Endophyte Strain NRRL 50072 producing volatile organics is a species of Ascocoryne

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A fungal endophyte (NRRL 50072) isolated from Eucryphia cordifolia from Patagonia that produces hydrocarbon derivatives is identified as an Ascocoryne sp. It was classified as Clonostachys rosea (Gliocladium roseum) strain C-13 = NRRL 50072 primarily based upon its morphological characteristics. The fungus produced slimy clumped conidia on verticillated conidiophores and red-pigmented particles in culture. However, DNA sequence analysis (ITS rDNA) suggested a 99% identity to Ascocoryne sarcoides rather than to fungi assigned to the group of Gliocladium-like anamorphs. Comparative genetic, biological and morphological studies confirmed that the anamorphic stage of an authenticated field-collected culture of Ascocoryne sarcoides AV-70 shares many of the same morphological, and genetic features as NRRL 50072, but the latter is unable to produce synnematal masses unless it is grown on a proper substratum. These data suggest that NRRL 50072 is most closely related to the asexual stage of A. sarcoides, namely a Coryne sp. Serial transfer of the A. sarcoides AV-70 resulted in major cultural changes in the fungus especially in the production of aerial hyphae, pigment production and the number of synnemata being formed. This report appropriately sets the taxonomic framework for further molecular biological and biochemical work on NRRL 50072 and related fungi.

Keywords: endophytes; hydrocarbons; rDNA; synnema; synnematal masses

Introduction

Examination of the volatile organic compounds (VOCs) produced by an endophytic isolate (NRRL 50072 =C-13) revealed the presence of several acids, alcohols, esters and ketone derivatives of alkanes and other assorted substances in the gas phase which were responsible for the biological activity of this organism (Strobel et al. 2008). The biological activity of VOCs has been observed in a number of fungi with the most potent activity observed in strains of Muscodor albus. This organism was originally used as a selection tool in successfully isolating strain NRRL 50072 (Strobel et al. 2001; Stinson et al. 2003). This series of compounds has not been observed in M. albus, thus the mode of action of its VOCs is probably not equivalent to that of M. albus.

One uncertainty about the previous work was the taxonomy of NRRL 50072. By morphology, the fungus was identified as Clonostachys rosea (=Gliocladium roseum). However, the 5.8 S, ITS1 and ITS2 rDNA sequences were nearly identical to Ascocoryne sarcoides (Jacq.) J.W. Groves &D.F. Wilson (anamorph Coryne dubia (Pers.)Gray) (Strobel et al. 2008). Various Gliocladium-like fungi are known as the imperfect stages of other wood inhabiting fungi such as Sphaerostilbella, Hypocrean and Nectria, but teleomorph genus Ascocoryne are not associated with Gliocladium-like anamorphs (Hanlin 1990). A series of attempts to get more morphological information on NRRL 50072, using various natural and synthetic media to encourage it to form teleomorphic or other synanamorph stages were unsuccessful. Thus, armed with the knowledge that the original isolate of NRRL 50072 was genetically related to A. sarcoides it was deemed important to find a wild type isolate of this organism in ecosystems for which it has been commonly reported and then determine its relationship to NRRL 50072 (Strobel et al. 2008). Ascocoryne species are most likely found in the fall seasons during periods of high moisture in northern and southern hemisphere temperate forests rich in species of the Fagaceae (beeches and oaks) and Pinaceae (pines firs and spruces). Ascocoryne sarcoides usually appears in the form of pink gelatinous masses that possess delicate apothecia on dead fallen trees suggesting that it is a saprophyte, although its trophic relationships remain unknown (Breitenbach and Kranzlin 1984; Bunyard et al. 2008). Ascocoryne species have long been known to inhabit living wood of a wide range of tree species and then upon the death of the host plant begin to grow and fruit on the dead wood (Basham 1973; Delatour and Sylvestre 1976; Roll-Hansen and Roll-Hansen 1976). Some evidence exits that the endophytic stage of A. sarcoides may antagonize and displace other wood-decay fungi (Basham 1975).

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Apothecia of *A. sarcoides* were found in abundance near Marysville in the Yarra Ranges, Victoria, Australia. The fungus was authenticated by morphological, and genetic techniques and then compared with NRRL 50072. Thus, the main purpose of this report is to demonstrate that NRRL 50072, reported to make hydrocarbon derivatives is an *Ascocoryne* sp. closely related to the anamorphic stage of *Ascocoryne sarcoides* (Strobel et al. 2008).

**Materials and methods**

**Fungal isolation, storage and culturing**

The culture of *A. sarcoides* (AV-70) used in this study was obtained from freshly growing ascocarps located on a dead tree stump of *Nothofagus cunninghamii* in the Yarra Ranges, Victoria, Australia (37° 29′ 415″ S° 145° 50 435″E). A purified culture of *A. sarcoides* was obtained from typical apothecial tissue on potato dextrose agar (PDA) and streaking multiple dilutions over the agar surface. The organism also grew in the presence of the volatile-antibiotic producing fungus, *Muscodor albus* which produces multiple VOCs with potent antibiotic activities (Strobel et al. 2001; Stinson et al. 2003; Strobel et al. 2007). The VOCs from *M. albus* effectively prevented the development of many contaminating microbes that may grow on the agar plates with *A. sarcoides* allowing for its ready isolation. Thus, the selective antimicrobial activity of *M. albus* may aid in the isolation of *Ascocoryne* spp. from other ascocarps associated with *Ascocoryne* spp. (Strobel et al. 2001). Eventually, pinkish red colonies developed that are characteristic of *A. sarcoides* (Groves & Wilson 1967; Delatauor and Sylvestre 1976; Seifert 1989). Picked colonies were restreaked to ensure purity.

The fungus was stored as mycelial agar plugs from PDA in 15% glycerol –70°C. The best storage condition, however, was obtained by growing *A. sarcoides* AV-70 on sterilized barley seed, drying the seed, and placing directly at –70°C. The fungus was deposited in the living Montana State University mycological collection as acquisition number –2369. The organism is also deposited in the Victoria Plant Pathology Herbarium as VPRI 41595. The ITS region of the AV-70 DNA was sequenced and deposited in GenBank as (GQ500107).

Bioactivity of AV-70 volatiles against the target oomycete, *Pythium ultimum* was tested on several media (Strobel et al. 2008). Each inhibition test was performed in triplicate and the data were analyzed to yield average inhibition and standard deviation information. Finally, *N. cunninghamii* stem (beech stem) medium produced by boiling 30 g of newly developing stem tissues in water for 30 min, filtering and then adding water to 1 L (Strobel et al. 2008).

*Ascocoryne sarcoides* strain AV-70 was also tested for changes in cultural phenotype during multiple serial transfers. Color, aerial mycelium, and synnematal mass production on oatmeal, cellulose and beech stem media were evaluated (Strobel et al. 2008). Experimentally, three whole mycelial transfer cultures were used and observed. All cultures were 8 weeks old at the time of the beginning of the experiment. Then cultures were transferred weekly and plates incubated at 22°C for 8 to 9 weeks prior to evaluation of phenotypic drift. Synnematal masses were counted in a 78 mm² area. Dry weights were measured from harvested and oven dried (35°C) aerial mycelia. In each case, cultures were transferred 11 times. Data were collected from each of the plates of any given specific transfer and then averaged and statistically analyzed.

**Scanning electron microscopy (SEM and ESEM)**

*A. sarcoides* AV-70 was grown on PDA, or autoclaved *N. cunninghamii* stems for several weeks and then was processed for SEM. The samples were slowly dehydrated in ethanol as previously described and critically point dried, coated with gold and examined with a FEI XL30 scanning electron microscope (SEM) (Stinson et al. 2003).

Because the spores and other fruiting structures of the fungus were fragile and easily disrupted, the organism was subjected to the relatively unique microscopic application which preserves the intact conidiophores with conidia. Thus, fresh or non-treated specimens were examined by environmental scanning microscopy (ESEM) and images were recorded with a FEI XL30 ESEM FEG. The temperature was 4°C with a chamber pressure which ranged from 5 to 6 Torr providing humidity up to 100% at the sample. All measurements of fungal structures made from SEM images (Strobel et al. 2007).

**Fungal DNA isolation and acquisition of ITS- 5.8S rDNA phylogenetics**

Mycelium grown on PD broth for 7 days was harvested and the genomic DNA extracted using DNeasy Plant and Fungi Mini Kit (Qiagen), according to the manufacturer’s directions. The ITS region of the fungus was amplified using PCR with the universal ITS primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (Ezra et al. 2004). All other procedures were carried out as previously described by Ezra et al., 2004. The DNA was sequenced at the W.M. Keck Facility at Yale University, and the sequences were submitted to GenBank. Sequences were compared to the GenBank data bases using BLAST.

Selected sequences were downloaded from Genbank and aligned with NRRL 50072 and AV-70 using
with a hymenial layer (150–200 μm) (Strobel, unpublished). It was apparent from the ESEM that individual conidiophores were highly branched with characteristic sub-globose to ellipsoidal conidia ranging from 2.2–3.1 × 0.7–1.0 μm on conidiophores that were highly branched with distinctive phialides (8–20 × 0.8–1.2 μm) (Figures 1B–E). It was apparent from the ESEM that individual conidia formed on highly branched phialidic conidiophores. This organism also formed individual classical synnema characteristic of the species (Figure 1C, D). The individual synnema and conidiophores fused to produce macroscopically visible large synnematal masses covered with a hymenial layer (150–200 μm in dia.) (Figure 1F) (Seifert 1989).

Results and discussion

Identification and characterization of Ascocoryne sarcoides strain AV-70

A. sarcoides AV-70 was obtained from a large fallen Notothofagus cunninghamii (Southern Beech) tree in a ravine near a stream bed. In this area many fallen beeches were present and nearly all displayed reddish gelatinous apothecia. A close visual examination of the apothecia (Figure 1A) revealed that they were identical to those described in the literature (Breitenbach, & Kränzlin 1984; Seifert 1989; Groves & Wilson 1967). When grown in pure culture the colony turned reddish purple after 10 days with a multitude of reddish purple particles deposited in the periphery of the fungal colony (Delatour and Sylvestre 1976; Seifert 1989; Chen and Huang 2004). Furthermore, within a week of incubation, the culture began to produce conidiophores with characteristic sub-globose to ellipsoidal conidia ranging from 2.2–3.1 × 0.7–1.0 μm on conidiophores that were highly branched with distinctive phialides (8–20 × 0.8–1.2 μm) (Figures 1B–E). It was apparent from the ESEM that individual conidia formed on highly branched phialidic conidiophores. This organism also formed individual classical synnema characteristic of the species (Figure 1C, D). The individual synnema and conidiophores fused to produce macroscopically visible large synnematal masses covered with a hymenial layer (150–200 μm in dia.) (Figure 1F) (Seifert 1989).

Comparative biology and phylogenetics of Ascocoryne sarcoides AV-70 and NRRL 50072

In culture, the two organisms (NRRL 50072 and AV-70) share cultural characteristics on common lab media, and both have nearly identical conidial morphologies including conidiophores with identical arrangement of the phialides (Figures 1B, 2A). Both produce mucilaginous conidial masses (Figures 1B, E) and reddish to purple pigment (identical between each via TLC chromatography which was probably the terphenylquinone, ascocorynin, (Chapman & Hall Chemical Data Base, http://library.dialog.com/blushsheets/ html/b0303.html) (Strobel, unpublished).

However, A. sarcoides AV-70 has an anamorphic stage which is Coryne dubia and this strain agrees well with various descriptions especially with its synnematal stages follows this description especially with its corresponding developing synnemal stages (Figure 1C, D & F) (Groves and Wilson 1967; Seifert 1989). The synnematal stages (individual synnema and synnematal masses – Figure 1C, F) were absent in NRRL 50072 grown on common culture media (PDA or oatmeal media). However, when isolate NRRL 50072 was grown on autoclaved stem pieces of its host Eucryphia cordifolia or Nothofagus spp. it produced a few synnematal masses, which were undeveloped (lacking a hymenial layer) (Figure 2B). This observation contrasted with the production of numerous synnematal masses in A. sarcoides strain AV-70 on host-derived as well as common laboratory media (Figure 1F). Eventually, conidiophores fused into synnema that then developed into synnematal masses on PDA and other media (Figure 1C, D, F). Also, when isolate NRRL 50072 was placed on sterile barley seeds, the organism produced normal synnematal masses of the type in Figure 1F. Under no conditions, however, did NRRL 50072 produce the classic individual synnema comparable to A. sarcoides AV-70 (Figure 1C, D). The production of the undeveloped synnematal masses in NRRL 50072 seemed to proceed via the fusing of a multitude of conidiophores which ultimately yielded abnormally formed synnematal masses bearing few conidia (Figure 2B). Thus, collectively the data suggest one or more biochemical factors in the support medium may stimulate the formation of normal or completely formed synnematal masses in NRRL 50072 (Figure 1F). However, it seems that it is entirely incapable of forming individual synnema as per A. sarcoides AV-70 (Figure 1C & D).

Morphological, biochemical, and molecular genetic evidence suggested that isolate NRRL 50072 was a fungus with a conidial state similar to, but probably not conspecific with that of Ascocoryne sarcoides (Figure 1B). It appears that its asexual cycle includes conidiophores, phialides and conidia like A. sarcoides, but lacks synnema production on common laboratory media.

Nevertheless, and not withstanding these differences, NRRL 50072 fits the description of a Coryne sp. because under some circumstances it produced a synnematal mass stage that was identical to the anamorphic stage of A. sarcoides- AV-70. Many fungi require one or more specific substances to stimulate specific fruiting structures. For example, Pestalotiopsis microspora required one or more specific plant components for development of the teleomorphic stage, Pestalosphearia hanssensii (Metz et al. 2000). Specifically, the substance (s) required to form this stage were in the nonpolar fractions of both hosts (Taxus spp.) and non-hosts alike (Pecan, Carya illinonensis) and barley, (Hordeum vulgare). In other cases, plant products, fungal compounds and primary metabolites such as cholesterol, can affect fruiting body formation in fungi (Hendrix 1964; Mirocha and Swanson 1983; Valent and Chumley 1991). Such products may have their origins within the
host plant or the fungus itself as it converts critical host precursors to the compounds needed for fruiting body formation.

Alignment and Bayesian analyses of publically available sequences of *Ascocoryne* species with a representative from each genus in the family Helitiaceae demonstrates that *Ascocoryne* more closely associates with members of the Family than to outgroups within the Orders (*Botryotinia* and *Sclerotinia*). The nodes denoting *Ascocoryne* as a distinct genus within Helotiaceae are perfectly supported (Bayes posterior of 1. Figure 3). This conclusion coincides well with a previous analysis of ITS sequences for *Ascocoryne* species (Bunyard et al. 2008). Both *A. sarcoides* AV-70 and NRRL 50072 (strain C-13) grouped within the *Ascocoryne* more closely associated with *A. sarcoides* than *A. cylichnium*. However, the node distinguishing these two species is not strongly supported (Bayes posterior of 0.73. Figure 3). Sequencing of more markers is necessary to confirm the species boundaries within the *A. sarcoides* clade.

**Fungal inhibitory properties of the VOCs of *A. sarcoides AV-70* on different media**

VOCs in isolate NRRL 5072 expressed antifungal activities against a number of organisms. Fungal inhibitory tests, using *Pythium ultimum*, had been successfully used to select the best media for volatile compound production.
in NRRL 50072 (Strobel et al. 2008). Likewise, comparable tests were applied to isolate \textit{A. sarcoides} strain AV-70 in order to determine if it too produced antifungal VOCs. AV-70 was grown on various media and then tested for the inhibition against \textit{P. ultimum}. The best media supporting antifungal activity were the oatmeal agar and the potato flakes/starch media. Host extract medium also supported the production of gases, but the volatiles were produced in low abundance. Apparently, \textit{A. sarcoides} AV-70 also emitted volatiles with antifungal activity comparable to that of NRRL 50072 (Strobel et al. 2008). Whether the mechanism of this antibiosis is related to previous observations on antagonisms of co-inoculated wood decay fungi by \textit{A. sarcoides} requires renewed investigation (Basham 1975).

**Phenotypic changes in \textit{A. sarcoides} AV-70 during serial transfers**

The appropriate handling of fungal cultures is critical for the stable and continued production of metabolic products. Major culture collections and industrial organizations utilize stored mother cultures (frozen under liquid nitrogen or at −70°C) in order to preserve the integrity and standard quality of inoculum. An alternative technique is to maintain fresh cultures by regular serial transfers of the fungal mycelium. Although, the approach may seem reasonable, it can be fraught with difficulties because continuous serial transfer of a culture can result in strains with permanent genetic or epigenetic changes leading to attenuated production of one or more products. Likewise, it is the serial transfer of plant-associated microbes can result in the loss of desirable properties of activities (Pinkerton & Strobel 1976). For example, the serial transfer of \textit{Bipolaris sacchari} (=\textit{Helminthosporium sacchari}) resulted in the complete loss of toxin production. The addition of certain plant products, originating in the sugarcane host of this fungus, into the medium restored the organism to its original state of toxin production. Thus, it was of interest to predict how cultures of \textit{A. sarcoides} react to the processes of serial transfer as it relates to culture phenotype. i.e. color, fruiting structures, aerial mycelium in order to ensure stable biosynthesis of various fungal products over time.

Several cultures of the original \textit{A. sarcoides} AV-70 were transferred, as agar plugs, on oatmeal agar weekly for 11 weeks. Consistently, there were noticeable changes in the cultures after the 4th transfer. For instance, the color of the cultures originally started as deep purple at the 1st transfer and most became creamy tan to brown at the 4th transfer and then mostly continued creamy-tan to mauve for the next 7 transfers (Figure 4). The number of pre-synnemal or synnemata masses per 78 mm² in the peripheral area of the Petri plate significantly increased from 3.2 ± 1.3 per 78 mm² from the original plates (8 weeks old) of \textit{A. sarcoides} AV-70 to 39.8 ± 19 by the 10th transfer (also 8 weeks old) (Figure 4). Finally, the aerial mycelial mass also increased during the course of the serial transfers with 20.8 ± 6.5 mg dry weight per oatmeal agar plate from the first transfer plate (8 weeks old) to 61 ± 18.4 on the 10th transfer plate (also 8 weeks old). Apparently, the serial transfer of this fungus on oatmeal agar caused a drift of culture pigmentation, aerial mycelial mass, and conidioaphore density.

When cellulose agar was used in the serial-transfer experiment, color changes in the medium were less evident over the course of the experiment, but synnematal masses increased noticeably in the latter transfers. Finally,
when the beech extract agar was used the pigmentation of the medium never drifted from the original pinkish coloration at the first transfer but synnematal mass production slightly increased during the later transfers starting as transfer 9.

Pigment changes of the serially transferred cultures in the oatmeal may be the result of the lost critical plant host signals that regulate the metabolism of the fungus that eventually dissipate in the transferred fungal mycelium and become lost after constant serial transfer. Thus, loss of fungal host signal interactions could affect the ability of the fungus to produce its pigments resulting in the loss of purple coloration in the growth medium. However, generally the numbers of synnematal masses developing in the latter transfers increased on all media tested suggesting that multiple factors regulate synnematal mass production in this fungus. One or more of these are definitely host related such as in isolate NRRL 50072 and its requirement for a plant product to make a synnematal mass (Figure 2B). Secondly, it seems that further transfers from the host with *A. sarcoides* AV-70 results in greater numbers of synnematal masses per mm² of the surface of the medium. This suggests that the numbers of synnematal masses being produced may also be related to one or more host products that suppress or limit production of asexual structures. As mentioned above, a comparable phenomenon has been observed in other fungi with the loss of pathogenicity and toxin production on a defined laboratory medium, but in some cases toxin production can be recovered when host factors, having been identified and synthesized, and added to the medium (Pinkerton & Strobel 1976).

However, in the case of *A. sarcoides*, re-establishment of an attenuated culture to its host did not cause a complete reversion to the original pigment phenotype.

Figure 3. The phylogenetic relationships of the *Ascocoryne* spp. Note that NRRL 50072 (C-13) and AV-70 form a monophyletic clade with other *Ascocoryne* spp. clearly distinct from other members of the family Helotiaceae. Node labels are Bayesian posterior probabilities.
These results may have some significance when studying secondary product formation in *A. sarcoides* because these processes may be regulated by plant-related factors. Therefore, future attempts at large bioreactor production with NRRL 50072 or other *Ascocoryne* strains should be conducted with properly stabilized and stored inocula.

**Conclusions**

The strain designated as NRRL 50072 used to produce hydrocarbon derivatives is a *Coryne* sp., the anamorphic form of an *Ascocoryne* species. This conclusion is supported by genetic, biological and morphological evidence presented in this report. However, because a large number of *Coryne* sp. (about 70 names www.Mycobank.org) still remain uncharacterized, and a modern monograph of *Ascocoryne* based on molecular data does not exist, it is not yet possible to determine whether NRRL 50072 is conspecific with an existing species or whether it is possibly something new. An authentic culture of *A. sarcoides* AV-70 was sensitive to the process of serial cultural transfer. Repeated transfers influenced pigmentation, synnematal mass and aerial mycelial production in this organism. One might predict that other strains from this group of fungi will behave similarly when serially transferred and therefore attempts to develop industrial processes for VOC or other products will need to consider methods to produce stable inoculum.

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