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Root Proteomic Analysis of Grapevine Rootstocks Inoculated with *Rhizophagus irregularis* and *Fusarium oxysporum* f. sp. *herbemontis*

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ABSTRACT: Grapevine decline and death caused by the pathogenic fungus *Fusarium oxysporum* f. sp*. herbemontis* is among the main phytosanitary problem for viticulture in southern Brazil. The eradication of infected plants is presently the most common procedure for disease control in vineyards. Inoculation with arbuscular mycorrhizal fungi is an option to reduce or neutralize the negative impacts of soil pathogenic microorganisms, but the mechanisms of plant response involved in this process are not yet completely elucidated. In order to better understand these mechanisms, an experiment was carried out to identify proteins related to plant defence induced by the mycorrhizal fungus after infection with the pathogenic fungus. We used the grapevine rootstocks SO4 and R110 (susceptible and resistant to the pathogenic fungus, respectively) inoculated or not inoculated with the mycorrhizal fungus *Rhizophagus irregularis*, and inoculated or not inoculated with *Fusarium oxysporum* f. sp*. herbemontis.* Growth of the rootstocks' shoot and root and presence of pathogenic symptoms were evaluated. The protein profiles of roots were characterized by two-dimensional electrophoresis and proteins were identified using mass spectrometry. The grapevine rootstocks inoculated with *R. irregularis* had higher biomass production and lower level of pathogenic symptoms. The R110 rootstock differentially accumulated 73 proteins, while SO4 accumulated 59 proteins. Nine plant-defence proteins were expressed by SO4 rootstock, and six were expressed by R110 rootstock plants. The results confirm the effect of mycorrhizal fungi in plant growth promotion and their potential for biological control against soil pathogenic fungus. Protein expression is dependent on rootstock characteristics and on the combination of plant material with the fungi.

Keywords: vitis, mycorrhiza, pathogenic fungus, defence response, proteins.

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INTRODUCTION

The main phytosanitary problem in southern Brazil is grapevine decline and death, caused by the soil pathogenic fungus *Fusarium oxysporum* Schlechtendahl emend. Snyder & Hansen f. sp. *herbemontis* Tochetto (FOH). The occurrence of this fungus is widespread in soils from this region, and the use of resistant grapevine rootstock is the main practice for control of the pathogenic attack on plants (Garrido et al., 2004; Sônego et al., 2005). Among the grapevine rootstocks available in southern Brazil, the SO4 rootstock (*Vitis berlandieri* × *V. riparia*) was commonly used in grapevine production due to its adaptability to different types of soils and climate, as well as to its vigour, which ensures high yield and fruit quality. However, SO4 was replaced, due to its susceptibility to FOH (Andrade et al., 1993; Dalla Costa et al., 2010; Cangahuala-Inocente et al., 2011), by Paulsen 1103, R99 and R110 (*V. berlandieri* × *V. rupestris*), and VR043-43 (*V. vinifera* × *V. rotundifolia*) grapevine rootstocks, resistant to FOH, which are currently the most recommended rootstocks in southern Brazil (Sônego et al., 2005; Souza et al., 2013). These rootstocks have low root development, and consequently low yield, but they have a long production cycle (Dalla Costa et al., 2010).

A sustainable option to prevent diseases from soil pathogens is the association between plants and beneficial microorganisms, especially when combined with other types of biological control (Azcón-Aguilar and Barea, 1996; Hol et al., 2014). Arbuscular mycorrhizal fungi (AMF) are soil fungi that establish a mutualistic interaction with plant roots. This symbiosis benefits plants by increasing mineral and water uptake, and also improves resistance against abiotic and biotic stress (Smith and Read, 2008). For this reason, AMF are known as a biological control tool for use in agriculture (Gianinazzi et al., 2010; Trouvelot et al., 2015). Many studies have shown that AMF improve plant resistance to soil pathogenic fungi, including *Fusarium* in grapevine rootstocks and tomato plants (Dalla Costa et al., 2010; Fierro-Coronado et al., 2013), and also act against nematodes in grapevine rootstocks (Hao et al., 2012). Inoculation of grapevine rootstocks with AMF is therefore an option to reduce or neutralize damage from soil-borne diseases. However, the mechanisms of plant response involved in this process have not yet been completely elucidated.

One of the possibilities to increase knowledge of this interaction is the identification of proteins related to interaction between fungi and plants, especially through use of two-dimensional electrophoresis (*2DE*), followed by mass spectrometry (MS) (Mehta et al., 2008). Asano et al. (2012) found an increase in proteins related to plant defence response in *Arabidopsis thaliana* leaves infected with *Fusarium sporotrichioides*. Dumas-Gaudot et al. (2004) and Recorbet et al. (2008) identified extra-radicular fungal proteins from *in vitro* AMF. Using *2DE* analyses, Aloui et al. (2011) showed an up-accumulation of shoot proteins in *Medicago truncatula* inoculated with AMF, and Cangahuala-Inocente et al. (2011) identified 39 differentially accumulated proteins in SO4 grapevine rootstock inoculated with AMF, including proteins related to plant defence. It is thus possible to identify plant proteins differentially expressed by inoculation with mutualistic or pathogenic fungi.

We hypothesized that AMF induces plant defence response after inoculation with the pathogenic fungus FOH, and that expression of plant defence response may be influenced by grapevine rootstock resistance or susceptibility to the pathogenic fungus. The aim of this study was to identify proteins related to plant defence induced by the AMF *Rhizophagus irregularis* after FOH infection in two grapevine rootstocks with different levels of resistance to the pathogenic fungus.

MATERIALS AND METHODS

Experimental design, biological material and plant growth

The experiment was arranged in a 2 × 2 × 2 factorial design containing two grapevine rootstocks (SO4 and R110), two mycorrhizal treatments (inoculated or not inoculated)

and two pathogenic fungus treatments (inoculated or not inoculated). The experiment, carried out in Florianópolis, southern Brazil, was repeated in time, with six replicates per treatment, consisting of a single plant each time.

SO4 and R110 rootstocks, from the plant collection of the Federal University of Santa Catarina, were micropropagated *in vitro* in DSD1culture medium (Lima da Silva and Doazan, 1995) containing 20 g L^{-1} of sucrose, 6.0 g L^{-1} of agar-agar and no plant growth regulators. Explants were kept between 45 and 60 days in a growth room at 25±1 °C with luminosity of 40 to 45 µmol m^{-2} s^{-1} and a 16 h d^{-1} photoperiod.

Inoculum of both fungi were obtained from material previously used by Dalla Costa et al. (2010) and kept at 4 °C until use. The AMF *Rhizophagus irregularis* (BEG 72, originally supplied by the French National Institute for Agriculture Research – INRA, Dijon, France) was applied as a soil-based inoculum consisting of a mixture of soil and the root system from the host plant (*Allium porrum*). The pathogenic fungus *Fusarium oxysporum* f. sp. *herbemontis* inoculum (CNPUV 287, originally supplied by the Brazilian Agricultural Research Corporation Centre for grape and wine - Embrapa Uva e Vinho) was kept in a sterile mixture of soil/fertilizer/sand. A sample from the FOH inoculum was added to sterile water and spread on petri dishes containing potato dextrose agar (PDA) culture medium, kept in a growth chamber for six days at 25±2 °C with luminosity of 150 µmol m^{-2} s^{-1} and 16 h d^{-1} photoperiod. Spores were collected by scraping the culture surface with a Drigalski spatula and adding sterile water to obtain a spore suspension with 1.0×10^{7} conidia mL^{1}.

After the *in vitro* growth period of the grapevine rootstocks, plants were transplanted to polystyrene trays with cells of 40 mL containing a sterile soil mixture consisting of organic substrate (Germina Plant, Turfa Fértil®), soil (*Nitossolo Vermelho Distroférrico* - Dystrophic Typic Hapludalf) and sand (2:1:2, v:v:v; Dalla Costa et al., 2010). Prior to transplant, all plants were trimmed to have 1.0-cm roots, 2.5 to 3.0 cm height and 3 to 4 leaves. For inoculation, 2.0 g of soil-based inoculum (50 to 150 spores) were placed near the plant roots, and non-inoculated plants received sterilized soil-based inoculum. Plants were kept in a growth chamber at 25± 2 °C, 98-100 % relative humidity, and luminosity of 150 µmol m^{-2} with a 16 h d^{-1} photoperiod. After 30 days, plants were transplanted into 400 mL plastic pots containing the same sterile soil mixture previously described; AMF treatments were reinoculated with 2.0 g of mycorrhizal AMF inoculum and non-inoculated plants received sterilized soil-based inoculum. The plants were grown under the same environmental conditions and watered daily with distilled water and once a week with Long Ashton nutrient solution (Smith et al., 1983) containing 10 % of the original P concentration. After 45 days, plant roots were cut to create infection points, and 1.0 mL of FOH spore suspension was placed in the soil near the roots of FOH treatments. The non-inoculated plants passed through the same procedure, with application of 1.0 mL of distilled sterile water. Subsequently, the plants were once more kept in a growth room for 14 days until harvest.

Mycorrhizal colonisation and plant growth analyses

Mycorrhizal colonization was assessed on the day the pathogenic fungus was inoculated to plants. Root pieces were taken from each plant and put in alcohol 50 °GL for storage. Mycorrhizal colonization was assessed as described by Trouvelot et al. (1986) after clearing and staining with trypan blue (Koske and Gemma, 1989).

Three plants (replicates) from each treatment were harvested 14 days after pathogenic fungus inoculation. Three plants from each treatment were kept in the growth chamber for 90 more days for future evaluation of disease symptoms. At harvest, root and shoot length and fresh biomass were measured. Root samples were taken for protein analyses.

Two-dimensional electrophoresis (2DE) and mass spectrometry (MS) analysis

For protein analyses, 2.0 g (fresh biomass) of roots from each treatment were ground to a fine powder in liquid nitrogen. Phenolic protein extraction and *2DE* analyses were conducted according to Cangahuala-Inocente et al. (2013). Protein concentrations were estimated according to Bradford (1976) and modified by Ramagli and Rodrigues (1985). A protein concentration of 500 µg from each sample was subjected to isoelectric focusing (first dimension) in an immobilized linear pH gradient 3-10 on 13 cm strips (IPG strips - GE Healthcare) on an Ettan-IPGPhor instrument (GE Healthcare) with a total of 35,000 Vh. After this step, the strips were equilibrated for 15 min in equilibration buffer (50 mmol L^{-1}, pH 8.8 Tris-HCl, 6 mol L^{-1} urea, 30 % (v/v) glycerol, 2 % (w/v) SDS and 0.002 % (w/v) bromophenol blue), containing 1 % (w/v) DTT and 2.5 % (w/v) iodoacetomide. The strips were attached to the top of 12 % polyacrylamide gels in a Hoefer SE 600 Ruby System (GE Healthcare) and run at 4 °C and 10 mA for 1 h, 20 mA for 1 h and 30 mA for 4 h (second dimension). The gels were stained with Coomassie Brillant Blue G250 solution according to Mathesius et al. (2001). Stained gels were scanned in an Image Scanner (GE Healthcare) and analysed with ImageMaster 2D Platinum 7.0 (GE Healthcare). The protein spots were identified and selected based on comparative analyses of the gels from non-inoculated and inoculated treatments ($p \leq 0.05$). Proteins were identified as unique, absent or up- or down-accumulated by AMF *R. irregularis* and/or by the pathogenic fungus *F. oxysporum* f. sp. *herbemontis*. Three gels were obtained from each treatment and biological replicate, resulting in a total of six gels per treatment.

The selected proteins spots from *2DE* gels were manually cut, destained with discoloration solution (50 % acetonitrile, 25 mmol L^{-1} ammonium bicarbonate), dried under a vacuum with pure acetonitrile and digested with porcine trypsin. The peptides were recovered with extraction solution (50 % acetonitrile, 5 % TFA), concentrated, dried, solubilized in 0.1 % trifluoroacetic acid and analysed in a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) AUTOFLEX II TOF-TOF instrument (Burker Daltonics). One µL of recrystallized HCCA matrix (Burker Daltonics) was dissolved (0.1 % trifluoracetic acid and 70 % acetonitrile) and spotted once on top of samples (Shevchenko et al., 1996). The MALDI-TOF mass spectra were acquired in a mass range of 1,000 Da to 3,200 Da. Protein identification was performed by the Mascot software database (http://www.matrixscience.com/) and subsequently by the NCBInr database to identify homologous proteins (http://www.ncbi.nlm.nih.gov/). The following parameters were used for the database search: fixed modifications of carbamidomethylation cysteine, taxonomy of Viridiplantae and trypsin as an enzyme. The molecular functions of proteins were confirmed on the UniprotKB database (http://www.uniprot.org/).

Evaluation of disease symptoms

The evaluation of disease symptoms caused by *F. oxysporum* f. sp. *herbemontis* was performed 90 days after inoculation with FOH, following the disease index method of Grigoletti Júnior (1985). Plants with an external and internal degree of infection between 1 and 2 are resistant to FOH; plants exhibiting degree 3 are tolerant; plants with a degree between 4 and 5 are susceptible to FOH.

Statistics

A factorial analysis of variance was performed for growth parameters, mycorrhizal colonisation, protein concentration and pathogenic symptoms. Mean separation of treatments with significance was done with specific post-hoc comparisons using the Student Newman Keuls method (SNK) at a significance level of 5 %. Statistical analyses were carried out using R version 3.2.1 software (The R Foundation, 2015).

RESULTS AND DISCUSSIONS

Mycorrhizal colonisation, plant growth, and disease symptoms

Arbuscular mycorrhizal fungi-inoculated grapevine rootstocks had a 100 % frequency of fungal infection and over 50 % intensity of root cortex colonisation, with no significant differences between SO4 and R110 rootstocks. In addition to high colonisation intensity, roots had arbuscules (over 50 % frequency), indicating mycorrhizal functionality. Shoot and root biomasses were significantly higher in both grapevine rootstocks inoculated with AMF in comparison to non-inoculated plants (Table 1). However, there were no differences between rootstocks in plant growth, and root length in non-inoculated rootstock was higher than in all other treatments. It has been shown that mycorrhizal plants may have fewer and shorter roots than non-mycorrhizal plants, since extraradicular AMF mycelium increases the volume of soil explored by the plant root system, allowing higher water and nutrient uptake (Locatelli et al., 2002). Therefore, these results confirm the ability of AMF to benefit plant growth, and the increase in shoot and root biomass is consistent with previous studies with grapevine rootstocks (Dalla Costa et al., 2010; Anzanello et al., 2011; Cangahuala-Inocente et al., 2011) or with other plant species (Lovato et al., 2006; Farzaneh et al., 2011; Cavagnaro et al., 2012; Evelin et al., 2012; Mohandas, 2012; Steinkellner et al., 2012). Anzanello et al. (2011) detected an increase in shoot and root biomass in SO4 and P1103 rootstocks inoculated with AMF, and Dalla Costa et al. (2010) also showed an increase in shoot biomass in SO4 rootstock inoculated with AMF. In a study using different varieties of tomatoes inoculated and not inoculated with AMF and inoculated and not inoculated with *F. oxysporum* f. sp. *lycopersici*, Steinkellner et al. (2012) detected a positive effect of AMF in plant biomass in some tomato varieties inoculated only with AMF and also when co-inoculated with both fungi.

Disease symptoms were less marked in grapevine rootstocks inoculated with both fungi than in plants inoculated only with FOH (Table 2). That suggests a bioprotective effect of AMF against FOH colonization in both grapevine rootstocks, which is more marked in the susceptible SO4 rootstock. Dalla Costa et al. (2010) also detected a decrease in disease symptoms in SO4 grapevine rootstocks co-inoculated with AMF and FOH. In two independent studies, Steinkellner et al. (2012) and Fierro-Coronado et al. (2013) showed

Table 1. Root, shoot and total fresh weight, and root length in grapevine rootstocks inoculated (Myc) and not inoculated (NM) with the mycorrhizal fungus *Rhizophagus irregularis* and inoculated (+FOH) or not inoculated (-FOH) with the pathogenic fungus *Fusarium oxysporum* f. sp. *herbemontis*. Mean for two rootstocks (SO4 and R110)

Treatment	Root fresh weight			Shoot fresh weight			Total fresh weight			Root length		
	-FOH	+FOH	Mean	-FOH	+FOH	Mean	-FOH	+FOH	Mean	-FOH	+FOH	Mean
	g									cm		
NM	0.85 bA	0.65 bA	0.75 bA	0.67 aA	0.61 aA	0.64 bA	1.52 bA	1.26 bA	1.39 b	24.5 aA	20.5 aA	22.5 a
Myc	1.44 aB	2.14 aA	1.79 aA	1.40 aA	1.79 aA	1.59 aA	2.85 aB	3.93 aA	3.39 a	18.6 aA	18.0 aA	18.3 b
Mean	1.15 A	1.40 A		1.04 A	1.20 A		2.18	2.60		21.5 A	19.3 B	

Means followed by the same lowercase letter in each column and by the same uppercase letter in each row are not significantly different (SNK, $p<0.05$).

Table 2. Internal and external disease symptom index (1 to 5) in grapevine rootstocks SO4 and R110 inoculated (Myc) or not inoculated (NM) with the mycorrhizal fungus *Rhizophagus irregularis* 90 days after inoculation with the pathogenic fungus *Fusarium oxysporum* f. sp. *herbemontis*

Treatment	Internal symptom			External symptom		
	SO4	R110	Mean	SO4	R110	Mean
NM	3.2 a	2.0 a	2.6 a	4.0 a	3.5 a	3.8 a
Myc	1.3 b	1.2 a	1.3 b	2.5 b	2.2 b	2.3 b

Means followed by the same letter in each column are not significantly different (SNK, $p<0.05$).

that tomato plants co-inoculated with AMF and *Fusarium oxysporum f. sp. lycopersici* had lower disease infection than plants inoculated only with the pathogenic fungus, indicating the bioprotective effect of AMF against that fungus. This bioprotective effect has been linked to a stimulation of plant defence mechanisms (Schenkluhn et al., 2010), which occur when either mutualistic or pathogenic microorganisms are inoculated. Identification of defence-related proteins may provide clues about the multiple mechanisms that may be involved.

Two-dimensional electrophoresis and mass spectrometry analysis

Total protein contents were higher in R110 grapevine rootstocks than in SO4, due to increases associated with AMF and FOH inoculation (Table 3). Simultaneously, *2DE* protein gels from both grapevine rootstocks showed differences in their protein profiles (Figure 1). The R110 grapevine rootstock yielded 73 differentially accumulated proteins, whereas

Table 3. Protein concentration in SO4 and R110 grapevine rootstock roots inoculated (Myc) or not inoculated (NM) with the mycorrhizal fungus *Rhizophagus irregularis* and inoculated (+FOH) or not inoculated (-FOH) with the pathogenic fungus *Fusarium oxysporum* f. sp. *herbemontis*, 60 days after transplanting and the second inoculation with mycorrhizal fungus

Treatment	SO4			R110		
	-FOH	+FOH	Mean	-FOH	+FOH	Mean
	µg mg^{-1} root					
NM	0.82 aA	0.73 aA	0.78 a	0.83 bB	1.12 aA	0.97 b
Myc	0.80 aA	0.76 aA	0.78 a	1.30 aA	1.49 aA	1.39 a
Mean	0.81 A	0.75 A		1.06 B	1.31 A	

Means followed by the same lowercase letter in each column and by the same uppercase letter in each row are not significantly different (SNK, $p<0.05$).

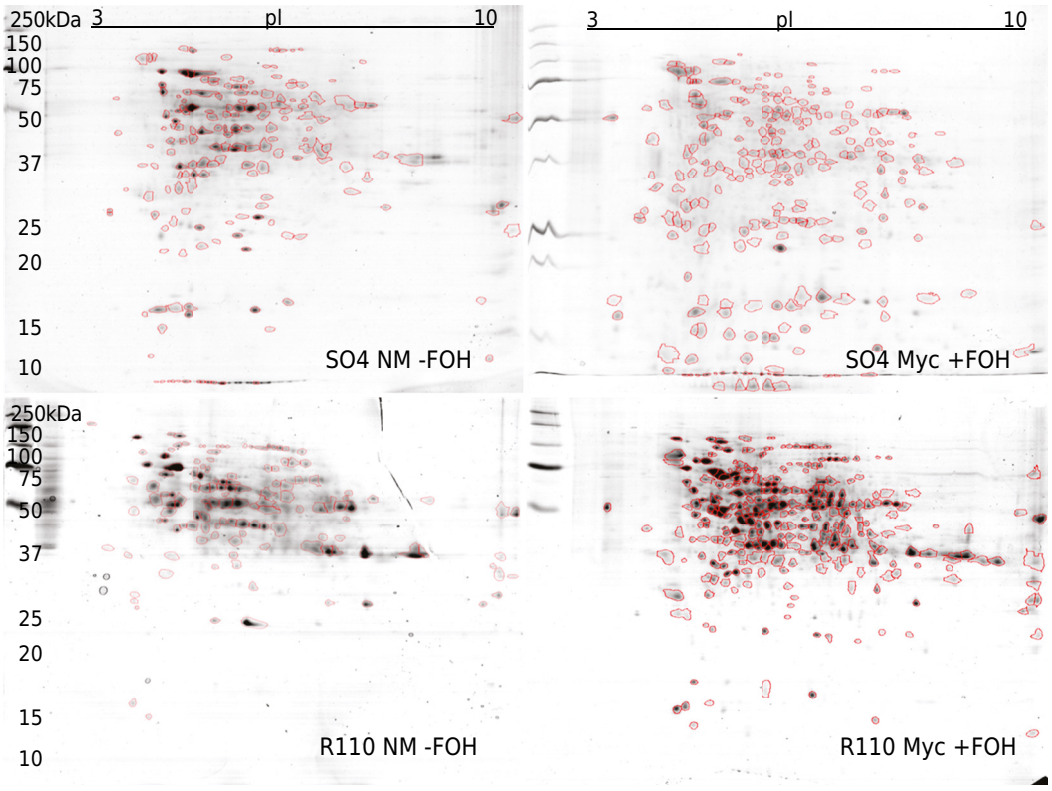

Figure 1. Representative image of *2DE* gel showing the differentially accumulated proteins (red circles) between the SO4 and R110 grapevine rootstocks and between the control treatment (NM -FOH) and co-inoculation with the mycorrhizal fungus *Rhizophagus irregularis* and the pathogenic fungus *Fusarium oxysporum f. sp. herbemontis* (Myc +FOH).

59 differentially accumulated proteins were found in the SO4 rootstock (Table 4). AMF and FOH inoculation, and also co-inoculation with both fungi, resulted in differences in protein accumulation. According to Anzanello et al. (2011), benefits of AMF depend on interaction between the fungus and the rootstock, which might explain the differences in protein profile. Concomitantly, differences in the protein profile may be related to the different levels of resistance from the grapevine rootstocks to FOH, also affecting the interaction between plant and fungi. Xu et al. (2015) also showed differences in the proteome of two cultivars of *Vitis amurensis* with different levels of resistance to a pathogenic fungus.

Mass spectrometry analysis revealed nine proteins related to defence response in SO4 grapevine rootstock (Table 5). Among them, two proteins (ID 252 and 256) were induced by AMF and also by FOH inoculation, and three proteins (ID 629, 655 and 670) were induced by co-inoculation with AMF and FOH. Meanwhile, four proteins (ID 2099, 2101, 2012 and 2143) were down-accumulated in all treatments, in comparison to the non-inoculated treatment. In general, those proteins were up-accumulated in response to AMF inoculation as well as to FOH inoculation. Plant colonisation by AMF also induces activation of the plant defence system, which is, however, readily suppressed, due to recognition of mutualistic symbiosis by the plant. Concomitantly, plants inoculated with AMF are capable of rapidly reactivating their defence system (priming) when facing a stress situation, resulting in enhanced resistance (Pozo and Azcón-Aguilar, 2007; Dalla Costa et al., 2010). Therefore, in the SO4 grapevine rootstocks co-inoculated with both fungi, AMF acted as a bioprotective agent against FOH.

The spot ID 252, “serine/threonine protein kinase A”, belongs to a class of plant resistance (R-) gene products that determine recognition of pathogen infection and trigger the plant defence system (Di Gaspero and Cipriani, 2003). Two cultivars of *Vitis amurensis* had the expression of R-genes enhanced in response to pathogenic fungus infection (Xu et al., 2015), and a similar process was found in the present study with AMF inoculation. The spot ID 256 is a pathogenesis-related protein 1 (PR-1 protein), stimulated by plant stress as a defence response (Edreva, 2005). The pathogenesis-related proteins (PR) are synthetized by numerous plants as a defence mechanism against various pathological processes, and these proteins are classified in different groups (Xu et al., 2015). The spot ID 629 is a cysteine-rich thionin protein, an antimicrobial peptide toxic to several

Table 4. Differentially accumulated proteins from the root system of two grapevine rootstocks (SO4 and R110) inoculated or not inoculated with the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* and inoculated or not inoculated with the pathogenic fungus *Fusarium oxysporum* f. sp. *herbemontis* (FOH), 60 days after transplanting and second inoculation with mycorrhizal fungus

Differential accumulation	Number of proteins	
	SO4	R110
Proteins induced only by AMF inoculation	5	11
Proteins induced only by FOH inoculation	4	-
Proteins induced only by AMF, associated or not with FOH co-inoculation	2	42
Proteins induced only by FOH, associated or not with AMF co-inoculation	4	2
Proteins induced by AMF, or FOH, or by co-inoculation	25	-
Up-accumulated proteins due to AMF or FOH inoculation or co-inoculation	5	3
Down-accumulated proteins due to AMF or FOH inoculation or co-inoculation	11	14
Proteins expressed only in non-inoculated plant roots	3	1
Total proteins	59	73

Table 5. Spot number identification from gel (Spot ID), protein coverage in % of protein length, accession number from database, experimental and theoretical isoelectric point (Exptl/theor pI), experimental and theoretical molecular weight (Exptl/theor MW) and protein name from database of SO4 and R110 grapevine rootstock roots inoculated or not inoculated with the mycorrhizal fungus *Rhizophagus irregularis* and inoculated or not inoculated with the pathogenic fungus *Fusarium oxysporum* f. sp. *herbemontis*

Spot ID	Coverage	Accession number	Exptl/ theor pI	Exptl/ theor MW	Protein name
	%				
			SO4		
252	22	B1AF67_POPTN	7.7/7.6	39/12	Serine/threonine protein kinase A (*Populus tremula*)
256	18	Q6K384_ORYSJ	7.5/12	43/8.0	Pr1-like protein (*Oryza sativa* Janponica Group)
629	21	A7REE7_ARATH	8.3/8.4	45/11	Plant thionin family protein (*Arabidopsis thaliana*)
655	20	I6Nk24_MEDSA	4.6/4.7	19/15	Caffeic-acid-O-methyltransferase, partial (*Medicago sativa* subsp. *caerulea*)
670	15	Q9XI32_ARATH	5.9/5.7	32/17	SNARE-like protein (*Arabidopsis thaliana*)
2099	39	D4P4K0-CITLA	5.3/5.0	44/26	Actin (*Citrullus lanatus*)
2101	13	Q6V1W8-AMATR	6.3/8.7	45/34	Antiviral/ribosome, inactivating protein (*Amaranthus tricolor*)
2102	12	PR2-PETCR	6.0/4.4	45/17	Pathogenesis-related protein 2
2143	22	F4YBE4_SOLNI	5.2/5.5	35/13	Ras-related protein (*Solanum nigrum*)
			R110		
1223	42	J9QUN6_9ROSI	6.2/4.6	53/5.0	Cinnamate 4-hydroxylase, partial (*Populus fremontii*)
1276	50	F6H9X1_VITVI	5.8/4.8	43/17	SKP1-like protein 4 (*Vitis vinifera*)
1370	100	Q66N69_VITVI	5.0/9.7	26/2.0	20S proteasome beta subunit (*Vitis vinifera*)
1447	26	D4N3S8_9ORYZ	3.8/4.5	45/12	Putative heat shock factor binding protein (*Oryza australiensis*)
1495	14	C5WZ03_SORBI	6.7/7.7	61/41	Hypothetical protein SORBIDRAFT_01g047930 (*Sorghum bicolor*)
2077	33	Q75T45_ORYSJ	4.5/4.8	66/17	Os12g0555000 (*Oryza sativa* Japonica Group)

biological systems, belonging to a group of proteins which include the pathogenesis related protein 13 (PR-13) (Nawrot et al., 2014). Epple et al. (1995) detected an increase in thionin concentration in seeds inoculated with *F. oxysporum* f. sp. *matthiolae*. The spot ID 2102 is also a pathogenesis-related protein belonging to group 2 (PR-2). The enzyme β-1,3-glucanase, a PR-2 protein, catalyses the pathogen cell wall polysaccharide β-1,3-glucan, thus preventing fungal diseases (Edreva, 2005). This enzyme has been detected in grapevine rootstocks challenged by *F. oxysporum* f. sp. *herbemontis* (Dalla Costa et al., 2010). Maxson-Stein et al. (2002) detected PR-2 proteins in apple trees

inoculated with a pathogenic fungus and sprayed with acibenzolar-S-methyl, leading to a decrease in pathogenesis symptoms. In summary, all these proteins are related to plant defence, and had their production enhanced by AMF inoculation.

Arbuscular mycorrhizal fungi (AMF) inoculation also affected other proteins, less directly linked to plant defence against pathogens. The spot ID 655, caffeic acid-O-methyltransferase, takes part in the lignin biosynthesis pathway, converting caffeic acid to ferulic acid during the phenylpropanoid pathway (Zhang et al., 2011). Plant cell wall lignification may also be triggered by plant defence mechanisms, preventing enzymatic hydrolysis and plant penetration by pathogenic fungi (Barros et al., 2015). The spot ID 670 is an integral membrane protein called SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptors) that mediates vesicle fusion between the organelles (Sanderfoot and Raikhel, 1999). SNARE proteins are located on the surfaces of vesicles and target membranes, and their main role is to identify the target organelle and mediate the fusion of vesicles with the organelle. Collins et al. (2003) identified that the SNARE complex mediates the expression of plant resistance genes against pathogenic fungus penetration. The spot ID 2099 is an actin, the main component of the plant cytoskeleton, contributing to cell maintenance. Actin may also act as a defence mechanism in the plant hypersensitive cell-death reaction against pathogenic infection. Chang et al. (2011) showed an increase in actin bundling and ensuing cell death in grapevines treated with phytoalexin. The spot ID 2143 corresponds to a superfamily of related proteins called Ras, constituted by small GTPase proteins. Their main action is on the signal transduction pathway coupling receptors to intracellular signals required for cell growth stimulation and differentiation, and also for actin polymerization (Hall, 1993). Therefore, accumulation of this protein could be directly related to accumulation of actin, as described above (spot ID 2099). The spot ID 2101 is an antiviral ribosome inactivating protein (Rip) related also to plant defence response against fungi. This protein is a toxin that inactivates the ribosome by cleavage of N-glycosidase at rRNA adenine, with consequent protein synthesis interruption, leading to cell death (Schrot et al., 2015).

Mass spectrometry of R110 grapevine rootstock extract revealed a smaller number of proteins related to plant defence response (Table 5). Among those six proteins, one of them (ID 1223) was down-accumulated in all treatments in comparison to non-inoculated plants. Four proteins (ID 1370, 1447, 1495 and 2077) were present in AMF-inoculated plants and also in plants with co-inoculation of AMF and FOH. Therefore, these four proteins were accumulated due to inoculation with AMF, since they were not detected in plants inoculated only with FOH. This suggests that in the co-inoculation treatment, the AMF acted as a bioprotective agent against inoculation with FOH, priming the plant defence response. Meanwhile, only one protein (ID1276) was induced by FOH inoculation and also by co-inoculation with AMF and FOH. In this case, this protein is only related to FOH inoculation.

The spot ID 1223 is a cinnamate 4-hydroxylase acting on electron transport and oxidoreductase activity. This enzyme catalyses the second step of the phenylpropanoid pathway, in which a wide range of plant defence response compounds are synthesized (Chen et al., 2014). The spot ID 1370 is an enzyme complex, 20S proteasome beta subunit, a component of the 26S proteasome. The proteasome degrades undesired or damaged proteins in the organism. Proteins are recognized by the proteasome via the signal molecule ubiquitin, attached to the proteins during the ubiquitination process, thus starting the ubiquitin proteasome pathway. This pathway contributes to cell proliferation, hormone responses, development, senescence and resistance to abiotic and biotic stress (Marques et al., 2009). The spot ID 1276 is a SKP1 protein acting on ubiquitination, and consequently also involved in the ubiquitin proteasome pathway (Zheng et al., 2002). Therefore, accumulation of this protein is directly related to spot ID 1370, and both act in plant defence response. Cangahuala-Inocente et al. (2011) showed up-accumulation of two proteins related to the proteasome complex and also

accumulation of ubiquitin in roots of SO4 grapevine rootstocks inoculated with the same AMF used in this study. Margaria and Palmano (2011) also showed up-accumulation of two proteins from the proteasome complex in *Vitis vinifera* (Nebbiolo) inoculated with phytoplasma. The accumulation of these two proteins in the R110 grapevine rootstock as well suggests a direct relation between the ubiquitin proteasome pathway and plant defence response by grapevine plants. The spot ID 1447 is a transcription factor called heat-shock factor binding protein. Under stress conditions, the heat shock factor induces the expression of heat shock proteins involved in plant defence response (Asthir, 2015). Mycorrhizal grapevine roots upregulated the expression of heat-shock proteins, due to inoculation with an ectoparasitic nematode (Hao et al., 2012). The spot ID 1495 was identified in the database with the molecular function of S-adenosyl-methionine-dependent methyltransferase activity. This protein catalyses the transfer of methyl groups S-adenosyl-methionine to other molecules (methylation) and is involved in biosynthesis of a wide variety of metabolites involved in plant defence, growth, and development (Joshi and Chiang, 1998). The biological process of the spot ID 2077 is described as plant defence response. Xu et al. (2011) described this protein as pathogenesis-related protein 10 (PR-10) and detected its increase in rice plants inoculated with the pathogenic fungus *Xanthomonas oryzae* pv*. oryzicola*, which damages rice shoots. Hao et al. (2012) detected upregulation of the PR-10 gene during AMF-induced bioprotection against nematode inoculation in grapevine roots, suggesting the priming effect of AMF to plant defence response, as observed in the present study in roots challenged by a fungus.

In summary, we detected a range of defence-related proteins that were expressed or upregulated in both grapevine rootstocks associated with either mutualistic or pathogenic fungi. There were a larger number of defence-related proteins in the susceptible SO4 rootstock than in the less susceptible 110 rootstock, suggesting that resistant or tolerant plants may block the infection process at several stages, resulting in less severe effects of endophytic fungi on protein expression in the root system. The results presented here show that the presence of the mutualistic and the pathogenic fungi results in a highly complex process, with a series of steps that must be taken into account in plant breeding and management.

CONCLUSIONS

The AMF *Rhizophagus irregularis* benefits grapevine rootstocks by improving plant growth and reducing damages caused by the pathogenic fungus *Fusarium oxysporum* f. sp*. herbemontis*.

Root protein profiles of grapevine rootstocks are dependent on their level of pathogen resistance.

Accumulation of proteins related to plant defence response in grapevine rootstocks varies with the combination of the rootstock, pathogen fungus, and arbuscular mycorrhizal fungus.

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