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MINI-REVIEW

Endophytic fungi: novel sources of anticancer lead molecules

Sheela Chandra

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Abstract Cancer is a major killer disease all over the world and more than six million new cases are reported every year. Nature is an attractive source of new therapeutic compounds, as a tremendous chemical diversity is found in millions of species of plants, animals, and microorganisms. Plant-derived compounds have played an important role in the development of several clinically useful anti-cancer agents. These include vinblastine, vincristine, camptothecin, podophyllotoxin, and taxol. Production of a plant-based natural drug is always not up to the desired level. It is produced at a specific developmental stage or under specific environmental condition, stress, or nutrient availability; the plants may be very slow growing taking several years to attain a suitable growth phase for product accumulation and extraction. Considering the limitations associated with the productivity and vulnerability of plant species as sources of novel metabolites, microorganisms serve as the ultimate, readily renewable, and inexhaustible source of novel structures bearing pharmaceutical potential. 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. They develop special mechanisms to penetrate inside the host tissue, residing in mutualistic association and their biotransformation abilities opens a new platform for synthesis of novel secondary metabolites. They produce metabolites to compete with the epiphytes and also with the plant pathogens to maintain a critical balance between fungal virulence and plant defense. It is therefore necessary that the relationship between the plants and endophytes during the accumulation of these secondary metabolites is studied. Insights from such research would provide alternative methods of natural product drug discovery which could be reliable, economical, and environmentally safe.

Keywords Endophytes · Podophyllotoxin · Taxol · Vinca alkaloids · Camptothecin

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Introduction

The search for natural products as potential anticancer agents dates back to 1550 BC, but the scientific period of this search is much more recent, beginning in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins (Cragg and Newman 2004; Srivastava et al. 2005). Plant-derived compounds have played an important role in the development of several clinically useful anticancer drugs. Vinblastine, vincristine, the camptothecin (CPT) derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and taxol are some of the clinically useful anticancer drugs (Fig. 1). Several promising new agents are in clinical developmental stage based on selective activity against cancer-related molecular targets, including flavopiridol and combretastin A4 phosphate (Cragg and Newman 2004). Production of a plant-based natural drug is produced at a specific developmental stage or under specific environmental condition, stress or nutrient availability. It is estimated that harvesting of 38,000 yew trees is required to generate 25 kg of taxol to treat 12,000 patients. One-kilogram paclitaxel is produced after extraction from 10,000-kg bark (Sohn and Okos 1998). Indiscriminate collection and cutting down of medicinal plants from the wild for extraction of products of interest has led to the extinction of certain number of species making them either vulnerable or critically endangered. The biotechnological approaches

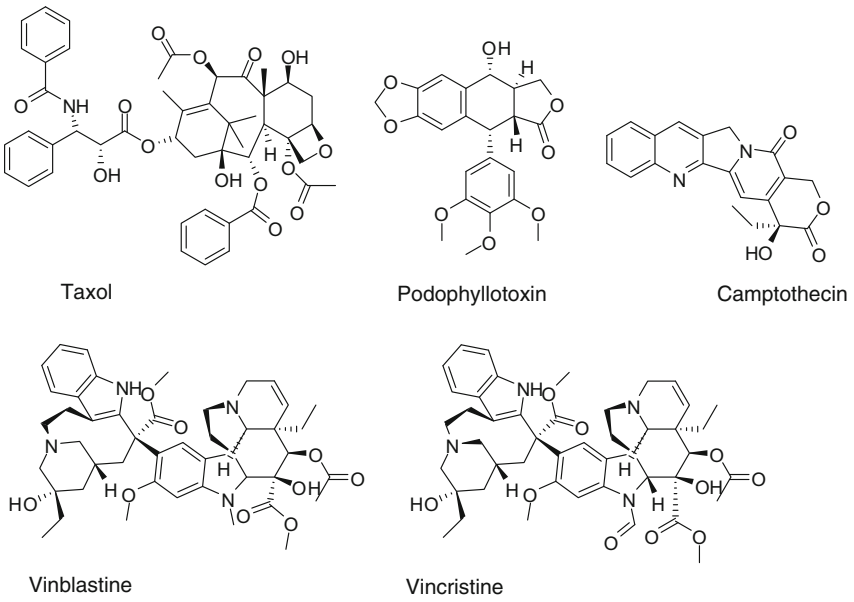

Fig. 1 Structures of some industrially relevant secondary metabolites used as anticancer compounds

involving plant cell and organ cultures and hairy root cultures appeared to fulfill the ever increasing demand up to a certain level. Different strategies have been used to increase the production of bioactive secondary metabolites in plant cell cultures. Strategies include screening and selection of high-producing cell lines, optimization of nutrient media for growth and production, organ culture, culture of immobilized cells, the use of biotic and abiotic elicitors, feeding of biosynthetic precursors, and scale up in bioreactors. Considering the limitations associated with the productivity and vulnerability of plant species as sources of novel metabolites, microorganisms serve as the ultimate, readily renewable, reproducible, and inexhaustible source of novel structures bearing pharmaceutical potential. Microorganisms, especially fungi, have long been regarded as an important source of active metabolites with promising anti-bacterial, anti-mycotic, and anti-viral activity.

Endophytes: alternative sources of secondary metabolites production

Endophyte refers to the fungi, yeast and bacteria which invade or live inside the tissues of plants without causing any disease or injury to them. They also promote growth of the host plant and the formation of secondary metabolites related to plant defense (Chandra et al. 2010). Endophytes have been found in all parts of plants including xylem and phloem (Petrini 1986). Endophytic fungi that grow within their plant hosts without causing apparent disease symptoms (Petrini 1991; Wilson 1995) are relatively unexplored and unattended as compared with soil isolates and plant pathogens. Endophyte residing in the plant host involves continual metabolic interaction between fungus and host. In comparison to fungal plant pathogens and fungal soil isolates, relatively few secondary metabolites have been isolated from endophytic fungi (Tan and Zou 2001). Tan and Zou (2001), Schulz et al. (2002), and Tejesvi et al. (2007) reviewed the diversity of metabolites isolated from endophytic fungi emphasizing their potential ecological role. These secondary metabolites of endophytic origin are synthesized via various metabolic pathways (Tan and Zou 2001), e.g., polyketide, isoprenoid, and amino acid derivation. Those isolated compounds belong to diverse structural groups, i.e., xanthones, steroids, isocumarines, phenols, quinones, furandiones, terpenoids, depsipeptides, and cytochalasines. In some cases, plant-associated fungi are able to make the same bioactive metabolites as the host plant itself. One of the best examples of this is the discovery of phytohormones "gibberellins" in *Fusarium fujikuroi* in the early 1930s (Kharwar et al. 2008). Almost all vascular plants including mosses, algae, and ferns are reported to harbor endophytic bacteria or fungi. Endophytic profile is more diversified in tropical areas. Arnold et al. (2000) isolated 418 endophyte morphospecies (estimated 347 genetically distinct taxa) from 83 healthy leaves of *Heisteria concinna* and *Ouratea lucens* in a lowland tropical forest of central Panama. The relationship between the plants and endophytic fungi during the accumulation of these secondary metabolites needs extensive research.

Fungal endophytes play important roles in the biosynthesis of secondary metabolites. Combination of inducing factors from both plants and endophytic fungi increased the accumulation of secondary metabolites in plants and fungi, respectively (Zhang et al. 2009; Li et al. 2009). Biosynthetic pathway studies reveal that plants and endophytic fungi have similar but distinct metabolic pathways for production of secondary metabolites (Jennewein et al. 2001). Independent production of taxol by endophytic fungi has been shown by the isolation of the gene 10-deacetylbaccatin-III-10-*O*-acetyl transferase from the endophytic fungus *Clasdosporium*

cladosporiodes MD2 isolated from *Taxus media* (yew species). This gene is involved in the biosynthetic pathway of taxol and shares 99 % identity with *T. media* (plant) and 97 % identity with *Taxus wallichiana* var. *marirei* (plant). Investigations revealed that the weight of roots, seedlings, and terpenoid production of *Euphorbia pekinensis* increased after they were inoculated with an endophytic *Phomopsis* species. Cytochemical analysis showed that the enzymatic activities of phenylalanine ammonia-lyase and 1-deoxy-D-xylulose 5-phosphate reductoisomerase in plant tissues were promoted upon the endophytic fungus colonization (Zhin-Lin et al. 2007). Artemisinin (antimalarial compound) content in hairy roots of *Artemisia annua* was increased from 0.8 to 1 mg g^{-1} dry weight by using elicitor treatment of mycelial extracts from the endophytic fungus *Colletotrichum* sp. (Wang et al. 2001a, 2002). A few studies showed that endophytes associated with non taxol producing plants have also been found to produce taxol. A novel endophytic taxol-producing fungus *Colletotrichum gloeosporioides* was isolated from the leaves of a medicinal plant, *Justicia gendarussa*, and it produced 163.4 μg/l of taxol (Gangadevi and Muthumary 2008). Studies of Wang et al. (2008) revealed the endophytic association of *Colletotrichum* species as endophytes most frequently isolated from *T. mairei*, and these have not yet been reported as endophytes of *Taxus* though they have been reported as common endophytes from other plants (Fröhlich et al. 2000; Larran et al. 2001; Photita et al. 2001; Cannon and Simmons 2002; Arnold et al. 2003). The production of deoxypodophyllotoxin (found in the host) by the cultured endophyte is an interesting observation. It demonstrates the transfer of gene(s) for accumulation of such products by horizontal means from the host plant to its endophytic counterpart. Further study will be interesting to explore the deoxypodophyllotoxin production and regulation by the cultured endophyte in *Juniperus communis* and in pure cultures. Cytotoxic active secondary metabolites cochliodinol have been produced from endophytic fungus *Chaetomium* species isolated from stem of *Salvia officinalis* (Debbab et al. 2009). Hence, symbiotic association and effects of plants and endophytes on each other during the production of other important pharmacological bioactive natural products such as CPT derivatives, vinblastine, and podophyllotoxin need to be explored. This could provide the framework for future natural product production through genetic and metabolic engineering (Karuppusamy 2009). Failure of exploiting the endophytic fungi rests on our current poor understanding of the evolutionary significance of these organisms and their dynamic interaction with their respective hosts. Research should focus on elucidating the molecular mechanisms during the establishment of plant-endophyte association for secondary metabolites production. The present review summarizes few potent anticancerous drugs, their mode of action, biotechnological approaches for their procurement, and endophytic species as novel sources of lead molecules.

Host–endophyte interaction

Endophytes develop special mechanisms to penetrate and reside in the host tissues in close association. They possess the exoenzymes necessary to colonize their hosts and they grow well in the apoplastic washing fluid of the host. When the roots are colonized, the association with the host may be mutualistic. These allow growth of the host and supply the endophyte with enough nourishment to extensively colonize the host's roots. It has been found that the concentrations of some plant defense metabolites are lower than in the control when the host is infected with a pathogen than with an endophyte (Schulz et al. 2002). There exists equilibrium between fungal virulence and plant defense (Fig. 2). If this balance is disturbed by either a decrease in plant defense or an increase in fungal virulence, disease develops. Endophyte synthesizes metabolites to compete with epiphytes, with pathogens to colonize the host, and also to regulate host metabolism in balanced association. Selection of host plant, screening, and utilization of potential endophytes involves studies on plant diversity, ethnobotany, and fungal taxonomy. The metabolic interactions of the endophyte with its host may favor the synthesis of some similar secondary metabolites. Plants and endophytic fungi through mutualism produce some similar secondary metabolites (Preeti et al. 2009). Endophytes experience long-term symbiotic relationships with their host plants, and many of them may produce bioactive substances as part of these relationships. They live in the same habitat, through long-term coexistence and direct contact, they have exchanged genetic material (Wang and Dai 2011; Nadeem et al. 2012).

In order to adapt to the ecological environment, plants have developed several mechanisms to overcome microbial diseases, including the production of several toxic substances. Some are present in healthy plants and some are synthesized during pathogenesis. Endophytes have a strong tolerance toward host's unique metabolites. The detoxification of these highly bioactive defense compounds is an important transformation ability of many endophytes which to a certain extent decides the colonization range of their hosts (Wang and Dai 2011). Biotransformation abilities (Zikmundova et al. 2002; Saunders and Kohn 2009) of

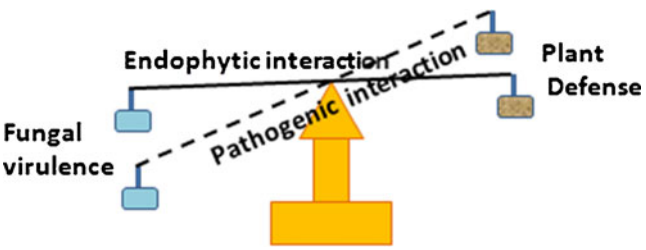

Fig. 2 Balanced antagonism between fungal virulence and plant defense

endophytes help in detoxification effects towards toxic metabolites produced by host plant and production of some novel bioactive secondary metabolites. Only with excellent biotransformation abilities, they can face the various external environments directly. It is believed that the structure types of active compounds produced by endophytes have been far beyond those produced by their host plants (Wang and Dai 2011). The former have become an important source of novel biologically active secondary metabolites. Improvement of existing drugs by modifying them with endophytes is another way of exploiting novel metabolites. For example, CPT which is a potent antineoplastic agent, is compromised in therapeutic applications due to its very low solubility in aqueous media and high toxicity. An endophytic fungus from the plant *Camptotheca acuminata* produces CPT (1), 9-methoxycamptothecin (2), and 10-hydroxycamptothecin (3) (Kusari et al. 2009b). Compounds (2) and (3) are two important analogues of compound (1) with lower toxicity and potential anticancer efficacy. Because of their effective biotransformation enzymes, endophytic fungi have been employed to change the three-dimensional conformation of compounds. Some researchers have tried to use endophytes to obtain more active substances. Studies of Borges et al. (2008), Agusta et al. (2005), and Verza et al. (2009) showed that different metabolites could be obtained by using different types of fungi, and those metabolite productions were stereoselective. Utilization of endophytes for region- and stereoselective production of novel products may allow us to obtain novel compounds that cannot be synthesized by chemical methods. In this sense, natural product drugs generated as microbial secondary metabolites exhibit a number of properties that make them excellent candidates for industrial processes (Tejesvi et al. 2007). The endophytes in culture can produce secondary metabolites in relatively high yield, when subjected to strain improvement program (Penalva et al. 1998). Endophytes are less studied than plant pathogenic fungi. Many groups of fungi in different biotopes are waiting to be explored and studied. Documented plant species should also be evaluated from the point of their distribution and taxonomy and also for their chemical or microbial profile.

Natural anticancer lead molecules and their production

Taxol

Taxol is a novel diterpenoid originally isolated from the stem bark of Pacific yew tree (*Taxus brevifolia* Nutt.) (Taxaceae) (Wani et al. 1971). The supply of taxol from the bark is limited (0.01–0.05 %) (Wheeler et al. 1992) because the plant is not abundantly found in nature (Cragg et al. 1993), and it also grows slowly taking several decades to increase a few inches in diameter (Flores and Sgrignoli 1991) and contains trace amounts of paclitaxel (0.01 % of dry weight of the bark) (Banerjee et al. 1996). The removal of the bark results in the death of the tree (Kwak et al. 1995). Several other species of *Taxus* like *Taxus baccata, Taxus cuspidata, Taxus canadensis, Taxus chinensis, Taxus x media, Taxus floridana, Taxus yunannensis, Taxus mairei, Taxus sumatrana*, and *Taxus wallichiana* (Majumder and Jha 2009) have been reported to produce taxol. Significant variation exists in taxane content among and within population and species. It is the world's first billion dollar anticancer drug. It has become a widely used anticancer drug in clinical treatments of advanced, progressive and drug refractory ovarian cancer (McGuire et al. 1989; Einzig et al. 1991; Markman 1991), and breast cancer (Holmes et al. 1991). Nowadays, it is also used for the treatment of lung (Ettinger 1992), head and neck (Forastiere et al. 1993), renal, prostrate, colon, cervix, gastric, and pancreatic cancers (Einzig et al. 1991; Arbuck et al. 1993; Roth et al. 1993; Brown et al. 1993). Besides this, it is also effective against noncancerous conditions like polycystic kidney diseases (Woo et al. 1994). Paclitaxel represents a new class of antineoplastic agents as it has a unique mode of action. Unlike other antimicrotubule agents like podophyllotoxin, colchicine, vinca alkaloids, and combretastatin which inhibit microtubule assembly, paclitaxel stabilizes microtubules against depolymerization; it promotes the polymerization of microtubules but inhibits depolymerization (Schiff et al. 1978; Horowitz et al. 1986). This unusual stability blocks the cells ability to disassemble the mitotic spindle during cell division; cells are blocked in the G2/M phase of the cell cycle (Schiff et al. 1978) and this finally leads to cell death. Due to the continuous growing market, current industrial production of taxol by semi-synthesis that consumes large amount of *Taxus* trees cannot meet the requirement of the market (Ji et al. 2006). Scientists all over the world have studied taxol production on various platforms including chemical synthesis, plant cell and tissue culture (Table 1) (Fett-Neto et al. 1992; Wang and Zhong 2002; Wang et al. 2007a, b, c; Khosroushahi et al. 2006; Croteau et al. 2006), endophytic fungi (reviewed by Visalakshi and Muthumary 2010), microbial fermentation, and so on (Jennewein and Croteau 2001; Frense 2007). Zhou et al. (2010) reviewed that microbial fermentation of endophytic fungi is a new and feasible approach to the production of taxol (Stierle et al. 1993; Lee et al. 1995; Li et al. 1996; Huang et al. 2001; Wang et al. 2000; Sun et al. 2008).

The first taxol-producing fungus *Taxomyces andreanae* was isolated in 1993 (Stierle et al. 1993). Table 2 shows varying yields of taxol production; to solve such a problem, current studies needs isolation and identification of high-taxol-producing cell lines, stable yield of taxol, as well as optimization of fermenting conditions. Strain improvement and optimization of the media of taxol-producing fungus *Fusarium maire* has been discussed by Xu et al. (2006). After the strain improvement andoptimization of the media, the yield of taxol increased from 20 to 225.2 μg/l. The

Table 1 Biotechnological approaches to produce taxol

Plant species/family	Type of culture	Yield	References
Taxus brevifolia Nutt. (stem and bark) Taxaceae	In vivo	0.01 % of dry weight of the bark	Banerjee et al. (1996)
Taxus cuspidata	Callus	0.020 % DW	Fett-Neto et al. (1992)
Taxus media	Cell suspension	115.2 mg/l	Yukimune et al. (1996)
Taxus chinensis	Elicitors *Aspergillus niger*	2-fold increase to control	Wang et al. (2001b)
T. x media var. Hicksii	Hairy root culture	Twice the amount of taxol than that in the bark of *T. brevifolia*	Furmanowa and Syklowska-Baranek (2000)
T. chinensis	Cell cultures in bioreactors	612 mg/l	Wang and Zhong (2002)

establishment of efficient transformation system of taxol-producing endophytic fungus EFY-21 (*Ozonium* sp.) from *T. chinensis* var. *mairei* led to improved taxol production. Table 2 summarizes a list of taxol-producing endophytic fungi.

Camptothecin

CPT, a pentacyclic quinoline alkaloid, a potent antineoplastic agent, was first isolated by Wall et al. (1966) from the wood of *C. acuminata* Decaisne (Nyssaceae), a plant native to mainland China. This alkaloid has been reported from several plant species (*Ophiorrhiza* species, *Ervatamia heyneana,* and *Merrilliodendron megacarpum*), with the highest yield found in *Nothapodytes nimmoniana* (Govindachari and Viswanathan 1972). It has a unique mechanism of action involving interference with eukaryotic DNA. It primarily targets the intranuclear enzyme DNA topoisomerase I (Topo I), which is required for the swiveling and relaxation of DNA during DNA replication and transcription. Numerous analogs have been synthesized as potential therapeutic

Table 2 A list of taxol-producing endophytic fungi (from 1993 to 2001 and 2010 to 2011); Zhou et al. (2010) lists taxol-producing endophytic fungi from 2001 to 2009

Host	Endophytic fungus	Yield(μg/l)	References
Taxus brevifolia	*Taxomyces andreanae*	0.024–0.05	Stierle et al. (1993)
Taxus yunnanensis	Unidentified	–	Qiu et al. (1994)
Taxus wallichiana	*Pestalotiopsis microspora*	0.06–0.07	Strobel et al. (1996)
Taxodium distichum	*P. microspora*	0.05–1.49	Li et al. (1996)
Wollemia nobilis	*Pestalotiopsis guepini*	0.17	Strobel et al. (1997)
Torreya grandifolia	*Periconia* sp.	0.03–0.83	Li et al. (1998)
Ginkgo biloba	*Alternaria* sp.	0.12–0.26	Kim et al. (1999)
Taxus chinensis var. *mairei*	*Tubercularia* sp.	185.4	Wang et al. (1999)
T. chinensis var. *mairei*	*Ozonium* sp.	4–18	Guo et al. (2006)
T. chinensis	*Fusarium solani*, Tax-3	163.35	Deng et al. (2009)
Pestalotiopsis versicolor	*Taxus cuspidata*	478	Kumaran et al. (2010)
T. chinensis var. *mairei*.	EFY-21 (*Ozonium* sp.)	–	Wei et al. (2010)
Taxus globosa	*Nigrospora* sp.	0.142–0.221	Ruiz-Sanchez et al. (2010)
Morinda citrifolia Linn.	*Botryodiplodia theobromae* Pat.	–	Pandi et al. (2010)
M. citrifolia	*Lasiodiplodia theobromae*	245	Pandi et al. (2011)
Aloe vera	*Phoma* species	73.66	Immaculate Nancy Rebecca et al. (2011)
Capsicum annuum	*Colletotrichum capsici*	687	Kumaran et al. (2011)
Taxus x media	*Cladosporium cladosporioides* MD2	–	Zhang et al. (2011)
T. chinensis var. *mairei*	*Didymostilbe* sp.	8–15	Wang and Tang (2011)
Plant debris	*Pestalotiopsis malicola*	186	Bi et al. (2011)
Taxus baccata	*Gliocladium* sp.	1,670 ng/200 ml	Sreekanth et al. (2011)

Table 3 Biotechnological approaches to produce camptothecin

Plant species (family)	Type of culture	Yield	References
Nothapodytes nimmoniana (root) (Icacinaceae)	In vivo	0.33 %/g DW	Padmanabha et al. (2006)
Ophiorrhiza rugosa var. *decumbens* (Rubiaceae)	Normal microshoots	0.311 mg/g DW	Vineesh et al. (2007)
	Albino microshoots	1.04 mg/g DW	Vineesh et al. (2007)
Ophiorrhiza kuroiwai	Aseptic microshoots	290.0 μg/g DW	Asano et al. (2004)
Ophiorrhiza liukiuensis	Aseptic microshoots	30.0 μg/g DW	Asano et al. (2004)
O. liukiuensis	Hairy root culture/crown gall formation	83.0±27.4 μg/g DW±SD	Asano et al. (2004)
O. kuroiwai	Hairy root culture/crown gall formation	219.3±31.44 μg/g DW±SD	Asano et al. (2004)
O. liukiuensis	Elicitors		Asano et al. (2004)
	MJ	Increased 1.3-fold	
	SA	Decreases	
	YE	Decreases	
O. kuroiwai	SA	Decreases	Asano et al. (2004)
	YE	Decreases	
	MJ	No effect	

agents. 10-hydroxycamptothecin as well as their synthetic derivatives 9-aminocamptothecin topotecan and irinotecan are potent antitumor and DNA Topo I inhibitory agents (Patel et al. 2010). CPT inhibits the replication of human immunodeficiency virus in vitro and is also shown to be effective in the complete remission of lung, breast, uterine, and cervical cancer (Kusari et al. 2009b). Identification of alternate species of plants like *Ophiorrhiza* species (Table 3) and development of tissue culture methods may be a suitable alternative for microshoots development and production of CPT. Studies of Roja (2008) revealed that micropropagated plantlets showed a higher alkaloid content compared with the normal plant. Chemical analysis of the different organs of the tissue cultured regenerated plant of *Ophiorrhiza rugosa* established in soil indicated 0.002 % dry weight of CPT in the roots, 0.011 % dry weight in the stems, 0.090 % dry weight in the leaves, and 0.015 % in the floral parts. Puri et al. (2005) first reported an endophytic fungus *Entrophospora infrequens* (Table 4) obtained from *Nothapodytes foetida* that had the ability to produce CPT. Amna et al. (2006) performed the kinetic studies of the growth and CPT accumulation of the endophyte *E. infrequens* in suspension culture and demonstrated that this endophyte would be a potential alternate microorganism source to produce CPT.

Vinca alkaloids

Vinblastine and vincristine are two natural alkaloids from *Catharanthus roseus* or *Vinca rosea* used as major drugs in the treatment of lymphoma and leukemia, respectively (Barnett et al. 1978). *C. roseus* L. (Apocynaceae) Madagascar Periwinkle is found to contain a very large number of alkaloids, about 100 of which have been isolated so far (Verpoorte et al. 1997; Hughes and Shanks 2002; Samuelsson 1999). The importance of this plant is due to the presence of two bisindole antitumor alkaloids, vinblastine and vincristine. The vinblastine and vincristine can lower the number of white blood cells. The antitumor alkaloids are produced in trace amounts (0.0003 % dry weight) in the roots. Studies of Balandrin and Klocke (1988) mentioned that about 500 kg of leaves are needed to produce just 1 g of purified vincristine. This means that 12–15 tons are required to produce 1 oz of drug (Taha et al. 2009). The high prices of these anticancer products, ranging from $1 million to $3.5 million/kg, have led to a widespread research interest in the scientists, over the past 25 years in the development of alternative sources for the production of these compounds

Table 4 Camptothecin-producing endophytic fungi and their host plant

Host	Endophytes	Yield	References
Nothapodytes foetida	*Entrophospora infrequens*	–	Puri et al. (2005)
N. foetida	*E. infrequens*	49.6 μg/g	Amna et al. (2006)
N. foetida	*Nodulisporium* sp.	5.5 μg/g	Rehman et al. (2009)
Camptotheca acuminata	Unidentified	–	Min and Wang (2009)
Camptotheca acuminate	*Fusarium solani*	–	Kusari et al. (2009b)
N. foetida	*Neurospora* sp.	–	Rehman et al. (2008)
Apodytes dimidiata	*F. solani*		Shweta et al. (2010)
Nothapodytes nimmoniana	*Botryosphaeria parva*	–	Gurudatt et al. (2010)

Table 5 Biotechnological approaches to produce vinca alkaloids

Plant species/family	Type of culture	Yield	References
Catharanthus roseus (Apocyanaceae)	In vivo	0.0003 % DW	Kalidass et al. (2010)
	Callus	Vincristine (20.38 mg/g)	Kalidass et al. (2010)
	Organogenesis (root from petiole)	20-fold vinblastine and 6-fold vincristine compared with natural petiole	Ataei-Azimi et al. (2008)
	Cell suspension cultures+elicitors (amino acids)	Vinblastine (0.0583 %) Vincristine (0.0425 %)	Taha et al. (2009)
	Hairy root culture (indole alkaloids)	2- to 3-fold higher than untransformed culture	Cau-uitz et al. (1994)

(Verpoorte et al. 1991). The vinblastine and vincristine (anticancerous drugs) prevent mitosis in metaphase and they bind to tubulin, thus prevents the cell from making the spindles it needs to divide. The cellular pharmacology (vincristine, vinblastine, and vindesine) used in cancer chemotherapy have not been clearly established. Their intracellular binding to tubulin with subsequent dissolution of microtubules and arrest of cells in mitosis are considered necessary to mediate their cytotoxic action (Creasey 1979). However, although these alkaloids have only minor structural differences and behave in the same way at the level of drug tubulin interaction (Himes et al. 1976; Owellen et al. 1977), their toxicity and spectrum of clinical activity differ considerably.

Plant cell and tissue cultures represent a promising source for valuable phytochemicals such as flavors, fragrances, and pharmaceuticals (Jacqueline et al. 1999). Cell suspension cultures could be used for the large-scale culturing of plant cells from which active agents can be extracted and prepared. However, the amount produced as well as the rate of production of useful metabolites in plant cell cultures is still very low. *C. roseus* cell cultures have been studied for producing these medicines or precursors catharanthine and vindoline for almost four decades but so far not commercially successful due to biological and technological limitations. The biosynthesis of vinblastine and vincristine in tissue culture systems has been elusive, due to the inability to synthesize vindoline, one of its precursors (O'Keefe et al. 1997). However, vindoline has been reported in transformed cell cultures of *C. roseus*, at low levels (O'Keefe et al. 1997). Factors such as tissue differentiation (Constabel et al. 1982; Hirata et al. 1987), light-activated regulation and/or development (De Carolis et al. 1990; De Luca et al. 1988), or both (Loyola-Vargas et al. 1992; Tyler et al. 1986; Vasquez-Flota et al. 1997), are considered important for the activity of the biosynthetic pathway to vindoline (Ataei-Azimi et al. 2008). However, Kalidass et al. (2010) (Table 5) reported in their studies that HPLC analysis of methanol extracts from callus cultures of *C. roseus* revealed that the cultures produced vincristine. The concentrations of the phytohormones alpha-naphthalene acetic acid and kinetin played a critical role in the production of vincristine.

Study of endophytes of *C. roseus*

Kharwar et al. (2008) isolated a total of 183 endophytic fungi representing 13 fungal taxa from leaf, stem, and root tissues of *C. roseus* from two different ecosystems in North India. Most of the isolates were hyphomycetes except one coelomycete and one ascomycete. It was found that root tissues were heavily colonized by genera such as *Alternaria*, *Cladosporium*, and *Aspergillus*. However, *Drechslera*, *Curvularia*, *Bipolaris*, *Alternaria*, and *Aspergillus* spp. were the dominant fungi isolated from leaf tissues (Kharwar et al. 2008). Studies on *C. roseus* endophytes (Table 6) revealed that *Alternaria* sp. and *Fusarium oxysporum* were isolated from phloem of the plant material and were responsible for production of vinca alkaloids.

Podophyllotoxin

Podophyllotoxin is a pharmaceutically active natural drug belonging to the chemical group of lignans. It is used as a precursor for the synthesis of important antitumour drugs like etoposide (VP-16-213) and teniposide (VM-26) which are used in the treatment of lung cancer, testicular cancer, a variety of leukemias and other solid tumors (Majumder and Jha 2009) Podophyllotoxin has been reported to occur both in gymnosperms (Cupressaceae) and angiosperms (Berberidaceae, Polygalaceae, Lamiaceae, and Linaceae). Commercially, podophyllotoxin is extracted from roots and rhizomes of two species of *Podophyllum—Podophyllum hexandrum*

Table 6 Fungal endophytes of *Catharanthus roseus* producing vincristine/vinblastine

Host	Endophyte	Compound/yield	Reference
C. roseus (Phloem)	*Alternaria* sp.	Vinblastine	Guo et al. (1998)
C. roseus (Phloem)	*Fusarium oxysporum*	Vincristine	Zhang et al. (2000)
C. roseus (leaves)	Unidentified	Vincristine 0.205 μg/l	Yang et al. (2004)

Table 7 Biotechnological approaches to produce podophyllotoxin

Plant species/family	Type of culture	Yield	References
Podophyllum peltatum Berberidaceae	In vivo	0.25 % in dry roots	Chattopadhyay et al. (2002)
Podophyllum hexandrum (roots and rhizomes)	In vivo	4 % in dry roots	Chattopadhyay et al. (2002)
P. peltatum	Callus	0.65 %	Kadkade (1982)
P. hexandrum	Callus	0.3 % (DW basis)	Van Uden et al. (1989)
P. hexandrum	Cell suspension (on addition of polyvinylpyrrolidone)	4.9 mg/l	Chattopadhyay et al. (2001)
P. hexandrum	Suspension culture	48.8 mg/l	Chattopadhyay et al. (2003a, b, c)
P. peltatum	Cell suspension	27 mg/l	Kutney et al. (1991)
P. hexandrum	Precursors (coniferin)	12.8-fold increase in content	Van Uden et al. (1990)
P. hexandrum	Bioreactor	0.19 mg l^{-1} day^{-1}	Chattopadhyay et al. (2002)
P. hexandrum	Hairy root culture	3-fold more than control	Giri et al. (2001)

Royle or the Indian *Podophyllum* and *Podophyllum peltatum* L. or the American *Podophyllum* of family Berberidaceae. *P. peltatum* is commercially inferior to *P. hexandrum* (Jackson and Dewick 1984) as the levels of podophyllotoxin in *P. peltatum* are lower than *P. hexandrum*. Podophyllotoxin content of rhizomes ranges between 0.36 and 1.08 % (on dry weight basis) (Nadeem et al. 2007). It functions as a mitotic inhibitor by binding reversibly to tubulin and inhibiting microtubule assembly (Cragg and Suffness 1988). Etoposide and its thiophene analog teniposide are structurally related to podophyllotoxin (Patel et al. 2010).

Due to ever increasing demand for podophyllotoxin, long juvenile phase and poor fruit setting ability of *P. hexandrum*, overexploitation, and lack of organized cultivations have made the plant "critically endangered"(Majumder and Jha 2009). However, new routes for total synthesis of podophyllotoxin have been discovered (Bush and Jones 1995; Berkowitz et al. 2000). But these are not economically feasible due to low yield. A lot of effort has been put in the past several years to improve its production from different podophyllotoxin producing plant species.

Agricultural production of *Podophyllum* has been unsuccessful since the plant requires proper climatic conditions (Moraes et al. 2001; Lee and Xiao 2003). Entire biochemical pathway, including key enzyme(s) and the genetic blueprint involved in podophyllotoxin biosynthesis, is not known yet. Other biotechnological ways for example, cell/tissue cultures and hairy root cultures have also not yielded desirable results (Table 7). Total chemical synthesis is also not feasible commercially (Damayanti and Lown 1998; Berkowitz et al. 2000). Hence, alternative approaches for production of podophyllotoxin through endophytic fungi are being vigourously explored. Several reports are available showing production of podophyllotoxin from endophytes of *P. hexandrm*, *P. peltatum*, *Juniperus recurva*, and *J. communis* L. Horstmann (Table 8). Current studies of Nadeem et al. (2012) reported maximum production of podophyllotoxin observed on day 8 (29 μg/g dry weight of mycelia). The discovery of fungal endophytes that produce active secondary metabolites has significant biological and commercial implications. For commercial production, the fungal culture can be scaled up to provide adequate production for new drug development. This

Table 8 Lists fungal endophytes producing podophyllotoxin

Host	Endophytes	Yield	References
Sinopodophyllum hexandrum (=*Podophyllum hexandrum*)	*Alternaria* sp.	–	Yang et al. (2003)
Juniperus vulgaris (=*Sabina vulgaris*)	*Alternaria* sp.	–	Lu et al. (2006)
P. hexandrum	*Trametes hirsuta*	30 μg/g	Puri Nazir and Chawla (2006)
Podophyllum peltatum	*Phialocephala fortinii*	0.5–189 μg/l	Eyberger et al. (2006)
S. hexandrum	*Alternaria neesex*	2.4 μg/l	Cao et al. (2007)
Juniperus recurva	*Fusarium oxysporum*	28 μg/g	Kour et al. (2008)
Juniperus communis L. Horstmann	*Aspergillus fumigatus* Fresenius	DPDT 0.04 μg/g dry mycelia and 3.0 μg/l broth	Kusari et al. (2009a)
P. hexandrum	*Fusarium solani*, P1	29.0 μg/g	Nadeem et al. (2012)

would reduce the load of harvesting wild populations of the source plant from natural habitat. Role of the fungus in the production of podophyllotoxin in *P. hexandrum* and regulation of its production needs further investigations (Eyberger et al. 2006; Nadeem et al. 2012).

Conclusion and future prospects

Endophytes live in the inner tissues of healthy plants, exhibit complex interactions with their hosts. During long coexistence process with their hosts, endophytes develop many significant and novel characteristics. In order to maintain stable symbiosis, endophytes secrete varieties of enzymes that contribute to colonization and growth. The unique habitats of endophytes make them more useful and selective in biological conversion, hence they have great potential for the synthesis of biologically active novel metabolites. The production of some similar bioactive natural secondary metabolites by the endophytes supports the theory that during the co-evolution of endophytes and their host plants, endophytes adapted themselves to their special microenvironments by genetic variation, including uptake of some host DNA into their own genomes (Germaine et al. 2004). This gene transfer might have led to the ability of certain endophytes to biosynthesize some phytochemicals originally produced by the host plant (Stierle et al. 1993). Tan and Zou (2001) further added that it is possible to isolate hundreds of endophytic species from a single plant, and among them, at least one generally shows host specificity. Research community should focus their effort on molecular studies of endophytes and optimization of fermentation conditions to scale up the production. Endophytes prove to be interesting and promising niches for production of bioactive secondary metabolites, needs to be exploited more.

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