MANIPULATING METABOLISM IN FILAMENTOUS FUNGI, UTILISING MEDIA VARIATION AND EPIGENETIC MODIFIERS

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OVERVIEW

- The fungal kingdom is incredibly diverse globally [1] - many underexplored species, especially in tropical regions [2]
- This is matched by metabolic diversity - fungi are talented metabolisers, responsible for the existence of many medicinal and important substances [3] (Fig. 1), though the secondary metabolite pool remains largely untapped [4]
- Problems in natural product discovery from fungi - Obtaining novel cultures, low yields, rediscovery, costly and time-consuming fermentation and isolation, silent biosynthetic gene clusters in laboratory conditions

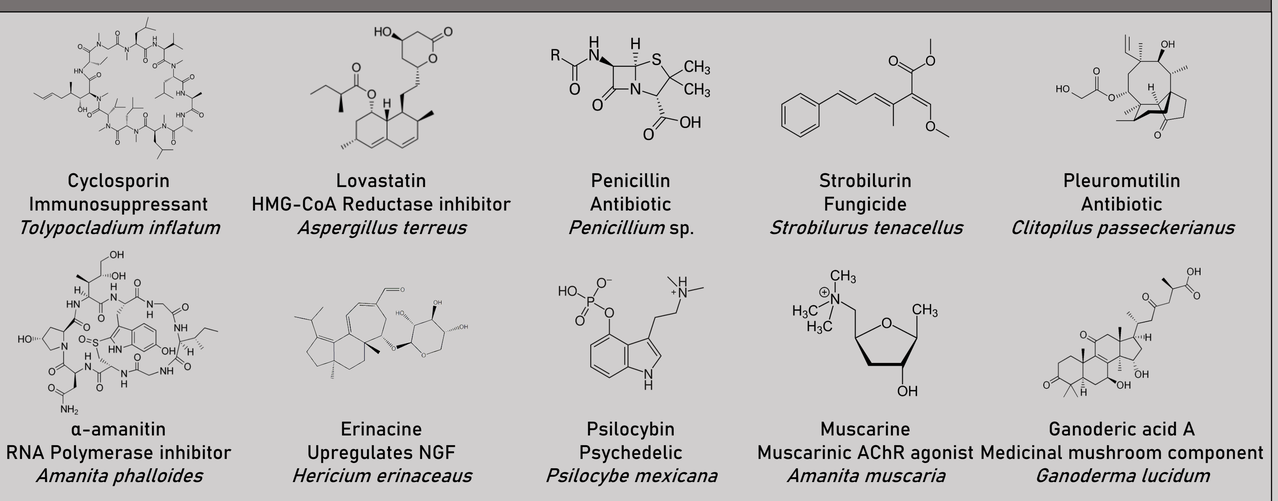

Figure 1. Examples of structurally diverse, medicinally and/or scientifically important fungal secondary metabolites, their uses, and the species from which they were discovered

- Regulation of secondary metabolism is finely controlled and involves particular conditions [5] (limiting nutrients, carbon/nitrogen source variation and concentration, temperature, pH, oxygenation, mineral composition etc)
- OSMAC approach can enhance metabolic exploration by manipulating fermentation and nutritional conditions, but secondary metabolite gene clusters are often silent under laboratory conditions [3]
- Epigenetic modifiers can be used to disrupt epigenetic regulatory mechanisms (DNA methylation, Histone modifications etc) Can influence secondary metabolism in laboratory conditions [6]
- In this work - media variation and epigenetic modifiers were employed to explore the metabolic potential of under-researched UK-native filamentous fungi (examples in Fig. 2 & 3), producing several potentially novel metabolites

- Hypocrelean fungi - rich sources of diverse secondary metabolites [7]
- Some genera contain under-researched Araneae-specific (Arachnogenous) entomopathogenic fungi, especially within *Cordycipitacea* [8]
- The largest genera are *Gibellula, Torrubiella* and *Akanthomyces* - some can be found in the UK
- 42 Metabolites to date from *Gibellula* - antibacterial, antiviral, cytotoxic [9] - None from European strains

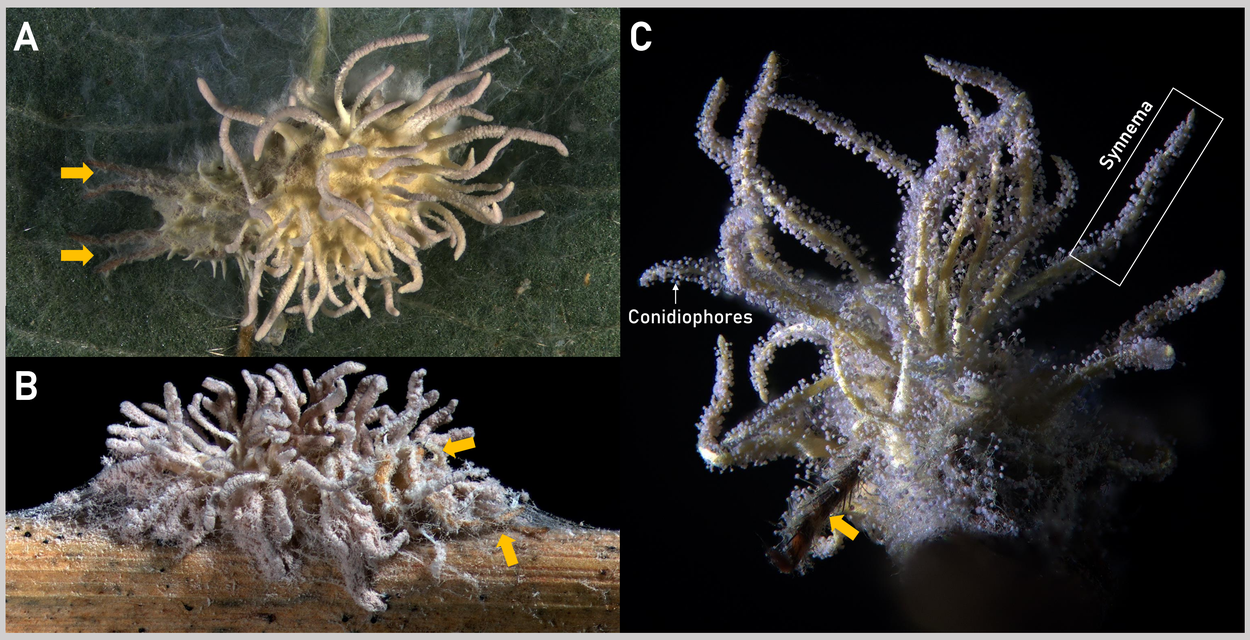

Figure 2. Arachnogenous fungi on unknown Araneae *spp.* (yellow arrows). Each produce abundant synnemata (conidiophore-bearing structures) which can be used to isolate cultures. A. Specimen on the underside of *Robus fruticosus* leaf, Devon, England. B-C. Specimens on *Phragmites sp.*, Norfolk, England.

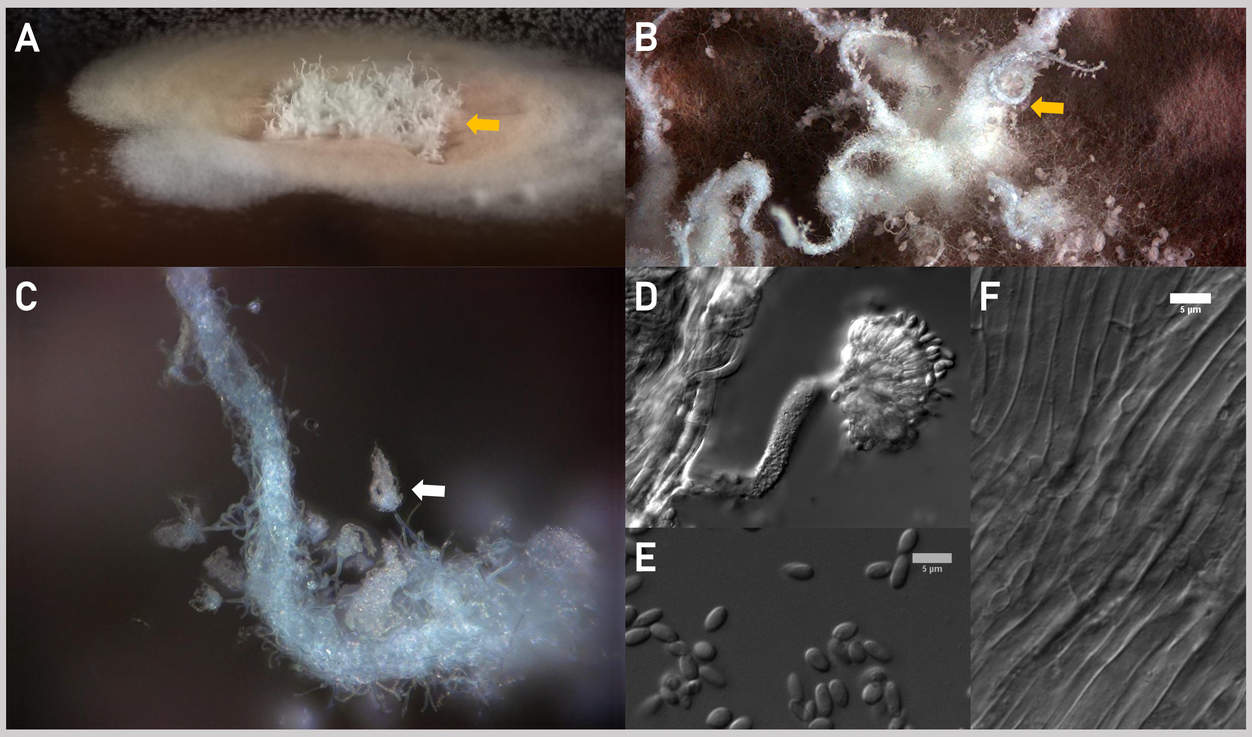

Figure 3. A-C. Culture obtained from specimen C (Fig. 2) on Potato Dextrose Agar (PDA), producing primitive synnemata (yellow arrows, A-B), bearing conidiophores (white arrow, C). D. Conidiophore from specimen C synnema. E. Conidia. F. Surface of synnema from Specimen C

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MEDIA SCREENING

- Fungal isolates were screened in several media (PDB, YM, ZM, Q6, Supplemeted Brown Rice) to examine metabolism in different nutritional conditions
- Biomass and media filtrate extracted separately – Often find differences between each (Fig. 4)
  - can aid purification
- Strains tend to grow slower in complex media (ZM, Q6) compared with simpler media (PDB, YM), but produce more varied metabolites

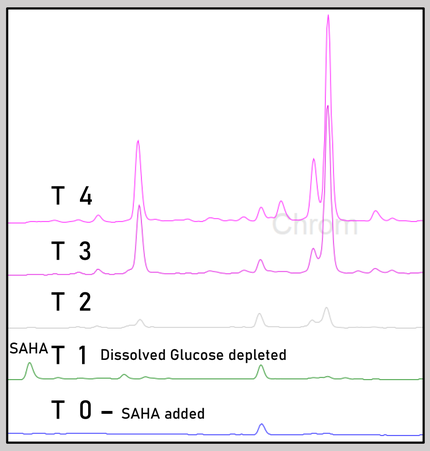


Figure 5. UV-Vis chromatograms of media filtrate extracts from a fungus grown in ZM media with Vorinostat (SAHA, a Histone Deacetylase [HDAC] Inhibitor). In this case, a group of metabolites are produced following depletion of glucose (Timepoint 1). T = Timepoint, each 48 hours apart

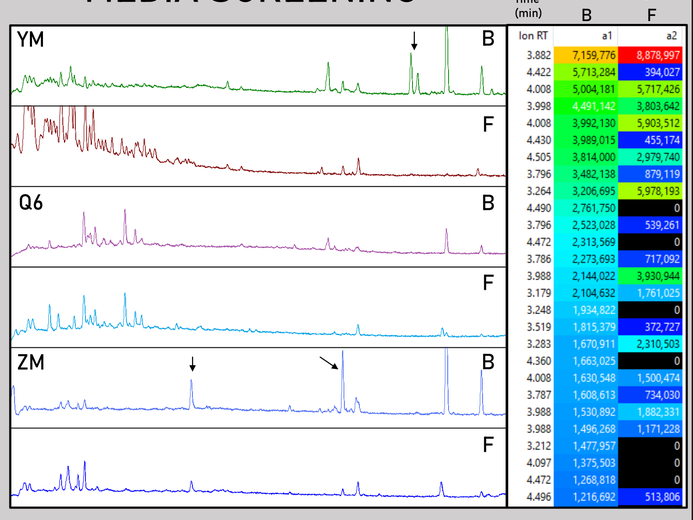
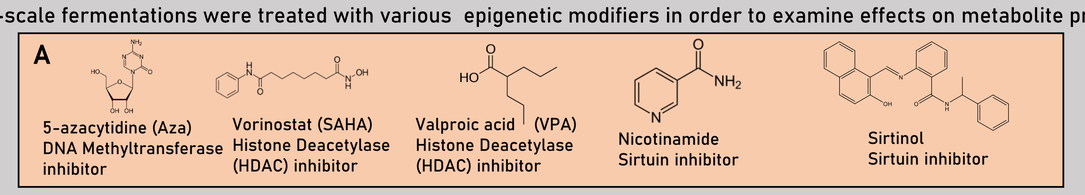


Figure 4. Example LCMS UV chromatograms (left) and ion intensities (right) from Biomass (B) and media filtrate (F) extracts of a fungal strain grown in several media (YM, Q6 and ZM). Arrows indicate differences between the media. Ion intensity differences highlight the variable distribution of substances between biomass and media filtrate

- Cultures were regularly sampled for monitoring of [glucose], pH and extraction of biomass and media filtrate for metabolite profiling via HPLC and LCMS
- This process generates chromatograms and LCMS data allowing determination of the time course of metabolite production under given conditions (e.g. Fig. 5)

EPIGENETIC MODIFIER SCREENING EXPERIMENTS

Small-scale fermentations were treated with various epigenetic modifiers in order to examine effects on metabolite profiles



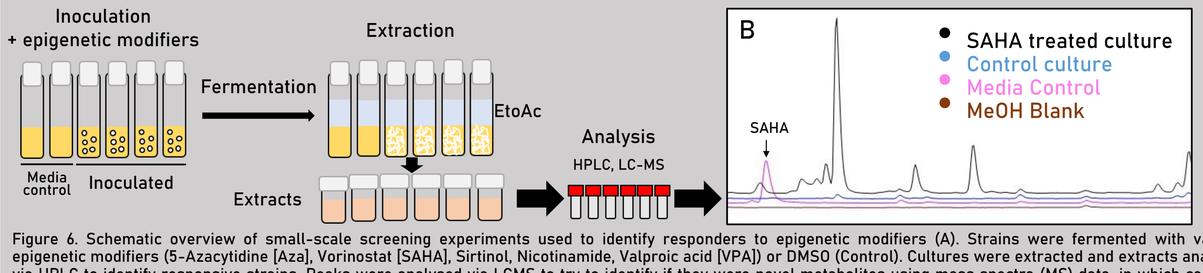


Figure 6. Schematic overview of small-scale screening experiments used to identify responders to epigenetic modifiers (A). Strains were fermented with various epigenetic modifiers (5-Azacytidine [Aza], Vorinostat [SAHA], Sirtinol, Nicotinamide, Valproic acid [VPA]) or DMSO (Control). Cultures were extracted and extracts analysed via HPLC to identify responsive strains. Peaks were analysed via LCMS to try to identify if they were novel metabolites using mass spectra (MS) data, in which case the fermentations were scaled up to allow isolation. The example chromatogram (B) shows upregulation of a group of metabolites by SAHA.

- Epigenetic modifiers can result in increased metabolite production (Fig. 6B)
- Responsive strains identified in small scale screening experiments before scaling up
- Important to check for biotransformation products of epigenetic modifiers
- Using this approach, cultures were screened for responses to SAHA, Aza, Sirtinol, Nicotinamide and VPA, alone and in combinations. Different groups were extracted and analysed via HPLC and LCMS (Fig. 7)
- In some instances, combination-dependent induction was observed when Sirtinol was combined with other modifiers (Fig. 7B) - peaks not detected with any of the modifiers alone

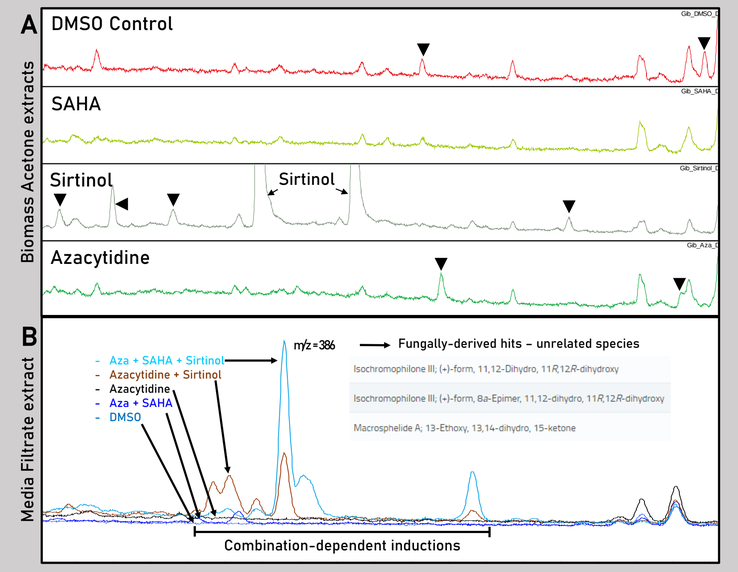


Figure 7. UV Chromatograms of Biomass (A) and Media Filtrate (B) extracts from YM media in the presence of Azacytidine, Sirtinol, SAHA, combinations, or DMSO (Vehicle Control).

DEREPLICATION, ISOLATION AND IDENTIFICATION

- Dereplication - prevents rediscovery - involves searching chemical databases (Dictionary of Natural Products, Metlin, Scifinder etc.) for hits - (m/z, MS^n fragmentation spectra, UV profiles, source organism etc)
- Following media & epigenetic screening, several fermentations were scaled up
- Metabolites were extracted from biomass and media filtrates via multi-step liquid-liquid extractions, and purified using flash chromatography and Preparative HPLC (Fig. 8)
- Using this approach, several promising metabolites/fractions have been produced, which are potentially novel or not currently known from these species
- Complete chromatographic purification and structural elucidation via NMR spectroscopy is ongoing

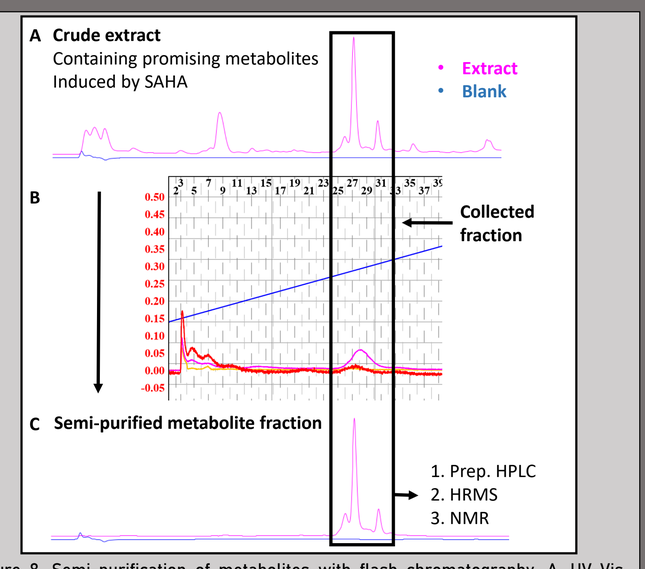


Figure 8. Semi-purification of metabolites with flash chromatography. A. UV-Vis chromatogram of crude media-filtrate extract. B. Flash chromatographic separation of crude extract. C. UV-Vis chromatogram of collected fraction showing closely eluting peaks, before further purification by preparative HPLC

CONCLUSIONS

- Vast diversity within fungal kingdom – likely to be many undiscovered secondary metabolites
- Underexplored fungal species screened in various media, +/- epigenetic modifiers - epigenetic responders identified
- Several promising metabolites produced, which may be novel or currently unknown from these species
- Fermentations and extractions scaled up to obtain large enough yields for structural elucidation
- First work with European *Gibellula sp*.
- Future work will complete metabolite purifications, structural elucidations and bioactivity assays

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