Revisión

Bol. Soc. Quím. Méx. **2007**, *1(1), 19-26*

ISSN 1870-1809

New hopes from endophytic fungal secondary metabolites

M. V. Tejesvi,[1] M. S. Nalini,[1] B. Mahesh,[1] H. S. Prakash,[1] K. R. Kini,[1] H. S. Shetty[1] and Ven Subbiah[2]*

[1] Department of Studies in Applied Botany and Biotechnology, University of Mysore, Manasagangotri, Mysore-570 006, Karnataka, India.

[2] PhytoMyco Research Private Limited, KIADB Industrial Park, Nanjangud-571 302, India and 1800 N. Greene Street, Greenville, NC 27834, U. S. A. E-mail: phytomyco@aol.com Tel: (252) 329-9200; Fax: (252) 329-9202

Abstract. The search for and exploitation of natural products and their properties has been the mainstay of biotechnology research. Natural product search and discovery from endophytes of medicinal plants represents a challenge to the biotechnologist. All the available evidence points to natural product discovery continuing strongly and accelerating as a consequence of new search strategies and innovative microbiology. In drug discovery, novel natural product chemotypes with interesting structures and biological activities continue to be reported. Without such discoveries, there would be a significant therapeutic deficit in several important clinical areas. The diverse range of biosynthetic pathways in plants, fungi and bacteria has provided an array of lead structures that have been used in drug development. This review highlights the importance of endophytes with desirable bioactivity, in a novel natural products screening programme.

Key words: Fungi, bacteria, yeasts, endophytes, natural products

Resumen. La búsqueda y la explotación de los productos naturales y de sus propiedades han sido de interés continuo en la investigación biotecnológica. El descubrimiento de nuevos productos naturales a partir de endófitos de plantas medicinales representan un reto para el biotecnólogo. Las evidencias disponibles indican que el descubrimiento de nuevos productos naturales se ha incrementado como consecuencia de la aplicación de nuevas estrategias de búsqueda y de procesos microbiológicos innovadores. Sin el descubrimiento de nuevos agentes terapéuticos, habría un severo déficit en numerosas áreas clinicas. La diversidad de rutas biosintéticas en plantas, hongos y bacterias ha proporcionado una amplia variedad de estructuras que se usan en el desarrollo de drogas. La presente revisión enfatiza la importancia de endófitos con bioactividad deseable, de utilidad práctica en el rastreo de nuevos productos naturales.

Palabras clave: Hongos, bacterias, levaduras, endófilos, productos naturales

Introduction

Drug resistance in bacteria, the appearance of life threatening viruses, breakdown of Severe Acute Respiratory Syndrome (SARS) and bird flu, recurring health problems in patients with organ transplants, and tremendous increase in the incidence of fungal infections in world's population only underscore our inadequacy to cope with these medical problems. Mankind is also facing difficulties in raising enough food on certain areas of the world to support local human populations. Environmental degradation, loss of biodiversity, and spoilage of land and water, also add to problems facing mankind. Endophytes, the microorganisms that reside in the tissues of living plants, are relatively unstudied and offer potential sources of novel natural products for exploitation in medicine, agriculture and the pharmaceutical industry [1]. Fungal endophytes have been found in healthy tissues of all the plants taxa studied to date [2-8] and it is their chemical diversity and not their biological diversity, that is largely responsible for the interest in these organisms. About 30% of the worldwide sales of drugs are based on natural products. In United States alone about 25% of prescribed drugs contain at least one active ingredient derived from plant material. Numerous examples from medicine demonstrate the innovative potential of natural compounds and their impact on progress in drug discovery and development.

Endophytes are microbes (fungi, bacteria and yeasts) that live within the plant tissue without causing any noticeable symptoms of disease. Endophytes have been found in all parts of plants including xylem and phloem [9]. The majority of the endophytes have been isolated from trees, but only a few herbaceous plants and shrubs have been examined for the presence of endophytes [1]. Endophytic fungi are being increasingly recognized as an ecological assemblage of microorganisms that may provide sources for new secondary metabolites with useful biological activities. Theoretically, the likelihood of discovery of new groups of secondary metabolites will be higher than with better-known groups of fungi, e.g. common genera of soil fungi [10].

Endophytes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural and industrial arenas. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about which higher plants to seek, study, and spend time isolating microfloral components. This may facilitate the product discovery processes. Although work on the utilization of this vast resource of poorly understood microorganisms has been initiated, it has already become obvious that an enormous potential for organism, product, and utilitarian discovery in this field holds exciting promise. This is witnessed by the discovery of a wide range of products, and microorganisms (Table 1). There are other characteristics of endophytic fungi that also render them desirable for manipulation in an industrial screening program. Most screening has focused on soil dwelling fungi, little attention has been directed toward endophytes. As a consequence, they have not been subjected to intensive screening programs, which suggest the vast majority largely remain undiscovered.

Table 1. List of natural products characterized from endophytes

SL No.	Plant host	Endophyte	Place of collection	Metabolite	Nature of metabolite	Bioactivity tested	Source
1.	*Artemisia annua* L. (Chinese herb)	*Colletotrichum* spp.	Nanjing, China	6-isoprenyl indole-3-carboxylic acid, 3β, 5α dihydroxy-6β-acetoxy-ergosta-7,22-diene and 3β, 5α dihydroxy-6β-phenyl acetyloxy-ergosta-7, 22-diene	UK*	Antimicrobial activity against human pathogenic fungi and bacteria, Fungistatic to plant pathogenic fungi	[25]
2.	*Artemisia mongolica* Fisch. ex Bess.	*Colletotrichum gloeosporioides*	Zijin mountain, Nanjing, China	Colletotric acid	Tridepside	Antibacterial and Antifungal (*Helminthosporium sativum*)	[26]
3.	*Bontia daphnoides* L.	*Nodulisporium* spp.	UK*	Nodulisporic acids	Indole diterpenes	Anti-insecticidal	[19]
4.	*Cinnamomum zeylanicum* Nees (Cinnamom tree)	*Muscodor albus*	Lancetilla botanical garden, La Ceibe, Honduras.	Volatile antimicrobials (1-butanol, 3-methyl acetate)	Ester	Antimicrobial *(Rhizoctonia solani, Ustilago hordei* and *F. solani* (basidiomycetes) *Cercospora beticola, Candida* spp. and *A. fumigatus* (human fungal pathogens) *Pythium ultimum* and *Phytophthora cinnamomi* (Oomycetes) Antibacterial (*E. coli, S. aureus, Micrococcus luteus* and *B. subtilis*)	[22]
5.	*Erythophelum chlorostachys* (Iron wood)	*Muscodor roseus*	UK*	Volatile antibiotics	UK*	Antibacterial and Antifungal	[23]
6.	*Grevillea pteridifolia* Knight (fern leaf tree)	*Muscodor roseus*	Northern Territory of Australia	Volatile antibiotics	UK*	Antibacterial and Antifungal	[24]
7.	*Maguireothamnus speciosus* (N. F. Brown) Steyern	*Seimatoantlerium tepuiense*	Tepuis of Venezuelan-Guyana border in south west Venezuela	Taxol®	Diterpenoid	UK*	[62]
8.	*Paullina paullinioides* (Liana)	*Muscodor vitigenus*	UK*	Naphthalene	UK*	Insect repellent against stem sawfly	[20,21]
9.	*Quercus suber* L. (cork oak)	*Diplodia mutila*	UK*	Diplopyrone	Tetrahydropyranpyran-2-one	Phytotoxic	[52]
10.	*Selaginella pallescens* (Pteridophyte)	*Fusarium* spp.	Guanacaste Conservation Area, Costa Rica.	CR377	Pentaketide	Antifungal, *C. albicans*	[27]
12.	*Taxus baccata* L.	*Acremonium* spp.	UK*	Leucinostatin A	UK*	Anti-oomycetes and anticancerous (melanoma G361, HT-144, Leukaemia cell lines HSB-2, K-562)	[15]
13.	*Taxus brevifolia* Nutt. (Pacific Yew)	*Pestalotiopsis microspora*	Bozenan, Montana, USA.	Pestalotiopsins A and B	Caryophyllene sesquiterpenes, 2 α –hydoxy-dimeninol and humulane	Sesquiterpenes,	[53,54,55]

Table 1. List of natural products characterized from endophytes (Continue).

SL No.	Plant host	Endophyte	Place of collection	Metabolite	Nature of metabolite	Bioactivity tested	Source
14.	*Taxus brevifolia* Nutt. (Pacific Yew)	*Taxomyces andreanae*	UK*	Taxol®	Diterpenoid	Anti-carcinogenic (P-388, P-1534, α-1210 murine leukaemia, Walker 256 carcinoma, sarcoma 180	[5]
15.	*Taxus mairie* (Chinese southern Yew)	*Tubercularia* spp.	Fujian province of South eastern main land, China	Taxol®	Diterpenoid	Anticancerous (P388 cells, KB cells)	[56]
16.	*Taxus wallachiana* (Nepalese Yew)	*Pestalotiopsis microspora*	Foothills of Himalyas.	Taxol®	Diterpenoid	Anti-carcinogenic	[51]
17.	*Taxus wallachiana* (Himalayan Yew)	*Phoma* spp.	Singhe-To, Khatmandu, Nepal.	Altersolanol A, 2- hydroxy- 6-methyl benzoic acid		Antibacterial (*Bacillus subtilis*)	[29]
18.	*Taxus wallachiana* (Himalayan Yew)	*Sporormia minima*, *Trichothecium* spp. and dimorphic fungus (unidentified)	Shivapuri, Khatmandu, Nepal	Paclitaxel	Diterpenoid	UK*	[57]
19.	*Terminalia morobensis* Coode	*Pestalotiopsis microspora*	Sepik river drainage system, Papua, New Guinea	Isopestacin	Isobenzofuranone	Antioxidant, antifungal (*Pythium ultimum*)	[16]
20.	*Terminalia morobensis* Coode	*Pestalotiopsis microspora*	Sepik river drainage system, Papua, New Guinea	Pestacin	1, 3, dihydro isobenzofuran	Antioxidant, antifungal (*Pythium ultimum*)	[17]
21.	*Torreya taxifolia* Arn	*Pestalotiopsis microspora*	UK*	Torreyanic acid	Quinone dimmer	Anticancerous and antibiotic	[58]
22.	*Tripterygium wilfordii* Hook.f.	*Cryptosporiopsis quercina*	UK*	Cryptocin	Tetramic acid	Antimycotic (*Pyricularia oryzae* and other plant pathogenic fungi)	[59]
23.	*Tripterygium wilfordii* Hook.f.	*Cryptosporiopsis quercina*	UK*	Cryptocandin	Lipopeptide	Antimycotic (*Sclerotinia sclerotiorum, Botrytis cineria*) Against human pathogens (*Candida albicans, Trichophyton* spp.)	[30]
24.	*Tripterygium wilfordii* Hook.f.	*Rhinocladiella* spp.	UK*	22-oxa-[12]-Cytochalasin	Alkoloid	Antitumor	[60]
25.	*Wollemia nobilis* (Wollemi pine)	*Pestalotiopsis guepinii*	Wollemi National Park near Sydney, Australia.	Taxol®	Diterpenoid	UK*	[15]
26.	*Fragraea bodenii* (oak tree)	*Pestalotiopsis jesteri*	Southern province, New Guinea	UK*		Antioomycetes	[31]
27.	*Torreya grandifolia*	*Periconia spp.*	Huangshan National Park, China	Taxol®	Diterpenoid	Anticancerous	[14]
28.	*Taxodium distichum* Rich	*Pestalotiopsis microspora*	Swamp forest, South California	Taxol®	Diterpenoid	Anticancerous	[13]
29.	*Tripterygium wilfordii* Hook.f.	*Fusarium Subglutinans*	UK*	Subglutinols A and B		Immunosuppressive	[18]

UK* – Unknown

Further, endophytic fungi colonize very specialized habitats, which means they have evolved special mechanisms for survival. This provides a likely explanation for the reason they represent a source of new secondary metabolites in biotechnology [10]. Random screening of chemically diverse molecules against a specific disease target discovers new pharmaceuticals. These molecules can either be extracted from living organisms (mainly plants and microbes) or produced synthetically. Nature provides an enormous variation of extremely complex molecules, infinitely more sophisticated than any molecule from any other source like synthetic and combinatorial chemistry [11]. It is very likely, given the extensive metabolic capabilities of microbes that fungal endophytes are the source of these drugs and that the plant is simply providing the proper environment for endophyte growth. This has been shown to be the case in the association between the Pacific yew and the endophytic fungus *Pestalotiopsis microspora* [12].

Endophytic microbes as a source of bioactive metabolites

An array of natural products has been characterized from endophytes, which includes anti-cancerous, anti-oxidants, anti-fungal, anti-bacterial, anti-viral, anti-insecticidal and immunosuppressants (Table I.). It is more common to find natural products with restricted taxonomic distributions in plants. For example, Taxol® appears to be restricted to yews (*viz.,* eleven species of the genus *Taxus*) but, interestingly, it has also been found in a number of different genera of fungal endophytes associated with yews and with endophytes from non-yew sources *viz*., *Taxodium distichum* [13], *Torreya grandifolia* [14], *Wollemia nobilis* [15]. The genetic origin of fungal Taxol® production has been speculated to have arisen by horizontal gene transfer from *Taxus* spp. to its endophytes [16]. If this is the case and the acquisition of the ability to produce certain metabolites by some microorganisms is by horizontal gene transfer from plant to microbial endophyte, the conservation of plant hosts and their indigenous microbial flora is of vital importance in the future search for new drugs. Anti-oxidant activity has been detected in Isopestacin and Pestacin isolated from *Pestalotiopsis microspora*, the endophytic fungus of *Terminalia morobensis* [16, 17]. The endophytic fungus, *Fusarium subglutinans* produces immunosuppressive compounds Subglutinols A & B. This was isolated from *Tripterygium willfordii* [18]. Anti-insecticidal compounds like Nodulisporic acids were isolated from *Nodulisporium* species, an endophyte of *Bontia daphnoides* [19]. Another insect repellent compound, Napthalene was isolated from *Muscodor vitigenus* colonizing a liana, *Paullina paullinioides* [11, 21]. A wide range of volatile antimicrobials produced by the endophytic fungi *Muscodor albus* and *Muscodor roseus* were isolated from *Cinnamomum zeylanicum* [22], *Erythophelum chlorostachys* [23] and *Grevillea pteridifolia* [24]. A number of antimicrobial compounds have been isolated to date from various endophytes, which include *Colletotrichum* species [25], *C. gloeosporioites* [26], *Fusarium* spp. [27], *Acremonium* spp. [28], *Phoma* spp. [29], *P. microspora* [16, 17], *Cryptosporiopsis quercina* [30], *P. jesteri* [31]. It can be concluded from Table 1, that a great deal of variation exists in the endophytic genera and the compounds isolated from indigenous plant species inhabiting the major rainforests of the world representing an undisturbed ecological niche.

Developing a productive microbial source for anti-cancerous, immunosupressants and anti-microbials not only would lower the cost of this effective anticancer agent but it also would help to make it more widely available. From 1983-1994, over 60% of all approved and pre-NDA stage cancer drugs were of natural origin as were 78% of all newly approved antibacterial agents [32]. The endophytic organism in culture can produce secondary metabolites in relatively high yield, particularly when subjected to strain improvement program [33]. It is feasible to isolate mutants that are more readily cultivated or generate additional product, or even a modified product with a higher therapeutic index [34]. Moreover, the metabolites they produce are largely generated by enzymatic pathways that have the potential to biosynthetically link existing structures to chemical adjuncts in a reproducible manner at yields that are acceptable for industrial use [35]. In this sense, natural products generated as microbial secondary metabolites exhibit a number of properties that make them excellent candidates for industrial processes.

Results and Discussion

Approaches for selection of endophytes

Herbal medicine is one of the oldest forms of health care known. Every plant on earth is known to harbor at least one endophytic microbe. When selecting medicinal plants for studying their unique mycoflora and the bioactives produced by them it is important to take into account the following:

1. Plant species already known to produce one or more notable drugs or that are of ethnobotanical or importance in traditional medicine.
2. Plants located in ecological settings that suggest microorganisms playing a role in protection against other microorganisms.
3. Plants growing in extreme conditions, e.g., high or low temperatures, aquatic environments, or high exposure to radiation or salt concentrations [36].

Once a biotechnological target has been identified, two questions follow: first, what might be the best organism or group of organisms to investigate? Second, what screening procedures should be used in order to detect the desired activity? The following approaches can be used for organism selection: (i) play the percentage game, e.g., endophytic fungi and actinomycetes for biopharmaceutins; (ii) make reference to taxa-chemistry and taxon-property databases of known

metabolites; (iii) focus on novel and neglected taxa; (iv) highlight isolates from unusual or little-explored ecosystems; (v) match the target with members of previously unscreened but known taxa.

Endophytes are known to reside in many plant species [37]. Very few medicinal plants have been studied for their endophytic fungi. Therefore, an attempt to isolate endophytic fungi of medicinal plants was undertaken with an ultimate objective of getting bioactive molecules of pharmaceutical and agricultural importance by our group. So far 310 endophytic fungal isolates were recorded from bark and twig segments of four medicinal plants namely viz., *Terminalia arjuna* W. & A., *Crataeva magna* (Lour.) DC., *Azadirachta indica* A. Juss., and *Holarrhena antidysenterica* (L.) Wall. ex. DC. These plants were selected based on their ethnopharmacological properties with special reference to target endothelin receptor antagonists. Among the endophytes, Mitosporic fungi represented a major group (82%) followed by Ascomycetes (15%) and Zygomycetes (3%). Some of the important endophytes isolated from the medicinal plants are shown in Fig.1.

The dominant fungi include *Pestalotiopsis*, *Myrothecium* and *Trichoderma* spp. Some of the endophytes isolated in our study are reported as potential sources of useful metabolites with immense value in agriculture, industry and therapeutics. These include *Fusarium subglutinans*, *Pestalotiopsis* spp., *Tubercularia* spp. and *Acremonium* spp. [18, 13, 50, 51].

Ethnopharmacological relevance and significance of endophytic fungi as new therapeutic agents

The number of plants with medicinal properties far exceeds the number of plants used as food source. For instance, Chinese herbalists have identified more than 5,000 medicinally important indigenous plants and the Amazon, the Golden triangle region of northern Thailand, the tropics of the Venezuela-Guyana border, and the teeming forests of central Africa, all have native human populations using indigenous plant resources for healing purposes. However, despite the huge biological potential of epiphytes and endophytes associated with these higher plants, these microorganisms have received little attention.

Taxol®, also known in the literature as Paclitaxel, is a novel diterpene (Fig. 2) that was isolated from the bark of the northwest Pacific Yew, *Taxus brevifolia* Nutt. in 1971 [38]. This product demonstrated moderate *in vivo* activity against P-388, P-1534 and l-1210 murine leukemia, the Walker 256 carcinosarcoma, Sarcoma 180, and Lewis lung tumor test system [39]. Despite its promise, there is a problem with Taxol®. This highly functionalized diterpene is isolated primarily from the inner bark of the relatively rare and slow growing pacific yew tree, *T. brevifolia*, and a few related species, in extremely small quantities (< 0.02% dry wt.). The emergence of Taxol® as an effective anticancer agent created a difficulty- there were simply not enough trees to supply the growing demands [38]. A mature pacific yew (100 years old) yields approximately 10 pounds of dry bark, so each collection required the sacrifice of 500 to 1500 trees [40]. Advanced preclinical and phase I clinical development of Taxol® required several collections ranging in size from 5,000 to 15,000 pounds of dry bark. Even with this estimate, however, it was clear that additional sources of Taxol® would be necessary. Several research groups have addressed the supply problem in a variety of ways. Although several strategies have been devised the most successful methods to date have been the use of plant tissue culture for reported yields with commercialization [41]. Stierle *et al*. [42] made an attempt at easing the supply dilemma focused on the discovery of a new biological source of the drug; an endophytic microbe colonizing the yew tree. They have isolated more than 300 fungi from the bark and needles of yews grown in Montana, USA. Out of the 300 fungi examined, *Taxomyces andreanae* was capable of producing 24 to 50 ng of Taxol® per litre.

From a practical view point, microbial fermentation as a means of producing bioactive substances has several advantages: (i) industrial production of a bioactive substances (like pharmaceuticals drugs) requires reproducible, dependable pro-

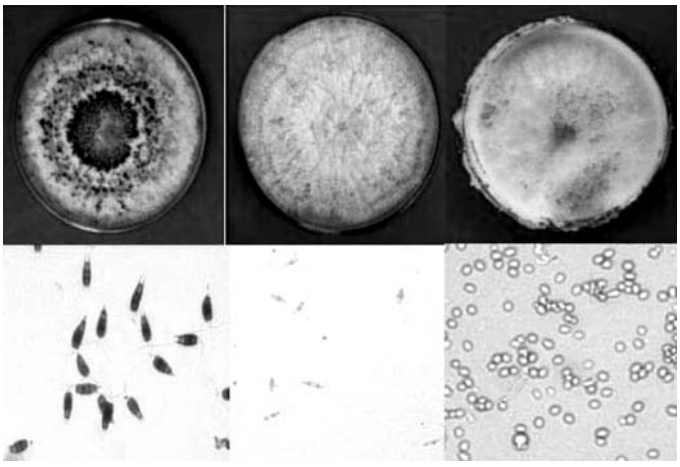

Fig. 1. Dominant endophytic fungal cultures isolated from the medicinal plants and their spores. a. *Pestalotiopsis* spp. b. *Myrothecium verrucaria*. c. *Trichoderma* spp.

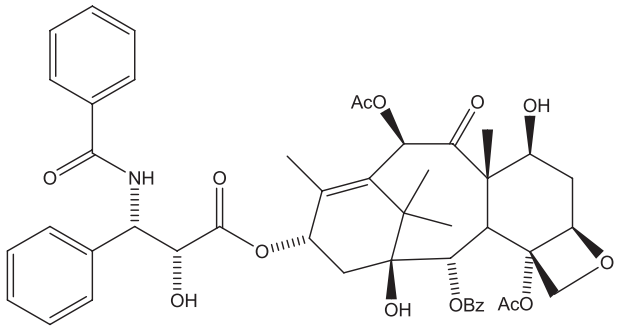

Fig. 2. Molecular Structure of Taxol.

ductivity. If the microbe is the source organism, it can be grown in tank fermentors as needed, producing a virtually inexhaustible supply; (ii) microorganisms typically respond favorably to routine culture techniques and tissue culture or growing plants requires either specialized techniques or months of growth before harvesting is feasible; (iii) product escalation is relatively easy in microorganisms. Directed changes in culture conditions can be explored indefinitely as a method of optimizing various biosynthetic pathways, which may lead to even more effective derivatives of lead compounds.

Microbes as sources of drug molecules

Considering the large and rapidly increasing awareness of economic potential of microbes, not only in the pharmaceutical and biotechnology industries, but also in relation to ecosystem function and maintenance, there is a pressing need to enhance our knowledge about them. The role of fungi was established early in history. Yeasts have been used in the making of bread and alcohol since the beginning of civilization and the discovery of Penicillin hit the press headlines in 1942 marking the beginning of a new approach to human health. Microbial metabolites have also contributed to fundamental biological science and fungi are involved in the industrial processing of more than 10 of the 20 most profitable products at the beginning of this century, e.g., three anti-cholesterol Statins, the antibiotic Penicillin and the immunosuppressant Cyclosporin A, have each a turn over in excess of $1 billion annually.

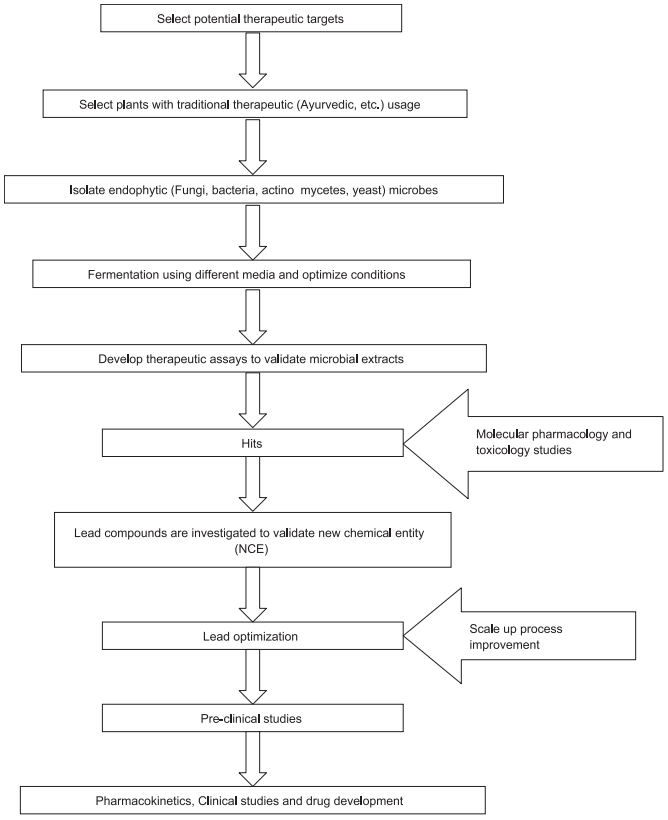

Fig. 3. The endophytes from ethnophamaceutically used plants as a source for new therapeutic leads.

Background and significance of endophytes

Medicinally important herbs and ethnopharmacologically used plants are wide spread in the Indian subcontinent and explain how people derive medicines from plants or other naturally occurring resources. The "discovery" that indigenous knowledge about medicinal plants may hold clues for curing diseases. Important monumental Ayurvedic works like Charakasamhita and Sushrutasamhita list nearly 700 plant drugs used in Ayurveda, Homeopathy, Siddha and Unani systems of medicine [43]. The list includes herbal remedies curing several diseases such as asthma, cardiovascular problems, diabetes, microbial infections and bronchitis. However, few plant species have been systematically studied for their endophytic microbes. All documented plant species should be evaluated from the point of their distribution and taxonomy and also for their chemical or microbial profile (Fig. 3).

Intelligent and efficient industrial screening of microorganism requires that a high diversity of organisms be maintained, while simultaneously minimizing redundancy among the taxa screened. This can only be achieved through an understanding of the floristic composition and pattern of colonization of the microorganisms within the particular ecological niche being sampled, i.e., What species are likely to inhabit the particular endophytic host and what is their relative abundance? How many species are likely to be found by sampling a single plant or individual trees? Does species richness and floristic composition vary among samples throughout a landscape? Does the isolation media or sampling of a particular plant organ influence the species recovered?

Endophytes are constantly exposed to intergeneric-genetic exchange with the host. This type of exchange would probably require an intimate association between the cells of the plant and its microbial associates. The recent example of the isolation of a potent anticancer agent, Taxol® producing fungus from its host plant, the yew tree [44,] and the classical example of the phytohormone producing fungus (*Gibberella fujikuroi*) from rice plant, suggests that a search for important chemical producing microorganisms should commence in the plant tissues. Most of the plants resist invading plant pathogens in part through the production of antimicrobial compounds; in some cases these compounds may be the product of the plant's associated microflora. Screening such plants for endophytic isolation may yield novel and interesting microbes. This provides a lead-directed approach in addition to random approach to screening.

Experimental

Isolation of endophytes from different parts of the plant

Most procedures for isolating endophytes are relatively simple and routine for anyone skilled in basic microbiological techniques. One of the critical needs for isolating endophytic fungi is obtaining fresh plant material. The need for preventing desiccation must be balanced against the need for adequate aeration; the former slows tissue death, whereas the later minimizes the growth of secondary contaminating fungi and bacteria. Prolonged transport in sealed plastic bags should be avoided if possible. Sturdy paper bags, wax paper bags, or ziplocked, perforated bags designed for vegetable storage work well for transport and temporary storage of most types of plant tissues. If plants are to be stored for long periods of time, especially in frost-free refrigerators, tissue desiccation will occur. However, it is possible to isolate a surprising number of fungal species even from desiccated woody tissues after freezer storage for more than a year [45].

A standard method utilizes dipping the plant material in both EtOH and NaOCl (bleach) for few minutes. The EtOH acts as a surfactant and the NaOCl is the actual sterilizing agent. The dilutions and immersion times in NaOCl vary with the type of tissue and host [46, 47, 48]. In general, woody tissues and leaves with thick cuticles are subjected to more stringent sterilization than more fragile ones. Tests have demonstrated that the series of EtOH-NaOCl-EtOH effectively kill thick-walled spores occurring in many common contaminant fungi. After the plant materials are surface sterilized, they are dissected to obtain epidermis, cambium, xylem, and phloem and plated on enriched and nutrition-depleted media to isolate different microbes; the plates are then incubated in a light chamber for three to four weeks [10, 49]. Chloramphenicol and Gentamycin are employed to inhibit bacterial contamination and the plates are monitored for the growth of the hyphal tips that can be isolated in pure form by growing them in mycological media. Several enriched media such as cornmeal or Brainheart infusion ensure the growth of thermally dimorphic fungi, while moderately enriched media such as Potato dextrose or Sabouraud dextrose, and nutritionally depleted media such as dextrin and other complex carbohydrate based media can be used to isolate interesting endophytes [10]. Cycloheximide or rose bengal are incorporated into the media to inhibit the rapidly growing saprophytic fungi, which can overgrow slow-growing fungi.

Conclusion

The microbial advantage in drug discovery is profound, offering several distinct advantages. The first is the negligible impact to the environment incurred in the collection process. Each microbe can produce metabolites with desirable bioactivity. If a microbial metabolite is considered as a drug candidate, the necessary additional material can be obtained by larger scale fermentation and by media manipulation to improve yields. In recent times, focus on plant research has increased all over the world and a large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. The secondary metabolites produced by endophytes associated with medicinal plants can be exploited for curing diseases. The development of drugs from endophytes with high potency and reasonable duration of action will offer much needed new remedies for acute and chronic human diseases. The naturally derived product will be nontoxic and inexpensive in the prevention of diseases. Microbes associated with plants, and other substrats merit a higher profile in future research programs related to the understanding, management, and sustainable use of biodiversity at a level appropriate both to their numbers and to their economic and environmental importance. The search for novel habitats from which isolates for screening may be derived is becoming a significant concern for the pharmaceutical and agricultural industries.

Acknowledgements

The authors wish to thank the Department of Biotechnology (DBT), Government of India, for the financial support.

References

1. Strobel, G.; Daisy, B. *Microbiol. Mol. Biol. Rev*. **2003,** *67*, 491.
2. Petrini, O.; Stone, J.; Carroll, F. E. *Can. J. Bot*. **1982,** *60*, 789.
3. Legault, D.; Dessureault, M.; Laflamme, G. *Can. J. Bot*. **1989,** *67*, 2052.
4. Schulz, B. U.; Wanke U.; Drager, S.; Aust, H. J. *Mycol. Res*. **1993,** *97*, 1447.
5. Rodrigues, K.F., in: Endophytic fungi in Grasses and Woody Plants, Redlin. S. C. and Carris, L.M., Eds., American Phytopathological Society Press, San Diego, California, **1996,** 121.
6. Fisher, P.J. *New Phytol*. **1996,** *132*, 119.
7. Lodge, D. J.; Fisher, P. J.; Sutton, B.C. *Mycologia* **1996**, *88*, 733.
8. Frohlich, J.; Hyde, K.D. *Biodiversity Conservation* **1999**, *8*, 977.
9. Petrini, O., In: Microbiology of the Phyllosphere, Fokkema, N. J., van den Heuvel, J. Eds., Cambridge University Press, Cambridge, **1986,** 175.
10. Bills, G. F.; Polishook, J.D. *Sydowia* **1992**, *44*, 1.
11. Petsko, G.A. *Nature* **1996,** *7*, 384 (suppl. 6604), 7.
12. Strobel, G. A.; Torczynski, R.; Bollon, A. *Plant Sci*. **1997,** *128*, 97.
13. Li, J.Y.; Strobel, G.; Sidhu, R.; Hess, W.M.; Ford, E.J. *Microbiol.* **1996,** *142*, 2223.
14. Li, J. Y.; Sidhu, R. S.; Ford, E.; Hess, W. M.; Strobel, G. A. *J. Ind. Microbiol*. **1998,** *20*, 259.
15. Strobel, G. A., Hess, W. M.; Li, J. Y.; Ford, E.; Sears, J.; Sidhu, R. S.; Summerell, B. *Aus. J. Bot*. **1998,** *45*, 1073.
16. Strobel, G.; Ford, E.; Worapong, J.; Harper, J.K., Arif, A.M.; Grant, D.M.; Fung, P.C.W.; Chau, R.M.W. *Phytochemistry* **2002,** *60*, 179.
17. Harper, J. K.; Barich, D. H.; Hu, J. Z.; Strobel, G. A.; Grant, D. M. *J. Org. Chem*. **2003,** *68*, 4609.

18. Lee, J.; Lobkovsky, E.; Pliam, N. B.; Strobel, G. A.; Clardy, J. *J. Org. Chem*. **1995,** *60*, 7076.
19. Bills, G.; Dombrowski, A.; Pelaez, F.; Polishook, J.; An, Z., 2002. In: Watling, R., Frankland, J. C., Ainsworth, A. M., Issac, S., Robinson, C. H. Eds., Tropical Mycology: Micromycetes, Vol. 2, CABI Publishing, New York, **2002,** 165.
20. Daisy, B. H.; Strobel, G.; Ezra, D.; Castillo, U.; Baird, G.; Hess, W. M. *Mycotaxon* **2002**, *84*, 39.
21. Daisy, B. H.; Strobel, G. A.; Castillo, U.; Ezra, D.; Sears, J.; Weaver, D.; Runyon, J. B. *Microbiol*. **2002,** *148*, 3737.
22. Strobel, G. A.; Dirksie, E.; Sears, J.; Markworth, C. *Microbiol*. **2001,** *147*, 2943.
23. Strobel, G. A. *Crit. Rev. Biotechnol*. **2002,** *22*, 315.
24. Worapong, J.; Strobel, G.; Daisy, B.; Castillo, U.F.; Gaird, G.; Hess, W. M. *Mycotaxon* **2002,** *81*, 463.
25. Lu, H.; Zou, W. X.; Meng, J.C.; Hu, J.; Tan, R. X. *Plant Sci*. **2000,** *151*, 67.
26. Zou, W. X.; Meng, J. C.; Lu, H.; Chen, G. X.; Shi, G. X.; Zhang, T. Y.; Tan, R. X. *J. Nat. Prod.* **2000,** *63*, 1529.
27. Brady, S. F.; Clardy, J. *J. Nat. Prod*. **2000**, *63*, 1447.
28. Nisbet, L .J.; Moore, M. *Curr. Opin. Biotechnol*. **1997,** *8*, 708.
29. Yang, X.; Strobel, G. A.; Stierle, A.; Hess, W. M.; Lee, J.; Clardy, J. *Plant Sci*. **1994,** *102*, 1.
30. Strobel, G. A.; Miller, R. V.; Miller, C.; Condron, M.; Teplow, D. B.; Hess, W. M. *Microbiology* **1999**, *145*, 1919.
31. Strobel, G.A.; Li, J.Y.; Ford, E.; Worapong, J.; Baird, G.I.; Hess, W. M. *Mycotaxon* **2000,** *76*, 257.
32. Concepcion, G.P; Lazaro, J.E.; Hyde, K.D. In: Ponting, S. B.; Hyde, K. D. Eds., Bio-exploitation of Filamentous Fungi, Fungal Diversity Press, Hong Kong, **2001,** 93.
33. Penalva, M.A.; Rowlands, R.T.; Turner, G. *Trends in Biotechnol.* **1998,** *16*, 483.
34. Piepersberg, W. *Crit. Rev. Biotechnol*. **1994,** *14*, 251.
35. Verdine, G. L. *Nature* **1996,** *384* (Suppl. 6604), 11.
36. Strobel, G.A.; Long, D.M. *Amer. Soc. Mycol. News* **1998,** *64*, 263.
37. Stone, J.K; Bacon, C.W.; White, J.F. Jr. in: Microbial Endophytes, Bacon C.W. and White J.F. Jr. Eds., Marcel Dekker, Inc, New York, **2000,** 3.
38. Wani, M. C.; Taylor, H.C.; Wall, M.E.; Coggon, P.; McPhail, A.T. *J. Amer. Chem. Soc.* **1971,** *93*, 2325.
39. Rowinsky, E. K.; Cazenave, L.A.; Donehower, R.C.; *J. Natl. Cancer Inst*. **1990,** *82*, 1247.
40. Cragg, G. M.; Snader, K. M. *Cancer Cells* **1991**, *3*, 233.
41. Fett-Nero, A.G.; DiCosmo, F.; Reynolds, W.F.; Sakata, K. *Bio/Technol*. **1992,** *10*, 1572.
42. Stierle, A.; Strobel, G.A.; Stierle D.; Grothaus, P.; Bignami, G. *J. Nat. Prod*. **1995,** *58*, 1315.
43. Yoganarasimhan, S. N. Medicinal plants of India. Interline Publishing Private Limited, Bangalore, **1996,** 644.
44. Stierle, A.; Strobel, G.; Stierle, D. *Science* **1993,** *260*, 214.
45. Bills, G. F., in: Systematics, Ecology and Evolution of Endophytic Fungi in Grasses and Woody Plants, Redlin, S. and Carris, L. M. Eds., APS Press, St. Paul, MN, **1996,** 31.
46. Carroll, G. C. and F. E. Carroll. *Can. J. Bot*. **1978**, *56*, 3034.
47. Dreyfuss, M; Petrini, O. *Botanica Helvetica* **1984,** *94*, 33.
48. Petrini, O., In: Microbiology of the Phyllosphere, Fokkema, N. J., van den Heuvel, J. Eds., Cambridge University Press, Cambridge, **1986,** 175.
49. Suryanarayanan, T.S. *The Mycol*. **1992,** *6*, 144.
50. Strobel, G. A.; Torczynski, R.; Bollon, A. *Plant Sci*. **1997,** *128*, 97.
51. Strobel, G.; Yang, X.; Sears, J.; Kramer, R.; Sidhu, R. S.; Hess, W. M. *Microbiology* **1996,** *142*, 435.
52. Evidente A.; Maddau L.; Spanu E.; Franceschini A.; Lazzaroni S.; Motta A. *J. Nat. Prod*. **2003,** *66*, 313.
53. Pulici, M.; Sugawara, F.; Koshino, H.; Uzawa, J.; Yoshida, S.; Lobkovsky, E.; Clardy, J. *J. Org. Chem*. **1996,** *61*, 2122.
54. Pulici, M.; Sugawara, F.; Koshino, H.; Uzawa, J.; Yoshida, S.; Lobkovsky, E.; Clardy, J. *J. Nat. Prod*. **1996,** *59*, 47.
55. Pulici, M.; Sugawara, F.; Koshino, H.; Uzawa, J.; Yoshida, S.; Lobkovsky, E.; Clardy, J. *J. Chem. Res*. **1996,** *N8*, 378.
56. Wang, J.; Li, G.; Lu, H.; Zheng, Z.; Huang, Y.; Su, W. *FEMS Microbiol. Lett.* **2000,** *193*, 249.
57. Shrestha, K.; Strobel, G. A.; Prakash, S.; Gewali, M. *Planta Med*. **2001,** *67*, 374.
58. Lee, J.C.; Strobel, G.A.; Labkovsky, E.; Clardy, J.C. *J. Org. Chem*. **1996,** *61*, 3232.
59. Li, J. Y.; Strobel, G. A.; Harper, J. K.; Lobkovsky, E.; Clardy, J. *Org. Lett*. **2000,** *2*, 767.
60. Wagenaar, M.; Corwin, J.; Strobel, G. A.; Clardy, J. *J. Nat. Prod*. **2000,** *63*, 1692.
61. Strobel, G.A.; Ford, E.; Li, J.Y.; Sears, J.; Sidhu, R. S.; Hess, W. M. *Syst. Appl. Microbiol*. **1999,** *22*, 426.