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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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Abstract

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

Keywords

AM symbiosis; microbial inoculum; grapevine; root transcriptome profile

Introduction

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

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

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

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

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

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

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

Materials and Methods

Plant material and growth conditions

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

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

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

RNA extraction and Illumina sequencing

For the RNASeq experiment, roots were harvested from the CTRL, FMOS and MICO plants after three months from the inoculation. Total root systems were chilled in liquid N₂ and RNA was extracted using the 'pine tree-method' (Chang et al. 1993) with the addition of 2% PVPP to the extraction buffer. For each growth condition, we used two biological replicates, each containing the pooled RNA from roots sampled from three plants. RNA quality and quantity controls have been performed using the Agilent 2100 Bioanalyzer. Ten micrograms of each RNA sample (RIN >8) were sent to HUGEF (Torino, Italy) where the libraries were produced and sequenced using an Illumina Genome Analyzer (Solexa). The six libraries were indexed, and single-end multiplexed sequencing was performed using 100 bp length reads. The reads obtained from Illumina HiSeq were processed using CASAVA pipeline version 1.8.2. (Illumina Inc, San Diego, CA, USA) and further checked for sequence quality with the fastQC application (ver. 0.10.1).

Bioinformatic methods

Expression profiling and differential expression analysis

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

Functional annotation and GO-enrichment analysis

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

Quantitative RT-qPCR validation

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

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

Phosphorous and potassium determination in roots

To determine P and K, about 2 mg of root system (4 plants for each condition) were dried for two days at 60°C, ground and digested at 95°C for 1 hour in 1 ml HNO₃ 6M, filtered using a glass filter and diluted with distilled water 1:6. The cations were determined with a Perkin Elmer Optima 7000 (Perkin Elmer, Norwalk, Connecticut, USA) inductively coupled plasma-optical emission spectrometer (ICP-OES). Standard solutions were prepared from concentrated stock solutions (Merck Titrisol or Sigma Aldrich). High purity water (HPW) produced with a Millipore Milli-Q system was used throughout. The reagents used were of analytical grade.

Statistical analyses

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

Results

Plant development and root features

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

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

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

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

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

A look at the whole root transcriptome and at the differentially expressed genes

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

was in different directions. Some DE transcripts were specific to a single condition (Table S5). In addition, for three DEGs, FPKM have been found only in MICO roots and not in the control, i.e. genes annotated as coding for a putative arachidonic acid-induced protein DEA1 (VIT_12s0035g02000), an unknown protein (VIT_03s0132g00060) and a no hit protein (VIT_13s0047g00580), although with low FPKM values (1,145; 0,697; 5,710 respectively).

Among the 10 most up-regulated genes, seven transcripts were common between to the two conditions (Table 2). The first, with a fold change of 2.85 and 3.64 in FMOS and MICO respectively, was a gene coding for a putative uroporphyrin III methylase (VIT_13s0064g01470). A corresponding (homolog) gene has been reported to be up-regulated in luxuriant (N+) treated *Eucalyptus* plants versus limited (N-) plants (Camargo et al. 2014), while in *Arabidopsis* roots a low expression has been found under Cd treatment (van de Mortel et al. 2008). VIT_03s0063g00370 and VIT_18s0001g03910, which code for a putative ferredoxin nitrite and a putative nitrate reductase, respectively, have a role in nitrate/nitrite assimilation, and might be regulated by nitrate, as previously demonstrated in *Arabidopsis* (Wang et al. 2003). In the same list, we also detected a gene encoding a putative nitric oxide reductase (VIT_06s0004g04400) as well as a gene coding for a nodulin belonging to the MtN21 family (VIT_01s0026g00550).

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

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

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

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

To better explore the novel transcriptomic data set, and to further understand the grapevine response to AM fungi, we studied in greater detail the expression profiles of genes described in the literature as specifically involved during AM symbiosis in legumes, rice and tomato (Fiorilli et al. 2009; Guether et al. 2009; Hoge Kamp et al. 2011; Handa et al. 2015; Fiorilli et al. 2015). AM symbiosis is mostly acknowledged for the improved nutrient exchange established between the two symbionts, and regulated by the activities of fine-tuned plant and fungal transporter genes (Casieri et al. 2013; Berruti et al. 2016a). In accord with this claim, a consistent group of plant transporters were identified as differentially expressed between treated (FMOS and/or MICO) and control plants (Table S6). One of them is an inorganic phosphate transporter gene (VIT_16s0050g02370) that shows homology with the mycorrhiza-inducible inorganic phosphate transporters such as LePT4 and OsPT11 (Table 2; Figure S3), and an oligopeptide transporter 4 (VIT_18s0001g07940). Several genes encoding for protein involved in the transport of molecules across cell membranes were also up-regulated in the same FMOS roots. Among them, genes coding for a putative potassium (K^+) transporter KUP1 (VIT_19s0027g01820), a sulphate transporter (VIT_05s0020g03970), a lysine histidine transporter (LHT, VIT_01s0011g03180), and an organic cation transport protein OCT1 (VIT_17s0119g00080) were exclusively up-regulated in FMOS roots. By contrast, other transporters were up-regulated in both the conditions: among them, two genes coding for putative nitrate transporters (VIT_17s0000g09470 and VIT_01s0127g00070) and a Zinc transporter 10 precursor (VIT_10s0042g01100) as well as three protease inhibitor/seed storage/lipid transfer protein (LTP) genes (Table 2). An additional VIT_09s0002g05660 sulphate transporter Sulp family was up-regulated under both treatments. In agreement with previous work (Zhang et al. 2010; Hoge Kamp et al. 2011), four ABC-transporters were identified as co-induced in FMOS roots (VIT_07s0031g02550; VIT_13s0074g00690; VIT_16s0098g00570; VIT_04s0008g04790), and only the last one was also up-regulated in MICO roots. Putative ammonium transporter genes (VIT_04s0008g05080, VIT_00s1818g00010, VIT_00s0179g00310, VIT_07s0031g02950) were significantly down-regulated in MICO roots as well as a gene corresponding to a putative NIP 1;2 (VIT_10s0003g01830).

The presence of AM fungi leads to relevant changes in the hormonal plant profile (Gutjahr 2014), including gibberellins, which are predicted to modulate their concentrations during the symbiosis. A gene coding for a DELLA protein GAI1 (VIT_17s0000g10300) was found as up-regulated in

FMOS and significantly down-regulated in MICO samples. Strigolactones not only represent a new class of plant hormones, but also may stimulate fungal branching while acting as pre-symbiotic molecules (Bonfante and Genre 2015). A gene involved in strigolactone (SL) biosynthesis, i.e. a carotenoid cleavage dioxygenase (CCD) 7 (VIT_15s0021g02190; *VvCCD7*) was found to be upregulated in FMOS samples. Interestingly, *VvCCD7* transcripts were not detected in MICO roots, consistent with the almost total absence of AM fungal colonization.

Homologs of nodulin genes (Denancè et al. 2014) were found to be differentially regulated in our datasets. In FMOS, genes encoding two MtN3 nodulins (VIT_16s0050g02540 and VIT_17s0000g00820) were up- and down-regulated respectively, while two MtN21 genes were up-regulated (VIT_03s0017g02310 and VIT_01s0026g00550). One of these last (VIT_01s0026g00550) was up-regulated also in MICO roots together with another MtN21 gene (VIT_04s0023g02730), while two others were down-regulated (VIT_13s0084g00090 and VIT_08s0040g02500).

Among genes potentially involved in arbuscule formation and fungal accommodation, two genes encoding CESA cellulose synthase (VIT_10s0003g01560 and VIT_07s0005g04110), a gene coding for a cellulose synthase IRX3 (VIT_11s0037g00530), a chitinase class III (VIT_16s0050g02210), and a subtilisin serine endopeptidase gene (VIT_15s0048g01200) were exclusively up-regulated in FMOS roots. A gene encoding a laccase (VIT_08s0007g01910) was also up-regulated only in FMOS, in addition to a metallothionein (VIT_08s0007g00330).

Both FMOS and MICO conditions elicited the expression of several TF genes belonging to different groups, while other members inside these families were down-regulated (Table S3; Table S4). In detail, genes belonging to GRAS, DOF, Zinc-finger, MYB and DREB transcription factor groups were found to be up-regulated in FMOS roots. Members inside these groups were also up-regulated in MICO samples. Additionally, it is worthwhile to note the regulation in AM-colonized roots of several circadian-regulated genes as well as genes potentially involved in the response to environmental stimuli (Table S3). Among them, the grapevine homolog to arachidonic acid-induced protein DEA1 (VIT_12s0035g02000) and a gene coding for a protein belonging to the RD22-like subfamily (VIT_04s0008g04150). By contrast, genes potentially involved in response to pathogens, such as stilbene synthase genes, were down-regulated in FMOS roots as well as four genes coding for Avr9/Cf-9 rapidly elicited protein 20. In contrast, no stilbene synthase genes were regulated in MICO roots.

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

high affinity nitrate transporter (VIT_06s0061g00310 and VIT_06s0061g00320), two additional nitrate transporters (VIT_11s0016g05170 and VIT_18s0001g11280), a sulphate transporter 1.3 (VIT_18s0001g04910), a ZIP family transporter (VIT_19s0085g00740), an arsenite transport protein (VIT_02s0025g03310), a Mg-chelatase subunit XANTHA-F (VIT_08s0007g08540) were up-regulated. Differently from the expression profile described in FMOS (Table S3; Table S4), genes coding for putative ammonium transporters, and a gene coding for a cationic amino acid transporter 2 (VIT_10s0003g04540) were down-regulated. Among genes involved in other pathways that potentially could be affected by the presence of bacteria (i.e. hormonal balance and defense; Vacheron et al. 2013; Drogue et al. 2014), an auxin response factor 3 (VIT_10s0003g04100) is specifically up-regulated in addition to a gene coding for the ABA 8'-hydroxylase CYP707A1 (VIT_02s0087g00710), which is a key catabolic enzyme and could be involved in the regulation of ABA level (Okamoto et al. 2006). Ethylene responsive factors genes (ERF), all were down-regulated in the MICO-treated roots, confirming previous results obtained in different beneficial plant-bacterium interactions (Verhagen et al. 2004; Drogue et al. 2014). Two genes encoding pore-forming toxins (VIT_07s0005g06090 and VIT_07s0005g06110) were specifically up-regulated in the MICO treatment.

Discussion

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

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

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

al. 2011; Fiorilli et al. 2015). Nevertheless, examples of transcriptomes with prevalent down-regulated genes already have been reported. Drogue et al. (2014) have analyzed four different plant-microbe combinations using two *Azospirillum* strains and two rice cultivars, and only in Nip_B510 combination the regulated genes were mainly down-regulated (203 up-regulated vs 2336 down-regulated). Interestingly, in a recent study focused on the transcriptome of mycorrhizal litchi roots, Shu, et al. (2016) found a number of down- (156) and up- (286) regulated genes, with a ratio between up- and down-regulated genes lower than other previous works. We cannot exclude that woody plants differently modulate their root transcriptome in the presence of beneficial microbes, or they may require a different and/or longer timing than herbaceous plants.

A single microbe versus a consortium

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

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

between the two treatments (FMOS and MICO) are involved in nitrogen metabolism, mainly in relation to nitrate, suggesting that in grapevine these beneficial root-associated microbes are particularly efficient in stimulating plant responses to nitrogen, which is an essential element for all grapevine processes ([http://www.awri.com.au/wp-](http://www.awri.com.au/wp-content/uploads/1_nutrition_nitrogen_fertilisation.pdf)

[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 other genes which are considered functional marker genes of the AM symbiosis and expected to be up-regulated in the presence of the AM fungus, such as for example ammonium transporter and NIP aquaporin genes (Guether et al. 2009b; Giovannetti et al. 2012), were found in AM-colonized grapevine roots although not significantly up- or down-regulated in the comparison with CTRL plants.

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

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

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

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

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

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

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

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

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

Conflict of interest

The authors declare that they have no conflict of interest.

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714

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

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

V.vinifera_Gene_id	Sample	UP/DOWN	Gene description
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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Legends

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

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

Fig. 3. Correlation between log2 relative expression values measured by RT-qPCR and RNA sequencing analyses.

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

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

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

Supplementary materials

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

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

Fig. S3. RT-qPCR validation of the relative expression for a subset of genes randomly selected from the RNAseq experiment in the FMOS versus CTRL (a) and MICO versus CTRL (b) comparison. Blue and red bars represent the relative expression (log2fold change) recorded in the RT-qPCR and RNAseq experiments, respectively. Asterisks indicate statistically significant data ($p < 0.05$).

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

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

Table S3. Gene expression data.

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

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

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

Figure 1

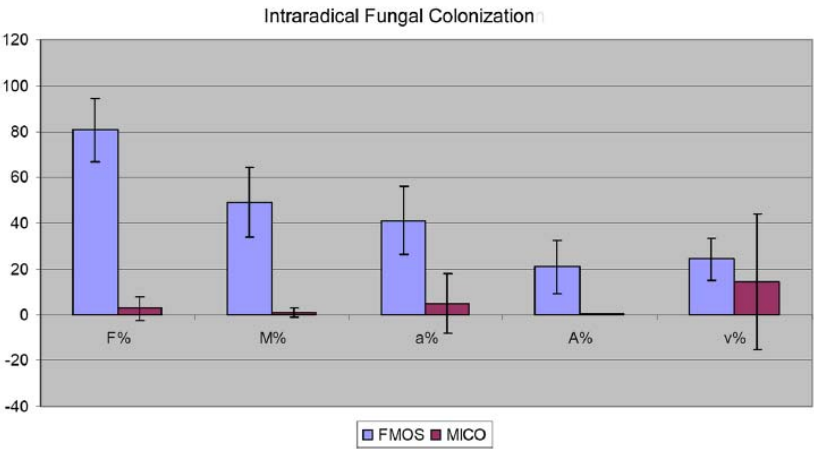


Figure 2

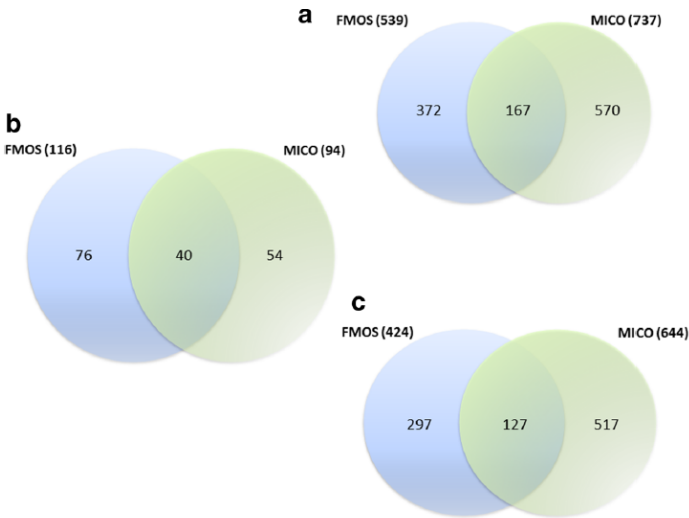


Figure 3

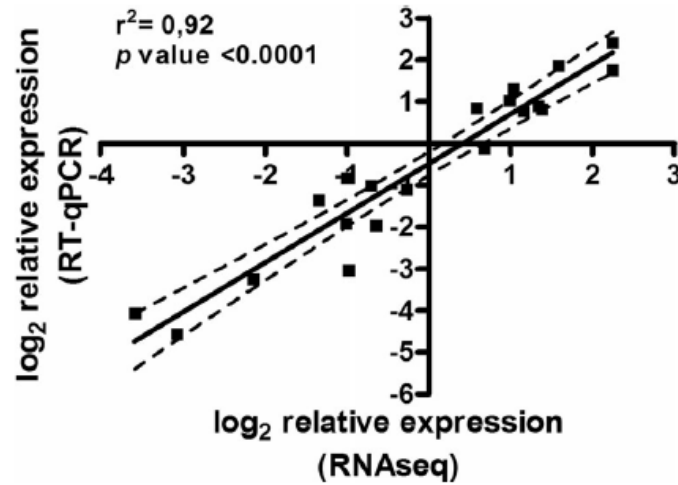


Figure 4

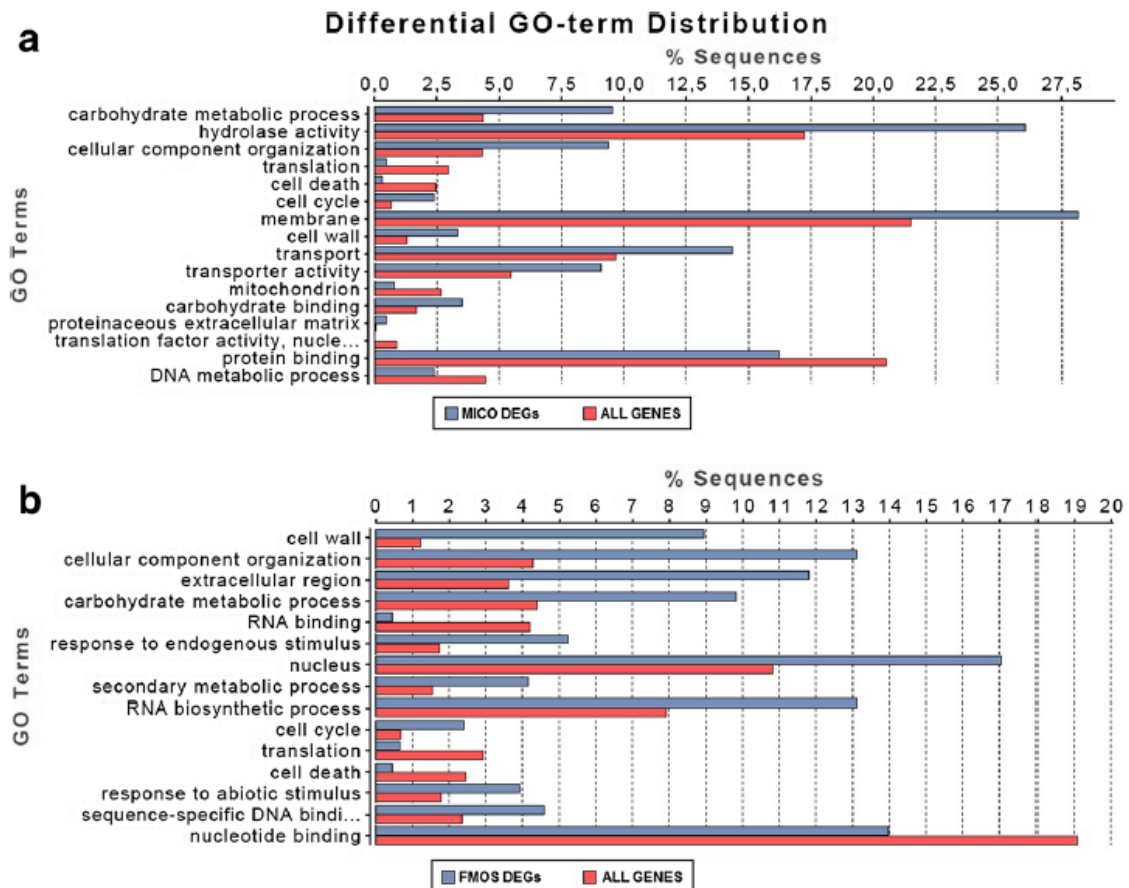


Figure 5

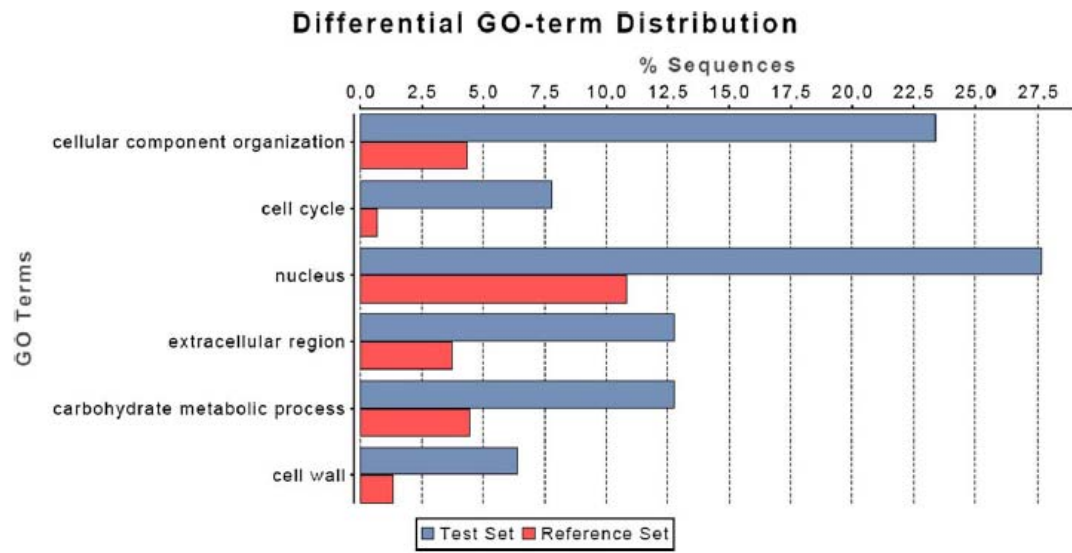


Figure 6

