Insights into process of isolation, characterization and biosynthesis of novel antifungal drugs from plant endophytes – A review

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Abstract

Recent reports on mucormycosis and other fungal infections in post covid cases highlights the search for new antifungal drug leads due to limitations of existing marketed antifungal agents. The overall process of development of antifungal agents is time consuming and needs large monetary investments. Therefore, process has been threatened by limited research in this particular area by major pharmaceutical industries, owing to the heavier monetary burden and low returns on investment'. In recent years microbial endophytes have emerged 'as promising substitute for plant secondary metabolites with potent antifungal, antibacterial, anticancer, insecticidal, and antioxidant properties. Endophytes hold the plausible application in pharmaceutical industries, due to their unique genetic and metabolic diversity as well as promising activities. However, there is little success in utilizing pharmaceutical potential of microbial endophytes. Pioneering research is enviable to establish and boost *in vitro* biosynthetic potential of microbial endophytes'. Although many valuable papers on endophytes have been published, we found no concise review that introduced and explained a workflow of endophytic research. The objective here is to outline the techniques that, in our view, should be followed in comprehensive studies on novel endophytes and their antifungal products, beginning from the isolation and screening of endophytes producing antifungal agent, through the production and purification of the novel antifungal compounds and finally, their characterization. This review on endophytes can assist not only to researchers whose studies are dedicated to antifungal producing endophytes, but can also be applied to other classes of bioactive products of endophytes.

Introduction

Novel antimicrobials are all time needed to control unavoidable and unpredictable occurrences of infectious diseases. Frequent emergence of new ailment, drug resistance in microbes, and remarkable increase in the occurrence of fungal infections among human population all over the globe are few of the frequent and serious problems encountered by human around the globe. Since late 1960's when antibiotic therapies were well practiced a rigorous rise in fungal infectious diseases were observed and they currently signify a worldwide health threat to all forms of life.

Currently one of a constant and serious threat to health of human is invasive fungal diseases. Approximately 1.5 million deaths all over the globe every year are owing to these fungal infections [1]. The fungal pathogens that most frequently cause fatal conditions have been the species of *Candida* and *Aspergillus*. Estimates in literature reveal 30-40 % mortality for the invasive candidiasis and 20-30% for the invasive aspergillosis [2]. Over the past several decades the frequency of disseminated candidiasis has increased noticeably. *Candida albicans* is frequently responsible for 'candidal septicemia' which may adversely affect important organs like liver, kidney, spleen ,lungs, heart, eyes and brain and it might also severely affect central nervous system. It is also responsible for causing oropharyngeal and cutaneous candidiasis. Prolonged uses of antibiotics, central venous catheterization and extended intensive care unit stay have shown to be associated with an increased risk of invasive candidiasm [3]. While, *A. fumigatus* is mainly responsible for around 90% of *Aspergillus* induced disorders in humans, the infections by other species for example *Aspergillus niger, A. flavus, A. terreus* along with *A. nidulans* had been as well reported [4]. Aspergillosis is the most frequent source of infectious pneumonic mortality in patients having heart, lung, pancreas, renal and bone marrow transplants [5]. At present, invasive aspergillosis is the greatest significant source of infection-related mortality in the patients with central nervous system or disseminated aspergillosis and for those with bone marrow transplant [6]. These infections are very common in long term corticosteroid treatment, immunodeficiency disorders related to AIDS, anticancer chemotherapy, cystic fibrosis, diabetes mellitus, organ transplants and aging population. Moreover, opportunistic fungal infections are increasing at a shocking rate. Some of the life threatening infections caused by fungi are Candidiasis, Cryptococcosis, Pneumocytosis, Aspergillosis and Zygomycosis.

Although extensive research is devoted to investigation, development and formulation of novel therapeutic drugs, currently there are only a restricted number of drugs available in market as antifungal agents. This may be because of the fact that, as compared to antibacterial drug, development of novel antifungal drug is more challenging since fungi are one of the eukaryotic organisms and many probable targets for antifungal therapy are also observed to be present in human beings with significant host toxicity risk

[7]. One more explanation is the overall practice of antimicrobial drug development is time consuming, moreover needs huge financial investments. As a result, this process has been threatened by limited investigation in this particular area by pharmaceutical industries, owing to heavier monetary burden in addition to little returns on investment. Actually merely four molecular classes of antifungal drugs targeting three different metabolic pathways in fungi are presently used in medical practice. These are polyenes, echinocandins, azoles, and fluoropyrimidine analogs [7]. Some additional classes, like allylamines and morpholines are used as only topical agents. However these antifungal drugs have a variety of shortcomings concerned with safety, toxicity, spectrum of activity, along with pharmacokinetic properties [1]. The emergence of novel strains which are resistant to the existing antifungal drug is one more issue that needs the attention to develop novel antifungal agents [8]. Acute and chronic side effects frequently are observed with use of existing antifungal drugs, and these are also reported to be immunosuppressive therefore the development of novel, efficient as well as safe antifungal agents, that too naturally occurring with novel mechanisms of action, is target of many researchers. Natural products serve as inspirational source for researchers by the virtue of their less toxicity, inexpensive, eco-friendly nature and high specificity. Most of existing antibiotics used currently in medical practice are either natural products or else their derivatives. Since plants are promising sources of valuable bioactive compounds, they may be significantly exploited by natural product based pharmaceutical industries. However as compared to animals and plants, microorganisms are potential sources for extraction of such bioactive natural compounds due to their high growth rate, capacity to grow on cheap raw material *in vitro*, and ease in genetic manipulation, etc. Natural product discovery has been encouraged by the findings of an important and valuable biomolecules like penicillin. Since the invention of penicillin, over 23,000 natural products have been discovered [9].

Many bacteria and fungi have potential to produce various novel compounds which may be either directly used as potent drugs or may be used as a lead structures meant for synthetic modifications. Among all classes of microorganisms, fungi are usually considered as the active producers of diverse antimicrobial compounds which have various agricultural and

pharmaceutical applications. Fungi are being used ever since five decades in production of, well known antibiotic such as penicillin, immunosuppressive drug like cyclosporine A, cholesterol reducing potential drugs like statins and several other drugs. Similarly, actinomycetes are long been considered as most important potential source of pharmacologically active agents, novel biologically active compounds, antibiotics and enzyme inhibitors.

Despite lot of microbes being studied there are still many more in the environment which are ignored or might have not been able to culture in laboratory condition. Usually fungi as well as actinomycetes are used for bioprospecting purpose because majority of antibiotics plus other therapeutics of microbial origin produced today are by using members from these groups. In contrast bacteria are mostly neglected from investigations as a producer of antimicrobials. Further in past, many bioactive compounds were investigated through screening programs using soil samples but now it is found that it is associated mostly with high rate of rediscovery of already known compounds and chances of obtaining novel chemical entities are very low.

To surpass this situation, best option is to use previously unexplored microbial niches. One such unexplored and less studied niche is healthy, green tissues of plants that harbor a rich, diverse microbial biota known as ‘endophytes’.

Endophytes: A potential source of antifungals

Endophytes are the microbes existing in inner tissues of the plants devoid of any visible disease symptoms in the host [10]. The ‘International Union for Conservation of Nature’ and Natural sources (IUCN) data reported that there are 2,97,326 species of plants and very minute number of species are considered for their associated endophytes. Each and every plant on this earth may act as host for one or more microbial endophytes which includes commonly bacteria, fungi and actinomycetes [11]. Further, host plant serves as a kind of selection system for production of bioactive compounds by endophytes with reduced toxicity for higher organisms. Thus, endophytes may possibly secrete a lot many of potent biologically active compounds of prospective use to agriculture, modern medicine, and allied industries. The literature survey of investigations on endophytes suggest that about a decade back, the studies mainly revolved around biodiversity, distribution of endophytic

assemblages, and ecological studies. This focus was then progressively shifted to bioactivities of endophytic fungi and now, studies of antimicrobial activities of endophytes are assuming considerable importance in scientific field. The breakthrough discovery of Paclitaxel or Taxol-an anti-cancer compound by *Taxomyces andreanae* obtained from *Taxus brevifolia* has directed the attention of researchers towards pharmaceutically important endophytic studies. Endophytes produce diverse type of bioactive compounds such as alkaloids, saponins, flavonoids, tannins, terpenoids, quinones, phenolic acids etc. and are known for various therapeutic applications like anticancer, antitumor, antidiabetic, antifungal, antiviral, antimicrobial, antimalarial, antioxidant activity [12]. These bio-active compounds might have diverse applications in both, research and applied fields of agriculture, medicine, food industry, as well as during pest management etc. One of report [13] by Newman and Cragg listed the drugs that are approved between years 1981 to 2014; suggest that maximum number of drugs were of microbial and or endophytic origin. The day is not far when endophytes will become significant and independent alternative sources of countless metabolites, sparing the parent host plants and will become an inexhaustible and unlimited source of valuable compounds using simple fermentation processes that too in short time. Investigation on endophytes for novel compounds may help to investigate novel drug leads for successful management of diseases in animals, plants and humans. Endophytes may find applications in other fields also, like biodegradation, biotransformation, fabricators of nanoparticles, biofuel and as an inexhaustible source of extracellular enzymes [12,14]. Microbial endophytes of medicinal plants act as prospective resource of natural products for use in oxidative stress as well as novel bio-active compounds [15]. Various nanoparticles synthesized by endophytic bacteria may emerge as one of novel research area in pharmaceutical engineering field [16]. Although abundant research was carried out on novel bioactives, endophyte research is still in budding stage since a tiny success has been achieved up till date in commercial production of such economically valuable compounds.

Furthermore, it is known that microorganisms as resource of a valuable product can be easily and more economically cultivated will help in effectively sinking market cost of product. Exponential advances in science and engineering, in the past few decades have

further established techniques like metabolic technology, microbial fermentation, chromatography and varieties of spectroscopy, etc. which have helped researchers to work into various unexplored regions of potential applications. By using mutation or gene manipulation and other recombinant DNA techniques, best quality endophytes can be chosen and their productivity can be increased by manipulating the relevant functional genes in their respective pathways for biosynthesis and culturing them *in vitro* for optimal production of significant bioactive compounds. Desired features may also be attributed to these bioactive compounds, and potential derivatives may be obtained from them. All these developments will surely lead to an unlimited supply of cheap, completely safe and naturally derived medicines, which will be easily available to each and every one in society. The entire process of obtaining such bioactive compounds will be in complete harmonization with the environment keeping a view to conserve and preserve the balance of eco-system by preserving the plants which may sustain it.

Endophytic population of medicinal plants may perhaps be promising source of antifungal substances. Novel antifungal agents produced by microbial endophytes of ethnomedicinal plants may be used not merely as drugs but also for other purposes like a pharmaceutical preservative in different formulations, against food-borne diseases, food-preservation, food-spoilage, as a antifungal component in wall paints etc.

Taking into consideration above discussed facts, and to identify proper potential of endophytes, an unambiguous understanding of ecological, biochemical, physiological, biotechnological, and chemical along with bioinformatics aspects is needed. Modern biotechnological techniques along with bioinformatics approaches may bridge a gap in endophytic research. The current review target to direct the attention of scientific community towards tapping potential of endophytes for antifungals as well as biotechnological methods used for exploitation of antifungal compounds. This principally summarizes past along with present research on microbial endophytes involving methods for their isolation, evaluation of their antifungal potential and their identification etc. A recent advance to study and characterize the endophytes using ‘omics’ methods along with high throughput sequencing are as well considered. This report furthermore focuses different methods used for characterization of antifungal compounds produced by endophytes with their

potential application. The present review may assist research scholars to acquire unambiguous understanding about exploiting endophytes for novel antifungals having potential applications in pharmaceutical industries. A better understanding about every aspect of endophyte for their bioactive potential will certainly help to make their commercial use in drug discovery.

Factors for plant selection and use of medicinal plants as better niche

Although, majority of endophytes exhibits the inherent and natural ability of synthesizing bioactive compounds, understanding methods along with rationale to choose the plants for bacterial as well as fungal endophytic association may offer better opportunities for isolation of novel microbial strain or to obtain novel therapeutics. The innovative and planned selection of plants helps in easy selection by narrowing down search and thus directs the study towards bioactivity screening. Isolation of bacterial endophytes are being attempted from variety of plants ranging from monocotyledonous, dicotyledonous plants, woody tree species and herbaceous crop plants as well as medicinal plants. Strobel and Daisy [17] had listed out rationale to facilitate the overall process of easy selection of plants as follows:

- Plants from unique and inimitable environmental settings, particularly with unusual biology, along with bearing novel survival strategies.
- Plants, which have reported ethnobotanical medicinal history. It has been suggested as therapeutic potential of a medicinal plant might not be merely due to compounds produced by it, but it is due to its associated endophytes.
- Plants which are endemic, having a remarkable longevity, and which have occupied a certain prehistoric land mass.
- Plants growing in region of enormous biodiversity.
- Plants surrounded by asymptomatic diseased plants.

Over the centuries, medicinal plants were used in almost all cultures as one of best source of therapeutic agents. Use of medicinal plant may be traced back to ancient agricultural societies, where native populations have utilized them as therapies for many diseases. In India, Ayurveda is the most earliest and living traditions, which is being practiced for treating different types of diseases and disorders. Plants that are having an ethno-botanical

history and are being used by traditional healthcare practitioners for various disease treatments may be the most excellent choice for the investigation of novel endophytes synthesizing potent bioactive antifungal metabolites having pharmaceutical importance. Further, genetic origin of potent bioactive compound has been predicted to be arising by horizontal gene transfer from host to its endophytes [18]. In fact during co-evolution process endophytes has undergone certain genetic changes together with DNA uptake of host plant into their own genome. Hence, some of endophytes have developed capacity to synthesize same 'phytochemicals' as like host plant [19]. Hence medicinal plants of indigenous origin would be screened for their endophytes and evaluated for their biological potential to manufacture several life-saving drugs required on daily basis by humans. The conservation of host plant along with native microflora is having crucial importance in process of investigation of novel drugs. In recent years a wide variety and a huge number of natural therapeutic products are reported to be obtained from fungal endophytes, however scanty information is available on both occurrence and potential significance of bacterial endophytes associated with medicinal plants [20]. While studies on medicinal properties of these plants have been carried out in great detail by many researchers, reports on the endophytic microbial population of these medicinal plants is lacking and this deserves a special attention for further development of microbial based biotechnological products along with variety of pharmaceutical formulations [21]. So in present review, endophytic population from medicinal plants is focused. The rationale behind selecting medicinal plants is, traditionally these plants are being particularly used for treatment of a variety of infections so it is reasonably correct to assume that therapeutic potential shown by host plant may also be observed in endophytes associated with such host plants, owing to genetic recombination occurring among endophyte and its host during evolutionary period. In fact it is supposed that, microbial endophytes existing in medicinal plants may imitate the chemistry of relevant host by producing same type of natural bioactive products or their derivatives which may sometimes even are more bioactive. This can be manifested from the fact that endophytes could produce podophyllotoxin, paclitaxel, camptothecine, hypericin, diosgenin, and vinblastine compounds same like their host plants. Such compounds may be useful in giving protection from pathogens. Now a

day's medicinal plants are regarded as a storehouse of microbial endophytes producing novel and potent metabolites of pharmaceutical and agricultural significance [22]. Further as per estimation by WHO, almost 80% of worldwide people from developing countries make use of traditional medicines which are generally derived from plants. However because of over exploitation of these medicinal plants along with 'other biotic interferences, many plants have now become critically endangered moreover they are on verge of extinction in near future. Since it is assumed that, these plant species may contain unique, distinct and potential endophytes 'with multifold application, research priority should be devoted to their study because disappearance of any of these host plant species will also cause disappearance of complete group of associated endophytes. Since extensive extraction procedures for valuable and potent bioactive compounds will have the risk of exhaustion of medicinal plants diversity, commercial collection of natural medicinal plants are legally regulated in many countries. Further plants, as compared to most drug producing microbes, exploited by the pharmaceutical industries, are often expensive and are difficult to propagate on commercial scale because of seasonal specificity, restricted availability of land for their cultivation and environmental competence etc. Moreover, national along with international regulations restrict the transport of many indigenous plant species [23]. However, if endophytes can produce similar rare and significant bioactive principles like host plant, it would not merely reduce the need to harvest slow growing and probably rare plants, but also will assist to conserve continually diminishing biodiversity of the globe.

During studies on endophytes of medicinal plants for obtaining antifungal compounds, some important points should be borne into mind such as season of collection, sampling from different plant parts of varied age, site of collection, sample size, type of culture media as well as surface sterilization protocol of plant tissues.

Sample collection

It is recommended that a fine physical condition of sampling site without plant infection is prerequisite for selecting target plant. Ideally identified target plant must be deposited in local herbarium as a voucher specimen. It is essential to note field data which includes location in terms of GPS, temperature, soil pH, soil type, humidity, light intensity, soil microbes, salinity etc. This may assist in correlating

information obtained and its effect on microbial endophytic population [24]. It is must to obtain fresh plant material for isolating endophytic microbes. Healthy and mature plants have to be selected for sampling of the plant tissues. Various segments of host plant like roots, leaves, stem, seeds and fruits should be excised randomly and separately with sterile blades. It is essential to collect plant specimens in a sterile, clean and dry zip lock covers with proper labeling and name tags. During its transport and storage it is essential to prevent desiccation by allowing adequate aeration; since the former slows tissue death, and later reduces growth of secondary contaminating fungi and bacteria. As far as possible prolonged transport in sealed plastic bags must be avoided. Use of sturdy paper bags, or zip locked, perforated bags, waxy paper are better for transport and temporary storage of plant tissues [25]. Storage for long periods, especially in frost-free refrigerators will cause tissue desiccation. Collected plant samples should be processed within 12 hours. Samples have to be stored at appropriate conditions and isolation of endophytes has to be initiated at the earliest when samples are fresh and free from contamination.

Sample size

Diversity of endophytic community depends on size and number of explant hence sample size is one of the critical factors in both, obtaining maximum endophytic diversity and in obtaining novel antifungal compounds. A competent isolation approach may aid in achieving highest endophytic diversity from a particular plant. It is recommended that, roughly 30 to 40 sampling units per tissue from 40 plants of a given species may yield approximately 80% endophytic fungi [26]. Conversely increase in recovery of endophytic isolates was observed when the explant size was reduced; indicating size of explants needs to be optimized for obtaining accurate endophytic diversity [27].

Effect of age on isolation

Age of plant is supposed to affect endophytic diversity. Elder plant part promotes more potential antifungal endophytes [28]. Increased number of microbial endophytes in mature leaves may be because of extensive period of tissue exposure to environment. Frequencies of endophyte establishment have found to be enhanced with leaf age [29]. This probably may be because of the fact that younger plant segments are

biochemically different from mature old plant segments.

Seasonal influence on microbial endophytic population

Endophytic population varies with change in climate of given region. Environmental conditions appear to considerably affect the endophytic bacterial [30] as well as fungal population present in host. A greater diversity of endophytic fungi was observed during rainy season [31]. It may be for the reason that high moisture along with temperature may be crucial for growth and dispersal of endophytic fungal spores [32]. Conversely, several reports suggest that in winter season, diversity was significantly higher than rainy and summer season [33]. In contrast diversity of bacterial endophytes in May was shown to be considerably lower than those in June, July and August [34]. Statistically it is reported and also proved that microbial endophytes are active and isolated more in number in rainy season as compared to winter and summer [35].

Isolation of Endophytes

Most significant and vital step while working with microbial endophytes is their successful isolation. Since endophytes are present in intercellular niches, they escape isolation procedure and many times epiphytes may overgrow. Proper surface sterilization of explant is essential to eliminate epiphytic population [36]. Isolation procedure must be sensitive adequate to recover maximum endophyte and must be strong sufficient to eliminate all epiphytes that are present on surface of explants. Most widely used isolation protocols combine surface sterilization of explants with either maceration of explants and streaking onto nutrient agar, or plating small sterilized segments onto nutrient agar. Isolation of endophytes requires a thorough surface sterilization of explant using disinfectants followed by their plating onto a suitable medium and incubation till the endophytic growth is initiated. It is essential to maintain sterility throughout procedure, and investigator should optimize procedure for each plant tissue, because sensitivity differs with species, age and surface properties. First step during this is washing explant in running tap water for 5 to 10 min to eliminate soil particles, dust and debris followed by treatment with a general disinfectant or strong oxidant and finally a sterile rinse [37]. Sodium hypochlorite to concentrations of 2-10% is frequently used disinfectant. Likewise efficient oxidants include 3% H_2O_2 and 2% $KMnO_4$. Ethanol (70-95%) and Tween 80 are commonly

used to assure surface sterilization of explants [38]. Samples need treatment with disinfectant like 70% ethanol and sodium hypochlorite (2-10%) stepwise for brief durations followed by washing with sterilized distilled water. Dilutions and period of exposure to sodium hypochlorite vary with tissue type and host [37]. Generally, leaves with thick cuticles and woody tissues need rigorous sterilization than fragile ones. It was proved that ethanol – sodium hypochlorite – ethanol series effectively kills thick walled spores of epiphytic contaminants. Concentration and immersion time in sterilants must be optimized since sterilants might possibly penetrate plant tissues thereby killing the endophytes and affecting the results. Instead of cutting, maceration of plant parts using sterile mortar and pestle homogenizer [39], by using a polytronhomogenizer [40] or blender in sterile buffer can help to isolate slow as well as fast growing endophytes [41]. To avoid toxicity of certain plant enzymes and metabolites to endophytes, use of EDTA or polyvinyl pyrrolidone is recommended [41]. Finally rinsing may be carried out by deionized sterile distilled water for 3-5 times, and may be blot dried under laminar air flow [42] and can be aseptically placed on suitable nutrient medium supplemented with antibiotics.

Types of culture media for isolation and incubation conditions

Selection of the culture medium is critical as it influence number as well as type of endophytes that is to be isolated [43]. Media plays a key role in growth as well as metabolite production. Frequently endophytes fail to grow on artificial media and sometimes it may unable to sporulate in *in vitro* condition. For fungal and bacterial isolation, medium should be supplemented with antibacterial agent and antifungal agent. Different workers have used variety of media for isolation of endophytic fungi e.g. Potato Dextrose Agar or Malt Extract Agar [44] supplemented or not with antibiotic agents. Various alterations of common culture media viz. potato dextrose agar [45], malt extract agar [46], Czapek's agar, oatmeal agar, synthetic nutrient-poor agar,V-8 agar, corn meal agar [47], Sabouraud Dextrose Agar, water agar, along with modified Melin-Norkrans medium, with varying factors such as temperature, light, pH, aeration as well as time of incubation along with addition of plant extract must be tried for best possible recovery of fungal strains [48,49]. Deka and Jha (49) showed a huge number may be isolated on media added with bark or leaf extracts.

Bills and Polishook [50] used selective media as, BOP (malt yeast-extract agar with benomyl and colony-restricting surfactants) and ACD (mycosel agar), MYE (malt-yeast extract) media. Combination of these selective media, allowed growth of plenty of rare species. Sometimes glycerol arginine medium [51], Starch milk agar, Glucose citrate agar, paraffin agar, corneal-malt agar, tryptone sugar agar, liquid culture medium M102, yeast extract glucose agar and chemically defined media have been also tested [52]. Though fungal media are usually slightly acidic, antibacterial agents, like oxytetracycline, penicillin, 'streptomycin sulfate, and/or novobiocin should be included [25]. Endophytic bacteria can be isolated by using medias like Nutrient agar [53], Tryptic Soya Agar and R2A [54], King's B medium [55], nutrient broth, yeast extract medium [40] or SC [56]. For isolation of endophytic bacteria antifungal substances like cycloheximide, ketoconazole, itraconazole, fungicides may be added or specific nutrients may be added to stimulate growth of desirable microbial groups. Inoculated plates may be sealed with a cling wrap and may be incubated at 25 ± 2^0C for 4-6 weeks under alternative periods of dark and light conditions. Upon initiation of a fungal colony, it may be transferred to PDA medium or Sabouraud Dextrose Agar medium and may be cultured for 3-6 weeks. Each fungal isolate may be purified by continuous culturing and may be finally maintained on PDA slants [57]. Whereas for bacterial isolation, plates can be incubated at 37 ± 2^0C for 3-5 days and later sub cultured to obtain pure isolates. For long-term preservation of microbial cultures cryopreservation is considered as ultimate method due to stability of secondary metabolite production and minimized genetic alterations [58].

Cultivation-independent methods

Almost 99% of microorganisms cannot be cultivated artificially and a variety of molecular techniques developed recently have proved to be valuable tools for studying diversity and functions of endophytic community in plant host [59]. Cultivation-dependent methods like plate counts miscalculate microbial numbers since they do not record viable but non-culturable cells, e.g. microorganisms with unknown growth requirements and obligate biotrophs. These can be identified and detected by using molecular methods wherein their DNA sequences can be compared with those in databanks'. Subjecting environmental samples directly for evaluation of mycobiomes helps to give exclusive facts of community

composition, diversity, taxonomy along with interactions [60]. Advanced methods such as metagenomics, metaproteomics, metatranscriptomics will help to know role of microbial communities and their interactions with plant host [61]. In metagenomics analysis of genetic material which is directly recovered from environmental samples such as plant tissue is carried out and it allows identification of unculturable species. Fingerprinting technique based on‘PCR-amplified 16S rRNA (bacteria) or 18S and 28S rRNA (fungi) genes allows analysis of composition of whole microbial community and their spatial and temporal variations in relation to environmental factors [62,63]. These techniques include denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) [63], Terminal Restriction Fragment Length Polymorphism (T-RFLP) [62] and PCR-Single-Strand- Conformation Polymorphism (SSCP) [64]. Now days such techniques are being successfully applied for analysis of endophytic communities. Due to limitations of individual methods one should not rely on single method [62,63], but should assess various characteristics, e.g. morphology, physiology as well as molecular outcome.

Localization of endophytes *in situ*

To understand endophyte-host interaction it is necessary to study localization of endophytes inside plant tissue that assist to detect inter or intracellular colonization (García et al. 2012). Direct microscopic visualization may help to allocate accurate location as well as colonization pattern of endophytes inside plant tissues, especially in leaves [65,66). Hiatt et al. [67], recommended use of an inexpensive, rapid tissue immunoblot technique which is coupled with histological staining and subsequently microscopic analysis for studying distribution of fungal endophytes. Recently autofluorescent protein techniques are used for studying biofilm formation and microbe–plant interactions [68]. These recent techniques are used to detect moreover count microbes in planta [69]. Once isolated, endophytes can be subjected to primary screening for antifungals.

Primary screening of endophytes for antifungal activity

In primary screening, a large number of endophytic isolates may be screened against microbial fungal pathogens to select the potent strains with capability to produce antifungal metabolites. Isolates which exhibit substantial antifungal activity can be further used for subsequent

secondary screening program. This method is considered as effective for differentiating bioactive isolates and will reduce laborious work of fermentation and antifungal activity programs. Preliminary screening can be good choice when large number of endophytes are to be evaluated where all endophytes may be primarily screened for their antifungal potential using different screening methods like cross streak plate method [70] and duel culture assay method [71,72].

Large-scale fermentation

Large scale cultivation is a pre-requisite for extraction of antifungal compounds which gets diffused into the culture medium and also for those produced inside the endophytes. This may be certainly accomplished by cultivating endophytes in broth cultures. Fermentation process of has to be optimized. Pure strain of endophyte may be inoculated into suitable optimized sterile broth media and allowed to grow for optimum time at optimum temperatures with or without periodical shaking at 120-150 rpm [73]. Subsequently it may be filtered through a sterile muslin cloth or centrifuged to obtain culture filtrate. Endophytic growth left behind and culture filtrate may be collected and stored for further analysis [74].

Methods for assessment of antifungal activity

Culture broth containing crude extracts of each endophyte should be tested through commonly used secondary screening methods like mycelial radial growth test, agar diffusion assay, disk diffusion assay etc [75,76]. The well-known techniques are broth or agar dilution and disk-diffusion methods. Whereas specially for antifungal testing, methods like poisoned food technique is used. Several methods were successfully used by many research workers like Agar disk-diffusion method [77], Agar well diffusion assay [78], Agar plug diffusion method [79], Cross streak method [70], Poisoned food method [80], Thin-layer chromatography (TLC)–bioautography [81], Agar diffusion [82], Direct bioautography [83], Agar overlay bioassay [81], Broth dilution method [84], Agar dilution method [85], Time-kill test [86], ATP bioluminescence assay [87] and flow cytofluorometric method [88].

Detection, isolation, purification and structural elucidation of antifungal compounds

Natural product research is a multidisciplinary approach. A plenty of researches have reported that endophytes

with no certain compound isolated showed antimicrobial activities, and some of which were significant [89]. Low yield of active molecules, synergistic bioactivity and presence of heavy load of undesirable compounds could hinder the pharmaceutical use of natural sources. A variety of in vitro techniques may be used for detection, isolation, purification and structural elucidation of antifungal compounds. Isolation and identification of potent antifungal agent from an active extract is preliminary and important step towards the drug discovery. Isolation and purification of actual molecule responsible for the activity is necessary for its structural elucidation. Structural elucidation of active molecule enables production of same molecule by synthetic chemistry or beneficial structural derivatizations and rationalization of mode of action. The development of compounds with better activity is possible only if the exact structure of the bioactive compound is known, which in turn help in conservation of natural resources by reducing the over dependency on depleting resources [90]. Still a plenty of research on endophyte is in screening stage and active constituents needs to be isolated. Separation of active constituents is supposed to be the next step once crude extracts of isolated endophytes are shown to possess antifungal activity against tested microbes. At times, instead of a single compound, synergistic activity of multiple compounds may be the cause for activity [90]. The biggest challenge still being faced is characterization of these bioactive compounds which involves sequential analysis using various techniques.

Secondary metabolite from endophytes may be extracted by using solvent partitioning or by other suitable methods as mentioned in table 1. For already known compounds, verification of spectroscopic data against authentic standard or by comparison with literature data helps to confirm its exact structure. Now a day an integrated interdisciplinary approach may be useful for the detection, identification and purification of antifungals. TLC followed by bioautography may be used for antifungal detection wherein, active extract would be separated by TLC and antimicrobial metabolites can be detected by spraying test microbes onto the resolved TLC plates followed by incubation. The zone of inhibition around a metabolite indicates antimicrobial activity [91]. Active components can be purified by using silica gel column chromatography [92]. Semi-preparative reverse phase HPLC has also been tried by many investigators for better

purification of semi pure fractions collected from silica gel column [93].

Identification of endophytes

The identification of selected endophyte is must after screening, as it is helpful in following endophytic workflow steps. Identification as well as characterization of endophytes is a critical step for diversity studies, population structure, dynamics and for production of bioactive compound. It involves a direct microscopic observation for obtaining details about colony morphology followed by detailed molecular studies involving genomic DNA extraction, amplification using suitable primers and its subsequent sequencing to assess their phylogenetic structure and evolutionary history. Fungal endophytes may be identified by morphological as well as molecular techniques. Endophytic fungi are usually subjected to microscopic observation using light microscope by staining with lactophenol cotton blue or phase contrast microscope with various magnifications in morphological methods .Preliminary information regarding colony morphology, color, fruiting bodies, spore and mycelial characteristics can be obtained through microscopic studies. These observations can be compared with the records from standard manuals of mycology [94] or else the cultural characteristics of fungal isolate like shape, colour, elevation of colony and spore characteristics [95] may be used for identification using online databases like Systematic Mycology and Microbiology Laboratory (ARS, USDA), Fungal Databases, Fungal Planet, MycoBank, Index Fungorum, Bibliography of Systematic Mycology, Tree of Life Web Project etc. In some cases isolates fails to sporulate during cultivation and so may be classified as sterile fungi or sterile mycelia [96]. Sometimes still endophytic fungi together with sterile fungi are ambiguous [27]. Therefore with the intention to overcome this drawback, alternative more reliable choice is molecular characterization [97]. On the other hand, uncultivable fungal endophytes may be isolated from explants by laser micro dissection and pressure catapulting method [98]. Internal Transcribed Spacer (ITS) is common sequencing technique for molecular identification of fungal species [99]. In this technique fungal endophytes are subjected to genomic DNA isolation and PCR, targeting adjacent sequences of 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS 4, 28S rRNA gene, using the ITS 1/4 primers. Large-scale research of micro fungi often depend on Basic Local Alignment Search Tool (BLAST) matches with National Center for Biotechnology

Information (NCBI) GenBank database for identification. To authenticate correctness of identification based on BLAST matches, taxonomic matches are compared at genus and family levels for similar isolates based on BLAST searches. The ITS rRNA sequence similarity above 98% helps in species identification whereas those between 95% to 98% is used for genus identification [100]. A new taxon may be proposed if endophytic fungus fails to display any close match in GenBank, thorough literature survey. Consensus tree is usually produced with sequences identified by BLAST search reflecting the highest identity and maximum query coverage. Finally, amplified ITS sequence of isolates may be submitted to NCBI GenBank database. A culture-independent DNA technique like Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restriction Fragment Length Polymorphism (T-RFLP) has been also developed for the exploration of complex microbial communities. Similarly for bacterial isolates, morphological, biochemical as well as molecular methods may be used. In morphological method isolated endophytic bacteria may be primarily characterized based on their colony characteristics, such as, size, shape, colour, margin, elevation, opacity, consistency, Gram nature and motility etc. In biochemical methods, conventional API-testing requires more time and is costly, as well as outdated. Currently bacterial isolates might be identified based on carbon source consumption profile using GP2 plates of Biolog. Whereas in molecular techniques 16S rRNA sequencing is selected principally for the identification along with classification of bacteria [101]. It is routinely used in polyphasic approach, describing any bacterial species or higher taxa [102]. Sequences obtained can be compared with existing databanks (e.g. GenBank, RDP, EMBL), by performing a BLAST search. Consequently, it is likely to assume quickly whether determined sequence is matching with known equivalent in databanks so as to establish similarity or otherwise and may lead to species identification. If not, the phylogenetic affiliation of sequence can be estimated easily to a class level. Generally, sequences need to be aligned to other sequences. The public alignment package ClustalW produces good alignments from scratch, but other programs are also available [103]. Now days a novel automated high-throughput MS-based approach is available which can identify hundreds of microbes in single experiment whereby bacterial biomarkers are being used. To identify bacteria at subspecies

level results can be compared with databases (e.g. Bruker BioTyper or SARAMIS). These are commonly used in clinical microbiology, and are expected to replace biochemical and molecular biology techniques in microbe identification in near future [104]. Isolation and screening for antifungal producers ends when selected strains are frozen and banked in a laboratory culture collection at -80ºC for further cultivation and analysis of antifungals.

Use of endophytes as a source of antifungal drugs

Endophytic microbes associated with medicinal plants are excellent source of valuable metabolites [105]. Novel fungus *Cinnamomum zeylanicum* was reported to be producing volatile organic compounds i.e. VOCs showing potent bioactivity. A mixture of VOCs containing alcohols, acids, ketones, esters, and lipids were found to be produced by *Muscoder albus* [106]. Antifungal lipopeptide, Cryptocandin A was obtained from endophytic *Cryptosporiopsis quercina* containing various unusual hydroxylated amino acids as well as 3 hydroxy-4-hydroxymethylproline active against *Candida albicans* and *Trichophyton sp* [107]. Fungal endophyte named *Phomopsis sp*. YM 311483 was found to be producing novel ten membered lactones active against *Aspergillus niger*, and *Fusarium sp*. and *Botrytis cinera* [108]. *Fusarium Spendophytic* from *Selaginella pollescens* was proved to be active against *Candida albicans* [17]. *Phoma sp.* produces volatile compounds that possess antifungal properties and are noticeably similar to methanolic extract of host plant. Medicinal plant associated fungal endophytes are more likely produce novel metabolites having pharmaceutical importance [109]. For instance, Gogoi et al [110] isolated fungal endophyte from *Taxus wallichiana* showing antifungal properties. Ecomycins produced by bacterial endophyte *Pseudomonas viridiflava,* is active against C*ryptococcus neoformans* and *C. albicans* [111]. As prospective reservoir but with a yet-unpredictable biodiversity, some chemically examined endophytes were found to be producers of strong fungicidal natural products with significant chemo-diversities [18]. Similarly a number of crude extracts from various culture broths were proved to possess antagonistic activity against pathogenic fungi and yeasts. Though research literature has abundant studies showing production of antifungals from endophytic fungi still many more are not yet investigated. These

include pentaketide [112], Glucoside derivatives – xylarosides [76], *T. brevicompactum* [113], Cytosporone B and C [108], *Cryptosporiopsis quercin* [17], Cryptocin, a tetramic acid [114], Pseudomycins [115], *L. theobromae* [116] and Ambuic acid [117]. Although majority of research is pertaining to fungal based production of antimicrobial compounds, several compounds active at low concentration against various human and animal pathogens and having low molecular weight are being isolated from bacterial endophytes. Various products of *Pseudomonas* species were reported to have activity against different types of fungi i.e. *Pythium ultimum*, *Rhozoctonia solani*, *Phytophthora capsici*, *Fusarium oxysporium* [118], *Aspergillus niger* and *Candida sp* [119]. Ecomycins, Pseudomycins, Kakadumycins, Munumbicins are some examples of recently discovered novel antibiotics from endophytic bacteria [11]. Such structurally diverse compounds possess potential therapeutic value that is why all over the globe researchers are more interested in studies related with endophytic bacteria for discovery of novel antifungals.

Future prospects and challenges

Medicinal plants have been considered as a rich source of natural products including antifungals that are used for treatment of many ailments; thus, are potential source for investigation of novel therapeutics. However owing to overexploitation of these resources along with other biotic interferences, several medicinal plants have ‘now become critically endangered moreover they are on verge of extinction in near future. Since it is assumed that, these plant species may port quite distinct and potential endophytes producing novel metabolites, research priority should be aimed to study them because disappearance of any of these plants will also cause disappearance of associated endophytes. Despite abundant research reports on antifungal compounds from microbial endophytes, still a tiny success is achieved in commercial production of these antifungals. Many endophytes are continually being discovered that are able to produce same bioactive compounds like their respective host plants or else novel drug leads, still, it requires a commercial implementation approach. So, it is essential to clarify biosynthetic pathways concerned with production of novel antifungals from endophytes for their further application in pharmaceutical field.

It has been reported that upon repeated sub-culturing in vitro endophytes losses their metabolic potential thereby found to be of useless commercial application. Up till date no study has claimed cost effective large scale production of antifungals from endophytes. Hence, it is necessary to establish a feasible method for industrial scale production of antifungals by cheaper way. Several modern techniques e.g metagenomics, 'genome mining, and *in silico* profiling of signature genes may be used to optimize production of antifungals from endophytes. Molecular and epigenetics-based protocols in conjunction with creating same natural habitat like host plant may trigger silent gene clusters and promote up-regulation of novel metabolites. Studies related with target gene sequences of endophytes may help to know their expression. Simulation of natural conditions such as co-cultivating two or more endophytic strains together, and/or addition of plant extract in the culture media' may stimulate production of novel antifungal metabolites. Additionally, cultivation of endophytic bacteria with fungi or other bacteria may give novel products that cannot be achieved when bacteria or fungi are cultivated alone. Therefore, extensive research on endophytes is necessary so as to develop appropriate co-culture system for continuous production of preferred antifungal compounds .Antifungal endophytes may contain some special genes probably arranged as an operon in highly contiguous clusters. This permits ready identification of signature genes or gene domains that are pathway-specific. These might further made use as probe for combinatorial biochemistry allowing generation of desirable structural analogs. This might synchronize the classical combinatorial chemistry. Studies related with bioinformatics will help to link molecular information to the metabolic profiles. This allows increasing yield of desired product by decoding exact steps at which the biosynthesis is constrained.'Upcoming omics technologies like genomics, metabolomics, proteomics, transcriptomics and associated knowledge might aid in studying biochemical along with physiological interaction of endophytes with host plant. This helps to achieve sustainable production of antifungal bioactives. It is must to know the mechanism of action, toxicity issues of novel antifungals from endophytes as well as preclinical and clinical studies, which can be achieved by systematic studies pertaining to biology, ecology, biotechnology, biochemistry and bioinformatics. Further endophyte derived antifungal compounds may be explored

for formulation of sprays for inhalation intended to treat fungal infections for example Aspergillosis in lungs, for treating superficial skin infections and sanitization, use in different novel drug delivery systems in addition to their applications in cosmetics and color paints. Although it is not easy to take an antifungal drug to the clinical stage due to several reasons first, many times it is associated with rediscovery of already known compound. Even same antifungal metabolite may be produced by different organisms. Most of the antifungal activities observed during screening of microbial products may not show desired spectrum or are not potent sufficient to make them attractive enough for industrial applications. Others are merely toxins that may affect mammalian cells, making them undesirable drug leads. Finally, when tested in animal models, many compounds with potential *in vitro* activity are proved to be inactive or only weakly active. This may be because of a variety of reasons associated with its pharmacokinetic properties, from poor absorption to inactivation by serum binding, high clearance rates, etc. In summary, although existing statistics suggest that the number of compounds showing antifungal activity is certainly huge, taking one of them to clinical trials is a very difficult task, and the level of resources required to make this effort should not be underestimated.

To conclude, bacterial and fungal endophytes have been proved to have great potential as an eco-friendly, natural resource that can be applied in antifungal agent production. However, there are still many issues to be addressed. An unambiguous understanding of plant endophyte interaction may be helpful in exploiting their true potential. Exhaustive studies related to effect of environmental factors and host plant help to obtain higher yield of antifungals. Modern biotechnological methods and‘sophisticated high throughput genomic/proteomic approaches assure to aid robust exploitation of secondary metabolite production by the vital endophytic communities. Apart from antifungal application of endophytes, other major fields of interest include biotransformation, biodegradation, phytoremediation, plant growth promoters, plant stress relievers, biocontrol agents, biocatalysts, biofuel production, nanoparticle production and agricultural importance etc. The upcoming use of endophyte-based nanoparticles has proved promising results for future drug development. This vast range of applications of endophytes particularly from medicinal plants itself speaks about

the depth and worth of this field that still needs to be explored. We hope that this review can stimulate the studies related with development of novel antifungal agents from endophytic strains.

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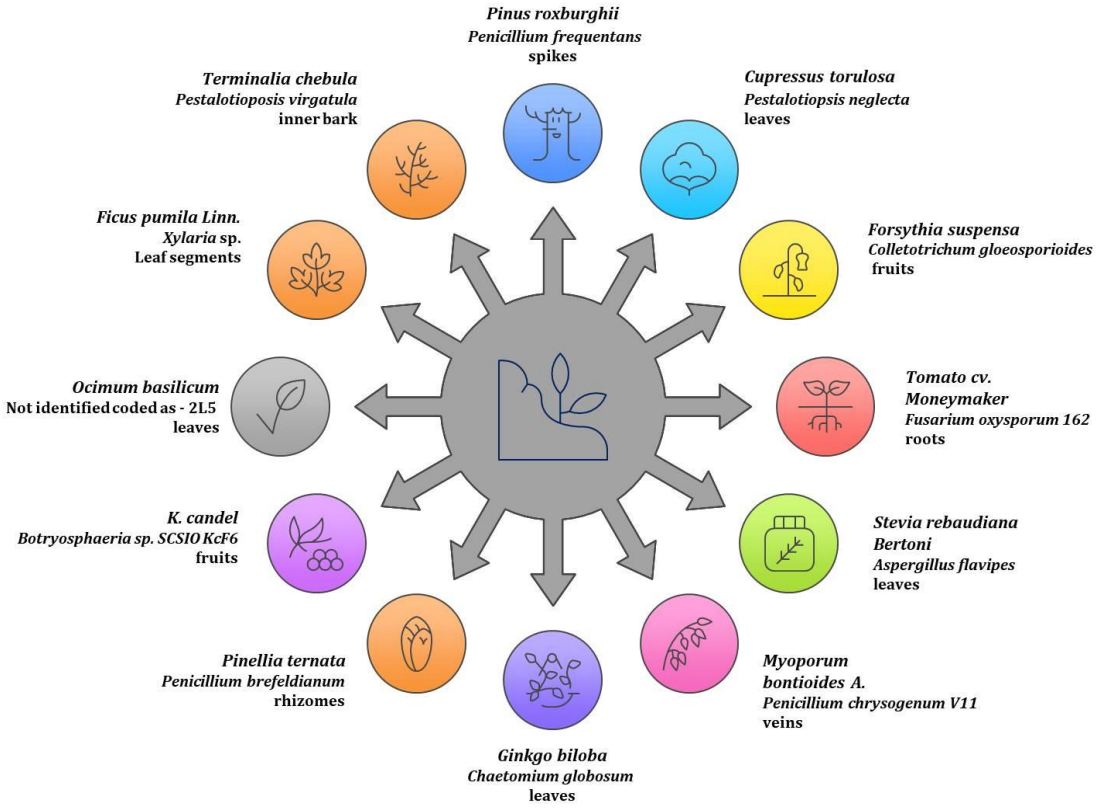

Table 1. Notable examples of extraction, isolation, purification and structural elucidation of antifungal agents from plants.

Sr. No	Name of plant	Plant part	Name of Endophyte	Compound isolated	Extraction, Isolation and/ purification	Structural elucidation	Reference
1	*Pinus roxburghii*	spikes	*Penicillium frequentans*	flavonoid and phenol	solvent partitioning with organic solvent like ethyl acetate, methanol, n-butanol, chloroform or hexane etc., ‘	GC-MS	Bhardwaj et al., 2015
2	*Cupressus torulosa*	Leaves	*Pestalotiopsis neglecta*	nonadecane, 1,2,3-propanetriol, 1-acetate, bis(2-ethylhexyl) phthalate and 4 Hpyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- and 5-hydroxymethylfurfural	By ethyl acetate and methanol for dissolution dimethyl sulphoxide (DMSO) or methanol used	GC-MS	Sharma et al., 2016
3	*Forsythia suspensa*	fruits	*Colletotrichum gloeosporioides*	phillyrin	ultrasound-assisted extraction with methanol and butanol	HPLC and HPLC-MS	Zhang et al., 2012
4	*tomato cv.*	roots	*Fusarium*	4-hydroxybenzoic acid,	methanol; acetone;		Bogner et al.,

	Moneymaker		*oxysporum 162*	indole-3-acetic acid (IAA) and gibepyrone D	petroleumether; petroleum ether-acetone gradient; petroleum ether and n-hexane-ethyl acetate gradient; ethyl acetate gradient; chloroform-methanol gradient; methanol acetone gradient and Semi-preparative RP-HPLC	HR-ESI-MS, 1 H-NMR, 13C-NMR, COSY-NMR and HMBC-NMR	2017
5	*Stevia rebaudiana Bertoni*	leaves	*Aspergillus flavipes*	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Acid precipitation method and TLC	GC-MS	Verma et al., 2014
6	*Myoporum bontioides A.*	vein	*Penicillium chrysogenum V11.*	penochalasin K, chaetoglobosin C, penochalasin I chaetoglobosin A	Size exclusion chromatography over Sephadex LH 20 and vacuum liquid chromatography on silica gel used for	1D, 2D NMR spectroscopic analysis and high resolution electrospray ionization mass	Zhu et al., 2017

					separation of compounds by using methanol water,petroleum ether-ethyl acetate	spectroscopy	
7	*Ginkgo biloba*	leaves	*Chaetomium globosum*	chaetomugilin D, , chaetomugilin A, chaetoglobosins A	bioassay-guided fractionation using ethyl acetate	COSY, NOESY, HMQC, and HMBC	Qin et al., 2009
8	*Pinellia ternata*	rhizome	*Penicillium brefeldianum*	indoloditerpene, 6,7-dehydropaxilline, indole-diketopiperazine, spirotryprostatin F, N-demethylmelearoride A, 8 alkaloids	Preparative reverse phase High Performance Liquid Chromatography (RP-HPLC) using a mobile phase methanol with 0.1% trifluoroacetatein water; methanol-water	positive HRESIMS, H NMR and 13C NMR, HMBCand ROESY, circular dichroism (CD) exciton chirality method	Gao et al., 2017
9	*K. candel,*	fruit	*Botryosphaeria sp. SCSIO KcF6*	botryosphaerin-A, botryosphaerin-B	silica gel column chromatography, semipreparative reversed-phase HPLC	1D and 2D-NMR and HRESIMS, circular dichroism	Ju et al., 2015

10	*0cimum basilicum*	leaves	*Not identified coded as - 2L5*	ergosterol and cerevisterol	silica gel column chromatography and recrystalisation	high-resolution 1- and 2-D NMR spectroscopy	Haque et al., 2005
11	*Ficus pumila Linn.*	leaf segments	*Xylaria sp.*	4- cyanomethoxy benzoic acid	silica gel column chromatography, HPLC	liquid chromatography–photodiode array detector–mass spectrometry [LC–PDA–MS])	Rakshith et al., 2013
12	*Terminalia chebula*	Inner bark	*Pestalotioposis virgatula*	9 Hydroxybenzo (C)oxepin-3[1H]-one	HPLC	HPLC-PDA-MS-SPE-NMR hyphenated system	Kesting et al., 2009

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