Exciting New Discoveries

In this section we will include innovative or exciting new discoveries in mycology. *Muscodor crispans* was discovered in the Bolivian Amazon as an endophyte of wild pineapple. In this paper it is described as a new species. This species has amazing potential in the pharmaceutical industry as it possesses remarkable bioactive properties against important human and plant pathogens as will be shown in subsequent publications.

Muscodor crispans, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon

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Muscodor crispans (isolate B-23) is described as a new species of *Muscodor*. It is an anamorphic sterilia endophytic fungus residing within the stem tissues of *Ananas ananassoides*, a wild pineapple in the Bolivian Amazon Basin. This strain is characterized by the production of a pinkish felt-like mycelium on potato dextrose agar (PDA) and other media under lighted conditions, but developing a whitish mycelium in the dark. The fungus produces no fruiting structures or spores of any kind when incubated on multiple synthetic or natural media. On PDA and other common laboratory media, its hyphae develop into regular undulating patterns and associated with them are cauliflower-like structures (3.5-14 µm). Analysis of the volatile organic compounds it produces in culture by GC/MS showed that *M. crispans* primarily produces a number of esters, alcohols, and small molecular weight acids, but no naphthalene or azulene derivatives as other members of this genus. The volatiles possess antibiotic properties making this organism potentially useful in a number of situations. A molecular genetic analysis of the ITS1, 5.8S rDNA, and ITS2 regions showed 100% similarity to *Muscodor albus*. *Muscodor albus*, *M. roseus* and *M. vitigenus* are each genetically related to xylariaceous taxa by virtue of ca. 95% sequence similarity to this group. Justification for the designation of a new species is primarily based on the novel phenotypic characters of isolate B-23 including its peculiar hyphal growth patterns (undulating hyphae), its reddish pigment production in the light, the odd cauliflower-like structures associated with its hyphae, and its unusual gaseous products. In spite of its 100% genetic similarity to the rDNA regions of *M. albus*, this organism is considered distinct because of the number and kind of its unusual phenotypic characteristics. The rDNA sequence data obtained represents well less than 1% of the total DNA of a fungus.

Key words: *Muscodor*, pineapple, rDNA, undulating hyphae

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Introduction

Due to the increased necessity for new and more effective antibiotics, the rise in fungal infections all over the world, and the problems associated with agricultural production and harmful pesticide residues; endophytes from rainforest plants are being studied for their

volatile antibiotic characteristics (Strobel *et al*., 2003). Endophytes are defined as microorganisms that live in the interstitial spaces of the living tissues of plants, but they are not generally considered to be parasitic (Azevedo *et al*., 2000; 2008; Sánchez Márquez *et al*., 2007; Wei *et al*., 2007). The medicinal, agricultural, and industrial applications of only a few of these microorganisms have been studied and many show commercial potential; however, due to deforestation, these highly valued microbes are also being destroyed and perhaps lost forever. The endophytes residing in these rainforest plants and even lichens are of great interest (Tejesvi *et al*., 2007; Li *et al*. 2007) Sometimes those which are biologically/ taxonomically unique are usually considered the most valuable since they often produce novel bioactive products (Sánchez Márquez *et al*., 2007). Species of *Pestalotiopsis* have received considerable attention because of their ability to produce several novel compounds (Strobel *et al*., 2000; Hu *et al*., 2007; Tejesvi *et al*., 2007; Wei *et al*., 2007).

Isolate B-23 was recovered from inside the tissues of a wild pineapple plant (*Ananas ananassoides*) growing in the Bolivian Amazon. Ultimately, it was shown to produce a mixture of volatile compounds having antibiotic activities. Using molecular techniques, isolate B-23 possessed sequence similarities to members of the genus *Muscodor*. These fungi are known to produce volatile organic compounds that can act as anti-microbials which are effective against both human and plant pathogens (Strobel *et al*., 2000). Members of *Muscodor* have been identified utilizing methods such as Phylogenetic Character mapping employing 18S rDNA plus ITS1, 5.8S rDNA sequence analyses. The sequences found in B-23 and other *Muscodor* spp. were BLAST searched in GenBank, and compared with other sequences (Bruns *et al*., 1991; Reynolds and Taylor 1993; Mitchell *et al*., 1995; Guarro *et al*., 1999; Taylor *et al*., 1999). Ultimately it was determined that these isolates are related to *Xylaria* (Worapong *et al*., 2001a,b). All isolated taxa that belong to *Muscodor* have similar characteristics, such as growing relatively slowly, possessing a felt-like mycelium, producing biologically active volatile compounds, and causing no harm to the plants in which they originally resided. Finally, they each share closely similar rDNA sequences (Ezra *et al*., 2004).

Although isolate B-23 shared all of the common features mentioned above, there were a number of different aspects to the taxon which distinguished it from all other *Muscodor* spp. and isolates. Thus, this report describes these unique characteristics and provides justification for the establishment of isolate B-23 as a new species.

The name proposed for this novel endophytic fungus is *Muscodor crispans*.

Materials and methods

Fungal isolation

Several small stems of *Ananas ananassoides* were taken from a plant growing in the Bolivian Amazon in March of 2007. They were collected in a savanna region adjoining the rainforest at 12º 40′ 07″ S and 68º′ 41′ 58″ W and were immediately transported to Montana State University for analysis. Several small (2-5 inch) pieces from the stems were cut and placed into 70% ethanol for 30 seconds under a laminar flow hood. A pair of sterile tweezers was used to hold the stems separately in the flame to remove excess alcohol. Then small pieces of inner tissue (beneath the bark) were excised and placed onto PDA with an actively growing *M. albus* isolate 620 on one side of the plate having a center well remove from it (Strobel *et al*., 2001). Effectively, this technique can be used to select for other isolates of *Muscodor* (Worapong *et al*., 2001a,b). During an incubation period of two weeks, the Petri plates were examined periodically for any fungal growth. Once hyphae were observed, the hyphal tips were aseptically cut out of the agar and placed on fresh PDA. Isolate B-23 was found in this manner. Several Petri plates (PDA) were used to determine if the fungus produced volatile antibiotics. This procedure included removing a 1-inch section of the agar from the middle of the plate, plating a plug of the B-23 isolate on one side and allowing it to grow for several days, and then plating test organisms on the other side of the gap (Strobel *et al*., 2001). The isolate (B-23) demonstrated the ability to produce volatile antibiotics, which either inhibited or killed the

fungi that were placed on the other side of the center well as test organisms, such as *Pythium ultimum*, *Sclerotinia sclerotiorum*, and others (Strobel *et al*., 2001).

Scanning electron microscopy

Scanning electron microscopy was preformed on isolate B-23 after procedures described by Castillo, *et al*. (2005). Agar pieces and host plant pieces supporting fungal growth were placed in filter paper packets then placed in 2% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4) with Triton X 100, a wetting agent, and aspirated for 5 minutes and left overnight. The next day they were washed in six 15 minutes changes in water buffer 1:1, followed by a 15 minutes change in 10% ethanol, a 15 minutes change in 30% ethanol, a 15 min change in 50% ethanol, five 15 minutes changes in 70% ethanol, and were then left overnight or longer in 70% ethanol. They were then rinsed six times for 15 minutes in 95% and then three 15 min changes in 100% ethanol, followed by three 15 minutes changes in acetone. The microbial material was critically point dried, gold sputtercoated, and images were recorded with an XL30 ESEM FEG in the high vacuum mode using the Everhart-Thornley detector. Hyphae were measured using Image J software (available online: http://rsb.info.nih. gov/ij/).

Fungal growth and storage

It was determined that B-23 did not produce spores or any other fruiting bodies when several pieces of carnation leaves were placed on top of actively growing B-23 to encourage spore production, and no such structures were observed after a week of incubation at 23°C. The fungus was also plated on several different media including Cellulose Agar (CA), Malt Agar (MA), and Corn Meal Agar (CMA) to determine if spore production of B-23 would be displayed. With the exception of a slower growth rate on some of the media, no other characteristics of B-23 appeared to be different, and no fruiting bodies or spores were observed.

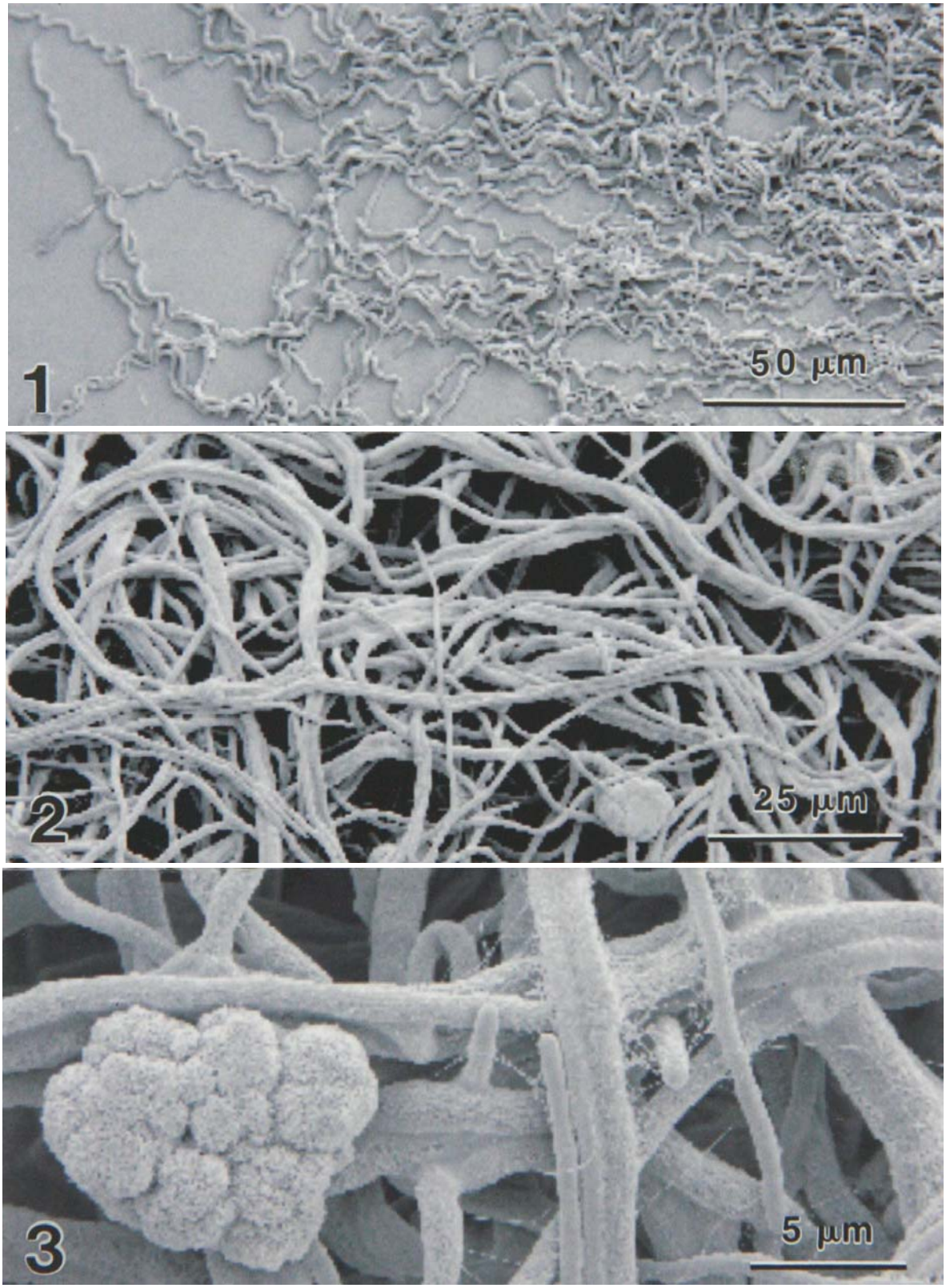
Several methods were used to store the isolated fungus as a pure culture, one of which was the filter paper technique. The fungus was also allowed to grow on PDA, and then it was cut into small squares which were placed into vials containing 15% glycerol and stored at –70°C. The fungus was also stored at 4°C by a similar method, using distilled water rather than glycerol. However the most effective method of storage was on infested sterile barley seed at –70°C.

Qualitative analysis of B-23 volatiles

The method used to analyze the gases in the air space above a 10 day old culture of the B-23 mycelium growing in Petri plates was comparable to that used on the original isolate of *M. albus* strain cz-620 (Strobel *et al*., 2001). First, a baked "Solid Phase Micro Extraction" syringe (Supelco) consisting of 50/30 divinyl-benzene/carburen on polydimethylsiloxane on a stable flex fiber was placed through a small hole drilled in the side of the Petri plate sporting the growth of B-23. The fiber was exposed to the vapor phase of the fungus for 45 min. The syringe was then inserted into the splitless injection port of a Hewlett Packard 6890 gas chromatograph containing a 30 m × 0.25 mm I.D. ZB Wax capillary column with a film thickness of 0.50 mm. The column was temperature programmed as follows: 30°C for 2 min followed to 220°C at 5°C/min. The carrier gas was ultra high purity Helium (local distributor) and the initial column head pressure was 50 kPa. Prior to trapping the volatiles, the fiber was conditioned at 240°C for 20 min under a flow of helium gas. A 30 sec injection time was used to introduce the sample fiber into the GC. The gas chromatograph was interfaced to a Hewlett Packard 5973 mass selective detector (mass spectrometer) operating at unit resolution. Data acquisition and data processing were performed on the Hewlett Packard ChemStation software system. Initial identification of the unknowns produced by B-23 was made through library comparison using the NIST database.

Fungal DNA isolation and acquiring ITS-5.8S rDNA sequence information

A 10 day old culture of B-23, growing on PDA, was used as a source of DNA after incubation at 25°C using the Rapid Homogenization: Plant leaf DNA Amplification Kit (Cartagen; Washington, USA). Some of the techniques used were comparable to those used



Figs 1-3. Scanning electron micrographs of *Muscodor crispans* isolated from *Ananas ananassoides*. **1.**Young hyphae at the colony edge showing the characteristic undulating young hyphal cells. **2.** Hyphal cells away from the colony edge showing fused hyphal cells. **3.** Fused hyphal cells and a cauliflower-like structure are shown. Scale Bars: 1 = 50 µm, 2 = 25 µm, 3 = 5µm.

to genetically characterize other *M. albus* isolates from Australia (Ezra *et al*., 2004a). Squares of the cultured mycelia (0.5 cm^2) were cut from one week old cultures. The agar was scraped from the bottom of the pieces, in order to exclude as much agar as possible. The pieces were placed into 1.5 ml Eppendorf vials and incubated for about 10 minutes at –80°C. The DNA was then extracted according to the instructions of the kit manufacturer. Extracted DNA was diluted (1:9) in double-distilled, sterile water and 1 µl samples were used for PCR amplification. The ITS1, 5.8S ITS2 rDNA sequence was amplified by the polymerase chain reaction using the primers ITS1 (TCCGTAGGTGAACCTGCGGG) and ITS4 (TCCTCCGCTTATTGATATGC). The PCR procedure was carried out in a 14 µl reaction mix containing 1 µl DNA extracted from the fungal culture (1:9 dilution), 0.5 µl primer ITS1 and 0.5 µl primer ITS4, 7 µl RedMixTM plus PCR mix with 1.5 mM $MgCl_2$ (Gene-Choice, Inc., Maryland, USA) and 5 µl ddH_2O PCR grade (Fisher Scientific, Wembley, Western Australia, Australia). The PCR amplification was performed in a Biometra personal cycler (Goettingen, Germany): 96°C for 5 minutes followed by 35 cycles of 95°C for 45 seconds, 50°C for 45 seconds and 72°C for 45 seconds, followed by a 72°C cycle for 5 minutes. The PCR products were examined using gel electrophoresis, on a 1.3% agarose gel for 30 minutes at 100V with TAE buffer (GelXLUltra V-2 from Labnet International, Inc., (Woodbridge, NJ, USA) or Wealtec GES cell system, from (Wealtec Inc., Georgia, USA). Gels were soaked in a 0.5 µg ml^{-1} ethidium bromide solution for 5 minutes and then washed in distilled water for 5 min. Gel imaging was performed under UV light in a Bio-Imaging System (model 202D; DNR-Imaging Systems, Kiryat Anavim, Israel). A ~ 500 bp PCR product was purified using the UltraClean PCR Clean Up DNA Purification Kit (MO BIO Laboratories, Inc., California, USA). Purified products were sent for direct PCR sequencing. Sequencing was performed on both strands of the PCR product using ITS1 and ITS4 primers. Sequencing was performed using a DYEnamic ET terminators on a MegaBACETM1000 analysis system (Danyel Biotech Ltd., Rehovot, Israel). Sequences were submitted to the GenBank on the NCBI web site (http://www. ncbi.nlm.nih.gov). Sequences obtained in this study were compared to the GenBank database using the BLAST software on the NCBI web site: http://www.ncbi.nlm.nih.gov/BLAST/).

Results and discussion

Fungal taxonomy

Muscodor crispans A.M. Mitch., Strobel, W.M. Hess, P.N. Vargas & Ezra, **sp. nov.** (Figs 1-3)

MycoBank: 511677

Etymology: The genus name, *Muscodor*, is taken from the Latin word which means musty. This is consistent with the quality of the odor produced by the first three isolates of the genus. The species name is – *crispans*, from the Latin meaning "curly, wavy." The hyphae of B-23 grow in regular undulating patterns (Fig. 1).

Fungus in natura cum *Ananas ananassoides* consociatus et est deuteromycete myceliis sterilibus pertinens. *Coloniae fungales* est rosae in vitro examinati in loco cum sol lux. *Sporae* velcorpora fructificantia sub statibus ullis non observata. *Hyphae* (0.6-2.7 µm) vulgo ramificantes et convolventes, fila stripformia et spiras perfectas (40 µm) formantes. *Hyphae novae* crescenti hyphae crescere undulatiforme sub omne orbi cum legum In vitro examiniter corpores colifloriform (3.5-14 µm) e repletus forma hyphae.

Fungus in nature is associated with *A. ananassoides* and is an anamorphic ascomycete with sterile mycelia. *Fungal colonies* whitish on all media when left out of direct sunlight. *Fungal colonies* pinkish on all media when put into direct sunlight. *Spores* or other fruiting bodies were not observed under any conditions. *Hyphae* (0.6-2.7 µm) commonly growing by branching, sometimes forming perfect coils (ca. 40 µm) and having cauliflower-like bodies (3.5-14 µm) associated with them. *Hyphae*, newly developing, grow in an undulating pattern when observed under all conditions with all of the media tested. *Mycelium* on PDA covers the plate in 3-4 weeks and produces a fruity odor.

The teleomorph of this fungus may be found in *Xylariaceae*, based on the similarity of the 18S rDNA gene sequence data between *M. crispans* and the family *Xylariaceae* in the GenBank database (Bruns *et al*., 1991; Reynolds and Taylor 1993; Mitchell *et al*., 1995; Guarro *et al*., 1999; Taylor *et al*., 1999). The molecular data from the 18S rDNA gene sequences of *M. crispans* show a 100% homology with *M. albus* isolate 620.

Holotype: The holotype is deposited in the living mycological culture collection of Montana State University as isolate No. 2347 (2/29/08). The collection is maintained in a metabolically inert state at –70°C. The organism was collected on *A. ananassoides* in the Bolivian Amazon in the Heath River area. The organism was successfully isolated from only one stem of this plant. It was collected by Percy Nunez Vargas and Gary A.Strobel. Both 18S rDNA and ITS sequences of *M. crispans* (B-23) have been submitted to GenBank with the assigned serial number-**EU195297**.

Teleomorph: Unknown.

Molecular biology of Muscodor crispans

The partial sequences of 18S rDNA, ITS1, 5.8S, and ITS2 have been demonstrated to be highly conserved regions of DNA and therefore very useful in the classification of organisms (Mitchell *et al*., 1995). These molecular distinguishing partial sequences of *M. crispans* were obtained and compared with the data in GenBank. After searching the 18S rDNA sequences, 525bp of *M. crispans* were subjected to an advanced blast search. The results showed 100% identity with 525 bp of *M. albus* (**AF324337**). Comparative analysis of the partial ITS 1&2 and 5.8S rDNA sequences of *M. crispans* hit ITS 1 and 2 of *M. albus* (AF324336), *M. roseus* (AY034664), *X. enteroleuca* CBS 651.89 (AF163033), *X. arbuscula* CBS 452.63 (AF163029), and *Hypoxylon fragiforme* (HFR246218) at 95, 95, 90, 90, and 91% homologies, respectively.

Fungal biology

The fungus produced a white mycelium on a water based medium. No fruiting structures or spores of any kind have been found under any laboratory conditions. Hyphae tend to intertwine to form rope-like coils. Other species of *Muscodor* also have this tendency (Worapong *et al*., 2001a; 2002). Newly developing hyphae tend to grow in an undulating fashion rather than the typical straight pattern and commonly intertwine to make coiled structures (Figs 1, 2). This pattern of growth may prove useful as a diagnostic tool in identifying this organism in *in-vivo* inoculation studies. The fungus also produces cauliflower-like structures that seem to be connected to the hyphae by small strands (Fig. 3). These bodies do not germinate under any conditions and thus appear not to be spores. This observation seems to be unique for *Muscodor* spp. and has not been noted as being present in any other fungal species in general. As measured by GC/MS, the fungus consistently produced alcohols, esters and small molecular weight acids, in the gas phase, when grown on PDA. Their presence mimics that of *M. albus*, but is not identical to it (Strobel *et al*., 2001). Some of these compounds include propanoic acid, 2-methyl; 1-butanol, 3-methyl, acetate; 1-butanol, and ethanol. Neither naphthalene nor azulene derivatives were produced by this organism when grown on PDA, which makes it different than all other *Muscodor* spp. that have been studied thus far. The odor produced by the fungus becomes noticeable after about 1 week and seems to increase with time up to and including at least three weeks. The volatiles of this fungus possess inhibitory and lethal bioactivity against a number of plant and human pathogens using the standard bioassay technique (Strobel *et al*., 2001). Details on the bioactivities of this interesting fungus will appear elsewhere.

Other, more classical features of *M. crispans* (isolate B-23) were also examined and compared to *M. albus*. *Muscodor crispans* produced a slow growing, dense, white colored mycelium on all media tested, unless it was placed in direct sunlight, which caused the mycelium to develop a light pink color. This contrasts to *M. albus* that produces a whitish mycelium on all comparable media and conditions tested (Worapong *et al*., 2001a). The young hyphae also grew in an undulating fashion, rather than the characteristic straight cable-like fashion as commonly observed with *M. albus* (Fig.1) (Strobel *et al*., 2001). No spores formed on any medium including ones containing the host plant material or carnation leaves. Hyphae varied in diameter (0.8-3.6 µm) and were often intertwined to make more complex structures and even hyphal coils (Figs 1-3). These hyphae were generally wider than those of *M. albus* (Worapong *et al*., 2001a).

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