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(Article begins on next page)

1 **Metabolomic adjustments in the orchid mycorrhizal fungus *Tulasnella calospora* during**  
2 **symbiosis with *Serapias vomeracea***

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25  
26 **Concise and informative title:**

27 Metabolomic adjustments during orchid mycorrhizal symbiosis

28

29

30 **SUMMARY**

- 31 • All orchids rely on mycorrhizal fungi for organic carbon, at least during early  
32 development. Orchid seed germination leads in fact to the formation of a protocorm, a  
33 heterotrophic postembryonic structure colonized by intracellular fungal coils, thought  
34 to be the site for nutrients transfer. The molecular mechanisms underlying mycorrhizal  
35 interactions and metabolic changes induced by this peculiar symbiosis in both partners  
36 remain mostly unknown.
- 37 • We studied plant-fungus interactions in the mycorrhizal association between the  
38 Mediterranean orchid *Serapias vomeracea* and the basidiomycete *Tulasnella*  
39 *calospora* using non-targeted metabolomics. Plant and fungal metabolomes obtained  
40 from symbiotic structures were compared with those obtained under asymbiotic  
41 conditions.
- 42 • Symbiosis induced profound metabolomic alterations in both partners. In particular,  
43 structural and signaling lipid compounds sharply increased in the external fungal  
44 mycelium growing near the symbiotic protocorms, whereas chito-oligosaccharides  
45 were identified uniquely in symbiotic protocorms.
- 46 • This work represents the first description of metabolic changes occurring in orchid  
47 mycorrhiza. These results - supported by transcriptomic data - provide novel insights  
48 on the mechanisms underlying the orchid mycorrhizal association and open intriguing  
49 questions on the role of fungal lipids in this symbiosis.

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51

52 **Keywords:** metabolomics, orchid mycorrhiza, *Serapias*, symbiosis, transcriptomics,  
53 *Tulasnella calospora*

54

55

## 56 INTRODUCTION

57 In nature, most land plants associate with symbiotic fungi to form mycorrhizae. Depending on  
58 the morphology of the association and the taxonomic position of the symbiotic partners, four  
59 major mycorrhizal types are formed, namely arbuscular, ecto-, ericoid and orchid mycorrhiza.  
60 Mycorrhizal fungi increase the host plant's ability to acquire mineral nutrients and to tolerate  
61 biotic and abiotic stresses. In exchange, the fungal partner receives photosynthesis-derived  
62 carbon (C) as energy source and takes advantage of a protected niche (Smith & Read, 2008).  
63 Orchids are peculiar because their minute seeds lack an endosperm and the symbiotic fungus  
64 provides the germinating seed and developing embryo with organic C, a strategy termed  
65 myco-heterotrophy (Leake, 1994), as well as other nutrients such as N and P (Cameron *et al.*,  
66 2006, 2007, 2008; Dearnaley & Cameron, 2017). Symbiotic seed germination leads to the  
67 formation of a heterotrophic orchid structure called protocorm (Rasmussen, 1995), in which  
68 intracellular hyphal coils (or *pelotons*) are formed and are thought to be responsible for the  
69 transfer of nutrients from the fungus to the host plant (Peterson & Farquhar, 1994).  
70 In the last years, the molecular bases underlying such peculiar plant-microbe interaction have  
71 been investigated (Yeh *et al.*, 2019). Gene expression profiling has identified fungal and plant  
72 genes putatively involved in signaling, symbiotic seed germination, mycoheterotrophy and  
73 plant defense (Zhao *et al.*, 2013; Perotto *et al.*, 2014; Kohler *et al.*, 2015b; Miura *et al.*, 2018;  
74 Lallemand *et al.*, 2019). Additionally, labeling experiments with stable isotopes (Cameron *et al.*,  
75 2008; Kuga *et al.*, 2014) and molecular analyses (Zhao *et al.*, 2013; Fochi *et al.*, 2017a)  
76 have focused on the nutrient exchanges between the symbionts.  
77 Metabolomics is an alternative approach to investigate metabolic changes in symbiosis.  
78 Through the determination of the low-molecular-weight complement of biological systems  
79 (Kluger *et al.*, 2015), metabolomics provides direct information on the biochemical status of  
80 cells. Although little is known on metabolite alterations in orchid mycorrhiza (OM), some  
81 plant secondary metabolites may play a role in the interaction. For example, the amount of  
82 lusianthrin, an antifungal stilbenoid initially identified in the orchid *Lusia indivisa* (Majumder  
83 & Lahiri, 1990), was found to be strongly increased in protocorm-like bodies of *Cypripedium*  
84 *macranthos* colonized by the mycorrhizal fungus, suggesting a role in plant defense (Shimura  
85 *et al.*, 2007). Similarly, symbiotic *Anacamptis morio* protocorms showed a higher  
86 concentration of the phytoalexin orchinol as compared to non-mycorrhizal protocorms  
87 (Beyrle *et al.*, 1995). Chang & Chou (2007) found that the content of some metabolites (i.e.,  
88 flavonoids, polyphenols, ascorbic acids, and polysaccharides) increased in mycorrhizal  
89 orchids, as compared to non-mycorrhizal plants.

90

91 Non-targeted metabolomics - i.e., a hypothesis-free analysis that aims to investigate the entire  
92 metabolome - represents a powerful tool to profile thousands of metabolites, especially in  
93 combination with pathway analyses (Fiehn *et al.*, 2000; Aharoni *et al.*, 2002; Schliemann *et*  
94 *al.*, 2008; Kårlund *et al.*, 2015). It has already been used to investigate plant-microbe  
95 interactions in legume root nodules (Zhang *et al.*, 2012), ectomycorrhizae (Tschapinski *et al.*,  
96 2014) and arbuscular mycorrhizae (Schliemann *et al.*, 2008; Laparre *et al.*, 2014; Rivero *et*  
97 *al.*, 2015). Here, we employed non-targeted metabolomics to investigate *in vitro* the  
98 mycorrhizal association between the Mediterranean orchid *Serapias vomeracea* and the  
99 basidiomycete *Tulasnella calospora* (Cantharellales). In particular, *S. vomeracea* seeds and *T.*  
100 *calospora* mycelium were grown together to form mycorrhizal orchid protocorms, and plant  
101 and fungal metabolite profiles were compared to those obtained when plant and fungus were  
102 cultivated separately as asymbiotic protocorms and free-living mycelium. We integrated  
103 metabolomic analyses with genomic information available for *T. calospora* (Kohler *et al.*,  
104 2015a) and our published transcriptomic data (Fochi *et al.*, 2017a). In addition to differences  
105 in the metabolite profiles of symbiotic and asymbiotic protocorms, the results revealed  
106 intriguing and unexpected differences in the lipid content of free-living and symbiotic *T.*  
107 *calospora* mycelium.

108

## 109 **MATERIAL AND METHODS**

110

### 111 ***Biological materials***

112

#### 113 **Free-living mycelium of *T. calospora***

114 *Tulasnella calospora* (AL13 isolate) mycelium was originally isolated from mycorrhizal roots  
115 of the terrestrial orchid species *Anacamptis laxiflora* in Northern Italy (Girlanda *et al.*, 2011)  
116 and was grown on solid 2% Malt Extract Agar (MEA) at 25°C for 20 days before use. Three  
117 plugs (6 mm diameter) of actively growing *T. calospora* mycelium were transferred onto a  
118 sterilized cellophane membrane placed on top of Oat Agar (OA, 0.3% milled oats, 1% agar;  
119 Fig. **1a, d**), the same used for symbiotic seed germination, in 11 cm *Petri* dishes (Schumann  
120 *et al.*, 2013). After 20 days at 25°C, the free-living mycelium (FLM) was collected,  
121 immediately frozen in liquid N<sub>2</sub> and stored at -80°C.

122

#### 123 **Symbiotic and asymbiotic germination of *Serapias vomeracea* seeds**

124 Symbiotic seed germination was obtained by co-inoculation of mycorrhizal fungus and orchid  
125 seeds in 9 cm *Petri* dishes, as previously described in Ercole *et al.* (2015). After surface  
126 sterilization, seeds were resuspended in sterile water and dropped on strips of autoclaved filter  
127 paper (1.5 x 3 cm) positioned on solid oat medium (0.3% (w/v) milled oats, 1% (w/v) agar). A  
128 plug of actively growing *T. calospora* mycelium was then placed in the center of each *Petri*  
129 dish and plates were incubated at 20°C in full darkness for 30 days (Fig. **1b**). Asymbiotic  
130 germination was obtained by placing surface-sterilized seeds directly on modified BM1  
131 culture medium (Van Waes & Debergh, 1986) at 20°C in darkness. Symbiotic protocorms  
132 (SYMB) were collected 30 days post-inoculation (dpi) and asymbiotic protocorms (ASYMB)  
133 120 dpi. Symbiotic germination was performed by placing the mycelial plug on autoclaved  
134 cellophane membrane, in order to collect the fungal mycelium (MYC) growing near to the  
135 protocorms (Fig. **1c-d**). MYC samples were harvested by carefully scraping the mycelium  
136 with a spatula. All samples were flash-freezed in liquid N<sub>2</sub> and stored at -80 °C.

137

#### 138 **Sample preparation for metabolomic analysis**

139 *S. vomeracea* symbiotic and asymbiotic protocorms and *T. calospora* mycelium were  
140 disrupted with TissueLyser (18Hz, 2 min, twice). Frozen powder samples (100 mg) were  
141 extracted with 1 ml of methanol:isopropanol:water (1:1:1, v/v) for 1 h at 4°C in constant  
142 shaking. Successively, the solution was centrifuged at 14,000 rpm for 15 min at 4°C and the  
143 supernatant was recovered, dried in a centrifugal evaporator (SpeedVac, Savant Inc, USA)  
144 and stored at -80°C. Before metabolomic analysis, the dried samples were dissolved in 200 µl  
145 of 50% acetonitrile in water and centrifuged at 14,000 rpm at 4°C for 10 min.

146

#### 147 ***UPLC-UHR-QqToF-MS measurements***

148 Ultra Performance Liquid Chromatography (UPLC) Ultra-High Resolution (UHR) tandem  
149 quadrupole/Time-Of-Flight (QqToF) mass spectrometry (MS) measurements were performed  
150 on an Ultimate 3000RS (ThermoFisher, Bremen, Germany) coupled to a Bruker Impact II  
151 with Apollo II source (ESI source) (Bruker Daltonic, Bremen, Germany). Chromatographic  
152 separation was achieved on a C<sub>18</sub> column (100 mm x 2.1 mm inner diameter with 1.7 µm  
153 particles, Fortis Technologies - Clayhill Industrial Park Neston Cheshire, UK). Eluent A was  
154 water with 0.1% of formic acid and eluent B was acetonitrile with 0.1% of formic acid.  
155 Gradient elution started with an initial isocratic hold of 0.5% B for 1 min, followed by an  
156 increase to 30% B in 15 min and a further increase to 80% B for 5 min. During the last 3 min,  
157 the initial conditions of 0.5% B were restored. The flowrate was 400 µl min<sup>-1</sup> and the column

158 temperature was continuously maintained at 40°C. The auto-sampler temperature was set to  
159 4°C. For each sample, two technical replicates were measured in both positive (+) and  
160 negative (-) ionization modes. Prior to sample analyses, quality control (QC) samples  
161 prepared from the aliquots of the different samples were injected for column conditioning.  
162 Mass calibration was achieved with 50 ml of water, 50 ml isopropanol, 1 ml sodium  
163 hydroxide, and 200 µl formic acid. The MS was operated under the following conditions: the  
164 nebulizer pressure was set to 2 bar, dry gas flow was 10 l min<sup>-1</sup>, dry gas temperature was  
165 220°C, a capillary voltage was set to 4000 V for the (+) and 3000 V for the (-) ionization  
166 mode and the endplate offset was 500 V. Mass spectra were acquired in a mass range of 50-  
167 1300 m/z in both (±) modes.

168

### 169 *Non-targeted metabolomic analysis*

170 Each MS spectrum file was separately imported into the GeneData Expressionist for MS  
171 software v13.5 (München, Germany) for peak peaking and alignment. The spectra were pre-  
172 processed by the following steps: i) chemical noise reduction, ii) retention time (RT)  
173 alignment, iii) identification of m/z features using the summed-peak-detection feature  
174 implemented in the GeneData software, iv) peaks not present in at least 10% of the mass  
175 spectra were discarded for isotope clustering, v) singletons (clusters with only one member)  
176 were discarded. The resulting peak matrix was exported, both (±) modes were combined, and  
177 the average peak intensity of both technical replicates was calculated and further used for  
178 statistical and annotation analyses. Mass features (m.f.) appearing in less than 75% of the  
179 biological replicate were removed from the data matrix. The resulting peak list was further  
180 used for annotation and statistical analysis. Metabolic annotation was achieved as before  
181 (Way *et al.*, 2013; Kersten *et al.*, 2013) using the portal MassTRIX3  
182 (<http://masstrix3.helmholtzmuellen.de/masstrix3/>). Compared to MassTRIX (Suhre &  
183 Schmitt-Kopplin, 2008; Wägele *et al.*, 2012), the updated version of MassTRIX3 contains all  
184 metabolites of KEGG (<http://www.genome.jp/kegg/>), the Human Metabolome Database  
185 (HMDB - <http://www.hmdb.ca>), ChemSpider (<http://www.chemspider.com/>), KNApSACk  
186 (<http://kanaya.naist.jp/KNApSACk/>), Lipidmaps (<http://www.lipidmaps.org/>) and PubChem  
187 (<https://pubchem.ncbi.nlm.nih.gov/>). Log<sub>2</sub> ratios of m.f. intensities (log<sub>2</sub>) were calculated for  
188 SYMB/ASYMB, SYMB/MYC, SYMB/FLM, and MYC/FLM to visualize metabolic up- or  
189 down-regulation. Putative molecular formulas were calculated from all m.f. using 4 ppm as a  
190 threshold. Molecular formulas were used to calculate H/C, O/C, N/C, P/C, S/C, N/P ratios for

191 the production of van Krevelen diagrams and for the multidimensional stoichiometric  
192 compound classification (MSCC) (Rivas-Ubach *et al.*, 2018).

193

#### 194 ***Pathway and Functional analyses***

195 Pathway analysis was performed by using the Pathway Omics Dashboard tools of BioCyc  
196 (<https://biocyc.org/>) (Paley *et al.*, 2017) on annotated metabolites, the concentrations of which  
197 changed significantly in MYC/FLM. We used MetaCyc v23.1 (<https://Metacyc.org>) as  
198 reference database (Caspi *et al.*, 2018). The biological function of the up/downregulated  
199 annotated metabolites in MCY/FLM were obtained from the KEGG, HMDB and Lipid Maps  
200 databases.

201

#### 202 ***Transcriptomic data***

203 Symbiotic and asymbiotic growth conditions used for these metabolomic studies were the  
204 same previously investigated by transcriptomics in Fochi *et al.* (2017a). Transcriptomic data  
205 are, however, missing for the MYC samples. The complete series of fungal and plant  
206 transcripts are available at GEO (GSE86968 and GSE87120, respectively).

207

#### 208 ***Statistical analysis***

209 Experiments were performed using four independent biological replicates. Metabolomic data  
210 were analyzed using Principal Component Analysis (PCA) and Orthogonal Partial Least  
211 Squares Regression (OPLSR) (SIMCA-P v13, Umetrics, Umeå, Sweden). The pre-processing  
212 of the data followed established procedures (Ghirardo *et al.*, 2005, 2012, 2016). Discriminant  
213 masses (Kaling *et al.*, 2015) between the different mycelia (MYC and FLM) and the  
214 protocorms (SYMB and ASYMB) were further tested for statistical significance using a false  
215 discovery rate (FDR) of 5% as previously described (Way *et al.*, 2013).

216

## 217 **RESULTS**

### 218 ***Impact of symbiosis on plant and fungal metabolomes***

219 The metabolome of symbiotic protocorms (SYMB) and *T. calospora* mycelium (MYC)  
220 collected near the symbiotic protocorms (Fig. **1a-b**) were compared with asymbiotic  
221 protocorms (ASYMB) and free-living mycelium (FLM) grown in pure culture on the same  
222 medium used for symbiotic seed germination (Fig. **1c-d**). We revealed a total of 24818  
223 metabolite-related mass features (m.f.), with the plant metabolome being more complex



224 (14722 m.f. for SYMB, 16213 for ASYMB) than the fungal metabolome (4376 m.f. for  
225 MYC, 3337 for FLM).

226 The number of common and specific m.f. in symbiotic and asymbiotic plant samples and  
227 mycelia is visualized in the Venn diagram (Fig. 2). The 521 m.f. common to all samples are  
228 most likely related to a “core metabolome” composed of primary metabolites found in both  
229 partners (Fig. 2). A relatively high number of m.f. (1265) was found in MYC samples but not  
230 in FLM ones, indicating accumulation of distinct metabolites in the fungal hyphae close to  
231 (but outside) the host plant. We found an overlapping metabolome composed of 8583 m.f. in  
232 SYMB and ASYMB orchid protocorms, but not in MYC or FLM samples, likely representing  
233 plant metabolites involved in general plant functions. Several m.f. were unique to symbiotic  
234 (SYMB, 3977) or asymbiotic (ASYMB, 5433) orchid protocorms. Although some of these  
235 unique m.f. likely represent plant metabolites regulated by symbiosis, some may be due to the  
236 different culture media required to obtain symbiotic and asymbiotic protocorms.

237 Metabolites uniquely found in MYC and SYMB samples (291 m.f.) or in MYC, SYMB, and  
238 FLM samples (315 m.f.) likely represent fungus-specific compounds, as they were not found  
239 in ASYMB samples. In addition, some unique metabolites in SYMB samples could originate  
240 from the mycorrhizal fungal partner colonizing symbiotic protocorm tissues. Indeed, fungal  
241 metabolites uniquely produced in symbiosis would group together with the SYMB-specific  
242 metabolites (Fig. 2).

243 In addition to unique and shared plant and fungal metabolites, the symbiotic plant-fungus  
244 interaction likely resulted in up- and downregulation of a broader set of metabolites. PCA of  
245 all m.f. abundances comprehensively visualized changes in metabolite levels and showed a  
246 highly diverse metabolic profile among samples (Fig. 3). Not surprisingly, the most  
247 considerable distance (46%), as seen by the first component (PC1), was between plant  
248 protocorms (ASYMB/SYMB) and fungal mycelium (MYC/FLM). A further and significant  
249 distance among data was described by PC2 (34%), which clearly separated SYMB from  
250 ASYMB samples and, to a lesser extent, by PC3 (7%), MYC from FLM samples.

251  
252 To gain insights into metabolites and metabolic pathways altered in the symbiosis, we  
253 performed an OPLSR analysis on the m.f., followed by database annotation of the  
254 discriminant masses (Kersten *et al.*, 2013). By doing so, we putatively annotated the m.f. that  
255 characterized the following sample pairs: SYMB/ASYMB, SYMB/MYC, SYMB/FLM, and  
256 MYC/FLM (Table S1). Additionally, we classified compounds based on their elemental  
257 compositions using the very recently developed multidimensional stoichiometric compound

258 classification (MSCC) approach (Rivas-Ubach *et al.*, 2018). This method avoids the  
259 limitations of actual database coverage, especially for less described organisms. Using MSCC  
260 in combination with van Kreveln diagrams, we visualized the significant global metabolic  
261 changes of the main compound categories (i.e., lipids, protein-related, amino sugars,  
262 carbohydrates, nucleotides, and phytochemical compounds) up/downregulated during plant-  
263 fungus symbiosis (Fig. 4). MSCC analysis highlighted the high abundance of lipids in MYC  
264 samples, as compared to FLM samples (Fig. 4a) The increased levels of lipids in MYC  
265 samples can also be seen in the van Krevelen diagram, where compounds with H/C ratio  
266  $\geq 1.32$  and O/C ratio  $\leq 0.6$  were strongly upregulated (Fig. 4c). Conversely, phytochemical  
267 compounds were downregulated in the MYC/FLM comparison (Fig. 4a). Importantly,  
268 carbohydrates (O/C ratio  $\geq 0.8$  and  $1.65 \leq H/C < 2.7$ ) were lower in MYC samples, indicating  
269 either a shift from carbohydrate metabolism to, for instance, lipid metabolism, or perhaps C  
270 transfer towards the mycorrhizal plant protocorms. The latter hypothesis would agree with the  
271 carbohydrates increase in SYMB protocorms (Fig. 4b, d).

272

### 273 ***Functional enrichment analysis in T. calospora***

274 Metabolic changes caused by symbiosis were clearly detected in the external hyphae of *T.*  
275 *calospora* (Fig. 4a) and represent an aspect of the interaction so far unexplored. We first used  
276 the Pathway Omics Dashboard tool of MetaCyc as unbiased analysis to visualize the overall  
277 metabolic changes of this fungus during its symbiotic interaction with the plant. When  
278 compared to FLM, the MYC metabolome was highly enriched in compounds involved in the  
279 synthesis of lipids, followed by cell-structures, hormones, carbohydrates, or compounds  
280 involved in metabolic regulation (Fig. 5). Conversely, cumulative changes in metabolites  
281 involved in secondary metabolism were found to be strongly downregulated in MYC samples,  
282 as compared to FLM.

283 Since MetaCyc was unable to classify ~70% of the annotated metabolites, we additionally  
284 investigated the chemical taxonomy and functions of regulated metabolites using data from  
285 the literature or available databases. This in-depth analysis showed that several compounds  
286 related to cell-structure and signaling were increased in MYC samples (Fig. 6, Table S1).  
287 ‘Lipids’ were still the most upregulated compounds in the external mycelium of *T. calospora*.  
288 Notable changes were also observed in several nitrogen-, oxygen- and sulfur-containing  
289 compounds (Fig. 6a). With respect to functions, symbiosis caused an overall increase in the  
290 amount of structural, signaling, and energy-related compounds in MYC samples, as compared  
291 to FLM samples, mainly related to lipids (Fig. 5-6b, Table S1). Among lipids (120

292 metabolites), the glycerophospholipids (68), fatty acyls (FA) (14) and isoprenoids (prenol  
293 lipids) (13) were strongly upregulated in MYC samples ( $\log_2 > 10$ ) (Table S1). Some  
294 nitrogen-containing organic compounds (29), organosulfur compounds (8), and  
295 phytochemical metabolites (14) involved in defense were also highly upregulated in MYC  
296 samples. On the other hand, fewer lipids (48) (FA, 10; isoprenoids, 9), but more nitrogen-  
297 containing organic compounds (41), organosulfur compounds (17), and, among  
298 phytochemical compounds (17), alkaloids (14) were significantly downregulated (Fig. 6).

299

### 300 *The external mycelium of T. calospora showed specific changes in lipid content*

301 The sharpest metabolomic differences between MYC and FLM samples were in the levels  
302 and compositions of lipids, in particular glycerophospholipids (GPL) and sphingolipids (Fig.  
303 **6, S1**, Table S1). Among 81 significantly upregulated GPL in MYC samples, the most  
304 upregulated GPL ( $\log_2 = 21.4$ ) was putatively annotated as lysophosphatidylethanolamine  
305 (LysoPE), a lipid metabolite involved in signaling. Notably, 18 glycerophosphoserines (GPS)  
306 were found to be strongly ( $\log_2 > 10$ ) upregulated, as compared to only one being  
307 downregulated, and the abundance of the GPL precursor palmitic acid was consistently  
308 increased ( $\log_2 = 9.1$ ). Among GPL, 13 phosphatidylcholines (PCs) and 9 phosphoinositides  
309 (PIs) were more abundant ( $\log_2 > 10$ ) in MYC samples than in FLM samples. Also, a  
310 glycerophosphocholine, putatively annotated as 1-palmitoyl-sn-glycero-3-phosphocholine  
311 (LPC(16:0)) was upregulated ( $\log_2 = 14.4$ ) in MYC samples. An essential intermediate in the  
312 biosynthesis of both triacylglycerols and GPL, and therefore involved in energy (storage and  
313 source) and structural metabolism, was the GPL phosphatidic acid PA(22:0/14:1(9Z)), also  
314 upregulated ( $\log_2 = 12.9$ ) in MYC samples. Highly upregulated in MYC samples were also the  
315 FA derivative of hydroxyeicosatetraenoic acid, 15-HETE ( $\log_2 = 21$ ), and the 8-  
316 hydroxyoctadeca-9Z,12Z-dienoic acid (8-HODE or laetiseric acid) ( $\log_2 = 13.85$ ), a FA having  
317 allelochemical functions. Moreover, the strong accumulation of sphingosine ( $\log_2 = 13.58$ ) and  
318 PI-Cer(d20:0/16:0) ( $\log_2 = 12.58$ ) indicate an increase in sphingolipid biosynthesis in MYC  
319 samples.

320 Direct integration of metabolomic and transcriptomic data was unfortunately not possible  
321 because previous transcriptomic analyses (Perotto *et al.*, 2014; Fochi *et al.*, 2017a) did not  
322 investigate the MYC condition. However, significant changes in the expression of fungal  
323 genes involved in lipid metabolisms (Table S2) were observed between SYMB and FLM  
324 samples. Among the annotated fungal genes most upregulated in symbiosis (fold change,  
325 'FC' > 10) were two members of the  $\text{Ca}^{2+}$ -independent phospholipase A<sub>2</sub> (Protein ID (#)

326 53822, #25657) and a myo-inositol-1-phosphate synthase (#72491), an essential enzyme for  
327 the biosynthesis of inositol containing phospholipids (PIs) and certain sphingolipid signaling  
328 molecules. Two fungal genes corresponding to phosphoinositide kinases (FC=4.6, # 26793,  
329 FC=2.4, # 28485) and three sphingosine N-acyltransferases, a key enzyme involved in  
330 sphingolipid biosynthesis, were upregulated in symbiosis (#18228, #79587, #18227) (Table  
331 **S2**). Conversely, a glucosylceramidase (#33445) was strongly downregulated. Several genes  
332 involved in FA metabolism through the Acyl-CoA coenzyme were also affected (Table **S2**),  
333 including two down-regulated genes coding for thiolases (#16280, #131995).  
334 Finally, we observed large changes of 29 isoprenoids in the MYC/FLM comparison (Table  
335 **S1**). Eight triterpenoids, one diterpene, and one tetraterpene were strongly upregulated (log2  
336 >10). Although transcriptional information on the MYC condition is not available, 5 *T.*  
337 *calospora* genes encoding terpenoid synthases were significantly upregulated in symbiotic  
338 protocorms (Table **S2**), two of them with FC >20 (#70959, #22905).

339

#### 340 ***Nitrogen-containing fungal compounds***

341 Nitrogen-containing (non-phospholipids) compounds were the second group of metabolites  
342 significantly affected in *T. calospora*, with a high proportion downregulated in MYC samples,  
343 as compared to FLM (Fig. **6a**, Table **S1**). Although most of these compounds could not be  
344 reliably annotated due to the constraints of the available databases, they indicate sharp  
345 changes in nitrogen metabolism in the fungus during symbiosis. Two of the few identified  
346 compounds with increased levels in MYC samples, as compared with FLM, were UDP-N-  
347 acetyl-D-glucosamine (UDP-GlcNAc) (log2=9.52) and dolichyl-N-acetyl-alpha-D-  
348 glucosaminyl-phosphate (log2=11.47), N-containing compounds essential for the biosynthesis  
349 of N-linked glycans, glycosylphosphatidylinositol (GPI)-anchored proteins, sphingolipids and  
350 glycolipids. UDP-GlcNAc can be polymerized to form chitin, a major component of the  
351 fungal cell wall. Short oligomers of chitin and chitosan, its deacetylated form, were found  
352 similarly enriched (log2 from 11.9 to 13.6) in SYMB when compared with either the MYC or  
353 the FLM samples, whereas no differences were observed between MYC and FLM samples  
354 (Table **S1**). Chitosan is produced through the activity of chitin deacetylase, and 3 chitin  
355 deacetylase genes (#174258, #26855, #107589), out of the 9 present in the *T. calospora*  
356 genome, were significantly upregulated in SYMB with respect to FLM (Table **S2**). By  
357 contrast, a single chitin synthase (#31299) was slightly upregulated in symbiosis (Table **S2**).  
358 Short chitin oligomers can be important signals in symbiosis and could also be generated  
359 from long chitin polymers by the activity of chitinases. The expression of both fungal and

360 plant chitinases was modified by symbiosis (Tables **S2-3**), some plant chitinases being  
361 strongly upregulated in symbiotic protocorms (TRINITY Contig Names:  
362 DN77284\_c0\_g1\_i3, DN5745\_c0\_g1\_i1, DN66370\_c0\_g1\_i1, DN62020\_c0\_g1\_i1).  
363 Comparing symbiotic and asymbiotic conditions, another primary class of regulated nitrogen-  
364 containing compounds was involved in amino acid metabolisms (Table **S1**). Accumulation of  
365 N-L-argininosuccinate was found in SYMB when compared to all other samples ( $\log_2 = 3.8$   
366 with ASYMB;  $\log_2 = 13.3$  with MYC and FLM). This compound is involved in arginine  
367 biosynthesis and fumarate formation, an essential intermediate of the TCA cycle.  
368 Unfortunately, the metabolomic study of symbiotic tissues (SYMB) is not an easy task  
369 because they contain both plant and fungal metabolites and assignment of most mass features  
370 to the symbionts is uncertain. Therefore, most amino acids and amino acid derivatives could  
371 not be assigned to the fungus or to the plant, with few exceptions. One was the putatively  
372 annotated ergothioneine, a naturally occurring metabolite of histidine exclusively found in  
373 some fungi and bacteria (Cumming *et al.*, 2018). The levels of ergothioneine were much  
374 higher in SYMB ( $\log_2=11.35$ ) than in MYC or FLM. The levels of hercynine, another fungal-  
375 specific and histidine related compound, were by contrast low ( $\log_2=-10.31$ ) in the SYMB vs  
376 MYC comparison. Transcriptomic evidence points to an important role of *T. calospora* in  
377 histidine biosynthesis during symbiosis, with three biosynthetic genes (#108905, #73648,  
378 #141375) being significantly upregulated in SYMB samples (Table **S2**).

379

### 380 **Organosulfur compounds**

381 Significant changes in sulfur-containing compounds were observed in *T. calospora* (Table  
382 **S1**), with 14 compounds being upregulated ( $\log_2>10$ ) and 18 downregulated ( $\log_2<-10$ ) in  
383 MYC samples, as compared to FLM. Similar to nitrogen-containing compounds, many  
384 organosulfur compounds could not be reliably annotated. An exception was S-  
385 adenosylmethioninamine, a decarboxylated derivative of S-adenosylmethionine (SAM)  
386 involved in polyamine biosynthesis (Pegg *et al.*, 1998). Notably, the amount of S-  
387 adenosylmethioninamine in MYC samples was sharply reduced ( $\log_2 = -11.05$ ), as compared  
388 to FLM, whereas SAM amount was sharply increased ( $\log_2 = 8.9$ ). SAM is a major source of  
389 methyl groups for reactions involving methylation. The substantial SAM accumulation in  
390 MYC samples (Table **S1**) suggests a role in symbiosis. Although SAM levels were similar in  
391 SYMB, MYC or FLM samples, transcriptomics revealed that the *T. calospora* SAM  
392 synthetase gene was upregulated ( $FC=4.29$ , #72837) in symbiosis (Table **S2**). Metabolomic

393 data further indicate a significantly lower ( $\log_2=-10.3$ ) SAM content in SYMB as compared  
394 to ASYMB (Table S1), suggesting down-regulation of the plant SAM in symbiosis.

395

## 396 **DISCUSSION**

397 Transcriptomics is the most common approach to indirectly investigate metabolic changes in  
398 symbiotic organisms because it reveals the contributions of both partners through changes in  
399 their gene expression. This approach was successfully used to investigate orchid mycorrhizal  
400 (OM) protocorms, symbiotic structures that contain a mixture of plant and fungal molecules  
401 that cannot be separated before molecular or biochemical analyses (Zhao *et al.*, 2013; Fochi *et*  
402 *al.*, 2017a; Miura *et al.*, 2018). However, although gene regulation is indicative of activation  
403 or repression of distinct biosynthetic pathways, transcriptional regulation of genes encoding  
404 enzymes does not necessarily reflect the final enzymatic activity, and there may be no direct  
405 association between metabolites and transcripts (Cavill *et al.*, 2016). Therefore, we used a  
406 non-targeted metabolomic approach to investigate metabolic changes in OM, and  
407 transcriptomic data were only used to corroborate metabolomic results. Metabolomics yielded  
408 particularly interesting results when the external mycelium of the OM fungus *T. calospora*  
409 growing near to symbiotic *S. vomeracea* protocorms (MYC) was compared with the free-  
410 living mycelium. All organic nutrients needed by the developing mycoheterotrophic  
411 protocorms are thought to be provided by the symbiotic fungus in the OM symbiosis. Thus,  
412 the metabolites identified in the MYC samples are most likely produced by *T. calospora* and  
413 differentially accumulated in the presence of the plant.

414

### 415 ***Symbiosis caused profound changes in the lipid content of T. calospora***

416 Lipids were the most prominent upregulated metabolites in the external *T. calospora*  
417 mycelium, as compared to asymbiotically-grown mycelium. Besides being major structural  
418 components of cell membranes, lipids provide critical biological functions as energy and  
419 carbon storage, in signaling, stress response and plant-microbe interactions (Siebers *et al.*,  
420 2016). Lipids have recently become an important topic in mycorrhizal research because a  
421 substantial increase in the amount of lipids was discovered in the hyphae of arbuscular  
422 mycorrhizal (AM) fungi during symbiosis (Keymer *et al.*, 2017). AM fungi are obligate  
423 biotrophs that fail in the *de novo* biosynthesis of fatty acids but become enriched thanks to  
424 lipid transfer from the plant. This is unlikely the case for OM fungi because the *T. calospora*  
425 genome contains the genetic machinery for lipid biosynthesis, and the increased lipid content  
426 in the external *T. calospora* hyphae more likely reflects endogenous lipid biosynthesis.

427 Phospholipids and sphingolipids are vital components of cell membranes and play key roles  
428 in signaling, cytoskeletal rearrangement, and in membrane trafficking (Meijer & Munnik,  
429 2003; Michell, 2008; Fyrst & Saba, 2010; Balla, 2013; Hou *et al.*, 2016; Singh & Del Poeta,  
430 2016; Hannun & Obeid, 2018; Blunsom & Cockcroft, 2020). In fungi, sphingolipids are  
431 important for hyphae formation (Oura & Kajiwara, 2010), regulating cell growth and  
432 differentiation (Obeid *et al.*, 2002), and cell division (Epstein *et al.*, 2012). In addition, lipid-  
433 derived molecules are essential for intra- and extra-cellular signaling and for defense against  
434 the proliferation of undesired microbes (Hou *et al.*, 2016; Siebers *et al.*, 2016; Singh & Del  
435 Poeta, 2016; Wang *et al.*, 2020). Lipid peroxidation of free fatty acids, acyl groups of  
436 triacylglycerols or galactolipids, is commonly activated to induce defense against pathogens.  
437 For instance, oxylipins are essential in signal transduction and in both induced systemic  
438 resistance (Wang *et al.*, 2020) and systemic acquired resistance (Siebers *et al.*, 2016).

439 Overall, we observed a generally increased level of several structural lipid constituents of cell  
440 membranes, such as glycerophospholipids (GPL), fatty acyls (FA), glycerolipids,  
441 saccharolipids and some of their metabolic precursors (e.g. palmitic acid and UDP-GlcNAc).  
442 Palmitic acid is one of the most common saturated fatty acids found in animals, plants and  
443 microorganisms, and the first fatty acid produced during lipogenesis (Sidorov *et al.*, 2014;  
444 Carta *et al.*, 2017). UDP-GlcNAc, an essential precursor of the fungal cell wall chitin, is also  
445 involved in the biosynthesis of sphingolipids and sulfolipids (Bowman & Free, 2006; Furo *et*  
446 *al.*, 2015; Ebert *et al.*, 2018). The most represented lipids upregulated in the external  
447 mycelium of *T. calospora* were GPL. Particularly, phosphatidylserines (PS) represented ca.  
448 41% of the up-regulated GPL compounds. PS are mostly restricted to the cytoplasmic  
449 membrane leaflet, and the covalent attachment of serine to the phosphate group creates a  
450 negative charge essential for targeting and functioning of several intracellular signaling  
451 proteins and for the activation of specific kinases, such as protein kinase C (Kay & Grinstein,  
452 2011). Sphingosine and PI-Cer(d20:0/16:0) are precursors of sphingolipids, also important  
453 components of fungal cell membranes (Meijer & Munnik, 2003; Hou *et al.*, 2016; Singh &  
454 Del Poeta, 2016). These compounds and other 9 PIs were strongly upregulated in the external  
455 *T. calospora* mycelium, likely reflecting the upregulation in symbiosis of phosphoinositide  
456 phosphatases and serine/threonine protein kinases, key enzymes involved in the biosynthesis  
457 of sphingolipids and glycerophosphoinositols (Balla, 2013; Hou *et al.*, 2016; Hannun &  
458 Obeid, 2018; Blunsom & Cockcroft, 2020).

459 Some membrane GPL also play essential roles in pathogenic and mutualistic interactions. For  
460 example, changes in membrane lipid compositions of rhizobia, including PS and PE,  
461 prevented the formation of nitrogen-fixing legume nodules (Vences-Guzmán *et al.*, 2008). In  
462 fungi, PS and PE have been correlated with *Candida albicans* virulence (Cassilly &  
463 Reynolds, 2018), and an increase in PS was observed during fungal differentiation in the  
464 phytopathogenic *Rhizoctonia solanii* (Hu *et al.*, 2017). Sphingolipids are also involved in  
465 plant-fungal interactions, and early intermediates of sphingolipid biosynthesis were found to  
466 be essential for normal appressoria development and pathogenicity of *Magnapothe oryzae*  
467 (Liu *et al.*, 2019).

468 In addition to structural membrane components, we found a strongly increased amount of  
469 lipids involved in signaling and defense in the external mycelium of *T. calospora*. The 1-18:1-  
470 lysophosphatidylethanolamine (LysoPE) belongs to the class of lysophospholipids, which  
471 serve essential signaling functions in plants and act as plant growth regulators (Meijer &  
472 Munnik, 2003; Cowan, 2006; Hou *et al.*, 2016). The FA 8-HODE (or laetiseric acid)  
473 originates from linoleic acid and is a bioactive oxylipin acting as a communication signal in  
474 plant-fungus interactions (Brodhun & Feussner, 2011; Christensen & Kolomiets, 2011). 8-  
475 HODE was first discovered in the basidiomycete *Laetisaria arvalis* as an allelochemical that  
476 suppresses growth of phytopathogenic fungi (Bowers *et al.*, 1986). 15-HETE, the  
477 hydroxylated fatty acid substrate for the oxylipin biosynthesis, is an intermediate of  
478 sphorolipids, extracellular glycolipids apparently necessary for signaling. The strong  
479 upregulation of 8-HODE (log<sub>2</sub>=13.8) and 15-HETE (log<sub>2</sub>=21) in MYC samples, as compared  
480 to FLM, indicates that the signaling apparatus in MYC samples is highly active during  
481 symbiosis. Interestingly, some Ca<sup>2+</sup> independent phospholipase A2 were among the most  
482 upregulated *T. calospora* genes (Table S2). This enzyme family plays important functions in  
483 membrane homeostasis, signal transduction, and virulence (Valentín-Berriós *et al.*, 2009).

484 Although we could hypothesize that the increased amount of structural membrane lipids in the  
485 fungal hyphae outside the mycorrhizal protocorm may simply reflect a stimulation of hyphal  
486 growth and a need for membrane biogenesis following symbiosis, the increase in potential  
487 membrane signaling molecules is intriguing. Also, several upregulated lipids in *T. calospora*  
488 contained phosphate, and it has been suggested by Plassard *et al.* (2019) that organic  
489 phosphate released by membrane lipids may be transferred to the plant in AM symbiosis.  
490 However, although organic phosphate transporters were identified in the genome of  
491 mycorrhizal fungi, including OM fungi (Plassard *et al.*, 2019), their occurrence in plants is to  
492 our knowledge unknown.



493

494 ***Nitrogen- and sulfur-containing organic compounds in the external *T. calospora* mycelium***

495 Compared to lipids (see above), more difficult to explain is the large percentage of nitrogen  
496 and sulfur-containing compounds downregulated in the same MYC samples (Fig. 6). In our  
497 system, the OM fungus likely provides the host with organic nitrogen, as suggested by the  
498 strong upregulation of some plant amino acid transporters in the mycorrhizal protocorms cells  
499 (Fochi *et al.*, 2017a,b). We could, therefore, speculate that depletion of some nitrogen-  
500 containing compounds in the external MYC mycelium may be the result of N transfer to the  
501 host. It is also possible that some of those non-annotated upregulated compounds are simply  
502 involved in the metabolism of nitrogen-containing lipids, such as glycerophospholipids and  
503 sphingolipids.

504 About sulfur, there is currently no information on its transfer to the host plant in OM. Among  
505 the few sulfur-containing compounds that could be reliably identified, S-adenosyl-  
506 methionine (SAM) was upregulated in MYC samples. SAM is the major methyl group donor  
507 for the methylation of DNA, RNA, proteins, metabolites, or phospholipids (Mato *et al.*,  
508 1997). Overexpression of SAM synthetase gene in *Aspergillus nidulans* had a substantial  
509 impact on development and secondary metabolism (Gerke *et al.*, 2012). Given the wide  
510 variety of target substrates of methyltransferases that use SAM as a methyl group donor, it is  
511 currently impossible to identify such targets in *T. calospora*.

512 Another notable sulfur and nitrogen-containing compound was ergothioneine (EGT)  
513 (Sheridan *et al.*, 2016). EGT occurs primarily in fungi, and no biosynthesis was detected so  
514 far in plants. Thus, it was possible to trace this compound in symbiotic protocorms, where it  
515 was highly induced ( $\log_2=11.35$ ) as compared to external or free-living mycelium.  
516 Ergothioneine exhibits powerful antioxidant properties, and biosynthetic deficiency in *A.*  
517 *fumigatus* mutants indicates a role for growth at elevated oxidative stress conditions (Sheridan  
518 *et al.*, 2016). Its accumulation in the symbiotic protocorm suggests that *T. calospora* is  
519 experiencing an oxidative environment and responds with the accumulation of antioxidants.

520

521 ***Chitin and chitin-derived metabolites in symbiosis***

522 Chitin is the main structural component of the fungal cell wall (Bowman & Free, 2006) and  
523 contains nitrogen in the form of N-acetyl glucosamine residues, joined by beta-(1,4) linkages.  
524 In addition to a structural role, chitin is a source of signaling molecules that regulate plant-  
525 microbe interactions (Sánchez-Vallet *et al.*, 2015). Chito-oligosaccharides with a degree of

526 polymerization of 6 to 8 act as signal molecules and are strong inducers of plant defense  
527 responses against pathogenic fungi because they are recognized by chitin-specific plant  
528 receptors (Pusztahelyi, 2018). The chitin oligomers accumulated in symbiotic *S. vomeracea*  
529 protocorms, as compared with MYC and FLM samples, were much smaller, with a degree of  
530 polymerization of 3 (chitotriose,  $\log_2=13.6$ ) and 2 (chitobiose,  $\log_2=11.9$ ). Chitin oligomers  
531 may originate by either a biosynthetic process or cleavage of a longer chitin polymer.  
532 Bacterial and fungal plant mutualists can synthesize chitin-derived signaling molecules to  
533 prepare their hosts for colonization (Sánchez-Vallet *et al.*, 2015). Alternatively, chito-  
534 oligosaccharides can be released from chitin by fungal and plant chitinases. Plant chitinases  
535 are involved in defense against fungal pathogens because they hydrolyze fungal cell wall  
536 chitinous components and release chitin oligomers that trigger the plant immune responses  
537 (Fukamizo & Shinya, 2019). Most plant chitinases are endochitinases that cleave randomly at  
538 internal sites in the chitin polymer, generating low molecular mass glucosamine multimers  
539 (Rathore & Gupta, 2015). Although we do not have direct evidence of the origin of the  
540 chitotriose and chitobiose compounds in *S. vomeracea* symbiotic protocorms, transcriptomic  
541 data support the hypothesis that they are generated by the activity of plant chitinases. In fact,  
542 only one of the two *T. calospora* chitin synthase genes expressed in symbiotic protocorms  
543 was slightly upregulated (FC=2, Table S2). Conversely, transcripts corresponding to plant  
544 chitinases belonging to GH18 and GH19 families were strongly upregulated in symbiotic  
545 protocorms (Table S3), in agreement with previous observations showing increased chitinase  
546 expression in symbiotic protocorms (Zhao *et al.*, 2013; Perotto *et al.*, 2014). Although plants  
547 produce endochitinases in response to phytopathogenic attacks (Kumar *et al.*, 2018), a role for  
548 chitinases in root symbioses has already been reported for AM and nodule symbioses. In AM  
549 roots, the strong expression of chitinases in arbusculated cells, mainly belonging to class III  
550 (GH family 18), is thought to reduce the amount of chitin elicitors released by the wall of a  
551 compatible symbiotic fungus (Kasprzewska, 2003; Hoge Kamp *et al.*, 2011; Grover, 2012).  
552 Interestingly, short oligomers of 2 to 5 N-acetyl glucosamine residues, similar to those found  
553 in this work, have been reported to actively promote AM colonization (Volpe *et al.*, 2020).  
554 Further studies are required to elucidate the involvement of *S. vomeracea* chitinases during  
555 the OM symbiosis.

556 Chitosan oligomers were also abundant in the SYMB samples. Chitosan is the deacetylated  
557 form of chitin and is not abundant in the cell wall of Basidiomycetes (Di Mario *et al.* 2008).  
558 It was therefore intriguing to find a similar enrichment of chitin and chitosan oligomers ( $\log_2$   
559 from 12.0 to 13.5) in symbiotic protocorms (Table S1), when compared with either the MYC

560 or the FLM samples. Chitosan is produced through the activity of chitin deacetylase and three  
561 *T. calospora* chitin deacetylase genes were significantly upregulated in the symbiotic  
562 protocorms, as compared with FLM (Table S2), supporting the hypothesis that chitin  
563 deacetylation is increased in symbiosis. Chitin deacetylase inactivates the elicitor activity of  
564 chitin oligomers because it converts them to ligand-inactive chitosan. Chitin deacetylation has  
565 been reported as a strategy of endophytic fungi and soil-borne pathogens to prevent chitin-  
566 triggered plant immunity (Cord-Landwehr *et al.*, 2016; Gao *et al.*, 2019). Also, chitin  
567 deacetylases are regulated during the interaction with plants in both ECM and AM fungi  
568 (Balestrini & Bonfante, 2014), suggesting a role during symbiosis establishment and  
569 functioning.

570

### 571 ***Current challenges of metabolomic studies of poorly described organisms***

572 Metabolomics is a powerful tool to investigate biological systems. Here, it provided a global  
573 profiling of the metabolites and it allowed the study of orchid mycorrhiza. We demonstrated a  
574 rearrangement of the metabolome and changes in compounds possibly related to structural,  
575 signaling, defense, and nutrient functions.

576 However, the metabolomic approach also showed some limitations. For example, several  
577 mass-features could not be annotated in the available database. This uncharacterized “dark  
578 matter” is surely an interesting chemical signature that contains crucial information. For  
579 instance, from the 291 and 315 mass-features uniquely found in MYC-SYMB or in MYC-  
580 SYMB-FLM (Fig. 2), respectively representing symbiosis-specific and constitutive fungal  
581 compounds, none could be reasonably matched in databases. Overall, there is still a severe  
582 limitation in metabolite annotation in non-targeted metabolomics study: only ~2% of spectra  
583 is currently found in databases (da Silva *et al.*, 2015). This is much less than for genomic  
584 studies, where annotation can reach ~80%. Further difficulties of metabolomic studies arise  
585 from the fact that metabolomics reports are usually focused on model organisms, hampering  
586 functional enrichment analysis of non-model organisms such as *T. calospora* and *S.*  
587 *vomeracea*. Orchids and the symbiotic fungus *T. calospora* are evolutionary distant to those  
588 organisms found in the database and, in the case of orchids, rich of yet unknown secondary  
589 metabolites (Sut *et al.*, 2017). For the current study, we used MetaCyc, the largest curated  
590 collection of metabolic pathways, and the most comprehensive reference database of  
591 metabolic pathways from all domains of life (Caspi *et al.*, 2018). It contains the experimental  
592 evidence of 457 pathways in a member of the taxonomic group fungi, from >54,000  
593 publications (Caspi *et al.*, 2019; Karp *et al.*, 2019). However, even when using such extensive

594 collection, 451 metabolites in the comparison MYC/FLM could not be matched to objects in  
595 the database, pointing to the abovementioned limitation in the reconstruction of the  
596 biochemical pathways of *S. vomeracea* and *T. calospora*. Nevertheless, despite the severe  
597 limitations in metabolite annotation and functional analysis, we could estimate the elemental  
598 formulas of detected mass features. Using an ultra-high mass resolution and following the  
599 “seven golden rules” (Kind & Fiehn, 2007), we could accurately measure the mass-to-charge  
600 ratios of the metabolome fingerprint and produce an excellent estimation of the metabolite  
601 elemental formula with a high probability (Kim *et al.*, 2006, probability of 98%). Atom ratios  
602 of compound elemental formulas can be visualized using van Krevelen diagrams for rough  
603 compound identification in chemical classes, although the limits defining those classes are  
604 overlapping among the compound categories. To overcome this issue, we employed the very  
605 recently developed multidimensional stoichiometric compound classification (MSCC)  
606 approach (Rivas-Ubach *et al.*, 2018). In this way, we successfully classified almost entirely  
607 the significant mass-features discriminant for the separation of MYC/FLM, MYC/SYMB,  
608 SYMB/ASYMB and overcame constrains of actual database.

609

610 In conclusion, we revealed profound changes in metabolite profiles in orchid mycorrhiza. The  
611 most interesting finding was the sharp adjustment of the lipid metabolism in the fungus *T.*  
612 *calospora* to the symbiosis. Although further and more sensitive targeted analyses are needed  
613 to elucidate the significance of these metabolic changes in symbiosis, our study demonstrates  
614 that the cross-link between metabolomic and transcriptomic data can pave the way for a more  
615 comprehensive understanding of the metabolic networks underlying orchid-fungus  
616 interactions.

617

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623 Martin (INRA, Nancy, France).

624

## 625 **AUTHOR CONTRIBUTIONS**

626 S.P., R.B. and J.P.S. conceived and designed the research. A.G., V.F. and B.L. conducted all  
627 wet lab experiments. A.G., J.P.S. and M.W. conducted data analyses. A.G., S.P., R.B. wrote  
628 the manuscript. All authors read and approved the manuscript.

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## 632 REFERENCES

633

634 **Aharoni A, Ric de Vos CH, Verhoeven HA, Maliepaard CA, Kruppa G, Bino R,**  
635 **Goodenowe DB. 2002.** Nontargeted metabolome analysis by use of Fourier Transform Ion  
636 Cyclotron Mass Spectrometry. *OMICS A Journal of Integrative Biology* **6**: 217–234.

637 **Balestrini R, Bonfante P. 2014.** Cell wall remodeling in mycorrhizal symbiosis: A way  
638 towards biotrophism. *Frontiers in Plant Science* **5**: 1–10.

639 **Balla T. 2013.** Phosphoinositides: Tiny lipids with giant impact on cell regulation.  
640 *Physiological Reviews* **93**: 1019–1137.

641 **Beyrle HF, Smith SE, Peterson RL, Franco CM. 1995.** Colonization of *Orchis morio*  
642 protocorms by a mycorrhizal fungus: Effects of nitrogen nutrition and glyphosate in  
643 modifying the responses. *Canadian Journal of Botany* **73**: 1128–1140.

644 **Blunsom NJ, Cockcroft S. 2020.** Phosphatidylinositol synthesis at the endoplasmic  
645 reticulum. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **1865**:  
646 158471.

647 **Bowers WS, Hoch HC, Evans PH, Katayama M. 1986.** Thallophytic allelopathy: Isolation  
648 and identification of laetisarinic acid. *Science* **232**: 105–106.

649 **Bowman SM, Free SJ. 2006.** The structure and synthesis of the fungal cell wall. *BioEssays*  
650 **28**: 799–808.

651 **Brodhun F, Feussner I. 2011.** Oxylipins in fungi. *FEBS Journal* **278**: 1047–1063.

652 **Cameron DD, Johnson I, Leake JR, Read DJ. 2007.** Mycorrhizal acquisition of inorganic  
653 phosphorus by the green-leaved terrestrial orchid *Goodyera repens*. *Annals of Botany* **99**:  
654 831–834.

655 **Cameron DD, Johnson I, Read DJ, Leake JR. 2008.** Giving and receiving: Measuring the  
656 carbon cost of mycorrhizas in the green orchid, *Goodyera repens*. *New Phytologist* **180**: 176–  
657 184.

658 **Cameron DD, Leake JR, Read DJ. 2006.** Mutualistic mycorrhiza in orchids: Evidence from  
659 plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera*

660 *repens*. *New Phytologist* **171**: 405–416.

661 **Carta G, Murru E, Banni S, Manca C. 2017.** Palmitic acid: Physiological role, metabolism  
662 and nutritional implications. *Frontiers in Physiology* **8**: 1–14.

663 **Caspi R, Billington R, Fulcher CA, Keseler IM, Kothari A, Krummenacker M,**  
664 **Latendresse M, Midford PE, Ong Q, Ong WK, et al. 2018.** The MetaCyc database of  
665 metabolic pathways and enzymes. *Nucleic Acids Research* **46**: D633–D639.

666 **Caspi R, Billington R, Keseler IM, Kothari A, Krummenacker M, Midford PE, Ong**  
667 **WK, Paley S, Subhraveti P, Karp PD. 2019.** The MetaCyc database of metabolic pathways  
668 and enzymes - a 2019 update. *Nucleic Acids Research* **1**: 1–9.

669 **Cassilly CD, Reynolds TB. 2018.** PS, it's complicated: The roles of phosphatidylserine and  
670 phosphatidylethanolamine in the pathogenesis of *Candida albicans* and other microbial  
671 pathogens. *Journal of Fungi* **4**: 28.

672 **Cavill R, Jennen D, Kleinjans J, Briedé JJ. 2016.** Transcriptomic and metabolomic data  
673 integration. *Briefings in Bioinformatics* **17**: 891–901.

674 **Chang DCN, Chou LC. 2007.** Growth responses, enzyme activities, and component changes  
675 as influenced by *Rhizoctonia* Orchid mycorrhiza on *Anoectochilus formosanus* Hayata.  
676 *Botanical Studies* **48**: 445–451.

677 **Christensen SA, Kolomiets M V. 2011.** The lipid language of plant-fungal interactions.  
678 *Fungal Genetics and Biology* **48**: 4–14.

679 **Cord-Landwehr S, Melcher RLJ, Kolkenbrock S, Moerschbacher BM. 2016.** A chitin  
680 deacetylase from the endophytic fungus *Pestalotiopsis* sp. efficiently inactivates the elicitor  
681 activity of chitin oligomers in rice cells. *Scientific Reports* **6**: 1–11.

682 **Cowan AK. 2006.** Phospholipids as plant growth regulators. *Plant Growth Regulation* **48**:  
683 97–109.

684 **Cumming BM, Chinta KC, Reddy VP, Steyn AJC. 2018.** Role of Ergothioneine in  
685 Microbial Physiology and Pathogenesis. *Antioxidants and Redox Signaling* **28**: 431–444.

686 **Dearnaley JDW, Cameron DD. 2017.** Nitrogen transport in the orchid mycorrhizal  
687 symbiosis – further evidence for a mutualistic association. *New Phytologist* **213**: 10–12.

688 **Ebert B, Rautengarten C, McFarlane HE, Rupasinghe T, Zeng W, Ford K, Scheller H**  
689 **V., Bacic A, Roessner U, Persson S, et al. 2018.** A Golgi UDP-GlcNAc transporter delivers  
690 substrates for N-linked glycans and sphingolipids. *Nature Plants* **4**: 792–801.

691 **Epstein S, Castillon GA, Qin Y, Riezman H. 2012.** An essential function of sphingolipids in  
692 yeast cell division. *Molecular Microbiology* **84**: 1018–1032.

693 **Ercole E, Adamo M, Rodda M, Gebauer G, Girlanda M, Perotto S. 2015a.** Temporal

694 variation in mycorrhizal diversity and carbon and nitrogen stable isotope abundance in the  
695 wintergreen meadow orchid *Anacamptis morio*. *New Phytologist* **205**: 1308–1319.

696 **Ercole E, Rodda M, Girlanda M, Perotto S. 2015b.** Establishment of a symbiotic in vitro  
697 system between a green meadow orchid and a Rhizoctonia-like fungus. *Bio-protocol* **5**: 1–7.

698 **Fiehn O, Kopka J, Dörmann P, Altmann T, Trethewey RN, Willmitzer L. 2000.**  
699 Metabolite profiling for plant functional genomics. *Nature Biotechnology* **18**: 1157–1161.

700 **Fochi V, Chitarra W, Kohler A, Voyron S, Singan VR, Lindquist EA, Barry KW,**  
701 **Girlanda M, Grigoriev I V., Martin F, et al. 2017a.** Fungal and plant gene expression in the  
702 *Tulasnella calospora* – *Serapias vomeracea* symbiosis provides clues about nitrogen  
703 pathways in orchid mycorrhizas. *New Phytologist* **213**: 365–379.

704 **Fochi V, Falla N, Girlanda M, Perotto S, Balestrini R. 2017b.** Cell-specific expression of  
705 plant nutrient transporter genes in orchid mycorrhizae. *Plant Science* **263**: 39–45.

706 **Fukamizo T, Shinya S. 2019.** Chitin/Chitosan-Active Enzymes Involved in Plant–Microbe  
707 Interactions. In: Yang Q, Fukamizo T, eds. *Targeting Chitin-containing Organisms. Advances*  
708 *in Experimental Medicine and Biology*. Singapore: Springer, Vol. 1142, 253-272.

709 **Furo K, Nozaki M, Murashige H, Sato Y. 2015.** Identification of an *N*-acetylglucosamine  
710 kinase essential for UDP-*N*-acetylglucosamine salvage synthesis in *Arabidopsis*. *FEBS*  
711 *Letters* **589**: 3258–3262.

712 **Fyrst H, Saba JD. 2010.** An update on sphingosine-1-phosphate and other sphingolipid  
713 mediators. *Nature Chemical Biology* **6**: 489–497.

714 **Gao F, Zhang B Sen, Zhao JH, Huang JF, Jia PS, Wang S, Zhang J, Zhou JM, Guo HS.**  
715 **2019.** Deacetylation of chitin oligomers increases virulence in soil-borne fungal pathogens.  
716 *Nature Plants* **5**: 1167–1176.

717 **Gerke J, Bayram Ö, Braus GH. 2012.** Fungal S-adenosylmethionine synthetase and the  
718 control of development and secondary metabolism in *Aspergillus nidulans*. *Fungal Genetics*  
719 *and Biology* **49**: 443–454.

720 **Ghirardo A, Heller W, Fladung M, Schnitzler J-P, Schroeder H. 2012.** Function of  
721 defensive volatiles in pedunculate oak (*Quercus robur*) is tricked by the moth *Tortrix*  
722 *viridana*. *Plant, Cell and Environment environment* **35**: 2192–2207.

723 **Ghirardo A, Sørensen HA, Petersen M, Jacobsen S, Søndergaard I. 2005.** Early  
724 prediction of wheat quality: analysis during grain development using mass spectrometry and  
725 multivariate data analysis. *Rapid communications in mass spectrometry* **19**: 525–532.

726 **Ghirardo A, Xie J, Zheng X, Wang Y, Grote R, Block K, Wildt J, Mentel T, Kiendler-**  
727 **Scharr A, Hallquist M, et al. 2016.** Urban stress-induced biogenic VOC emissions impact

728 secondary aerosol formation in Beijing. *Atmospheric Chemistry and Physics* **15**: 2901–2920.

729 **Girlanda M, Segreto R, Cafasso D, Liebel HT, Rodda M, Ercole E, Cozzolino S,**  
730 **Gebauer G, Perotto S. 2011.** Photosynthetic Mediterranean meadow orchids feature partial  
731 mycoheterotrophy and specific mycorrhizal associations1. *American Journal of Botany* **98**:  
732 1148–1163.

733 **Grover A. 2012.** Plant Chitinases: Genetic Diversity and Physiological Roles. *Critical*  
734 *Reviews in Plant Sciences* **31**: 57–73.

735 **Hannun YA, Obeid LM. 2018.** Sphingolipids and their metabolism in physiology and  
736 disease. *Nature Reviews Molecular Cell Biology* **19**: 175–191.

737 **Hogekamp C, Arndt D, Pereira PA, Becker JD, Hohnjec N, Küster H. 2011.** Laser  
738 microdissection unravels cell-type-specific transcription in arbuscular mycorrhizal roots,  
739 including CAAT-Box transcription factor gene expression correlating with fungal contact and  
740 spread. *Plant Physiology* **157**: 2023–2043.

741 **Hou Q, Ufer G, Bartels D. 2016.** Lipid signalling in plant responses to abiotic stress. *Plant*  
742 *Cell and Environment* **39**: 1029–1048.

743 **Hu W, Pan X, Abbas HMK, Li F, Dong W. 2017.** Metabolites contributing to *Rhizoctonia*  
744 *solani* AG-1-IA maturation and sclerotial differentiation revealed by UPLC-QTOF-MS  
745 metabolomics. *PLoS ONE* **12**: 1–16.

746 **Kaling M, Kanawati B, Ghirardo A, Albert A, Winkler JB, Heller W, Barta C, Loreto F,**  
747 **Schmitt-Kopplin P, Schnitzler J-PP. 2015.** UV-B mediated metabolic rearrangements in  
748 poplar revealed by non-targeted metabolomics. *Plant, cell & environment* **38**: 892–904.

749 **Kårlund A, Hanhineva K, Lehtonen M, Karjalainen RO, Sandell M. 2015.** Nontargeted  
750 metabolite profiles and sensory properties of strawberry cultivars grown both organically and  
751 conventionally. *Journal of Agricultural and Food Chemistry* **63**: 1010–1019.

752 **Karp PD, Billington R, Caspi R, Fulcher CA, Latendresse M, Kothari A, Keseler IM,**  
753 **Krummenacker M, Midford PE, Ong Q, et al. 2019.** The BioCyc collection of microbial  
754 genomes and metabolic pathways. *Briefings in Bioinformatics* **20**: 1085–1093.

755 **Kasprzewska A. 2003.** Plant chitinases - Regulation and function. *Cellular and Molecular*  
756 *Biology Letters* **8**: 809–824.

757 **Kay JG, Grinstein S. 2011.** Sensing phosphatidylserine in cellular membranes. *Sensors* **11**:  
758 1744–1755.

759 **Kersten B, Ghirardo A, Schnitzler J, Kanawati B, Schmitt-Kopplin P, Fladung M,**  
760 **Schroeder H. 2013.** Integrated transcriptomics and metabolomics decipher differences in the  
761 resistance of pedunculate oak to the herbivore *Tortrix viridana* L. *BMC genomics* **14**: 737.



762 **Keymer A, Pimprikar P, Wewer V, Huber C, Brands M, Bucerius SL, Delaux P, Klingl**  
763 **V, Wang TL, Eisenreich W. 2017.** Lipid transfer from plants to arbuscular mycorrhiza fungi.  
764 **6:e29107.**

765 **Kim S, Rodgers RP, Marshall AG. 2006.** Truly ‘exact’ mass: Elemental composition can be  
766 determined uniquely from molecular mass measurement at ~0.1 mDa accuracy for molecules  
767 up to ~500 Da. *International Journal of Mass Spectrometry* **251**: 260–265.

768 **Kind T, Fiehn O. 2007.** Seven Golden Rules for heuristic filtering of molecular formulas  
769 obtained by accurate mass spectrometry. *BMC Bioinformatics* **8**: 1–20.

770 **Kluger B, Lehner S, Schuhmacher R. 2015.** Metabolomics and secondary metabolite  
771 profiling of filamentous fungi. In: Zeilinger S, Martín J, García-Estrada C, eds. *Biosynthesis*  
772 *and Molecular Genetics of Fungal Secondary Metabolites Volume 2. Fungal Biology.* New  
773 York: Springer, 81–101.

774 **Kohler A, Böcker U, Shapaval V, Forsmark A, Andersson M, Warringer J, Martens H,**  
775 **Omholt SW, Blomberg A. 2015a.** High-throughput biochemical fingerprinting of  
776 *Saccharomyces cerevisiae* by Fourier transform infrared spectroscopy. *PLoS ONE* **10**: 1–22.

777 **Kohler A, Kuo A, Nagy LG, Morin E, Barry KW, Buscot F, Canbäck B, Choi C,**  
778 **Cichocki N, Clum A, et al. 2015b.** Convergent losses of decay mechanisms and rapid  
779 turnover of symbiosis genes in mycorrhizal mutualists. *Nature Genetics* **47**: 410–415.

780 **Kuga Y, Sakamoto N, Yurimoto H. 2014.** Stable isotope cellular imaging reveals that both  
781 live and degenerating fungal pelotons transfer carbon and nitrogen to orchid protocorms. *New*  
782 *Phytologist* **202**: 594–605.

783 **Kumar M, Brar A, Yadav M, Chawade A, Vivekanand V, Pareek N. 2018.** Chitinases—  
784 Potential candidates for enhanced plant resistance towards fungal pathogens. *Agriculture* **8**.

785 **Lallemand F, Martin-Magniette ML, Gilard F, Gakière B, Launay-Avon A, Delannoy É,**  
786 **Selosse MA. 2019.** *In situ* transcriptomic and metabolomic study of the loss of photosynthesis  
787 in the leaves of mixotrophic plants exploiting fungi. *Plant Journal* **98**: 826–841.

788 **Laparre J, Malbreil M, Letisse F, Portais JC, Roux C, Bécard G, Puech-Pagès V. 2014.**  
789 Combining metabolomics and gene expression analysis reveals that propionyl- and butyryl-  
790 carnitines are involved in late stages of arbuscular mycorrhizal symbiosis. *Molecular Plant* **7**:  
791 554–566.

792 **Leake JR. 1994.** Tansley Review No. 69. The biology of myco-heterotrophic (‘saprophytic’)  
793 plants. *New Phytol.* **127**: 171–216.

794 **Liu X-H, Liang S, Wei Y-Y, Zhu X-M, Li L, Ping-Ping Liu B, Zheng Q-X, Zhou H-N,**  
795 **Zhang Y, Mao L-J, et al. 2019.** Metabolomics Analysis Identifies Sphingolipids as Key

796 Function in *Magnaporthe oryzae*. *American Society For Microbiology* **10**: 1–18.

797 **Majumder PL, Lahiri S. 1990.** Lusianthrin and lusianthridin, two stilbenoids from the  
798 orchid *Lusia indivisa*. *Phytochemistry* **29**: 621–624.

799 **Di Mario F, Rapanà P, Tomati U, Galli E. 2008.** Chitin and chitosan from Basidiomycetes.  
800 *International Journal of Biological Macromolecules* **43**: 8–12.

801 **Mato JM, Alvarez L, Ortiz P, Pajares MA. 1997.** S-adenosylmethionine synthesis:  
802 Molecular mechanisms and clinical implications. *Pharmacology and Therapeutics* **73**: 265–  
803 280.

804 **Meijer HJG, Munnik T. 2003.** Phospholipid-Based Signaling in Plants. *Annual Review of*  
805 *Plant Biology* **54**: 265–306.

806 **Michell RH. 2008.** Inositol derivatives: Evolution and functions. *Nature Reviews Molecular*  
807 *Cell Biology* **9**: 151–161.

808 **Miura C, Yamaguchi K, Miyahara R, Yamamoto T, Fuji M, Yagame T, Imaizumi-**  
809 **Anraku H, Yamato M, Shigenobu S, Kaminaka H. 2018.** The mycoheterotrophic  
810 symbiosis between orchids and mycorrhizal fungi possesses major components shared with  
811 mutualistic plant-mycorrhizal symbioses. *Molecular Plant-Microbe Interactions* **31**: 1032–  
812 1047.

813 **Obeid LM, Okamoto Y, Mao C. 2002.** Yeast sphingolipids: Metabolism and biology.  
814 *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* **1585**: 163–171.

815 **Oura T, Kajiwara S. 2010.** *Candida albicans* sphingolipid C9-methyltransferase is involved  
816 in hyphal elongation. *Microbiology* **156**: 1234–1243.

817 **Paley S, Parker K, Spaulding A, Tomb JF, O'Maille P, Karp PD. 2017.** The omics  
818 dashboard for interactive exploration of gene-expression data. *Nucleic Acids Research* **45**:  
819 12113–12124.

820 **Pegg AE, Xiong H, Feith DJ, Shantz LM. 1998.** S-adenosylmethionine decarboxylase:  
821 Structure, function and regulation by polyamines. *Biochemical Society Transactions* **26**: 580–  
822 586.

823 **Perotto S, Rodda M, Benetti A, Sillo F, Ercole E, Rodda M, Girlanda M, Murat C,**  
824 **Balestrini R. 2014.** Gene expression in mycorrhizal orchid protocorms suggests a friendly  
825 plant-fungus relationship. *Planta* **239**: 1337–1349.

826 **Peterson RL, Farquhar ML. 1994.** Mycorrhizas: Integrated development between roots and  
827 fungi. *Mycologia* **86**: 311–326.

828 **Plassard C, Becquer A, Garcia K. 2019.** Phosphorus Transport in Mycorrhiza: How Far Are  
829 We? *Trends in Plant Science* **24**: 794–801.

830 **Pusztahelyi T. 2018.** Chitin and chitin-related compounds in plant–fungal interactions.  
831 *Mycology* **9**: 189–201.

832 **Rasmussen HN. 1995.** *Terrestrial orchids: from seed to mycotrophic plant* (Cambridge, Ed.).  
833 Cambridge, UK: Cambridge University Press.

834 **Rathore AS, Gupta RD. 2015.** Chitinases from bacteria to human: properties, applications,  
835 and future perspectives. *Enzyme Research* **2015**: 1–9.

836 **Rivas-Ubach A, Liu Y, Bianchi TS, Tolić N, Jansson C, Paša-Tolić L. 2018.** Moving  
837 beyond the van Krevelen diagram: A new stoichiometric approach for compound  
838 classification in organisms. *Analytical Chemistry* **90**: 6152–6160.

839 **Rivero J, Gamir J, Aroca R, Pozo MJ, Flors V. 2015.** Metabolic transition in mycorrhizal  
840 tomato roots. *Frontiers in Microbiology* **6**: 598.

841 **Sánchez-Vallet A, Mesters JR, Thomma BPHJ. 2015.** The battle for chitin recognition in  
842 plant-microbe interactions. *FEMS Microbiology Reviews* **39**: 171–183.

843 **Schliemann W, Ammer C, Strack D. 2008.** Metabolite profiling of mycorrhizal roots of  
844 *Medicago truncatula*. *Phytochemistry* **69**: 112–146.

845 **Schumann U, Smith NA, Wang MB. 2013.** A fast and efficient method for preparation of  
846 high-quality RNA from fungal mycelia. *BMC Research Notes* **6**: 71.

847 **Sheridan KJ, Lechner BE, Keeffe GO, Keller MA, Werner ER, Lindner H, Jones GW,  
848 Haas H, Doyle S. 2016.** Ergothioneine biosynthesis and functionality in the opportunistic  
849 fungal pathogen, *Aspergillus fumigatus*. *Scientific Reports* **6**: 1–17.

850 **Shimura H, Matsuura M, Takada N, Koda Y. 2007.** An antifungal compound involved in  
851 symbiotic germination of *Cypripedium macranthos* var. *rebunense* (Orchidaceae).  
852 *Phytochemistry* **68**: 1442–1447.

853 **Sidorov RA, Zhukov A V., Pchelkin VP, Tsydendambaev VD. 2014.** Palmitic acid in  
854 higher plant lipids. In: *Palmitic Acid: Occurrence, Biochemistry and Health Effects*. Nova  
855 Science Publishers, Inc., 124–144.

856 **Siebers M, Brands M, Wewer V, Duan Y, Hölzl G, Dörmann P. 2016.** Lipids in plant–  
857 microbe interactions. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*  
858 **1861**: 1379–1395.

859 **da Silva RR, Dorrestein PC, Quinn RA. 2015.** Illuminating the dark matter in  
860 metabolomics. *Proceedings of the National Academy of Sciences of the United States of*  
861 *America* **112**: 12549–12550.

862 **Singh A, Del Poeta M. 2016.** Sphingolipidomics: An important mechanistic tool for studying  
863 fungal pathogens. *Frontiers in Microbiology* **7**: 1–14.

864 **Smith S, Read D. 2008.** *Mycorrhizal symbiosis*. Cambridge, UK: Academic Press.

865 **Suhre K, Schmitt-Kopplin P. 2008.** MassTRIX: mass translator into pathways. *Nucleic*  
866 *acids research* **36**: 481–484.

867 **Sut S, Maggi F, Dall’Acqua S. 2017.** Bioactive Secondary Metabolites from Orchids  
868 (Orchidaceae). *Chemistry and Biodiversity* **14**: e1700172.

869 **Tschaplinski TJ, Plett JM, Engle NL, Deveau A, Cushman KC, Martin MZ, Doktycz**  
870 **MJ, Tuskan GA, Brun A, Kohler A, et al. 2014.** *Populus trichocarpa* and *Populus deltoides*  
871 Exhibit Different Metabolomic Responses to Colonization by the Symbiotic Fungus *Laccaria*  
872 *bicolor*. *Molecular Plant-Microbe Interactions* **27**: 546–556.

873 **Valentín-Berríos S, González-Velázquez W, Pérez-Sánchez L, González-Méndez R,**  
874 **Rodríguez-Del Valle N. 2009.** Cytosolic phospholipase A: a member of the signalling  
875 pathway of a new G protein  $\alpha$  subunit in *Sporothrix schenckii*. *BMC Microbiology* **9**: 1–16.

876 **Vences-Guzmán MA, Geiger O, Sohlenkamp C. 2008.** Sinorhizobium meliloti mutants  
877 deficient in phosphatidylserine decarboxylase accumulate phosphatidylserine and are strongly  
878 affected during symbiosis with alfalfa. *Journal of Bacteriology* **190**: 6846–6856.

879 **Volpe V, Carotenuto G, Berzero C, Cagnina L, Puech-Pagès V, Genre A. 2020.** Short  
880 chain chito-oligosaccharides promote arbuscular mycorrhizal colonization in *Medicago*  
881 *truncatula*. *Carbohydrate Polymers* **229**: 115505.

882 **Van Waes JM, Debergh PC. 1986.** In vitro germination of some Western European orchids.  
883 *Physiologia Plantarum* **67**: 253–261.

884 **Wägele B, Witting M, Schmitt-Kopplin P, Suhre K. 2012.** Masstrix reloaded: Combined  
885 analysis and visualization of tran-scriptome and metabolome data. *PLoS ONE* **7**: 1–5.

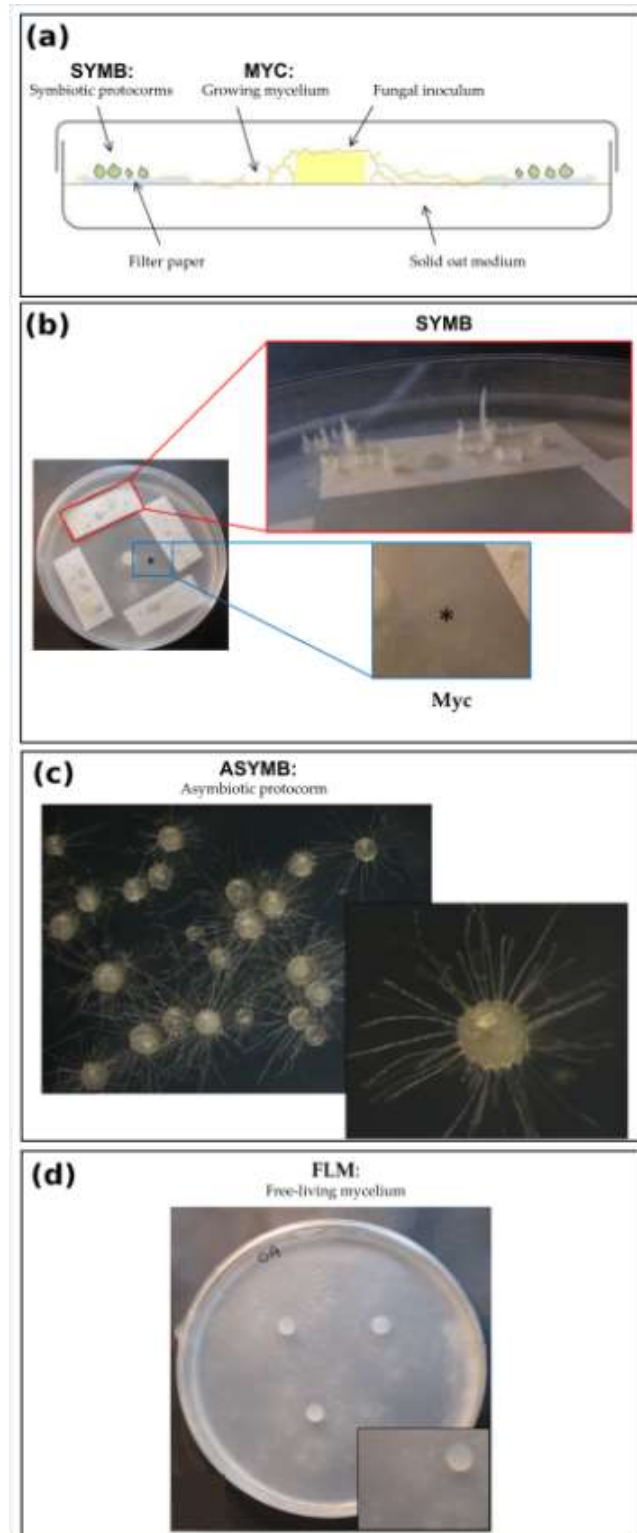
886 **Wang K Der, Borrego EJ, Kenerley CM, Kolomiets M V. 2020.** Oxylipins Other Than  
887 Jasmonic Acid Are Xylem-Resident Signals Regulating Systemic Resistance Induced by  
888 *Trichoderma virens* in Maize. *The Plant cell* **32**: 166–185.

889 **Way D, Ghirardo A, Kanawati B, Esperschütz J, Monson RK, Jackson RB, Schmitt-**  
890 **Kopplin P, Schnitzler J-P. 2013.** Increasing atmospheric CO<sub>2</sub> reduces metabolic and  
891 physiological differences between isoprene- and non-isoprene-emitting poplars. *The New*  
892 *phytologist* **200**: 534–546.

893 **Yeh CM, Chung KM, Liang CK, Tsai WC. 2019.** New insights into the symbiotic  
894 relationship between orchids and fungi. *Applied Sciences* **9**: 1–14.

895 **Zhang N, Venkateshwaran M, Boersma M, Harms A, Howes-Podoll M, Den Os D, Ané**  
896 **JM, Sussman MR. 2012.** Metabolomic profiling reveals suppression of oxylipin biosynthesis  
897 during the early stages of legume-rhizobia symbiosis. *FEBS Letters* **586**: 3150–3158.

898 **Zhao MM, Zhang G, Zhang DW, Hsiao YY, Guo SX. 2013.** ESTs analysis reveals putative  
899 genes involved in symbiotic seed germination in *Dendrobium officinale*. *PLoS ONE* **8**:  
900 e72705.  
901  
902



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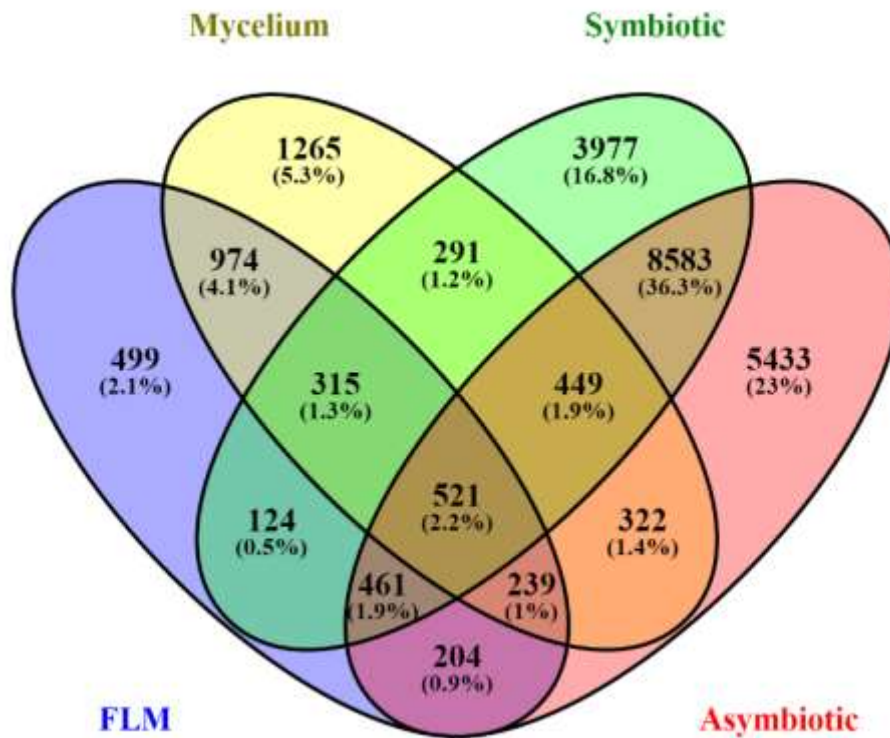
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**Figure 1:** (a) Schematic representation of the *in vitro* symbiotic germination system of *Serapias vomeracea* seeds with the orchid mycorrhizal fungus *Tulasnella calospora* (redraw from (Ercole *et al.*, 2015b)). (b) Symbiotic seed germination in Petri dishes; Mycorrhizal symbiotic protocorms (SYMB) of *S. vomeracea* (red box) and fungal mycelium (MYC) growing near the symbiotic protocorms (blue box) after 30 days of co-incubation. (c) Asymbiotic protocorms (ASYMB) grown on BM1 medium 120 days after sowing. (d) Free-living mycelium (FLM) of *T. calospora* grown on oat medium (OA) at 20 dpi.

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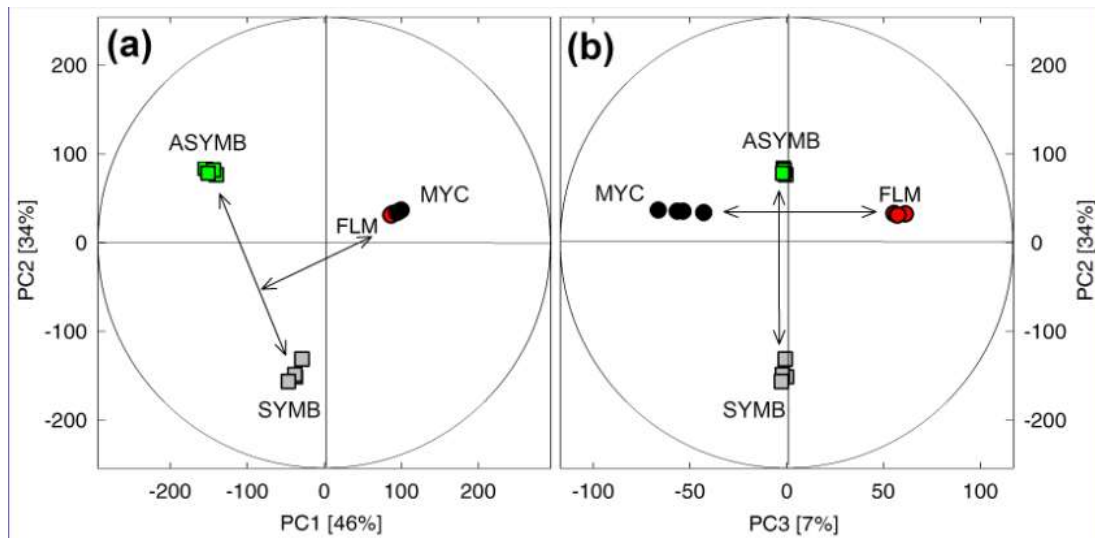
916 **Figure 2:** Venn diagram of specific and shared mass features (m.f.) occurring and  
917 overlapping in symbiotic (SYMB) and asymbiotic (ASYMB) *S. vomeracea* protocorms, *T.*  
918 *calospora* free-living mycelium (FLM) and mycelium growing near symbiotic protocorms  
919 (MYC).

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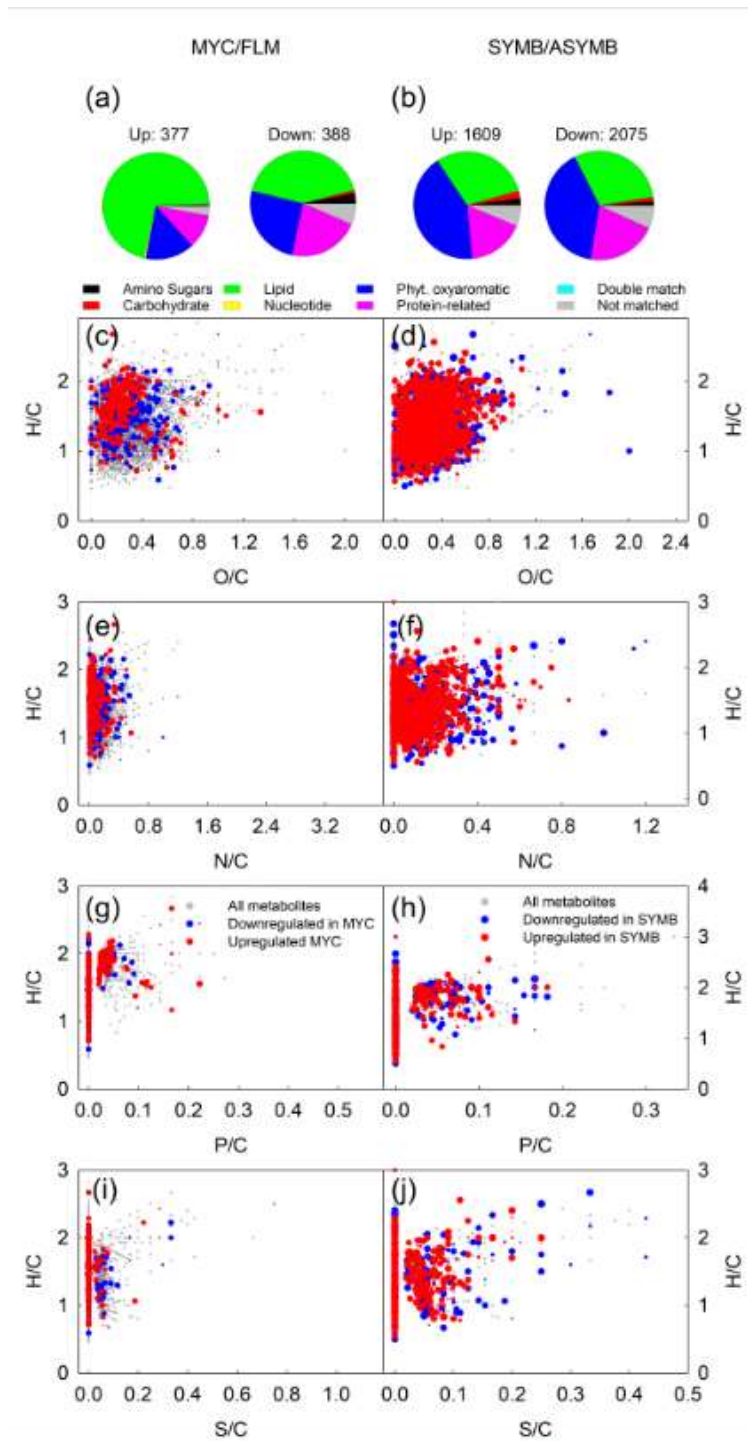
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927 **Figure 3:** Score plots of principal components analysis (PCA) of all mass features detected by  
928 non-targeted metabolomics. (a) Principal component (PC) 1 vs. PC2 shows the metabolic  
929 distances between *T. calospora* AL13 growing as free-living mycelium (FLM) or collected  
930 near the symbiotic protocorms (MYC) and between *S. vomeracea* symbiotic (SYMB) and  
931 asymbiotic (ASYMB) protocorms. (b) PC3 depicts metabolic differences between MYC and  
932 FLM. The variances explained by each PC are given in parentheses. Ellipses denote the  
933 Hotelling's  $T^2$  confidence interval of 95%. N = 4 biologically independent replicates. FLM,  
934 red circles; MYC, black circles; ASYMB, green square; SYMB, grey square.

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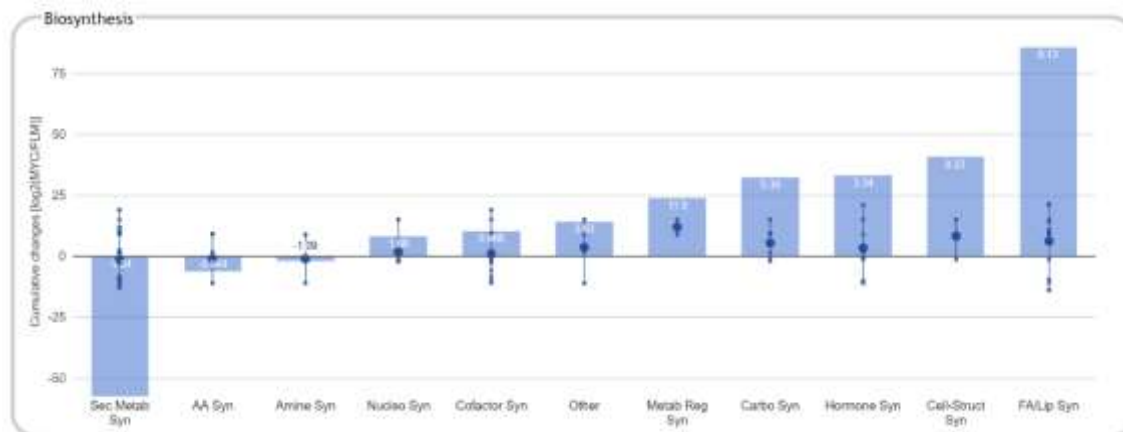


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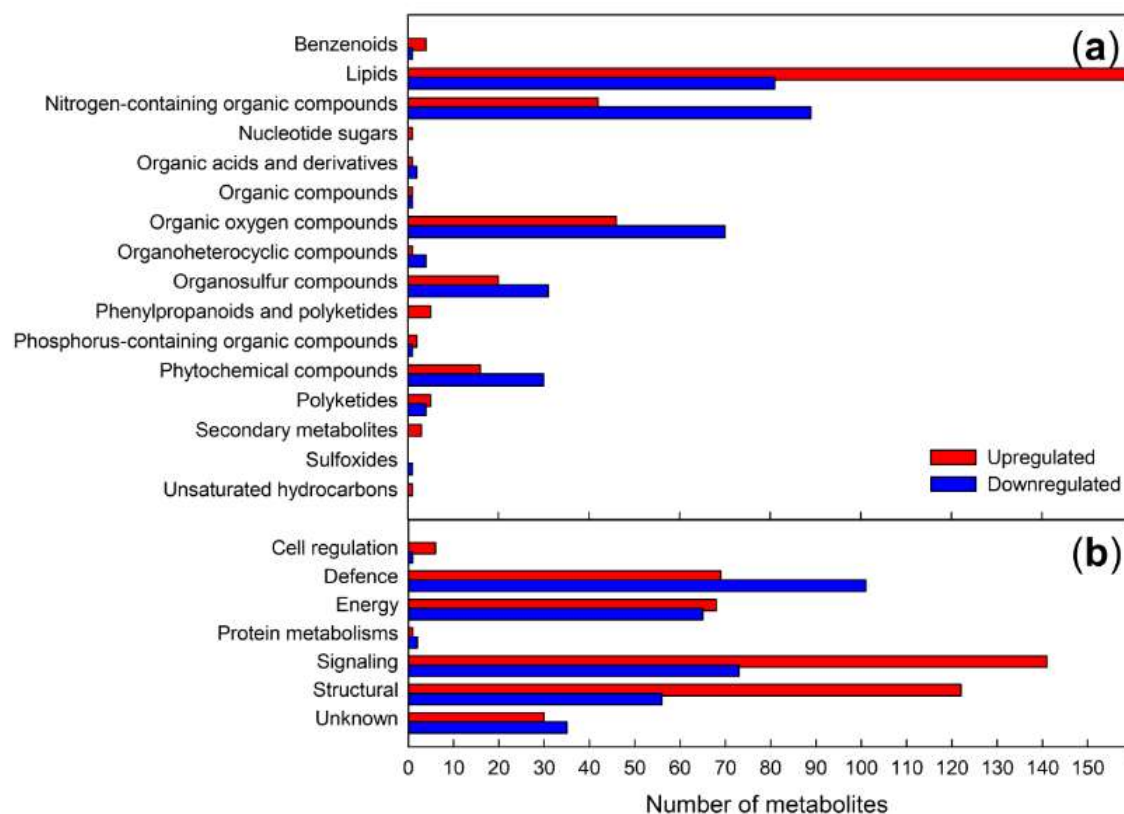
939 **Figure 4:** (a-b), Multidimensional stoichiometric compound classification (MSCC), and van  
 940 Krevelen diagrams (c-j) showing all metabolites (in grey) and statistically up- (in red) or  
 941 down- (in blue) regulated metabolites in asymbiotic and symbiotic conditions. Abbr. MYC,  
 942 fungal mycelium growing near the mycorrhizal protocorms; FLM, asymbiotic free-living  
 943 mycelium; SYMB, symbiotic orchid protocorms; ASYMB, asymbiotic orchid protocorms.  
 944 The magnitude of up- and down-regulated metabolites are depicted in (c-j) with different  
 945 symbol sizes (larger symbols represent stronger up/downregulation), using  $\sqrt{(\log_2(x))/2}$  for  
 946 upregulated and  $\sqrt{-\log_2(x)/2}$  for downregulated metabolites, where x is MYC/FLM (in c, e,  
 947 g, i) or SYMB/ASYMB (in d, f, h, j).

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**Figure 5:** Cumulative changes of significantly differently produced compounds on the metabolisms of MYC samples, compared to FLM. MYC, fungal mycelium growing near the mycorrhizal protocorms; FLM, asymbiotic free-living mycelium. The functional classes are based on the MetaCyc pathway ontology (<https://metacyc.org/>) and the graph constructed using the Omics Dashboard (Paley *et al.*, 2017).



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960 **Figure 6:** Changes of metabolites in the *T. calospora* mycelium. The number of metabolites  
961 grouped according to their (a) chemical taxonomy and (b) the biological functions of the up-  
962 (in red) and downregulated (in blue) metabolites in MYC samples, as compared to FLM. A  
963 comprehensive list is given in Table S1. The classification is based on KEGG, HMDB and  
964 Lipid Maps databases. Unknown organic compounds were classified based on the following  
965 priority of their atom compositions: S>P>N>O. For multifunction metabolites, the functions  
966 were added to different groups.

## ***Supporting Information***

Article title: **Metabolomic adjustments in the orchid mycorrhizal fungus *Tulasnella calospora* during symbiosis with *Serapias vomeracea***

Authors: Andrea Ghirardo, Valeria Fochi, Birgit Lange, Michael Witting, Jörg-Peter Schnitzler, Silvia Perotto, Raffaella Balestrini

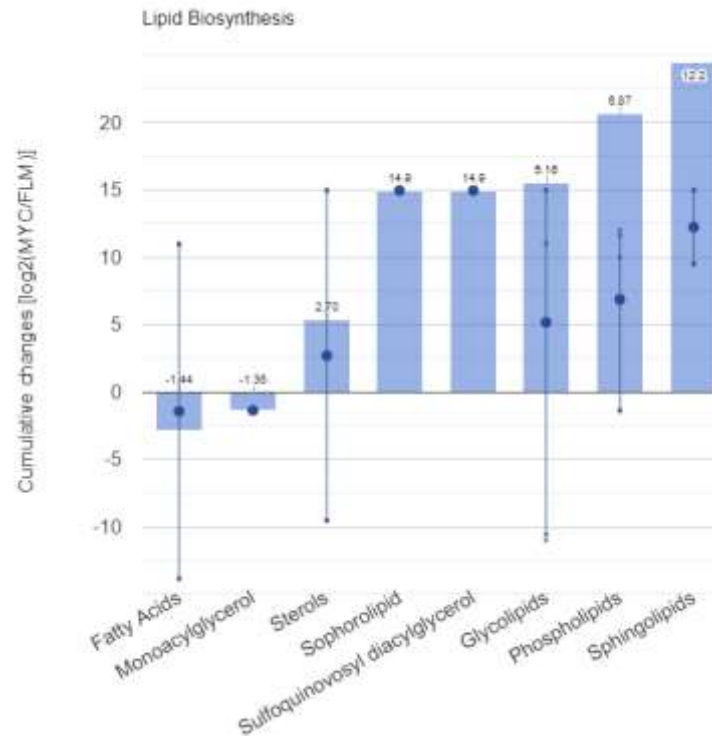
The following Supporting Information is available for this article:

**Fig. S1** Cumulative changes in lipid biosynthesis on the metabolisms of fungal mycelium (MYC) compared to free-living mycelium (FLM).

**Table S1** Metabolomic annotation. (*attached*)

**Table S2** Gene expression in *Tulasnella calospora*.

**Table S3** Gene expression in *Serapias vomeracea*.



**Fig. S1 Cumulative changes of significantly differently produced compounds involved in lipid biosynthesis on the metabolisms of MYC samples, compared to FLM.** MYC, fungal mycelium growing near the mycorrhizal protocorms; FLM, asymbiotic free-living mycelium. The functional classes are based on the MetaCyc pathway ontology (<https://metacyc.org/>) and the graph constructed using the Omics Dashboard (Paley *et al.*, 2017).

**Table S1** Excel file containing all the significant annotated molecular formulas of LC-MS measurements. (*online material*)

**Table S2 Gene expression in *Tulasnella calospora* (Fochi et al., 2017a).** Only genes significantly upregulated (FC>2, p-value<0.05) or downregulated (FC<0.5, p-value<0.05) in the comparison between symbiotic and asymbiotic conditions are reported.

Metabolism	Protein ID	Mean Read Count		SYMB/FLM comparison		Protein definition
		FLM	SYMB	Fold Change	FDR p-value*	
Glycerophospholipid /FA metabolism	53822	0.76	21.15	27.83	9.47E-09	Ca <sup>2+</sup> -independent phospholipase A2
	72491	10.37	185.29	17.87	4.07E-69	Myo-inositol-1-phosphate synthase
	25657	1.26	20.38	16.17	6.76E-08	Ca <sup>2+</sup> -independent phospholipase A2
	223254	5.91	33.53	5.67	4.22E-09	Lipid phosphate phosphatase
	244232	1.53	7.54	4.93	0.029349	Ca <sup>2+</sup> -independent phospholipase A2
	235323	46.03	222.55	4.83	1.45E-52	Lipid phosphate phosphatase
	69758	23.18	93.42	4.03	3.5E-11	Lysophospholipase
	113249	2.25	8.69	3.86	0.020451	Phosphate acyltransferase
	24893	19.26	56.86	2.95	6.86E-09	Lipid phosphate phosphatase
	34211	45.10	129.35	2.87	1.67E-08	Acyl-CoA synthetase
	25656	25.32	66.37	2.62	7.91E-09	Ca <sup>2+</sup> -independent phospholipase A2
	63963	14.25	33.85	2.38	0.000603	Predicted lipase
	241659	12.18	27.88	2.29	0.001902	Lysophosphatidic acid acyltransferase
	48469	83.70	185.09	2.21	1.03E-17	Acyl-CoA synthetase
	12116	30.59	66.71	2.18	1.27E-06	Phosphatidylinositol transfer protein
	65651	44.02	20.76	0.47	1.4E-05	Putative phosphoinositide phosphatase
	55914	42.26	19.11	0.45	8.4E-06	Predicted phospholipase
	16280	30.48	13.67	0.45	0.000304	3-oxoacyl CoA thiolase
	79164	64.39	28.44	0.44	8.25E-09	Peroxisomal long-chain acyl-CoA transporter
	218567	77.64	33.27	0.43	4.74E-11	Very-long-chain acyl-CoA dehydrogenase
	245357	31.93	13.53	0.42	0.000157	Enoyl-CoA hydratase
	227101	10.18	4.26	0.42	0.035275	Long chain fatty acid acyl-CoA ligase
	245109	11.19	4.30	0.38	0.015401	Enoyl-CoA hydratase
	131995	244.62	94.03	0.38	8.86E-10	3-oxoacyl CoA thiolase
	25831	75.79	27.28	0.36	3.33E-06	Enoyl-CoA isomerase
	244385	17.74	6.20	0.35	0.000882	Mitochondrial/plastidial beta-ketoacyl-ACP reductase
	240581	135.39	46.56	0.34	2.66E-14	Peroxisomal multifunctional beta-oxidation protein

	243150	183.98	61.97	0.34	2.82E-08	Lipid phosphate phosphatase
	222821	49.06	16.50	0.34	0.001611	Triglyceride lipase-cholesterol esterase
	14918	52.55	16.97	0.32	2.16E-11	Acyl-CoA:diacylglycerol acyltransferase (DGAT)
	234265	85.95	27.57	0.32	0	Peroxisomal long-chain acyl-CoA transporter
	72780	62.38	15.32	0.25	5.32E-05	Ca <sup>2+</sup> -independent phospholipase A2
	243148	69.94	12.78	0.18	0	Lipid phosphate phosphatase
	191699	109.84	15.95	0.15	0	Predicted lipase
	244713	106.19	11.89	0.11	0	Acyl-CoA:diacylglycerol acyltransferase (DGAT)
	47248	465.71	29.63	0.06	0.000681	Phosphatidylserine decarboxylase
	18228	2.62	18.56	7.06	6.93E-06	Sphingosine N-acyltransferase.
	79587	12.31	52.25	4.24	2.43E-08	Sphingosine N-acyltransferase.
	18227	19.67	40.03	2.04	0.000892	Sphingosine N-acyltransferase.
	33445	40.84	4.17	0.10	0	Glucosylceramidase.
	27319	0.16	6.70	41.88	0.00235578	17 beta-hydroxysteroid dehydrogenase type 3. HSD17B3
	112707	0.61	22.49	36.87	1.51E-07	17 beta-hydroxysteroid dehydrogenase type 3. HSD17B3
	15520	5.31	66.76	12.57	2.16E-23	C-4 sterol methyl oxidase
	15617	7.84	84.02	10.72	3.16E-28	C-4 sterol methyl oxidase
	227917	3.01	23.74	7.89	1.24E-07	Hydroxymethylglutaryl-CoA reductase (NADPH).
	13385	18.94	104.95	5.54	4.54E-27	Sterol C5 desaturase
	97990	7.50	22.58	3.01	0.00048427	C-8.7 sterol isomerase
	37203	11.20	29.36	2.62	0.00206051	Hydroxymethylglutaryl-CoA reductase (NADPH).
	242907	22.80	57.27	2.51	3.03E-07	3-oxo-5-alpha-steroid 4-dehydrogenase.
	228549	13.32	29.56	2.22	0.0018794	3-keto sterol reductase
	245385	40.68	83.47	2.05	0.00927699	C-8.7 sterol isomerase
	20568	117.07	52.51	0.45	1.23E-07	START domain-containing proteins involved in steroidogenesis/phosphatidylcholine transfer
	76927	26.93	5.47	0.20	1.61E-08	Steroid reductase
	113659	122.22	22.29	0.18	0	Steroid reductase
	70959	1.95	52.49	26.92	1.74E-11	Terpenoid synthase
	22905	4.72	105.71	22.40	7.32E-20	Terpenoid synthase
	23789	3.16	29.45	9.32	9.57E-10	Terpenoid synthase
	145950	14.53	128.65	8.85	1.01E-40	Terpenoid synthase
	240449	8.90	27.97	3.14	4.75E-05	Cis-prenyltransferase
	214286	18.16	44.90	2.47	0.005619	Terpenoid synthase
	119731	15.07	31.99	2.12	0.010037	Phytoene dehydrogenase-related protein
	19151	66.18	29.56	0.45	7.26E-09	Prenyltransferase/squalene oxidase
	27794	11.86	4.01	0.34	0.015927	Peroxisomal phytanoyl-CoA hydroxylase

**Sphingolipid metabolism**

**Steroid metabolism**

**Terpenoid metabolism**



<b>S-adenosyl-L-methionine metabolism</b>	27796	137.39	45.42	0.33	0	Peroxisomal phytanoyl-CoA hydroxylase
	214327	41.06	7.31	0.18	6.86E-15	Terpenoid synthase
	228858	2.92	44.79	15.34	0.001399	SAM-dependent methyltransferases
	244998	32.75	166.80	5.09	7.45E-41	SAM-dependent methyltransferases
	72837	215.01	922.27	4.28	2.94E-39	S-adenosylmethionine synthetase
	21107	2.84	8.71	3.06	0.045065	SAM-dependent methyltransferases
<b>Chitin metabolism</b>	174258	1.32	27.07	20.51	1.76E-05	Chitin deacetylase
	26855	4.78	63.15	13.21	5.78E-12	Chitin deacetylase;
	107589	29.31	109.37	3.73	2.86E-18	Chitin deacetylase
	31299	11.46	23.48	2.05	0.0142	Chitin synthase
	33089	14.53	6.95	0.48	0.0257	Chitin deacetylase
<b>Histidine biosynthesis</b>	108905	17.67	144.87	8.201	5.7E-42	ATP phosphoribosyltransferase
	73648	15.20	70.89	4.663	1.77E-09	Histidinol dehydrogenase
	141375	12.16	39.12	3.217	5.88E-07	Phosphoribosylformimino-5-aminoimidazole carboxamide ribonucleotide (ProFAR) isomerase

\* P-value = 0 indicates values <1E-70

**Table S3 Expression of *S. vomeracea* contigs in symbiotic (SYM) and asymbiotic (ASYMB) protocorms.** Contigs obtained in the *de novo* assembly were annotated by BlastX against the *A. thaliana* proteome.

Metabolism	Trinity Contig Name	Mean read count		SYMB/ASYMB comparison		<i>A. thaliana</i> Gene Id	Putative function in <i>A. thaliana</i>	score	e-value	percent identity
		SYMB	ASYMB	Fold Change	P-value					
<b>Chitin metabolism</b>	TRINITY_DN77284_c0_g1_i3	226.85	0.00	SYM*	1.58E-07	AT5G24090.1	Chitinase A	203	2.00E-20	69.2
	TRINITY_DN5745_c0_g1_i1	26.04	0.00	SYM*	0.015799	AT1G02360.1	Chitinase family protein	263	1.00E-28	63.4
	TRINITY_DN66370_c0_g1_i1	17.42	0.18	95.70	1.62E-06	AT5G24090.1	Chitinase A	786	5.00E-103	58.2
	TRINITY_DN62020_c0_g1_i1	45.79	1.40	32.60	1.21E-10	AT1G02360.1	Chitinase family protein	715	2.00E-92	64.1
<b>S-adenosyl-L-methionine metabolism</b>	TRINITY_DN95258_c0_g1_i1	10.59	0.03	343.40	0.005566	AT5G66430.1	SAM-dependent methyltransferases	369	3.00E-42	46.9
	TRINITY_DN44325_c0_g1_i1	21.46	0.62	34.79	4.62E-12	AT5G04370.2	SAM-dependent methyltransferases	103	2.00E-06	48.8
	TRINITY_DN75761_c2_g1_i1	19.99	0.98	20.45	1.42E-10	AT2G14060.1	SAM -dependent methyltransferases	287	8.00E-30	46.2
	TRINITY_DN67911_c0_g1_i2	93.74	5.30	17.68	3.2E-22	AT4G34050.1	SAM -dependent methyltransferases	639	2.00E-80	57
	TRINITY_DN75761_c1_g1_i1	5.52	0.43	12.77	0.014476	AT5G38020.1	SAM -dependent methyltransferases	292	6.00E-30	45.5
	TRINITY_DN75761_c0_g1_i3	16.36	1.43	11.44	5.58E-07	AT3G11480.1	SAM -dependent methyltransferases	273	4.00E-29	46.8
	TRINITY_DN75761_c2_g7_i1	11.85	1.09	10.82	2.8E-05	AT5G04370.2	SAM -dependent methyltransferases	120	4.00E-08	50.9
	TRINITY_DN75761_c1_g1_i3	16.55	2.62	6.31	0.001078	AT5G38020.1	SAM -dependent methyltransferases	298	1.00E-30	46
	TRINITY_DN67911_c0_g1_i1	86.46	17.49	4.94	2.52E-15	AT4G34050.1	SAM -dependent methyltransferases	635	1.00E-80	57
	TRINITY_DN75761_c2_g6_i1	8.50	1.87	4.55	0.041527	AT5G04370.1	SAM -dependent methyltransferases	123	2.00E-08	43.6

	TRINITY_DN69539_c1_g2_i2	29.21	8.28	3.53	0.001477	AT5G19530.1	SAM -dependent methyltransferases	1226	5.00E-167	68
	TRINITY_DN76586_c0_g1_i2	12.57	3.88	3.24	0.024737	AT2G43940.1	SAM -dependent methyltransferases	727	2.00E-93	61.8
	TRINITY_DN77952_c0_g2_i1	28.17	9.65	2.92	7.06E-05	AT4G00750.1	SAM-dependent methyltransferases	2064	0	63
	TRINITY_DN73756_c1_g13_i1	25.50	9.85	2.59	0.001217	AT4G10440.1	SAM -dependent methyltransferases	2293	0	68.6
	TRINITY_DN74865_c4_g1_i1	17.96	7.01	2.56	0.01841	AT5G64030.1	SAM -dependent methyltransferases	2213	0	66.2
	TRINITY_DN75661_c0_g2_i3	23.33	9.96	2.34	0.041245	AT4G26220.1	SAM -dependent methyltransferases	177	9.00E-17	67.4
	TRINITY_DN75699_c0_g12_i1	4.43	16.55	0.27	0.000675	AT2G32170.1	SAM -dependent methyltransferases	159	1.00E-13	61.9
	TRINITY_DN77162_c3_g1_i3	4.74	30.85	0.15	2.12E-12	AT4G00750.1	SAM -dependent methyltransferases	1078	3.00E-140	56.5
	TRINITY_DN76265_c0_g7_i1	0.74	6.63	0.11	0.007444	AT1G23360.1	SAM -dependent methyltransferases	101	3.00E-06	94.7

*Note:* SYM\*, uniquely expressed in symbiotic conditions.