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Production of the Alkaloid Swainsonine by a Fungal Endosymbiont of the Ascomycete Order Chaetothyriales in the Host *Ipomoea carnea*

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Supporting Information

ABSTRACT: Some plant species within the Convolvulaceae (morning glory family) from South America, Africa, and Australia cause a neurologic disease in grazing livestock caused by swainsonine. These convolvulaceous species including *Ipomoea carnea* contain the indolizidine alkaloid swainsonine, an inhibitor of α-mannosidase and mannosidase II, and polyhydroxy nortropane alkaloids, the calystegines which are glycosidase inhibitors. Swainsonine has been shown to be produced by a fungal endosymbiont in legumes of the *Astragalus* and *Oxytropis* genera, where it causes a similar neurologic disease in grazing livestock called locoism. Here we demonstrate that *I. carnea* plants are infected with a fungal endosymbiont that was cultured from its seeds and which produced swainsonine in pure culture but not the calystegines. The same fungal endosymbiont was detected by PCR and by culturing in *I. carnea* plants containing swainsonine. The fungal endosymbiont belongs to the Ascomycete order Chaetothyriales. Plants derived from fungicide-treated seeds lacked swainsonine, but calystegine concentrations were unaltered.

KEYWORDS: *Ipomoea carnea, Convolvulaceae, secondary metabolites, indolizidine alkaloid, swainsonine, calystegines, endosymbiont, vertically transmitted, Chaetothyriales, locoweed, fungal endophyte*

INTRODUCTION

Swainsonine (**1**) (Figure 1), an indolizidine alkaloid with significant physiological activity, is an alpha-mannosidase and mannosidase II inhibitor that alters glycoprotein processing and causes lysosomal storage disease.[1−3] Swainsonine is the toxic principle in a number of plant species worldwide and causes severe toxicosis in livestock grazing these plants.[1,4−6] Consumption of these plants by grazing animals leads to a chronic disease characterized by weight loss, depression, altered behavior, decreased libido, infertility, and death.[7] Swainsonine-containing plants are estimated to cause tens of millions of dollars in livestock losses annually.[8]

Swainsonine occurs sporadically in three diverse plant families: Fabaceae (Fabales), Malvaceae (Malvales), and Convolvulaceae (Solanales). In the Convolvulaceae, some *Ipomoea* and *Turbina* species, including *I. carnea*, *I. riedelli*, *I. sericophylla*, and *T. cordata*, have been reported to contain **1**.[6,9,10] Only a single species of Malvaceae, *Sida carpinofolia*, has been reported to contain **1**.[11] Lastly, some *Astragalus*, *Oxytropis*, and *Swainsona* species of the legume family (Fabaceae) have also been reported to contain **1**.[1,4,12]

Like **1**, the calystegines, polyhydroxy nortropane alkaloids, occur sporadically among a number of species of the Convolvulaceae and Solanaceae plant familes.[13] Calystegines are glycosidase inhibitors and may contribute to the toxicity of plants that contain them.[5,9,10] Some convolvulaceous species including *I. carnea* and *I. riedelli* are reported to contain both **1** and calystegine B_1, B_2, and C_1, (**2**−**4**) (Figure 1), while others such as *I. sericophylla* and *T. cordata* are reported to contain **1** but do not contain **2**−**4**.[6,10,13,14] The sporadic occurrence of some natural products such as **1** and the calystegines in plants brings into question their biosynthetic origin and significance as chemotaxonomic markers. Three mechanisms could explain the sporadic occurrence of natural products such as **1** in unrelated taxa:[15] first, the biosynthetic pathways of a natural product may have originated multiple times over evolutionary history; second, the genes responsible for the biosynthesis of a natural product may have been horizontally transferred between distantly related taxa;[16] or third, the natural product may be produced by microbes associated with a range of plants.

In the genera *Astragalus* and *Oxytropis*, fungal endophytes of the ascomycete genus *Undifilum* (Pleosporales),[17,18] previously described as *Embellesia* species,[19] have been reported to be responsible for production of **1**.[20] *Undifilum* spp. are vertically transmitted endophytes[21,22] and concentrations of **1** in the host plant are correlated to the amount of endophyte.[23,24] In addition to *Undifilum*, **1** is produced by two other fungi: a pathogen of red clover (*Trifolium pratense*), *Rhizoctonia leguminicola* (Cantharellales), a Basidiomycete, that causes black patch disease[25] and the entomopathogen *Metarhizium anisopliae* (Hypocreales), an Ascomycete, that attaches to the

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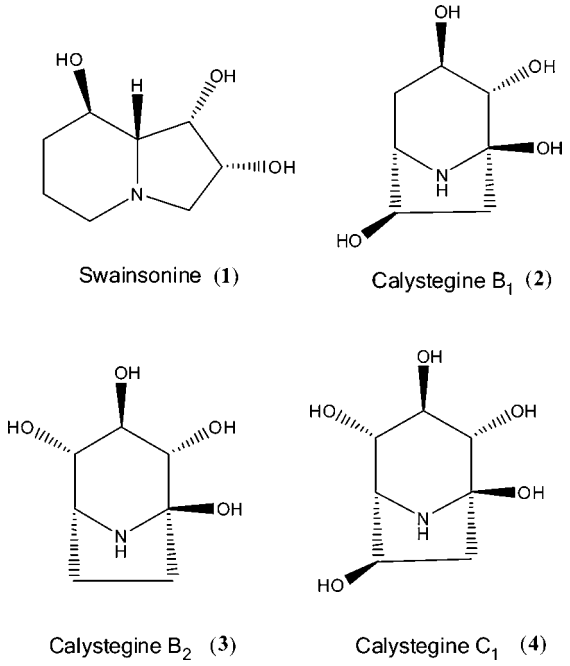

Figure 1. Structures of the indolizidine alkaloid swainsonine (**1**), and the polyhydroxyl nortropane alkaloids, calystegine B_1 (**2**), calystegine B_2 (**3**), and calystegine C_1 (**4**).

outside of an insect, grows internally, and causes death.[26] Swainsonine therefore occurs in disjunct fungal and plant orders.

Based upon the association of **1** and fungi we hypothesized that convolvulaceous species containing **1** are host to a fungal endosymbiont that produces **1**. Here we report the examination of *I. carnea*, a plant of pantropical distribution that contains **1** and the calystegines, **2**–**4**, for endophytes that may be responsible for production of some or all of the metabolites **1**–**4**. Specifically, we addressed the following questions: (1) Do *I. carnea* plants derived from fungicide-treated seeds contain **1** and/or **2**–**4**? (2) Do *I. carnea* seeds contain a fungal endosymbiont that produces **1** and/or **2**–**4**?

MATERIALS AND METHODS

Plant Material. *Ipomoea carnea* (Jacq.) subsp. *fistulosa* (Mart. ex Choisy) seeds were collected in April 2011 near the veterinary hospital of the University of Campina Grande, Campus of Patos in the city of Patos, Paraiba, Brasil (S 7° 04′ 02″ W 37° 16′ 53″), and were deposited at the Intermountain Herbarium at Utah State University (UTC 00260470). *Ipomoea carnea* seeds were scarified and imbibed overnight in water. Plants (n = 25) derived from the above-mentioned seeds were grown in the greenhouse with a 16 h photoperiod and day/night temperatures of 25 °C/20 °C. Leaves from the plant were harvested and frozen at −80 °C. Subsequently, the harvested leaves were freeze-dried and ground. Swainsonine (**1**) and DNA were extracted from this plant material for further analyses.

Fungicide Treatment. *Ipomoea carnea* seeds (n = 15) were scarified and imbibed overnight in 0.9% pyraclostrobin (BASF, Research Triangle Park, NC) solution to determine if a seed-associated fungus is present. Pyraclostrobin, a strobilurin class fungicide, has a mode of action shown to be effective against a broad range of fungal species including a large number of Ascomycetes. Following treatment, seeds were potted and the resulting plants were grown in the greenhouse.

Fungal Cultures. Fungal endosymbiont cultures (n = 3) were obtained from seeds of *I. carnea* by the followings means: scarified seeds were surface sterilized in a mixture of 70% ethanol, 30% bleach, and 0.01% Triton X-100 (Promega, Madison, WI) solution for 5 min with constant agitation, followed by a second step of 30% bleach and 0.01% Triton X-100 for 5 min, and a third step wherein the seeds were rinsed two times in sterile water. Seeds were bisected using aseptic technique and the halves were placed on potato dextrose agar (Becton, Dickinson and Company, Sparks, MD) and placed in the dark at 24 °C for 2–3 weeks. Subsequently, equal portions of the culture were subcultured onto preweighed sterile filter paper that was placed on potato dextrose agar plates. After 41 days the culture plus filter paper and the agar were removed separately and freeze-dried. The mass of the fungal culture was calculated by subtracting the mass of the filter paper alone from the mass of the culture plus filter paper. Swainsonine amounts were determined for the fungal culture plus filter paper and agar to determine the percent **1** produced by the endosymbiont. A voucher specimen of the endosymbiont isolated from seeds of *I. carnea* was deposited at the fungal collection of the Intermountain Herbarium at Utah State University (UTC 00260470). Immature leaves of *I. carnea* (folded) were surface sterilized as described above, after which the leaf was unfolded and placed with the upper surface face down on the surface of a Petri plate (n = 3). Culturing conditions for leaves were the same as those described for seeds.

Swainsonine and Calystegine Analysis. Swainsonine was extracted using a modification of the procedure described by Gardner and Cook.[27] Dried plant material (50 mg), freeze-dried mycelia plus filter paper (whole), and freeze-dried agar (150 mg) were extracted in 2% acetic acid for 18 h with agitation. After extraction, samples were centrifuged and an aliquot from the extraction was diluted into 20 mM ammonium acetate in a 1 mL autosampler vial. Samples were analyzed by LC-MS/MS to quantitate **1** as previously described.[28] For endophyte samples, total **1** in the freeze-dried agar, and mycelia plus filter paper was calculated and expressed as a percent of the mycelial mass. The detection limit of **1** was 0.001% of dry weight using this extraction procedure.

A subset of samples (n = 8 per treatment, samples were selected using a stratified approach based on concentration of **1**) was analyzed for **2**–**4**. In brief, a 0.5 mL aliquot of the acetic acid extract was added to a strata-XC (30 mg) SPE column that was prerinsed with methanol and water (2 mL each). The SPE columns were rinsed again with water and methanol (2 mL) and the calystegines were eluted with 3 mL of ammoniated methanol (1 to 5 dilution of methanol saturated with NH_3). The extract was evaporated to dryness and 1.0 mL of methanol added. A 200 μL aliquot was added to an autosampler vial and evaporated to dryness. Pyridine (200 μL) and BSTFA silylation reagent (50 μL) were added and the sample was heated (60 °C) for 30 min. Samples were diluted to 1.0 mL with chloroform and then analyzed by GC-MS for **1** (tri-TMS derivative) and calystegine-TMS derivatives using previously described methods.[28] The concentration of each **2**–**4**, was estimated based on the peak area versus the peak area for **1** for which the concentration was predetermined by LC-MS. Additionally the GC-MS method described above was used to verify the identification of **1** in the fungal endophyte.

Light Microscopy. Epiphytic mycelia were visualized using an acetate peel technique modified from White et al.[29] The adaxial leaf surface was stained with a 2:1 solution of 1% aqueous aniline blue and 85% lactic acid.[30] The stain was allowed to set for 1 min, after which it was gently rinsed off and the leaf was allowed to dry. Next, a thin coat of clear nail polish was painted over the stained mycelia, allowed to set and then peeled off and mounted in polyvinyl-lacto-glycerol. Images were captured by a Nikon DXM1200C digital camera on an Eclipse E600 Microscope (Nikon Instruments, Melville, NY).

Scanning Electron Microscopy (SEM). The specimens (whole leaves) were fixed in 2.5% glutaraldehyde, dehydrated in a graded series of ethanol and critical point dried in a Balzers CPD30 unit using CO_2. The mounted leaf pieces were coated with gold/palladium in a Polaron ES100 sputter coater and examined with a JEOL JSM-5800 scanning electron microscope (JEOL Company, Tokyo, Japan) at the Indiana Molecular Biology Institute.

DNA Extraction. DNA was extracted from freeze-dried, ground plant material (~20 mg) and mycelia from cultures (~20 mg) using the DNEasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Extractions

were performed according to the manufacturer's instructions. DNA was quantified with the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

PCR Primers. The PCR primers ITS1F[31] and ITS4[32] were used to amplify the internal transcribed spacer of the rDNA (ITS). NSSU97A[33] and NS24[31] were used to amplify the small subunit of the rDNA (SSU). NMS1 and NMS2[34] were used to amplify a portion of the small subunit of the mitochondrial rDNA (mtSSU). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

PCR. All PCR was performed with a Bio-Rad Dyad PCR detector (Bio-Rad Laboratories Inc., Hercules, CA). Thermal cycling conditions for the fungal endosymbiont were as follows: (1) for ITS, an initial denaturation step for 3 min at 94 °C, followed by 40 cycles of 33 s at 94 °C, 50 s at 57 °C, and 30 s at 72 °C; (2) for SSU, an initial denaturation step for 3 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 60 s at 45 °C, and 30 s at 72 °C; and (3) for mtSSU, an initial denaturation step for 3 min at 94 °C, followed by 40 cycles of 33 s at 94 °C, 50 s at 54 °C, and 30 s at 72 °C. A final extension of 5 min at 72 °C was performed for all programs. Each PCR reaction had a total volume of 50 μL containing 50 ng (5 μL of a 10 ng/μL stock) of total DNA. GoTaq DNA polymerase (Promega Corporation, Madison, WI) was used in reaction conditions recommended by the manufacturer. PCR products were resolved on a 1% agarose gel containing ethidium bromide at 118 V for 20 min and visualized under UV illumination. Agarose gels were visualized and analyzed with a Kodak Image Station 2000RT imager and its software (Eastman Kodak, Rochester, NY).

DNA Sequencing. PCR products were prepared for sequencing with the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The ITS region was sequenced using both the forward and reverse primers used for amplification. DNA sequencing was performed at Eton Bioscience, San Diego, CA and the Indiana University Molecular Biology Institute, Bloomington, IN.

Phylogenetic Analysis. A composite sequence of the internal transcribed spacer of the rDNA (ITS), small subunit of the rDNA (SSU), and small subunit of the mitochondrial rDNA (mtSSU) was generated using Sequencher v4.9 (GeneCodes Corporation, Ann Arbor, MI) for 19 sequences from different species in the Chaetothyriomycetidae. Alignment of the sequences, phylogram generation using the NJ method[35] and bootstrap testing using 1000 replicates[36] were conducted in MEGA5.[37] The evolutionary distances were computed using the Maximum Composite Likelihood method[38] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 2051 bases in the final data set.

Data Analysis. All statistical comparisons were done using ANOVA in Sigma Stat 3.1 with a posthoc test of significance using the Bonferroni correction. A p-value of <0.01 was considered to be statistically significant. Mean values reported are ± the standard error. Bar plots were constructed using Sigma Stat 3.1.

RESULTS AND DISCUSSION

Previous phytochemical investigations have shown that *I. carnea* contains **1** and the calystegines, **2**−**4**.[14] Plants derived from fungicide-treated seeds contained no detectable **1**, while control plants derived from seeds that were not treated with a fungicide had a mean **1** concentration of 0.03 ± 0.004% (Figure 2), ranging from being not detected to 0.07%. This resulted in a significant difference for concentrations of **1** between control plants and plants derived from fungicide-treated seeds (P < 0.001). The calystegines, **2**−**4**, occurred in plants derived from fungicide-treated seeds at mean relative concentrations of 0.03, 0.08, and 0.008% respectively, and in plants from nonfungicide treated seeds at 0.05, 0.08, and 0.01%, respectively (Figure 2). There was no significant difference (P > 0.35) in relative concentrations of **2**−**4**, between control plants and plants derived from fungicide-treated seeds (Figure 2). These

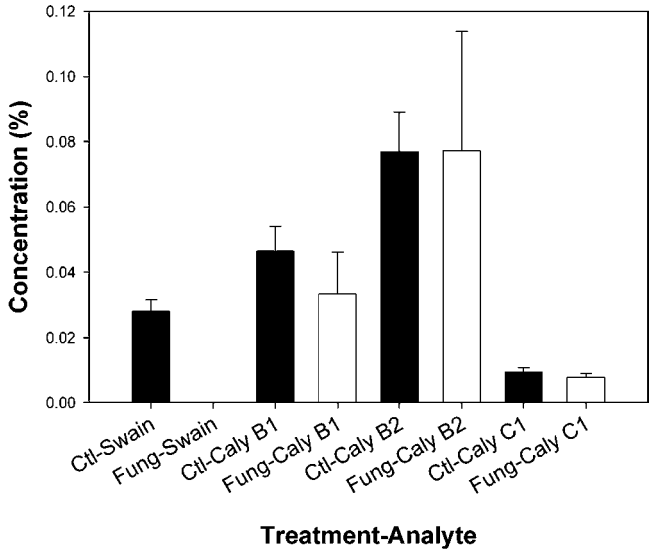

Figure 2. Concentrations (%) of swainsonine (swain) and the calystegines (caly) in *Ipomoea carnea* control plants (Ctl) and plants derived from fungicide-treated seeds (Fung).

concentrations are consistent with previous reports for *I. carnea* and concentrations of **1** in some locoweeds.[12,14] These results suggest that a fungal presence was necessary for **1** to be detected from *I. carnea*, but that fungal presence did not influence **2**−**4** production implying these alkaloids were made by the plant.

The lack of **1** in plants derived from fungicide-treated seeds suggested the presence of a seed-transmitted fungal endosymbiont capable of producing **1**. A fungal endosymbiont was cultured from surface sterilized *I. carnea* seeds. The fungal endosymbiont produced **1** *in vitro* as verified by GC-MS[39] (Figure 3) and LC-MS/MS.[28] Mean concentrations of **1** *in vitro* were 2.0 ± 0.8% (n = 3) with a range of 0.7−3.3%. The fungal endosymbiont did not produce detectable **2**−**4** *in vitro* as verified by GC-MS.[39] Endosymbiont isolates from *I. carnea* were brown to light brown at margin of growth (data not shown) and were slow growing on potato dextrose agar. Endosymbiont isolates had a radial growth rate of 0.17 ± 0.01 mm/day, a total radial growth of 7.1 ± 0.5 mm, and a total biomass of 34.3 ± 2.1 mg (41 days on potato dextrose agar). These growth rates were similar to those reported for swainsonine-producing *Undifilum* species.[17,18]

Macroscopic inspection of the upper leaf surface revealed the presence of whitish mycelia on all the control plants that contained **1** but no detectable mycelia on plants derived from fungicide-treated seeds or control plants where **1** was not detected (Figure 4). Microscopic inspection of the leaf surface further confirmed the presence of mycelia on the control plants and their absence among plants derived from fungicide-treated seeds (Figures 5 and 6). Upon further analysis using SEM, mycelia appeared to be loosely associated with secretory glands present on the leaf surface (Figure 6). To determine if this fungus present on the leaf surface of plants that contained **1** and absent on plants derived from fungicide-treated seeds was the **1** producing endosymbiont cultured from seeds, fungal hyphae were scraped from the surface of the leaf and the ITS sequence was amplified. The amplified PCR products were sequence verified to be identical to the fungal endosymbiont cultured from seeds. Additionally, the endosymbiont isolated from seeds of *I. carnea* was detected *in planta* using two other

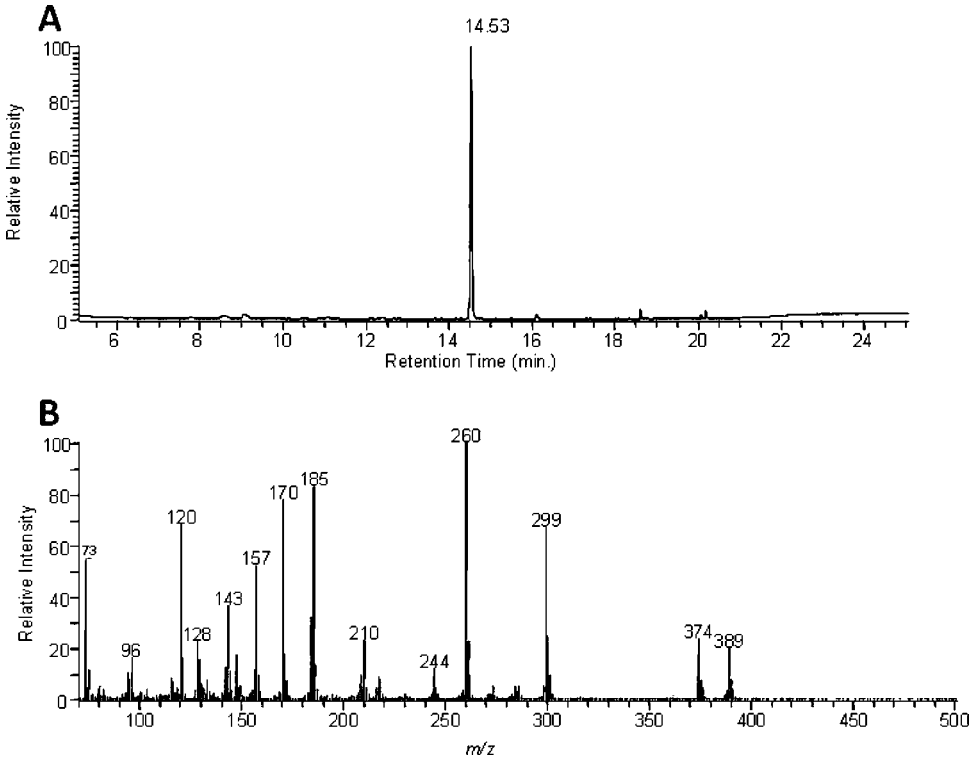

Figure 3. (A) GC-MS chromatogram and (B) mass spectrum of swainsonine, as the tri-TMS derivative (R_t = 14.53 min) produced from the endosymbiont of *Ipomoea carnea*.

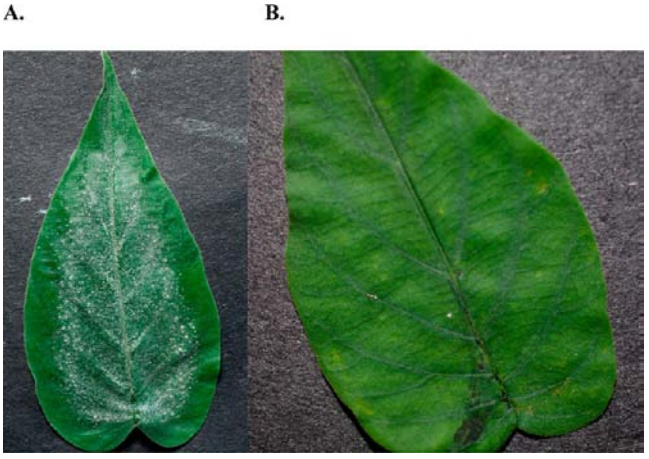

Figure 4. Photograph of *Ipomoea carnea* leaves from plants derived from (A) nontreated seeds and (B) fungicide-treated seeds.

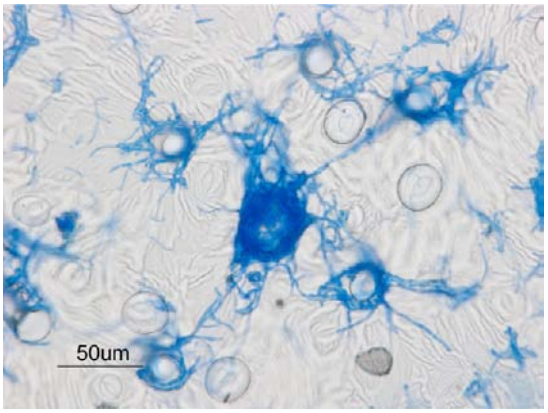

Figure 5. Micrograph of *Ipomoea carnea* leaves from plants derived from nontreated seeds. Leaves were stained with aniline blue to reveal fungal mycelia (Size bar = 50 μm).

approaches. First, a fungus morphologically similar to the endosymbiont cultured from *I. carnea* seeds was cultured from leaves of *I. carnea* containing **1** and the ITS sequence was amplified. Second, the endosymbiont was detected by PCR through amplification of the ITS in leaf samples of *I. carnea* where **1** was detected. In each case the amplified PCR products were sequence verified to be identical to the fungal endosymbiont cultured from seeds. In summary, these results provide evidence that the endosymbiont isolated from *I. carnea* seeds is seed transmitted to the plant like vertically transmitted endophytes, and produces epiphytic mycelia on the upper leaf surface.

An alignment of a composite sequence of the internal transcribed spacer of the rDNA (ITS), small subunit of the rDNA (SSU), and small subunit of the mitochondrial rDNA (mtSSU) for 19 species of the Ascomycete sub class Chaetothyriomycetidae including the isolates (n = 3) from *Ipomoea carnea* resulted in a 2051-character data set with 904 (44.1%) variable characters and 555 (27.1%) variable informative characters. All three isolates of the endosymbiont from *I. carnea* yielded identical sequences of ITS, SSU, and mtSSU. Neighbor joining analysis resulted in placement of the endosymbiont isolated from *I. carnea* in the ascomycete order Chaetothyriales (Figure 7). The order Chaetothyriales represents nonlichenized Ascomycetes composed of two families, the Chaetothyriaceae and Herpotrichiellaceae.[40,41] The Herpotrichiellaceae family in the phylogenetic tree has a bootstrap support of 100 (Figure 7) and is represented by *Chaetothyriales* species TRN247, *Capronia* species WUC102,

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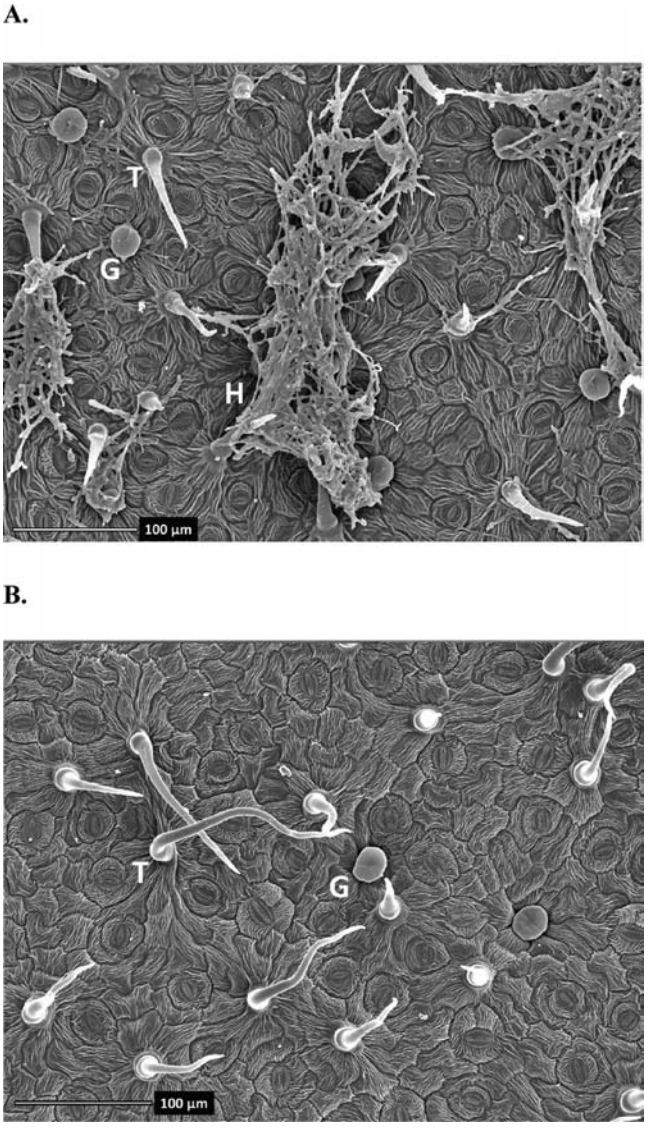

Figure 6. SEM micrograph of *Ipomoea carnea* leaves from plants derived from (A) nontreated seeds and (B) fungicide-treated seeds. The letters G, H and T correspond to the peltate secretory glands, fungal hyphae, and trichomes, respectively (Size bar = 100 μm).

three *Exophiala* species, two *Cladophialophora* species, and *Fonsecaea monophora*. The Chaetothyriaceae family is represented by *Conioporium perforans* while other members on the tree with the exception of outgroup (Pyrenulales) are represented by unclassified Chaetothyriales species. The endosymbiont from *I. carnea* is distantly related to the Herpotrichiellaceae, the unclassified Chaetothyriales species, and *Conioporium perforans* as seen by the branch length. Its precise placement on the tree is uncertain due to the lack of bootstrap support (Figure 7). Based on its placement in the tree we suspect the endosymbiont from *I. carnea* is a member of the Chaetothyriaceae. In general, the Chaetothyriaceae are epiphytes and/or saprophytes associated with plants as well as a number of slow-growing melanized fungi inhabiting rocks.[40,41] On the other hand, the Herpotrichiellaceae are opportunistic human pathogens in the sexual stage (teleomorph) while in the asexual stage (anamorph), they are saprophytes growing on decaying wood, plants, or other substrates.[40,41]

In addition to **1**, ergot alkaloids have been shown to occur in select *Ipomoea* species.[15,42] Species of fungi in the genus *Periglandula* are members of the Clavicipitaceae (Ascomycota: Hypocreales) that produce ergot alkaloids and are associated with certain morning glories. *Periglandula* spp. are vertically transmitted through seeds, and produce ephemeral epiphytic mycelia on the upper surfaces of young leaves.[15,43] Similarly, the phylogenetically disjunct endosymbiont of the order Chaetothyriales isolated from *I. carnea* produces **1**, is vertically transmitted through seeds, and produces epiphytic mycelia on the upper leaf surface, although it appears to persist longer than epiphytic mycelia produced by *Periglandula*. The endosymbiont isolated from *I. carnea* does not appear to be horizontally transmitted. Both control plants and those derived from fungicide-treated seeds growing next to each other in the greenhouse have maintained the same chemical phenotype for greater than a year and macroscopic inspection of the leaf surface does not reveal the presence of any hyphae on the fungicide-treated plants as reported here. We find it striking that distantly related fungi (Hypocreales and Chaetothyriales) that produce different alkaloids (ergot alkaloids and **1**) have converged upon a similar life history within the same plant family. Also noteworthy is that plant families containing ergot alkaloids, including the monocotyledonous grasses (Poaceae) and sedges (Cyperaceae) and the dicotyledonous morning glories (Convolvulaceae), are associated with clavicipitaceous fungi, while two of the plant families containing **1**, the legumes (Fabaceae) and morning glories (Convolvulaceae), are associated with two phylogenetically disjunct groups of fungi, the Pleosporales and Chaetothyriales. Most examples of vertically transmitted fungi that produce bioactive chemicals are known from having adverse effects on livestock, but this life history strategy may be much more common in nature than currently known.[44] Lastly, while we refer to the fungus from *I. carnea* as an endosymbiont, the term fungal endophyte can also apply as it is commonly used in the literature to describe a variety of symbiotic plant-associated fungi, including those that also produce epiphytic structures.[45]

In summary, *I. carnea* plants derived from fungicide-treated seeds lack **1** while control plants contain **1**. Calystegine concentrations did not differ between control plants and plants derived from fungicide-treated seeds, suggesting these compounds are produced endogenously by the plant and are not influenced by the presence of the fungal endosymbiont. A fungal endosymbiont was isolated from seeds and leaves of *I. carnea* that produces **1** *in vitro* and no detectable **2**−**4**. This endosymbiont belongs to the Ascomycete order Chaetothyriales and is phylogenetically distinct from other members of this fungal family described to date. It is possible that other *Ipomoea* species reported to contain **1** may contain an endosymbiont related to the one described herein as is the case for related *Undifilum* spp. found across *Astragalus* and *Oxytropis* locoweed host plants.[17,18] Given what we now know about the fungal origin of **1** in the Fabaceae and *I. carnea,* the occurrence of **1** in other species of the Convolvulaceae and at least one species in the Malvaceae could be explained by the presence of a swainsonine-producing endosymbiont, but this remains to be investigated. In total, this study provides another example of a bioactive secondary metabolite produced by a vertically transmitted fungal symbiont.[44] The extent to which this association plays a role in determining host fitness of *I. carnea* should be of special interest going forward.

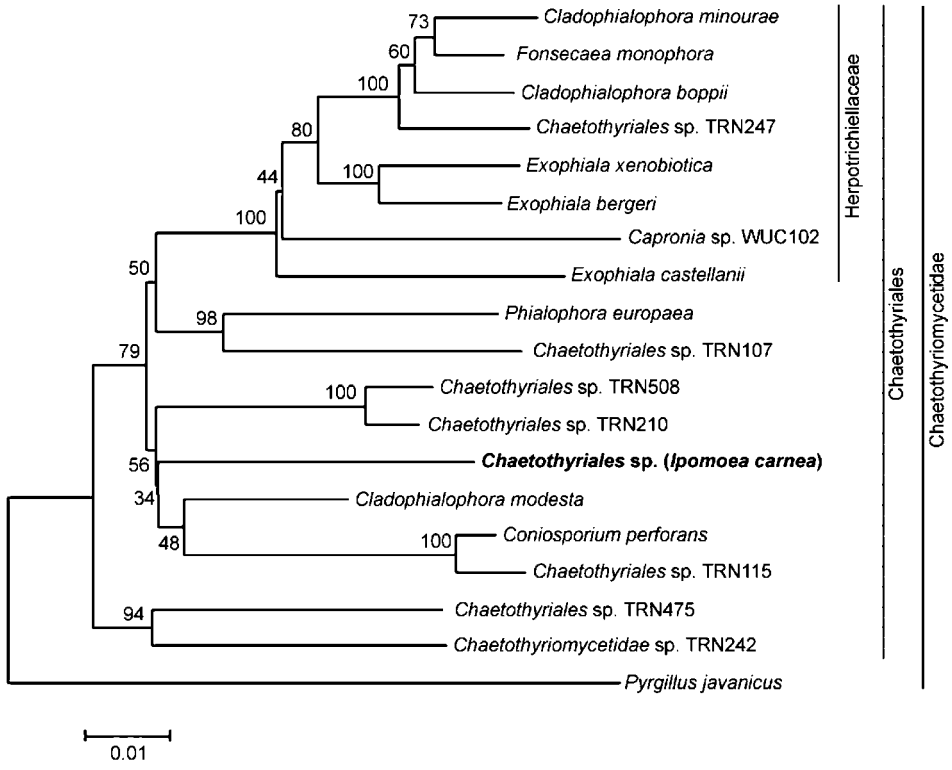

Figure 7. Neighbor joining bootstrap consensus tree showing phylogenetic placement of the endosymbiont isolated from *Ipomoea carnea*. The scale indicates the number of base substitutions per site.

■ ASSOCIATED CONTENT

Supporting Information

Table S1. Isolates used for phylogenetic analyses, their sources, and GenBank accession numbers. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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