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Cold-induced changes in mineral content in leaves of *Coffea* spp. Identification of descriptors for tolerance assessment

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Abstract

Temperature and mineral nutrition are major environmental factors regulating plant growth and development. Yet, cold impact on mineral contents and the ability of the plants to perform changes in specific elements as a part of the acclimation process received little attention. Using five *Coffea* genotypes previously characterized concerning their cold sensitivity, a mineral analysis was performed considering macro (N, P, K, Ca, Mg, and S) and micro (Na, Fe, Mn, Zn, Cu, and B) nutrients in order to predict their importance in cold tolerance. The results showed a cold-induced dynamics of mineral nutrients in recently mature leaves. The less cold sensitive Icatu and partially Catuaí accumulated N, Ca, Mn, Cu, and Zn with potential implications in the maintenance of photosynthetic performance, the reinforcement of the antioxidative defense system, lipid metabolism, and the expression of cold regulated genes, thus constituting interesting traits to evaluate the cold acclimation ability. After a principal component analysis (PCA), N, Fe, Mn, and Cu were further confirmed as strong candidates for an early cold tolerance evaluation due to their dynamics and to specific roles in the activities of superoxide dismutase (Cu), ascorbate peroxidase (Fe), and photosystem II (Mn).

Additional key words: cold sensitivity, coffee genotypes, mineral dynamics, principal component analysis, tolerance markers.

Introduction

Exposure to low positive temperatures has a strong impact on plant organs and the metabolic pathways. Photosynthesis is especially impaired as consequence of multiple effects, namely reduction of stomatal conductance, changes in the structure and composition of the pigment complexes, decreased photochemical efficiency of photosystems, thylakoid electron transport, and enzyme activities, as well as modifications in the biophysical properties of thylakoid lipids and in carbon metabolism, allocation, and partitioning (Öquist 1982, Morcuende *et al.* 1996, Harwood 1998, Adams *et al.* 2002, Ensminger *et al.* 2006, Partelli *et al.* 2011). That is also associated with imbalances between energy absorption and its use by metabolic sinks as enzymatic reactions involved in C, N, and S reduction are more affected than light energy absorption, transfer, and transformation (Logan 2005, Ensminger *et al.* 2006).

An adequate mineral-nutrient status of plants plays a critical role in the acclimation ability to environmental changes (Marschner 1995) including future climate scenarios (Lynch and St. Clair 2004). In most plant species, cold restricts water and nutrient uptake by roots (Ensminger *et al.* 2006) what might affect the nutrient status and the growth rate of the above-ground plant organs (Aphalo *et al.* 2006). Such lowered mineral uptake could also interfere with the contents of some trace metals that are decisive to the photosynthetic electron transport in O_2-evolving organisms (*e.g.*, Fe, Mn, and Cu; Raven *et al.* 1999) and to oxidative stress control (*e.g.*, Fe, Cu, Mn, and Zn) (Mills *et al.* 2008, Pedas *et al.* 2008, Gao *et al.* 2009). Also changes in some nutrient uptakes will affect others. For instance, a balanced supply of N

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Abbreviations: APX - ascorbate peroxidase; Chl - chlorophyll; OEC - oxygen evolving complex of PS II; PCA - principal component analysis; PS - photosystem; ROS - reactive oxygen species; RH - relative humidity; Rubisco - ribulose-1,5-bisphosphate carboxylase/oxygenase; SOD - superoxide dismutase.
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determines the uptake and translocation rates of P, K, and S (Paal *et al.* 1981, McGrath and Zhao 1996, Groot *et al.* 2003).

Environmental stresses often restrict resource availability whereas successful acclimation sets in motion processes that restore supply (Geiger *et al.* 1996) towards homeostasis. Plant eco-physiology and biochemistry studies regarding the responses to harsh environmental conditions have dealt almost exclusively with changes in content and activities of molecules considered important to acclimation. Among them, there are compounds that could promote osmotic adjustment (*e.g.*, sugars and amino acids), anti-oxidative protection (*e.g.*, carotenoids and enzymes from the ascorbate-glutathione cycle), photo-chemical energy use (*e.g.*, components of the photo-synthetic apparatus), as well as gene expression. Although the protective effect of an adequate mineral nutrition to cold injury has been long reported (Dunn 1937), apart from experiments that relate fertilization to cold sensitivity/tolerance, the stress impact on cell mineral homeostasis has surprisingly received uneven little attention taking into account the wide range of important roles of minerals in the cell. These include the presence in biomolecules (in their structure and/or as co-factors) and their action as compatible osmolytes, ROS scavenging, *etc*. (Marschner 1995, Ramalho *et al.* 1995, 1999, Raven *et al.* 1999, Cakmak 2005a, Pedas *et al.* 2008, Waraich *et al.* 2011). Cold detrimental effects are particularly strong in tropical and sub-tropical plants (Bauer *et al*. 1985, Belintani *et al.* 2012), namely in coffee, as growth, development, and photosynthesis are strongly impaired, with the latter ceasing almost completely at 5 - 10 ºC (Barros *et al.* 1999, Ramalho *et al.* 2003, Batista-Santos *et al.* 2011). Since coffee crop is being spread towards new cultivation areas, unfavorable temperatures increasingly become a limiting factor to growth and yield (Bauer *et al.* 1985, DaMatta and Ramalho 2006). Yet, amongst coffee genotypes, some differences were identified at physiological and biochemical levels resulting in contrasting abilities to cold acclimation. That was particularly related to the strengthening of cellular antioxidative capability and the maintenance of adequate membrane fluidity through qualitative changes of the lipid matrix of membranes (Campos *et al.* 2003, Ramalho *et al.* 2003, Fortunato *et al.* 2010, Batista-Santos *et al.* 2011, Partelli *et al.* 2011). Nonetheless, in coffee, as in other plants, an adequate leaf availability of specific minerals (*e.g.* N and Ca^{2+}) is determinant to a high photosynthetic performance and to the strengthening of protective mechanisms that allow the plants to cope with environmental constraints, namely high irradiance, cold, and drought (Ramalho *et al.* 1998, 2000, DaMatta and Ramalho 2006, Pompelli *et al.* 2010).

The aim of this study was to provide insights concerning the relation of content of minerals and change on cold impact and its role in the different cold tolerance exhibited by different *Coffea* genotypes, with Icatu and Apoatã showing the lowest and highest cold sensitivities, respectively (Campos *et al.* 2003, Ramalho *et al.* 2003, Fortunato *et al.* 2010, Batista-Santos *et al.* 2011). It is hypothesized that the changes in content of some minerals, occurring along cold imposition in recently mature coffee leaves, can constitute an important trait to the expression of cold acclimation. Therefore, these characteristics could constitute "tracers/markers" for cold tolerance/sensitivity to be used in an early screening of plants before the implantation in the field. To assess the validity of such assumptions, a wide evaluation of the leaf mineral concentrations was performed in plants submitted to the same nutrient availability, upon a gradual cold exposure, using genotypes previously characterized for their cold sensitivity, particularly in what concerns the photosynthetic functioning.

Materials and methods

Plants, growth conditions, and cold imposition: Five *Coffea* cultivars with contrasting cold tolerance were used, with Icatu (and partly Catuaí) displaying lower cold sensitivity, Apoatã presenting the worst global performance, and *C. dewevrei* and Piatã showing an intermediate behavior in most of the studied parameters (Campos *et al*. 2003, Ramalho *et al*. 2003, Batista-Santos *et al*. 2011, Partelli *et al*. 2011). Briefly, *Coffea arabica* L. cv. Catuaí (IAC 81) and Icatu (IAC 2944 - *C. canephora* Pierre *ex* Froehner × *C. arabica*), both highly appreciated as producers, as well as *C. canephora* cv. Apoatã (IAC 2258) and *C. dewevrei* De Wild.& Durand, both used in breeding programs, and Piatã (IAC 387 - *C. dewevrei* × *C. arabica*), an hybrid with good yield potential, were grown for 1.5 years in a greenhouse at Oeiras, Portugal (lat. N 38º 42´ 02´´; long. E 10º 41´ 14´´) in 10-dm^3 pots in a substrate consisting of a mixture of soil, peat, and sand (3:1:3, v/v/v). Each plant was fed with 150 cm^3 of a complete nutrient solution (Hoagland and Snyder 1933), with pH adjusted to 6.0, given monthly from March to September (higher growth season) and every 2 months from October to February (lower growth season). A supplementary 50 cm^3 of a 5 mM NH_4N0_3 solution was provided every 2 weeks along the higher growth season and every month in the rest of the year in order to provide an adequate N supply (Ramalho *et al*. 2000). During the growth season, the plants were transferred into walk-in growth chambers (*10000 EHHF*, *ARALAB*, Albarraque, Portugal) with a 12-h photoperiod, day/night temperatures of 25/20 ºC, relative humidity of 65 - 70 %, and irradiance of 750 - 850 µmol m^{-2} s^{-1} and left there for 12 d to acclimate to these new conditions. Plants were then successively submitted to: *1*) a gradual temperature decrease (0.5 ºC d^{-1}) from 25/20 ºC to 13/8 ºC, over 24 d, to allow cold acclimation; *2*) then to 3-d chilling cycle (3 × 13/4 ºC), where the

plants were exposed to 4 ºC along the night and in the first 4 h in the morning followed by a rise to 13 ºC applied throughout the rest of the diurnal period; and *3*) rewarming period of 7 d at 25/20 ºC, to allow recovery (for further detail see Fortunato *et al*. 2010, Batista-Santos *et al*. 2011).

Leaf material was collected from 8 - 10 plants per genotype in each of the 5 independent biological experiments from the two top pairs of recently mature (completely expanded) leaves of both orthotropic and plagiotropic branches in the upper part of the plant grown under similar irradiance. That allowed comparison to physiological and biochemical data obtained from the same leaf type and conditions in previous experiments (Ramalho *et al.* 2003, Fortunato *et al.* 2010, Batista-Santos *et al.* 2011, Partelli *et al*. 2011). A separate group of plants was maintained at 25/20 ºC along the experimental period (*ca*. 43 d), where it was found that physiological parameters and minerals stayed unchanged from the beginning to the end of the experiment (data not shown). Therefore, the changes observed along the experiment will be a consequence of cold exposure and not to a result of a mere nutrient evolution along time.

Nutrients analysis: Determinations were made in the 2 top pairs of the most recent mature leaves from each branch in 8 - 10 plants per genotype. Total N content was determined in 100 mg of dry material by the micro Kjeldahl method. Digestion was performed in 3 cm^3 of H_2SO_4. A mixture of potassium sulphate and red mercuric oxide (1:10) was used as catalyst, and salicylic acid was included to reduce nitrate (Correia 1984).

For the determination of P, 3 cm^3 of concentrated nitric acid was added to 200 mg of dry mass and that mixture submitted to 60 °C for 30 min and then 150 °C for 60 min. Concentrated perchloric acid (1.5 cm^3) was then added and the sample submitted to 220 °C for 60 min. The P content was evaluated by the method of vanadate-molybdate (Chapman and Pratt 1961) using a *Unicam SP8 400* spectrophotometer (*Pye Unicam*, Cambridge, U.K.) for colorimetric readings at 420 nm.

For K, Ca, Mg, Na, Fe, Mn, Zn, Cu, and B determination, 1 g of dry material was mineralized by incineration at about *ca.* 550 ºC and then digested by nitric acid (Vandecasteele and Block 1993). A *Unican model 939* absorption unit equipped with a hollow cathode lamp was used for determinations.

Sulphur content was determined by inductively coupled plasma atomic emission spectrometry using a *Atomscan 2000* (*Jarrell-Ash, Fisher Scientific Company,* Waltham, USA) sequential spectrometer, after nitric acid digestion (Röhl *et al*. 1984).

Maximal activities of chloroplast antioxidative enzymes and of photosystem II: The activity of chloroplast Cu/Zn-superoxide dismutase (Cu/Zn-SOD, EC1.15.1.1) was determined after chloroplast isolation from *ca*. 15 g of leaf fresh mass. The assay was performed as described for *Coffea arabica* in Ramalho *et al*. (1998). Chloroplast ascorbate peroxidase (APX, EC1.11.1.11) activity was evaluated after chloroplast isolation from 4 g (leaf f.m.) as was described previously (Ramalho *et al.* 1998). Enzyme activity was assayed through ascorbate consumption (monitored at 290 nm) using a coefficient of absorbance of 2.8 mM^{-1} cm^{-1} for calculations.

The electron transport rates associated with photosystem (PS) II including the oxygen evolving complex (OEC), $H_2O \rightarrow$ DCPIP, were measured polarographically in sub-chloroplast fractions from freshly cut leaf material (*ca*. 4 - 5 g), using an O_2 electrode (*LW2*, *Hansatech*, Kings Lynn, UK), at 25 ºC and irradiance of *ca*. 4 000 μmol m^{-2} s^{-1} given by a Björkman lamp (*Hansatech*) as previously described in Ramalho *et al.* (1999).

Statistical analysis: Data were statistically analyzed through a two-way *ANOVA* ($P \leq 0.05$) after which a Tukey's mean comparison test was performed for a 95 % confidence level. In order to examine the relation between minerals and the temperature shift, data from the measured mineral content was normalized with respect to the control temperature, log2-transformed and standardized before producing a pair-wised correlation matrix subjected to eigenvalue decomposition to identify orthogonal components of the original matrix and generate a principal component analysis (PCA) plot using the *NTSYSpc v. 2.01b* package (Rohlf 1989).

Results

The analysis of the cold impact on the content of macro- (N, P, K, Ca, Mg, and S) and micro-nutrients (Na, Fe, Mn, Zn, Cu, and B) was carried out in completely expanded, recently mature leaves. Significant effects were observed between genotypes, between temperatures, and for the interaction. Thus, in order to select some of these elements as markers to cold sensitivity/tolerance screening, the mineral changes within each genotype were compared with their known cold sensitivity that are as extensively characterized from physiological to molecular levels (Ramalho *et al*. 2003, Fortunato *et al*. 2010, Batista-Santos *et al.* 2011, Partelli *et al*. 2011).

Considering N content, Icatu was the solely genotype showing a significant increase of N with cold exposure (that was maintained afterwards) with a maximal rise of 15 % at 13/8 ºC. Apoatã, *C. dewevrei*, and Piatã revealed an opposed trend with maximal reductions of *ca*. 10, 7, and 9 %, respectively, at 13/8 ºC or upon chilling, followed by a recovery to control values by the end of the experiment (Table 1). Catuaí showed small variation upon cold and somewhat higher values thereafter. Piatã and *C. dewevrei* consistently showed the lowest values.

For P content, a generalized decrease was observed under cold exposure with the minimal values registered already at 18/13 ºC for Apoatã, Catuaí, and Icatu, and at 13/8 ºC for the other 2 genotypes. Reductions ranged from 11 % (Apoatã) up to 32 % (Catuaí). Apoatã, *C. dewevrei*, and Piatã recovered to their control values by the end of the experiment. On the other hand, Catuaí and Icatu showed significant reductions at 18/13 ºC and maintained quite stable values thereafter. Among *Coffea* genotypes, the lowest P leaf content was consistently found in Piatã along the experiment often accompanied by *C. dewevrei* whereas the highest values were usually detected in Catuaí and Icatu.

The K content increased *ca*. 8 % in Apoatã and Piatã showing maximum values by the end of the acclimation period (13/8 ºC). Yet, Icatu presented the highest K values along the experiment closely followed by Catuaí and Apoatã (except at 25/20 ºC) whereas *C. dewevrei* and Piatã kept the lowest ones.

Calcium revealed significant decreases in Apoatã from 13/8 ºC onwards with a decrease of 16 % after 4 ºC exposure. In contrast, Icatu showed moderately higher Ca content from 18/13 ºC onwards with the highest increase (26 %) at that temperature whereas the other 3 genotypes showed some fluctuations. Icatu and Catuaí consistently showed the lowest Ca content along the experiment.

Along cold exposure, the Mg content decreased in Apoatã, *C. dewevrei*, and Icatu significantly at 13/8 ºC and after chilling. Catuaí showed only a non-significant decrease whereas Piatã maintained quite close values during the experiment. *C. dewevrei* showed the lowest Mg content in the experiment (except at 18/13 ºC).

During cold exposure, the content of S in the leaves of Apoatã did not vary significantly. For Icatu, *C. dewevrei*, and Piatã, a non-significant lowering tendency was found. Catuaí was the only genotype where S was significantly reduced (13/8 ºC) but by the end of the experiment, no differences were found for all genotypes when compared to their respective controls.

Concerning micronutrients (Table 2), Na strongly increased in Catuaí and Icatu until 13/8 ºC (55 and 22 %, respectively, as compared to their controls) showing also the highest absolute values. The other genotypes showed only marginal changes until 13/8 ºC but upon chilling all

Table 1. Leaf content of macronutrients in *Coffea* genotypes under control conditions (25/20 ºC), at the middle (18/13 ºC), and by the end of the acclimation period (13/8 ºC), after 3 chilling cycles (3 × 13/4 ºC) and after 7 d of recovery (Rec 25/20 ºC). Each value represents the mean of 4 - 5 replicates. Different letters indicate significant differences between temperatures within the same genotype (a, b, c, d) or between genotypes for the same temperature (r, s, t, u) for 95 % confidence level.

Mineral	Genotype	25/20 ºC	18/13 ºC	13/8 ºC	3 × 13/4 ºC	Rec 25/20 ºC
N [mg g^{-1}(d.m.)]	Apoatã	33.5 ar	30.7 br	31.5 br	30.3 br	34.7 ar
	Catuaí	29.5 bs	30.0 abrs	28.3 bs	28.9 brs	31.8 as
	C. dewevrei	28.6 abst	28.3 abcs	26.5 cst	26.9 bcst	29.7 at
	Icatu	27.0 ct	30.1 brs	31.0 abr	29.2 br	32.2 as
	Piatã	27.2 at	26.0 abt	24.7 bt	26.1 abt	26.2 abu
P [mg g^{-1}(d.m.)]	Apoatã	1.65at	1.47br	1.55brs	1.52bs	1.65ar
	Catuaí	2.19ar	1.49cr	1.51cs	1.64br	1.64br
	C. dewevrei	1.46bcu	1.49b r	1.29dt	1.38cdt	1.56ars
	Icatu	1.87as	1.50cr	1.62br	1.51cds	1.58bcrs
	Piatã	1.54au	1.36bs	1.32bt	1.38bt	1.52as
K [mg g^{-1}(d.m.)]	Apoatã	24.6 cs	24.1 cr	26.7 as	26.2 abr	25.1 bcs
	Catuaí	29.0 ar	24.0 cr	26.3 bs	26.2 br	26.1 brs
	C. dewevrei	20.9 at	19.6 at	19.9 au	20.6 at	20.2 au
	Icatu	30.2 ar	24.3 cr	28.3 br	27.3 br	27.0 br
	Piatã	22.0 bt	22.4 abs	23.8 at	23.0 abs	22.0 bt
Ca [mg g^{-1}(d.m.)]	Apoatã	13.6 as	14.0 ar	11.9 bs	11.4 bt	11.6 bt
	Catuaí	9.3 bt	10.1 au	9.3 bu	9.9 abu	9.5 abu
	C. dewevrei	15.5 ar	14.6 br	15.8 ar	14.6 br	15.2 abr
	Icatu	9.0 dt	11.3 at	10.7 abt	10.2 bcu	9.8 cu
	Piatã	13.5 as	12.4 bcs	12.1 cs	12.9 abs	13.6 as
Mg [mg g^{-1}(d.m.)]	Apoatã	3.31ar	3.42ar	2.94bst	2.95bs	2.97bs
	Catuaí	2.83as	2.63at	2.78at	2.79as	2.80as
	C. dewevrei	2.47abt	2.65at	2.33bcu	2.21ct	1.97ct
	Icatu	3.16ar	3.05bs	2.99bcs	2.85bcs	2.79cs
	Piatã	3.19abr	3.09bs	3.28abr	3.28abr	3.34ar
S [mg g^{-1}(d.m.)]	Apoatã	2.52ar	2.47ars	2.40ars	2.54ar	2.26ar
	Catuaí	2.90ar	2.65abr	2.35brs	2.46abr	2.48abr
	C. dewevrei	2.48ar	2.14as	2.14as	2.20ar	2.25ar
	Icatu	2.93ar	2.43ars	2.71ar	2.45ar	2.53ar
	Piatã	2.45ar	2.26ars	2.33ars	2.11ar	2.47ar

Table 2. Leaf content of micronutrients in *Coffea* genotypes under control conditions (25/20 ºC), at the middle (18/13 ºC), and by the end of the acclimation period (13/8 ºC), after 3 chilling cycles (3 × 13/4 ºC) and after 7 d of recovery (Rec 25/20 ºC). Each value represents the mean of 4 - 5 replicates. Different letters indicate significant differences between temperatures within the same genotype (a, b, c, d) or between genotypes for the same temperature (r, s, t, u) for 95 % confidence level.

Mineral	Genotype	25/20 ºC	18/13 ºC	13/8 ºC	3 × 13/4 ºC	Rec 25/20 ºC
Na [mg g^{-1}(d.m.)]	Apoatã	1.01dr	1.45br	1.01ds	1.23cs	1.79ar
	Catuaí	0.78cs	0.61dt	1.21br	1.12bt	1.49at
	C. dewevrei	0.47cu	0.56bct	0.64bu	1.46ar	0.22du
	Icatu	0.99dr	1.39br	1.21cr	1.20cst	1.67as
	Piatã	0.65dt	0.72cds	0.77bct	0.87bu	1.53at
Fe [µg g^{-1}(d.m.)]	Apoatã	85.6 abs	76.7 br	94.3 ar	85.2 abs	59.5 ct
	Catuaí	88.4 as	83.7 abr	83.1 abs	77.2 bst	83.3 abs
	C. dewevrei	88.2 as	40.2 bct	39.8 bcu	44.8 bu	32.5 cu
	Icatu	123.1 ar	65.5 cs	102.5 br	97.9 br	93.6 br
	Piatã	72.1 bt	85.5 abr	64.7 bct	71.8 bt	55.9 ct
Mn [µg g^{-1}(d.m.)]	Apoatã	22.5 as	21.5 abt	20.0 abt	18.4 bt	20.5 abs
	Catuaí	24.8 cs	35.8 bs	34.9 br	43.4 ar	35.1 br
	C.dewevrei	14.3 at	12.1 au	12.3 au	10.3 au	11.6 at
	Icatu	29.0 cr	43.2 ar	36.5 br	33.4 bs	34.0 br
	Piatã	25.0 abrs	25.2 at	25.6 as	20.9 bt	21.0 bs
Zn [µg g^{-1}(d.m.)]	Apoatã	20.4 br	13.8 cst	19.2 bs	25.3 ar	13.1 ctu
	Catuaí	15.7 cs	19.0 br	24.5 ar	18.5 bs	25.2 ar
	C. dewevrei	10.9 abt	12.1 at	12.2 at	12.0 at	9.3 bv
	Icatu	15.6 ds	16.2 cds	18.4 bcs	19.9 abs	21.5 as
	Piatã	13.2 ast	12.1 at	12.4 at	12.2 at	11.3 auv
Cu [µg g^{-1}(d.m.)]	Apoatã	6.64bcr	4.95cr	7.95abr	9.31ar	8.57abr
	Catuaí	5.78abrs	5.30br	6.86abrs	6.98abs	7.48ars
	C. dewevrei	4.96brs	5.50br	4.70bt	9.08ar	5.21bt
	Icatu	4.43bs	4.41br	7.23ars	7.88ars	7.01arst
	Piatã	5.52brs	5.01br	5.64bst	8.23ars	6.05bst
B [µg g^{-1}(d.m.)]	Apoatã	26.4 ar	25.7 as	26.1 ar	25.1 ar	23.7 as
	Catuaí	26.6 ar	23.3 brs	25.7 abr	26.0 abr	27.4 ar
	C. dewevrei	15.8 as	13.4 abu	12.8 abu	12.8 abu	11.8 bu
	Icatu	24.0 ar	20.5 bs	21.5 abs	20.8 bs	20.5 bt
	Piatã	15.1 as	16.6 at	16.6 at	15.9 at	17.7 at

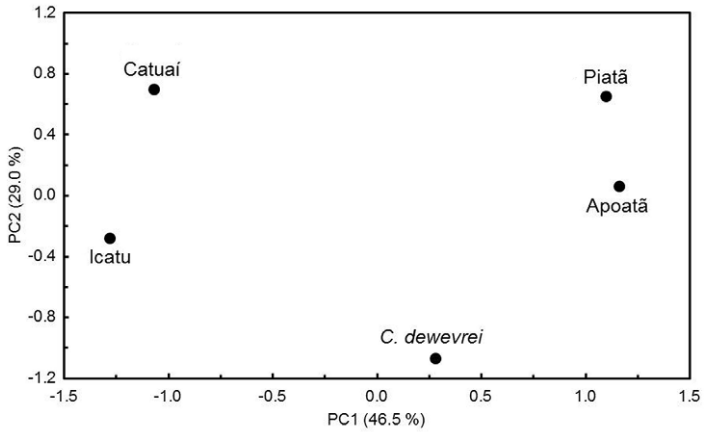

Fig. 1. Following a principal component analyses (PCA) of nutrient content/dynamics, in planes defined by the first and second components (PC_1 and PC_2) using the global projection of the experimental variables (temperatures: 25/20ºC - control conditions; 18/13ºC and 13/8ºC - middle and the end of the acclimation period; 3 × 13/4ºC - after 3 chilling cycles; Rec 25/20ºC - after 7 d under rewarming conditions) and cases (*Coffea* genotypes: Apoatã, Catuaí, *C. dewevrei*, Icatu, and Piatã) interactions for all nutrients considered together.

genotypes revealed significant increases of this element. Except for *C. dewevrei*, all genotypes further accumulate Na in leaf tissues during the recovery period.

Along the gradual cold imposition, Fe content in

C. dewevrei strongly declined to *ca*. half of the initial value after chilling exposure and to 37 % of the control value by the end of the experiment. This genotype also displayed the lowest values along the experiment except under control conditions. Although Icatu revealed a diminished amount of Fe at 18/13 ºC, it showed a clearly higher Fe content than the other genotypes. Catuaí, Apoatã, and Piatã displayed only small fluctuations of Fe content during cold exposure but the last two showed reductions by the end of the experiment.

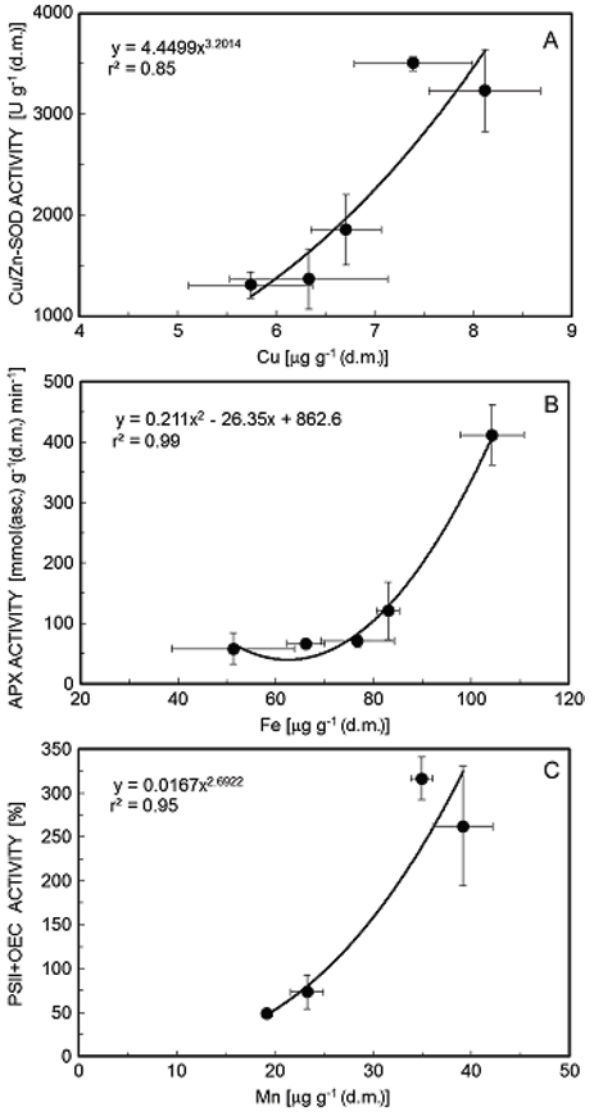

Fig. 2. Establishment of the best correlations between Cu content and Cu/Zn-SOD activity (*A*), Fe content and APX activity (*B*), and Mn content and the activity variation of PSII + OEC. Each point represents the mean ± SE (n = 4 - 5) of the values of plants acclimated to different temperatures (25/20, 18/13, 13/8, and 3 × 13/4 ºC) and after rewarming for each genotype (Apoatã, Catuaí, *C. dewevrei*, Icatu, and Piatã). Exception in *C*, in which *C. dewevrei* was not included due to lack of results.

Upon cold exposure, the Mn content in Apoatã, *C. dewevrei*, and Piatã gradually declined, with minimal values observed after chilling representing decreases of *ca*. 18, 28, and 16 %, respectively, as compared to the initial values. Instead, Catuaí and Icatu had significant Mn increases from 18/13 ºC onwards including the recovery period. Maximal increases were observed at 18/13 ºC for Icatu (49 %) and after chilling exposure for Catuaí (75 %). These two genotypes also showed the highest Mn content along the entire experiment whereas *C. dewevrei* (followed by Apoatã) kept the lowest values.

In some genotypes, Zn followed a similar trend to Mn. Significant increases were detected in Catuaí and Icatu upon cold and in the recovery period. By this time, they had 60 and 38 % higher values, respectively, doubling the content of most of the other genotypes. Piatã and *C. dewevrei* maintained stable values along cold exposure, slightly decreasing thereafter, and showed always the lowest Zn values. Apoatã did not present a clear trend with a strong decrease at moderately low temperature (18/13 ºC), an increase at 13/8 ºC and upon chilling exposure, followed by a decrease afterwards.

Concerning Cu contents, higher values were observed at 13/8 ºC and/or upon chilling in all genotypes with increases between 21 % in Catuaí (the only not significant) and 83 % in *C. dewevrei*. Enhanced values were still detected by the end of the experiment, significantly only in Icatu.

Finally, B was quite stable along the experiment, although *C. dewevrei* presented a gradual decrease which became significant by the end of the experiment. Piatã and *C. dewevrei* showed the lowest values along the entire experiment.

In order to evaluate which minerals could be used as proxy to cold tolerance evaluation, a principal component analysis (PCA) was performed. Using all the studied elements and taking into account the entire experimental period (Fig. 1), a clear separation of Icatu from Apoatã (the less and highest cold sensitive genotypes) was obtained, explained by both PC1and PC2 that comprised 75.5 % of total variance. The distinct dynamics of individual minerals also gave good separations of Icatu/ Apoatã, namely for N, Mn, Fe, and Cu (data not shown), to which accounted 35.9 % (PC2), 70.8 % (PC1), 96.3 % (PC1+PC2) and 81.3 % (PC1) of the total variance for those minerals in the same order.

To further clarify the possible relation between some of these minerals and its possible impacts on biochemical parameters, some correlations considering Cu, Fe, and Mn were performed. Strong correlations were found between Cu and the Cu/Zn-SOD activity (Fig. 2*A*), between Fe and APX activity (Fig. 2*B*), and between Mn and PS II activity including the (OEC) (Fig. 2*C*). It agrees with the known function of these minerals in these enzymes and in the functioning PS II.

Discussion

Combinations of soil nutritional problems with other environmental stress factors are responsible for severe losses in crop production worldwide (Cakmak 2005a) with drought, temperature (low and high), and mineral availability being the major environmental factors limiting plant growth and development (Garmash 2005). Still, in the framework of the acclimation processes, long-term adjustments of the internal content of several minerals occur, contributing to stress tolerance and assuring plant survival and productivity under limiting conditions (Cakmak 2005a). Under such conditions, the absorption of light energy may exceed its use for CO_2 fixation causing overexcitation of chloroplast structures and enhancing the production of highly reactive oxygen species (ROS) with negative effects on cellular structures and metabolism (Foyer *et al.* 2002, Logan 2005, Fortunato *et al.* 2010, Kayihan *et al*. 2012). Therefore, an adequate supply of mineral nutrients is decisive to maintain the efficiency of the photosynthetic metabolism, the imbalance of the mineral status greatly affects the plant ability to cope with stressful conditions, exacerbating photooxidative damage, and limiting plant performance (Marschner 1995, Cakmak 2005a).

Before the onset of cold conditions (25/20 ºC), no macronutrient deficiency was found as compared to leaf usual content in *C. arabica* (Carvajal 1984, Malavolta 1993, Ramalho *et al.* 1995) and *C. canephora* (Bragança *et al.* 2007). However, due to cold stress, all nutrients changed significantly reflecting a cold-driven dynamics. That was somewhat unexpected considering that some of the minerals are known by their low mobility within the plant (*e.g*., Ca) and that cold induced stomata closure (Ramalho *et al.* 2003, Batista-Santos *et al.* 2011), what implicates a limitation on water, mineral, and metabolite translocation within the soil-plant-atmosphere continuum. Plants exposed to environmental stresses may require additional supplies of minerals (among them N, K, Mg, Ca, and Zn) to minimize the adverse effects (Cakmak 2005a) as it was the case of cabbage where such mineral rise has been related to the ability to cope with low temperature (Öncel 1988). In this context, we aimed at identifying nutrient changes that might be related to the cold sensitivity of coffee genotypes knowing that Icatu (and partially Catuaí) had the best global performance whereas Apoatã (and often *C. dewevrei*) showed cold sensitivity (Ramalho *et al.* 2003, Fortunato *et al.* 2010, Batista-Santos *et al.* 2011, Partelli *et al.* 2011).

Nitrogen is one of the most important nutrients as it is a component of proteins, DNA, and Chl, being widely related to plant stress endurance (Carvajal 1984). Under N-adequate supply, *C. arabica* plants are able to cope with excessive irradiation, allowing photosynthesis to be maintained and the triggering of photoprotective mechanisms (Fahl *et al.* 1994, Ramalho *et al.* 1998, 1999, 2000, Pompelli *et al.* 2010). Leaf N accumulation occurred in Icatu from the beginning of the cold stress (Table 1) and, despite the severe reducion of C-assimilation in all genotypes, no significant impact of cold stress on the Rubisco content (Ramalho *et al.* 2003) and the PS activities (Batista-Santos *et al.* 2011) was observed in this genotype. Our results agree with the data found for cabbage, where an increased N content aided the development of acclimation in a frost unaffected cultivar, whereas N content decreased in a sensitive one (Öncel 1988). Such increased cold tolerance that accompanied N increase might be related to higher protein synthesis, namely of enzymes related to antioxidative mechanisms (Marschner 1995) implicating the expression of *COR* genes (Xin and Browse 2000, Hewezi *et al.* 2006). In this way, the increased N availability in Icatu would endorsed a higher protein synthesis linked to repair processes (*e.g*., at PS levels) and an effective strengthening of photoprotective and antioxidative mechanisms (Ramalho *et al.* 2003, Fortunato *et al.* 2010) protecting the photosynthesis apparatus and allowed a faster recovery after the cold period (Ramalho *et al* 2003, Batista-Santos *et al*. 2011).

Phosphorus is the main element involved in storage and transfer of energy, since it is a part of ATP and NAD/NADP molecules. It is also involved in the regulation of enzymes, transport of sugars, and it is a component of nucleic acids and phospholipids (Carvajal 1984, Marschner 1995, Mengel and Kirkby 2001). During cold acclimation and hardiness of cold tolerant cabbage plants, a P decline was reported, related to a slow metabolism (Öncel 1988). That could be also the case in all *Coffea* genotypes, since the decreased P content upon cold accompanied the decline in the photosynthetic activity (Ramalho *et al.* 2003, Batista-Santos *et al.* 2011). Moreover, knowing that cold acclimation responses (*e.g.* in Icatu) include a preferential *de novo* synthesis of phospholipids (Partelli *et al.* 2011), it can be inferred that the P reduction did not interfere with the observed phospholipids synthesis.

Potassium plays an important role in plant survival under environmental stresses being essential for many physiological processes, such as photosynthesis (*e.g*., by controlling stomata aperture), translocation of photosynthates into sink organs, osmotic adjustment, as well as for activation of enzymes (Carvajal 1984, Marschner 1995, Bohnert and Sheveleva 1998, Mengel and Kirkby 2001, Bragança *et al.* 2007). Also, an improvement of K-nutrition status under stress conditions seems to lower ROS production by membrane-bound NAD(P)H oxidases, since K reduces the activity of these enzymes and maintains photosynthetic electron transport (Cakmak 2005b). By increasing K supply, a protection against low-temperature in several plants was promoted (Cakmak 2005b, Walker *et al.* 2008) and, therefore, increase in K content during cold acclimation can be expected (Öncel 1988). The less cold sensitive Icatu (and partially Catuaí) did not show K increase but presented the highest K content under control conditions and maintained somewhat higher content (together with Apoatã) along

the experiment, contrary to what happened to *C. dewevrei* and Piatã. That can contribute to the lower (Icatu) and higher (*C. dewevrei*) oxidative stress (Fortunato *et al.* 2010).

Calcium is particularly important in coffee plants, being the 3rd absorbed element in *C. arabica* and the 2nd in *C. canephora*, corresponding up to 12 and 31 % of total macronutrients, respectively (Ramalho *et al.* 1995, Bragança *et al.* 2007), what agrees with our values that were between *ca*. 12 % (*C. arabica*) and 22 % (*C. dewevrei*). This essential nutrient is required for normal plant growth and development being involved, namely in stabilization of cell walls and membranes and in facilitating root extension (Ramalho *et al.* 1995, Mills *et al.* 2008). Since it is required at almost all stages of plant development, Ca also participates in plant adaptation to drought, cold, and salinity (Mills *et al.* 2008, Song *et al.* 2008) by mediating stress responses during injury, recovery, and acclimation (Waraich *et al.* 2011). Also it is a second messenger of paramount importance that regulates polar growth of cells and couples a wide range of extracellular stimuli to a vast array of intracellular responses (Bragança *et al.* 2007, Mills *et al.* 2008). The addition of Ca^{2+} chelators prevents cold acclimation and the expression of *COR* genes (Tähtiharju *et al.* 1997) whereas the addition of Ca^{2+} ionophore induces the expression of cold acclimation-specific genes at 25 °C (Monroy and Dhindsa. 1995). Although Ca is a quite immobile mineral and stomata conductance was reduced, Ca increased as much as *ca*. 25 % at 18/13 ºC solely in Icatu, what could have contributed to the higher expression of genes implicated in the cold acclimation process (Fortunato *et al.* 2010, Batista-Santos *et al.* 2011). In addition, Ca is required for the normal functioning of PS II and to obtain high rates of O_2 evolution in the thylakoid lumen (Ghanotakis and Yocum 1990, Raven *et al.* 1999) protecting the Chl through stabilization and aggregation of LHC (Ramalho *et al.* 1995), as suggested to occur in Icatu (Ramalho *et al.* 2003, Fortunato *et al.* 2010, Batista-Santos *et al.* 2011). In contrast, Ca decreased in the cold sensitive Apoatã which showed severe photosystem impairments and a lower gene expression, further supporting the Ca role in alleviating cold stress in coffee plants.

Magnesium affects growth and development, namely through the activation of enzymes (*e.g.*, ATPases, RNA polymerase, protein kinases, and Rubisco), Chl synthesis (its deficiency promotes interveinal leaf chlorosis). Also low Mg content reduces phloem export of photosynthates to sink organs causing sugar accumulation in leaves and, therefore, restriction of photosynthetic C-metabolism (Marschner 1995, Bragança *et al.* 2007, Waraich *et al.* 2011).

Sulphur integrates protein structure (through the cysteine and methionine amino acid constitution) and the antioxidant glutathione (Carvajal 1984, Marschner 1995) which is particularly abundant in chloroplasts (Asada 1994, Perl-Treves and Perl 2002).

However, in coffee, Mg and S did not have a decisive effect on cold acclimation, as Icatu and Apoatã showed similar decline and absolute values, close to adequate levels (Ramalho *et al.* 1995, Bragança *et al.* 2007).

As for macronutrients, the micronutrient leaf content was fairly adequate at 25/20 ºC (Carvajal 1984, Malavolta 1993, Ramalho *et al.* 1995), although for Apoatã, Fe, Mn, Cu, and B could be considered somewhat below the optimal level as compared to *C. canephora* cv. Conilon (Bragança *et al.* 2007). Cold tolerance was related to leaf K and/or Na accumulation in *Atriplex halimus* (Walker *et al.* 2008), what might have occurred for Na in *Coffea* genotypes. Na increased during the cold acclimation period only in Icatu, presenting also the highest absolute values (together with Catuaí) at 13/8 ºC. Yet, upon chilling exposure and afterwards, all genotypes (except *C. dewevrei* in the recovery period) showed enhanced Na content (suggesting that plants may be prepared to further cold exposure) limiting its use as a cold discriminatory mineral.

Iron is involved in photosynthetic and respiratory pathways as Fe bound tightly to polypeptides catalyzing redox reactions (Raven 1990). This mineral is the quantitatively most important trace metal involved in thylakoid reactions of oxygenic organisms, since linear electron flow from H_2O to $NADP^+$ involves PS II (2 - 3 Fe), cyt b_6f (5 Fe), PS I (12 Fe), and ferredoxin (2 Fe) (Raven *et al.* 1999), what justifies that *ca*. 80 % of plant cell Fe is located in the chloroplasts (Marschner 1995, Bragança *et al.* 2007). In fact, Fe is also an activator of several enzymes among them peroxidases and catalases and is a structural component of Fe-SOD (found in chloroplasts) that, together with Mn-SOD (located in mitochondria and peroxisomes) and Cu/Zn-SOD (present in cytosol, chloroplasts, mitochondria, peroxisomes, glyoxisomes, and extracellular space), integrates the first line of defenses against ROS (Halliwell and Gutteridge 1989, Asada 1994, Raven *et al.* 1999, Gao *et al.* 2009, Kayihan *et al.* 2012). In fact, Fe also promotes the accumulation of ascorbate peroxidase transcripts (Vansuyt *et al.* 1997), induces APX activity at a post-transcriptional stage (Ishikawa *et al.* 2003), and affects the expression/activities of APX under cold (Gao *et al.* 2009). The enhancement of SOD and APX activities contributes to plant stress tolerance in coffee plants subjected to high irradiance, N deficiency (Ramalho *et al.* 1998), drought (Lima *et al.* 2002), and cold (Fortunato *et al.* 2010). Although the changes of Fe content along the experiment did not show a clear relation to a possible cold sensitivity, *C. dewevrei* showed the strongest fall whereas Icatu revealed the highest Fe content amongst genotypes (except at 18/13 ºC). Since Cu and Fe cold-induced changes revealed a strong correlations, respectively, to SOD activity (Fig. 2*A*) and the availability of Fe showed a similar relationship with APX activity (Fig. 2*B*),, these minerals might have complementary roles favouring the aptitude for ROS removal by promoting the simultaneous enhancement of Cu/Zn-SOD and APX observed only in Icatu (Fortunato *et al*. 2010).

On the other hand, Mn is firmly associated with the OEC of PS II, where it is required for water splitting and O_2 evolution (Raven *et al.* 1999, Pedas *et al.* 2008). Mn is also present in Mn-SOD and is an activator of several enzymes, such as peroxidases and enzymes from N metabolism (Raven 1990, Bragança *et al.* 2007), decarboxylases and dehydrogenases in the Krebs cycle (Marschner 1995, Mills *et al.* 2008), phenylammonia lyase in the shikimic acid pathway, and several glycosyltransferases in the Golgi apparatus (Mills *et al.* 2008, Pedas *et al.* 2008). Therefore, Mn is an essential trace element in all stages of plant development (Mills *et al.* 2008) justifying that its deficiency in coffee provokes low yield and loss of plant vigour (Bragança *et al.* 2007). Considering that Mn-deficient plants were reported to be more susceptible to low-temperatures (Marschner 1995), that Mn improves protection against oxidative stress (Gao *et al.* 2009), and that it is required to PS II functioning (Raven *et al.* 1999, Pedas *et al.* 2008), it was remarkable that its highest content and increase occurred in Icatu and Catuaí, whereas the other genotypes showed decrease. Therefore, the correlation between Mn content and PS II activity (Fig. 2C) fully agrees with the 2.5 fold increased activity of PS II (Batista-Santos *et al.* 2011) and the absence of ROS increase (Fortunato *et al*. 2010) upon cold conditions in Icatu. Mn also plays an important role in the biosynthesis of fatty acids and carotenoids (Marschner 1995, Mills *et al.* 2008). Hence, it could have contributed to the highest carotenoid content (Ramalho *et al.* 2003, Batista-Santos *et al.* 2011) and to the lipid dynamics (Partelli *et al.* 2011) observed mostly in Icatu and, partially, in Catuaí. In this context, the Mn content increases and its highest content showed by the tolerant Icatu (and Catuaí) by opposition to what happened in the sensitive *C. dewevrei* and Apoatã suggested that Mn might be a good candidate to evaluate cold sensitivity in coffee.

Zn and Cu are other important microelements to cell functioning. Cu is essential for sugar and N metabolisms as well as for lignin synthesis needed for cell wall strength. Cu is a part of the plastocyanin which is involved in thylakoid electron transport and also plays an important role in the activity of Cu/Zn-SOD (Carvajal 1984, Marschner 1995, Raven *et al.* 1999, Bragança *et al.* 2007, Gao *et al.* 2009). Zn is needed for the activities of carbonic anhydrase, Cu/Zn-SOD, RNA polymerase, and a large number of dehydrogenases, affecting also several enzymes of the sugar metabolism (Marschner 1995, Raven *et al.* 1999, Bragança *et al.* 2007). Mn and Zn presented a rising tendency along the experiment in Icatu and Catuaí whereas *C. dewevrei* and Piatã maintained their values which were simultaneously the lowest ones. Also Cu content tended to increase in all genotypes at 13/8 ºC and/or upon chilling. Thus, the increased activity of chloroplast Cu/Zn-SOD observed at 13/8 ºC and/or after chilling exposure in Icatu, Catuaí, and Apoatã (Fortunato *et al.* 2010) could be associated with the simultaneous increase of Zn and Cu, and was further supported by the correlation between Cu/Zn-SOD activity and Cu content (Fig. 2*A*) or Cu + Zn content (not shown). That agrees with the improved cold tolerance in cucumber promoted by a higher Mn^{2+}, Cu^{2+}, or Zn^{2+} availability in the nutrient solution. Such higher cold tolerance was related to enhanced activities (and gene expression) of Cu/Zn-SOD and Mn-SOD (Gao *et al.* 2009, Kayihan *et al*. 2012) as well as to reduced accumulation of MDA and lower electrolytic leakage (Gao *et al.* 2009, Belintani *et al* 2012). Also the production of ROS linked to the NADPH-dependent oxidase is stimulated by drought, chilling, and salinity but can be reduced by Zn (and K) that interfere with this enzyme activity, thus preventing ROS formation (Cakmak 2005a). Thus, by showing levels below adequate supply of Mn and Cu, Apoatã cold acclimation might have been restricted.

In plants, B is associated with cell wall formation, water relations, phenol and N metabolisms, as well as with meristem activity (Carvajal 1984, Marschner 1995, Waraich *et al.* 2011) but does not seem to be determinant to the cold acclimation. In fact, although Piatã and *C. dewevrei* presented the lowest values along the experiment, B leaf content did not show appreciable changes.

Complementary, a PCA test was performed taking into account all the studied minerals (Fig. 1). That confirmed that mineral content and dynamics could be envisaged as a proxy tools for cold sensitivity evaluation in coffee plants since a clear separation of the less (Icatu and Catuaí) and more (Apoatã) cold sensitive genotypes was obtained. Furthermore, similar separations were obtained through PCA tests for some individual minerals (data not shown), among them N, Mn, Cu, and Fe which can be elected as good candidates for cold tolerance evaluation.

N evaluation using PCA became quite strengthened probably reflecting the wide use of this element in the plant metabolism (see above). Therefore, the higher N-accumulation ability of Icatu, when compared to the other genotypes (Table 1), would promote the energy metabolism and the acclimation processes (Öncel 1988, Marschner 1995).

The other three selected minerals are the micro-nutrients Cu, Mn, and Fe that, apart from their wide range of cellular roles, are components of SOD, thus being directly involved in the oxidative stress protection. Among these 3 elements, Cu relation was explained by *ca*. 81 % of total variance allowing a clear separation of Icatu from the genotype Apoatã with the worst cold acclimation and antioxidative system performances. On the other hand, for Fe and Mn, the separations were explained by 96.1 % (Fe) and 71 % (Mn) of total variance implying a very high accuracy. Likewise, it should be emphasised that only Icatu clearly showed parallel Mn, Cu, and Zn increases along the acclimation period and upon chilling exposure accompanied by the highest Fe content, what would allow a reinforcement of the activity of SOD and APX as well as a higher efficiency of PS II (Fig. 2).

Conclusions

Under cold stress, an important mineral content dynamics occurred in recently mature and fully expanded leaves in the studied coffee cultivars. That was fairly surprising since some of these minerals are considered to display low mobility within the plant and because such changes occurred in a context of severe restriction of the transpiration flow due to negligible stomatal conductance at 13/8 ºC and upon chilling in all genotypes. The dynamics of some of those nutrients could have a positive contribution to the acclimation mechanisms and, therefore, to the lower cold sensitivity of Icatu (and partially Catuaí). The N enhancement upon cold would support *de novo* protein synthesis (*e.g.*, of antioxidative enzymes) and repair of components damaged by the cold-exacerbated oxidative stress allowing also a quicker recovery after stress removal, especially the photosynthetic functioning. Specific roles in the preservation of photosystem stability and functioning could have occurred, namely the triggering of *COR* genes (Ca) whereas Fe (due to the larger content), Mn, Zn, and Cu (due to their increase) could have favored the expression and/or activity of SOD (Cu, Zn, Mn and Fe) and APX (Fe), enhanced carotenoid and lipid synthesis, promoted PS II activity (Mn), and protected thylakoid electron transport (Cu). Altogether, these processes would contribute to promoting ROS control and to preserving photosynthetic performance as previously observed in Icatu. Despite known important roles in cell functioning, P, K, Mg, S, Na, and B did not present clear relations to cold tolerance although Na consistently increased along the acclimation period only in Icatu. The PCA suggests N, Fe, Mn, and Cu as predictors to cold sensitivity evaluation in *Coffea*. In conclusion, an adequate availability of several minerals and their dynamics (particularly the simultaneous enhancement of N, Cu, Zn, and Mn, and the highest Fe content) would contribute to the acclimation process in *Coffea* spp. constituting a useful tool to assess cold tolerance.

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