Induction of the sexual stage of Pestalotiopsis microspora, a taxol-producing fungus

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Pestalotiopsis microspora, isolate NE-32, is an endophyte of the Himalayan yew (Taxus wallichiana) that produces taxol, an important chemotherapeutic drug used in the treatment of breast and ovarian cancers. Conditions were determined to induce the perfect stage (teleomorph) of this organism in the laboratory as a critical first step to study inheritance of taxol biosynthetic genes. The perfect stage of Pestalotiopsis microspora NE-32 forms in a period of 3–6 weeks on water agarose with dried yew needles at 16–20 °C with 12 h of light per day. Morphological analysis of the teleomorph and sequencing of the 18S rDNA indicates that Pestalosphaeria hansenii is the perfect stage of Pestalotiopsis microspora. Only certain plants (e.g. yews, some pines, pecan, oat and some barley cultivars) allow the production of perithecia. Exhaustive methylene chloride extraction of yew (Taxus cuspidata) needles removes their capacity to induce production of perithecia. The methylene chloride extract is able to induce formation of perithecia by strain NE-32 in a bioassay system utilizing the sterilized sheaths of the Cholla cactus (Opuntia bigelovii) spine, indicating that a chemical compound(s) in yew stimulates the formation of the perfect stage. This hydrophobic plant compound(s) has been designated the perithecial-stimulating factor (PSF). The data suggest that plant products may play a role in regulating the biology of endophytic microbes.

Keywords: Pestalotiopsis, Pestalosphaeria, taxol, teleomorph, sex factor

INTRODUCTION

A common fungus present in tropical and semitropical plants is Pestalotiopsis microspora. Although this organism is a widespread saprophyte on bark and decaying plant material, it is also found as an endophyte in many plant species. We have consistently isolated this organism, over the years, from a wide variety of plant families and from every major area on the earth where rainforests exist. Despite its prevalence, the role of this fungus in plant ecology remains unclear. However, our studies of this organism suggest it has potential as a model organism for biological and biochemical studies in the laboratory. The fungus appears to be genetically diverse in that almost every isolate has distinctive biochemical and phenotypic traits (Li et al., 1996). Under laboratory conditions it can take up heterologous DNA, add telomeric DNA, express the heterologous DNA and replicate it independently of chromosomal DNA (Long et al., 1998). It has been suggested that comparable activity in this fungus may exist in nature, allowing it to readily adapt to a new plant by incorporating plant DNA into its own genome (Strobel et al., 1996a; Li et al., 1996). One isolate of Pestalotiopsis microspora, designated NE-32, has enormous potential utility because it is a producer of taxol (Strobel et al., 1996a, b).

Because of our interest in studying the genetics of the taxol biosynthetic pathway, we pursued conditions to obtain the perfect stage of Pestalotiopsis microspora isolate NE-32. Pestalosphaeria, the teleomorph of Pestalotiopsis, has been observed in culture after lengthy (5–6 month) incubation periods on potato dextrose agar (PDA) (Zhu et al., 1991), a time frame that is not amenable to routine laboratory investigation. The teleomorph of NE-32 had never been produced in our

Abbreviations: ddH2O, double-distilled water; MeCl, methylene chloride; PDA, potato dextrose agar; PSF, perithecial-stimulating factor.
The GenBank accession numbers for the sequences determined in this work are: Pestalotiopsis microspora NE-32 18S rDNA, AF104356; Pestalosphaeria hansenii ATCC 48245 18S rDNA, AF242846.
laboratory on the carnation leaf agar used to generate the anamorph. Because the perfect stage of *Pestalotiopsis* spp. has been reported on their respective host plants (Barr, 1975; Hyde, 1996; Nag Raj, 1985; Shoemaker & Simpson, 1980), we used needles of *Taxus* spp. (yew, the natural host of *Pestalotiopsis microspora* NE-32) to produce sexual fruiting structures. Additionally, work on basidiomycetes showed that adequate light and aeration, low (15 °C) temperatures, and nitrogen and carbon limitation were critical in promoting sporogenesis in these organisms (Croan *et al.*, 1997). In this study we therefore used nutrient-poor water agarose and examined temperature and lighting conditions to generate the perfect stage of *Pestalotiopsis microspora*. The teleomorph was found to be consistent with *Pestalosphaeria hanseni*, one of eight *Pestalosphaeria* species (Hyde, 1996) found in the Amphisphaeriaceae (*sensu stricto*) family (Kang *et al.*, 1999).

*Pestalosphaeria* has also been cultured in the laboratory by growth on specific plant materials such as rusted-rose leaves (Hansen *et al.*, 1956) or mangrove leaf sections (Chang & Chang, 1990). We found several plant materials besides yew needles that were amenable to perithecial induction. Although these plants, including gymnosperms such as pine and cereal grains such as oat and barley, are not easily categorized, it seemed possible that they have in common a compound(s) that may be responsible for stimulation of perithecial growth. We developed a bioassay to test plant extracts for their ability to stimulate induction of the sexual stage. In the present paper, we report that a methylene chloride (MeCl) extract of yew needles was able to induce formation of perithecia in NE-32, suggesting the presence of a lipid-like perithecial-stimulating factor(s). These results demonstrate that *Pestalotiopsis microspora* NE-32 now has the potential for serving as a fungal model for sexual interconversion studies.

**METHODS**

**Sources of fungi used in testing.** The primary organism used in this study was *Pestalotiopsis microspora* isolate NE-32, anamorph, originally obtained as an endophyte of *Taxus wallichiana* near Kathmandu, Nepal (Strobelt *et al.*, 1996a). Other *Pestalotiopsis microspora* isolates (our laboratory catalogue nos 98, 99, 203, 210, 216, 227 and 228) were obtained as endophytes from various parts of the world. All other *Pestalotiopsis* spp. were purchased from the Centraalbureau voor Schimmelcultures (CBS) in Baarn, the Netherlands. Each isolate was tested for its ability to develop acervuli of *Pestalosphaeria* (Hyde, 1996) found in the Amphisphaeriaceae (*sensu stricto*) family (Kang *et al.*, 1999).

Testing conditions for formation of perithecia. Water agarose plates containing 15% agarose (Life Technologies) were prepared with ddH₂O. Plant parts (stems, leaves, seeds and seed husks) were collected from various geographical locations as indicated. The wild barley cultivars were obtained from Dr Isaac Barash, University of Tel Aviv, Israel. γ-Irradiated carnation leaves, commonly used to support the development of acervuli of *Pestalotiopsis* spp., were supplied by the Fusarium Lab at Pennsylvania State University. All agarose plates were sealed with a ribbon of Parafilm. Incubation, unless otherwise indicated, was in 12 h light/12 h dark cycles at 20 °C. The light source was a 20 W cool-white fluorescent light (General Electric) and the intensity was 25 μE m⁻² s⁻¹ at the surface of the agarose plate. Plates held in the dark were wrapped in aluminium foil. Numbers of fruiting bodies (acervuli and perithecia) were counted per 10 mm² representing randomly selected areas of the plant tissues encompassed by the field of view of a binocular microscope (Vision Engineering). A simple scoring system was used to represent the numbers of fruiting structures present per 10 mm². In the case of acervuli: 0, no fruiting bodies; +, 2–7; ++, 7–10; ++++, > 10. In the case of perithecia: 0, no fruiting bodies; +, 1–5; ++, 5–10; +++, 10–20; +++++, > 20.

To determine optimum conditions for production of perithecia, *Pestalotiopsis microspora* isolate NE-32 growing from a single conidium was used to inoculate the centre of agarose plates with either air-dried and autoclaved yew needles (*Taxus cuspidata*) or γ-irradiated carnation leaves. Plates were placed at 16, 20 or 25–27 °C under various light conditions for up to 6 weeks. To test the ability of different *Pestalotiopsis* spp. to produce perithecia, isolates were grown on agarose with air-dried and autoclaved yew needles at 20 °C with 12 h dark/12 h light cycles. Production of perithecia was checked at 6 weeks. Since only the NE-32 control produced perithecia in this period, incubation was continued and plates were checked periodically for up to 5 months.

Whole leaves, leaf parts or stem fragments of various plants were studied for their ability to cause the formation of fungal fruiting bodies. Freshly harvested plant materials were left untreated, oven-dried at 110 °C for 10 h or naturally air-dried over a period of weeks. All plant materials tested were wrapped in aluminium foil and autoclaved for 30 min. *Pestalotiopsis microspora* NE-32 growing from a single conidium was used to inoculate the centre of a water agarose plate which also contained 5–10 of the sterilized plant fragments being tested.

To determine if perithecial formation was due to an extractable compound(s) present in yew needles, intact needles (1 g) of *T. cuspidata* were soaked in ddH₂O, 95% ethanol or MeCl. After 48 h of soaking, the needles were removed from each solvent and air-dried. The treated needles were wrapped in aluminium foil and autoclaved for 30 min, placed on agarose in the presence of *Pestalotiopsis microspora* NE-32 and incubated at 20 °C with 12 h light/12 h dark cycles for 6 weeks. To ensure that solvent residue did not inhibit fungal growth, control plates were prepared with yew needles and carnation leaves that had been soaked in the solvents for 30 min and air-dried before use. Conidia from these experiments were germinated on PDA (Difco) to verify their viability.

**Taxol quantitation assay.** Fungi were grown in 500 ml M1D medium supplemented with 1 g soytone l⁻¹ as previously described (Strobelt *et al.*, 1996a). For each fungus, a MeCl extract of the culture fluid was prepared and taxol levels were measured by a competitive inhibition enzyme immunoassay as previously described (Li *et al.*, 1996).

**Preparation of plant extracts.** *T. cuspidata* needles (2-3 g) were placed in 60 ml ddH₂O and homogenized for 30 s. The insoluble material was removed by filtration through six layers of cheesecloth and the soluble materials (crude extract) were separated over a Dowex 50 H⁺ (1.5 × 20 cm) column into cationic, anionic and neutral fractions. Extract was loaded and allowed to enter the column and the resin was subsequently rinsed with 5 ml ddH₂O. The eluate was the neutral
fraction. The cationic fraction was eluted from the column with 5 ml 6 M trifluoroacetic acid. The anionic fraction was obtained by passage of the Dowex 50 H+ column run-through over a 1×4 cm column of Dowex 1 (formate form) followed by 5 ml ddH2O rinse and elution with 5 ml 6 M formic acid. All fractions were dried on a rotary evaporator unit and weighed. To prepare the MeCl extract, needles of T. cuspidata (1 g) were soaked in a sealed container for 48 h in 30 ml MeCl. This organic solvent extract was decanted from the plant material, dried on a rotary evaporator and the residue was weighed. In addition, a portion of the T. cuspidata organic extract was separated chromatographically on a 15 cm column of silica gel. It was eluted sequentially with 50 ml volumes of neat chloroform and chloroform/methanol (successively 99:1, 95:5, 90:10 and 80:20, v/v). Each fraction was dried and the residue weighed. Identical extracts were made from the leaves of Carya illinonensis (pecan).

Cholla sheath bioassay. Plant preparations extracted by various solvent extraction methods were dissolved in methanol to the concentration indicated and 5 µl was aseptically applied via a 10 µl Hamilton syringe to the inside of previously autoclaved sheaths of Cholla cactus (Opuntia bigelovii) spines (Fig. 1a, b). The spine sheaths were harvested from a Cholla cactus specimen in the desert region of Southern Utah. The sheaths served to meet the thigmotropic requirement of Pestalotiopsis microspora to produce its perfect stage while also serving as a reservoir for the chemical extracts. The sheaths were collected by carefully pulling them away from individual Cholla cactus spines. The sheath has an inside diameter slightly larger than the outside diameter of a 10 µl Hamilton syringe tip. The solutions were injected into individual spine sheaths, which were then placed in a positive-air-flow hood for 1–2 h to allow solvent evaporation. The prepared sheaths were then placed on agarose plates inoculated with Pestalotiopsis microspora NE-32. Fungal fruiting structures (both acervuli and perithecia) were counted weekly for 6 weeks. Spine sheaths without plant extract produced neither acervuli nor perithecia over the course of any experimental period.

Statistical analysis. Raw data were used to calculate the mean and standard deviation for each experiment. The number of replicates for each experiment is noted in the appropriate table legend. Analysis of variance of raw data was done at a significance level of 0.05, using fitted treatment means with multiple comparisons based on the Newman–Keuls method.

Scanning electron microscopy. Representative specimens of fungal fruiting structures (both acervuli and perithecia) were harvested from individual plant materials. The plant parts containing such structures were placed directly into 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2–7.4 (Upadhyay et al., 1991). Tissue samples were fixed for at least 2 h, rinsed three times (15 min each) in 0.1 M sodium cacodylate buffer (pH 7.2–7.4), and dehydrated in an ethanol dilution series (Dykstra, 1993). Samples were critical-point dried and sputter-coated with gold, and photographed with a JEOL 6100 scanning electron microscope.

Light microscopy. Samples were prepared as previously described (Hess, 1966; Spurr, 1969). Sections were cut with diamond knives using an RMC model MTX ultramicrotome. Sections (2–4 µm thick) were mounted on glass slides and stained in aqueous 1% toludine blue O, 1% azure II and 1% sodium borate (Hess & Seegmiller, 1988). After the slides were dried, they were placed in xylene and cover slips were sealed with Permount.

Comparison of 18S rDNA sequences. For DNA isolation, Pestalotiopsis microspora NE-32 and Pestalosphaeria bannensis ATCC 48245 (Shoemaker & Simpson, 1981) were grown in potato dextrose broth in 15 ml tubes for 24–48 h at 23 °C. The mycelium was harvested by centrifugation and washed twice with sterile ddH2O. Total genomic DNA was extracted by the methods of Lee & Taylor (1990). A 1732 bp fragment of the 18S rDNA gene from each fungus was amplified by PCR as a single fragment with the primers UK4F (5′-CTGCTGGTATGCTGCCCCG-3′) and UREV (5′-GYTACCTTGTACCGACTT-3′). PCR was performed in a 50 µl reaction containing 0.1 µg genomic DNA, 0.4 µM each primer, 0.16 mM each of the four dNTPs and 5 U Taq polymerase (Promega) in a buffer of 10 mM Tris/HCl (pH 9.0 at 25 °C), 50 mM KCl, 3 mM MgCl2, 0.1% Triton X-100. Amplification was for 30 cycles (45 s at 94 °C, 55 s at 53 °C, 90 s at 72 °C). The PCR products were gel-purified and desalted using the QuickStep PCR purification kit (Edge BioSystems). Ten to forty nanograms of PCR product was sequenced using ABI Prism BigDye terminator chemistry (Perkin-Elmer) and reactions were resolved on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). Nucleotide sequences were determined on both strands (Pestalotiopsis microspora NE-32 18S rDNA partial gene, GenBank accession no. AF104356; Pestalosphaeria bannensis ATCC 48245 18S rDNA partial gene, GenBank accession no. AF242846) and aligned using CLUSTAL W (version 1.74).

RESULTS

Fungal identification

The primary fungus used in this study was the conidial–acervular hyphomycetous fungus Pestalotiopsis microspora NE-32 (Fig. 2a, b). This isolate was originally
identified as *Pestalotiopsis microspora* by Dr B. Sutton of the Commonwealth Mycological Institute, UK. All other unidentified *Pestalotiopsis* spp. isolates used in this study are very similar if not identical in conidium size and morphology to those in the original description of *Pestalotiopsis microspora* NE-32 (Strobel et al., 1996a). The teleomorphic stage of *Pestalotiopsis microspora* NE-32 formed on several selected plant materials and was thoroughly studied in order to establish its relatedness to other ascogenous fungi. The perithecia forming on yew needles and several other plant species tested were amphigenous, yellowish brown, and surrounded by a thick brown thallus. Perithecia appeared mostly isolated, although sometimes clustered, on the leaf surface. Generally, they were semi-immersed in host tissue with an erumpent neck and were globose to subglobose, up to 200 μm in diameter, 330–450 μm high, and hirsute at the base of the erumpent neck. The perithecial wall was composed of brown layers of ‘angularis textura’ cells and yellowish brown ‘prismatica textura’ cells making up the neck of the perithecium. The ostiole was 50–75 μm in diameter and appeared to rip open. Older perithecia possessed agglutinated masses of ascospores clustered as a ball at the tip of the ostiole (Fig. 2c). The asci were uniloculate, cylindrical with a short stipe or pedicel having an apical apparatus with two amyloid inclusions (Fig. 2d). They were octosporous, 99–108 × 6·4 μm and were intermixed with single 4–6-septate, smooth–walled attenuated paraphyses that were 1·6–3·2 μm wide × 112–114 μm long (Fig. 2d). The ascospores were uniseriate, ellipsoidal, 2-septate (occasionally with one and four

**Fig. 2.** Primary fruiting structures of *Pestalotiopsis microspora* and *Pestalosphaeria hansenii*: (a) an acervulus of *Pestalotiopsis microspora*; (b) appendage-bearing conidiospores of *Pestalotiopsis microspora*; (c) a perithecium of *Pestalosphaeria hansenii* with an agglutinated mass of ascospores; (d) the asci of *Pestalosphaeria hansenii* located within a perithecium.
Anamorph conversion of *Pestalotiopsis microspora*

![Image](https://via.placeholder.com/150)

**Fig. 3.** Fruiting structures of *Pestalosphaeria hansenii*: (a) ascospores of *Pestalosphaeria hansenii*; (b) numerous perithecia of *Pestalosphaeria hansenii* produced on sterilized pecan leaves; (c) poorly formed perithecium on oat seed husks; (d) perithecium formed on a Cholla spine sheath injected with a MeCl extract of yew needles.

Septa) (Fig. 3a). The central cell was dark brown and the adjoining cells were pale to amber brown. The wall was smooth and slightly constricted at the septa. Ascospores were 4.8–6.4 × 12.8–16.9 µm in size (Fig. 3a).

These observations indicated that the teleomorph of *Pestalotiopsis microspora* NE-32 was identical in all respects except perithecial size to the ascomycetous fungus *Pestalosphaeria hansenii* (Shoemaker & Simpson, 1981). The cultured perithecia we observed from NE-32 were taller (450 µm vs 280 µm) than *Pestalosphaeria hansenii*, with a wider ostiole (50–75 µm vs 24 µm) and slightly longer and thinner asci. However, observations on the structure of the *Pestalosphaeria hansenii* teleomorph were made on specimens gathered from the wild, and we examined artificially cultured NE-32 perithecia. It is likely that the more controlled laboratory environment and lack of competing organisms allowed the fruiting structures to grow to a larger size in culture than in nature. The anamorph of *Pestalotiopsis microspora* NE-32 was virtually identical in all respects to the anamorph of *Pestalosphaeria hansenii*. The 18S rDNAs from NE-32 and *Pestalosphaeria hansenii* (ATCC 48245, the authentic culture of *Pestalosphaeria hansenii* described by Shoemaker & Simpson, 1981) were sequenced. When 1732 bp, representing at least 90% of the 18S rDNA sequences, were aligned they were found to be identical in both isolates.

**Conditions for the induction of the sexual stage**

Carnation leaves have commonly been used to induce fungal fruiting structures (Nelson *et al.*, 1983). While this substrate routinely induces formation of acervuli in NE-32, carnation leaves were ineffective in inducing the
**Table 1. Conditions for the induction of Pestalospaeria hansenii from Pestalotiopsis microspora**

_Pestalotiopsis microspora_ mycelium was used to inoculate agarose plates with air-dried and autoclaved yew ( _Taxus cuspidata_ ) needles or γ-irradiated carnation leaves. After an incubation time of 6 weeks, plates were scored from 0 to + + + for presence of acervuli (with the maximum number of acervuli being > 10 per 10 mm²) and perithecia (with the maximum number of perithecia being > 20 per 10 mm²). Twenty leaves were observed for each data point.

<table>
<thead>
<tr>
<th>Support</th>
<th>Light regime</th>
<th>Temperature</th>
<th>Acervuli</th>
<th>Perithecia</th>
<th>Acervuli</th>
<th>Perithecia</th>
<th>Acervuli</th>
<th>Perithecia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yew needle</td>
<td>Dark</td>
<td>16 °C</td>
<td>0/ +</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1 h d⁻¹ for 5 d</td>
<td>20 °C</td>
<td>+ +</td>
<td>0</td>
<td>+ +</td>
<td>0</td>
<td>+ +</td>
<td>0</td>
</tr>
<tr>
<td>Yew needle</td>
<td>12 h d⁻¹</td>
<td>25 °C</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>0/ +</td>
</tr>
<tr>
<td>Carnation leaf</td>
<td>12 h d⁻¹</td>
<td></td>
<td>+ + +</td>
<td>0</td>
<td>+ + +</td>
<td>0</td>
<td>+ + +</td>
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</tr>
</tbody>
</table>

perfect stage of the fungus. However, the needles of _Taxus_ spp. (yew), the natural host of NE-32, were able to support formation of perithecia (Table 1). Once it was realized that the perfect stage of _Pestalotiopsis microspora_ could be induced on yew needles, the optimal conditions for perithecial growth were more stringently defined. _Pestalotiopsis microspora_ was grown on water agarose plates with either carnation leaves or yew needles under a variety of culture conditions. Unlike acervuli, which formed readily under a wide range of circumstances, perithecia formed only under defined temperature and lighting conditions (Table 1). Furthermore, acervuli formed quickly and could be seen as little as 5 d, whereas perithecia required at least 3 weeks, and optimally 6 weeks, to develop.

Temperature and light were critical factors in culturing perithecia. The optimum temperature was at 16–20 °C; few or no perithecia formed at 25 °C (Table 1). By contrast, acervuli formed on both carnation leaves and yew needles at all temperatures examined, although an increase in temperature appeared to favour formation of acervuli. Similarly, acervuli formed under all lighting conditions tested, although only a few small acervuli formed in complete darkness. No perithecia were produced in the absence of light, or with exposure to a minimum light period of 1 h per day for 5 d. The fungus only produced perithecia when grown at 16 °C or 20 °C on yew needles with at least 12 h light per day (Table 1).

Media conditions were also modified to ascertain their effect on formation of perithecia. Both agar and highly purified agarose, as well as water of different purities, were tested for their effect on the production of the NE-32 sexual stage. While agarose proved to be slightly better than agar, changes in the purity of the water used to make the media did not affect production of perithecia. Both tap and distilled water proved equally effective in inducing perithecia. Sodium acetate induces budding and sporulation in yeasts (M. Ettayebi, personal communication, 1999). However, the presence of sodium acetate at 8 g l⁻¹ did not induce formation of perithecia (data not shown). Additionally, the moisture content of the plant material also did not appear to be critical. Freshly harvested yew needles were nearly as effective as naturally dried needles, while oven-dried needles had slightly diminished perithecia-inducing properties (data not shown). Thus, while the asexual stage formed readily on both yew needles and carnation leaves in our initial study, only yew needles were effective in inducing the perfect stage. This result strongly suggested that while environmental conditions such as light and temperature were important for perfect stage induction, so too was the nature of the supporting plant material.

In order to determine if the perfect stage of other _Pestalotiopsis_ spp. could be induced in a similar manner, a number of _Pestalotiopsis_ isolates were examined for production of perithecia under the optimal conditions defined for _Pestalotiopsis microspora_ NE-32. The level of taxol production, as measured by an immunoassay of culture-media extract, was also determined for each isolate. Of the isolates, which included _Pestalotiopsis microspora_ specimens as well as other _Pestalotiopsis_ spp., from four continents, only NE-32 was convertible to its teleomorph in the presence of _T. cuspidata_ needles (Table 2). Conversion to the sexual stage did not appear to be directly related to the taxol biosynthetic pathway, as both taxol-producing and non-producing isolates failed to generate the teleomorph under the conditions that favoured production of the perfect stage of NE-32 (Table 2). The other isolates may require different conditions to produce the perfect stage in the laboratory.

Although yew needles effectively induced formation of perithecia, there was some precedent in the literature (Hansen et al., 1956; Chang & Chang, 1990) that suggested that other plant materials might also do this. We used _Pestalotiopsis microspora_ NE-32 as the test organism to determine if other plant materials could induce the formation of perithecia. Leaves from various plant families and geographical locations were tested as...
Table 2. Induction of perithecia in selected Pestalotiopsis isolates

<table>
<thead>
<tr>
<th>Pestalotiopsis isolate</th>
<th>Source country</th>
<th>Source plant</th>
<th>Laboratory culture no.</th>
<th>Perithecia (max. per 10 mm²)</th>
<th>Taxol (ng L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. microspora</em></td>
<td>Nepal</td>
<td><em>Taxus wallachiana</em></td>
<td>NE-32</td>
<td>20</td>
<td>2923</td>
</tr>
<tr>
<td><em>P. microspora</em></td>
<td>USA</td>
<td><em>Taxodium distichum</em></td>
<td>203</td>
<td>0</td>
<td>1488</td>
</tr>
<tr>
<td><em>P. microspora</em></td>
<td>USA</td>
<td><em>Taxodium distichum</em></td>
<td>210</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Pestalotiopsis sp.</td>
<td>PR China</td>
<td><em>Cephalotaxus\ sp.</em></td>
<td>98</td>
<td>0</td>
<td>930</td>
</tr>
<tr>
<td>Pestalotiopsis sp.</td>
<td>PR China</td>
<td><em>Cephalotaxus\ sp.</em></td>
<td>99</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Pestalotiopsis sp.</td>
<td>Australia</td>
<td><em>Wollemia nobilis</em></td>
<td>216</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Pestalotiopsis sp.</td>
<td>Australia</td>
<td><em>Wollemia nobilis</em></td>
<td>227</td>
<td>0</td>
<td>ND</td>
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<tr>
<td>Pestalotiopsis sp.</td>
<td>Australia</td>
<td><em>Wollemia nobilis</em></td>
<td>228</td>
<td>0</td>
<td>128</td>
</tr>
<tr>
<td><em>P. microspora</em></td>
<td>Florida, USA</td>
<td>‘Bath towel’</td>
<td>CBS 364.54</td>
<td>0</td>
<td>92</td>
</tr>
<tr>
<td><em>P. neglecta</em></td>
<td>UK</td>
<td><em>Taxus baccata</em></td>
<td>CBS 200.65</td>
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<td>0</td>
</tr>
<tr>
<td><em>P. funereoides</em></td>
<td>Unknown</td>
<td><em>Juniperus\ sp.</em></td>
<td>CBS 175.25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

supports for the formation of fruiting structures (Table 3). All the *Taxus* spp. examined induced formation of perithecia, but *T. cuspidata* appeared to be the most effective in this regard. Several pines, but not all, were also effective inducers. Surprisingly, one of the best inducers was the leaf of the pecan tree, *Carya illinoinensis* (Fig. 3b). On this material, the fungus produced the greatest number of perithecia per mm² of any of the plants tested and each perithecium appeared perfectly developed. In contrast, barley (*Hordeum vulgare*) and oat (*Avena sativa*) seed husks induced formation of perithecia, but these fruiting bodies appeared incompletely developed as contrasted to those on yew or pecan (compare Fig. 2c to Fig. 3c). They seemed to be less erumpent, had a shorter perithecial neck and were slightly misshapen. Acervuli formed on each of the plant materials tested except *Pinus ponderosa* and the spine sheaths of the Cholla cactus, *Opuntia bigelovii* (Table 3). On these two plants, neither acervuli nor perithecia were able to form.

The observed variability in the ability of different *Taxus* spp. needles to induce the perfect stage suggested that perhaps even closely related plant substrates could vary in their ability to support the fungal teleomorph. The availability of a set of wild barley cultivars allowed us to investigate this possibility. The seed husks of 12 cultivars were tested for their ability to induce formation of perithecia in *Pestalotiopsis microspora* under optimum conditions. The cultivars showed a wide range of variation in perithecial induction, from none to an average of more than eight perithecia per seed husk, depending on the cultivar (data not shown). Individual seed husks of some cultivars varied greatly in their ability to support perithecial formation. Because of this wide variation, no one cultivar was better within statistical significance in inducing the teleomorph. The reason for this fluctuation remains unclear, but suggests that the factor that supports perithecial formation is highly specific to individual species or cultivars.

**Plant extracts and induction of the sexual stage**

The above data collectively indicated that the leaves of certain plants under appropriate conditions will induce the formation of the teleomorph of *Pestalotiopsis microspora* NE-32. Because perfect-stage fruiting structures appeared on some plant materials but not others, it seemed possible that these plants contained one or more compounds that were responsible for the induction phenomenon. To check this possibility, batches of *T. cuspidata* needles were soaked for 48 h in one of several solvents, dried, and then assayed for their ability to induce formation of perithecia in NE-32 on agarose plates under optimum conditions. Needles soaked in MeCl or ethanol were not supportive of formation of perithecia, whereas untreated or water-extracted needles did allow the teleomorph to form (20 ± 5.1 and 8.5 ± 6.6 perithecia per needle, respectively; means ± SD n = 4). To ensure that spore formation or fungal growth was not inhibited by residue from the organic solvents themselves, yew needles and carnation leaves were soaked in ethanol and MeCl for 30 min, air-dried and used to culture NE-32 on agarose plates. The fungus grew normally and produced numerous acervuli with viable conidia in the presence of both ethanol and MeCl-treated plant materials.

**The perithecial-stimulating factor (PSF)**

Because yew needles lost the ability to support perithecial formation after extraction with MeCl, the extract was tested for compound(s) that could in turn stimulate.
**Table 3. Relative ability of various plant species to induce production of perithecia in Pestalotiopsis microspora**

*Pestalotiopsis microspora* isolate NE-32 was inoculated onto agarose plates with the air-dried and autoclaved plant material. After an incubation time of 6 weeks, plates were scored from 0 to +++ for presence of acervuli (with the maximum number of acervuli being >10 per 10 mm²) and perithecia (with the maximum number of perithecia being ≥20 per 10 mm²). Unless otherwise noted, all plant parts tested were leaves.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Origin</th>
<th>Acervuli (per 10 mm²)</th>
<th>Perithecia (per 10 mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No plant</td>
<td>–</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Yucca baccata</td>
<td>SW Utah</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Larrea tridentata</td>
<td>SW Utah</td>
<td>+ +</td>
<td>0</td>
</tr>
<tr>
<td>Quercus tubinella</td>
<td>SW Utah</td>
<td>+ + +</td>
<td>0</td>
</tr>
<tr>
<td>Artemisia tridentata</td>
<td>SW Utah</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Taxus brevifolia</td>
<td>N Montana</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>Taxus baccata</td>
<td>N Morocco</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>Taxus cuspidata</td>
<td>S Montana</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Carya illinonensis</td>
<td>SW Utah</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pinus aristata</td>
<td>Central Utah</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Equisetum arvense</td>
<td>Montana</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td>Israel</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>Opuntia bigelovii</td>
<td>SW Utah</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Opuntia bigelovii</td>
<td>SW Utah</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ephedra viridis</td>
<td>SW Utah</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Acacia baileyana</td>
<td>Utah</td>
<td>+ +</td>
<td>0</td>
</tr>
<tr>
<td>Pinus flexilis</td>
<td>SW Montana</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>Pinus ponderosa</td>
<td>W Montana</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pinus monophylla</td>
<td>SW Utah</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>Gymnocladus dioica</td>
<td>Central Utah</td>
<td>+ + +</td>
<td>0</td>
</tr>
<tr>
<td>Cedrus atlantica</td>
<td>Morocco</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Arceuthobium pusillum</td>
<td>SW Utah</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Dianthus caryophyllus</td>
<td>Pennsylvania</td>
<td>+ + +</td>
<td>0</td>
</tr>
<tr>
<td>Avena sativa</td>
<td>Montana</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>Platanus occidentalis</td>
<td>Utah</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Perithecial formation in the absence of the yew needle itself. To test the extract, a bioassay was developed using the sheaths of Cholla cactus spines. These hollow sheaths, which do not support either acervuli or formation of perithecia themselves (Table 3), can serve as a reservoir to bring extracted plant compounds into contact with the fungus. A water (crude) extract of ground *T. cuspidata* needles was dried and the residue was resuspended in methanol. A portion of the crude extract was also fractionated over an ion-exchange column. Neither the crude extract nor any of the fractions were able to stimulate perithecial formation in the bioassay, although acervuli did form (Table 3). In a similar manner, a MeCl extract of *T. cuspidata* needles was tested. Both the 10 x and 100 x concentrated MeCl extract induced production of perithecia (Table 4). These perithecia produced viable ascospores on Cholla spine sheaths (Fig. 3d). The MeCl extract from yew needles was further fractionated on a silica gel column by sequentially developing the column with 100% chloroform and chloroform/methanol in the following ratios: 99:1, 95:5, 90:10 and 80:20 (v/v). None of these fractions possessed the PSF, as no perithecia were produced in the Cholla sheath bioassay. Each fraction, however, did have the ability to induce the production of acervuli (Table 4). Interestingly, production of acervuli also increased in the MeCl fractions that stimulated production of perithecia, which might indicate that the PSF is not a definitive ‘sex factor’. However, production of acervuli appeared to increase with exposure to several organic and aqueous *T. cuspidata* extracts, and rates of formation of acervuli also varied with exposure to different temperatures and light (Tables 1 and 4) without a corresponding increase in perithecia formation.
Table 4. Ability of various fractions of T. cuspidata needles to induce the formation of acervuli and perithecia

Chemical fractions were tested in the Cholla sheath bioassay as described in Methods. Each result is the mean of two to five independent experiments. Statistically significant differences (0.05 significance level) in acervuli (†) or perithecia (‡) are indicated.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Material per sheath (µg)</th>
<th>Acervuli (per sheath)</th>
<th>Perithecia (per sheath)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td>Crude (H₂O) extract</td>
<td>0.29</td>
<td>1.5±1.5</td>
<td>0±0</td>
</tr>
<tr>
<td>Crude (10×)</td>
<td>2.9</td>
<td>1.5±1.5</td>
<td>0±0</td>
</tr>
<tr>
<td>Crude (100×)</td>
<td>29</td>
<td>2.3±1.2</td>
<td>0±0</td>
</tr>
<tr>
<td>Anionic</td>
<td>125</td>
<td>13.8±1.8*</td>
<td>0±0</td>
</tr>
<tr>
<td>Cationic</td>
<td>45</td>
<td>8.0±4.8*</td>
<td>0±0</td>
</tr>
<tr>
<td>Neutral</td>
<td>490</td>
<td>9.0±3.4*</td>
<td>0±0</td>
</tr>
<tr>
<td>MeCl (crude)</td>
<td>0.19</td>
<td>0.8±0.7</td>
<td>0±0</td>
</tr>
<tr>
<td>MeCl (10×)</td>
<td>1.9</td>
<td>7.5±1.5*</td>
<td>2.5±1.5†</td>
</tr>
<tr>
<td>MeCl (100×)</td>
<td>19</td>
<td>7.5±0.5</td>
<td>1.5±1.5‡</td>
</tr>
<tr>
<td>Chloroform</td>
<td>4.5</td>
<td>11.0±1.0*</td>
<td>0±0</td>
</tr>
<tr>
<td>Chloroform/MeOH, 99:1</td>
<td>46</td>
<td>7.0±4.0</td>
<td>0±0</td>
</tr>
<tr>
<td>Chloroform/MeOH, 95:5</td>
<td>36</td>
<td>5.0±1.0</td>
<td>0±0</td>
</tr>
<tr>
<td>Chloroform/MeOH, 90:10</td>
<td>20</td>
<td>5.3±2.0</td>
<td>0±0</td>
</tr>
<tr>
<td>Chloroform/MeOH, 80:20</td>
<td>15</td>
<td>8.0±2.2*</td>
<td>0±0</td>
</tr>
</tbody>
</table>

† Clusters with MeCl 10× extract but is not statistically significant at 0.05 significance level.

perithecia under these conditions. Thus it seems unlikely that an increase in acervuli along with perithecia signals that a general stimulus has been observed. Rather, it appears that acervuli are produced under a wide variety of stimuli, but there is a factor in the MeCl extract that specifically stimulates perithecial growth.

Since various plants, especially pecans, effectively induced formation of perithecia, the PSF appeared to be present in plants other than yews. The bioassay was thus used to test a similar set of pecan leaf extracts. However, none of these extracts stimulated the formation of perithecia (data not shown). Pecan has not been reported as a natural host for Pestalotiopsis microspora. T. cuspidata, both because it strongly induces production of perithecia and because this stimulating factor can be extracted, may serve as the best starting material from which to attempt isolation of the PSF, although pecan remains a viable alternative.

Interconversion between the teleomorph and the anamorph

Ascospores obtained from the yew (T. cuspidata) and barley seed husks as well as those acquired from the Cholla spine sheaths were germinated. The mycelium resulting from these ascospores effectively interconverted from the teleomorphic state (ascospores) back to the anamorphic stage of the fungus. This was accomplished by taking the mycelium from germinated ascospores and transferring it to γ-irradiated carnation leaves. The ascospore-derived cultures always produced acervuli with their complement of conidiospores (Fig. 2a, b) within 1–3 weeks.

DISCUSSION

Many ascomycetous fungi readily form the perfect stage in culture. However, telemorphs of Pestalotiopsis spp. grown in the laboratory are much rarer and the specific triggers for their production have not been elucidated. Some past attempts to produce the perfect stage of Pestalotiopsis in the laboratory have failed (Barr et al., 1975) while in other reports, the isolates required either months of growth or specific plant substrates to form perithecia (Zhu et al., 1991; Chang & Chang, 1990; Hansen et al., 1956). In other fungi, certain plant-extract agar (such as wheat flour or rice bran) have served to induce the perfect stage (Valent & Chunley, 1991; Tan & Chang, 1989; Yildiz, 1998). Compounds such as tannic acid and caffeine (Tan & Chang, 1989), and even electric impulses (Meguro et al., 1988), have been shown to increase yields of fruiting bodies in edible fungi that already fruit in culture. Fungal compounds can also control the interconversion phenomenon. The control of sexual development by fungal sex hormones and morphogens is well documented (Siddiq et al., 1989; Gooday & Adams, 1993). Other examples include the fungal compound zearealenone, which can stimulate Fusarium roseum to produce its teleomorph Gibberella zeae (Mirocha & Swanson, 1983; Wolf & Mirocha, 1977), and sterols such as cholesterol, which stimulate production of oogonia in certain oomycetes (Hendrix,
1964). However, specific plant-derived compounds that facilitate the interconversion of a fungal anamorph to its teleomorph have yet to be described. We found conditions to readily induce production of the perfect stage of Pestalotiopsis microspora isolate NE-32, and suggest that the teleomorph of Pestalotiopsis microspora is Pestalosphaeria hanseni, based on both genetic and phenotypic evidence. The present paper is the first report of the induction of the perfect stage in a taxol-producing fungus as well as the first demonstration that the teleomorph can be produced using plant extracts in a model system.

Our evidence suggested that a specific strain of Pestalotiopsis microspora, NE-32, had the unusual quality of converting to its sexual stage under certain lighting, temperature and media conditions (Tables 1 and 2). Although Pestalotiopsis microspora is a robust taxol producer, formation of perithecia did not appear to be directly linked to the taxol biosynthetic pathway, as other isolates which produce taxol failed to produce perithecia under the conditions tested. It remains to be seen whether culture conditions that stimulate perithecial induction also affect rates of taxol biosynthesis.

Because only specific plants (including Taxus spp., several pines, barley, oat and especially pecan) had the ability to induce the teleomorphic stage (Table 3), it seemed likely that these plant materials contained a specialized compound(s), which we designated the PSF, that was responsible for stimulating production of the perfect stage. Because the induction phenomenon was limited to specific plants, common plant compounds, such as the sterols implicated in interconversion between the asexual and sexual stage in oomycetes (Hendrix, 1964), would not be expected to be good candidates for the PSF molecule(s). Our experiments showed that the substance(s) in plants responsible for the induction of the perfect stage was lipid-like, since extraction of the yew needles with organic solvents destroyed their ability to support perithecial induction, and a MeCl extract of these needles possessed the PSF activity. We were unable to further purify the PSF by silica gel column chromatography, perhaps because the PSF consists of multiple compounds that were separated during the chromatography process, or because it adhered to the column matrix and was lost during the purification process.

Until it is possible to actually isolate and characterize one or all of the components of the PSF, our understanding of the biochemistry of this phenomenon must remain uncertain. The data are certainly intriguing, suggesting that a fungal endophyte may utilize a plant-derived compound to complete its teleomorphic stage. Although conditions to stimulate perfect-stage production in NE-32 did not translate to other Pestalotiopsis isolates, the availability of a purified PSF may in the future circumvent this problem and allow induction of the teleomorph in the laboratory for isolates which otherwise have been recalcitrant in forming fruiting structures. Additional studies will be required to determine if the PSF is limited to stimulating NE-32, or if, once purified to some degree, it will serve as a general sex factor.

Our evidence is the first indication that the interaction of Pestalotiopsis microspora with its plant host may be a symbiotic one. Although there is one report of a Pestalosphaeria sp. as a serious pathogen on coconut (Sathiarajan & Govindan, 1989), it appears unlikely that Pestalotiopsis microspora is a potenpathogen of its host, Taxus wallachiana, as there were no symptoms of disease on the tree specimen from which NE-32 was isolated.

The direct genetic benefit of having the perfect stage of Pestalotiopsis microspora is that it will allow us to begin to study inheritance in this fungus. Since this organism is one of the best fungal taxol producers known, we have the opportunity to better study taxol metabolism and the inheritance of the taxol biosynthetic genes. However, the fungus appears to be homothallic and it is not clear if genetic outcrossing will be possible. We will need to develop genetically marked mutants and test them for sexual compatibility to investigate this possibility. We are continuing to test Pestalotiopsis microspora isolates from around the world in the hopes of finding one or more that is compatible for mating with NE-32, although we have not been successful to date. This would allow a complete complement of genetic studies, including octad analysis, to begin.

Pestalotiopsis microspora appears to be a ubiquitous organism in global rainforest systems. Of the hundreds of tropical and subtropical rainforest plants that we have examined in our laboratory, it has been isolated as an endophyte from the stems, leaves, flowers and fruits of virtually all. This prevalence may suggest an important role for this fungus in the ecosystem of the forest, and as such we have dubbed this organism ‘the E. coli of the temperate and tropical rainforest systems’. Its relationship to plants seems to be either as a weak pathogen invading aged leaves or as an endophytic symbiont functioning with the higher plant in as-yet mysterious ways. Besides its possible importance in nature, Pestalotiopsis microspora has great potential in the pharmaceutical industry as a taxol producer and in the laboratory as a model biological system for the study of telomeres. Despite its prevalence in the forest and its potential utility in the laboratory, this microbe is poorly understood. The demonstration that we can induce the perfect stage of Pestalotiopsis microspora in the laboratory will allow us to further understand the genetics and biology of this highly important organism.

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