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Taxol from fungal endophytes and the issue of biodiversity

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Fungi represent one of the most understudied and diverse group of organisms. Commonly, these organisms make associations with higher life forms and may proceed to biochemically mimic the host organism. An excellent example of this is the anticancer drug, taxol, which had been previously supposed to occur only in the plant genus *Taxus* (yew). However, taxol has been reported in a novel endophytic fungus—*Taxomyces andreanae*, but also has been demonstrated to occur in a number of unrelated fungal endophytes including *Pestalotia, Pestalotiopsis, Fusarium, Alternaria, Pithomyces, Monochaetia* and others. Thus, this report presents information on the presence of taxol among disparate fungal genera, and uses these observations as an additional argument to support efforts to study fungal endophytes and preserve their associated host plants.

Keywords: fungi; mycology; taxol; *Taxus*

Introduction

Certainly, one of the most significant groups of organisms on earth remaining to be studied are the fungi [9]. Many fungi occupy thousands of unsuspected niches in nature including cohabitation with other larger and more imposing life forms including higher plants [2]. The mechanisms of association with these higher plant forms can vary from mutualistic to pathogenic or to some form of commensalism [2,3]. Given the large numbers and varieties of higher plants, the numbers and kinds of their associated microfungi must be enormous. In his presidential address to the British Mycological Society in 1990, Hawksworth estimated that only 5% of the world's fungi are currently known [10]. Certainly, some of the major niches of the remaining unknown fungi to be found, named and examined are those associated with higher plants. This work should be done, not only for the sake of taxonomy, but also for potentially useful and important products made by the organisms. The rationale for this approach are examples known where certain representative plant-associated microbes make each of the known classes of phytohormones [6]. It is also well established that certain compounds from higher plants are useful to man's industry and well-being. Thus, if plant microbes have the potential, via co-evolution or genetic transfer from their hosts to make phytohormones, they may also have the ability to make other critically important compounds such as antifungal agents, and antibiotics.

Our experiences, as presented in this report, primarily with one plant genus—*Taxus* (yew) illustrate these points. There are possibly 11 species of yew trees [9]. Yews grow primarily in wet, shaded mountainous regions of the Northern hemisphere [9].

Taxol, an important anticancer drug, has been found in each of these species [20]. In spite of semisynthetic production methods, taxol remains an expensive drug. It is especially targeted for use in breast and ovarian cancers. A microbial source for taxol that is as productive as the other fungal-derived antibiotics would be extremely helpful, more cost effective, and more widely available.

Because few fungi associated with yew have been deposited in culture collections, we sampled small yew twigs from each of the world's yew species in order to enlarge our collections [4]. Invariably, *Taxus* is a rich source of endophytic fungi. *Taxus brevifolia* from Glacier National Park, Montana, yielded the fungus *Taxomyces andreanae*, the first established case of a non-yew source of taxol [17]. Soon, hundreds of different fungi were obtained from *T. brevifolia* and at least ten different fungi (unidentified) were shown to produce taxol via an immunological method [16]. Also, hundreds of fungal endophyte species have been obtained recently from European, Asiatic, and other North American yews. As a whole, yew is a microbiological treasure trove of novel and interesting microbes interacting with each other and the plant. Of peculiar interest is the fact that *Taxomyces andreanae* has not been isolated from any yew other than the original tree from Glacier National Park, Montana. Also, some of the more common tree endophytes such as *Trichoderma* spp are common to yew, while *Penicillium* spp (common to the pines) as endophytes are uncommon in yew. Further, the yews growing in more subtropical locations such as the lower slopes of the Himalayas, Philippines, Florida and South China harbor a complex of *Pestalotia* spp/*Pestalotiopsis* spp [18]. Endophytes of yew roots, mature stems, berries and leaves remain to be characterized and studied.

Of immediate concern, however, is to find one or more fungi that produce more taxol than the *ca* 50 ng L^{-1} demonstrated in liquid cultures of *T. andreanae* [17]. Obviously, a major task has been to define growing conditions for the yew endophytes, obtain pure cultures, and then screen each culture for traces of taxol. Tens of fungus cultures have

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been subjected to taxol-screening methods and several fungi have been found that make taxol. Thus, the subject of this report is to review the current status of fungal-produced taxol and to report fungal species inhabiting European and Asiatic yews, and also those recently discovered from a non-yew source (*Taxodium distichum*) which make it.

Endophytic fungi from yew

Endophytic fungi are those microbes that seem to have established a relationship with a higher plant and are found within the living tissues of the plant. The relationship between the fungus and the plant is usually asymptomatic [2,3]. Microbe–plant relationships that occur either on the plant or in dead or dying tissues are usually considered as epiphytic or saprobic. In a search for taxol-producing fungi we initially examined living yew tissues (cambium, sapwood, and phloem) for the presence of endophytes. The rationale is that living tissues would perhaps be the best source of potential taxol producers since some plant–microbe relationship would have necessarily been established [17].

In order to eliminate epiphytic microbes, we routinely surface treat with 70% ethanol for several minutes until the solution evaporates from the plant [18]. Then with a sharp blade, the other tissues (dead cells, bark) are removed which exposes the living bark (phloem, cambium, xylem) tissues which lie beneath. Using sterilized forceps, particles of these living tissues are placed on Petri plates containing water agar. Within a few days fungi and bacteria may grow from the tissue pieces. For our studies, only filamentous fungi are of interest. These organisms are subsequently purified by a hyphal tip technique and stored in tubes containing distilled H_2O at 4° C. Those stored for longer periods are placed in 15% glycerol in H_2O and frozen at −70° C [18]. Over the past several years hundreds of individual endophyte species have been isolated from local populations of *T. brevifolia* and placed on deposit at the MSU Mycological Collection, the CBS in Baarn, Holland, and the NRRL in Peoria, IL [4]. On the other hand, we have isolated at least 20–50 endophytic fungal species from each of the other *Taxus* spp.

Taxol from endophytes

Typically, we grow endophytes on semi-synthetic media; high in sucrose, low in phosphate, with a soytone nitrogen source along with minor elements and vitamins from a small amount of yeast extract [18]. To be certain that taxol from the host plant is not being carried through the fungal mycelium via the initial transfer from the source tree, we do at least 4–5 serial hyphal tip transfers prior to fermentation on any fungus suspected of making taxol. We also process uninoculated media (minus the test fungus) for an ultimate check for the presence of taxol. These are important considerations (controls) in assuring confidence in any final outcome suggesting that taxol is being produced by a given fungus [17].

Production of fungal taxol ranges from 50 ng to 10–50 μg. Taxol is positively identified via its co-chromatographic mobilities with authentic taxol in a multitude of thin layer chromatographic systems [17,18], its reactivity with the vanillin/sulfuric acid spray reagent (blue-gray) [1], its characteristic UV absorption spectrum (Figure 1), and its characteristic NMR and mass spectra. In working with μg quantities of taxol, we have observed a relative ease in confirming the identity of taxol via electrospray mass spectroscopy (Figure 2). Typically, taxol accepts either a proton or a sodium ion to produce peaks at $[M + H]^+ = 854$ and $[M + Na]^+ = 876$ [17,18]. Spectra can be obtained from less than μg quantities of taxol (Figure 2).

Finding a taxol-producing fungus from a myriad of yew endophytes was originally a laborious task since each fungus had to be grown in 1-L batches, then extracted with an organic solvent, the residue subjected to flash chromatography, and the column effluent co-chromatographed on a thin layer plate along with authentic taxol as a reference compound. Then several successive preparative thin layer chromatographic systems were used to concentrate the putative taxol [16]. Now, with the availability of taxol-specific monoclonal antibodies, the taxol screening can easily be done on the fungal medium residue after the first organic solvent extraction step, with an error of ± 1 ng [7,12].

The presence of fungal taxol has been shown for the

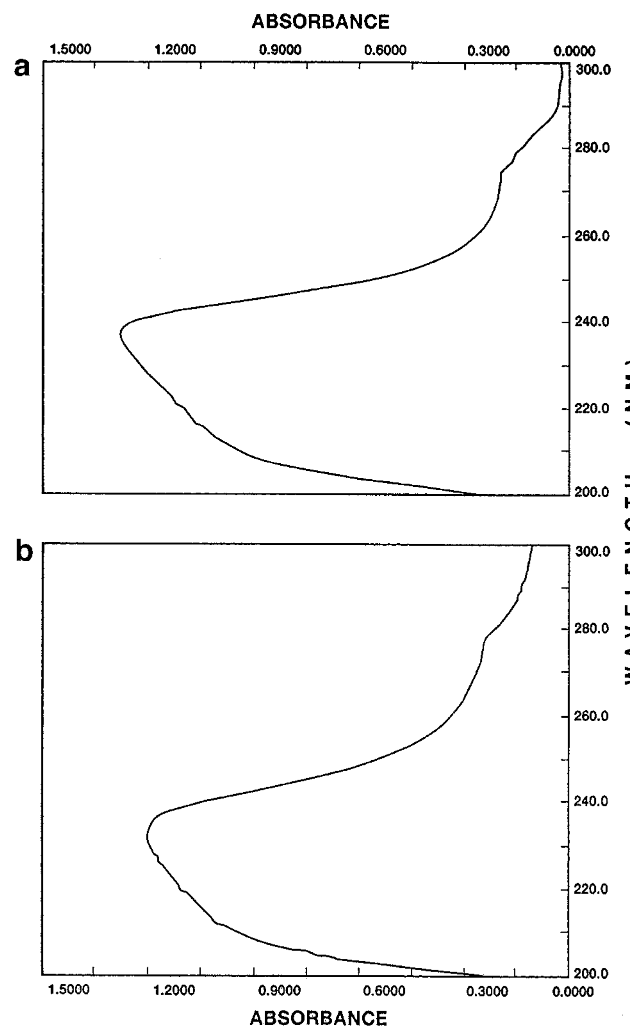

Figure 1 UV spectrum of authentic taxol (a) and fungal taxol (*P. microspora*) (b).

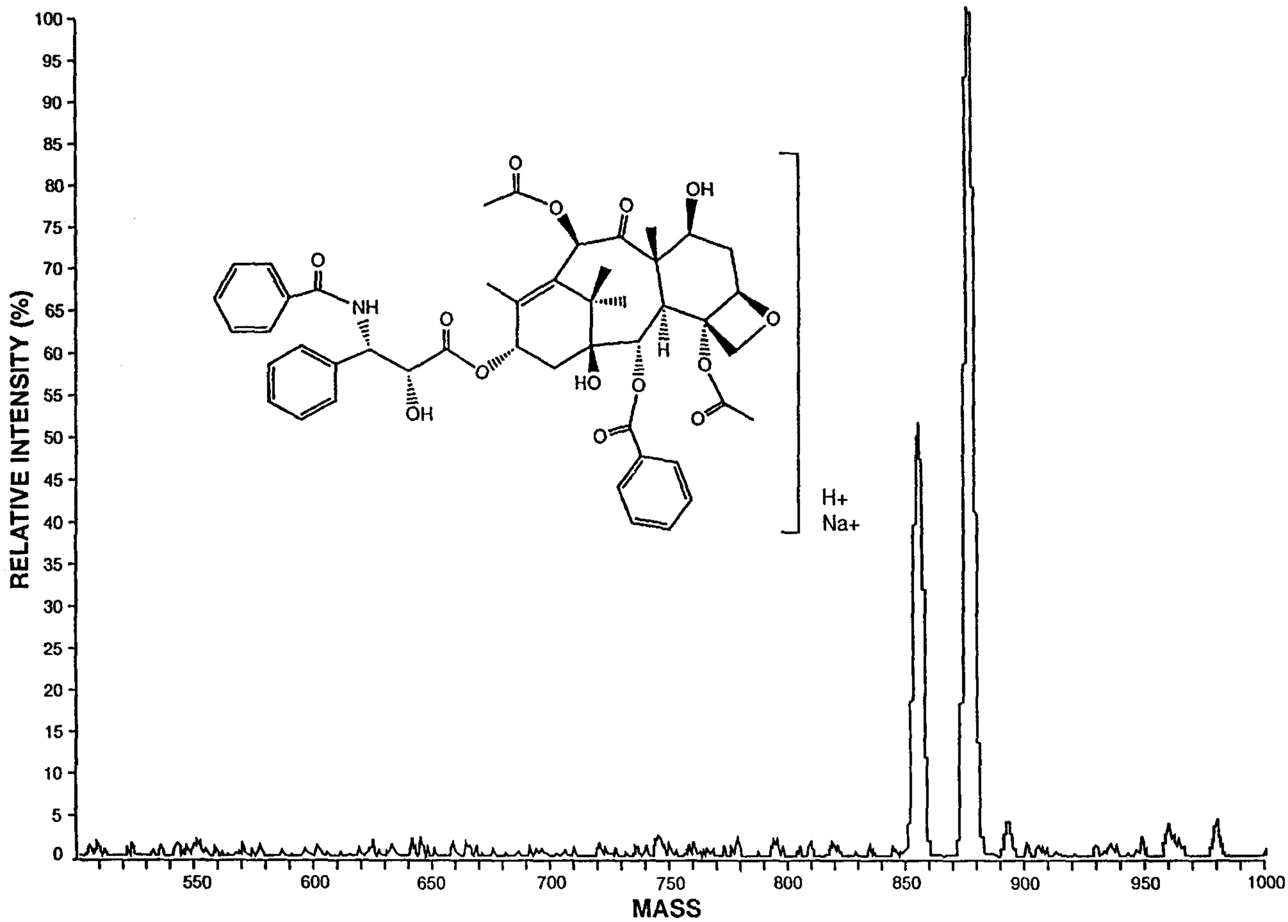

Figure 2 An electrospray mass spectrum of fungal taxol (*P. microspora*). Note characteristic peaks at 854 $[M + H]^+$ and 876 $[M + Na]^+$.

fungi *Taxomyces andreanae* [17], and *Pestalotiopsis microspora* [18]. A number of other endophytic fungi from *T. brevifolia* have given a positive indication of taxol production via immunological evidence, including multiple strains of *Penicillium* sp, *Pestalotiopsis* sp and *Truncatella* sp [16].

The taxol-producing fungi from yews

More than 160 different endophytic fungal species were obtained from *Taxus* spp that are common to Europe, Asia and North America (besides *T. brevifolia*). Each fungus was grown in a standard medium for 3 weeks and subsequently extracted with CH_2Cl_2 [18]. The residue obtained after extraction was subjected to the standardized immunoassay technique employing specific monoclonal antibodies to taxol after an initial indication from thin layer chromatography that taxol might be present [18]. At least seven of these fungi consistently (three tests on three different cultured extracts) tested positive for taxol (Table 1). The amount of taxol present in these cultures varied from 95 to 1081 ng L^{-1}. No substantial (>10 ng L^{-1}) taxol-producing fungi were found from *T. floridana* or *T. canadensis*. Endophytic fungi obtained from *T. chinensis* (Asiatic yew) and *T. globosa* were not examined. In several cases, however, fungal extracts gave a positive reaction for other taxanes with monoclonal antibodies specifically reactive against the taxane skeleton [7]. This suggests the presence of taxol derivatives/precursors such as baccatin III in fungi.

Table 1 Endophytic fungi producing taxol

Culture reference No.	Taxus source	Fungus	Taxol L^{-1} [a]
Tbp-2	*T. baccata*	*Monochaetia* sp	102
Tbp-9	*T. baccata*	*Fusarium lateritium*	130
Ja-69	*T. cuspidata*	*Alternaria* sp	157
Ja-73	*T. cuspidata*	*Pestalotiopsis microspora*	268
Ne-32	*T. wallachiana*	*Pestalotiopsis microspora*	500
P-96	*T. sumatrana*	*Pithomyces* sp	95
Tbx-2[b]	*T. baccata*	*Pestalotia bicilia*	1081

[a]Taxol measured quantitatively using monoclonal antibodies.
[b]This fungus was obtained from the stem—xylem, while all others were from the phloem/cambium.

Identification of taxol production by these fungi was confirmed by several methods. In each case, putative taxol was obtained by preparative thin layer chromatography in four successive solvent systems after an initial separation of the crude CH_2Cl_2 extract on a 1- × 5- cm silica flash column [18]. The putative taxol was then chromatographed in at least five thin layer chromatographic systems to yield a product with the same R_F and color reaction with vanillin/sulfuric acid as authentic taxol [1]. The putative taxol prepared by chromatography also reacted positively in the monoclonal antibody reaction [7].

In the case of taxol from *P. microspora*-Ne32, hundreds of micrograms had been prepared and tested with a multitude of techniques including FAB and electrospray mass spectroscopy, proton nuclear magnetic resonance spectroscopy and UV analyses. In each case the spectrum was identical to that of authentic taxol [14,18,20]. In addition, ^{14}C-taxol was isolated from *P. microspora* that had been administered phenylalanine-^{14}C and other precursors [18].

The fungi yielding taxol were identified (Table 1) after sporulation on gamma-irradiated carnation leaves supported on water agar [15]. The spores were examined and photographed with a light microscope and, after fixation, by scanning electron microscopy using standard techniques [19]. Artist's drawings were also made (Figures 3 and 4). The fungus (Tbx-2), *Pestalotia bicilia* meets the definition of a *Pestalotiopsis* with its three-colored internal cells and two hyaline terminal cells, but this species has not yet been transferred to the genus *Pestalotiopsis* (Dr B Sutton, Commonwealth Mycological Institute, Surrey, UK, personal communication). The other fungi listed in Table 1 each definitely match the specific fungal genus listed in all respects eg spore morphology, color, and method of sporulation (Figures 3 and 4).

Other fungi producing taxol

Taxol in yews may be an important defense mechanism for this tree throughout their range, yews prefer damp shaded conditions at altitudes of 1–3000 meters [9]. Commonly, oomycetous root-infecting fungi also prefer these environments. Taxol effectively halts the growth of all common pathogenic oomycetes including species of *Pythium, Phytophthora* and *Aphanomyces* [21]. Since a wide range of deuteromycetous fungi make taxol, it is conceivable that they could range freely as endophytes in plants sharing the same environment as yew. For this reason, we sampled bald cypress (*Taxodium distichum*) in a swamp garden of South Carolina. Over 25 isolates of endophytes were found in several small limbs from one tree. Of these, 16 isolates were identified on a morphological basis as *Pestalotiopsis microspora*, but only two were identical in all respects to each other. Of these 16 *P. microspora* isolates, nine produced taxol [12]. Thus, it seems taxol-producing fungi may be found not only in yews, but from other plant species that may share the same environmental requirements as yew.

The genetic origin of fungal taxol production has been speculated to have arisen by horizontal gene transfer from *Taxus* spp to its endophytes [17]. This idea has some precedence in the well-known gene transfer occurring between *Agrobacterium tumefaciens* and *A. rhizogenes* with higher plants [8]. Little documentation exists for gene transfer from a higher plant to an endophyte or parasite. Alternatively, of course, fungi may have independently evolved systems for taxol production.

Conclusion

It seems apparent from our limited experiences in working with *Taxus* species, having about 11 representative species worldwide, that the concept of studying and preserving nature's biodiversity not only holds true for the plant—but the myriad of other organisms associated with the plant. The endophytes present in yew stems are numerous, diverse, and have barely been studied both taxonomically and biochemically. Yet-unstudied are the microbes of yew living in association with its non-living heartwood (xylem), seeds, seed coats, roots, leaves, and large trunk-sized stems. Based on the information presented in this review, the importance of such studies not only lies in the possibilities of new taxonomic discoveries, but in the potential for finding new sources for extremely valuable life-saving drugs such as taxol. We also surmise that the presence of taxol in yew might be related to the protective effects rendered by this compound against root-invading fungi [21]. From a teleological point of view, other fungi (some endophytes) may use the same strategy of producing taxol to assure their own survival not only in yew, but as endophytes in plants other than *Taxus* sp [12]. Furthermore, given the extremely competitive environment in which microbes live, it would seem that many more 'taxol-like' compounds are being made by plant endophytes to give them a competitive edge.

The loss of one tree in man's process of destroying the tropical rainforest, not only means the destruction of one organism (a macrophyte), but the loss of what might be hundreds of, as yet, unstudied and unknown microbes that may have more benefits to industrial microbiology, human health and well-being, than the original plant itself. An examination of the botanical record reveals that there are probably more than 300 000 different higher plant species on the earth. A mere 150 of these have entered the world's commerce, and only 10 stand between man's existence and starvation. Critical drugs have come from the botanical cornucopia, including morphine, vincristine, emetine, atropine, reserpine, digitoxin, and quinine. Many of these drugs were originally found by shamans, herbalists, and sorcerers and given to their ill patients as concoctions, brews, or powdered plant parts. Furthermore, an impressive 70% of the anticancer drugs now known have been found in tropical forests [6]. Yet, it seems that in the Amazon alone, only 1% of the plants have been studied chemically, and 90% have not even had a cursory examination for their potential medicinal properties [5]. To our knowledge there is not one documented case showing that any of the well established plant-derived medicinals actually has its origin with one or more of the endophytes associated with that plant. An exception to this case might be fungal taxol, but it is obvious that the yields of taxol from fungi are too low to account for its presence in *Taxus* spp. Besides, it has now been firmly established that axenic tissue cultures of yew do produce taxol and related taxanes [8]. Nevertheless, given our success in finding fungi producing taxol, it seems likely that other endophytes exist in the myriad of the world's medicinal plants. These endophytes may either make the identical plant medicinal or mimic the plant product with one or more other compounds that are structurally related, or similar to the medicinal. Such is the case with the subglutinols that we have recently described from *Fusarium subglutinans*, an endophyte of a well-known Chinese medicinal plant that possesses immunosuppressive properties [11,13]. The subglutinols also have immunosuppressive

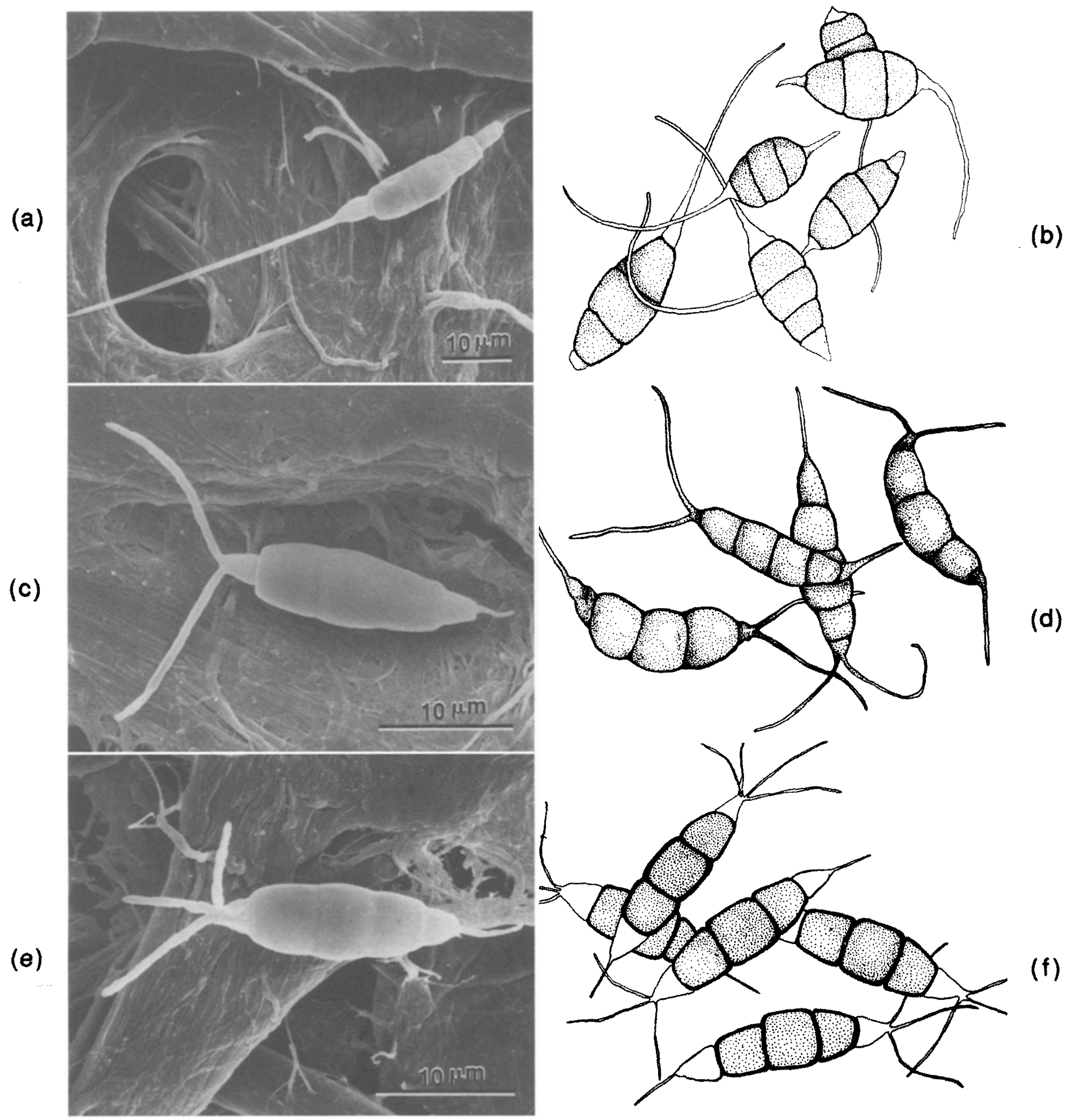

Figure 3 (a) and (b) *Monochaetia* spp isolated from *T. baccata*. (a) Scanning electron micrograph of a spore on filter paper. (b) Drawing of spores. (c) and (d) *Pestalotia bicilia* isolated from *T. baccata*. (c) Scanning electron micrograph of a spore on filter paper. (d) Drawing of spores. (e) and (f) *Pestalotiopsis microspora* isolated from *T. cuspidata* and *T. wallachiana*. (e) Scanning electron micrograph of a spore on filter paper. (f) Drawing of spores.

activities and have certain structural similarities to the known active principles in this plant [11]. The number of known plants that have medicinal properties far exceeds the number of plants used as food sources. There are over 5000 medicinally important plants in China alone. The Amazon, the golden triangle region of Northern Thailand, the tropics of the Venezuelan-Guyana, and the teeming forests of central Africa each has native human populations using its plant resources for healing pruposes. It is absolutely surprising that so much biological potential in the form of epiphytes and endophytes associated with these higher plant forms has never been investigated. What is more disheartening is the prospect that we may never have an opportunity to study these organisms as they are rapidly being destroyed by land-clearing activities including burning, deforestation, and mining interests. A major effort should be mounted to recover and preserve these microbes before they are lost, and the best strategy is to preserve the forest and systematically study it.

(a) (c) (e) (b) (d) (f)

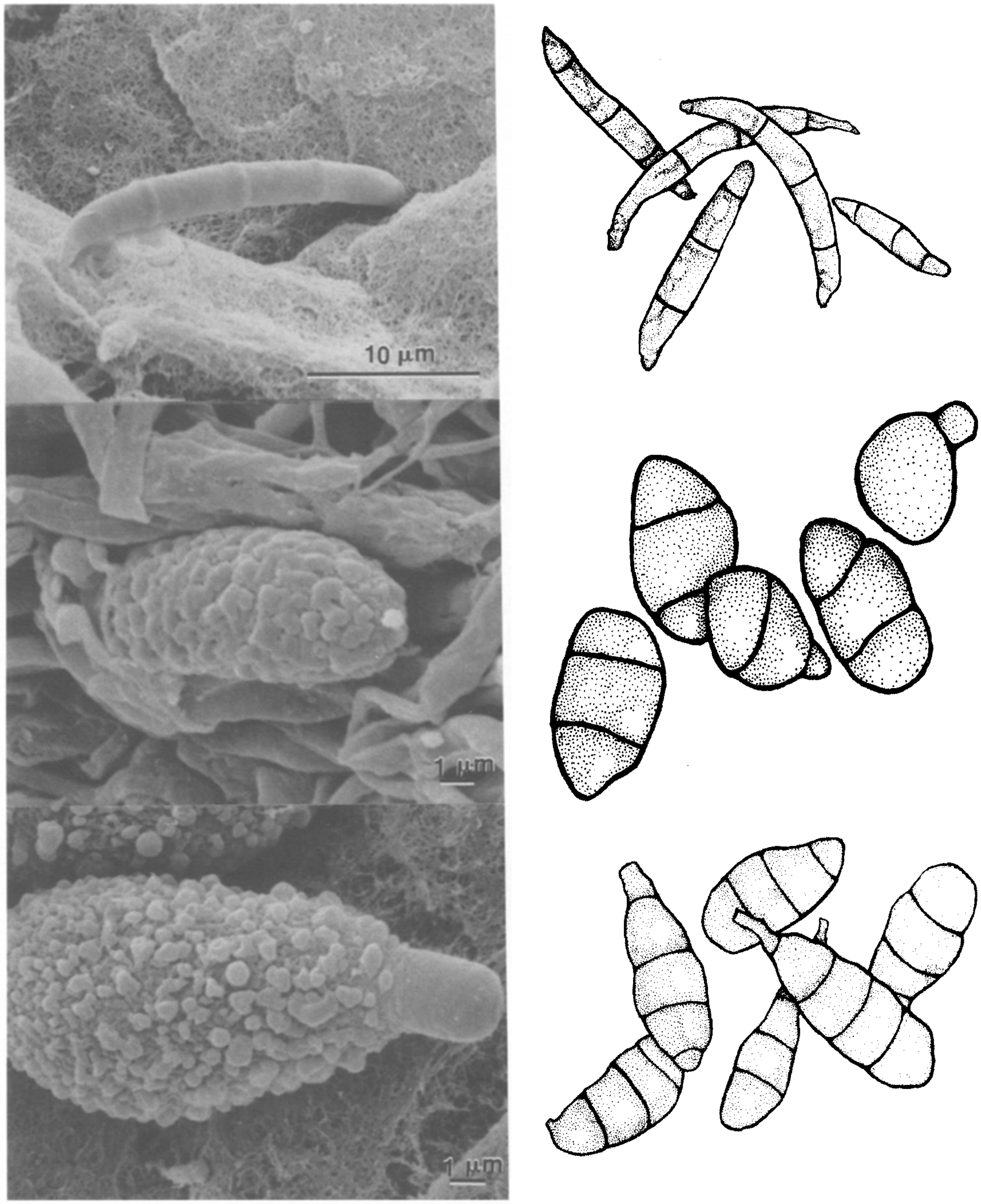

Figure 4 (a) and (b) *Fusarium lateritium* isolated from *T. baccata*. (a) Scanning electron micrograph of a spore on a leaf surface. (b) Drawing of spores. (c) and (d) *Pithomyces* sp isolated from *T. sumatrana*. (c) Scanning electron micrograph of a spore on a leaf surface. (d) Drawing of spores. (e) and (f) *Alternaria* sp isolated from *T. cuspidata*. (e) Scanning electron micrograph of a spore on a leaf surface. (f) Drawing of spores.

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