The use of chemical agents for surface sterilization is the most widely accepted method today. The most commonly used surface sterilant is sodium hypochlorite (NaOCl). Mercuric chloride is another common sterilizing agent, its shortcoming being a bit more toxic than sodium hypochlorite (Sieber, 2002). 40% formaldehyde and 70-90% ethanol is also used. Apart from directly using these surface sterilants, there is also a common procedure in which plant parts are pretreated with certain surfactants. Some accepted surfactants by the scientific community are Tween 80, Tween 20 and Triton X-100 (Sahu et al., 2022). Ensuring complete surface sterilization is crucial, if not it can lead to contamination (Reinhold-Hurek and Hurek, 2011). Surface sterilant, if used beyond a particular time period can kill the endophytes as well (Lundberg et al., 2012). Hence the particular surface sterilant used, concentration of the sterilant and the time of exposure vary with plants and the part of the plant used for culture of the endophyte.

When the leaf is used as the plant material, it is rinsed well with tap water for a few minutes to remove the debris on the surface of the leaf. Then it is washed well using double distilled water and is further subjected to treatment with 70% ethanol and 4% sodium hypochlorite solution. Afterwards using sterile double distilled water, the leaf is rinsed well for about 3 times (Palanichamy et al., 2018 and Tiwari et al., 2014). If the plant material is a root, initially soil and other debris is removed by washing thoroughly under running tap water. Pieces of root are then treated with ethanol (96%), sodium hypochlorite (5%) and then ethanol (96%) for different time periods (Larran et al., 2007). For nodules being plant material, surface sterilization is carried out by using 92.8% ethyl alcohol followed by treatment with 3% hydrogen peroxide (Fred et al., 1928).

3. Culture Conditions and Medium

For endophyte isolation, segments of plant parts can be directly planted on the medium after surface sterilization (Wenndt et al., 2021) or the segments can be macerated using mortar and pestle with or without a buffer. Phosphate saline buffer is generally used. Macerated samples can be subjected to serial dilutions before plating so as to improve the chances of obtaining individual colonies (Gupta et al., 2022).

All activities such as the final surface sterilization procedures, blotting plant parts after surface sterilization, and cutting or maceration of the plant segments need to be carried out inside a laminar airflow chamber (LAF) wiped with 70% ethanol which provides a sterile working environment.

The culture medium used varies with the endophyte of interest. For isolation of fungal endophytes, a medium made of potato dextrose agar (PDA) [1000ml: 200g potato infusion; 20g dextrose; 20g agar; pH 5.6±0.2] is often used. The medium is also supplemented with antibiotics such as streptomycin (250µg/ml). The sole purpose of this supplemented streptomycin is to inhibit the growth of bacteria along the fungal endophytes. Thus, contamination due to undesired organisms can be avoided to an extent. Incubation for 5-7 days at about 25°C will yield the growth of the endophytes (Manoharan et al., 2019) Malt extract agar (MAE) [1000ml: 30g malt extract; 5g mycological peptone, 15g agar; pH 5.6±0.2] is also widely used for fungal endophyte culture (Yu et al., 2022). This medium can be supplemented with streptomycin sulfate (0.05g/100ml of medium) which will inhibit bacterial growth. Plates are then incubated at 28°C (ALkahtani et al., 2020). The period of incubation may vary with the endophyte of interest.

Tryptone soy agar (TSA) [1000ml: 15g casein peptone; 5g soya peptone; 5g sodium chloride; 5g agar; pH 7.3±0.2] is a widely accepted medium for the culture of bacterial endophytes. Macerated samples are placed on TSA plates and incubated at 28±2°C for 3 days to obtain colonies of bacterial endophytes (Bhutani et al., 2021). Luria - Bertani agar (LB) [1000ml: 10g peptone; 5g yeast extract; 5g sodium chloride; 15g agar; 7.5±0.2 pH] and nutrient agar (NA) medium [1000 ml: 3g beef extract; 5g peptone; 8g sodium chloride; 15g agar; 6.8±0.2 pH] are also extensively used for culture of bacterial endophytes. Cultures are incubated at 30°C in dark for 5 days (Yu et al., 2022). Medium used can be supplemented with nystatin or cycloheximide (60µg/ml) to suppress the growth of fungus. Growth of a particular type of endophyte can also be encouraged with supplements provided in the medium used. Use of nalidixic acid (60µg/ml) and K₂Cr₂O₇ (60µg/ml) will encourage growth of actinomycetes and suppress the growth of other fast-growing bacteria (Passari et al., 2015).

Pure cultures of the bacterial or fungal endophytes obtained through the aforementioned methods can be obtained by streaking them on fresh agar plates made with similar medium (Pola et al., 2018). The purified endophyte isolates can be stored for long term in slope culture made of similar medium in refrigerator at 4°C (El-Sayed et al., 2021)

III. IDENTIFICATION

1. Morphological Identification

A single bacterium on a culture medium divides in an organized pattern, starting from the centre towards the periphery, to form a visible structure that we call as a colony. Secretion of various molecules is involved in the establishment of specific colony patterns for a particular species. (Ben-Jacob and Levine, 2005; Branda et al., 2005; Fleming et al., 2007) These patterns can be of immense help in the preliminary identification of bacteria. Some of the major characteristics considered are size, shape, odour, texture, elevation, margins and pigmentation. Diameter is measured to assess size. When a colony is less than 1 mm in diameter it is said to be pinpoint, small if it is 2-3 mm, medium when 4-5 mm, and large when size is greater than 5 mm. Colonies can also acquire various shapes such as circular, irregular, rhomboid, rhizoid, filamentous or umbonate. Umbonate is the shape where there is a protuberance at the centre. Bacterial colonies can also have curdy, pungent, fruity, putrid, buttery or blechy odour. Each will be a characteristic feature of a particular colony. Texture is how one perceives the surface of a colony. For a bacterial colony, it can be smooth,

transparent, opaque, rough, translucent or wrinkled. As bacteria form colonies on flat solid medium the colony can obtain different surface elevations. That is, the pattern in which the colony is raised on the flat surface will be different. It can be flat, raised or convex. The entire colony will be raised in the 'raised' pattern but in the convex pattern, the central portion will be more raised than the margins. Colony margins can be entire, irregular or filamentous. Bacterial colonies tend to produce different pigments. According to the particular pigment produced, colonies can generally have yellow, golden yellow, whitish or orange colouration. Each bacterial colony will have a unique property regarding to each of these aspects and thus it can be used for morphological identification (Smibert et al., 1994)

Identification of fungi up to the species level based on their morphological features can be done using certain universal keys (Raper et al., 1965; Domsch et al., 1980; Booth, 1971). Hyphal morphology, characteristics of mycelia and spores need to be studied for this (Carmichael et al., 1980). Fungal morphology is studied both microscopically and macroscopically. The structure of the colony and its growth rate are macroscopic characters. The shape and size of conidia and spores come under microscopic characteristics (Rabha et al., 2016). The colours formed by fungal colonies are also specific. Colours can be named according to 'A Mycological Colour Chart' by Rayener (1970). But a lot of the endophytic fungi when grown in a culture medium do not produce conidia or spores which makes morphological identification almost completely out of consideration (Corrêa et al., 2014)

Fungal endophytes can be classified in many ways. Endophytes in grasses are classified as clavicipitaceous and those in vascular and nonvascular plants as non-clavicipitaceous (Rodriguez et al., 2009). They can be sexual or asexual, vertically or horizontally transmitted, necrotrophs or biotrophs, symptomatic or asymptomatic and root or foliar endophytes (Brem and Leuchtmann, 2001; Saikkonen et al., 2002; Delaye et al., 2013; Pinto et al., 2000; Behie et al., 2015)

Bacteria are often classified under Gram-negative and Gram-positive categories based on the result of Gram staining. Differential staining is carried out in the Gram staining procedure. The two major stains used are safranin and crystal violet. Bacteria, heat-fixed on a glass slide, is stained with crystal violet for 1 minute. This is washed off with tap water and then treated with Gram's iodine for 1 minute which acts as a mordant. After washing off the mordant the slide is treated with 95% alcohol which is a decolorizing agent. Gram-positive bacteria will retain the colour of crystal violet but the Gram-negative bacteria will lose the colour and take up the colour of safranin added after washing the alcohol off (Cappuccino and Sherman, 1992)

The external appearance of endophytes can be visualized using a scanning electron microscope (SEM). Bacterial cultures are washed and suspended in a phosphate buffer. A drop of this is placed and allowed to dry on a carbon disc which is again washed with a phosphate buffer and fixed with 2% glutaraldehyde. This is again treated with 1% osmium tetroxide and an ethanol series. These dehydrated bacterial films are coated with gold before subjecting to SEM (Hayat, 1989)

2. Molecular Identification

At the genus level, the identification of endophytes is possible through the amplification of conserved rDNA sequences. These sequences are different for bacteria and fungi. The region is called internal transcribed spacer (ITS) in fungi and 16S in bacteria (Deutsch et al., 2021). Initially, the DNA needs to be isolated for which pure cultures of bacteria and fungus are to be prepared. LB broth can be used for bacteria and PDA plates for fungus. The LB broth is subjected to centrifugation to obtain pellets and the fungal hyphae can be weighed out to extract DNA. Extraction of DNA can then be performed using specific DNA extraction kits from manufacturers such as ZYMO Research, DNA Wizard Kit Promeg or Gen Elute Bacterial Genomic DNA Kit Sigma (Deutsch et al., 2021; Mousa et al., 2015; Elfiati et al., 2022). The extracted DNA needs to be quantified before amplification. A nanodrop machine is used for this purpose (Mousa et al., 2015)

The rDNA region is amplified using PCR from this extracted DNA. Suitable primers have to be selected for amplification. Fungal-specific primers such as ITS4 and ITS5 can be used for amplification of the ITS region in fungus (White et al., 1990). Various primers are used for amplification of 16S rDNA in bacteria such as the universal primers 16SF and 16SR (Gupta et al., 2022). The PCR products are extracted by gel electrophoresis, purified and then sequenced. Fragments can be purified with E.Z.N.A. Gel Extraction Kit (Omega, United States) and sequenced based on platforms such as Illumina Nova 600 (Illumina, San Diego, United States) (Yu et al., 2022). Methods such as Sanger sequencing are also used to sequence PCR products (Elfiati et al., 2022).

The genome sequencing of endophytes, besides identification up to genus level, also has the potential to reveal the direct or indirect role of endophytes in plant growth promotion, pharmacognostic properties and other metabolic activities (Kaul et al., 2016). There are certain genomic features that enable certain microbes to live as endophytes, without causing any apparent harm to the plant, but other closely related species to be pathogens. Studies through comparative genomics can reveal such genetic determinants. Production of lipopolysaccharides and adhesins were reported as potential molecular factors contributing to this fine line between endophytism and parasitism by Monteiro et al. (2012).

It is not always feasible to study the gene in individual endophytes after isolation, as a vast majority do not belong to the class of culturable endophytes. Metagenomic studies can be used to get past such obstacles. DNA of the whole population is extracted and then the contents are analyzed (Sessitsch et al., 2012). It is also important to study the expression of these genes which can be achieved through transcriptomics or proteomics. Transcriptomics study the different transcriptional products of the gene which gives an insight into how the gene is differentially expressed (Kaul et al., 2016). Advancement of technology has brought about proteomics which studies the proteins expressed by particular organisms (Wilkins et al., 1995).

Whole genome sequencing of the endophytes has the immense potential to shed light on the taxonomical aspects and the evolutionary aspects of endophytic microbes. The already processed sequences of endophytes which can be used for comparative studies are available on domains such as NCBI, FungiDB and the like (Salvi et al., 2022)

The sequences can be searched with BLAST (Basic Local Alignment Search Tool) for the identification of endophytic fungi or bacteria (Chapla et al., 2020; Mousa et al., 2015). The function of BLAST is to find similar regions between the sequence of interest and sequences in databases such as GenBank. Both protein sequence and nucleotide sequence can be searched this way. Using the similarity between the sequences, organisms can be identified up to the genus level.

Another important step in the identification of endophytes is the construction of phylogenetic trees. Phylogenetic trees are very important in revealing the evolutionary history of organisms. That is, when two taxa are said to have common characters, we can infer their derivation from a common ancestor as well as predict the characters present in the ancestors (Telford et al., 2003). Phylogenetic trees can be constructed from molecular data through 4 major steps such as acquiring of sequences, alignment of sequences, estimation of tree and presentation of tree. Trees can be estimated through the neighbour-joining method, UPGMA Maximum Parsimony, Bayesian Inference and Maximum Likelihood. MEGA is a software that can perform all these required steps. The two algorithms used by MEGA are ClustalW and MUSCLE (Hall, 2013)

IV. CHARACTERIZATION

1. Based on Plant Growth Promotion Activities

Endophytes take part in plant growth regulation through different mechanisms such as the production of metabolites that improve nutrient uptake or regulation of molecules such as abscisic acid, auxins, cytokinins, jasmonates and strigolactones (Reinhold-Hurek and Hurek, 2011; Brader et al., 2014; Santoyo et al., 2016; Shahzad et al., 2016).

A. Phosphate Solubilization

Plants require various nutrients for their growth which are absorbed from the soil. The better the absorption, the better will be the plant growth. Some of the major macronutrients are potassium and phosphorus. Sometimes the soluble form of these nutrients that can be absorbed by the plant will be limited. Endophytes have the capability of macronutrient solubilization. Verma et al. (2015) reported various *Bacillus* species that are involved in potassium, phosphorus and zinc solubilization.

Pikovskaya's agar plates developed by Pikovskaya (1948) can be used for evaluating phosphate solubilization. It is calculated as a phosphate solubilization index (PSI). Premono et al. (1996) have put forward the equation for PSI as

$$PSI = \frac{\text{Colony diameter} \pm \text{halozone diameter}}{\text{Colony diameter}}$$

Quantification of the solubilized phosphate can be done using NBRIP medium (Fiske and Subbarow, 1925)

B. IAA Production

IAA (indole acetic acid) is a common phytohormone that endophytes can produce which is directly involved in cell division, elongation, differentiation and light responses (Bhutani et al., 2018). The application of IAA-producing bacterial endophytes to crops can increase yield significantly (Duca et al., 2014). Salkowski method explained by Tang and Bonner (1948) can be used for the estimation of indole acetic acid. It is a calorimetric method where absorbance is measured at 530 nm (Gordon and Weber, 1951).

C. ACC Deaminase Activity

Ethylene is a plant hormone involved in senescence. The precursor of ethylene is a molecule known as ACC (1-aminocyclopropane-1-carboxylic acid), which is degraded by the enzyme ACC deaminase. Thus, ACC deaminase can downregulate the formation of ethylene which in turn leads to plant growth promotion (Glick, 2015). Glick (2014) reports various endophytes have the ability to produce ACC deaminase. The formation of ethylene is generally upregulated under drought or saline stress, affecting plant growth (Saleem et al., 2007). Thus, ACC deaminase is involved in stress alleviation and enhancement of plant fitness (Hardoim et al., 2008). Belimov et al. (2009) reported a decrease in xylem ACC concentration in *Pisum sativum* upon inoculation with the *Variovorax paradoxus*.

Penrose and Glick (2003) put forward a protocol for testing ACC deaminase. Bacteria is grown in a DFS medium having 2g (NH₄)₂SO₄ for 72h at 28 and 100 rpm. 50µl of liquid culture having glucose and citric acid, and 50µl DFS medium with 3mM ACC were mixed. Absorbance is measured at 590 nm. The ACC deaminase activity can be measured by comparing this absorbance with the absorbance of a selected blank (Ghyselinck et al., 2013)

D. Siderophore Production

Siderophores are those molecules that are capable of making iron molecules more available to the plants when the conditions are iron limiting (Tan et al., 2006; Szilagyi-Zecchin et al., 2014). Siderophores also prevent phytopathogens from binding with iron. Thus, siderophores can import protection from pathogen attack. Endophytes producing siderophores are also reported to help plants alleviate stressed conditions (Rani et al., 2022)

Louden et al. (2011) enumerated the test for siderophore production. Chrome azurol medium (CAS) is used here. 1mL of sterile PBS is prepared, using which a dense bacterial suspension is prepared. This is then spotted on the CAZ medium. Halo zones will appear indicating siderophore production which can be measured after about 2,4,7,10 days of incubation.

E. HCN Production

Hydrogen cyanide (HCN) is involved in systemic resistance in plants. Through biogenic cyanogenesis they can inhibit the growth of phytopathogens (Swarnalakshmi et

al., 2019). Endophytes from *Glycine max* have been reported to inhibit phytopathogens such as *Sclerotium rolfsii*, *Rhizoctonia solani*, *Colletotrichum truncatum* and the like (Dalal et al., 2015).

For the detection of HCN, the method proposed by Lorck (1948) is used. Pathogens such as *Aspergillus niger* and *Fusarium oxysporum* grown on PDA medium can be used to study the production of HCN through its ability to inhibit fungal growth (Kumar et al., 2015)

F. Stress Tolerance

The ability of endophytes to help plants alleviate biotic and abiotic stresses have been previously reported (Jha et al., 2012). The role of endophytes in imparting stress tolerance to plants has triggered studies related to the possible use of such endophytes in alleviating stress in plants due to climatic changes (Hacquard et al., 2017; Bennet and Classen, 2020; Jansson and Hofmockel, 2020). Waqas et al. (2012) reported endophytes having a role in alteration of abscisic acid, jasmonic acid and salicylic acid levels that are changed due to salt and drought stress in rice. Endophytes isolated from Pokkali variety of rice and inoculated in IR-64 variety, reduced overall splicing events in the plant which is considered to be part of salt stress tolerance (Sampangi-Ramaiah et al., 2019). Thus, endophytes are potential biocontrol options with minimal damage to the environment (Verma et al., 2021).

2. Based on Pharmacognostic Property

A. Antioxidant Property

In humans, chronic diseases such as cancer, diabetes, septic shock, cardiovascular disease, atherosclerosis, stroke and other degenerative diseases can be caused due to the deprivation in the structure and function of biomolecules like nucleic acid, lipids, enzymes and proteins, which in turn is a result of oxidative destruction, either by exogenous agents such as smoking, organic solvents, pesticides, ionizing radiations or by agents such as reactive oxygen species (ROS), which includes hydrogen peroxide, superoxide anion, hydroxyl radical, singlet oxygen etc, (Fang et al., 2002). Oxidative destruction can be brought down by natural antioxidant compounds through radical scavenging, which improves the functioning of the immune system (Tan et al., 2018). There is equilibrium between antioxidants and the generated number of free radicals in the human body (Mau et al., 2002). Cytotoxicity, mutation, cell death and oxidative damage can be the result of overproduction of reactive oxygen species (ROS) (Kohen and Nyska 2002). Bioactive antioxidant compounds such as gallic acid, rutin, euphorbin - A, B, C, D etc. are produced by the endophytic fungus residing in the plant *Euphorbia hirta* (Kumari et al., 2021).

To monitor the presence of antioxidant compounds in crude extract, TLC bioautography can be done (Belaqziz et al., 2017). DPPH standard methodology is used to perform in vitro antioxidant activity (Shen et al., 2010). In this method, methanolic solution with crude extract and purified compounds was added with the required amount of DPPH solution (0.2mM). Positive standard antioxidant compounds are ascorbic acid (AA) and quercetin (QR), the percentage of antioxidant activity of purified compounds

was compared to this. Using a UV-visible spectrophotometer, optical density was measured at 517 nm after 30 minutes incubation in the dark. Triplicates of all the samples were taken in this experiment. DPPH free radical inhibition and optical density shows an inverse relation. EC₅₀ value (Effective concentration) of purified compounds was calculated (Gautam et al., 2022).

$$\begin{aligned} & \% \text{ of free radical inhibition} \\ & = \frac{[\text{Absorbance of control} - \text{Absorbance of test}]}{\text{Absorbance of control}} \times 100 \end{aligned}$$

Another major antioxidant assay is ABTS methods, in which 2,2'-azino-bis(ethylbenzthiazoline - 6 - sulfonic acid (ABTS⁺) free radical cations are scavenged by antioxidant compounds (Kaaniche et al., 2019). Equal volume of 2.45 mM potassium persulfate and 7 nM ABTS solution are mixed to form radical cation. Then they are kept for incubation in the dark for 15 min at room temperature. To acquire an absorbance of 0.700 ± 0.05 at 734 nm, ethanol is used to dilute ABTS⁺ solution. The mixture of sample of various concentration, 2mL ethanol and 1mL of ABTS⁺ was vortexed for 30 seconds to estimate the antioxidant potential of partially purified compounds. Absorbance of the mixture is measured at 734 nm using a spectrophotometer. Positive controls were ascorbic acid and quercetin. Calculated the IC₅₀ value of purified extract (Gautam et al., 2022).

B. Anticancer Property

Cancer is a kind of disease, which makes a cell grow in an uncontrolled, abnormal way and forms a tumor, which is a mass of abnormal cells (Bonita et al., 2006). Cancer is either caused due to aging, mutation among genes, weakening of the immune system, hormonal problems or due to external factors such as lifestyle, smoking, alcohol and exposure to radiations such as UV (Pandi et al., 2013). Among fatal non communicable diseases, after cardiovascular condition, cancer holds the second position (Bonita et al., 2006). In developing countries, the death rate caused due to cancer is increasing every day. As a solution to this problem, discovery of novel cancer drugs is necessary. Numerous studies have revealed that medicinally important compounds are produced by plants in which endophytic fungus resides. These endophytes in plants can be the reason for its medicinal properties. Many secondary metabolites production inside the host plant occurs due to plant - endophyte interaction. The anticancer activity could be controlled by the secondary metabolites isolated from the endophytes inhabiting the host plant (Jalgaonwala et al., 2017).

Anticancer properties can be studied by sulforhodamine (SRB) assay. DMEM with 10% heat inactivated fetal bovine serum and 1% penicillin or streptomycin are used to culture human cancer cells of lung (LU - 1), prostate (PC - 3) and breast (MCF - 7). 5% CO₂ (humidified atmosphere) and culture is incubated at 37⁰C.

Percentage of surviving cells is measured as the ability of cultured cells to grow rapidly in test extract and afterwards using sulforhodamine B (SRB), total protein content is assessed. Into the wells of microplate with test samples, cells are added. 50% cold aqueous TCA (Trichloroacetic acid) is used to attach the cells to the plastic substratum.

Plates were washed in tap water and air dried only after the incubation for 30 minutes at 4⁰C. TCA fixed cells are dissolved in 1% aqueous acetic acid for 30 minutes after staining with 4% sulforhodamine B dye. 1% aqueous acetic acid is used to remove excess SRB dye. 10 mM unbuffered tris base at pH 10 is used to solubilise bound dye. Absorbance is determined at 515 nm, after orbital shaking of the plate for 15 minutes. Equal number of cells were added to several wells of the microplate for zero-day control, in each occurrence and kept for 30 minutes incubation at 37⁰C. Cells are attached with TCA. Subtracting zero-day control from absorbance gives the average absorbance value. Nonlinear regression analysis obtained by plotting percentage survival versus concentration is used to calculate IC₅₀ value. Ellipticine is used as toxicity control (Wu et al., 2015).

C. Anti-Inflammatory Property

Inflammation is an immune response to pathogens, damaged cells, toxic compounds or radiations that can lead to tissue damage (Chen et al., 2017). One of the major events reported to take place during inflammation is the denaturation of proteins leading to the release of contents in the lysosome (Chou, 1997). Thus, Urumbil and Anilkumar (2021) infer that through the inhibition of protein denaturation, inflammation can be controlled and treated. Medications currently available for treating inflammation are reported to have toxic effects such as gastrointestinal bleeding (Wongrakpanich et al., 2018) which makes the discovery of less toxic alternate sources such as endophytes, for anti-inflammatory drug development all the more important.

Weber et al. (2004) isolated the first endophytic metabolite with anti-inflammatory properties from the plant *Erythrina crista-galli* produced by the endophyte *Phomopsis sp.* The isolated compound was phomol. Anti-inflammatory assays can be carried out both in vivo and in vitro. *In-vitro* assays include protein denaturation assay (Sangeetha and Vidhya., 2016). Another important one is heat-induced hemolysis. In this assay, human blood is collected, centrifuged and washed in is saline to prepare a suspension. Bacterial extract is added with this and is incubated at 56⁰C for 30 minutes. The absorbance of the supernatant is measured at 560 nm. Standard drugs such as diclofenac sodium (MERCK) were used to calculate the percentage of inhibition. Inflammatory studies can also be done using cell lines and through gene expression. Carrageenan-induced acute paw oedema and formalin induced paw oedema can be used for in-vivo analysis (Urumbil and Anilkumar, 2021).

D. Antimicrobial property

The number of fungal infections and resistance to antifungal agents in pathogens has been increasing over the past 20 years (Pfaller et al., 2015; Lamoth et al., 2018). Fungal diseases are also a major threat to the agricultural sector (Fisher et al., 2012) causing diseases such as vascular wilts (Yadeta and Thomma, 2013). Endophytes have been reported as potential anti-microbial biocontrol agents in crop plants against pathogenic microbes (Mohamad et al., 2018).

One of the major methods used for antimicrobial study in endophytic bacteria is the agar well diffusion method (Schillinger and Lucke, 1989). The endophytes are first

grown in NA broth. This is centrifuged for 10 minutes at 1000 rpm after shaking it in an orbital shaker at 200 rpm for 48 hours. Supernatant is used as the test sample. Pathogenic bacterial culture is spread on NA plates uniformly and four wells with 7 mm diameter are made on the petri plate to which 2 test samples and 2 negative controls are added. Zone of inhibition is measured for analysis of bacterial activity. For antifungal activity, fungal pathogens are placed on the edge of the PDA medium and the test sample is loaded in a single well made out at the center of the plate. Zone of inhibition is measured and compared against a control (Devi et al., 2021).

CONCLUSION

Endophytes are a promising source of worthwhile discoveries, in the field of medicine and agriculture. Almost all plants are inhabited by endophytic treasure troves and yet the studies in this direction are not adequate enough. This makes the isolation, identification and characterization of endophytes a very important endeavor. Isolation requires particular culture media for bacterial and fungal endophytes. Composition of the media is very important to procure desired cultures. Identification needs to be carried out after isolation. The most sought-after practice is the molecular method using 16Sr DNA or ITS sequencing. It gives more sophisticated and reliable results compared to the conventional morphological or biochemical methods. After identification the endophytes need to be characterized for their plant growth promoting or pharmacognostic properties. Numerous reports suggest the role of endophytes in amplifying stress tolerance in plants, which can be used to aid plants in acclimatizing to climatic changes quicker. Cultivation of agricultural crops in stressed conditions can also be made more profitable. Plant growth promoting properties such as IAA production, ACC deaminase activity, siderophore production, phosphate solubilization and drought and salinity stress needs to be evaluated for this. The endophytes also need to be characterized for their pharmacognostic properties such as antioxidant, anticancer, antimicrobial, anti-inflammatory and hepatoprotective activities which will give us an idea of how the endophytes can be utilized in further studies for the development of novel drugs.

Table 1: Same Recently Reported Endophytic Fungi and Bacteria

Endophytic fungus	Plant	Reference
<i>Nigrospora oryzae</i>	<i>Bacopa monnieri</i>	Soni et al.,2021
<i>Alternaria alternata</i>		
<i>Aspergillus terreus</i>		
<i>Nigrospora aurantiaca</i>	<i>Melaleuca leucadendra</i> Linn.	Monzote et al 2020
<i>Aspergillus fumigatus</i>	<i>Myricaria laxiflora</i>	Xue et al., 2021
<i>Chaetomium globosum</i>		
<i>Alternaria alternata</i>	<i>Hordeum vulgare</i> L.	Shadmani 2021
<i>Microdochium bolleyi</i>		
<i>Bipolaris zeicola</i>		
<i>Fusarium redolens</i>		
<i>Fusarium tricinctum</i>		
<i>Fusarium monlifforme</i>		

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<i>Clonostachys rosea</i>		
<i>Epicoccum nigrum</i>		
<i>Phyllosticta</i>	<i>Eucalyptus exserta</i>	Mao et al., 2021
<i>Penicillium</i>		
<i>Eutypella</i>		
<i>Purpureocillium</i>		
<i>Talaromyces</i>		
<i>Lophiostoma</i>		
<i>Cladosporium</i>		
<i>Pestalotiopsis</i>		
<i>Chaetomium</i>		
<i>Fusarium</i>		
<i>Gongronella</i>		
<i>Scedosporium</i>		
<i>Pseudallescheria</i>		
<i>Taxomyces andreanae</i>	<i>Taxus brevifolia</i>	Ejaz et al., 2020
<i>Chaetomium globosum</i>	<i>Ulva pertusa</i>	Cui et al., 2010
<i>Penicillium brasilianum</i>	<i>Melia azedarach</i>	Ejaz et al., 2020
<i>Rhytidhysterium rufulum</i>	<i>Bruguiera gymnorrhiza</i>	Chokpaiboon et al., 2016
<i>Penicillium sp</i>	<i>Limonium tubiflorum</i>	Aly et al., 2011
<i>Fusarium lateritium</i>	<i>Taxus baccata</i>	Ejaz et al., 2020
<i>Trichoderma asperellum</i>	<i>Soybean plant</i>	Sallam et al., 2021
<i>Penicillium oxalicum</i>	<i>Ficus carica L.</i>	Abdou et al., 2021
<i>Aspergillus ochraceus</i>	<i>Sargassum kjellmanianum</i>	Cui et al., 2010
<i>Bartalinia robillardoides</i> <i>Tassi</i>	<i>Aegle marmelos Correa ex roxb</i>	Ejaz et al., 2020
<i>Irpex hynoides</i>	<i>Rhizophora mucronata</i>	Bhimba et al., 2011
<i>Pestalotiopsis guelpinii</i>	<i>Wollemia nobilis</i>	Ejaz et al., 2020
<i>Fusarium proliferatum</i>	<i>Ficus carica L.</i>	Abdou et al., 2021
<i>Trichoderma longibrachiatum</i>	<i>Soybean plant</i>	Sallam et al., 2021
<i>Alternaria alternata</i>	<i>Ficus carica L.</i>	Abdou et al., 2021
<i>Cladosporium cladosporio</i>	<i>Taxus media</i>	Ejaz et al., 2020
<i>Aspergillus versicolor</i>	<i>Petrosia sp</i>	Lee et al., 2010
<i>Fusarium oxysporum</i>	<i>Rhizophora annamalayana</i>	Ejaz et al., 2020
<i>Chaetomium globosum</i>	<i>Ficus carica L.</i>	Abdou et al., 2021
<i>Trichoderma atroviride</i>	<i>Soybean plant</i>	Sallam et al., 2021
<i>Pestalotiopsis microspore</i>	<i>Taxodium distichum</i>	Ejaz et al., 2020
<i>Aspergillus neoniger</i>	<i>Ficus carica L.</i>	Abdou et al., 2021
<i>Phyllosticta citricarpa</i>	<i>Citrus medica</i>	Ejaz et al., 2020

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<i>Trichoderma harzianum</i>	<i>Ilex cornuta</i>	Chen et al., 2007
<i>Penicillium janthinellum</i>	<i>Melia azedarach</i>	Marinho et al., 2005
<i>Curvularia geniculata</i>	<i>Catunaregam tomentosa</i>	Chomcheon et al., 2010
<i>Phomopsis cassia</i>	<i>Cassia spectabilis</i>	Silva et al., 2005
<i>Aspergillus fumigates</i>	<i>Juniperus communis L.</i>	Kusari et al., 2009
<i>Alternaria alternata</i>	<i>Elymus dahuricus and Triticum aestivum</i>	Qiang et al., 2019

Endophytic Bacteria	Plant	Reference
<i>Bacillus cereus</i>	<i>Solanum melongene</i>	Achari and ramesh 2019
<i>Pseudomonas</i>	<i>Alkanna tinctoria Tausch</i>	Rat et al., 2021
<i>Azotobacter chroococcum</i>	<i>Arnebia hispidissima</i>	Singh and Sharma, 2016
<i>Bacillus pumilus</i>	<i>Oryza sativum</i>	Khan Z. et al., 2016
<i>Xanthomonas</i>	<i>Alkanna tinctoria Taush</i>	Rat et al., 2021
<i>Inquilinus</i>	<i>Alkanna tinctoria Taush</i>	Rat et al., 2021
<i>Bacillus siamensis</i>	<i>Cicer arietinum L.</i>	Gorai et al.,2021
<i>Pseudomonas putida</i>	<i>Arabidopsis thaliana</i>	Sheoran et al., 2016
<i>Variovorax</i>	<i>Alkanna tinctoria Taush</i>	Rat et al., 2021
<i>Bacillus subtilis</i>	<i>Pennisetum glaucum</i>	Sangwan et al., 2021
<i>Pantoea</i>	<i>Alkanna tinctoria Taush</i>	Rat et al., 2021
<i>Stenotrophomonas</i>	<i>Alkanna tinctoria Taush</i>	Rat et al., 2021
<i>Pseudomonas aeruginosa</i>	<i>Solanum nigrum</i>	Shi et al., 2016
<i>Cystobacter</i>	<i>Piper longum</i>	Mishra et al., 2021
<i>Bacillus methylotrophicus</i>	<i>Potentilla fulgens</i>	Thokchom et al., 2017
<i>Leptotrichia</i>	<i>Piper longum</i>	Mishra et al., 2021
<i>Streptomyces mutabilis</i>	<i>Triticum aestivum</i>	Toumatia et al., 2016
<i>Rubricoccus</i>	<i>Piper longum</i>	Mishra et al., 2021

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