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**Impact of an arbuscular mycorrhizal fungus versus a mixed microbial inoculum on the transcriptome reprogramming of grapevine roots**

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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3 **Impact of an arbuscular mycorrhizal fungus *versus* a mixed microbial inoculum on the**  
4 **transcriptome reprogramming of grapevine roots**

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27 for Figure 3 preparation and Samuele Voyron for the Bioanalyzer analysis.

28 **Abstract**

29 Grapevine, cultivated for both fruit and beverage production, represents one of the most  
30 economically important fruit crops worldwide. With the aim of better understanding how grape  
31 roots respond to beneficial microbes, a transcriptome sequencing experiment has been performed to  
32 evaluate the impact of a single arbuscular mycorrhizal (AM) fungal species (*Funneliformis*  
33 *mosseae*) versus a mixed inoculum containing a bacterial and fungal consortium, including different  
34 AM species, on Richter 110 rootstock. Results showed that the impact of a single AM fungus and of  
35 a complex microbial inoculum on the grapevine transcriptome differed. After three months, roots  
36 exclusively were colonized after the *F. mosseae* treatment, and several AM marker genes were  
37 found to be up-regulated. The mixed inoculum led only to traces of colonization by AM fungi, but  
38 elicited an important transcriptional regulation. Additionally, the expression of genes belonging to  
39 categories such as nutrient transport, transcription factors, and cell wall-related genes was  
40 significantly altered in both treatments, but the exact genes affected differed in the two conditions.  
41 These findings advance our understanding about the impact of soil beneficial microbes on the root  
42 system of a woody plant, also offering the basis for novel approaches in grapevine cultivation.

43

44 **Keywords**

45 AM symbiosis; microbial inoculum; grapevine; root transcriptome profile

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## 56 **Introduction**

57 Grapevine, cultivated since the dawn of civilization for the production of fruit, juice and wine,  
58 represents one of the most economically important fruit crops worldwide, with widespread  
59 cultivation (77,181,122.00 Mt produced in 2013; <http://faostat.fao.org>) and high commercial value  
60 (Vivier and Pretorius 2002). It has become a model organism for fruit trees, as mirrored by the two  
61 genome-sequencing projects developed on grape (Jaillon et al. 2007; Velasco et al. 2007).

62 The molecular regulation occurring during berry development has been investigated using several  
63 high-throughput technologies (Zenoni et al. 2010; Fasoli et al. 2016). Additionally, in the last years,  
64 several studies investigated different aspects related to water transport and water deficit impact  
65 (Perrone et al. 2012; Chitarra et al. 2014; Tombesi et al. 2015; Corso et al. 2015) as well as to the  
66 interactions with pathogens (Milli et al. 2011; Dal Santo et al. 2013; Vitali et al. 2013; Pantaleo et  
67 al. 2016).

68 Only a few papers so far have been published on transcriptomics in *Vitis spp.* roots. Du et al. (2014)  
69 recently studied the root transcriptome, using the Affymetrix *V. vinifera* genome array, to verify the  
70 impact of phylloxera attack in a resistant rootstock (140Ru) and in the susceptible cultivar “Crimson  
71 Seedless”. Because of the ongoing climate change in wine-growing regions, the selection of  
72 rootstocks tolerant to several biotic and abiotic stresses is considered a crucial factor for developing  
73 sustainable agriculture. As a consequence, next generation viticulture is aimed to select appropriate  
74 rootstocks bred from several *Vitis* species (Corso and Bonghi 2014; Flexas et al. 2009).

75 Grapevine is highly responsive to local environmental conditions and vineyard management  
76 practices. In this context, Anesi and colleagues (2015) have suggested a genome plasticity in  
77 relation to environment, overall known as terroir, that characterizes a specific vineyard and impacts  
78 grape and wine quality. Soil qualities, rootstocks, location, climatic factors, and soil management  
79 have been reported to influence grape development and fruit and wine quality (Koundouras et al.  
80 2006; de Andrés-de-Prado et al. 2007; Marè et al. 2013). The transcriptome variation in relation to  
81 different soils and rootstocks recently has been studied in leaves of the scion *cv.* Pinot noir through  
82 a microarray approach, suggesting a link among soil composition, rootstock and gene expression  
83 (Marè et al. 2013). Today, viticulturists aim to produce high quality wine, increasing profit from the  
84 land and reducing agronomic inputs, through encouraging natural soil beneficial organisms  
85 (Trouvelot et al. 2015).

86 In this changing context, many Italian wines are now labelled as "organic wines" and are produced  
87 by introducing commercially available microbial inoculants to the soil. These inoculants include  
88 bacteria belonging to the genera *Bacillus*, *Pseudomonas*, *Streptomyces* and biocontrol fungi such as  
89 *Trichoderma spp.* and/or arbuscular mycorrhizal (AM) fungi (Pinto and Gomes 2016). The latter

90 microbes are among the most relevant soil organisms that colonize the roots of most land plants,  
91 where they facilitate mineral nutrient uptake from the soil in exchange for plant-assimilated carbon  
92 (Bonfante and Genre 2010). It is already known that vineyard soils support indigenous AM fungi  
93 (AMF) and it is well established that grapevine roots are colonized by native AMF (Balestrini et al.  
94 2010; Trouvelot et al. 2015). The use of molecular approaches, including metagenomics and/or  
95 Next Generation Sequencing (NGS) techniques, has offered new information about the AMF  
96 assemblages that live in symbiosis with this important, typical Mediterranean fruit crop (Schreiner  
97 and Mihara 2009; Balestrini et al. 2010; Lumini et al., 2010; Holland et al. 2014). By contrast, the  
98 molecular basis underlying the interactions between grapevine and AM fungi still has to be  
99 elucidated. While RNAseq techniques have been used to study transcriptome profiles in AM-  
100 colonized roots from herbaceous plants such as tomato, rice and *Lotus japonicus* (Ruzicka et al.  
101 2012; Fiorilli et al. 2015; Handa et al. 2015), information is scarce on woody crops. Recently,  
102 transcriptome data have been obtained from litchi (*Litchi chinensis* Sonn.) roots, identifying  
103 transcripts involved in the interaction with AM fungi under carbohydrate starvation (Shu et al.  
104 2016).

105 With the aim to better understand how grape roots respond to beneficial microbes, a transcriptome  
106 experiment has been performed to evaluate the impact of a single AM fungal species (*Funneliformis*  
107 *mosseae*) versus a mixed inoculum containing a bacterial and fungal consortium on Richter 110  
108 rootstock.

109

## 110 **Materials and Methods**

### 111 **Plant material and growth conditions**

112 Pinot noir plants grafted on 110 Richter rootstock have been prepared at Roero Viti Vivai  
113 (<http://www.roerovitivivai.it>). In detail, after that rootstock developed roots, grafted plants were  
114 grown in pots with a natural soil collected in vineyards of Val d'Aosta, Italy (characterized by 81%  
115 sand, 16% loam and 3% clay; pH 7.70; organic matter content 3.73 g/100g; cation exchange  
116 capacity 15.47 meq/100g), previously sterilised. The same sterilised soil was supplemented with an  
117 inoculum of *Funneliformis mosseae* (formerly *Glomus mosseae*) Gerd. & Trappe (BEG 12)  
118 purchased from MycAgro Lab for single species inoculation (FMOS; 30% inoculum/soil for each  
119 plant), or with the mixed inoculum MICOSAT F® Vite (CCS-Aosta) containing a microbial  
120 consortium including AM fungi (MICO; 30 g for each plant). In detail, FMOS inoculum consisted  
121 of spores, extraradical mycelium, sorghum mycorrhizal roots and sorghum growth substrate and  
122 each plant has been inoculated with about 1000 propagules. MICO inoculum, as stated in the

123 website (<http://www.micosat.it/portfolio/fertilizzante-micosat-f-vite/>), contains: *Trichoderma viride*,  
124 *T. harzianum*, *Pochonia chlamidosporia*, *Streptomyces* spp. ST60, *Streptomyces* spp. SB14,  
125 *Streptomyces* spp. SA51, *Bacillus subtilis* BA41, *Pseudomonas fluorescens* PN53, *Pseudomonas*  
126 spp. PT65, *Glomus* spp. GB67, *Glomus mosseae* GP11, *Glomus viscosum* GC41 in the percentage  
127 of 40% crude inoculum (AM fungi) and 21.6% bacteria and saprotrophic fungi. In parallel, control  
128 plants (CTRL) have been prepared using only the sterilized soil. Plants were grown in greenhouse  
129 conditions from July to October 2013 under natural light and temperature, with drip irrigation for  
130 one hour every 15 days with slight intensification in days of high heat peaks. At least 33 plants for  
131 each of the three considered conditions (CTRL, FMOS, MICO) were used. At the end of the  
132 experiment, thirty randomly chosen 1-cm-long root segments per plant were treated in 10% KOH  
133 for four hours at room temperature, stained with 0.1% cotton blue in lactic acid and then fungal  
134 colonization was quantified according to the Trouvelot system (Trouvelot et al. 1986) using the  
135 MYCOCALC software. Root segments, obtained from at least fifteen plants, were analysed. The  
136 remaining roots were stored at -80°C until molecular analysis.

137 To evaluate fresh and dry root weight under the different conditions, five entire root systems for  
138 each treatment have been weighted (fresh weight, FW), dried at 60°C for three days and weighted  
139 again (dry weight, DW).

140

#### 141 **RNA extraction and Illumina sequencing**

142 For the RNASeq experiment, roots were harvested from the CTRL, FMOS and MICO plants after  
143 three months from the inoculation. Total root systems were chilled in liquid N<sub>2</sub> and RNA was  
144 extracted using the 'pine tree-method' (Chang et al. 1993) with the addition of 2% PVPP to the  
145 extraction buffer. For each growth condition, we used two biological replicates, each containing the  
146 pooled RNA from roots sampled from three plants. RNA quality and quantity controls have been  
147 performed using the Agilent 2100 Bioanalyzer. Ten micrograms of each RNA sample (RIN >8)  
148 were sent to HUGEF (Torino, Italy) where the libraries were produced and sequenced using an  
149 Illumina Genome Analyzer (Solexa). The six libraries were indexed, and single-end multiplexed  
150 sequencing was performed using 100 bp length reads. The reads obtained from Illumina HiSeq were  
151 processed using CASAVA pipeline version 1.8.2. (Illumina Inc, San Diego, CA, USA) and further  
152 checked for sequence quality with the fastQC application (ver. 0.10.1).

153

## 154 **Bioinformatic methods**

### 155 *Expression profiling and differential expression analysis*

156 To determine gene expression levels, reads were mapped against *Vitis vinifera* 12x genome using  
157 TopHat version 2.0.12 with default parameters, and alignments were processed with Cufflinks  
158 version 2.2.0 (Trapnell et al. 2013). Cuffdiff was used to detect differentially expressed genes, and a  
159 false discovery rate (FDR) of 0.05 (Benjamini and Hochberg 1995) was set as a significance  
160 threshold. Sample clustering based on Jensen–Shannon distances between conditions and replicates  
161 was performed using the R package CummeRbund (Goff et al. 2013).

### 162 *Functional annotation and GO-enrichment analysis*

163 *Vitis vinifera* gene annotations were retrieved from the VitisNet portal  
164 (<https://www.sdstate.edu/ps/research/vitis/pathways.cfm>; Grimplet et al. 2012). To identify protein  
165 domains and CAZyme domains, *V. vinifera* predicted proteins were annotated with PFAM release  
166 27 and dbCAN v.3 databases using Hmmer version 3.1b1 with default parameters. GO-terms and  
167 KEGG pathways annotation were performed with Blast2GO version 2.8 using default parameters  
168 (Conesa et al. 2005). To detect significantly enriched GO-terms in differentially expressed genes  
169 (DEGs), two-tailed Fisher Exact Tests were performed and an FDR of 0.05 was set as a significance  
170 threshold.

### 171 **Quantitative RT-qPCR validation**

172 All the RNA samples were treated with the Turbo DNA-free™ kit (Ambion, Austin, TX, USA) for  
173 RT-qPCR analyses according to the manufacturer's instructions. The RNA samples were submitted  
174 to a control reverse-transcription PCR to check for the absence of DNA contamination using the  
175 One Step RT-PCR kit (Qiagen) and primers specific for the grapevine elongation factor 1- $\alpha$  gene  
176 (*VvEF1- $\alpha$* , Reid et al. 2006; Table S1). First strand cDNA was synthesized from 500 ng of total  
177 RNA with the Superscript II reverse transcriptase kit (Invitrogen) following the manufacturer's  
178 instructions. At the end of the reaction, the cDNA was diluted to 1:3 for the gene expression  
179 analysis. Gene specific primers (Table S1) were designed using Primer 3  
180 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>). Quantitative RT-PCR (RT-qPCR) reactions were  
181 carried out in a 48-well StepOne™ Real time PCR system instrument (Applied Biosystems), in a  
182 final volume of 20  $\mu$ l, containing 10  $\mu$ l of 23 iQ SYBR Green Supermix, 4  $\mu$ l of primers 3  $\mu$ M, 5  $\mu$ l  
183 of water and 1  $\mu$ l of cDNA template. The PCR program consisted of a holding stage (95°C for 10  
184 min) and 40 cycles of 95°C for 15 sec. and 60°C for 1 min. A melting curve (55-95°C with a  
185 heating rate of 0.5°C per 10 sec. and a continuous fluorescence measurement) was recorded at the



186 end of each run to assess amplification product specificity. All the reactions were performed with  
187 three technical replicates and three biological replicates. A portion of the grapevine *VvEF1- $\alpha$*  gene  
188 was used as the housekeeping gene for normalization (Chitarra et al. 2014), by subtracting the CT  
189 value of *VvEF1- $\alpha$*  from the CT value of the candidate gene resulting from the  $\Delta$ CT. The expression  
190 ratios were calculated without the PCR efficiency correction from equation  $2^{\Delta\Delta CT}$ ; where  $\Delta\Delta$ CT  
191 represents the  $\Delta C_T$  sample –  $\Delta C_T$  control. Before calculating the  $\Delta C_T$ , the technical replicates were  
192 checked for their  $C_T$  value uniformity and for outliers, which led to the exclusion of any standard  
193 deviations above 0.2. The primer names and corresponding sequences are listed in Table S1.

194

### 195 **Phosphorous and potassium determination in roots**

196 To determine P and K, about 2 mg of root system (4 plants for each condition) were dried for two  
197 days at 60°C, ground and digested at 95°C for 1 hour in 1 ml HNO<sub>3</sub> 6M, filtered using a glass filter  
198 and diluted with distilled water 1:6. The cations were determined with a Perkin Elmer Optima 7000  
199 (Perkin Elmer, Norwalk, Connecticut, USA) inductively coupled plasma-optical emission  
200 spectrometer (ICP-OES). Standard solutions were prepared from concentrated stock solutions  
201 (Merck Titrisol or Sigma Aldrich). High purity water (HPW) produced with a Millipore Milli-Q  
202 system was used throughout. The reagents used were of analytical grade.

203

### 204 **Statistical analyses**

205 All the data were subjected to statistical analysis using SYSTAT 10 software, applying the non-  
206 parametric Kruskal-Wallis test adopting a probability level of  $P < 0.05$ .

207

## 208 **Results**

### 209 **Plant development and root features**

210 After 3 months of growth under greenhouse conditions, all the plants presented a similar vegetative  
211 development (not shown). Fresh and dry root weight were evaluated (Figure S1): no statistically  
212 significant differences in root biomass were found among the three treatments (FMOS, MICO,  
213 CTRL) nor in root P and K concentration, where slightly higher values were recorded in FMOS  
214 plants (Figure S1).

215 The roots of the plants inoculated with the mixed inoculum (MICO) did not present a significant  
216 AMF colonization, with only three plants presenting traces of AM fungal colonization (Table S2): a

217 morphological quantification revealed a mean number of colonized fragments (F) of 2.6% and a  
218 mean intensity of mycorrhization (M) of 0.88 % (Figure 1). AM fungal colonization was observed  
219 in the FMOS roots, with a mean number of colonized fragments of 80.66 % (F) and a mean  
220 intensity of mycorrhization (M) of 48.93% (Figure 1). Arbuscules (a%) and vesicles were highly  
221 variable; their percentage ranged from 2.66 to 41.13 and from 4.86 to 21.45, respectively. No  
222 colonization structures were detected in the CTRL roots (not shown).

223

#### 224 **Analyses of RNA-Seq data: read number, transcriptome coverage and total expressed genes**

225 Sequencing of RNA samples produced on average 20,147,903 of reads per sample (Table 1).  
226 Sequencing reads ranging from 12 to 32 million for each sample (Table 1) were mapped on the *Vitis*  
227 *vinifera* genome obtaining on average ~93.2% overall alignment rate. Based on the sum of  
228 transcript lengths, as reported in the current V1 annotation (39,893,396 bp that does not currently  
229 account for transcript isoforms), we reached an average transcriptome coverage of 24x for each  
230 replicate.

#### 231 **A look at the whole root transcriptome and at the differentially expressed genes**

232 A total of 9,593 genes were expressed in all samples when using a cut-off value of RPKM > 0 to  
233 declare a gene as expressed. Based on expression values, the samples clustered by condition (Table  
234 S3, Figure S2). In order to identify genes involved in root development we mined the keyword  
235 "root" from the gene descriptions of *Vitis vinifera* V1 annotation thus obtaining 57 candidate genes.  
236 Among them, a total of 33 genes were expressed in all our samples setting a cut-off value of RPKM  
237 > 0 to declare a gene as expressed. This data set comprises genes that seem to be specific to the  
238 root, as they are reported to be involved in different root developmental processes looking at GO  
239 descriptions and Blast2GO (ver. 3.3) results (not shown). As a second step, transcriptional changes  
240 were determined by comparing *F. mosseae*-colonized roots (FMOS) and those inoculated with the  
241 mixed inoculum (MICO) with un-inoculated control (CTRL) samples. This analysis revealed 539  
242 and 737 differentially expressed genes (DEGs) in FMOS and MICO plants, respectively (Figure  
243 2a). The mixed inoculum led to the regulation of a higher number of genes compared to the AMF  
244 inoculum, and a large proportion of them (85%, on average) were down-regulated in both  
245 treatments *versus* the control condition. Only about 30% of the FMOS DEGs were found in  
246 common with those of MICO roots, suggesting that the AM fungus and the mixed inoculum have  
247 led to different transcriptome profiles. Most of them presented a common trend in the two  
248 conditions (Table S4), but some instances were observed in which regulation in the two treatments

249 was in different directions. Some DE transcripts were specific to a single condition (Table S5). In  
250 addition, for three DEGs, FPKM have been found only in MICO roots and not in the control, i.e.  
251 genes annotated as coding for a putative arachidonic acid-induced protein DEA1  
252 (VIT\_12s0035g02000), an unknown protein (VIT\_03s0132g00060) and a no hit protein  
253 (VIT\_13s0047g00580), although with low FPKM values (1,145; 0,697; 5,710 respectively).

254 Among the 10 most up-regulated genes, seven transcripts were common between to the two  
255 conditions (Table 2). The first, with a fold change of 2.85 and 3.64 in FMOS and MICO  
256 respectively, was a gene coding for a putative uroporphyrin III methylase (VIT\_13s0064g01470). A  
257 corresponding (homolog) gene has been reported to be up-regulated in luxuriant (N+) treated  
258 *Eucalyptus* plants versus limited (N-) plants (Camargo et al. 2014), while in *Arabidopsis* roots a low  
259 expression has been found under Cd treatment (van de Mortel et al. 2008). VIT\_03s0063g00370  
260 and VIT\_18s0001g03910, which code for a putative ferredoxin nitrite and a putative nitrate  
261 reductase, respectively, have a role in nitrate/nitrite assimilation, and might be regulated by nitrate,  
262 as previously demonstrated in *Arabidopsis* (Wang et al. 2003). In the same list, we also detected a  
263 gene encoding a putative nitric oxide reductase (VIT\_06s0004g04400) as well as a gene coding for  
264 a nodulin belonging to the MtN21 family (VIT\_01s0026g00550).

265 The expression of 14 genes randomly selected from those identified in the RNA-seq experiment was  
266 successfully validated by RT-qPCR (Figure S3 and Figure 3).

267 To have an overview of the regulation of the main metabolic processes and signalling pathways  
268 involved in the different comparisons, we conducted GO enrichment analysis. Figure 4 shows the  
269 enriched GO terms specific for FMOS and for MICO, while the GO terms over-represented in both  
270 growth conditions in response to AM fungus and the mixed inoculum are represented in Figure 5.  
271 Differentially expressed transcripts were grouped in functional classes, on the basis of the specific  
272 biological process in which they were involved (Table S4; Table S5; Figure 4a, b). Several GO  
273 terms were over-represented and among them transport and transporter activity categories were  
274 over-represented in MICO roots in addition to cell wall, membrane, cell component organization  
275 (Figure 4a). Among the over-represented genes in FMOS roots, response to endogenous stimulus,  
276 response to abiotic stimulus, nucleus, RNA biosynthetic process, and cell cycle were annotated  
277 (Figure 4b). Six over-represented functional GO classes were found in common between the two  
278 different treatments: cellular components organization, cell cycle, nucleus, extracellular region,  
279 carbohydrate metabolic process, cell-wall (Figure 5). CAZymes domains analysis showed that  
280 MICO sample expressed genes contains several annotated CAZymes domains which are  
281 represented also in the whole *V. vinifera* dataset, such as glycosyltransferases (GT), glycoside

282 hydrolases domains (GH), carbohydrate-binding modules (CBM) and carbohydrate esterases (CE)  
283 (Figure 6).

284

### 285 **Grapevine transcripts: specific responses to the AM fungus *versus* the mixed inoculum**

286 To better explore the novel transcriptomic data set, and to further understand the grapevine response  
287 to AM fungi, we studied in greater detail the expression profiles of genes described in the literature  
288 as specifically involved during AM symbiosis in legumes, rice and tomato (Fiorilli et al. 2009;  
289 Guether et al. 2009; Hoge Kamp et al. 2011; Handa et al. 2015; Fiorilli et al. 2015). AM symbiosis is  
290 mostly acknowledged for the improved nutrient exchange established between the two symbionts,  
291 and regulated by the activities of fine-tuned plant and fungal transporter genes (Casieri et al. 2013;  
292 Berruti et al. 2016a). In accord with this claim, a consistent group of plant transporters were  
293 identified as differentially expressed between treated (FMOS and/or MICO) and control plants  
294 (Table S6). One of them is an inorganic phosphate transporter gene (VIT\_16s0050g02370) that  
295 shows homology with the mycorrhiza-inducible inorganic phosphate transporters such as LePT4  
296 and OsPT11 (Table 2; Figure S3), and an oligopeptide transporter 4 (VIT\_18s0001g07940). Several  
297 genes encoding for protein involved in the transport of molecules across cell membranes were also  
298 up-regulated in the same FMOS roots. Among them, genes coding for a putative potassium (K<sup>+</sup>)  
299 transporter KUP1 (VIT\_19s0027g01820), a sulphate transporter (VIT\_05s0020g03970), a lysine  
300 histidine transporter (LHT, VIT\_01s0011g03180), and an organic cation transport protein OCT1  
301 (VIT\_17s0119g00080) were exclusively up-regulated in FMOS roots. By contrast, other  
302 transporters were up-regulated in both the conditions: among them, two genes coding for putative  
303 nitrate transporters (VIT\_17s0000g09470 and VIT\_01s0127g00070) and a Zinc transporter 10  
304 precursor (VIT\_10s0042g01100) as well as three protease inhibitor/seed storage/lipid transfer  
305 protein (LTP) genes (Table 2). An additional VIT\_09s0002g05660 sulphate transporter Sulp family  
306 was up-regulated under both treatments. In agreement with previous work (Zhang et al. 2010;  
307 Hoge Kamp et al. 2011), four ABC-transporters were identified as co-induced in FMOS roots  
308 (VIT\_07s0031g02550; VIT\_13s0074g00690; VIT\_16s0098g00570; VIT\_04s0008g04790), and  
309 only the last one was also up-regulated in MICO roots. Putative ammonium transporter genes  
310 (VIT\_04s0008g05080, VIT\_00s1818g00010, VIT\_00s0179g00310, VIT\_07s0031g02950) were  
311 significantly down-regulated in MICO roots as well as a gene corresponding to a putative NIP 1;2  
312 (VIT\_10s0003g01830).

313 The presence of AM fungi leads to relevant changes in the hormonal plant profile (Gutjahr 2014),  
314 including gibberellins, which are predicted to modulate their concentrations during the symbiosis. A  
315 gene coding for a DELLA protein GAI1 (VIT\_17s0000g10300) was found as up-regulated in

316 FMOS and significantly down-regulated in MICO samples. Strigolactones not only represent a new  
317 class of plant hormones, but also may stimulate fungal branching while acting as pre-symbiotic  
318 molecules (Bonfante and Genre 2015). A gene involved in strigolactone (SL) biosynthesis, i.e. a  
319 carotenoid cleavage dioxygenase (CCD) 7 (VIT\_15s0021g02190; *VvCCD7*) was found to be  
320 upregulated in FMOS samples. Interestingly, *VvCCD7* transcripts were not detected in MICO roots,  
321 consistent with the almost total absence of AM fungal colonization.

322 Homologs of nodulin genes (Denancè et al. 2014) were found to be differentially regulated in our  
323 datasets. In FMOS, genes encoding two MtN3 nodulins (VIT\_16s0050g02540 and  
324 VIT\_17s0000g00820) were up- and down-regulated respectively, while two MtN21 genes were up-  
325 regulated (VIT\_03s0017g02310 and VIT\_01s0026g00550). One of these last  
326 (VIT\_01s0026g00550) was up-regulated also in MICO roots together with another MtN21 gene  
327 (VIT\_04s0023g02730), while two others were down-regulated (VIT\_13s0084g00090 and  
328 VIT\_08s0040g02500).

329 Among genes potentially involved in arbuscule formation and fungal accomodation, two genes  
330 encoding CESA cellulose synthase (VIT\_10s0003g01560 and VIT\_07s0005g04110), a gene coding  
331 for a cellulose synthase IRX3 (VIT\_11s0037g00530), a chitinase class III (VIT\_16s0050g02210),  
332 and a subtilisin serine endopeptidase gene (VIT\_15s0048g01200) were exclusively up-regulated in  
333 FMOS roots. A gene encoding a laccase (VIT\_08s0007g01910) was also up-regulated only in  
334 FMOS, in addition to a metallothionein (VIT\_08s0007g00330).

335 Both FMOS and MICO conditions elicited the expression of several TF genes belonging to  
336 different groups, while other members inside these families were down-regulated (Table S3; Table  
337 S4). In detail, genes belonging to GRAS, DOF, Zinc-finger, MYB and DREB transcription factor  
338 groups were found to be up-regulated in FMOS roots. Members inside these groups were also up-  
339 regulated in MICO samples. Additionally, it is worthwhile to note the regulation in AM-colonized  
340 roots of several circadian-regulated genes as well as genes potentially involved in the response to  
341 environmental stimuli (Table S3). Among them, the grapevine homolog to arachidonic acid-induced  
342 protein DEA1 (VIT\_12s0035g02000) and a gene coding for a protein belonging to the RD22-like  
343 subfamily (VIT\_04s0008g04150). By contrast, genes potentially involved in response to pathogens,  
344 such as stilbene synthase genes, were down-regulated in FMOS roots as well as four genes coding  
345 for Avr9/Cf-9 rapidly elicited protein 20. In contrast, no stilbene synthase genes were regulated in  
346 MICO roots.

347 Among differentially expressed genes in MICO roots, we again found a consistent core of genes  
348 involved in nutrient transport, but interestingly several of them had a different ID than those for  
349 FMOS, revealing a specific response to the inoculum (Table S3; Table S4). Among them, two

350 high affinity nitrate transporter (VIT\_06s0061g00310 and VIT\_06s0061g00320), two additional  
351 nitrate transporters (VIT\_11s0016g05170 and VIT\_18s0001g11280), a sulphate transporter 1.3  
352 (VIT\_18s0001g04910), a ZIP family transporter (VIT\_19s0085g00740), an arsenite transport  
353 protein (VIT\_02s0025g03310), a Mg-chelatase subunit XANTHA-F (VIT\_08s0007g08540) were  
354 up-regulated. Differently from the expression profile described in FMOS (Table S3; Table S4),  
355 genes coding for putative ammonium transporters, and a gene coding for a cationic amino acid  
356 transporter 2 (VIT\_10s0003g04540) were down-regulated. Among genes involved in other  
357 pathways that potentially could be affected by the presence of bacteria (i.e. hormonal balance and  
358 defense; Vacheron et al. 2013; Drogue et al. 2014), an auxin response factor 3  
359 (VIT\_10s0003g04100) is specifically up-regulated in addition to a gene coding for the ABA 8'-  
360 hydroxylase CYP707A1 (VIT\_02s0087g00710), which is a key catabolic enzyme and could be  
361 involved in the regulation of ABA level (Okamoto et al. 2006). Ethylene responsive factors genes  
362 (ERF), all were down-regulated in the MICO-treated roots, confirming previous results obtained in  
363 different beneficial plant-bacterium interactions (Verhagen et al. 2004; Drogue et al. 2014). Two  
364 genes encoding pore-forming toxins (VIT\_07s0005g06090 and VIT\_07s0005g06110) were  
365 specifically up-regulated in the MICO treatment.

366

## 367 **Discussion**

368 In this work we have developed new transcriptomic data sets that illustrate the main pathways  
369 activated in grapevine roots as well as those elicited by beneficial microbes. Although grapevine is  
370 a woody plant with economic relevance for berry production, limited attention has been given so far  
371 to its root system and how its transcriptome responds to AM fungi and Plant-Growth Promoting  
372 Bacteria (PGPBs). In addition, grapevine is characterized by a secondary growth pattern, but, with a  
373 few exceptions (Shu et al. 2016), all the transcriptional profiles following microbial colonization so  
374 far available refer to herbaceous crop plants.

375 In our experimental set up we investigated the impact of both a single AM fungus (*Funneliformis*  
376 *mosseae*), which is considered a symbiotic fungus for many crops, and a microbial consortium,  
377 which is commercialized as suitable for grapevine.

378 The two data sets derived from plants inoculated with microbes reveal some interesting similarities:  
379 they both present a limited number of differential expressed genes and a higher number of down-  
380 regulated genes in respect to previous papers on the transcriptome profiles in AM- and/or PGPB-  
381 colonized roots. Many previous experiments demonstrated that up to 60-70% of genes were up  
382 regulated during AM symbiosis (Guether et al. 2009; Fiorilli 2009; Handa et al. 2015; Hoge-kamp et

383 al. 2011; Fiorilli et al. 2015). Nevertheless, examples of transcriptomes with prevalent down-  
384 regulated genes already have been reported. Drogue et al. (2014) have analyzed four different plant-  
385 microbe combinations using two *Azospirillum* strains and two rice cultivars, and only in Nip\_B510  
386 combination the regulated genes were mainly down-regulated (203 up-regulated vs 2336 down-  
387 regulated). Interestingly, in a recent study focused on the transcriptome of mycorrhizal litchi roots,  
388 Shu, et al. (2016) found a number of down- (156) and up- (286) regulated genes, with a ratio  
389 between up- and down-regulated genes lower than other previous works. We cannot exclude that  
390 woody plants differently modulate their root transcriptome in the presence of beneficial microbes,  
391 or they may require a different and/or longer timing than herbaceous plants.

392

### 393 **A single microbe versus a consortium**

394 Overall, the analysis of the generated data sets revealed that the impact on the gene expression of a  
395 single AM fungal species and of a complex microbial inoculum on the grapevine transcriptome was  
396 diverse. The differences in transcriptome profiles mirrored morphological observations (Figure 1)  
397 showing a good AM colonization in FMOS and only traces of AM fungal hyphae in MICO roots.  
398 Despite the complex microbial inoculum label indicated the presence of AM fungal propagules, the  
399 AM colonization was found in low amount. This is in agreement with previous data obtained using  
400 similar microbial formulations produced by the same company: a very low AM fungal colonization  
401 intensity was detected in *Camellia japonica* rooted cuttings (Berruti et al. 2013), and the AM fungal  
402 taxa inoculated failed to colonize maize roots and lacked soil persistence (Berruti et al. 2016b).  
403 However, the mixed inoculum provided us the opportunity to test a complex condition, because – as  
404 in the soil – grapevine was in contact with multiple microbes. Looking at the genes significantly  
405 regulated in FMOS and MICO roots, genes belonging to the same categories (e.g. nutrient transport,  
406 TF, cell wall metabolism) have been found to be up-regulated in both conditions, but several of  
407 them had different IDs, suggesting a specific response to the specific inoculum.

408 The AM fungus activates many of the AM-symbiosis markers that are at the moment considered the  
409 functional signatures of the symbiosis (Guether et al. 2009a; Gomez et al. 2009; Hogekamp et al.  
410 2011). Among them major attention can be directed to a gene coding for a protein that shows a high  
411 identity with LePT4, a phosphate transporter that probably also acts as a sensor of phosphate  
412 availability in the soil and inside the root environment (Volpe et al. 2015). However, several up-  
413 regulated nutrient transporter genes (12) were identified, in agreement with that reported in *Lotus*  
414 *japonicus* where 43 nutrient transporters were identified as up-regulated in the transcriptome of  
415 mycorrhizal roots (Guether et al. 2009a). In our work, several of the common up-regulated genes

416 between the two treatments (FMOS and MICO) are involved in nitrogen metabolism, mainly in  
417 relation to nitrate, suggesting that in grapevine these beneficial root-associated microbes are  
418 particularly efficient in stimulating plant responses to nitrogen, which is an essential element for all  
419 grapevine processes ([http://www.awri.com.au/wp-](http://www.awri.com.au/wp-content/uploads/1_nutrition_nitrogen_fertilisation.pdf)  
420 [content/uploads/1\\_nutrition\\_nitrogen\\_fertilisation.pdf](http://www.awri.com.au/wp-content/uploads/1_nutrition_nitrogen_fertilisation.pdf)). In contrast, transcripts corresponding to  
421 other genes which are considered functional marker genes of the AM symbiosis and expected to be  
422 up-regulated in the presence of the AM fungus, such as for example ammonium transporter and NIP  
423 aquaporin genes (Guether et al. 2009b; Giovannetti et al. 2012), were found in AM-colonized  
424 grapevine roots although not significantly up- or down-regulated in the comparison with CTRL  
425 plants.

426 Nodulin genes, first described as legume genes involved in root nodule symbiosis development and  
427 also reported as up-regulated in AM symbiosis, showed a differential regulation between the two  
428 datasets. Twelve MtN3/saliva/SWEET genes (Sugars Will Eventually be Exported Transporters)  
429 have been reported in *Vitis vinifera*, while 23 MtN21/EamA-like/UMAMIT genes (Usually  
430 Multiple Acids Move In and out Transporters) have been found (Denancè et al. 2014). Recently,  
431 plant SWEETs have been shown to be involved in the feeding of pathogenic microbes, and an  
432 important role for SWEET transporters during the mycorrhizal symbiosis has been suggested,  
433 although further analyses are needed to clarify their role during symbiosis (Manck-Götzenberger  
434 and Requena 2016).

435

436 As expected, a core of differentially regulated genes involved cell wall-related genes. The induction  
437 in mycorrhizal roots of a large number of genes related to membrane dynamics and cell wall  
438 metabolism is well documented, consistent with the concept that plant cells have an active role in  
439 fungus accommodation *via* membrane proliferation and cell wall construction (Balestrini and  
440 Bonfante, 2014). Here, three genes encoding three putative cellulose synthase, two CESA and one  
441 IXR3 respectively, have been found to be up-regulated only in the presence of the AM fungus.  
442 While CesA proteins are part of the cellulose synthase complex in higher plants (Taylor 2008), and  
443 in *L. japonicus*, transcripts of a putative cellulose synthase, *LjCesA*, have been demonstrated to  
444 accumulate in arbusculated cells, IXR3 (AtCesA7) has been reported as required for secondary wall  
445 cellulose synthesis in *Arabidopsis* (Richmond and Sommerville 2000). Genes putatively involved in  
446 lignin biosynthesis (Barros et al. 2015), such as a putative cinnamoyl-CoA reductase and a  
447 cinnamyl alcohol dehydrogenase gene, also were found to be up-regulated in the presence of  
448 the AM fungus. Because these genes already have been described during plant-PGPR (Plant Growth



449 Promoting Rhizobacteria) interactions (Vacheron et al. 2013), but never as AM-inducible, we  
450 suggest that they may take part in a response that is specific for woody hosts.

451 Another novel set of genes which has never been reported as AM-inducible concerns circadian-  
452 related genes (Carbonell-Bejerano et al. 2014). They have been deeply investigated in model plants  
453 like *Arabidopsis*, but also have been reported as crucial in ecologically relevant symbioses such as  
454 corals living with their photosynthetic algae (Sorek et al. 2014). Circadian clock-related genes have  
455 been identified in the grapevine genome and oscillation in their expression has been correlated with  
456 the daily oscillatory changes in the berry transcriptome at late ripening stages (Carbonell-Bejerano  
457 et al. 2014).

458 It already was reported that PGPBs can affect plant physiology and growth, including root system  
459 architecture, and that these modifications are accompanied by changes in plant transcriptome  
460 profiles (Vacheron et al. 2013). An impact on the root transcriptome has been reported in PGPB-  
461 treated roots with several bacterial models, and the differences in the regulated transcripts were  
462 mainly related to the used species/strain (Verhagen et al. 2004; Weston et al. 2012; Vacheron et al.  
463 2013; Drogue et al. 2014; Spaepen et al. 2014). Interestingly, an *Azospirillum* strain (B510) that can  
464 colonize the outer layer of rice root tissue leads to a repression of a wider set of genes involved in  
465 stress response and defence than a strain that was shown to colonize only the rice-root surface  
466 (Drogue et al. 2014). Indeed, some plant-associated bacteria are known as ISR (Induced Systemic  
467 Resistance)- bacteria, while others directly promote plant growth, thus leading to different plant  
468 gene expression profiles. In our study, a mixed inoculum containing different bacteria  
469 species/strains has mirrored a natural environment (where plants encounter different bacterial  
470 species). Genes belonging to different categories (e.g. transcriptional regulation, nutrient transport,  
471 hormonal balance, cell wall metabolism) have been identified as regulated in PGPB-treated roots,  
472 showing an impact on different root processes, in agreement with previous transcriptome studies. In  
473 addition, genes encoding pore-toxin proteins were found to be up-regulated in the MICO condition.  
474 These proteins are the most common bacterial cytotoxins and are required for virulence in a large  
475 number of important pathogens. Interestingly, pore-forming proteins with remarkably similar  
476 structures to pore-forming toxins (PFTs) are found in vertebrates and constitute part of their  
477 immune system (Dal Peraro and van der Goot 2016). The functions of these proteins has remained  
478 unclear in higher plants, but their transcription levels were greatly increased under biotic stress  
479 (Shao et al. 2015).

480 In conclusion, with an experimental set up which has allowed the plants to grow in a comparable  
481 way irrespective of the microbial inoculum, we found that AM fungi may elicit in grapevine most  
482 of the responses which have already been characterized in crop and herbaceous plants. This is a

483 further confirmation that the symbiotic pathway operating in the plants as a consequence of the AM  
484 presence is very ancient and conserved irrespective of a plant's phylogenetic position.

485 The mixed inoculum led to a very low colonization by AMF, but elicited an important  
486 transcriptional regulation, which, as a consequence, probably can be assigned predominantly to the  
487 presence of the PGPBs.

488 Because grapevines live in association with multiple bacterial and fungal communities (Trouvelot et  
489 al. 2015; Pinto and Gomes 2016), our data offer a starting point to dissect the grapevine response  
490 both to a single microbe and to a mixed inoculum, offering a basis for the development of novel  
491 approaches in vineyard practices.

492

493

#### 494 **Conflict of interest**

495 The authors declare that they have no conflict of interest.

496

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715 **Table 1.** Read number and alignment summary.

<b>Description</b>	<b>Total number of reads</b>	<b>Total alignments</b>	<b>Total unique aligned reads</b>	<b>Total aligned bases</b>	<b>Uniquely aligned mapping reads</b>
<b>CTRL 1</b>	19,445,924	17,649,261	16,238,647	882,463,050	15,358,770
<b>CTRL 2</b>	32,353,647	28,847,668	26,381,472	1,442,383,400	24,908,376
<b>FMOS 1</b>	26,154,442	23,614,153	21,657,536	1,180,707,650	20,483,703
<b>FMOS 2</b>	12,134,327	11,470,488	10,513,263	573,524,400	9,949,366
<b>MICO 1</b>	15,730,900	15,332,942	14,077,415	766,647,100	13,344,301
<b>MICO 2</b>	19,006,336	18,441,962	16,874,712	922,098,100	15,954,651

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718 **Table 2.** List of the 10 genes most up- or down-regulated in FMOS and MICO conditions, in  
719 comparison with control plants.

<b>V.vinifera_Gene_id</b>	<b>Sample</b>	<b>UP/DOWN</b>	<b>Gene description</b>
VIT_01s0026g00550	FMOS	up	nodulin MtN21 family
VIT_03s0063g00370	FMOS	up	Nitrite reductase
VIT_13s0064g01290	FMOS	up	basic helix-loop-helix (bHLH) family
VIT_13s0064g01470	FMOS	up	uroporphyrin III methylase
VIT_16s0050g02540	FMOS	up	nodulin MtN3 family
VIT_05s0062g01160	FMOS	up	pectinesterase family
VIT_06s0004g04400	FMOS	up	nitric-oxide reductase, cytochrome b-containing subunit I
VIT_18s0001g03910	FMOS	up	nitrate reductase 2 (NR2)
VIT_14s0068g01580	FMOS	up	basic helix-loop-helix (bHLH) family
VIT_08s0007g01910	FMOS	up	Laccase
VIT_01s0026g00550	MICO	up	nodulin MtN21 family
VIT_03s0063g00370	MICO	up	Nitrite reductase
VIT_13s0064g01290	MICO	up	basic helix-loop-helix (bHLH) family
VIT_13s0064g01470	MICO	up	uroporphyrin III methylase
VIT_18s0001g03910	MICO	up	nitrate reductase 2 (NR2)
VIT_14s0068g01580	MICO	up	basic helix-loop-helix (bHLH) family
VIT_17s0000g05620	MICO	up	integral membrane HPP family protein
VIT_04s0023g03540	MICO	up	Thaumatococcus SCUTL1
VIT_06s0004g04400	MICO	up	nitric-oxide reductase, cytochrome b-containing subunit I
VIT_10s0003g04880	MICO	up	Ferredoxin:nadp+ Oxidoreductase PETH
VIT_02s0025g02650	FMOS	down	Cellulase CEL2
VIT_05s0020g02170	FMOS	down	Sugar transporter ERD6-like 16
VIT_05s0020g03740	FMOS	down	lipid transfer protein
VIT_12s0028g02800	FMOS	down	isoflavone methyltransferase/ Orcinol O-methyltransferase 1 oomt1
VIT_15s0046g01600	FMOS	down	acidic endochitinase (CHIB1)
VIT_14s0060g00760	FMOS	down	galactinol synthase
VIT_03s0038g02800	FMOS	down	cyclin B2;4
VIT_17s0053g00990	FMOS	down	alpha-expansin 1 precursor
VIT_01s0011g06180	FMOS	down	blight-associated protein p12 precursor
VIT_02s0012g00830	FMOS	down	Expansin-like B1
VIT_02s0025g02650	MICO	down	Cellulase CEL2
VIT_02s0234g00010	MICO	down	gibberellin 20-oxidase
VIT_07s0104g01230	MICO	down	Auxin response factor 2
VIT_10s0116g01620	MICO	down	lyase
VIT_14s0068g01610	MICO	down	DELLA protein RGL1 (RGA-like protein 1)
VIT_07s0104g00360	MICO	down	early-responsive to dehydration
VIT_13s0019g02560	MICO	down	subtilisin protease C1
VIT_00s0665g00020	MICO	down	Carrier protein, Mitochondrial

VIT_12s0055g00950	MICO	down	receptor-like kinase 902
VIT_10s0003g05390	MICO	down	FAD-linked oxidoreductase 1

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721 **Legends**

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723 **Fig. 1.** Colonization rate in grapevine roots after treatment with MICO and FMOS. F%, Frequency  
724 of mycorrhiza in the root system; M%, Intensity of the mycorrhizal colonization in the root system;  
725 a%, Arbuscule abundance in mycorrhizal parts of root fragments; A%, Arbuscule abundance in the  
726 root system; v%, vesicle abundance in mycorrhizal parts of root fragments.

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728 **Fig. 2.** Venn diagrams of differentially-expressed genes (DEGs). **a)** Venn diagram of all DEGs. **b)**  
729 Venn diagram of up-regulated DEGs. **c)** Venn diagram of down-regulated DEGs.

730 **Fig. 3.** Correlation between log<sub>2</sub> relative expression values measured by RT-qPCR and RNA  
731 sequencing analyses.

732 **Fig. 4.** GO enrichment in differentially-expressed genes (DEGs). **a)** Biological process enriched  
733 GO-terms for MICO samples. **b)** Biological process enriched GO-terms for FMOS samples.

734 **Fig. 5.** GO enrichment in differentially-expressed genes (DEGs). Biological process enriched GO-  
735 terms for DEGs in common between FMOS and MICO samples against the whole reference *V.*  
736 *vinifera* gene space.

737 **Fig. 6.** The numbers of CAZymes functional domains identified in FMOS and MICO samples. On  
738 the rows, PL=polysaccharide lyases, GT=glycosyltransferases, GH=glycoside hydrolases,  
739 CE=carbohydrate esterases, CBM=carbohydrate-binding modules and AA= auxiliary activities.

740 **Supplementary materials**

741 **Fig. S1.** Fresh and dry root weight and P and K determination. Data are expressed as a mean  $\pm$  SD.

742

743 **Fig. S2.** Dendrogram showing the clustering of RNA-seq samples in two major sub-groups based  
744 on their expression signatures.

745 **Fig. S3.** RT-qPCR validation of the relative expression for a subset of genes randomly selected  
746 from the RNAseq experiment in the FMOS versus CTRL (a) and MICO versus CTRL (b)  
747 comparison. Blue and red bars represent the relative expression (log<sub>2</sub>fold change) recorded in the  
748 RT-qPCR and RNAseq experiments, respectively. Asterisks indicate statistically significant data  
749 (p<0.05).

750 **Table S1.** List of the oligonucleotides used for RT-qPCR experiments.

751 **Table S2.** Colonization rate, in grapevine roots after treatment with MICO and FMOS, for each  
752 plant.

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754 **Table S3.** Gene expression data.

755 **Table S4.** Differential expression analysis results and functional annotation of common DEGs in  
756 FMOS and MICO samples.

757 **Table S5.** Differential expression analysis results and functional annotation of DEGs specific of  
758 FMOS or MICO samples.

759 **Table S6.** Transporters related DEGs differentially expressed in MICO and FMOS (above) and  
760 specific for one of the two samples (below).

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Figure 1

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Figure 2

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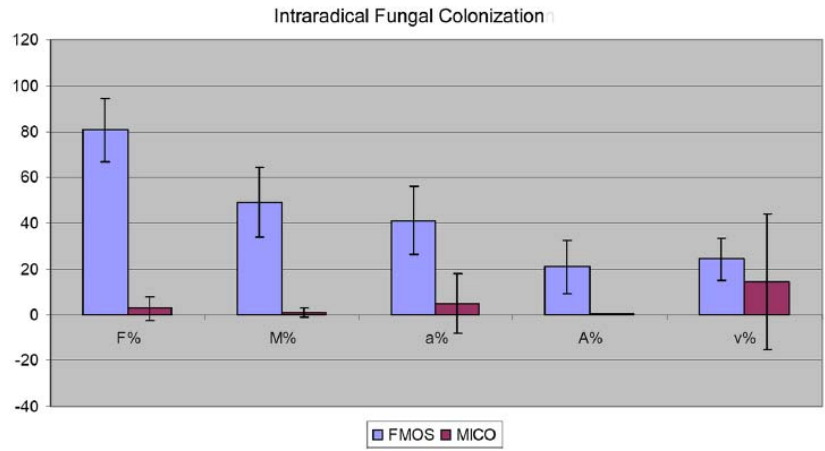
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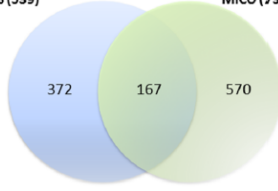
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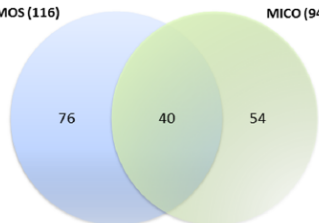


■ FMOS ■ MICO

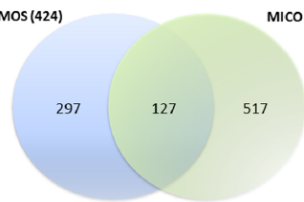
**a** FMOS (539) MICO (737)



**b** FMOS (116) MICO (94)



**c** FMOS (424) MICO (644)



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Figure 3

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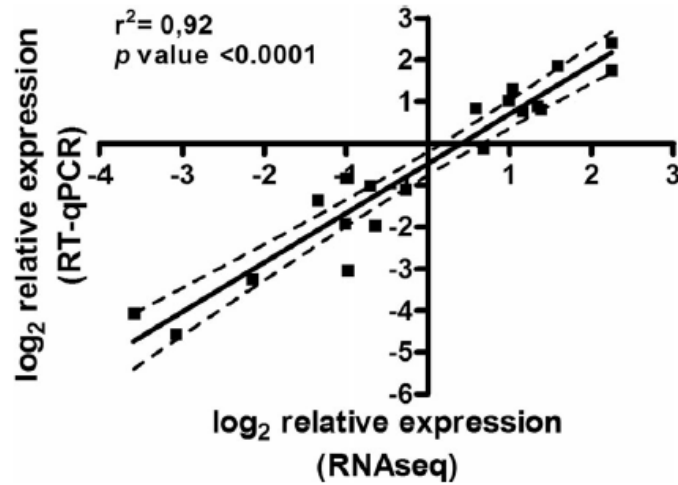
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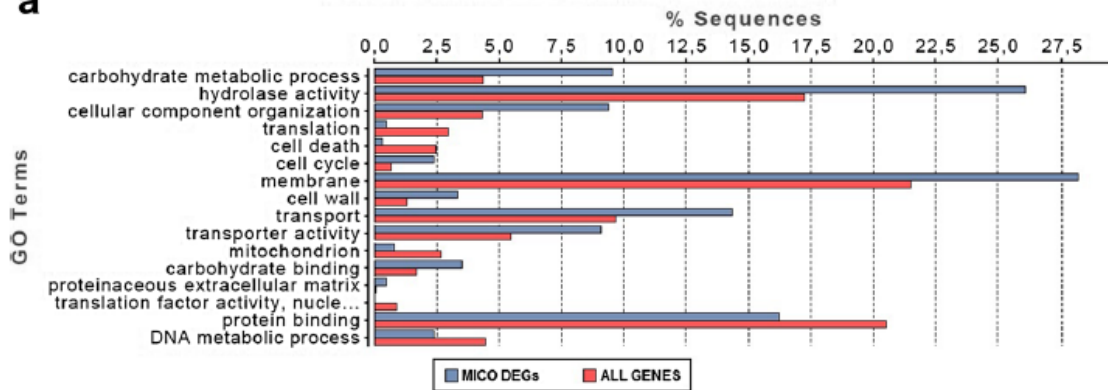
Figure 4

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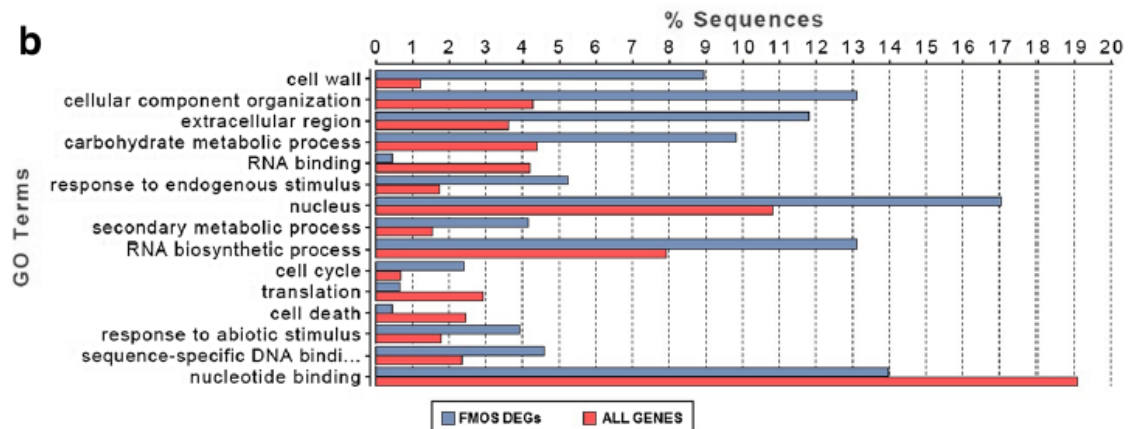
**a**

### Differential GO-term Distribution



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**b**



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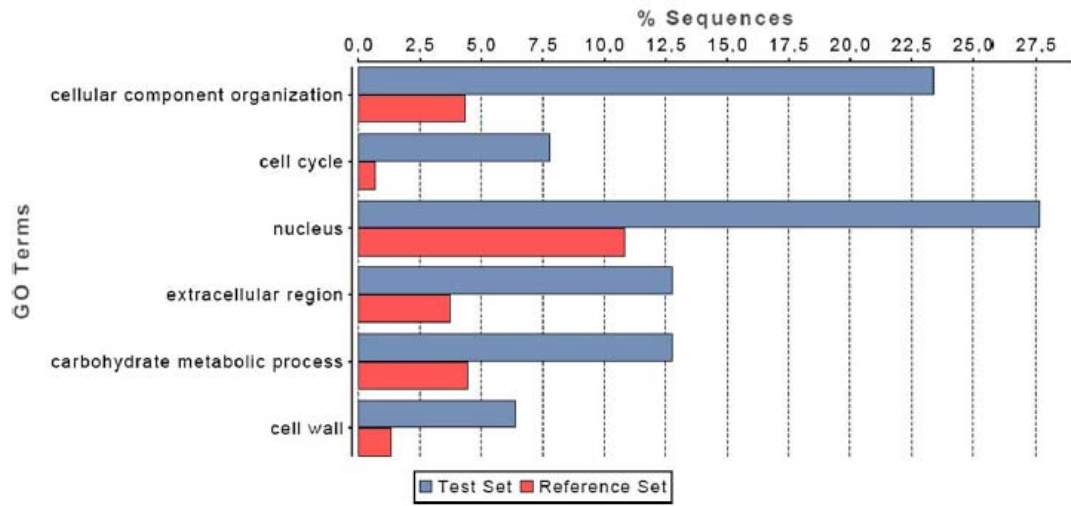
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Figure 5

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Differential GO-term Distribution

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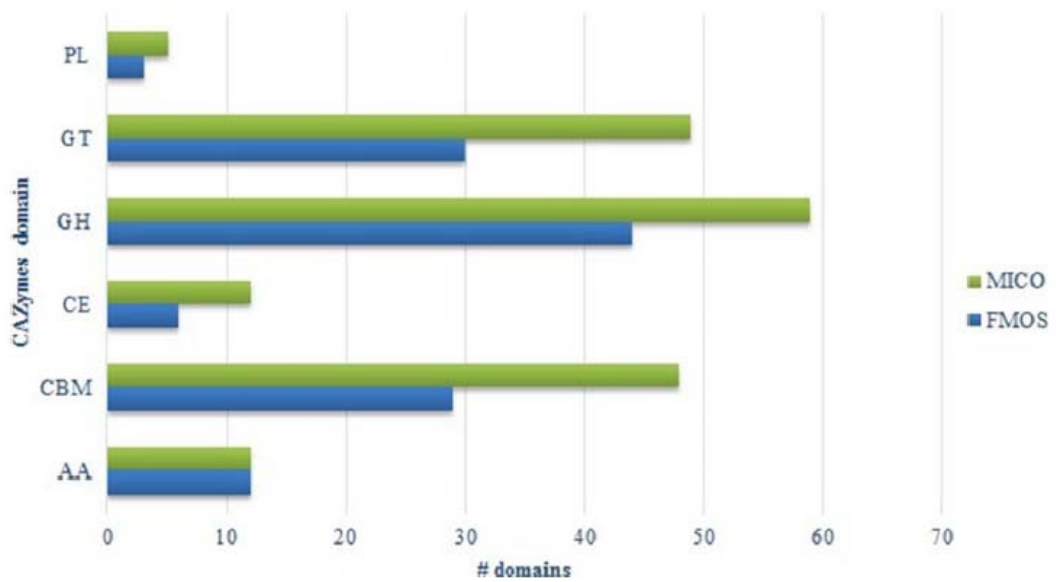
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Figure 6

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