Hyperdiverse, bioactive, and interaction-specific metabolites produced only in co-culture suggest diverse competitors may fuel secondary metabolism of xylarialean fungi

Mario E.E. Franco[a,b]§, Megan N. Nickerson[a], Benjamin P. Bowen[c], Katherine Louie[c], Trent R. Northen[c], Jana M. U'Ren[a,b*#]

[a]BIO5 Institute, University of Arizona, Tucson, AZ, USA

[b]Department of Biosystems Engineering, University of Arizona, Tucson, AZ, USA;

[c]U.S. Department of Energy, The Joint Genome Institute, Berkeley National Labs, Berkeley, CA, USA

Running Head: Competitor-specific bioactive xylarialean metabolites

#**Corresponding author:** jana.uren@wsu.edu

***Present address**: Department of Plant Pathology, Washington State University, Pullman, WA, USA

§Present address: Sustainable Plant Protection Programme, Institute of Agrifood Research and Technology (IRTA), Lleida, Spain

Abstract

Xylariales is one of the largest and most ecologically diverse fungal orders. Xylarialean fungi are well-known for their chemical diversity, reflecting a hyperdiversity of biosynthetic gene clusters (BCGs), even compared to other bioactive fungi. Enhanced secondary metabolism appears linked to the number of horizontal gene transfer (HGT) events and gene duplications, which is highest in the clade that also has a greater ability to both degrade lignocellulose as saprotrophs and interact with a wider variety of plant and lichen hosts as symbiotic endophytes. Thus, one hypothesis for BGC diversification in this clade is that diverse competitive interactions—in both their free-living and symbiotic life stages—may exert selective pressure for HGT and a diverse metabolic repertoire. Here, we tested this hypothesis using untargeted metabolomics to examine how pairwise co-culture interactions between seven xylarialean fungi influenced their metabolite production. Overall, we detected >9,000 features, including putatively anti-microbial, insecticidal, and medicinal compounds. In total, 6,115 features were over-represented in co-cultures vs. 2,071 in monocultures. No features occurred in all 21 interactions, and only 39% of features occurred in >10 different co-culture combinations. Each additional co-culture interaction resulted in an 11 to 14-fold increase in metabolite richness. Consistent with HGT, metabolite profiles did not reflect phylogenetic relationships. Overall, the diversity and specificity of metabolites support the role of widespread and diverse competitive interactions to drive diversification of xylarialean metabolism. Additionally, as plant hormones were only detected in co-culture, our results also reveal how competition may influence the outcome of endophytic symbioses.

45 **Importance**

46 Saprotrophic and endophytic xylarialean fungi are among the most prolific producers of
47 bioactive secondary metabolites, which are often encoded by sets of physically clustered genes
48 (i.e., biosynthetic gene clusters). Secondary metabolites have numerous industrial uses as
49 antibiotics, pharmaceuticals, and insecticidal toxins, but their ecological importance for
50 competitive and symbiotic interactions with other fungi, bacteria, and plants is not fully
51 understood. Here, we examined how co-culture interactions with ecologically and genetically
52 diverse competitors influence the metabolite production of seven xylarialean strains. Among 21
53 pairwise interactions, we detected >9,000 metabolite features, 67% of which were produced only
54 in specific co-cultures and not in monocultures. Over 8,000 features were unclassified at any
55 level and likely represent novel bioactive compounds. Our data illustrate that competitor identity
56 and ecology significantly impact xylarialean fungal secondary metabolite production, key
57 information that will enable us to further harness the immense biotechnological potential of these
58 fungi.

59 **Introduction**

60 As both plant symbionts and saprotrophs that can catabolize a wide range of organic
61 substrates, fungi are a critical component of all ecosystems (1, 2). As osmotrophs, competition
62 for shared resources can drive fungal metabolic diversification, especially the production of
63 secondary metabolic (SM) compounds that provide an ecological advantage (3, 4). Fungal SMs
64 can determine virulence against plants and animals, enhance nutrient acquisition and defense
65 against predators and abiotic stressors, and facilitate intra-organismal communication and
66 quorum sensing (e.g., (4–7)). Genes involved in fungal SM production are frequently encoded by
67 biosynthetic gene clusters (BGCs) that can evolve through gene duplication and/or horizontal
68 gene transfer (HGT) (e.g., (8, 9)).

69 Xylarialean fungi, especially species of Xylariaceae *sensu lato* (*s.l.*) (10, 11), are among
70 the most prolific fungal SM producers (12, 13). Described species are primarily saprotrophs or
71 pathogens of woody hosts (14–16); however, numerous species have been cultured as endophytic
72 symbionts of diverse plants and lichens (e.g., (17–19)). Endophytic xylarialean fungi appear to
73 be host and substrate generalists: closely related strains have been cultured from phylogenetically
74 diverse hosts, as well as both living and decomposing plant tissues (17). A previous study found
75 that xylarialean endophytes with higher BGC diversity and rates of HGT formed symbiotic
76 associations with a greater diversity of hosts and displayed a greater capacity to degrade leaf
77 litter (11). Generalist endophytes thus interact with both a greater number of hosts, as well as
78 potentially with diverse microbes *in planta* and/or during a free-living saprotrophic stage, which
79 may promote greater SM diversification compared to more ecologically specialized species (11)
80 (see also (20)).

81 Here, to better understand the evolutionary and ecological factors influencing SM
82 diversification in xylarialean fungi, we used untargeted metabolomics to study the metabolic

83 interactions in all pairwise combinations of seven xylarialean fungal strains whose BGC
84 diversity was previously studied (11) (Fig. 1). Although performed *in vitro*, co-culturing can
85 induce the production of bioactive compounds not typically produced by either microorganism
86 when grown without competitors (21–23). For xylarialean fungi, co-culture has proven to be a
87 valuable tool to trigger the production of bioactive SMs (24–27). More recently, co-culturing
88 paired with mass spectrometry has led to discoveries of novel SMs with applications in the
89 pharmaceutical, food, and agronomic industries (21, 23, 28).

90 Our main goal was to directly compare the diversity, composition, and bioactivity of
91 small molecules secreted by fungi in monoculture vs. co-culture, explicitly examining the impact
92 of fungal ecological mode, BGC content, and phylogenetic position on the resulting metabolome
93 diversity and composition. Specifically, we tested the following hypotheses: (i) a strain's BGC
94 content and SM production would be positively correlated; (ii) phylogenetic distance between
95 strains would influence SM production in co-culture, with greater competition and SM
96 production occurring in interactions between close relatives (21, 22), and (iii) each strain will
97 produce a unique suite of metabolites during each interaction, reflecting selection for BCG
98 diversification in response to diverse microbial competitors in different hosts and substrates.

99

100 **Results**

101 ***Fungal interactions.*** Overall, 17 of 21 (80%) pairwise co-culture combinations resulted in
102 deadlock growth between strains (i.e., fungal hyphae approached each other, but growth was
103 arrested). Deadlock usually resulted in a clear space between fungal hyphae representing a
104 potential zone of inhibition, but in some co-cultures, hyphae of each pair appeared to nearly

105 touch, yet without a clear zone (Supplemental Figs. 1-7). Only four co-culture pairs resulted in
106 the invasion of one strain over another: three cases involved *X. arbuscula* invading other strains,
107 and the fourth instance was *B. mediterranea* overgrowing *P. punctata*.
108
109 ***Results of Untargeted Metabolomics.*** Across all 84 fungal samples and controls, liquid
110 chromatography coupled with high-resolution tandem mass spectrometry (LC-MS/MS) detected
111 47,316 ions with unique mass-to-charge ratio (m/z) and retention time (RT) values (i.e., features)
112 (Table 1). Approximately 20% of features (n = 9,762) were significantly more abundant in
113 fungal cultures compared to negative extraction and media controls (Table 1; Supplemental
114 Table 1), but only 955 of 9,762 features were assigned to a Superclass (Supplemental Fig. 8).
115
116 ***Influence of co-culture vs. monoculture on metabolite production.*** Pairwise statistical analyses
117 of all 21 co-cultures compared to their respective monocultures (Fig. 2) identified a total of 6,141
118 features that were significantly over-represented in either co-culture and/or monoculture
119 (hereafter, "dysregulated") (Table 2). Among these dysregulated features, 4,070 were unique to
120 co-cultures (i.e., only over-represented in co-culture), and 26 features were unique to
121 monocultures (i.e., only over-represented in monoculture). Including features over-abundant in at
122 least one co-culture or monoculture (i.e., “ambiguous”; see Fig. 2), our pairwise analyses
123 identified a total of 6,115 features that were significantly more abundant in at least one co-
124 culture vs. monoculture. In comparison, 2,071 features were more abundant in at least one
125 monoculture vs. its respective co-cultures (Table 2).
126 Consistent with our hypothesis, the mean number of features significantly more abundant
127 in at least one co-culture was 2.2X higher than the mean number of features significantly over-
128 represented in at least one monoculture (Fig. 3A; Table 2). Moreover, rarefaction analysis

129 illustrates each additional different co-culture interaction increases metabolite richness 11 to 14-
130 fold (i.e., 1,279 to 4,037 additional metabolites) compared to similar comparisons between
131 monocultures (Fig. 3B). Although the number of features over-represented in co-cultures did not
132 differ significantly between fungi of various phylogenetic distances (Fig. 3C), co-cultures
133 involving *P. punctata* resulted in a 1.8X increase in the mean number of over-represented
134 features regardless of partner (Fig. 3D).

135 Although co-cultures consistently resulted in a greater number of over-represented
136 features compared to monocultures, the magnitude of the difference was variable among
137 different pairwise comparisons (Fig. 4). For example, we observed a 6-fold increase in
138 significant features for the co-culture of *P. punctata* and *X. flabelliformis* NC1011 vs. either
139 strain's monoculture. However, there was only a 1.2X increase in the number of features for the
140 co-culture of *X. arbuscula* vs. *N. diffusa* compared to either monoculture (Table 2). The latter
141 pair also had the fewest total features in co-culture (n = 1,278). The highest number of features
142 significantly over-represented in any co-culture was 4,817 (*P. punctata* vs. *X. flabelliformis* CBS
143 116.85), whereas the highest number of features over-represented in any monoculture was 1,315
144 for two *X. flabelliformis* strains compared to their co-culture (Table 2).

145

146 ***Influence of competitor identity on co-culture metabolism.*** Among the 6,115 features that were
147 over-abundant in co-culture vs. the respective monocultures, none were universally over-
148 abundant in all 21 co-culture combinations, and only one feature was shared among 20 of 21
149 pairs (Fig. 5A). Instead, we found that only 8.4% of over-abundant features (n = 514) occurred in
150 at least 15 of 21 interactions, and only 30.9% (n = 1,891) of features were shared across 11-14
151 interaction pairs. Instead, the majority of features (60.7%; n = 3,710) were shared among 10 or
152 fewer interactions. In addition, 245 (4.0%) over-abundant features were unique to a single co-

153 culture pair, including 190 features that were unique to the co-culture of *X. flabelliformis*
154 NC1011 and *B. mediterranea* (Fig. 4A). Concomitant with the general lack of shared metabolites
155 among different fungal interactions, a heatmap of feature frequency in co-cultures of each strain
156 illustrates that the production of most metabolite features occurred infrequently (Fig. 5B). Yet,
157 while most features were rare, a subset of features were consistently observed in the co-cultures
158 of some strains (e.g., 533 features that occurred in all co-cultures with *P. punctata* as a partner)
159 (Fig. 5B).

160 Similar to the general lack of overlap among features over-abundant in co-culture
161 compared to monoculture across all 21 interactions, comparisons of feature overlap for a single
162 strain in co-culture with six other strains interactions compared to the corresponding
163 monoculture revealed a majority of features occurred in only one or two co-culture combinations
164 per focal strain, although the percentage of shared features varied by focal strain (Supplemental
165 Fig. 9). The fraction of features unique to one particular interaction pair ranged from 620 in all
166 co-cultures with *P. punctata* (12% of total features in this strain) to 1,428 in all co-cultures with
167 *X. flabelliformis* NC1011 (53% of total features in this strain). In general, co-cultures with any of
168 the three *X. flabelliformis* strains resulted in a relatively high number of features specific to these
169 interaction pairs (Supplemental Fig. 9).

170 Although the phylogenetic relationship or genetic distance between strains did not appear
171 to explain the number of shared features (see Fig. 3C), we observed other patterns. For example,
172 the same 880 features were found among *X. arbuscula* co-cultures with either two (n = 432) or
173 three (n = 448) of the *X. flabelliformis* strains (Supplemental Fig. 9). However, in contrast to
174 other strains that had largely unique metabolite profiles when interacting with different
175 competitors, 1,096 features were shared among all *P. punctata* co-culture combinations,
176 regardless of the partner (Supplemental Fig. 9). For *B. mediterranea*, 18% percent of features (n

177 = 686 of 3,776 total) were shared in interactions between all other strains except *P. punctata*
178 (Supplemental Fig. 9).
179
180 ***Links between metabolites and fungal genomes.*** Using MAGI 1.0 (29) we identified 135
181 features (out of 6,141 total) that were linked to genes/BGCs in the genomes of all seven strains
182 (Table 3). Overall, these 135 features were linked to 2,651 genes via 487 different InChIKey
183 strings (Table 3; Supplemental Tables 2-3). Additionally, 14.3% of linked genes (i.e., 379 genes
184 that were linked to 83 features) were previously found to reside within the boundaries of 300
185 different BGCs (based on data from (11)). Among these 379 genes, 78 were within BGCs with at
186 least one significant hit against the MIBiG repository (Table 3) (11). Furthermore, by leveraging
187 previous analyses of HGT in the genomes of these strains (11), we found that 20 of these genes
188 (8 previously identified as part of a BGC) were potentially acquired via HGT from Bacterial
189 donors (Supplemental Table 3).
190
191 ***Co-culturing and the production of bioactive compounds***. In addition to the greater number of
192 features produced in co-cultures vs. monocultures, numerous features that were over-abundant in
193 co-cultures were putatively identified as bioactive compounds (Table 4; Supplemental Table 4).
194 In general, sesquiterpenes were one of the most prevalent bioactive compound classes produced
195 by these seven fungal strains in co-culture (Table 4). Feature hits include matches to parthenolide
196 (30), alantolactone (31), polygodial (32), zerumbone (33), steviol (34), and valerenic acid (35),
197 nootkatone, which have been reported to have anti-microbial, anti-fungal, insecticidal, anti-
198 inflammatory activity. Numerous features also had hits to mycotoxins such as alternariol,
199 diacetoxyscirpenol, culmorin, and the cytochalasins C, D, and J (36, 37), that all pose a serious
200 threat to human health and food safety (12, 13, 21–23).

201 Overall, we detected numerous features with putative matches to compounds typically
202 identified in plants, including hormones, terpenes, and toxins. Features identified as the plant
203 hormones jasmonic acid (JA) and abscisic acid (ABA) occurred in at least one co-culture of each
204 of the seven strains (71% and 90% of all potential pairwise interactions) (Supplemental Table 4).
205 In general, features with matches to ABA were linked to a single, unclustered gene in each of the
206 genomes; however, in one case, the feature was linked to the ABA gene within a putative BGC
207 in *P. punctata* (Supplemental Table 3) (38). In at least one co-culture of each strain, we observed
208 features with matches to the monoterpene carvone (Table 4), a component of dill and carraway
209 essential oils with insecticidal and insect feeding-deterrent properties (38). Fungal features with
210 hits to carvone were linked to numerous fungal genes, including some genes that reside within
211 BGCs (Supplemental Table 3).
212 Numerous features had hits to bioactive compounds with reported antimicrobial activity
213 (Table 4, Supplemental Table 4), such as the potent and non-toxic Gram-positive antibacterial
214 compounds platencin and platensimycin from *Streptomyces platensis* (28, 39). Features
215 identified as platencin were predominately found in co-cultures of *B. mediterranea*, whereas
216 features linked to platensimycin were detected in all co-cultures of *X. flabelliformis* CBS 116.85
217 and *X. flabelliformis* NC1011. A feature identified as mitochondrial F1F0 ATP synthase inhibitor
218 oligomycin B from *Streptomyces* spp. (40), occurred in four co-cultures involving *P. punctata*. A
219 feature with hits to the antibiotic malioxamycin from *Streptomyces lydicu* (41) was exclusively
220 detected in all co-cultures of *P. punctata*. Features matching the antibacterial and antifungal
221 compound, asperlactone from *Aspergillus ochraceus* (42), were detected in all co-cultures of *B.*
222 *mediterranea* and *N. diffusa*, as well as in co-cultures with *X. flabelliformis* CBS 123580. The
223 broad-spectrum antibiotic 2,4-diacetylphloroglucinol (DAPG), which is considered the primary
224 determinant of the biological control activity of *Pseudomonas fluorescens* F113 (43), was

225 frequently observed in co-cultures with the three different strains of *X. flabelliformis*. Lastly,
226 features matching the antifungal compound griseofulvin and its structural analogs,
227 dechlorogriseofulvin and griseofulvic acid, were observed in numerous different co-culture
228 combinations (Fig. 6; Supplemental Fig. 10). In general, features matching griseofulvin occurred
229 rarely in monocultures (Table 4); however, for some cultures, features structurally related to
230 griseofulvin were significantly more abundant in monocultures compared to some of their co-
231 cultures (e.g., *B. mediterranea*, *P. punctata*, *X. arbuscula*, *X. flabelliformis* CBS 116.85, and *X.*
232 *flabelliformis* NC1011) (Fig. 6).

233 **Discussion**

234

235 ***Co-culture interaction phenotypes not related to metabolite production.*** Xylarialean fungi,
236 especially endophytic strains, are known to be prolific producers of bioactive compounds (12,
237 13, 44). Consistent with these reports, the majority of our co-culture interactions resulted in the
238 formation of an inhibitory halo or a deadlock between strains, indicative of the production of
239 inhibitory metabolites such as griseofulvin (see below). While the majority of co-culture
240 interactions appeared to inhibit the growth of both strains, one strain (*X. arbuscula*) overgrew its
241 competitor in three of six interactions. However, overgrowth did not appear to substantially alter
242 the number of features over-represented in co-culture compared to monoculture. In addition, the
243 dung saprotroph *P. punctata*, which showed no visible growth on the media, still appeared to
244 strongly inhibit the growth of *Nemania diffusa*, as well as three *X. flabelliformis* strains.
245 Interactions with *P. punctata* also resulted in a significantly higher number of features in co-
246 culture. Thus, our results suggest that the total number of metabolites produced or the fraction of
247 features unique to particular co-culture interactions, as well as potentially the number of
248 bioactive compounds, may not be easily predicted based on fungal growth or degree of

249 inhibition/antagonism between strains. Future work will need to examine how xylarialean fungal
250 interaction phenotypes are altered by particular SMs, their cellular distribution or secretion,
251 and/or regulatory changes (21, 25).
252
253 ***Influence of competition on the evolution of xylarialean fungal secondary metabolic diversity.***
254 Xylarialean fungi are of particular interest due to their bioactivity and chemical diversity (12,
255 13), which reflects a significantly higher richness and diversity of BCGs in xylarialean genomes
256 compared to other filamentous fungi, including highly bioactive genera such as *Aspergillus* and
257 *Penicillium* (11). Such enhanced secondary metabolism, which is linked to the number of HGT
258 events and gene duplications (45), appeared greatest in the Xylariaceae *s.l.* clade that also has a
259 greater ability to degrade lignocellulose as saprotrophs, as well as interact with a wide variety of
260 plant and lichen hosts as endophytes (11). Thus, one hypothesis for the diversification of BGCs
261 in xylarialean fungi is that diverse competitive interactions in both their free-living and
262 symbiotic life stages may exert selective pressure for both the HGT of BGCs and the
263 maintenance of a diverse BGC repertoire (11).
264 Species that have evolved to occupy a specific ecological niche have also evolved to
265 compete with one another, *sensu* Gause's competitive exclusion principle (46). For example, the
266 composition of fungal communities in decaying wood strongly reflects combative interactions
267 between diverse basidiomycete fungi (47). In fungi, antagonistic interactions often occur
268 primarily through the production of SM in the presence of the competing organism (23). Thus,
269 our experiment was designed to assess whether xylarialean fungi exhibit a differential metabolic
270 response to different competitors, which would provide evidence to support the hypothesis for
271 BGC diversification for ecological generalists. In contrast, if co-culturing resulted in the
272 production of similar metabolite profiles regardless of competitor identity, or no up-regulated

273 response to competition (i.e., no differences between co-culture and monoculture), competition
274 may not be the major factor driving xylarialean BGC diversity.

275 In support of the role of competition against different competitors driving the
276 diversification of diverse xylarialean BGCs, xylarialean fungi had a significantly higher number
277 of over-represented metabolites in co-culture vs. monoculture. Overall, each additional co-
278 culture interaction resulted in an 11 to 14-fold increase in metabolite richness compared to
279 monocultures. Compared to monocultures, co-cultures had a higher fraction of compounds
280 classified as lipids, accompanied by a corresponding decrease in the fraction of organic acids or
281 organoheterocyclic compounds. Additionally, rather than a shared metabolic response to all
282 competitors, we found that zero features occurred in all 21 co-culture combinations and <40% of
283 features occurred in more than 10 different co-culture combinations (out of 21 possible).
284 Similarly, analyses of each strain vs. all others demonstrated that the majority of features were
285 found in only one or two of the six possible co-culture combinations.

286 Contrary to our hypothesis that more closely related fungi would be more likely to
287 compete for similar resources, resulting in the production of a greater number of metabolites in
288 co-culture, we found no clear relationship between intra-strain phylogenetic distance and either
289 the number of metabolites they produced in co-culture or the similarity of their metabolomes
290 (i.e., metabolomes did not cluster by phylogeny). The unpredictable distribution of shared
291 metabolites among different co-culture pairs, as well as the observation that distantly related
292 fungi produce similar metabolite features, aligns with the high rate of HGT of BGCs reported for
293 xylarialean fungi, including numerous BGCs for the production of bioactive compounds (11). In
294 addition, consistent with the well-known bioactivity and high number of BGCs among strains of
295 *X. flabelliformis* (11), we observed that co-cultures of any strain with any of the three *X.*

296 *flabelliformis* strains typically resulted in a large number of features specific to these interaction
297 pairs.
298 Conversely, although the genome of *P. punctata* has fewer predicted BGCs compared to
299 stains of *Xylaria* and *Nemania*, all co-cultures with *P. punctata* as a partner resulted in a
300 significantly higher mean number of over-abundant features. As other six strains were either
301 isolated as endophytes or saprotrophs of wood/bark, the significant impact of *P. punctata* on
302 metabolite diversity may reflect its distinct ecological niche as a saprotroph of animal dung.
303 Herbivorous animal dung is a nutrient-rich, spatially limited, and ephemeral resource that
304 contains mycoparasites, bacteria, fungi, and invertebrates (48, 49). As a late colonizer of dung,
305 *P. punctata* has potentially evolved the ability to synthesize antagonistic metabolites to displace
306 already established organisms (50, 51). However, despite the higher number of over-abundant
307 metabolites, *P. punctata* produced the fewest number of unique features in co-culture: all *P.*
308 *punctata* co-cultures contained the same 1,096 features regardless of the partner. The lack of
309 unique metabolites potentially reflects the slightly lower BGC diversity within *P. punctata*,
310 which may be related to its role as a substrate specialist on dung, rather than a generalist
311 saprotroph or endophyte.
312
313 ***Co-culture and the production of bioactive compounds.*** Previous experiments show that
314 fungal co-culturing will 'turn on' the synthesis of bioactive compounds that are typically not
315 produced in pure culture (or only at low levels) (21, 23). While a Petri dish does not accurately
316 reflect nature, co-culturing has led to the discovery of novel chemical diversity and their
317 ecological roles (23, 28, 52, 53). In this present study, we found that for all strains, co-culture
318 resulted in a substantially higher number of features with hits to bioactive compounds with
319 antiviral, bactericidal, fungicidal, insecticidal, herbicidal, and nematicidal activity (Table 4;

320 Supplemental Table 4). Microorganisms employ secondary metabolites for niche exploitation
321 and colonization (5–7, 54). Thus, our observation that features related to broad-spectrum
322 antimicrobial compounds were over-abundant in co-culture rather than monoculture is consistent
323 with xylarialean fungi producing bioactive compounds as a mechanism to compete for resources
324 within complex microbial communities often found in decomposing organic matter and soils (21,
325 54, 55). Although the majority of putatively bioactive features were produced in co-culture, some
326 features related to bioactive compounds were observed in monocultures, suggesting the potential
327 for constitutive production of some secondary metabolites (e.g., griseofulvin; Fig. 6).
328 In previous studies of interactions between basidiomycete white rot fungi (*Trametes*
329 *coccinea* with either *Leiotrametes lactinea* or *T. versicolor*), the zone where competing
330 mycelium physically connected was darkly pigmented due to melanin production (56).
331 Interactions between the wood-decay fungi *Stereum hirsutum* and two *Coprinus* spp. also
332 produce melanin (57). In these studies, melanin production appears related to the production of
333 extracellular enzymes (e.g., laccase) that can convert phenolic compounds to reactive quinone
334 compounds (57, 58). Oxidative polymerization of quinone compounds produces melanin and
335 melanin-like compounds that act as physical barriers to competitors (57, 58). Here, we observed
336 a dark pigmentation in the co-culture interaction zones of *X. arbuscula*, especially with *N. diffusa*
337 or *X. flabelliformis* CBS 123580 (Supplemental Fig. 4), and on nutrient-rich media xylarialean
338 cultures often become very dark over time (see (17)) and *Xylaria* can cause spalting in wood
339 (59). Consistent with the numerous melanin BCGs in xylarialean genomes (11), we detected a
340 feature matching the melanin precursor molecule, 1,8-dihydroxynaphthalene (59), yet it was not
341 significantly over-abundant in co-culture compared to monocultures. Thus, while previous
342 studies on basidiomycete fungi show the production of large amounts of melanin in co-culture

343 (57, 58), the strains examined here appear to produce bioactive SMs as a defensive mechanism
344 rather than invest in melanin production.
345 In addition to their roles in complex microbial communities found in litter, wood, or
346 soils, the production of antimicrobial or cytotoxic compounds by xylarialean fungi may be
347 related to symbiotic interactions. Previously, it was noted that there was a positive relationship
348 between the number of BGCs and endophytic host breadth (11), consistent with fungal secondary
349 metabolites directly interacting with host plants, similar to toxin production by fungal pathogens
350 (60). However, endophytes can also produce antagonistic secondary metabolites that protect their
351 hosts from pathogens and pests (7, 61). For example, the entomopathogenic endophyte
352 *Beauveria bassiana* protects its hosts from insects (62). Here, in co-culture xylarialean fungi
353 produced a metabolite feature similar to the insecticidal sesquiterpenoid nootkatone described
354 from plants such as *Callitropsis nootkatensis* and grapefruit (63). In addition, the seven
355 xylarialean fungi produced metabolite features putatively identified as steviol glycosides and the
356 phytohormones ABA and JA. Numerous symbiotic fungi and bacteria produce plant
357 phytohormones that can influence plant responses to abiotic stressors and modulate plant growth
358 and development (64). For example, some strains of *Botrytis cinerea* can produce ABA (65).
359 Likewise, certain fungi, such as *Lasiodiplodia theobromae*, are capable of producing JA at high
360 levels (66). Interestingly here xylarialean fungi produced ABA only in co-culture, which
361 suggests a potential role for microbial interactions in stimulating the production of compounds
362 that impact host plants during symbiosis. Future studies are necessary to accurately identify these
363 features detected here and determine their role in endophyte-host interactions.
364
365 ***Importance of integrating genomic and metabolomic data.*** Although our untargeted approach
366 was aimed at understanding phylogenetic and ecological factors that influence metabolite

367 diversity in xylarialean fungi and does not provide the unambiguous identification of methods
368 such as NMR (67), numerous features over-abundant in co-culture were also linked to genes or
369 BGCs related to the production of secondary metabolites. Our results also suggest that even
370 fungi with significant genomic variation in BGC gene content and identity may be capable of
371 producing structurally related compounds with similar antifungal activity. For example, we
372 established connections between genes and features identified as griseofulvin and its structural
373 analogs (dechlorogriseofulvin and griseofulvic acid) for all strains except *P. punctata*. In the
374 present study, despite no known BGCs related to griseofulvin found in their genomes (11),
375 monocultures of *B. mediterranea* and *X. arbuscula* contained features with hits to griseofulvin
376 and its analogs.

377 Although we observed griseofulvin production in a few monocultures, it was primarily
378 observed in co-cultures, consistent with griseofulvin's antifungal activity against competitors (68,
379 69). A previous co-culture study of an endophytic *X. flabelliformis* strain with *Aspergillus*
380 *fischeri* also resulted in deadlock due to the production of griseofulvin (25). In that study,
381 griseofulvin was completely extruded into the media in co-culture; yet in monoculture, the
382 compound accumulated in outermost mycelia (25). Here, the visible inhibitory zone observed in
383 the majority of *X. flabelliformis* interactions are consistent with antifungal secretion into the
384 media, as was griseofulvin detection in media surrounding some monocultures. Further targeted
385 metabolomics research will be necessary to confirm that these strains produce griseofulvin, as
386 well as transcriptomics to identify the genes/BCGs that are upregulated during griseofulvin
387 production. There remain significant challenges to link diverse diverse xylarialean metabolites to
388 BCGs as >90% of metabolite features we detected here were not identified at the SuperClass
389 level and previously, only 25% of 6,879 putative BCGs predicted in 96 xylarialean genomes had
390 hits to characterized BCGs in MiBIG (11).

391

392 ***Conclusions***. The results of the present study are consistent with our previous hypothesis that
393 secondary metabolite diversity in xylarialean fungi is higher in strains that are ecological
394 generalists (11). Here, we found xylarialean strains that are substrate and host generalists
395 deployed a unique metabolic response for different competitors, consistent with their high BGC
396 diversity. In contrast, although ecological specialist dung saprotroph *P. punctata* was very
397 metabolically active and appeared to inhibit the growth of other fungi, rather than unique
398 responses to competitors in co-culture, it produced a very similar suite of metabolites regardless
399 of competitor identity. The fact that the *P. punctata* genome contains >60 predicted BGCs,
400 which is higher than typical Pezizomycotina fungi (Fig. 1), suggests that total BCG number
401 cannot predict a fungus' response to competitors. However, future studies should include
402 additional strains with diverse ecological modes to assess how ecological roles and BGC
403 diversity impact fungal metabolomes.

404 In addition to providing novel information on the evolutionary and ecological factors that
405 may influence secondary metabolism within xylarialean fungi, our results further emphasize the
406 incredible bioactivity of xylarialean fungi and their potential in the pharmaceutical, agricultural,
407 food, and cosmetic industries. Many of these putatively identified compounds also have been
408 attributed to antiproliferative, anti-inflammatory, antioxidant, antiallergic, and sedative
409 properties, as well as mycotoxins, actin-binding cytochalasins, and phytohormones. Obtaining
410 bioactive compounds is typically expensive and can have negative environmental impacts. For
411 example, although parthenolide can be obtained from its traditional plant sources, such processes
412 are neither sustainable nor prepared to meet the growing market demand (70). Thus, the potential
413 for fungi to provide an alternative source for some bioactive compounds would be both
414 economically and environmentally beneficial. Finally, the fact that the majority of the features in

415 this study were unidentified, even at the Superclass level, indicates a large number of novel
416 putative bioactive compounds. Future work will be necessary to accurately identify and
417 characterize the activity of the diverse metabolites identified here.
418
419 **Materials and Methods**
420
421 ***Fungal strain selection and co-cultivation experiment***
422 BCG content and phylogenetic position were used to select seven strains of Xylariaceae *s.l.* for
423 co-culturing and metabolomics (11) (Fig. 1; Supplemental Tables 5-7). A ~6 mm diameter agar
424 plug (removed from the growing edge of a 2-week-old pure culture of each strain using a sterile
425 transfer tube) was placed on a 100 mm Petri dish with minimal media containing glucose either
426 alone (i.e., monoculture) or in combination another strain (i.e., co-culture). Three replicate plates
427 were inoculated for each monoculture and co-culture interaction. After one month at room
428 temperature under ambient light/dark, defined interactions had occurred among the majority of
429 fungal pairs and each plate was photographed (Supplemental Figs. 1-7). Each co-culture
430 interaction was scored as either deadlock (i.e., visible inhibition zone) or invasion (i.e., one
431 fungus grew over the other) following (47).
432
433 ***Metabolite extraction and LC-MS/MS***
434 As the majority of co-cultures had no mycelial contact, we harvested only media from the entire
435 clear space between the hyphae of each strain (~4-7 cm x 1 cm) using a sterile scalpel. In three
436 cases, one strain overgrew the other, and we harvested a similar area of agar/mycelium in the
437 interaction zone containing mycelia of both strains. For monocultures, a similar area of
438 mycelium/agar was removed from the growing mycelial edge. A similar amount of media was

439 also harvested from each negative control plate. Media/tissue was immediately flash-frozen in
440 liquid N and stored at -80°C until lyophilized for 48 h. Freeze-dried samples were homogenized
441 to a fine powder in a 2 ml tube containing stainless steel beads (Qiagen, Hilden, Germany) at
442 1,400 RPM for 30-sec intervals (maximum of 90 sec). Samples were extracted in MeOH and
443 resuspended in methanol with an internal standard (ISTD) mix of isotopically labeled
444 compounds (71), filtered, and analyzed on an Agilent 1290 UHPLC inline with a Thermo
445 QExactive Orbitrap HF mass spectrometer (Thermo Scientific, San Jose, CA), implementing JGI
446 LC-MS/MS ESI methods (72, 73). Normal phase and reverse chromatography were performed (3
447 uL injections) with randomized injection order. Blanks (methanol only) were run between each
448 sample.
449 A list of features was generated with MZmine (74), removing isotopes, adducts, and
450 features without MS/MS. The most intense fragmentation spectrum for each feature was
451 uploaded to Global Natural Products Social Molecular Networking (GNPS) (75) and analyzed
452 using the Feature-Based Molecular Network (FBMN) workflow. Peak heights were normalized
453 using a global normalization approach adapted from NormalyzerDe (76). Only features that were
454 significantly more abundant (log2FC = 2; adjusted P-value <0.05) in cultures vs. controls were
455 kept for analyses (77). MetaboAnalyst 5.0 (78) was used to perform a data integrity check and
456 data filtering using Interquartile Range (IQR). Features from each chromatography column and
457 ionization mode were combined and compared between (i) each co-culture pair and their
458 corresponding monocultures and (ii) all pairwise combinations of monocultures (see Fig. 2). To
459 connect features to putative genes/BGCs, MAGI 1.0 (29) was run with default setting using
460 significant features and predicted genes of each strain (11).
461
462 **Data Availability**

463 Raw metabolomics data is deposited in the Mass spectrometry Interactive Virtual Environment
464 (http://massive.ucsd.edu/) with the accession number MSV000093277. For non-polar
465 metabolites Feature Based Molecular Networking (FBMN) (results in positive and negative
466 ionization modes (with minimum cosine score of 0.7 and at least 3 matching fragment ions for
467 both library matches) are available at:
468 https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=3b3f57d427b54d9fb5317bb94b73509b and
469 https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=b3f3b9c0a43f46ceb140aa99ff2d0a20. For
470 polar metabolites, FBMN results in positive and negative ionization modes are available at
471 https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=e54e61e735f04bae9f55cd26cb25b7b5 and
472 https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ec1f5d74e6c349a284e69a1f3f897476.
473 Genome data for all seven strains are available at JGI Mycocosm
474 (https://mycocosm.jgi.doe.gov/mycocosm/home).

475 **Acknowledgments**

476 Funding for the project was provided by the DOE JGI Large-scale Community Science Project
477 (Grant no. 503506 to JMU: https://doi.org/10.46936/10.25585/60001144) conducted by the U.S.
478 Department of Energy Joint Genome Institute (https://ror.org/04xm1d337), a DOE Office of
479 Science User Facility, that is supported by the Office of Science of the U.S. Department of
480 Energy operated under Contract No. DE-AC02-05CH11231. MEEF was funded by the
481 University of Arizona Office for Research, Innovation, and Impact and BIO5 Postdoctoral
482 Fellowship Program. The authors thank Drs. M. Tfaily and J.H. Wisecaver for helpful discussion
483 on the project; L.P. Moore for laboratory assistance; C. Orlando Ayala-Ortiz for sharing R
484 scripts; and G. Sensibar and D. Odum at Tucson High Magnet School for generating the time-

lapse of fungal interactions as part of their Southern Arizona Regional Science and Engineering Fair (SARSEF) project. The authors have no competing interests.

Tables

Table 1. Number of features combined and for each column type and ioniziation mode after processing.

Number of features	Combined	HILIC-Z +	HILIC-Z -	C18 +	C18 -
Raw data	47,316	8,181	7,556	21,282	10,297
Not significantly different from extraction and negative controls	37,554 (79.4%)	7,422 (90.7%)	5,988 (79.2%)	17,237 (81.0%)	6,907 (67.1%)
Significantly more abundant in the fungal cultures than controls*	**9,762**	**759**	**1,568**	**4,045**	**3,390**
Classified at Superclass level	955 (9.8%)	176 (23.2%)	150 (9.6%)	495 (12.2%)	134 (4.0%)

* Features that were used for all down-stream analyses after removal of duplicated InChI keys, keeping those with the best MQScore value.

491

Table 2. Number of features that were significantly greater (i.e., log2FC, adjusted P<0.05) in either co-culture vs. monoculture for all 21 pairwise combinations. Features derived from both chromatographies and ionization modes were combined into a single dataset and analyzed together.

Fungal Strain A	Fungal Strain B	Number of features more abundant in co-culture than one or both monocultures					Number of features more abundant in either co-culture or monoculture (i.e., "ambiguous")			Number of features more abundant in monoculture than co-culture				
		AB > A, and AB > B	AB > A	AB > B	Total (only co-culture)	Total co-culture (including ambiguous)	AB > B and A > AB	AB > A and B > AB	Total Ambiguous	A > AB, and B > AB	A > AB	B > AB	Total (only monoculture)	Total monoculture (including ambiguous)
Biscogniauxia mediterranea AZ0048*	*Poronia punctata* CBS180.79	186	363	2,467	3,016	3,064	2	46	48	2	11	150	163	211
Nemania diffusa NC0034	*Biscogniauxia mediterranea* AZ0048	56	500	1,330	1,886	1,992	70	36	106	2	93	204	299	405
Nemania diffusa NC0034	*Poronia punctata* CBS180.79	289	416	2,861	3,566	3,727	108	53	161	7	94	136	237	398
Nemania diffusa NC0034	*Xylaria flabelliformis* NC1011	51	1,022	738	1,811	1,888	11	66	77	2	24	75	101	178
Nemania diffusa NC0034	*Xylaria flabelliformis* CBS123580	52	697	1,345	2,094	2,186	22	70	92	2	19	54	75	167
Xylaria arbuscula FL1030	Biscogniauxia mediterranea AZ0048	164	526	1,578	2,268	2,290	0	22	22	4	20	75	99	121
Xylaria arbuscula FL1030*	*Nemania diffusa* NC0034	22	676	525	1,223	1,278	23	32	55	0	21	35	56	111
Xylaria arbuscula FL1030*	*Poronia punctata* CBS180.79	114	325	3,183	3,622	3,705	30	53	83	6	24	174	204	287
Xylaria arbuscula FL1030*	*Xylaria flabelliformis* NC1011	167	1,187	690	2,044	2,049	0	5	5	0	1	25	26	31
Xylaria arbuscula FL1030	*Xylaria flabelliformis* CBS123580	128	554	1,190	1,872	1,894	1	21	22	3	14	86	103	125
Xylaria flabelliformis CBS116.85	*Biscogniauxia mediterranea* AZ0048	148	414	2,485	3,047	3,159	75	37	112	1	77	167	245	357
Xylaria flabelliformis CBS116.85	*Nemania diffusa* NC0034	11	279	1,339	1,629	1,731	78	24	102	1	75	129	205	307
Xylaria flabelliformis CBS116.85	*Poronia punctata* CBS180.79	248	249	4,204	4,701	4,817	72	44	116	0	28	78	106	222
Xylaria flabelliformis CBS116.85	*Xylaria arbuscula* FL1030	39	407	1,641	2,087	2,153	48	18	66	0	31	15	46	112
Xylaria flabelliformis CBS116.85	*Xylaria flabelliformis* NC1011	4	62	1,267	1,333	1,338	0	5	5	0	1	70	71	76
Xylaria flabelliformis CBS116.85	*Xylaria flabelliformis* CBS123580	11	81	2,221	2,313	2,328	13	2	15	0	16	57	73	88
Xylaria flabelliformis NC1011	*Biscogniauxia mediterranea* AZ0048	336	757	2,337	3,430	3,456	5	21	26	1	57	57	115	141
Xylaria flabelliformis NC1011	*Poronia punctata* CBS180.79	103	321	3,424	3,848	3,906	8	50	58	0	16	113	129	187
Xylaria flabelliformis CBS123580	*Biscogniauxia mediterranea* AZ0048	241	1,026	1,403	2,670	2,691	18	3	21	3	149	21	173	194
Xylaria flabelliformis CBS123580	*Poronia punctata* CBS180.79	337	623	2,440	3,400	3,541	115	26	141	0	153	24	177	318
Xylaria flabelliformis CBS123580	*Xylaria flabelliformis* NC1011	30	1,597	172	1,799	1,799	0	0	0	4	37	5	46	46
Total Number of Unique Features for Each Category		**2,748**	**65**	**1,257**	**4,070**	**6,115**	**272**	**55**	**2,045**	**2**	**8**	**16**	**26**	**2,071**

* Indicates fungal strains that overgrew competitor in co-culture. See Supplemental Figs. 1-7 for images.

492

Table 3. Number of features and related compound gene-gene connections using MAGI 1.

Number of features (both columns and ionization modes combined):	**Co-culture and/or monoculture***	**Co-culture > monoculture****	**Monoculture > co-culture*****
Significantly different by treatment	6,141	6,115	2,071
MAGI 1, input features	783	741	251
MAGI 1, input compounds (InChI keys)	1,060	1,048	537
MAGI 1, unsearched compounds (InChI keys)	573	568	278
MAGI 1, features connected to compounds (InChI keys)	135	135	57
MAGI 1, genes connected to compounds (InChI keys)	2,651	2,601	584
MAGI 1, features connected to genes in SMGCs (antiSMASH)	83	82	27
Unique SMGC IDs	300	295	78
MAGI 1, features connected to genes in BCGs (using antiSMASH) with significant hits to MIBiG (KnownClusterBlast)	55	55	16
Unique MIBiG IDs	78	78	38

*Features that were significantly over- or under-represented in either mono- or co-culture (i.e., dysregulated).

**Includes features only over-represented in co-culture (AB>A; AB>B; or AB>A and AB>B), as well as ambiguous features (AB>A and B>AB; AB>B and A>AB).

***Includes features only over-represented in monoculture (B>AB; A>AB; or A>AB and B>AB), as well as ambiguous features (AB>A and B>AB; AB>B and A>AB)

493

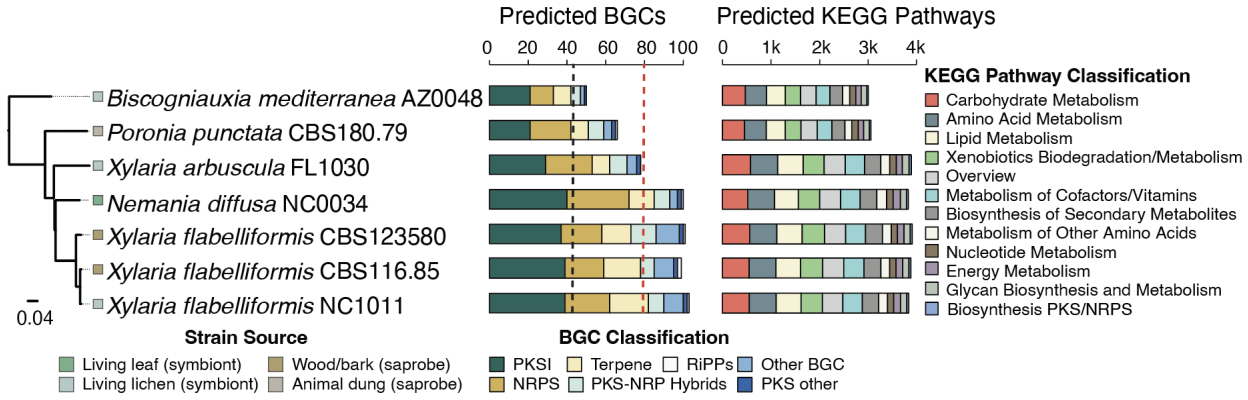
Table 4. Subset of the putatively bioactive compounds with MQScores >0.90, their frequency in xylarialean co-culture vs. mono-culture, biological activities, uses, and previously reported biological sources. See Supplemental Table 7 for more detailed information.

Compound name	Compound Category*	Observed Biological Activities*	Previously Described Natural Source*	Frequency in all co-culture pairwise analyses	Frequency in all co-culture pairwise analyses	Feature(s) MQScore
Nootkatone	Sesquiterpenoid	M, C, A, I, O	Plants	0.95	0.00	0.988043-0.413273
Perillaldehyde	Aldehyde	M, C, A	Plants	0.71	0.00	0.99322-0.592918
Chamazulene	Sesquiterpenoid	M	Plants	0.67	0.00	0.939767-0.743369
Orcinol β-D-glucoside	Glycoside	M	Plants	0.62	0.00	0.951372-0.740981
(-)-Myrtenal	Monoterpenoid	M, C	Plants	0.62	0.00	0.992069-0.679613
(+)-Carvone	Monoterpenoid	M, C, A, I, P	Plants	0.57	0.00	0.977081-0.972748
L-Carvone [(A)]	Monoterpenoid	M, C, A, I	Plants	0.57	0.00	0.901109-0.883955
ar-Turmerone	Sesquiterpenoid	M, C, A, I, O	Plants, Lichen	0.52	0.00	0.960267-0.815509
Neoabietic acid	Diterpenoid	A	Plants	0.19	0.00	0.998189-0.972936
α-Cyperone	Sesquiterpenoid	M, C, A	Fungi, Plants	0.95	0.02	0.960776-0.400954
Cytochalasin D	Cytochalasin	M, C, A	Fungi	0.90	0.02	0.935687-0.407988
Abietic acid	Diterpenoid	M, A	Algae, Plants	0.62	0.02	0.994925-0.414036
Lumichrome	Riboflavin metabolite	P	Algae, Bacteria, Fungi, Plants	0.29	0.02	0.95404-0.429184
Jasmonic acid	Oxo monocarboxylic acid	P	Algae, Fungi, Plants	0.71	0.05	0.994482-0.403317
Dehydrocostus lactone	Guaianolide sesquiterpene lactone	O	Bacteria	0.95	0.07	0.920102-0.416364
Parthenolide	Sesquiterpene lactone	M, C, A	Fungi, Plants	0.95	0.07	0.971081-0.405395
Galiellalactone	Lactone	C, A	Fungi	0.90	0.07	0.909601-0.415254
Steviol	Ent-kaurane diterpenoid	P	Plants	0.86	0.07	0.920258-0.478784
Bisabolol oxide A	Sesquiterpene	I	Animals, Plants	1.00	0.10	0.978746-0.458545
Zerumbone	Sesquiterpenoid	M, C, A, I	Plants	1.00	0.10	0.969946-0.40489
Costunolide	Germacranolide	M, C, A	Plants	0.95	0.12	0.925914-0.412583
Alternariol	Benzochromenone	C, O	Fungi, Lichen	0.71	0.12	0.902518-0.524149
Norlichexanthone	Xanthone	M, C, A, O	Fungi, Lichen, Plants	0.71	0.12	0.945321-0.428965
Thymoquinone	Benzoquinones	M, C, A	Plants	0.62	0.12	0.934129-0.879387
Lecanoric acid	Benzoate ester	M, C, A	Fungi, Lichen	0.43	0.12	0.983711-0.81159
Orsellinic acid	Phenol	M	Fungi, Lichen	0.43	0.12	0.985166-0.888573
Valerenic acid	Monocarboxylic acid	M	Plants	1.00	0.14	0.965617-0.428295
Polygodial	Sesquiterpenoid	M, C, A, I	Animals, Plants	1.00	0.14	0.977564-0.42917
(-)-Globulol	Sesquiterpenoid	A	Fungi, Plants	0.90	0.14	0.946199-0.798093
Cedrol	Sesquiterpenoid alcohol	M, C, A, I	Fungi, Plants	0.71	0.14	0.983602-0.797492
Patchouli alcohol	Sesquiterpenoid tertiary alcohol	M, C, A, I, O	Plants	0.71	0.14	0.995816-0.804617
α-Bisabolol	Monocyclic sesquiterpene alcohol	M, C	Algae, Fungi, Plants	0.67	0.14	0.949284-0.621228
Farnesol [(A)]	Sesquiterpenoid	M, C, I, O	Animals, Fungi, Plants	0.67	0.14	0.952341-0.582818
β-Elemene	Sesquiterpenoid	M, C, A, I	Animals, Fungi, Algae, Plants	0.67	0.14	0.915714-0.816806
Stachydrine	Proline and derivatives	M, C	Animals, Fungi, Algae, Plants	0.52	0.14	0.980021-0.745809
Griseofulvin [(A)]	Benzofuran	A	Fungi	0.90	0.19	0.97691-0.536526
(-)-Caryophyllene oxide	Epoxide	M, C, I	Plants	1.00	0.21	0.984298-0.400756
Malioxamycin	Valine derivate	A	Bacteria	0.29	0.21	0.930222-0.410817
Alantolactone	Sesquiterpene Lactone	M, C, A, I	Plants	1.00	0.24	0.952116-0.405555

* Information from Chemical Entities of Biological Interest (ChEBI; https://www.ebi.ac.uk/chebi/). **(A) Antimicrobial**: anti-oomycete, antibacterial, antidermatophyte, antifungal, antileishmanial, antinematodal, antiviral, anti-HIV, anti-trypanosomal; **(C) Cytotoxic**: anti-tumor, herbicidal, mycotoxin, phytotoxic; **(I) Insecticidal**: anti-tick, insect antifeedant; **(M) Medicinal:** anti-acetylcholinesterase, anti-inflammatory, anti-osteoporotic, anti-psoriasis, anti-anxiety, anti-depressant, anti-pyretic, anti-venom, anti-epileptic, anti-osteoporosis, anxiolytic, cardioprotective, hepatoprotective, immunosuppressant, nephroprotective, neuroprotective, acaricidal, analgesic, anthelmintic; **(P) Plant-Interaction:** legume-rhizobia symbiosis, plant-growth promoting molecule, potato sprouting inhibitor, plant sweetener, phytohormone; **(O) Other:** antioxidant, aroma-flavor, fragrance, quorum-sensing, virulence factor, spore germination/fungal inhibitor

494

495 **Figures**



496

497

498 **Figure 1. Phylogenetic relationships and metabolic gene content for the seven ecologically**

499 **diverse xylarialean fungal strains used in this study**. To examine the impact of phylogenetic

500 distance on metabolic production in co-culture we selected three closely related strains of

501 *Xylaria flabelliformis,* along with four additional strains of Xylariaceae s.l. that were

502 increasingly phylogenetically distant from *X. flabelliformis* (11). *Xylaria flabelliformis* was

503 chosen as a focal species due to the highest number of BGCs and previous untargeted

504 metabolomics studies that detected bioactive metabolites in NC1011 (11). Different colored

505 boxes next to each strain name indicate its substrate of isolation in either living tissues

506 (symbiont) or decomposing tissues (saprobe). Stacked bars indicate the number and classification

507 of predicted biosynthetic gene clusters (BGCs; the black and red dashed lines show the mean

508 number of BCGs in genomes of other Pezizomycotina and Xylariaceae *s.l.,* respectively) and

509 KEGG pathways in each strain (11). See Supplemental Tables 5-7.

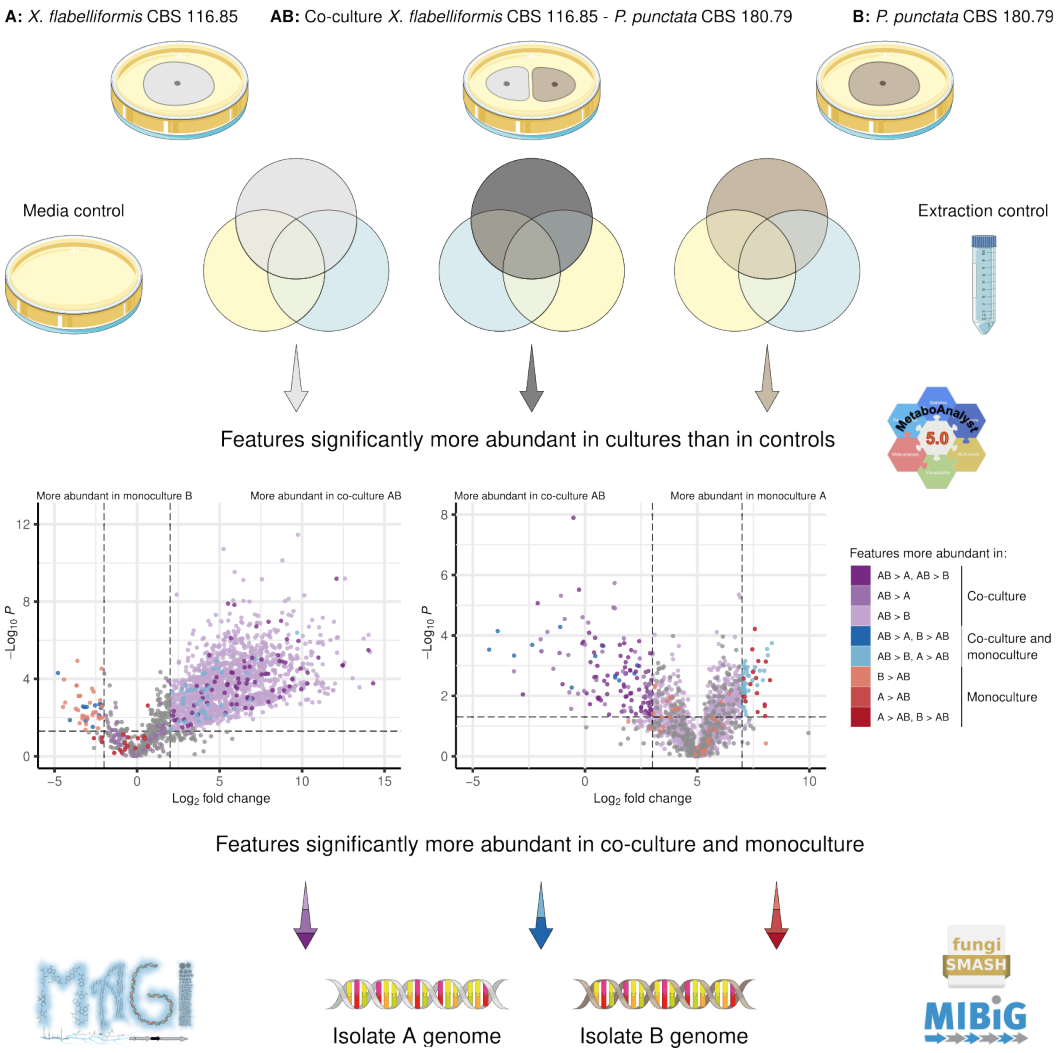


Figure 2. Schematic of analysis workflow to identify metabolite features significantly more abundant in co-culture or monoculture for 21 different pairwise comparisons. In total, there were three replicate plates for each pairwise co-culture (n = 63), each of the seven monocultures (n = 21) for a total of 84 samples. Negative controls included three media-only plates (extracted in parallel) and extraction controls. Features from each monoculture (above A or B) and co-culture (A+B) whose abundance was log2FC greater than controls were selected to perform pairwise comparisons of each co-culture (A:B) against each corresponding monoculture (A or B) using a univariate volcano plot analysis in MetaboAnalyst (criteria >log2 fold-change (log2FC),

520 p-value < 0.05) (Table 1). Given the nature of the pairwise comparisons, some features could not
521 be unambiguously assigned to either category: they were more abundant in co-culture compared
522 to at least one of the monocultures, but also more abundant in at least one of the monocultures
523 compared to a co-culture (i.e., AB > B and A > AB; AB > A and B > AB; hereafter,
524 "ambiguous" features) (see Table 2). MAGI 1.0 (29) was executed with all significantly
525 dysregulated features in co-cultures relative to each of their corresponding monocultures against
526 genes in the corresponding genome of each strain (i.e., 4 compound sets with 2 gene sets) (11).
527 Compound-gene connections ('MAGI score' ≥ 2, a 'reciprocal score' ≥ 1, and labeled as 'direct')
528 were then compared to predicted BGCs and annotations from MiBIG (11) (Table 3). Data from
529 both chromatography columns and ionization modes were combined for analyses (with
530 duplicates removed).

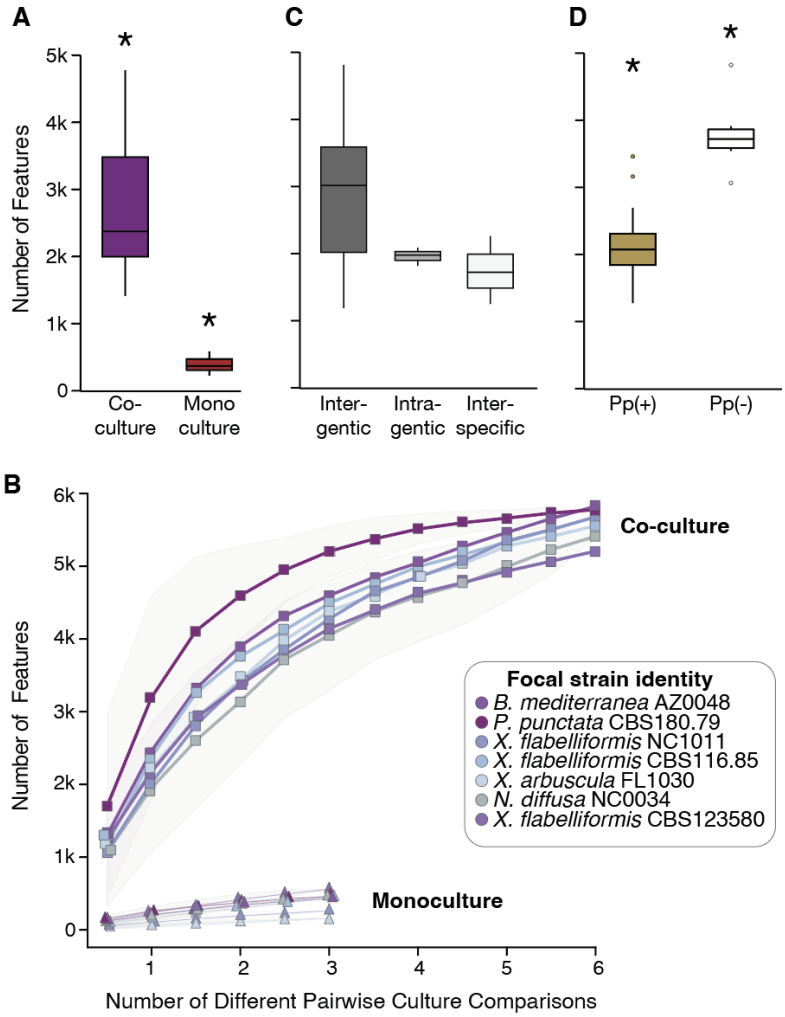


Figure 3. Statistical comparisons of metabolite richness between co-cultures and monocultures. (**A**) Interquartile box plots comparing the median number of significantly over-abundant metabolites produced in all pairwise co-culture comparisons vs. all monoculture comparisons. Asterisks indicate significant differences after t-test of means ($t_{20.6} = 11.56$, $P < 0.0001$). (**B**) Rarefaction curves of metabolite richness as a function of the number of comparisons to other cultures when grown in either co-culture or monoculture. (**C-D**) Interquartile box plots comparing the median number of metabolites that were significantly over-abundant in co-culture as a function of the taxonomic relationships among different pairs. (**C**) Intergenic: pairwise comparisons of strains in four different genera (*Xylaria*, *Nemania*, *Poronia*,

541 and *Biscognauxia*); Intragenic: pairwise comparisons of four *Xylaria* spp.; Interspecific: pairwise

542 comparisons of three *X. flabilliformis* strains). ANOVA found no significant differences: $F_{2,18}$ =

543 2.60, P = 0.1000. (**D**) Pairwise co-culture comparisons either with (Pp+) or without (Pp-) strain

544 *P. punctata* CBS 180.79. Asterisks indicate significant differences after t-test of means ($t_{9.5}$ =

545 −5.84, P = 0.0002).

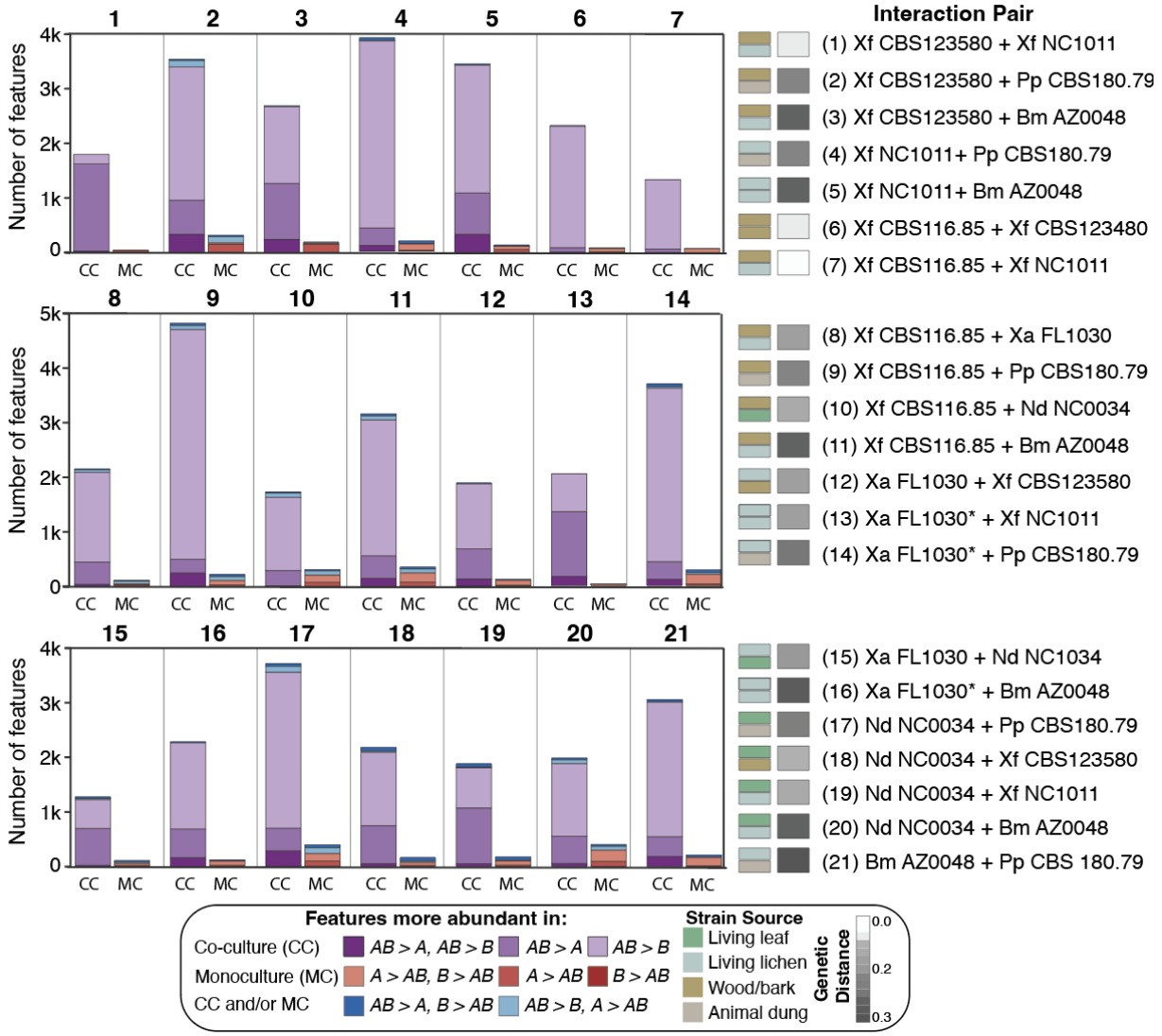


Figure 4. Stacked bar plots with the total number of features that were identified as significantly more abundant in either co-culture (CC) or monoculture (MC) for all 21 interaction pairs. Bars are colored by the statistical methods used to assign features to either treatment, as shown in Fig. 2. Ambiguously defined features (defined as CC and/or MC) are also indicated. Each numbered plot corresponds to one co-culture interaction pair (abbreviated strain names are listed to the right). Asterisks (*) next to *X. arbuscula* (Xa FL1030) indicate three cases where this strain overgrew its competitor in co-culture. For each interaction pair, boxes to the left of the name indicate either the substrate source of each strain or the pairwise genetic distance

555 between genomes of each strain (11). Metabolites were compared after one month of growth
556 either in co-culture or monoculture on *Aspergillus* minimal medium at room temperature under
557 ambient light/dark conditions. Features derived from both chromatography columns and
558 ionization modes were combined into a single dataset and analyzed together (Supplemental
559 Table 1).

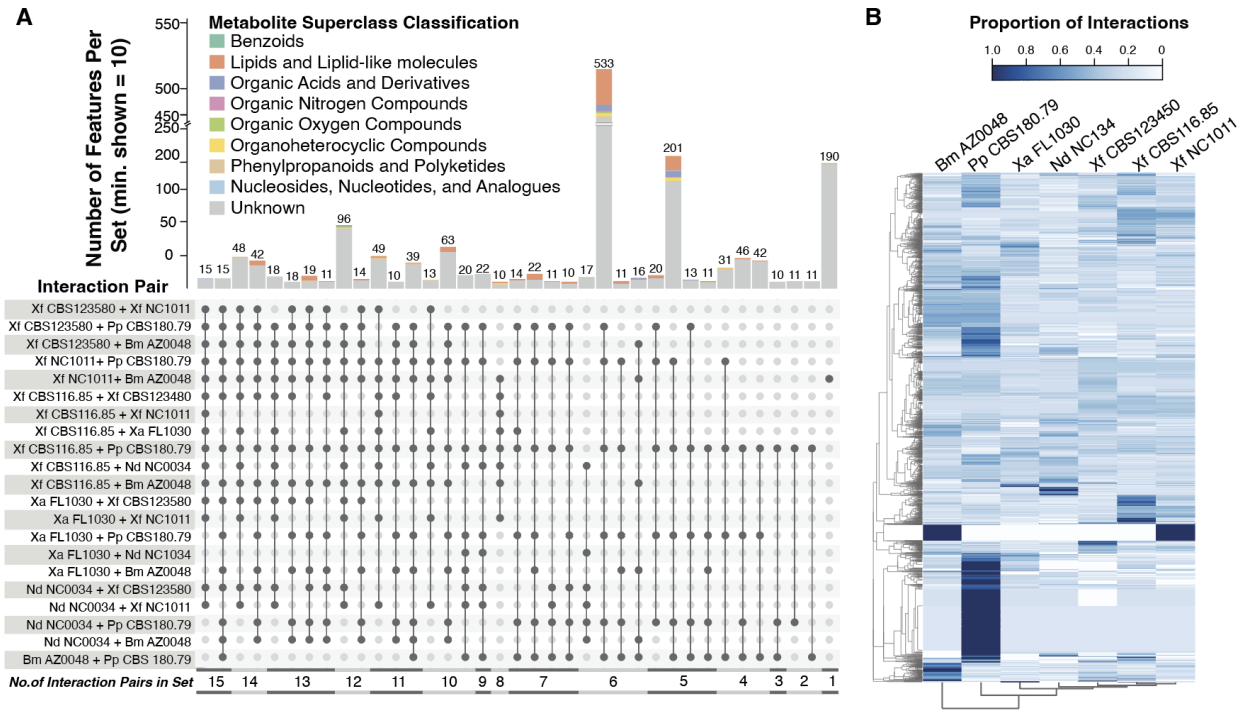


Figure 5. Overlap and frequency of features over-represented in co-culture vs. monoculture. (A) UpSet plot showing the distribution of features that were over-abundant in co-culture vs. monocultures across all 21 interaction pairs. The height of each stacked bar indicates the number of features found (i.e., "intersection" size) for different interaction pair(s). Vertical black lines connect features shared between >1 pair. Dot color indicates whether a feature is present (black) or absent (grey) for each interaction pair. Colors in each stacked bar correspond to feature classifications within each set at the 'Superclass level' using the ClassyFire. Interactions with <10 features are not displayed for readability. **(B)** Heatmap based on the proportion of co-cultures (columns) where a feature (row) was found in co-culture for a particular strain (i.e., number of occurrences out of six different co-cultures). A proportion equal to 1.0 (dark blue) thus corresponds to compounds always detected in co-cultures with that strain, suggesting it may be the compound producer. Compounds and fungal strains were grouped using hierarchical clustering (complete) using Euclidean distance (see also Supplemental Fig. 11,

574 where strains are instead ordered by their phylogenetic relationships). Features derived from both
575 chromatography columns and ionization modes were combined into a single dataset and
576 analyzed together with duplicates removed.

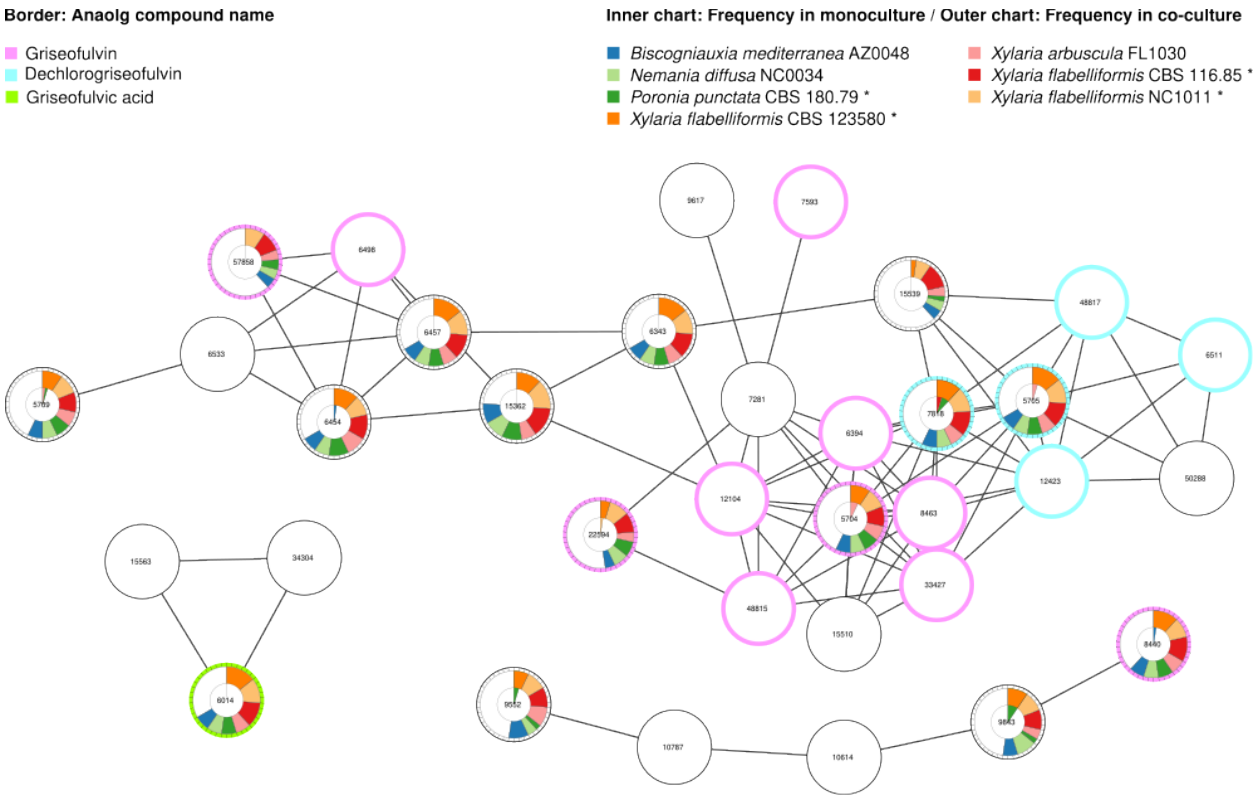


Figure 6. Compounds structurally related to griseofulvin were frequently detected among co-cultures yet rarely observed in monocultures. Network showing some features structurally related to griseofulvin. Each node represents a feature with its ID in the center. The border is colored by the analog compound identified in the database: griseofulvin. The outer and inner charts show the frequency with which the feature was detected as significantly more or less abundant (respectively) in co-culture than monoculture, colored by fungal strain. Asterisks indicate strains with BGCs matching the griseofulvin biosynthetic gene cluster from *Penicillium aethiopicum* at the MIBiG repository (BGC0000070). Nodes were positioned by a pre-fuse force-directed layout algorithm, and edges between two nodes were weighted by the Cosine score. Ions derived from the C18 chromatography and positive ionization mode.

589 **References**

590 1. Naranjo-Ortiz MA, Gabaldón T. 2019. Fungal evolution: diversity, taxonomy and
591 phylogeny of the Fungi. Biol Rev Camb Philos Soc 94:2101–2137.

592 2. Peay KG, Kennedy PG, Talbot JM. 2016. Dimensions of biodiversity in the Earth
593 mycobiome. Nat Rev Microbiol 14:434–447.

594 3. Richards TA, Talbot NJ. 2013. Horizontal gene transfer in osmotrophs: playing with public
595 goods. Nat Rev Microbiol 11:720–727.

596 4. Naranjo-Ortiz MA, Gabaldón T. 2019. Fungal evolution: major ecological adaptations and
597 evolutionary transitions. Biol Rev Camb Philos Soc 94:1443–1476.

598 5. Bills GF, Gloer JB. 2016. Biologically active secondary metabolites from the fungi.
599 Microbiol Spectr 4.

600 6. Keller NP. 2019. Fungal secondary metabolism: regulation, function and drug discovery.
601 Nat Rev Microbiol 17:167–180.

602 7. Spiteller P. 2015. Chemical ecology of fungi. Nat Prod Rep 32:971–993.

603 8. Gluck-Thaler E, Haridas S, Binder M, Grigoriev IV, Crous PW, Spatafora JW, Bushley K,
604 Slot JC. 2020. The architecture of metabolism maximizes biosynthetic diversity in the
605 largest class of Fungi. Mol Biol Evol 37:2838–2856.

606 9. Wisecaver JH, Slot JC, Rokas A. 2014. The evolution of fungal metabolic pathways. PLoS
607 Genet 10:e1004816.

608 10. Wendt L, Sir EB, Kuhnert E, Heitkämper S, Lambert C, Hladki AI, Romero AI, Luangsa-
609 ard JJ, Srikitikulchai P, Peršoh D, Stadler M. 2018. Resurrection and emendation of the
610 Hypoxylaceae, recognised from a multigene phylogeny of the Xylariales. Mycol Prog
611 17:115–154.

612 11. Franco MEE, Wisecaver JH, Arnold AE, Ju Y-M, Slot JC, Ahrendt S, Moore LP, Eastman
613 KE, Scott K, Konkel Z, Mondo SJ, Kuo A, Hayes RD, Haridas S, Andreopoulos B, Riley R,
614 LaButti K, Pangilinan J, Lipzen A, Amirebrahimi M, Yan J, Adam C, Keymanesh K, Ng V,
615 Louie K, Northen T, Drula E, Henrissat B, Hsieh H-M, Youens-Clark K, Lutzoni F,
616 Miadlikowska J, Eastwood DC, Hamelin RC, Grigoriev IV, U'Ren JM. 2022. Ecological
617 generalism drives hyperdiversity of secondary metabolite gene clusters in xylarialean
618 endophytes. New Phytol 233:1317–1330.

619 12. Becker K, Stadler M. 2021. Recent progress in biodiversity research on the Xylariales and
620 their secondary metabolism. J Antibiot 74:1–23.

621 13. Helaly SE, Thongbai B, Stadler M. 2018. Diversity of biologically active secondary
622 metabolites from endophytic and saprotrophic fungi of the ascomycete order Xylariales. Nat
623 Prod Rep 35:992–1014.

624 14. Hsieh H-M, Lin C-R, Fang M-J, Rogers JD, Fournier J, Lechat C, Ju Y-M. 2010.
625 Phylogenetic status of Xylaria subgenus *Pseudoxylaria* among taxa of the subfamily
626 Xylarioideae (Xylariaceae) and phylogeny of the taxa involved in the subfamily. Mol
627 Phylogenet Evol 54:957–69.

628 15. Stadler M, Læssøe T, Fournier J, Decock C, Schmieschek B, Tichy H-V, Peršoh D. 2014. A

629 polyphasic taxonomy of *Daldinia* (Xylariaceae)1. Stud Mycol 77:1–143.

630 16. Hsieh H-M, Ju Y-M, Rogers JD. 2005. Molecular phylogeny of *Hypoxylon* and closely
631 related genera. Mycologia 97:844–865.

632 17. U'Ren JM, Miadlikowska J, Zimmerman NB, Lutzoni F, Stajich JE, Arnold AE. 2016.
633 Contributions of North American endophytes to the phylogeny, ecology, and taxonomy of
634 Xylariaceae (Sordariomycetes, Ascomycota). Mol Phylogenet Evol 98:210–232.

635 18. Davis EC, Franklin JB, Shaw AJ, Vilgalys R. 2003. Endophytic *Xylaria* (Xylariaceae)
636 among liverworts and angiosperms: phylogenetics, distribution, and symbiosis. Am J Bot
637 90:1661–1667.

638 19. Brunner F, Petrini O. 1992. Taxonomy of some *Xylaria* species and xylariaceous
639 endophytes by isozyme electrophoresis. Mycol Res 96:723–733.

640 20. Scott K, Konkel Z, Gluck-Thaler E, Valero David GE, Simmt CF, Grootmyers D, Chaverri
641 P, Slot J. 2023. Endophyte genomes support greater metabolic gene cluster diversity
642 compared with non-endophytes in *Trichoderma.* PLoS One 18(12):e0289280

643 21. Bertrand S, Bohni N, Schnee S, Schumpp O, Gindro K, Wolfender J-L. 2014. Metabolite
644 induction via microorganism co-culture: a potential way to enhance chemical diversity for
645 drug discovery. Biotechnol Adv 32:1180–1204.

646 22. Netzker T, Flak M, Krespach MK, Stroe MC, Weber J, Schroeckh V, Brakhage AA. 2018.
647 Microbial interactions trigger the production of antibiotics. Curr Opin Microbiol 45:117–
648 123.

649 23. Knowles SL, Raja HA, Roberts CD, Oberlies NH. 2022. Fungal-fungal co-culture: a primer
650 for generating chemical diversity. Nat Prod Rep 39:1557–1573.

651 24. Sica VP, Rees ER, Tchegnon E, Bardsley RH, Raja HA, Oberlies NH. 2016. Spatial and
652 temporal profiling of Griseofulvin production in *Xylaria cubensis* using mass spectrometry
653 mapping. Front Microbiol 7:544.

654 25. Knowles SL, Raja HA, Wright AJ, Lee AML, Caesar LK, Cech NB, Mead ME, Steenwyk
655 JL, Ries LNA, Goldman GH, Rokas A, Oberlies NH. 2019. Mapping the fungal battlefield:
656 using *in situ* chemistry and deletion mutants to monitor interspecific chemical interactions
657 between Fungi. Front Microbiol 10:285.

658 26. Knowles SL, Raja HA, Isawi IH, Flores-Bocanegra L, Reggio PH, Pearce CJ, Burdette JE,
659 Rokas A, Oberlies NH. 2020. Wheldone: characterization of a unique scaffold from the
660 coculture of *Aspergillus fischeri* and *Xylaria flabelliformis*. Org Lett 22:1878–1882.

661 27. Fricke J, Schalk F, Kreuzenbeck NB, Seibel E, Hoffmann J, Dittmann G, Conlon BH, Guo
662 H, Wilhelm de Beer Z, Vassão DG, Gleixner G, Poulsen M, Beemelmanns C. 2023.
663 Adaptations of *Pseudoxylaria* towards a comb-associated lifestyle in fungus-farming
664 termite colonies. ISME J 17:733–747.

665 28. Selegato DM, Castro-Gamboa I. 2023. Enhancing chemical and biological diversity by co-
666 cultivation. Front Microbiol 14:1117559.

667 29. Erbilgin O, Rübel O, Louie KB, Trinh M, Raad M de, Wildish T, Udwary D, Hoover C,
668 Deutsch S, Northen TR, Bowen BP. 2019. MAGI: A method for Metabolite Annotation and
669 Gene Integration. ACS Chem Biol 14:704–714.

670 30. Seca AML, Silva AMS, Pinto DCGA. 2017. Chapter 9 - Parthenolide and Parthenolide-like
671 Sesquiterpene lactones as multiple targets drugs: current knowledge and new developments,
672 p. 337–372. *In* Atta-ur-Rahman (ed.), Studies in Natural Products Chemistry. Elsevier.

673 31. Liu X, Bian L, Duan X, Zhuang X, Sui Y, Yang L. 2021. Alantolactone: A sesquiterpene
674 lactone with diverse pharmacological effects. Chem Biol Drug Des 98:1131–1145.

675 32. Kipanga PN, Demuyser L, Vrijdag J, Eskes E, D'hooge P, Matasyoh J, Callewaert G,
676 Winderickx J, Van Dijck P, Luyten W. 2021. Investigating the antifungal mechanism of
677 action of polygodial by phenotypic screening in *Saccharomyces cerevisiae*. Int J Mol Sci
678 22.

679 33. Ibáñez MD, Sánchez-Ballester NM, Blázquez MA. 2022. Healthy zerumbone: From natural
680 sources to strategies to improve its bioavailability and oral administration. Plants 12:5.

681 34. Ceunen S, Geuns JMC. 2013. Steviol glycosides: chemical diversity, metabolism, and
682 function. J Nat Prod 76:1201–1228.

683 35. Murphy K, Kubin ZJ, Shepherd JN, Ettinger RH. 2010. *Valeriana officinalis* root extracts
684 have potent anxiolytic effects in laboratory rats. Phytomedicine 17:674–678.

685 36. Cook D, Gardner DR, Pfister JA. 2014. Swainsonine-containing plants and their
686 relationship to endophytic fungi. J Agric Food Chem 62:7326–7334.

687 37. Burkin AA, Kononenko GP. 2015. Metabolites of toxigenic fungi in lichens of genera
688 *Alectoria*, *Bryoria*, *Evernia*, *Pseudevernia*, and *Usnea*. Biology Bulletin 42:296–301.

689 38. Tripathi AK, Prajapati V, Kumar S. 2003. Bioactivities of l-carvone, d-carvone, and

690 dihydrocarvone toward three stored product beetles. J Econ Entomol 96:1594–1601.

691 39. Fleming A. 1929. On the antibacterial action of cultures of a *Penicillium*, with special
692 reference to their use in the isolation of *B. influenzae*. Br J Exp Pathol 10:226–236.

693 40. Dame ZT, Islam MT, Helmke E, von Tiedemann A, Laatsch H. 2016. Oligomycins and
694 pamamycin homologs impair motility and induce lysis of zoospores of the grapevine downy
695 mildew pathogen, *Plasmopara viticola*. FEMS Microbiol Lett 363.

696 41. Takeuchi M, Inukai M, Enokita R, Iwado S, Takahashi S, Arai M. 1980. Malioxamycin, a
697 new antibiotic with spheroplast-forming activity. I. Producing organism, fermentation,
698 isolation and characterization. J Antibiot 33:1213–1219.

699 42. Torres M, Balcells M, Sala N, Sanchis V, Canela R. 1998. Bactericidal and fungicidal
700 activity of *Aspergillus ochraceus* metabolites and some derivatives. Pestic Sci 53:9–14.

701 43. Nowak-Thompson B, Gould SJ, Kraus J, Loper JE. 1994. Production of 2,4-
702 diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can J
703 Microbiol 40:1064–1066.

704 44. Macías-Rubalcava ML, Sánchez-Fernández RE. 2017. Secondary metabolites of endophytic
705 *Xylaria* species with potential applications in medicine and agriculture. World J Microbiol
706 Biotechnol 33:15.

707 45. Rokas A, Mead ME, Steenwyk JL, Raja HA, Oberlies NH. 2020. Biosynthetic gene clusters
708 and the evolution of fungal chemodiversity. Nat Prod Rep 37:868–878.

709 46. Chapman RN, Gause GF. 1935. The Struggle for Existence. Ecology 16:656.

710 47. Boddy L. 2000. Interspecific combative interactions between wood-decaying
711 basidiomycetes. FEMS Microbiol Ecol 31:185–194.

712 48. Bills GF, Gloer JB, An Z. 2013. Coprophilous fungi: antibiotic discovery and functions in
713 an underexplored arena of microbial defensive mutualism. Curr Opin Microbiol 16:549–
714 565.

715 49. Bruns TD. 2019. The developing relationship between the study of fungal communities and
716 community ecology theory. Fungal Ecol 39:393–402.

717 50. Wicklow DT, Hirschfield BJ. 1979. Evidence of a competitive hierarchy among
718 coprophilous fungal populations. Can J Microbiol 25:855–858.

719 51. Ceci A, Angelini P, Iotti M, Lalli G, Leonardi M, Pacioni G, Perrone L, Pioli S, Siniscalco
720 C, Spinelli V, Venturella G, Wagensommer RP, Zotti M, Persiani AM. 2021. Values and
721 challenges in the assessment of coprophilous fungi according to the IUCN Red List criteria:
722 the case study of *Poronia punctata* (Xylariales, Ascomycota). Plant Biosyst 155:199–203.

723 52. Arora D, Gupta P, Jaglan S, Roullier C, Grovel O, Bertrand S. 2020. Expanding the
724 chemical diversity through microorganisms co-culture: current status and outlook.
725 Biotechnol Adv 40:107521.

726 53. Xu S, Li M, Hu Z, Shao Y, Ying J, Zhang H. 2023. The potential use of fungal co-culture
727 strategy for discovery of new secondary metabolites. Microorganisms 11:464.

728 54. Fox EM, Howlett BJ. 2008. Secondary metabolism: regulation and role in fungal biology.
729 Curr Opin Microbiol 11:481–487.

730 55. Karlovsky P. 2008. Secondary metabolites in soil ecology, p. 1–19. *In* Secondary
731 Metabolites in Soil Ecology. Springer Berlin Heidelberg, Berlin, Heidelberg.

732 56. Dullah S, Hazarika DJ, Goswami G, Borgohain T, Ghosh A, Barooah M, Bhattacharyya A,
733 Boro RC. 2021. Melanin production and laccase mediated oxidative stress alleviation
734 during fungal-fungal interaction among basidiomycete fungi. IMA Fungus 12:33.

735 57. Peiris D, Dunn WB, Brown M, Kell DB, Roy I, Hedger JN. 2008. Metabolite profiles of
736 interacting mycelial fronts differ for pairings of the wood decay basidiomycete fungus,
737 *Stereum hirsutum* with its competitors *Coprinus micaceus* and *Coprinus disseminatus*.
738 Metabolomics 4:52–62.

739 58. Rayner ADM, Griffith GS, Ainsworth AM. 1995. Mycelial Interconnectedness, p. 21–40. *In*
740 The Growing Fungus. Springer Netherlands, Dordrecht.

741 59. Morris H, Smith KT, Robinson SC, Göttelmann M, Fink S, Schwarze FWMR. 2021. The
742 dark side of fungal competition and resource capture in wood: Zone line spalting from
743 science to application. Mater Des 201:109480.

744 60. Li J, Cornelissen B, Rep M. 2020. Host-specificity factors in plant pathogenic fungi. Fungal
745 Genet Biol 144:103447.

746 61. Schulz B, Boyle C, Draeger S, Römmert A-K, Krohn K. 2002. Endophytic fungi: a source
747 of novel biologically active secondary metabolites. Mycol Res 106:996–1004.

748 62. Sui L, Lu Y, Zhou L, Li N, Li Q, Zhang Z. 2023. Endophytic *Beauveria bassiana* promotes
749 plant biomass growth and suppresses pathogen damage by directional recruitment. Front
750 Microbiol 14:1227269.

751 63. Fan J, Liu Z, Xu S, Yan X, Cheng W, Yang R, Guo Y. 2022. Non-food bioactive product
752 (+)-nootkatone: Chemistry and biological activities. Ind Crops Prod 177:114490.

753 64. Egamberdieva D, Wirth SJ, Alqarawi AA, Abd Allah EF, Hashem A. 2017. Phytohormones
754 and beneficial microbes: essential components for plants to balance stress and fitness. Front
755 Microbiol 8:2104.

756 65. Izquierdo-Bueno I, González-Rodríguez VE, Simon A, Dalmais B, Pradier J-M, Le Pêcheur
757 P, Mercier A, Walker A-S, Garrido C, Collado IG, Viaud M. 2018. Biosynthesis of abscisic
758 acid in fungi: identification of a sesquiterpene cyclase as the key enzyme in *Botrytis*
759 *cinerea*. Environ Microbiol 20:2469–2482.

760 66. Eng F, Marin JE, Zienkiewicz K, Gutiérrez-Rojas M, Favela-Torres E, Feussner I. 2021.
761 Jasmonic acid biosynthesis by fungi: derivatives, first evidence on biochemical pathways
762 and culture conditions for production. PeerJ 9:e10873.

763 67. Moco S. 2022. Studying Metabolism by NMR-Based Metabolomics. Front Mol Biosci
764 9:882487.

765 68. Oxford AE, Raistrick H, Simonart P. 1939. Studies in the biochemistry of micro-organisms:
766 Griseofulvin, C(17)H(17)O(6)Cl, a metabolic product of *Penicillium griseofulvum* Dierckx.
767 Biochem J 33:240–248.

768 69. Aris P, Wei Y, Mohamadzadeh M, Xia X. 2022. Griseofulvin: an updated overview of old
769 and current knowledge. Molecules 27.

770 70. Shi Y, Dong T, Zeng B, Yao M, Wang Y, Xie Z, Xiao W, Yuan Y. 2022. Production of
771 plant sesquiterpene lactone parthenolide in the yeast cell factory. ACS Synth Biol 11:2473–

2483.

71. Katherine B. Louie, Suzanne Kosina, Thomas Harwood, Meghana Faltane, Marie Lynde, Benjamin P. Bowen, Trent Northen. 2024. JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Polar HILIC-Z. Protocols.io. dx.doi.org/10.17504/protocols.io.kxygxydwkl8j/v1

72. Katherine B. Louie, Suzanne Kosina, Thomas Harwood, Meghana Faltane, Marie Lynde, Benjamin P. Bowen, Trent Northen. 2024. JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Nonpolar C18. Protocols.io. dx.doi.org/10.17504/protocols.io.ewov19r27lr2/v1

73. Katherine B. Louie, Suzanne Kosina, Thomas Harwood, Meghana Faltane, Marie Lynde, Benjamin P. Bowen, Trent Northen. 2024. JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Nonpolar C18. Protocols.io. dx.doi.org/10.17504/protocols.io.ewov19r27lr2/v1

74. Pluskal T, Castillo S, Villar-Briones A, Oresic M. 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinformatics 11:395.

75. Wang M, Carver JJ, Phelan VV, Sanchez LM, Garg N, Peng Y, Nguyen DD, Watrous J, Kapono CA, Luzzatto-Knaan T, Porto C, Bouslimani A, Melnik AV, Meehan MJ, Liu W-T, Crüsemann M, Boudreau PD, Esquenazi E, Sandoval-Calderón M, Kersten RD, Pace LA, Quinn RA, Duncan KR, Hsu C-C, Floros DJ, Gavilan RG, Kleigrewe K, Northen T, Dutton RJ, Parrot D, Carlson EE, Aigle B, Michelsen CF, Jelsbak L, Sohlenkamp C, Pevzner P,

793 Edlund A, McLean J, Piel J, Murphy BT, Gerwick L, Liaw C-C, Yang Y-L, Humpf H-U,
794 Maansson M, Keyzers RA, Sims AC, Johnson AR, Sidebottom AM, Sedio BE, Klitgaard A,
795 Larson CB, P CAB, Torres-Mendoza D, Gonzalez DJ, Silva DB, Marques LM, Demarque
796 DP, Pociute E, O'Neill EC, Briand E, Helfrich EJN, Granatosky EA, Glukhov E, Ryffel F,
797 Houson H, Mohimani H, Kharbush JJ, Zeng Y, Vorholt JA, Kurita KL, Charusanti P,
798 McPhail KL, Nielsen KF, Vuong L, Elfeki M, Traxler MF, Engene N, Koyama N, Vining
799 OB, Baric R, Silva RR, Mascuch SJ, Tomasi S, Jenkins S, Macherla V, Hoffman T,
800 Agarwal V, Williams PG, Dai J, Neupane R, Gurr J, Rodríguez AMC, Lamsa A, Zhang C,
801 Dorrestein K, Duggan BM, Almaliti J, Allard P-M, Phapale P, Nothias L-F, Alexandrov T,
802 Litaudon M, Wolfender J-L, Kyle JE, Metz TO, Peryea T, Nguyen D-T, VanLeer D, Shinn
803 P, Jadhav A, Müller R, Waters KM, Shi W, Liu X, Zhang L, Knight R, Jensen PR, Palsson
804 BO, Pogliano K, Linington RG, Gutiérrez M, Lopes NP, Gerwick WH, Moore BS,
805 Dorrestein PC, Bandeira N. 2016. Sharing and community curation of mass spectrometry
806 data with Global Natural Products Social Molecular Networking. Nat Biotechnol 34:828–
807 837.

808 76. Willforss J, Chawade A, Levander F. 2019. NormalyzerDE: Online tool for improved
809 normalization of omics expression data and high-sensitivity differential expression analysis.
810 J Proteome Res 18:732–740.

811 77. Ayala-Ortiz C. 2024. Scripts for analysis of LC-MS/MS data (Version 2.0.0). Zenodo
812 https://doi.org/10.5281/zenodo.14194276.

813 78. Pang Z, Chong J, Zhou G, de Lima Morais DA, Chang L, Barrette M, Gauthier C, Jacques
814 P-É, Li S, Xia J. 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and

815 functional insights. Nucleic Acids Res 49:W388–W396.