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The metabolic effects of mercury during the biological cycle of vines (*Vitis vinifera*)

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Abstract Mercury (Hg) is a major environmental pollutant that can be disposed to the environment by human activities, reaching crops like vineyards during irrigation with contaminated waters. A 2-year study was performed to monitor Hg variations during reproductive and vegetative stages of vines after Hg supplementation. Variations were focused on total Hg concentration, the molecular weight of Hg fractions and Hg-proteins associations in roots, stems and leaves. Total Hg concentrations increased during reproductive stages and decreased during vegetative stages. Variations in length of these stages were observed, according to an extension of the vegetative period. Six months post Hg administration, in roots, stems and leaves, initial Hg proteic fractions of 200 kDa were catabolized to 66 kDa fractions according to a transition from reproductive to vegetative stages. However, 24 months after Hg supplementation, the 66 kDa Hg proteic fraction was continuously determined in a prolonged senescence. Accordingly, the identified proteins associated to Hg show catabolic functions such as endopeptidases, hydrolases, glucosidases and nucleosidases. Stress associated proteins, like peroxidase and chitinase were also found associated to Hg. During the reproductive periods of vines, Hg was associated to membrane proteins, such as ATPases and lipid transfer proteins, especially in roots where Hg is absorbed.

Keywords Mercury · Proteins · Vines · Biological cycle

Introduction

Mercury (Hg) is a toxic heavy metal and an important environmental pollutant (Li et al. 2016a, b). It can be disposed to the environment mainly by industrial activities such as combustion, mining and manufacturing (Miotto et al. 2014). Hg has a long biological half-life and can accumulate in living organisms (Peralta-Videa et al. 2009). In humans, Hg can affect nervous, digestion and immune systems, being the main pathway of the food chain into human bodies through bio-magnification (Miotto et al. 2014).

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Plants take up essential and non-essential elements from soils in response to concentration gradients induced by selective uptake of ions by roots, or by diffusion of elements in the soil (Peralta-Videa et al. 2009). Plants can accumulate toxic elements like Hg that can be ingested by humans or biota. Absorption of toxic elements leads to a number of biochemical reactions in plants like the increase of reactive oxygen species and the displacement of protein cationic centers (Zhou et al. 2016). These proteins can be categorized into diverse functional classes, related to metabolic processes, photosynthesis, stress response, protein fate, energy metabolism, signaling pathways and immunosuppression (Liu et al. 2013). Despite the fact that effects of metal stress proteins expression and functionality have been described in plants, research describing specific metal associations to proteins is scarce.

There is a need of analytical techniques to reach systems-level approaches to understand how environmental metals interact with plants proteomes. To this purpose, size exclusion chromatography has been successfully applied to identify and isolate Hg proteic fractions in plant organs of Hg treated plants (Spisso et al. 2013; Yathavakilla and Caruso 2007). In water hyacinth (*Eichhornia crassipes*), the identified fractions were collected for protein identification and they were associated specifically with chloroplast and mitochondria proteins, related to photosynthesis, carbon fixation and electron transport chain in leaves (Pacheco et al. 2014).

Viticulture represents an important agricultural practice in many countries (Komárek et al. 2010). The irrigation water quality is an important variable defining the transportation of metals and other elements necessary for vine growth from soil toward the plant (Fabani et al. 2009). The annual growth cycle of fruiting grapevines is divided into a vegetative and a reproductive period (Keller 2015). These periods can be altered by stress factors such as drought, nutrient deficiency or metal excess (Mohr et al. 1995). Alteration of these periods can affect grapes productions by vines, with an impact on wine industry. Deeper insights of Hg metabolism in vines can conduct to develop strategies to counteract Hg contamination in vineyards.

The metal uptake capacity of *Vitis vinifera* has been reported before (Chopin et al. 2008; Leita et al. 1998; Todic et al. 2006), as well as a risk assessment of Hg irrigation with contaminated water (Spisso et al. 2013). Vines uptake inorganic Hg and translocate it from roots, through stems, to leaves transforming it to organic forms. In stems and leaves, Hg − S associations suggested a possible protein or peptide binding. Phytochelatins are peptides with sulfhydryl groups generated by plants in response to metal stress (Cobbett and Goldsbrough 2002). Later studies probed the presence of phytochelatins-Hg complexes in vines (Spisso et al. 2014).

The present research monitors Hg distribution in vine plants for a 2 years period after a single time Hg supplementation. To achieve this goal, plant organs like roots, stems and leaves, were collected at different periods after supplementation. Total Hg variations were evaluated. Hg-proteins associations were identified according to their molecular weight and Hg-S associations. Hg-S fractions were collected, and the specific proteins associated to Hg were determined by proteomics approaches. Biochemical pathways affected by Hg through protein binding in vines are discussed, along with Hg fractions variations during the annual growth cycle of vines.

Experimental

Hg administration to vines

Two groups of six plants, control and treated, were used to carry out the experiments under green house. The Hg treated group was administered with 100 mg L^{-1} Hg^{2+} for 4 days. One week after administration, roots, stems and leaves were sampled for analysis. Sampling continued 6, 12 and 24 months after Hg administration. Specific information regard plants cultivation, Hg administration and sampling can be observed in Sect. 1.1 of Supplementary Material.

Sample treatment and analysis

For total Hg determinations, samples were digested by microwave acid digestion (MAD). After liquid nitrogen homogenization, extractions of Hg fractions from samples were carried out with a solution of SDS-Tris and phenylmethylsulfonylfluoride solution.

Total Hg analysis were carried out by Inductively Coupled Plasma Mass Spectrometry (ICP MS). Proteic fractions were analyzed by Size Exclusion

Chromatography coupled to ICP MS (SEC-ICP MS). To this end wide molecular weight range column was used. Mercury and Sulfur were monitored for Hg proteic fractions identification, the last one as protein marker. Once Hg proteic fractions were identified, they were collected for protein identification. Protein identification was performed as follows: proteins were enzymatically digested with trypsin, and the resulting peptides were analyzed by nanoLC-ESI-ITMS^2. Proteins were identified by introducing the results to a MS/MS ion search engine. Specific details of Hg extraction and Hg analysis are depicted in Sect. 2 of Supplementary Material.

Results and discussion

Total Hg variations during the biological cycling of *Vitis vinifera*

Hg concentration in plant organs changes according to the annual growth cycles of vines, as observed in Fig. 1. Accordingly, after 7 days of Hg supplementation, vines showed a higher Hg concentration in roots compared to above ground organs like stems and leaves. Table 1 shows the different concentration values, which agree with previous studies (Spisso et al. 2013, 2014). Concentration in aerial organs is significantly lower, associated to a defense mechanism of plants, being Hg sequestered by roots to avoid damages to aerial organs (Peralta-Videa et al. 2009).

Six months after Hg administration, Hg concentration in roots, stems and leaves decreased, in agreement with vegetative period of vines. Lower Hg concentrations found in leaves are correspondent with a mechanism of Hg elimination in plants by releasing

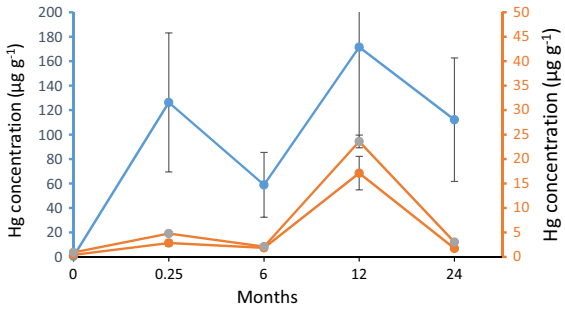

Fig. 1 Variations of total Hg concentrations in roots, stems and leaves of vines during a two years period. (Color figure online)

Table 1 Hg concentration in vine organs after Hg administration during a biological cycle

Months	Hg concentration (ug g^{-1})*		
	Roots	Stems	Leaves
0	0.6 ± 0.3	0.42 ± 0.2	0.5 ± 0.3
0.25	126.31 ± 49.2	2.84 ± 0.3	1.95 ± 1.1
6	58.95 ± 31.3	1.85 ± 0.9	0.26 ± 0.1
12	171.57 ± 70.9	17.13 ± 9.3	6.48 ± 4.1
24	112.2 ± 53.6	1.74 ± 0.9	1.3 ± 0.6

*n = 6

it in leaves via stomata to the atmosphere (Greger et al. 2005). However after 12 months of Hg administration, its concentrations rise again. This increase in Hg absorption can be attributed to a shift from vegetative, to reproductive periods in vines, or from dormant to active growth, being restored the metabolic processes of vines (Keller 2015). Dead material from vines after senescence could also contribute to sustain Hg pool in soil by metal turnover (Zheng et al. 1997). This necromass becomes important to metal budget of soil (Duarte et al. 2010). In addition, Hg in soil could not be leached by external factors since plants were cultivated in pots.

After 24 months of Hg supplementation, Hg concentrations in plant organs decreased, but basal Hg concentrations were not reach. This observation can be attributed to an inability of vines to remove Hg effectively before growth cycle ends. After 12 months of Hg supplementation, vines were in reproductive period and an increase of Hg absorption was observed. However, at 24 months, *Hg absorption did not increase again as expected for reproductive cycles, suggesting that this cycle is delayed, or the vegetative period is extended*. These effects of Hg in the biological cycle of vines, may decrease grape production rates caused by the extension of the vegetative period.

Variations of mercury proteic fractions during the biological cycle of vines

Proteins metabolism was monitored by SEC-ICP MS according to the different stages of growth cycle. SEC differentiates proteins according to their molecular weight. ICP MS analyzed sulfur as protein marker, and

Hg to determine associations of this metal to different proteins. Extracts of roots, stems and leaves were analyzed and results can be observed in Figs. 2, 3 and 4, respectively.

Roots

During the reproductive period, there is an active metabolism of vines. These processes can be observed in Fig. 2, since at 7 days after Hg supplementation, different molecular weight proteins fractions of 200, 66 and 29 kDa can be observed according to S signal. Hg is associated to protein fractions of 150–66 kDa. Fractions of 669 kDa correspond to remains of membranes or organelles formed during the extraction, since the molecular weight is too high to be considered as proteins. After 6 months of Hg supplementation vines were in vegetative period, and proteins of 200 kDa, with less S signal, are being catabolized to smaller fractions of < 66 kDa. At this point, Hg signal decreases according to a lower metabolism rate of vines, being associated to 150–66 kDa proteic fractions. At 12 months after supplementation, Hg levels raise again similar to the reproductive stage of vines, *however proteins distribution continues as vegetative periods*, being proteic fractions below 66 kDa more abundant. This observation sustain the idea that an extension of senescence and vegetative period occurs after Hg stress. Similar results were observed at 24 months after Hg supplementation. Similar results has been observed in rice roots where the related protein expression levels changed in response to Hg stress (Clemens and Ma 2016). In addition, proteins catabolism in plants not only regulates protein processing and intracellular protein levels, but removes abnormal or damaged proteins from the cell, formed by oxidation under metal stress (Kang et al. 2015).

Stems

In Fig. 3 it is observed that at 7 days after Hg supplementation, this metal is bound to 443 and 150 kDa proteins. Sulfur fractions correspond mostly to < 66 kDa, however fractions of 66, 200 and 443 kDa can also be observed, correspondent with the reproductive period of vines. After 6 months and associated to vegetative period, proteic fractions of 200 and 66 kDa were determined, the latest in higher intensity. Similar observations were made at 12 months after Hg supplementation, only a < 66 kDa proteic fraction could be found, as if vegetative period was sustained in time. Compare to roots, no significant increase in Hg signal was observed at this stage. *This could be associated to an active transport of Hg from roots to leaves, through stems, with no accumulation.* After 24 months of Hg administration, proteic fraction of 150 kDa and < 66 kDa are observed again, comparable with reproductive periods.

Leaves

Since vine plants were grown in greenhouse conditions, plants never reached a complete vegetative period, where leaves are lost completely. For this reason, leaves could be sampled at every studied period. In Fig. 4, at 7 days after Hg supplementation during reproductive period, proteic fractions of 66 and 29 kDa were observed according to sulfur signal. Hg was associated to the 66 kDa fraction. At 6 months after Hg supplementation during vegetative period, proteic fractions of 150 and 66 kDa were determined and Hg was associated to the 150 kDa fraction, but at lower concentrations. The Hg signal decrease observed in leaves is in good agreement with Hg decrease observed in roots. At 12 months Hg signal rise again and it is associated to 150 kDa proteins. Quite the opposite, Hg signal decreased again at 24 months and no associations of proteins could be observed, except for the 669 kDa fraction. This Hg association was observed during all the sampled periods. In leaves, no catabolism of 150 kDa fractions to < 66 kDa fraction was detected, being related to a continued photosynthesis where chlorophyll was not decomposed under greenhouse conditions.

Proteins associated to Hg during the biological cycle of vines

Mercury has a particularly high affinity for thiol groups and coordination to cysteines is the dominant mechanism for Hg–protein interactions (Li et al. 2016b). The path of Hg from the soil into vegetative and reproductive organs of the plant are dependent of transporters, metal-handling proteins, and low-molecular-weight metal chelators (Clemens 2006). Several studies have reported changes in proteins functions

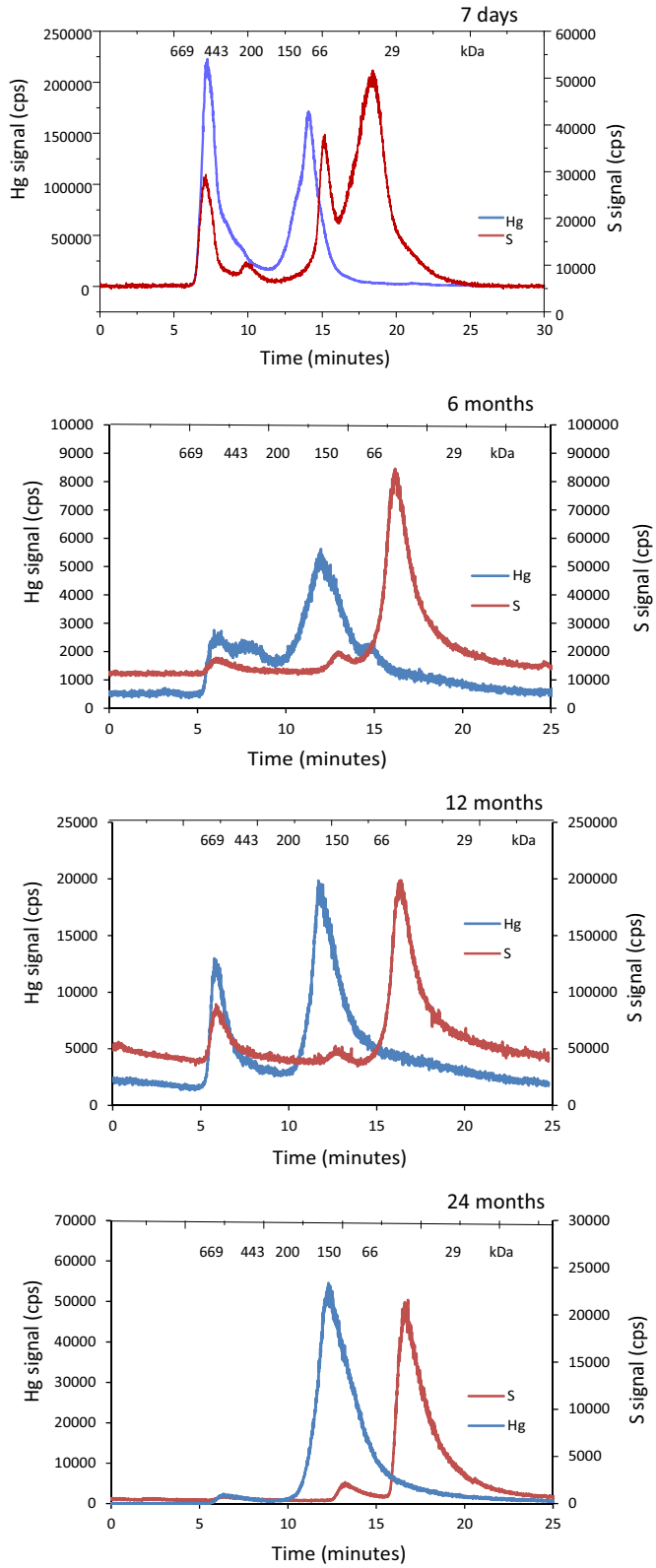

Fig. 2 SEC-ICPMS chromatogram of root extracts from vines at different periods after Hg supplementation. Molecular weight markers can be observed at the top of the figure. (Color figure online)

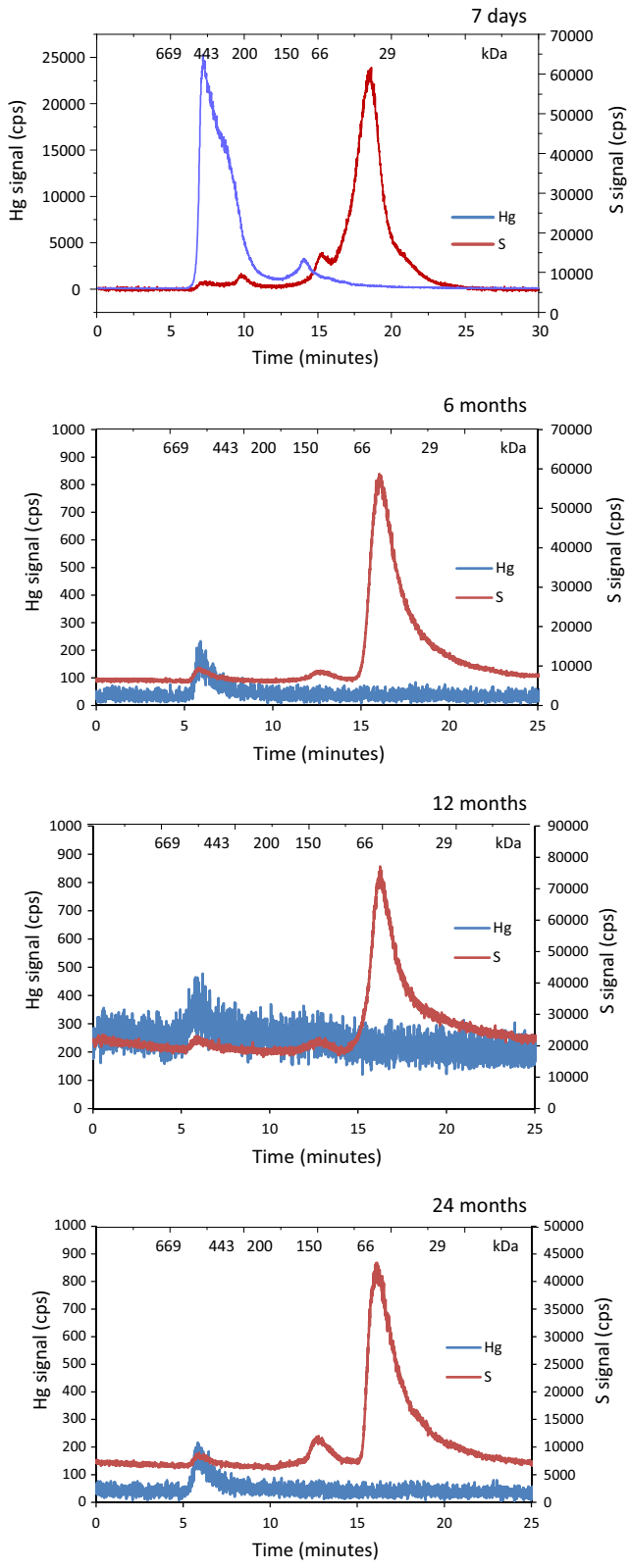

Fig. 3 SEC-ICPMS chromatogram of stems extracts from vines at different periods after Hg supplementation. Molecular weight markers can be observed at the top of the figure. (Color figure online)

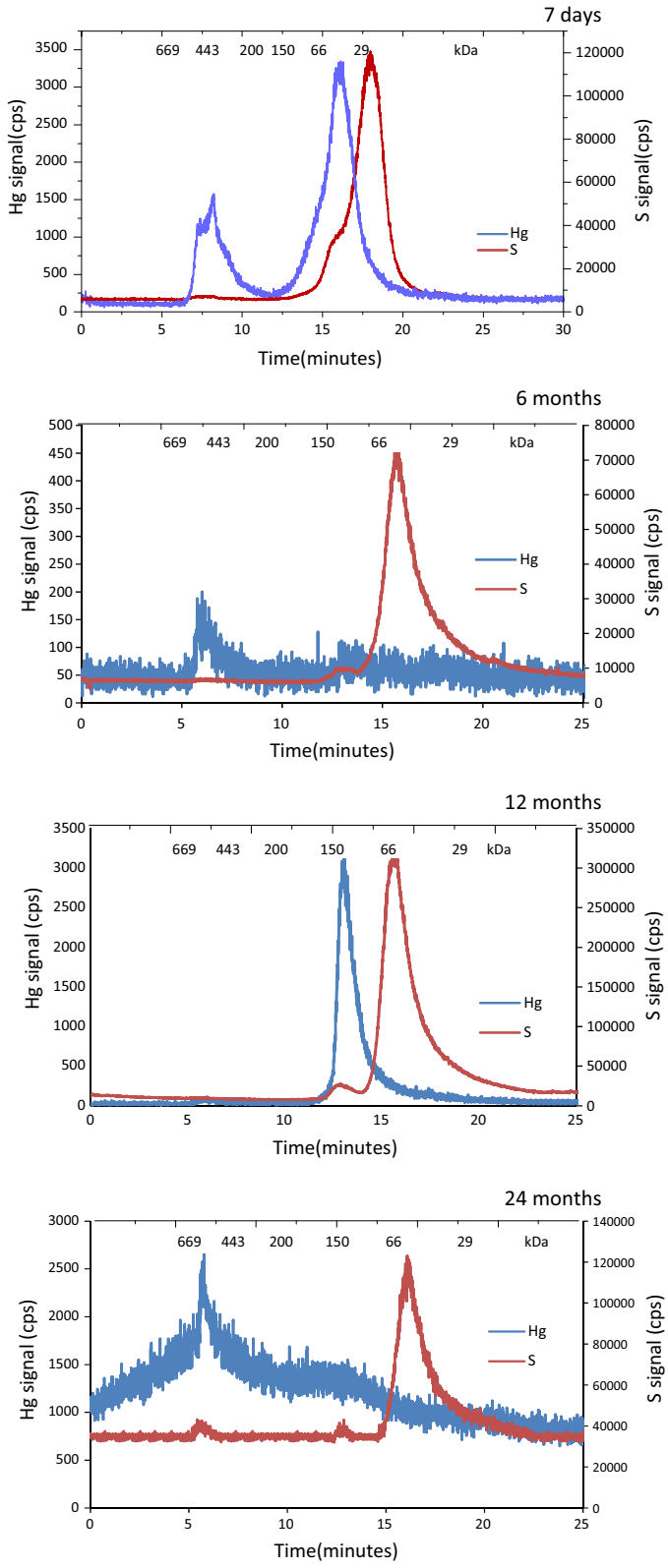

Fig. 4 SEC-ICPMS chromatogram of leaves extracts from vines at different periods after Hg supplementation. Molecular weight markers can be observed at the top of the figure. (Color figure online)

(Chen et al. 2012) and expression (Li et al. 2016a; Liu et al. 2013) in plants, related to Hg stress. To contribute to identify proteins associated to Hg in vine plants, proteic fractions from SEC were collected and enzymatically digested. Proteins were identified by peptide mapping, and results can be observed in Table 2, according to vine organs and sampling time after Hg supplementation. Protein function, as well as cellular location, according to UniProt database, are also depicted.

ATPase

Proteins associated to Hg with ATPase activity, coupled to transmembrane movement of substances, were found in roots, at 0.25 and 6 months after Hg administration. This protein is located in cell and mithocondrial membranes. At early stages of Hg administration, during reproductive period of vines, Hg is absorbed at elevated rates, explaining Hg association to this type of protein. However, at 6 months after Hg administration, plants are in a vegetative period, and Hg absorption remains, probably due to an alteration of the growth cycle by metal stress. In plants, heavy metal–transporting ATPases were described. ATPases function as integral membrane proteins coupling ATP hydrolysis to metal cation transport (Clemens and Ma 2016). A sufficiently high concentration of Hg can reduce ATP production and water and solutes transport across membranes (Kang et al. 2015). Hg up-regulates ATP dependent transporters in rice seedlings (Chen et al. 2014). This Hg-ATPase association was also found in stems 12 months after administration related to an active transport of Hg from roots to leaves through stems during reproductive period of vines for elimination.

Endopeptidases

Proteolytic enzymes play a key role in plant physiology. They not only maintain the protein pool of the cell, but also are involved in various intra- and extracellular processes like leaf senescence, breakdown of storage proteins in germinating seeds (Kembhavi et al. 1993), development and ripening of fruits, regulatory mechanisms, among others. Tissues with high metabolic rate have shown elevated endopeptidases activity (Pande et al. 2006). In roots, two different serine-type endopeptidase inhibitors were found associated to Hg, 0.25 months after Hg administration, when vines were metabolically active. These proteins are expressed in response to wounding and defoliation. Twelve months after Hg administration a cysteine-type endopeptidase was found associated to Hg. *This protein plays an important role in plant immunity* since it possesses four Hg-binding sites, with the motive CXXC (where C is cysteine, and X is any amino acid) (Li et al. 2016b).

Peroxidases (POD)

These type of proteins have metal binding functions through CXXC and CCXX amino acidic sequences, and response to oxidative stress. They are secreted to the extracellular interstice. PODs were found in roots associated to Hg in almost every stages of the biological cycle of vines. A remarkable decrease in PODs expression in response to Hg stress was observed, consistent with other studies where POD activity was inhibited by heavy metals in horseradish hairy roots (Li et al. 2016b). In addition, an up regulation of POD in wheat seedlings roots was found in response to Hg stress (Kang et al. 2015).

Hydrolases

Proteins related to carbohydrate metabolism were also associated to Hg in vines, specifically hydrolases of O-glycosyl compounds. These proteins were identified in roots and leaves, organs with elevated metabolic rates, at 0.25 and 24 months after Hg supplementation, in reproductive and vegetative periods of vines, respectively. The effects of Hg stress on carbohydrates have been described in literature. Proteins related to carbohydrate metabolism were affected in wheat seedlings (Kang et al. 2015), decreasing in leaves in 71.0%. In rice roots, Hg induced biochemical and proteomic changes in carbohydrate metabolism (Chen et al. 2012). *Hg effects on carbohydrates metabolism in vines can affect wine production due to a lower carbohydrate concentration in grapes.*

Glucosidases

Glucan endo-1,3-β-glucosidase is an hydrolytic enzyme that attack the cell wall of plants pathogens (Mohr et al. 1995), and is an anchored component of

Table 2 Proteins associated to Hg in vines according to biological cycle

Period (months)	Entry	Protein	Function	Location
Roots				
0.25	F6GU68	Putative uncharacterized protein	ATPase activity, coupled to transmembrane movement of substances	Membrane, mitocondrial membrane
	D7T7B5	Putative uncharacterized protein	Serine-type endopeptidase inhibitor activity, response to wounding	Not indicated
	F6GUF3	peroxidase	Metal ion binding, response to oxidative stress	Extracellular, secreted
	Q6YEY6	Protease inhibitor	Serine-type endopeptidase inhibitor activity, response to wounding	Not indicated
	F6I685	Putative uncharacterized protein	Hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process	Not indicated
6	A7PQW3	Glucan endo-1,3-beta-glucosidase	Implicated in the defense of plants against pathogens	Anchored component of plasma membrane
	A5AJB3	Putative uncharacterized protein	Chitinase activity, cell wall macromolecule catabolic process	Intracellular
	Q850K5	Non-specific lipid-transfer protein	Plant non-specific lipid-transfer proteins transfer phospholipids as well as galactolipids across membranes. May play a role in wax or cutin deposition in the cell walls of expanding epidermal cells and certain secretory tissues	Not indicated
	F6GU68	Putative uncharacterized protein	ATPase activity, coupled to transmembrane movement of substances	Membrane, mitocondrial membrane
	F6GUF3	Peroxidase	Metal ion binding, response to oxidative stress	Extracellular, secreted
12	F6GUF3	Peroxidase	Metal ion binding, response to oxidative stress	Extracellular, secreted
	F6H3E6	Putative uncharacterized protein	Catalytic activity, nucleoside metabolic process	Not indicated
	F6HMA2	Putative uncharacterized protein	cysteine-type endopeptidase activity, proteolysis involved in cellular protein catabolic process	Not indicated
24	A5AJB3	Putative uncharacterized protein	chitinase activity, cell wall macromolecule catabolic process	Intracellular
Stems				
12	F6GU68	Putative uncharacterized protein	ATPase activity, coupled to transmembrane movement of substances	Membrane, mitocondrial membrane
Leaves				
0.25	A7PQW3	Glucan endo-1,3-beta-glucosidase	Implicated in the defense of plants against pathogens	Anchored component of plasma membrane

Table 2 continued

Period (months)	Entry	Protein	Function	Location
24	A7PQW3	Glucan endo-1,3-beta-glucosidase	Implicated in the defense of plants against pathogens	Anchored component of plasma membrane
	A5AYL3	Putative uncharacterized protein	Hydrolase activity, hydrolyzing O-glycosyl compounds, carbohydrate metabolic process	Not indicated
	D7SSN5	Chitotriosidase-1	Chitinase activity, carbohydrate metabolic process	Extracellular

the membrane. This protein was found associated to Hg in roots and leaves of vine plants at reproductive and vegetative stages. *In this way Hg might be affecting vines capacity to defend against patogens, increasing its vulnerability to infections.* Glucosidases also play a fundamental role in the breakdown of cell wall polysaccharides and allows sugar to be available for senescing cells (Mohapatra et al. 2010). Plants under metal stress showed accelerated senescence symptoms (Gómez-Sagasti et al. 2016).

Chitinase

The major function of chitinase is the catalysis of the hydrolytic cleavage of the β-1,4-glycoside bond of N-acetylglucosamine (Li et al. 2016b). It is also associated with a defense mechanism against a variety of pathogens as well as abiotic stresses, such as osmotic, dehydration, low temperature and wound stress. Oligosaccharide can act as a signal for defense responses in plants. Chitinase can work indirectly by releasing oligosaccharide to activate plant defense response (Li et al. 2016b). Chitinases have shown a different expression in response to methyl-mercury and inorganic mercury stress in rice roots. In vines, an intracellular putative uncharacterized protein related to cell wall macromolecule catabolic processes was found associated to Hg in roots, during vegetative period. In leaves, at the same period of the growth cycle, chitriosidase-1 was found associated to Hg. However this chitinase is extracellular related to carbohydrate metabolic processes.

Lipid transfer proteins

A non-specific lipid-transfer plant protein, transferring phospholipids and galactolipids across membranes, was found associated to Hg in roots during vegetative period of vines. This protein may play a role in wax or cutin deposition in the cell walls of expanding epidermal cells and certain secretory tissues. Hg ion has been reported to bind directly with lipid–protein complexes of membranes, resulting to an exposed lipid–protein complexes (Puzon et al. 2014).

Nucleosidase

Nucleosides after being import into cells, can be phosphorylated to nucleotides. Nucleotides are major energy carriers and they are building blocks of nucleic acids (DNA and RNA) (Möhlmann et al. 2010). In vines, a nucleosidase was found associated to Hg in roots at vegetative stages. Nucleosidases are hydrolytic enzymes producing D-ribose and purine base.

Hg extended senescence in vines and this was reflected on its associations to catabolic proteins, like endopeptidases, hydrolases, glucosidases and nucleosidases. Stress associated proteins, peroxidase and chitinase, were found associated to Hg and identified. On the other hand, during reproductive periods of vines, Hg was found associated to membrane proteins, such as ATPases and lipid transfer proteins, especially in roots where Hg is absorbed.

Conclusions

The effects of mercury on vines metabolism were studied. Elevated Hg uptake was observed in vines during reproductive stages. This elevated metabolic rate was also reflected in the different molecular weight Hg fractions determined. However during vegetative stages, high molecular weight fractions were catabolized to lower molecular weights. In

addition, an extension in time of the vegetative period was observed. An alteration of this period in vines can negatively affect grapes production.

The identification of proteins associated to Hg showed effects on carbohydrates metabolism and defense against pathogens in vines. *Hg tropism towards these metabolic pathways can affect wine production due to a lower carbohydrate concentration in grapes and an increased vulnerability of the plant to infections.* Deeper insights of Hg metabolism in vines can conduct to develop strategies to counteract Hg contamination in vineyards.

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