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Antioxidative responses of cell suspension cultures of two *Coffea arabica* varieties to low aluminum levels at pH 5.8

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ABSTRACT - (Antioxidative responses of cell suspension cultures of two *Coffea arabica* varieties to low aluminum levels at pH 5.8). The effects of aluminum (Al) on the activities of antioxidant enzymes and ferritin expression were studied in cell suspension cultures of two varieties of *Coffea arabica*, Mundo Novo and Icatu, in medium with pH at 5.8. The cells were incubated with 300 μM Al^{3+}, and the Al speciation as Al^{3+} was 1.45% of the mole fraction. The activities of superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) were increased in Mundo Novo, whereas glutathione reductase (GR) and guaiacol peroxidase (GPOX) activities remained unchanged. SOD, GR, and GST activities were increased in Icatu, while CAT activity was not changed, and GPOX activity decreased. The expression of two ferritin genes (*CaFer1 and CaFer2*) were analyzed by Real-Time PCR. Al caused a downregulation of *CaFER1* expression and no changes of *CaFER2* expression in both varieties. The Western blot showed no alteration in ferritin protein levels in Mundo Novo and a decrease in Icatu. The differential enzymes responses indicate that the response to Al is variety-dependent.
Key words: aluminum toxicity, antioxidant enzymes, ferritin, oxidative stress

RESUMO - (Resposta antioxidante de células em suspensão de duas variedades de *Coffea arabica* submetidas à baixa concentração de alumínio em pH 5.8). Foram estudadas a atividade de enzimas antioxidantes e a expressão da ferritina em culturas celulares de duas variedades de *Coffea arabica*, Mundo Novo e Icatu, tratadas com alumínio (Al) em meio de cultura com pH 5.8. As células foram incubadas com 300 μM Al^{3+}, e a especiação do Al para Al^{3+} foi de 1.45%. Para Mundo Novo, a atividade da superóxido dismutase (SOD), catalase (CAT) e glutationa S-transferase (GST) aumentou, enquanto que a atividade da glutationa redutase (GR) e da guaiacol peroxidase (GPOX) permaneceu inalterada. Para Icatu, a atividade da SOD, GR e GST aumentou, a atividade da CAT não se alterou e da GPOX diminuiu. Foram analisadas a expressão de dois transcritos da ferritina (*CaFer1 e CaFer2*) por meio de PCR em Tempo Real. O Al provocou queda na expressão do *CaFER1* e nenhuma alteração em *CaFER2* nas duas variedades. Não houve alteração no acúmulo de ferritina para Mundo Novo, mas uma diminuição para Icatu. A resposta diferencial das enzimas indica que a resposta antioxidante é variedade dependente.
Palavras-chave: enzima antioxidante, estresse oxidativo, ferritina, toxicidade do alumínio

Introduction

Many types of abiotic and biotic stresses induce the formation of reactive oxygen species (ROS) in plant cells (Boscolo *et al*. 2003). ROS are potentially dangerous molecules, whose production must be finely controlled in cells as they have a remarkable capacity to cause dramatic physiological damage (Gratão *et al*. 2005). However, the biosynthesis of ROS molecules cannot be completely eliminated due to their functions as signalling molecules, being important parts of plant defence against abiotic and biotic stresses (Strozycki *et al*. 2003, Gratão *et al*. 2005,).

To minimise the damaging effects of ROS, plant cells possess protection systems based on

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both non-enzymatic and enzymatic antioxidant defences (Scandalios 2005). Glutathione (GSH), cysteine, the hydroquinones, mannitol, vitamins C and E, the flavonoids, some alkaloids, and β-carotene are examples of non-enzymatic defences, while enzymatic antioxidant defences include enzymes such as catalase, peroxidases and dismutases, and enzymes of the ascorbate-glutathione cycle, among others (Azevedo *et al*. 1998, Scandalios 2005). Both non-enzymatic and enzymatic antioxidant defences work to efficiently reduce excess ROS production under normal conditions, and without them, the toxic oxygen species can have harmful effects, such as lipid peroxidation and protein and DNA oxidation, leading, in many cases, to cell death (Boscolo *et al*. 2003, Strozycki *et al*. 2003).

Aluminum (Al) is not a transition metal and cannot catalyze redox reactions; however, the Al^{3+}, the most toxic of the soluble forms of Al, is involvement in the induction of oxidative stress in many plant species (Yamamoto *et al*. 2003). In general, for soils with pH > 5.5, Al concentration is lower than 37 μM, but with the pH decreases Al is released into soil solution (Ramírez-Benítez *et al*. 2008). Al is the major growth-limiting factor for plants on acid soils, and at micromolar concentrations it can inhibit plant root growth (Kochian *et al*. 2002). Due these aspects, Al toxicity is becoming an economically important issue, because acid soil comprise up 70% of the world's arable lands, limiting the growth of important food crops and causing severe food losses (Kochian *et al*. 2002, Chen *et al*. 2010). The major recognised symptom of Al toxicity is the inhibition of root growth, but it can also provoke a reduction in respiration and ATP synthesis (Delhaize & Ryan 1995, Yamamoto *et al*. 2003).

Some plant species and cultivars have developed tolerance strategies to Al toxicity, as increasing the antioxidant enzymes activities and changing the expression of various genes induced by Al presence (Pereira *et al*. 2011). Al induces the expression of genes that have already been characterised in wheat, maize, sugarcane, tobacco and *Arabidopsis* (Simonovicova *et al*. 2004, Pereira *et al*. 2011). Richards *et al*. (1998) observed high levels of peroxidase (PER) mRNA in *A. thaliana* exposed to Al during 48 h (50 μm Al^{3+}), and low of glutathione S-transferase (GST) and superoxide dismutase (SOD) mRNAs. In the same study, catalase (CAT) mRNA declined during Al stress. In tobacco cells it was also observed that the activities of peroxidases are induced by Al stress (Ezaki *et al*. 1996). Pea plants (*Pisum sativum* L.) submitted to Al treatment had an increase of genes encoding the enzymes GST, CAT, SOD, and ascorbate peroxidase (Panda & Matsumoto 2010). Several other genes are induced by Al but in general they are related stress responses, like oxidative stress, pathogen infection, heat shock, metal stress, and hormone treatment (Ezaki *et al*. 2001).

Ferritin is a protein found in all the living kingdom, except in yeast, that can store up to 4,500 iron (Fe) atoms in their central cavities (Briat *et al*. 2010b). In animal cells, ferritin is involved in the response against stresses resulting from ultraviolet irradiation, phorone (a glutathione-depleting drug), and excess iron (Vile & Tyrrell 1993, Cairo *et al*. 1995, Déak *et al*. 1999). In plants, ferritin gene expression seems be modulated by different environmental factors, and many reports have suggested that it plays an important role in protecting cells against oxidative stress (Ravet *et al*. 2009, Briat *et al*. 2010b). The iron excess results to an increase in ROS, provoking oxidative stress. The synthesis of ferritin is induced by iron, H_2O_2, NO or ozone application, and high light intensity, and antagonized by antioxidant molecules (Briat *et al*. 2010a).

Coffea is the most economically important genus of Rubiaceae family and is a very important crop in several tropical countries (Ramírez-Benítez *et al*. 2009). In Brazil, one of the largest coffee exporter in the world, 40% of coffee plantations are established in soils with low pH (3.7 to 5.0), with high Al content (Rodrigues *et al*. 2011). Although Al toxicity is a problem for coffee-producing countries, there are only a few reports about tolerance and susceptibility of coffee plantations to high Al concentration in soil or nutrient solution (Pavan & Bingham 1982, Rodrigues *et al*. 2011). Some recent studies reported that soil superficial layer corrected with lime and fertilizers allows the normal growth of aerial parts of coffee tree cultivated in Al-rich subsoils, although had affected root distribution with depth (Rodrigues *et al*. 2011). Ramírez-Benítez *et al*. (2008) have analyzed the induction of organic acids in *C. arabica* cells treated with 100 μM $AlCl_3$, these organic compounds are considered to be a major factor in plant Al resistance. Pinheiro *et al*. (2004) have studied the roles of antioxidant enzymes in coffee plants in response to drought, but no study has been carried out trying to establish a relationship between Al toxicity, ferritin induction, and the mechanisms of antioxidant defense in this crop.

For many reports of Al toxicity and plant antioxidant responses, highly stress-inducing metal concentrations are used, and consequently, significant metabolic changes are a rule. For most of crops, the maintenance of a soil pH ranging from 5 to 6 is recommended, but even at this pH, soluble toxic Al may still be available in very low concentrations. Therefore, the aim of this work was to study the effects of low Al concentrations on the enzyme antioxidant system, including ferritin, in coffee cell suspension cultures. Because we wanted to isolate the effects of Al from low pH soil, the medium pH was kept at 5.8, and the Al concentration was increased to a level (300 μM Al^{3+}) that was higher than the dose used in the literature for pH 4.3 (200 μM $AlCl^3$) (Ramírez-Benítez *et al*. 2009).

Material and methods

Coffee cell suspension culture - Leaves of the third and fourth leaf pairs of *Coffea arabica* L. var. Mundo Novo (IAC 388-1) and the tolerant hybrid Icatu (IAC 404-5; *C. arabica* × *C. canephora*) were used to produce the cell cultures. These varieties are widely cultivated in Brazil. Icatu was included in this study because, although not confirmed in several field trials, it has been suggested as an Al-tolerant variety (Rodrigues *et al*. 2011). The explants were maintained in CIM (callus inducing medium) solid medium (Neuenschwander & Baumann 1992) containing MS salts (Murashige & Skoog 1962) at pH 5.8 and supplemented with 10 mg L^{-1} thiamine-HCl, 100 mg L^{-1} inositol, 30 g L^{-1} sucrose, 4 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D. Calluses that were produced in 12-13 weeks in the dark and presented with a pale-yellow colour and a friable aspect were desegregated, transferred to 30 ml of liquid CIM medium in a 250 ml Erlenmeyer flasks and maintained at 100 rpm in the dark at 25 ± 2 °C. Every week, half of the cells from the flask were transferred to a new flask containing 15 ml of fresh CIM medium. At this stage, a forceps was used to eliminate large aggregates to obtain uniform cell suspensions formed by small homogeneous aggregates.

Aluminium cell treatments - To induce Al stress, seven-day-old coffee suspension cells were suction-dried, and approximately 4 g of cells were weighed and transferred to a new flask, containing 50 ml of fresh liquid CIM medium with or without 300 μM Al^{3+} as $AlK(SO_4)_2.12H_2O$. The flasks were incubated for 72 h (100 rpm in the dark at 25 ± 2 °C), and the cells were harvested by vacuum filtration on filter paper, washed with 100 mL of 500 mM NaCl to remove any loosely associated Al and then washed with abundant distilled deionised water. Washed cells were immediately frozen in liquid nitrogen and stored at -80 °C for further analyses. Small portions of the cells were freeze-dried for lipid peroxidation determinations. Previous results have shown that under the cultivation conditions used here, seven-day-old coffee cells are in the exponential growth phase (Filippi *et al*. 2007).

Aluminium concentration in the cells - The cells were harvested by filtration, washed with 500 mM NaCl (to desorb Al trapped in the cell walls) and distilled water, dried (60 °C for 76 h), weighed and ground to a powder in a mortar with a pestle. Al concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES; JobinYvon, JY50P) after HNO_3-$HClO_4$ digestion. To obtain an estimate of the free Al^{3+} in the medium, we used the Geochem software (http://www.plantmineralnutrition.net/Geochem/Geochem%20Download.htm), applying only the information regarding the mineral composition.

Real-Time PCR Analyses of ferritin genes - RNA extraction, cDNA synthesis and quantitative real-time PCR analysis of *CaFER1* (GenBank GQ913984) and *CaFER2* (GenBank GU001880) were carried out essentially as described by Bottcher *et al*. (2011). Ribosomal protein rpl39 was used as an endogenous control. Three technical replicates were obtained for each of three biological replicates of each sample and PCR reactions in the absence of template were also performed as negative controls for each primer pair. Data were analyzed using the threshold cycle (Ct), which is the fractional cycle number at which a fixed amount of DNA is formed. The relative gene expression was presented using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Total protein extraction and Western blot analysis - The coffee cells harvested by filtration were subjected to total protein extraction. Initially, the cells were homogenised in 5 ml 100 mM HEPES (pH 7.0), 2 mM EDTA, 5 mM $MgCl_2$, 2% ascorbic acid and 10 mM 2-mercaptoethanol. The homogenate was incubated for 30 min on ice and then centrifuged

(27,000 × *g* for 25 min at 4 °C), and the supernatant was filtered in a PD10 Sephadex G25 column (Amersham Biosciences). The proteins were eluted with 20 mM HEPES (pH 7.0), and the total soluble protein was quantified (Bradford 1976), using bovine serum albumin (Sigma) as the standard. The Western blot analysis were carried out essentially as described by Bottcher *et al*. (2011). The polyclonal anti-ferritin antibody was kindly supplied by Dr. Janette Palma Fett (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). The quantification was carried out by measuring the intensity of the bands in relation to the control, using the ImageJ software (http://rsbweb.nih.gov/ij/).

Enzyme extraction and assays - Samples were extracted as for the Western blot analysis and stored at -80 °C until the superoxide dismutase (SOD, EC 1.15.1.1), guaiacol peroxidase (GPOX, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), glutathione S-transferase (GST, EC 2.5.1.18) and glutathione reductase (GR, EC 1.6.4.2) activity analyses. Total CAT activity was determined as described by Azevedo *et al*. (1998). SOD, GR and GPOX activities were determined as described by Gomes-Junior *et al*. (2006a). Activity staining following non-denaturing PAGE was carried out for total SOD activity (Gomes-Junior *et al*. 2006a). GST activity was determined as described by Habig & Jakoby (1981).

Lipid peroxidation - Measurements were taken from a 0.25 g tissue sample, in which the amount of malondialdehyde (MDA) was determined by the thiobarbituric acid-reactive substances (TBARS) (Cakmak & Horst 1991).The concentration of MDA was calculated, using an extinction coefficient of 155 $mmol^{-1}\ cm^{-1}$.

Statistics - The one-factor analysis of variance (ANOVA) test was used to assess differences in *CaFER1* and *CaFER2* gene expression between Al-treated and non-treated cells and to analyse TBARS concentrations and CAT, GR, GPOX and GST activities. When a significant variation was found, the Tukey test was used as a post hoc comparison to adjust the *p* values. Al accumulation data were expressed as the standard error of the mean (± SEM) of three independent replicates. The statistical significance was set to an α level of 5%, and the analysis was carried out with the BioEstat 3.0 program (Ayres *et al*. 2003).

Results

Aluminium content in the cells and free Al^{3+} in the medium - Mundo Novo cells treated with Al accumulated 4,500 mg Al kg^{-1} dry weight, while Icatu accumulated 4,350 mg Al kg^{-1}. Al was not detected in the control cells. Free Al^{3+} in the medium was calculated, using the Geochem software, and it was 1.43%, corresponding to 4.35 µM Al^{3+}.

Lipid peroxidation - No statistical differences were observed in lipid peroxidation among Mundo Novo and Icatu control and Al-treated cells. The values ranged from 3.67 to 4.00 nmol g^{-1} (figure 1).

RT-qPCR and Western blot analyses of ferritin - Al induced a downregulation of *CaFER1* expression in suspension cells of the varieties Mundo Novo ($p < 0.05$, figure 2A) and Icatu ($p < 0.05$, figure 2B) of *C. arabica*. An approximately five-fold decrease was observed for Mundo Novo and a two-fold decrease for Icatu compared to their respective controls. No significant changes were detected for *CaFER2* mRNA levels in Mundo Novo ($p = 0.0582$, figure 2A) and Icatu ($p = 0.7655$, figure 2B). The Western blot analyses did not show alterations in the protein ferritin levels in the Mundo Novo cells (figure 3A), but a decrease of 1.66-fold (relative intensity to the control) was observed in the Icatu cells (figure 3B). Relative abundance of ferritn protein in coffee cells treated with Al was obtained by densitometric analysis.

Enzyme activities – In some ways, the activities of all antioxidant enzymes were altered by Al, and differences were observed between the two coffee

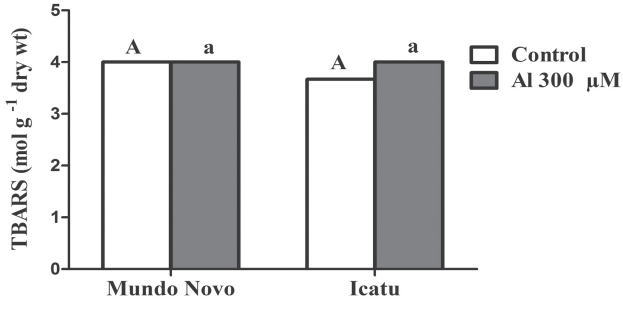


Figure 1. Thiobarbituric acid-reacting substances (TBARS) content in varieties of Mundo Novo and Icatu suspension cells grown for 72 h with 300 µM Al. Means with the same letter are not significantly different ($p < 0.05$) by the Tukey's test.

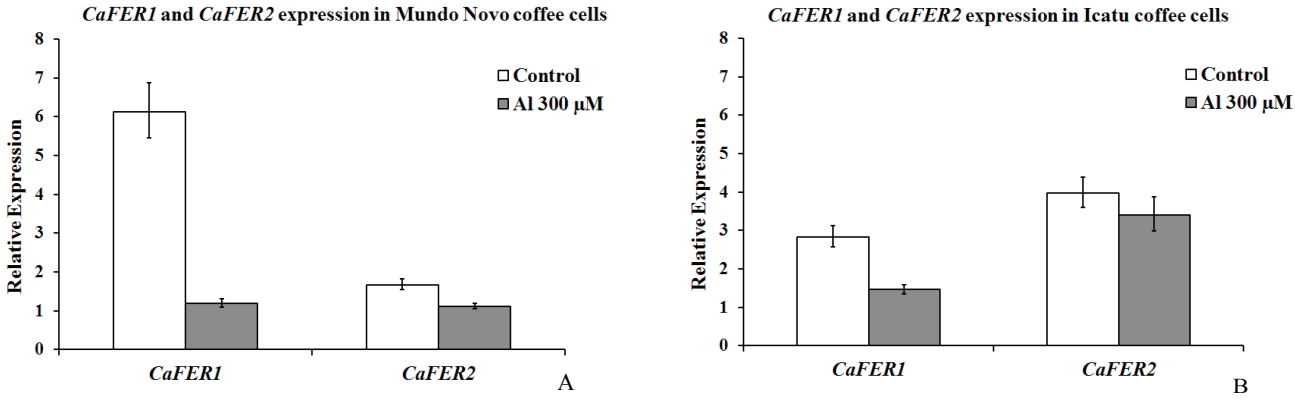


Figure 2. Quantitative PCR analysis of mRNA from the coffee ferritin genes *CaFer1* and *CaFer2* in Mundo Novo (A) and Icatu (B) varieties treated with 300 µM Al compared to the constitutively expressed rpl39 gene. Columns represent gene expression average values obtained from three independent replicates and vertical bars indicate minimum and maximum relative expression values of three independent replicates.

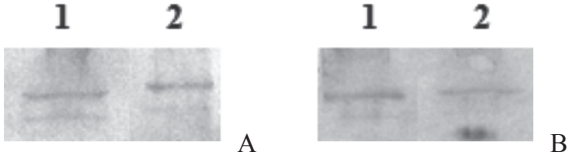


Figure 3. Western Blot analysis of ferritin protein in Mundo Novo (A) and Icatu (B) cells, using a polyclonal anti-ferritin antibody. All reactions were performed using 20 µg of total protein extracts. Lane 1 – control; lane 2 – 300 µM Al.

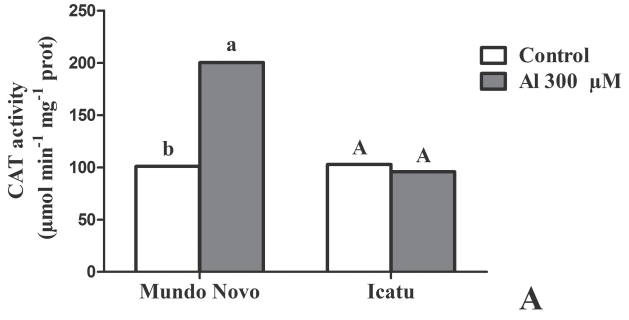
varieties (figure 4). The CAT activity in Mundo Novo was responsive to the presence of the Al, showing a 1.9-fold increase, while no variation was observed in the Icatu cells (figure 4A). The SOD activity assessed by non-denaturing PAGE revealed one major band whose activity was shown to increase in both coffee cells when treated with Al (figure 4B), but the response was more intense in the Mundo Novo cells. The total GR activity showed no significant increase in the Mundo Novo cells treated with Al, but in the Icatu cells, the activity increased by approximately 1.6-fold compared to the control cells (figure 4C). GPOX activity did not show significant variation in the Mundo Novo cells exposed to Al, while a 1.4-fold decrease was observed in the Icatu cells (figure 4D). GST levels increased in the cell suspensions of both varieties, exhibiting 1.6- and a 3.1-fold increases in the cells of Mundo Novo and Icatu, respectively (figure 4E).

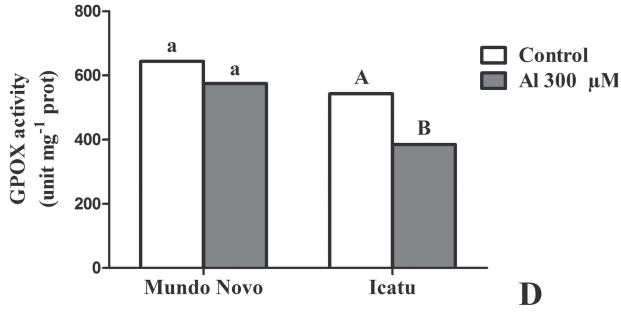
Discussion

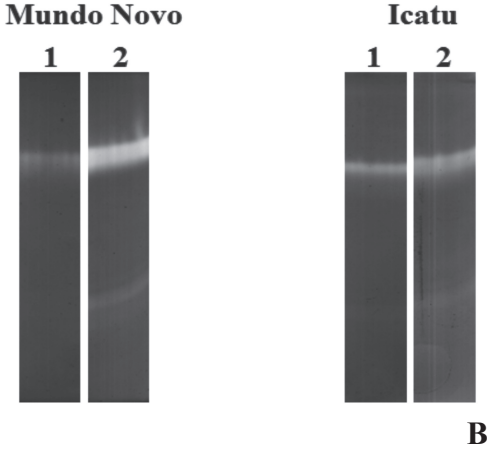
In alkaline, neutral or slightly acidic soils, Al exists in forms that are not harmful to plants, but at pH < 5.5, it increases in solubility (as Al^{3+} speciation) and may be toxic to plants once it is taken up by the root cells, producing negative effects on plant growth and developmental processes (Goodwin & Sutter 2009).

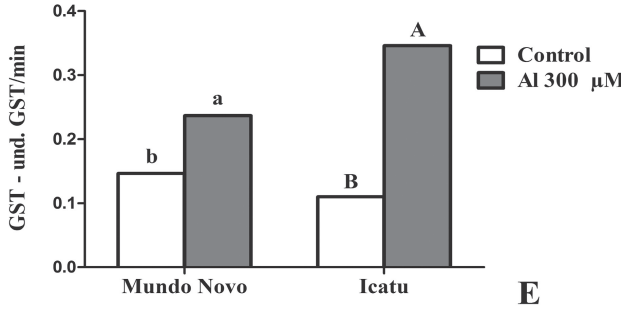
Root growth inhibition, reduction in water absorption and nutrient uptake (Delhaize & Ryan 1995), ROS production and lipid peroxidation (Cakmak & Horst 1991) are characteristic responses to Al stress. Even very low Al levels may cause significant metabolic responses in plants. Cucumber roots exposed to low levels of Al (1 µM and 10 µM of $Al_2(SO_4)_3$) at pH 4.0 showed increased CAT and SOD activities and lipid peroxidation. Increased H_2O_2 levels were observed only after exposure to higher concentrations of Al (Pereira *et al*. 2010).

Martinez-Estevez *et al*. (2001b) performed a detailed study on the effects of Al on the growth of coffee cell suspensions. Initially, they determined the free Al concentration in the media at pH 4.3 and 5.8 and found that at 25 µM $AlCl_3$, the metal concentration were approximately 17.3 and 7.8 µM, respectively. At the higher concentration tested (100 µM), the values were 64.5 and 19.5 µM, respectively. The concentration of free Al was also determined for intermediate concentrations of $AlCl_3$ (50 and 75 µM). When Al was not included in the medium but the pH was kept at 4.3, cells grew more slowly than in the control, kept at pH 5.8. Next, Martinez-Estevez *et al*. (2001b) used pH 4.3 medium to study the Al toxicity of coffee cell cultures (in a 14 day culture cycle) exposed to a wide range of metal concentrations (25-1,000 µM $AlCl_3 \simeq$ 5.05-202 µM Al^{3+}) and observed









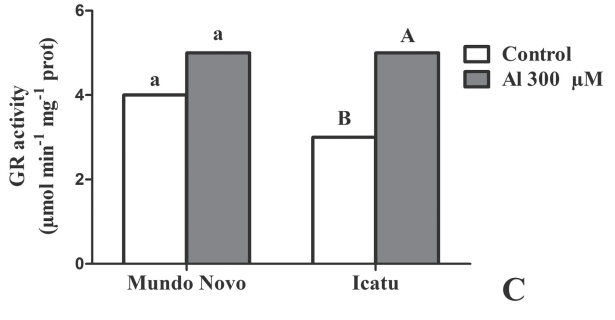


Figure 4. Specific activity of (A) catalase (CAT). Activity staining for (B) superoxide dismutase (SOD) following native PAGE of extracts of cultured coffee cells; lane 1, bovine SOD standard; lane 2, control (zero Al); lane 3, 72 h of growth in 300 µM of Al. Specific activity of (C) glutathione reductase (GR); (D) guaiacol peroxidase GOPX and (E) glutathione S-transferase (GST) in coffee cells grown for a 72 h period in 300 µM of Al. Means with the same letter are not significantly different ($p < 0.05$) by the Tukey's test.

that cell growth diminished in a dose-responsive way, and the lethal dose (LD50) was found to be 25 µM AlCl3 (5.05 µM Al^{3+}).

Here, we used 300 µM of Al^{3+} and no visible alterations was observed indicating cell death, which was probably because the medium pH was set 5.8 and consequent low Al speciation. However, cells absorbed Al (4,350-4,500 mg kg^{-1} cells, equivalent to 161-167 mM) and the concentration of Al^{3+} determined in the medium using the Geochem software was 4.35 µM Al^{3+}, close to the LD50 observed by Martinez-Estevez *et al*. (2001b). Martinez-Estevez *et al*. (2001b) did not determine the Al concentration in the coffee cell suspension exposed to 25 µM at pH 4.3 but staining with the Al indicator dye Morin followed by UV-light observation showed a very intense Al concentration in the cells. Therefore, considering that cell suspensions provide better surface contact with the medium, it seems that 300 µM Al compensated for the low speciation as Al^{+3} caused by the 5.8 pH level.

Despite the alterations in the enzyme activities

observed here, it was not possible to detect any difference in TBARS concentrations (figure 1), a typical sign of Al toxicity in plants indicating membrane lipid peroxidation. A similar situation was already observed in coffee cell suspensions exposed to Ni (Gomes-Junior *et al*. 2006a) and Cd (Gomes-Junior *et al*. 2006b). While at high metal concentrations (305 μM Cd and 225 μM Ni) there was a significant increase in TBARS, at low metal concentration (30.5 μM Cd and 22.5 μM Ni) TBARS levels remained low, although changes were observed in antioxidant enzyme activities. According to Gomes-Junior *et al*. (2006a, 2006b), the oxidative stress tolerance at low Cd and Ni concentrations indicated by unaltered TBARS values may be due to a rapid and significant increase in the activities of some of the major antioxidant enzymes, such as APX, CAT, SOD and GR, suggesting therefore an effective protection.

Besides a role as a putative Fe-storage protein, studies on plant ferritin functions have indicated a probable link between this protein and protection against oxidative stress (Briat *et al*. 2010b). Ferritin in animals has also been documented to bind Al atoms, which might be a cellular protection mechanism, guarding against eventual accumulated oxidative damage (Sakamoto *et al*. 2004). The reports with animals exhibited results indicating that the number of Al atoms can vary from 10 to 160 per ferritin molecule (Briat & Lebrun 1999).

Several recent reports have described the potential of ferritin to act as a cytoprotective antioxidant, which suggests that in addition to being an important Fe source for plant nutrition and maintaining Fe homeostasis, this protein also plays relevant roles in plant defence against oxidative stress. The close interaction between Fe homeostasis and ROS has been well-characterised in bacteria and animals, in which the regulation of Fe homeostasis has been shown to be modulated by oxidative stress (Ravet *et al*. 2009).

A possible role for coffee ferritin involving response against oxidative stress provoked by Al was excluded because in both coffee varieties, ferritin expression decreased (figures 2A and 2B). We also did not observe any accumulation of the ferritin protein in Al-stressed cells; instead, we observed a decrease in Icatu (figure 3). On the other hand, ferritin gene expression increased as a response to high level of Fe in coffee cell cultures (Bottcher *et al*. 2011).

It is known that metals induce oxidative stress, and the involvement of Al in this type of plant response has been previously suggested, even though Al itself is not a transition metal and cannot catalyze redox reactions (Yamamoto *et al*. 2002, Vitorello *et al*. 2005). It has also been shown that low concentrations of Al are sufficient to generate ROS and provoke oxidative stress in plants (Richards *et al*. 1998, Yamamoto *et al*. 2002). We have analysed the responses of key antioxidant enzymes that have been previously shown to respond to metal-induced oxidative stress in plants (Gratão *et al*. 2005). SOD activity is responsible for the degradation of the superoxide radical ($O_2^{\bullet-}$), producing O_2 and H_2O_2. Here, total SOD activity on non-denaturing PAGE showed an increased activity for both coffee varieties (figure 4B), indicating that Al exposure induces the formation of $O_2^{\bullet-}$ in a quantity greater than that which the pre-existing SOD can remove (Boscolo *et al*. 2003). Al was shown to enhance SOD mRNA levels in the root tips of Arabidopsis (Richards *et al*. 1998), the roots and shoots of rice seedlings (Sharma & Dubey 2007) and the roots of sorghum (Peixoto *et al*. 1999).

Unfortunately we did not measure the activity of ascorbate peroxidase (E.C. 1.11.1.11), the main H_2O_2 scavenging in plant cells. Less effectively, CAT is also responsible for the degradation of hydrogen peroxide (H_2O_2). The increase in CAT activity suggests the existence of elevated Al-enhanced H_2O_2 production in suspension cells, but this increase was observed only in Mundo Novo, remaining unaltered in Icatu cells exposed to Al (figure 4A). CAT activity was also inhibited significantly in the root cells of *Allium cepa* L. exposed to Al (Achary *et al*. 2008). In Al-stressed rice seedlings, CAT did not appear to be an efficient scavenger of H_2O_2 when higher concentrations of this metal were used (Ma *et al*. 2007, Sharma & Dubey 2007). CAT, SOD, and APX are important ROS-scavenging enzymes in plants. The balance among these enzymes is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide (Pereira *et al*. 2011).

The enzyme GPOX participates in different metabolic reactions, such as lignin biosynthesis, cell wall cross-linkage, auxin degradation, disease resistance, oxidative stress and converting H_2O_2 to water (Gratão *et al*. 2005). There was no increase observed in GPOX activity in the cells of Mundo Novo; however, a decrease was observed in Icatu (figure 4D). These results might indicate that H_2O_2 degradation occurred in Mundo Novo mainly due to

the activation of CAT, while the activities of other peroxidases are involved in H_2O_2 detoxification in Icatu. For instance, other enzymes, such as GR (figure 4C) and GST (figure 4E) have both exhibited significantly increased activities in response to Al in Icatu. The increased GST activity was also observed for Mundo Novo; however, it was not to the same extent, whereas changes in GR activities in Mundo Novo were not significantly different between the Al and control treatments. GST enzymes conjugate a co-substrate that contains a reactive electrophilic centre to glutathione in the reduced form (GSH) and form S-glutathionylated reaction products that are transported to the vacuole, detoxifying toxic substances (Kumari *et al*. 2008). These enzymes help to protect cells from oxidative stress. GST has been observed to be upregulated in *Arabidopsis* and rice that have been exposed to Al (Ezaki *et al*. 2004, Yang *et al*. 2007). The GR and GST results clearly indicate that Al is inducing the enhanced activities of the enzymes, which is occurring more dramatically in Icatu. The increased GR activity in Icatu also indicates the need to maintain glutathione in the reduced form, providing a favourable redox status to avoid the oxidative damage of the cells (Gomes-Junior *et al*. 2006a, Sharma & Dubey 2007).

The differences observed between Mundo Novo and Icatu with regard to ferritin expression and enzyme activities may be related to their genetic backgrounds. The Icatu lineage used here is an advanced generation of a hybrid between *C. arabica* and *C. canephora* that was backcrossed six times with the former until the agronomical and beverage quality attributes had been rescued. Icatu inherited its resistance to the leaf coffee rust from *C. canephora*, which is caused by *Hemileia vastatrix* Berk et Br. (Carvalho 1988). While *C. arabica* is tetraploid (2n = 44) and is found in the storey of Ethiopian forests, *C. canephora* is diploid and, among *Coffea* species, it has the widest natural distribution area in tropical African forests (Gomez *et al*. 2009). To hybridise the two species, chromosomes in *C. canephora* were duplicated using colchicine (Monaco *et al*. 1975). In comparison with Catuaí, a dwarf *C. arabica* variety resulting from a cross between Mundo Novo and the dwarf variety Caturra, Icatu seedlings that had been slowly acclimated to cold showed the greatest ability to control oxidative stress damage by enhancing enzyme activities (Cu, Zn-SOD and ascorbate peroxidase), increasing the contents of antioxidant molecules (ascorbate, alpha-tocopherol and chlorogenic acids), lowering the reactive oxygen species contents (H_2O_2 and $OH^{\bullet}$) and also increasing the expression of genes coding for GR, dihydroxy ascorbate reductase and class III and IV chitinases (Fortunato *et al*. 2010).

Therefore, our results suggest that the ability of coffee cells to support low Al concentrations seems to be related to its capacity to switch on antioxidant defences. Additionally, both varieties studied here showed differential responses, providing evidence that the induction or inhibition of specific antioxidant enzymes in response to Al is variety-dependent. Finally, these results indicate that ferritin may not be involved in the prevention of oxidative stress damage provoked by Al excess in coffee cell suspensions.

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