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Agricultural contamination: Effect of copper excess on physiological parameters of potato genotypes and food chain security

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Summary

Areas with a history of cupric fungicide application accumulate copper (Cu), which may be toxic to plants and might result in food chain contamination. This work aimed to study the effects of Cu contaminated vineyard soils (2.2, 5, 36.3, 67, 95.7, 270.5 and 320.70 mg Cu kg⁻¹ soil) on three potato genotypes and its potential risk to human health, during the fall and spring growing seasons. The increase of Cu concentration in leaves was dependent on external Cu concentrations and development stage of the leaves. There were genotypic differences for both growth and biochemical parameters including high accumulation of Cu in tubers among the genotypes. Therefore, Cu stress triggered a defense mechanism against oxidative stress in potato plants; and the magnitude of Cu stress was depended on the genotype and the plant physiological status. In addition, these results provide evidence that potato antioxidants are not sufficient to prevent biological damage caused by Cu toxicity, and that potato cultivation in areas with high Cu levels is not recommended due to low production and potential risk to human health.

Keywords: antioxidant system, copper toxicity, heavy metals, phosphorus, *Solanum tuberosum*.

Introduction

On the basis of nutritional requirement and potential toxicity to plants, different categories of metals have been distinguished. Some metals are essential to cell metabolism with specific functions and have unknown toxic effects, except at extremely high concentrations (KHATUN et al., 2008; WANG et al., 2010). However, some elements may either have unknown metabolic function or are highly toxic even at low levels. The iron (Fe), zinc (Zn) and copper (Cu) are essentials for higher plants and animal life, and these metals may be toxic at higher concentrations (YRUELA, 2005; SEIDEL et al., 2009). This phenomenon is one of the most interesting to consider from the perspective of cellular regulation due to the need to maintain intracellular metals in a relatively constant concentration despite the variation in the supply of metal nutrients and the potential for toxicity (THIELE, 1992).

Similar for humans, both deficiency and toxicity of Cu in plants may result in the reduction of biological function. The amplitude of functions and effects on different organisms related to Cu are dependent on Cu concentration and oxidation status. Soils naturally contain Cu, but, in some cases, the available Cu content may be insufficient for healthy crop growth. Moreover, there are many soils with high contents of available Cu, thus rendering them potentially

toxic to plants (SUDO et al., 2008; WARNE et al., 2008).

Recently, the impact of heavy metal pollution, such as Cu contamination resulting from anthropogenic inputs, has caused concerns due to Cu persistency in soil, economic loss from a reduction in crop production (ADREES et al., 2015) and its impact on the security of the food chain. The vineyard soils from the southern region of Brazil are an example of antropogenic pollution caused by agricultural practices. These soils have received successive applications of Bordeaux mixture (CuSO₄·5H₂O Ca(OH)₂) to protect the vineyards from fungal diseases. Susceptibility to Cu toxicity as well as the capacity to accumulate this element varies greatly with plant species. For instance, alfalfa and barley crops are highly tolerant to Cu stress, but rice and potato crops are less tolerant (JONES, 1998).

In this sense, there might be many differences among genotypes for the same species. As an example, other works have previously demonstrated that some potato genotypes are tolerant to aluminum (Al) with lower oxidative stress than Al-sensitive genotypes as demonstrated not only by a lower production of ROS (H₂O₂) and lipid peroxidation but also by the presence of more efficient enzymatic and non-enzymatic antioxidant systems (TABALDI et al., 2009a). Differences in Al and cadmium accumulation as well as mineral elements were also observed (TABALDI et al., 2009b). Thus, the aim of this work was to characterize the general biochemical and physiological aspects of Cu toxicity in three potato genotypes (*Solanum tuberosum*) grown on vineyard soils from the southern region of Brazil, as well as evaluate the tuber production and Cu accumulation of different genotypes and its impact on the security of the food chain.

Materials and methods

Plant materials and growth conditions

This study consisted of two experiments conducted with vineyard soils in a greenhouse during the fall (March to May) and the spring (September to November) growing seasons.

During the fall growing season the SMIE040-6RY and SMINIA793101-3 genotypes were evaluated. Due to the contrasting response to Cu excess between the genotypes, a second experiment was proposed with SMIF212-3 and SMINIA79310-3 genotypes cultivated during the spring growing season.

For both experiments, seven soils were collected from southern Brazil. Four soils from vineyards located at Serra Gaúcha (from vineyards at Embrapa Uva and Vinho experimental areas in Bento Gonçalves, RS) classified as Humic Cambisols. These soils were named Cambi C, Cambi VN1, Cambi VN2 and Cambi VN3, respectively (VN indicates that the soils were collected from vineyards). For purposes of comparison, Cambi C was used as a

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control because it was collected under a native forest. Cambi C + PK was created by correcting the levels of P and K (55 mg kg⁻¹ and 50 mg kg⁻¹ respectively) based on the results of the soil analysis and according to CQFS-RS/SC (Comissão de química e fertilidade do solo) (CQFSRS/SC, 2004). Three soils were collected from vineyards in Campanha Gaúcha (from commercial vineyards located in the municipality of Santana do Livramento, RS) and were classified as Ultisols. These soils were named Ulti, Ulti VN1 and Ulti VN2, respectively. Ulti was used as a control and was collected from a seedling production area with a history of no application of cupric fungicides. The concentrations of P, Cu, K, Zn and Fe of these soils are presented at Tab. 1.

For the assays, 3 kg of each soil was air-dried and placed in pots with a capacity of 5 kg. In each pot, one tuber with a diameter of 2 to 3 cm and an average weight of 8.4 g was sown. Throughout cultivation, the soil humidity was maintained at 80% of field capacity with daily irrigation with distilled water. Throughout the cultivation, two applications of nitrogen (N) totalizing 70 mg kg⁻¹ were applied to the soil. The experimental design was completely randomized with six replicates per treatment.

The experiments were conducted in a greenhouse from March to May (fall growing season) and from September to November (spring growing season), with eight plants of each genotype cultivated per treatment (soil used).

Cu tissue concentration

Cu tissue concentration was determined in dried plant tissue (between 0.01 and 0.25 g) digested with 5 mL of concentrated HNO₃. Sample digestion was performed in an open digestion system using a heating block Velp Scientific (Milano Italy). The Cu concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-EOS) using a PerkinElmer Optima 4300 DV (Shelton, USA) equipped with a cyclonic spray chamber and a concentric nebulizer.

Soil analysis

The soils were analyzed for particle size distribution of the soil constituents according to the pipette method (EMBRAPA, 1997).

The determination of pH was performed with water in a 1:1 ratio according to the methodology proposed by TEDESCO et al. (1995).

The concentration of soil organic matter (OM) was analyzed by wet oxidation using potassium dichromate in a sulfuric acid medium (0.2 M), and the determination of OM was according to EMBRAPA (1997). The total contents of Cu and Zn in the soil samples were obtained according to method N° 3050B (US EPA, 1996). The extraction of available Cu (CuEDTA) and Zn (ZnEDTA) was performed using 0.01 mol L⁻¹ Na₂-EDTA/1.0 mol L⁻¹ ammonium acetate with the pH level adjusted to 7.0 according to CHAIGNON et al. (2003).

The extraction of available P and exchangeable K was performed with the Mehlich 1 solution (0.05 mol L⁻¹ HCl + 0.0125 mol L⁻¹ H₂SO₄) according to MURPHY and RILEY (1962). The exchangeable cations (Ca, Mg and Al³⁺) were extracted with a 1.0 mol L⁻¹ KCl solution (EMBRAPA, 1997). The concentration of Al³⁺ was determined by an acid-base titration with a 0.0125 mol L⁻¹ NaOH solution, and the concentrations of Ca and Mg were determined by atomic absorption spectroscopy (AAS) (TEDESCO et al., 1995).

Growth and biochemical parameters

At the end of the growth cycle, plants were harvested and divided into shoots, tubers, roots and stolons. The effects of Cu toxicity on potato plant growth were evaluated using the following parameters: average fresh weight of tubers and number of tubers per plant.

For all biochemical assays, the fourth expanded leaf of each plant was collected during the fall growing season, and the third and fourth expanded leaves were collected during the spring growing season. The samples were collected during tuber initiation (approximately 30 days after emergence) and near the end of the cycle (approximately 65 days after emergence). Once collected, samples were immediately placed in liquid nitrogen and pulverized to a fine powder using a porcelain mortar.

For the first experiment, concentrations of the following components were measured: TBARS and H₂O₂. These analyses were not completed for the SMIE040-6RY genotype grown in Cambi VN2, Cambi VN3 and Ulti VN2 because there were no expanded leaves at tuber initiation. At the second collection (approximately 65 days after emergence), plants grown in these soils still did not have expanded leaves, so all the leaves present in each plant grown in Cambi VN2,

Tab. 1: Chemical and physical properties of vineyard soils with application of Cu-based fungicides.

Parameters	Cambi C+PK	Cambi C	Cambi VN1	Cambi VN2	Cambi VN3	Ulti	Ulti VN1	Ulti VN2
Sand g kg ⁻¹	346	346	298	345	320	675	661	705
Silt g kg ⁻¹	391	391	373	353	370	260	264	205
Clay g Kg ⁻¹	263	263	329	302	310	65	75	90
pH H ₂ O	5.8	5.8	5.2	5.3	5.3	5.5	5.3	5.2
M.O. g kg ⁻¹	34.3	33.9	27.3	37.9	35.9	11.2	12.1	9.2
Al cmolc kg ⁻¹	0	0	0.05	0.02	0.03	0	0.06	0.03
CEC ef cmolc kg ⁻¹	8.8	8.6	6	8	8	2.4	2.2	1.6
CEC 7 cmol kg ⁻¹	11.5	11.4	10.2	12.5	11.8	4.6	5.4	4.4
Cu EDTA mg kg ⁻¹	5	5	95.7	270.5	320.7	2.2	36.3	67
Cu total mg kg ⁻¹	29	29.8	183	480.3	490.3	11.3	51.6	73.1
Zn EDTA mg kg ⁻¹	2	2	14	18	21	2	7	10
Zn total mg kg ⁻¹	60.6	59.2	81	84.6	81.8	8.2	10.8	16.4
Mn mg kg ⁻¹	280	270	210	210	180	90	85	87
P mg kg ⁻¹	18.2	4.8	37	19	27	47.1	75	60
K mg kg ⁻¹	130	110.9	260	100	110	129	100	110
Ca cmolc kg ⁻¹	4.7	4.6	3.7	5.9	5.6	1.4	1.3	1
Mg cmolc kg ⁻¹	3.7	3.7	1.6	1.9	2.1	0.6	0.6	0.4

VN3 and Ulti VN2 were collected for the biochemical assays. A second experiment was proposed with additional analyses, including enzymatic and non-enzymatic analyses, to better characterize the potato response to Cu exposure during the spring growing season.

Estimation of lipid peroxidation and hydrogen peroxide (H₂O₂)

The level of lipid peroxidation products was estimated following the method of El-MOSHATY et al. (1993) by measuring the concentration of malondialdehyde (MDA) as an end product of lipid peroxidation by reaction with thiobarbituric acid (TBA).

The H₂O₂ concentration of potatoes was determined according to LORETO and VELIKOVA (2001). Approximately 0.05 g of the frozen sample was homogenized in 2 mL of 0.1% (w/v) TCA. The homogenate was mixed with 0.5 mL of a 10 mM potassium phosphate buffer (pH 7.0) and 1.0 mL of 1 M KI, and the mixture was centrifuged at 12,000 g for 15 min at 4 °C. The H₂O₂ concentration of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve.

Enzyme activities of antioxidant system

Frozen samples of leaves were used for the enzyme analysis. The samples were composed of 0.6 g of tissue homogenized in 2.0 mL of a 0.05 M sodium phosphate buffer (pH 7.8) containing 1 mM EDTA and 2% (w/v) PVP. The homogenate was centrifuged at 13,000 g for 20 min at 4 °C. The supernatant was used for enzyme activity and protein content assays (ZHU et al., 2004).

Catalase (CAT) activity was assayed following the method of AEBI (1984) with slight modifications. The activity was determined by monitoring the disappearance of H₂O₂ by measuring the decrease in absorbance at 240 nm of a reaction mixture containing 15 mM H₂O₂ in a potassium phosphate buffer (pH 7.0) and 30 mL of extract with a final volume of 2.0 mL.

Ascorbate peroxidase (APX) was measured according to ZHU et al. (2004). The reaction mixture consisted of a total volume of 2 mL of a 25 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H₂O₂ and 100 µL of enzyme extract.

The activity of the enzyme superoxide dismutase was determined according to MISRA and FRIDOVICH (1972). Approximately 0.2 g of the oat's shoot fresh matter was homogenized in 5 mL potassium phosphate buffer (100 mM, pH 7.8) containing 0.1 mM EDTA, 0.1% Triton X-100 (v/v) and 2% PVP (w/v). The extract was filtered using filter paper and then centrifuged at 22,000 g for 10 min at 4 °C. The reaction mixture reached a final volume of 1 mL, containing glycine buffer (pH 10.5), 17 mL of epinephrine (1 mM), and the enzymatic material. Epinephrine was the last component to be added. Adrenochrome formation in the 4 min following the addition of epinephrine was recorded at 480 nm. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions.

Ascorbic acid (AsA) and non-protein thiol groups (NPSH) concentrations

Frozen samples were homogenized in a solution containing 50 mM Tris-HCl and 10% Triton X-100 (pH 7.5), and the samples were then centrifuged at 2,600 rpm for 10 min. The supernatant was removed, and 10% TCA was then added at a ratio of 1:1 (v/v) to the supernatant followed by centrifugation (2600 rpm for 10 min). The supernatant was used to determine AsA and NPSH concentration. AsA determination was performed as described by JACQUES-SILVA et al. (2001). NPSH concentration in the potatoes plants was measured spectrophotometrically with Ellman's reagent (ELLMAN, 1959).

Protein determination

For all the enzyme assays, protein was measured by the Coomassie Blue method according to BRADFORD (1976) using bovine serum albumin as a standard.

Multivariate analysis

Initially, data from plants cultivated in different seasons were transformed by ranking on a scale ranging from 1 to 10. The average value of the evaluated parameters corresponded to 5 on the scale with 1 being the lowest assessed value and 10 being the highest assessed value. The average data were analyzed using CANOCO® statistical software (version 4.5, Fa. Biometris). The data matrix was submitted to PCA analysis to compound variables, thus providing information about the factors responsible for these patterns.

Results and discussion

Effects of Cu toxicity on tissue Cu concentration and growth parameters

The general human population is exposed to Cu through inhalation, consumption of food and water, and dermal contact with air, water, and soil that contains Cu. The primary source of copper intake is the diet. However, the amount of Cu in the diet usually does not exceed the average dietary requirements (RDAs) for Cu. In the United States, the Institute of Medicine has set a consumption limit for copper of 10 mg/day based on a critical endpoint of liver damage (ABERNATHY and ROBERTS, 1994; GOLDBERGER, 2003). In the present study the maximum Cu concentration in tubers was 18.67 mg Cu kg⁻¹ of fresh tuber (for SMIF212-3 plants cultivated in Cambi VN3; data not shown) being the average Cu concentration around 13 mg Cu kg⁻¹ of fresh tuber in Cu contaminated soil. In this view, considering a diet based on potato, these tubers could easily exceed the RDA for Cu to human dietary. After nutritional requirements are met, there are several mechanisms that prevent Cu overload. However, the excess Cu absorbed can result in a number of adverse health effects including liver and kidney damage, anemia, immune toxicity, and developmental toxicity. Many of these effects are related with oxidative damage to membranes or macromolecules (BREMNER, 1998).

The results of the present study (Tab. 2) also demonstrated that higher Cu exposures led to increases in Cu concentration in leaves tissues, which has been previously reported for distinct plant species by other authors (SUDO et al., 2008; GIROTTO et al., 2016). Additionally, the tuber accumulation index (TAI) (Tab. 3) was higher in Cambi VN2, VN3 and Ulti VN2 treatments regardless of the tested genotype. Remarkably, SMINIA 793101-3 and SMIF 212-3 genotypes showed similar response in TAI terms, while SMIE 040-6RY genotype had lower TAI values as compared to SMINIA 793101-3 under Cu exposure.

During the fall growing season, SMIE040-6RY plants showed the highest sensitivity to Cu excess in growth terms (Fig. 1, 2 and 3). The response to Cu toxicity included plants without expanded leaves and lacking tuber production. Remarkably, the Cu concentration in leaf tissues of plants without expanded leaves grown in Cambi VN2 and Cambi VN3 did not differ from the Cu concentration of plants grown in Cambi C and Ulti controls, but plants with expanded leaves grown in soils with Cu contamination (Cambi VN1, Ulti VN1 and Ulti VN2) had significant increases in Cu tissue concentrations (Tab. 2).

Despite the plant development stage, the fitointoxication itself may reduce the transport and consequent accumulation of Cu, once there is a drastic reduction of absorption and translocation of water and solutes from soil as a result to the growth inhibition by Cu stress. Previous studies have reported a correlation between Cu excess and ethylene production (MAKSYMIEC, 2007), which is mainly associated

Tab. 2. Effect of increasing Cu level on leaves Cu concentration in potato genotypes grown in vineyards soils.

SOIL TREATMENT	Cu concentration ($\mu\text{g/g}$ dry weight)			
	<i>Fall growing season</i>			
	SMINIA 793101-3	SMIE 040-6RY	SMINIA 793101-3	SMIE 040-6RY
	<i>tuber initiation</i>		<i>18 days before harvesting</i>	
Cambi C+PK	3.40 \pm 0.09 dA	3.80 \pm 0.09 dA	3.67 \pm 0.05 eB	4.34 \pm 0.09 cA
Cambi C	7.23 \pm 0.3 cA	6.10 \pm 0.09 cA	10.00 \pm 0.2 dA	11.23 \pm 0.09 bA
Cambi VN1	11.00 \pm 0.3 bA	12.00 \pm 0.5 bA	19.13 \pm 0.4 bA	20.45 \pm 0.2 aA
Cambi VN2	20.01 \pm 0.1 a	ND*	29.00 \pm 0.3 aA	14.45 \pm 0.4 bB
Cambi VN3	15.34 \pm 0.2 ab	ND*	28.55 \pm 0.3 aA	13.00 \pm 0.4 bB
Ulti	3.90 \pm 0.09 dB	5.20 \pm 0.06 cdA	4.69 \pm 0.09 eB	9.76 \pm 0.3 bA
Ulti VN 1	17.00 \pm 0.1 aA	19.35 \pm 0.1 aA	25.10 \pm 0.2 aA	21.97 \pm 0.1 aB
Ulti VN 2	14.23 \pm 0.1 b	ND*	16.49 \pm 0.3 cA	18.66 \pm 0.2 aA
	<i>Spring growing season</i>			
	SMINIA 793101-3	SMIF 212-3	SMINIA 793101-3	SMIF 212-3
	<i>tuber initiation</i>		<i>15 days before harvesting</i>	
	Cambi C+PK	3.40 \pm 0.09 dA	3.80 \pm 0.08 dA	3.00 \pm 0.07 eB
Cambi C	5.70 \pm 0.3 cA	6.80 \pm 0.1 cA	10.70 \pm 0.2 cdA	10.50 \pm 0.3 dA
Cambi VN1	15.70 \pm 0.6 abA	16.50 \pm 0.3 bA	19.01 \pm 0.3 bA	18.00 \pm 0.1 bA
Cambi VN2	20.00 \pm 0.2 aA	21.00 \pm 0.1 aA	25.50 \pm 0.1 aA	24.00 \pm 0.2 aA
Cambi VN3	18.50 \pm 0.2 aA	18.55 \pm 0.2 aA	23.00 \pm 0.1 aA	24.50 \pm 0.2 aA
Ulti	3.30 \pm 0.09 dA	2.30 \pm 0.09 eB	6.80 \pm 0.3 dA	4.70 \pm 0.09 eB
Ulti VN 1	9.00 \pm 0.5 bcB	15.00 \pm 0.4 bA	13.00 \pm 0.5 cB	25.50 \pm 0.2 aA
Ulti VN 2	13.34 \pm 0.4 bA	11.50 \pm 0.09 bB	14.32 \pm 0.2 cA	14.00 \pm 0.4 cA

Data represent the mean \pm S.D. of six different replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype and collect ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level and collect ($p < 0.05$) ND* (data not available).

Tab. 3: Effect of increasing Cu level on tuber accumulation index (TAI)* in potato genotypes grown in vineyards soils.

SOIL TREATMENT	<i>Fall Growing Season</i>	
	SMINIA 793101-3	SMIE 040-6RY
Cambi C +PK	8.53 \pm 2.09 fA	7.68 \pm 0.13 eA
Cambi C	19.52 \pm 0.08 eA	10.44 \pm 0.24 dB
Cambi VN1	40.89 \pm 1.48 cA	21.43 \pm 1.48 cB
Cambi VN2	31.90 \pm 0.15 dA	28.25 \pm 0.92 bB
Cambi VN3	69.22 \pm 2.42 aA	40.78 \pm 2.13 aB
Ulti	11.94 \pm 1.34 fA	11.00 \pm 0.61 dA
Ulti VN1	31.34 \pm 0.07 dA	23.48 \pm 2.13 cB
Ulti VN2	53.90 \pm 2.17 bA	45.30 \pm 1.43 aB
	<i>Spring Growing Season</i>	
	SMINIA 793101-3	SMIF 212-3
Cambi C +PK	22.10 \pm 0.71 cA	11.13 \pm 0.09 fB
Cambi C	23.81 \pm 0.60 cA	18.71 \pm 0.87 eB
Cambi VN1	28.29 \pm 2.28 bA	18.72 \pm 2.18 eB
Cambi VN2	40.11 \pm 2.40 aA	42.22 \pm 2.31 bA
Cambi VN3	33.98 \pm 3.91 bA	20.47 \pm 0.20 deB
Ulti	15.62 \pm 0.22 dB	28.09 \pm 0.21 cA
Ulti VN1	23.46 \pm 1.08 cA	22.48 \pm 1.12 dA
Ulti VN2	41.21 \pm 0.82 aB	55.17 \pm 1.07 aA

Data represent the mean \pm S.D. of six different replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype and collect ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level and collect ($p < 0.05$). TAI* (total Cu concentration in plant tissue / produced tuber Cu concentration).

with growth retardation in plants (POTTERS et al., 2007), thus agreeing with the data of the present study.

The genotypes SMINIA 793101-3 and SMIF 212-3 were able to expand leaves and produce tubers in all tested soils (Fig. 1 and 2). In a previous works of our research group these genotypes were classified as P use efficient, while SMIE 040-6RY was classified as P non-use efficient. In view of the internal requirement of the plant nutrient, efficiency is generally defined as the biomass produced per unit of nutrient applied to the soil, which depends on two main components: efficiency of acquisition and utilization (BAILIAN et al., 1991). These components may mitigate Cu effects through a good nutrition, which reflects in a superior biomass produced and consequently dilute the metal in the tissues.

In plants grown under high Cu levels but also with suitable level of P and high K concentrations (Cambi VN1), tuber production was not negatively affected and had significant increases as compared to Cambi C and Ulti (Fig. 1 and 2). Potato plants are responsive to K fertilization with effects observed in tuber number and tuber weight per plant (ADHIKARY and KARKI, 2006), which together to high P levels may explain the mitigation of Cu toxicity effects on plant growth and the decrease of Cu in the leaf tissues in Cambi C + PK as compared to Cambi C.

Importantly, the present study had two controls for Cambi soils. A positive control (Cambi C + PK), without Cu accumulation and with appropriate nutrient concentrations for the tested culture, as well as a negative control (Cambi C), a soil also with no Cu accumulation but with P deficiency. This comparison was important for assessing the genotype variability in relation to P nutrition and for comparison of oxidative stress and growth under Cu contamination and nutritional deficiency.

Regardless of the significant difference in Cu content or concentration, there was a great reduction in the growth of plants

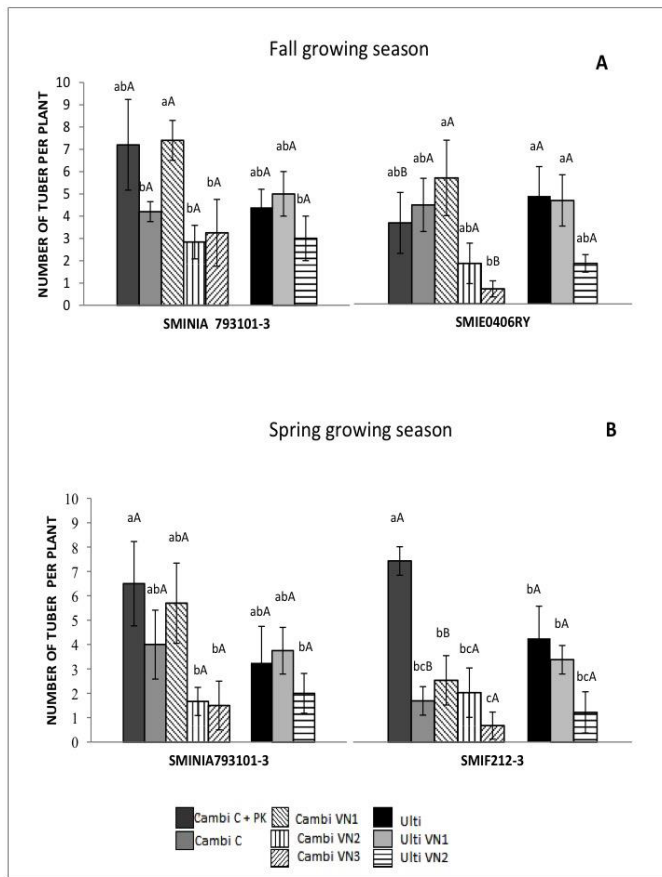


Fig. 1: Effect of increasing Cu levels on number of tubers per plant at fall (A) and spring (B) growing seasons. Data represent the mean \pm S.D. of six replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype and growing season ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level ($p < 0.05$).

cultivated in Ultisols with Cu accumulation, even when Cu was below the established threshold of 200 mg kg^{-1} (CONAMA, 2009), which was similar to the effects observed in Cambisols with Cu toxicity. The behavior of metals in soils is influenced by attributes of the soil solid phase, type of adsorbent (organic matter, silicate minerals, iron oxides, manganese, and phosphate groups) and geochemical conditions of the solution (ALLOWAY, 1995). Thus, it is possible to infer that the value established for the Cu threshold is not suitable for sandy soils, such as Ultisols.

The numbers of tubers per plant were similar between the genotypes in the same growing season (Fig. 1). There was an important effect regarding the growing season, with the spring growing season corresponding to fewer but heavier tubers while fall growing season resulted in higher number of smaller tubers (Fig. 2). These data demonstrated that the type of tuber produced during the fall growing season in this experimental system is less acceptable for commercial production due to the small size and average weight (LI, 2008).

An important factor to ensure a good productivity of tubers, in addition to the season and fertilization, is the quality of seed tubers, because seed tubers are responsible for the initial plant nutrition and they can also transmit diseases to the plant (BADONI and CHAUHAN, 2010). In this view, the utilization of homogeneous seed tubers may help to differentiate the genotypes according to initial nutrition requirements. During the fall growing season SMINIA793101-3 plants had a higher absorption of seed tubers in soils with Cu

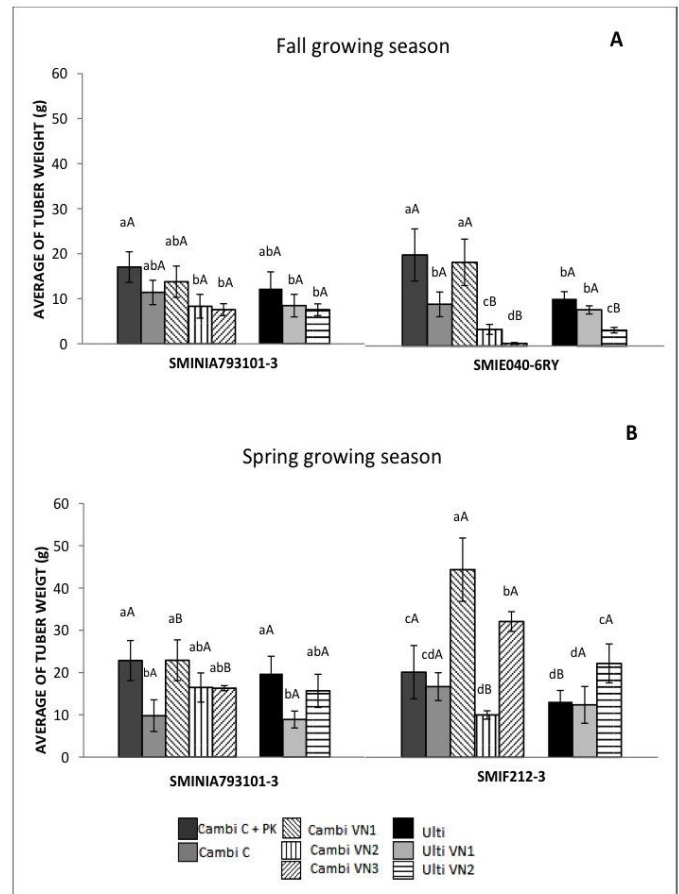


Fig. 2: Effect of increasing Cu levels on average of tuber weight at fall (A) and spring (B) growing seasons. Data represent the mean \pm S.D. of six replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype and growing season ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level and growing season ($p < 0.05$).

accumulation, while SMIE040-6RY plants responded with an increase of seed tubers biomass under the same treatments (Fig. 3). This response might be correlated to mineral use efficiency and to a possible higher tolerance to Cu toxicity, since these genotypes were also described as contrasting for P use efficiency (FARIAS et al., 2013).

Estimation of lipid peroxidation and hydrogen peroxide concentration

The main site of attack by any redox active metal in a plant cell is usually the cell membrane, and oxidation damage can cause a variety of harmful effects, including lipid peroxidation (ISLAM et al., 2008). In the present study, there were differences in MDA levels between seasons, harvests and genotypes (Fig. 4). During the fall growing season, there was an increase in MDA concentration in leaves during the plant cycle in all tested soils. This data was partially in agreement with results reported by GIROTTO et al. (2016), who tested *Avena sativa* grown in the same soil types.

The increase in MDA levels may be a result of increased levels of H_2O_2 , which occurred in plants grown in Cambi C, Cambi VN2, Cambi VN3, Ulti VN1 and Ulti VN2, thereby indicating the excessive accumulation of Cu in the soil. The H_2O_2 is an oxidant that can cause cellular damage, such as carbonylation of proteins, or even cell death (BIENERT et al., 2006). In addition, Cu can catalyze the formation of

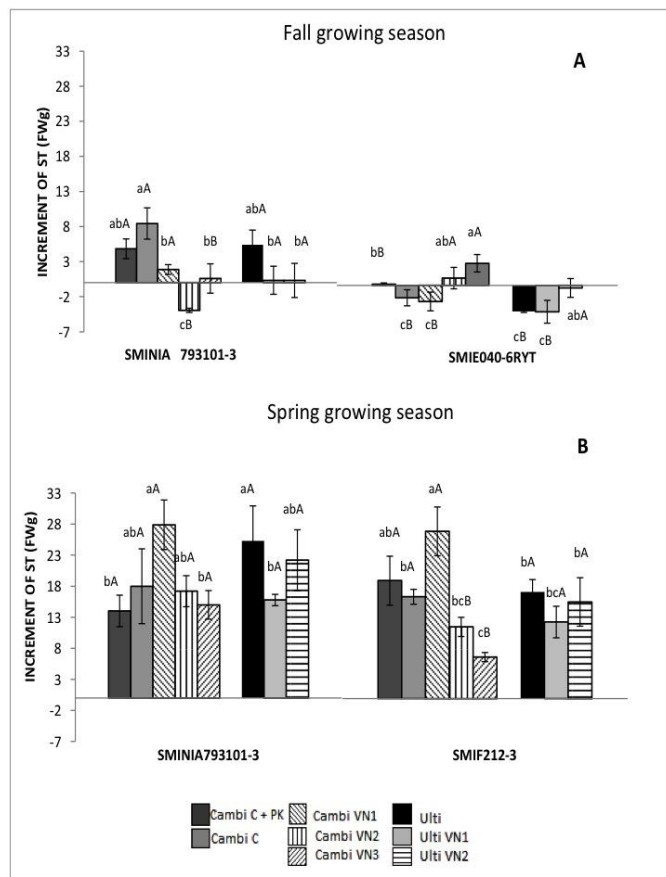


Fig. 3: Effect of increasing Cu levels on seed tuber (ST) increment per tuber at fall (A) and spring (B) growing seasons. Data represent the mean \pm S.D. of six replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype and growing season ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level and growing season ($p < 0.05$).

hydroxyl radicals (OH \cdot) from the non-enzymatic chemical reaction between superoxide (O $_2^-$) and H $_2$ O $_2$ (Haber-Weiss reaction). The main mechanism of Cu toxicity involves the Fenton reaction catalyzed by the presence of Cu, which is characterized by the production of hydroxyl radicals from superoxide and hydrogen peroxide in the presence of free Cu ions in the cell (BRIAT and LEBRUN, 1999). TAMÁS et al. (2004) suggested that the function of this elevated H $_2$ O $_2$ formation in tissues may be due to the increase in cell wall loosening. Interestingly, at the second harvest, genotypes SMINIA 793101-3 and SMIF 212-3 showed a higher increase in H $_2$ O $_2$ levels when grown in Cambi VN2 and Cambi VN3, while the genotype SMIE 040-6RY had the highest H $_2$ O $_2$ level when grown in Cambi C (Fig. 5). Additionally, regardless of the genotype tested, the H $_2$ O $_2$ concentrations were significantly higher in plants grown in Cambi C as compared to Cambi + PK, which may be attributed to the low availability of P in soil. These results were consistent with those obtained by KUMAR TEWARI et al. (2004), who showed that low levels of available P and exchangeable K in soil caused increased MDA levels in maize plants and increased H $_2$ O $_2$ concentrations in mulberry plants (BRANCO-NEVES et al., 2017).

Enzyme activities of antioxidant systems

The presence of excess Cu can cause oxidative stress in plants and, subsequently, cause an increase in antioxidant responses (YRUELA,

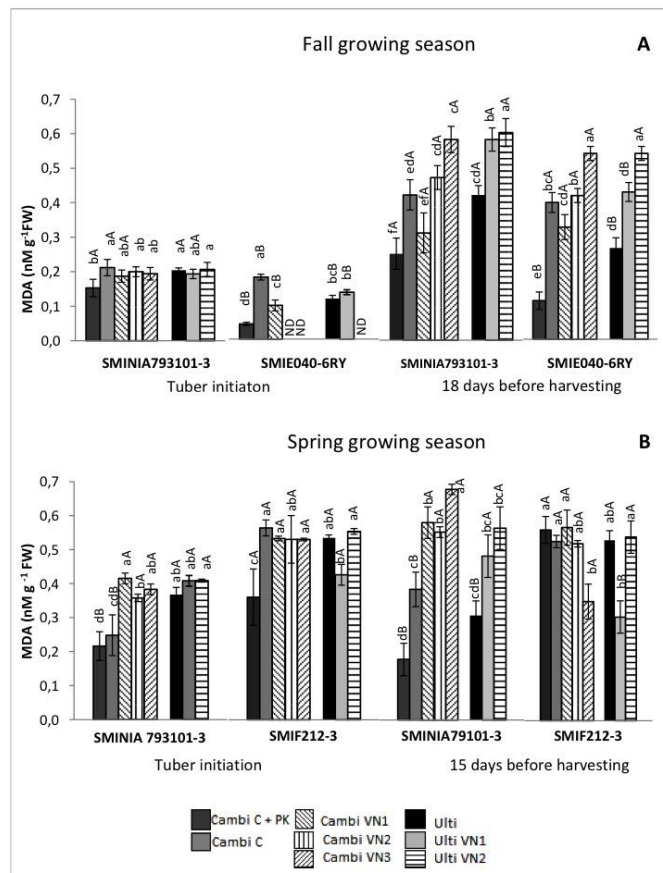


Fig. 4: Effect of increasing Cu levels on leaf lipid peroxidation in different development stages (tuber initiation and 15/18 days before harvesting) of different potato genotypes at fall (A) and spring (B) growing seasons. Data represent the mean \pm S.D. of six replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level ($p < 0.05$).

2005). Accordingly, the present study showed a significant increase in the activity of SMINIA793101-3 SOD tissue activity at both harvests with increased Cu levels in Ulti and Cambi soils as compared to Cambi C (Fig. 6). The increase in SOD activity is usually attributed to an increase in superoxide radical concentration, which is due to de novo synthesis of enzyme proteins (VERMA and DUBEY, 2003) resulting from the induction of SOD genes by superoxide-mediated signal transduction (FATIMA and AHMAD, 2005).

According to MITTLER (2002), the different affinities of APX (l M range) and CAT (mM range) for H $_2$ O $_2$ suggest that they belong to two different classes of H $_2$ O $_2$ -scavenging enzymes where APX may be responsible for the fine modulation of ROS for signaling and CAT may be responsible for the removal of excess ROS during stress. In the present study, the CAT activity was increased with Cu contamination with the exception of Ulti control because its CAT activity was higher than that of Ulti VN1 at the first collection time (Fig. 6). Interestingly, in SMINIA793101-3 plants, both CAT and APX had high activity under Cu contamination. However, CAT was more related to Ulti soils, and APX was more related to Cambi soils. Moreover, CAT activity was increased with the maturation of plants. In contrast, APX had the highest activity at tuber initiation.

On the other hand, the APX and CAT activities of SMIF212-3 plants grown in Cambi soils were increased with high Cu levels at the second collection time (Fig. 9B and C). These results suggested that the CAT

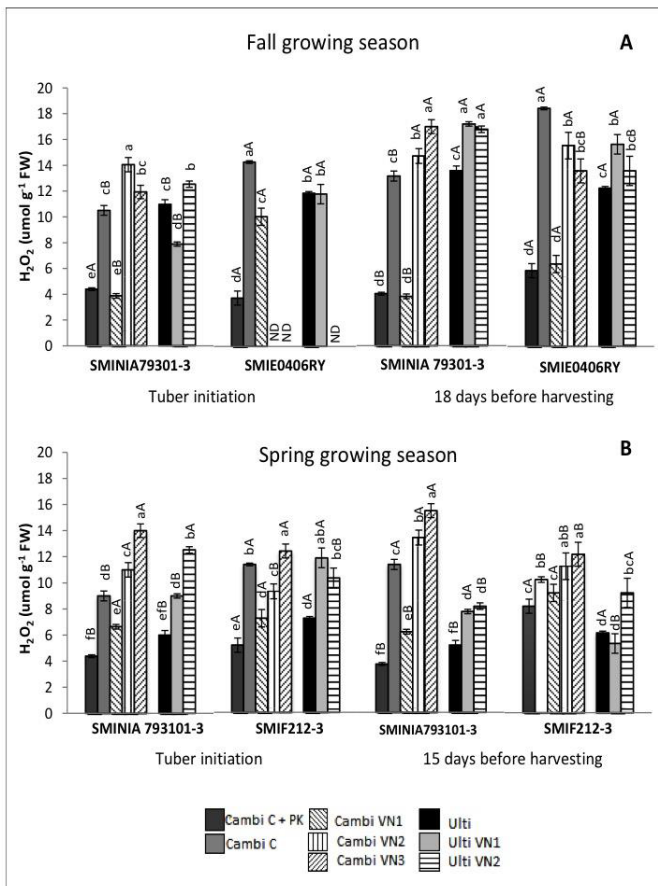


Fig. 5: Effect of increasing Cu levels on leaf H_2O_2 concentration in different development stages (tuber initiation and 15/18 days before harvesting) of different potato genotypes at fall (A) and spring (B) growing seasons. Data represent the mean \pm S.D. of six replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level ($p < 0.05$).

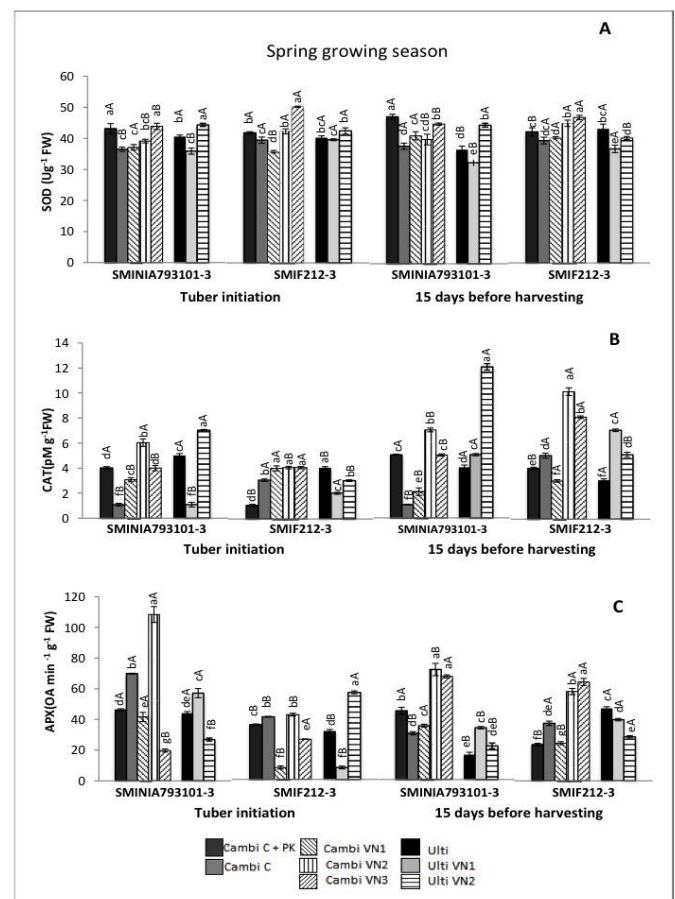


Fig. 6: Effect of increasing Cu levels on SOD (A), CAT (B) and APX (C) leave activities in different development stages (tuber initiation and 15/18 days before harvesting) of different potato genotypes at spring growing season. Data represent the mean \pm S.D. of six replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level ($p < 0.05$).

and APX activities (Fig. 6A and 6B) were more effective than SOD activity to scavenge the excess of ROS (Fig. 6A, 6B and 6C). APX is extremely sensitive to ascorbate concentrations. APX loses stability and its activity declines with low AsA contents (SHIGEOKA et al., 2002). However, the lower activity of APX did not correlate with the lower concentration of AsA in this experiment.

Non-enzymatic antioxidants

The AsA concentrations of SMINIA793101-3 plants in both Cambi and Ulti soils increased with higher Cu concentrations as compared to Cambi C and Ulti controls (Fig. 7), and the AsA concentrations of SMIF212-3 plants were only significantly increased in Cambi VN2 and Cambi VN3 at the first harvest and in Ulti VN1 at the second harvest. AsA concentrations can be varied considerably between tissues, and they depend on the physiological status of the plant and on environmental factors (SMIRNOFF, 1996). AsA is an essential constituent of higher plants that has key roles in antioxidant defense, cell division and cell elongation (HOREMANS et al., 2000). Therefore, AsA concentration is generally higher in younger tissues than in older ones, and AsA accumulates in actively growing tissues, such as meristems. In addition, photosynthetic tissues have high AsA concentrations (HOREMANS et al., 2000). However, in the present study, the AsA concentration did not considerably varied between

the collection times and genotypes.

The NPSH concentrations in SMINIA793101-3 plants were higher in Cambi VN2 and Cambi VN3 as compared to Cambi C at both collection times, but the NPSH concentrations resulting from plants grown in these soils were less than those resulting from plants grown in Cambi + PK and Cambi VN1 at tuber initiation. In Ulti soils, the NPSH concentrations in SMINIA793101-3 plants only gradually increased with Cu increments at the second collection time (Fig. 7B). Together, these data suggested an initial suppression of NPSH levels followed by an increase in response to Cu concentration in plant maturation, which has been previously described by ALI et al. (2006) in *Panax ginseng* plants. However, SMIF212-3 plants had lower NPSH values with high Cu concentrations even at maturation.

NPSH is affected by the presence of several metals (XIANG and OLIVER, 1998). Conversely, MURPHY and TAZI (1995) tested 10 *Arabidopsis* ecotypes for Cu tolerance with the expression of 2 metallothionein genes (MT1 and MT2) and NPSH levels, and they reported that MT1 is uniformly expressed in all tested treatments and that MT2 is Cu inducible in all 10 ecotypes. Moreover, they reported that ecotypes with higher levels of NPSH have an inducible tolerance mechanism.

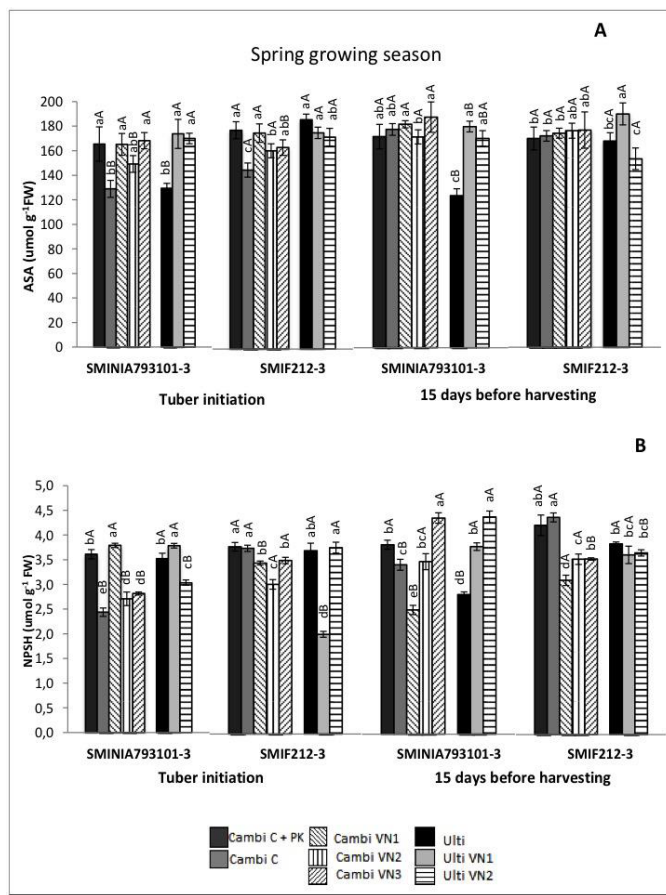


Fig. 7: Effect of increasing Cu levels on AsA (A) and NPSH (B) leave concentrations in different development stages (tuber initiation and 15/18 days before harvesting) of different potato genotypes at spring growing season. Data represent the mean ± S.D. of six replicates. Different lowercase letters indicate significant differences between Cu levels in the same potato genotype ($p < 0.05$). Different capital letters indicate significant differences between potato genotypes at the same Cu level ($p < 0.05$).

Multivariate analysis

The results obtained by the PCA based on a correlation matrix of different soils in relation to biochemical parameters of potato plants showed that genotypes were clustered by response to Cu contamination and soil fertility. However, there were distinct patterns of response between the tested genotypes and between the harvests (Fig. 8 and 9).

At the first harvest there was a common pattern of response related to H₂O₂ concentration and the activity of CAT and SOD, which were related to soils with high Cu content (Cambi VN2, Cambi VN3 and Ulti VN2) and also associated with P deficiency (Cambi C) for SMINIA 79101-3 genotype (Fig. 8 and 9). Interestingly, the other soil treatments were clustered together, in which Cambi VN1 and Ulti VN1 were primarily responsible for the explanation of a group of variables (protein, AsA, MDA and NPSH). Therefore, the principal component analysis promoted the separation of different treatments, exclusively under high concentrations of Cu and P deficiency, suggesting that in biochemical terms, during the early stage of plant development, the genotype SMINIA 79101-3 only responds in situations of marked toxicity or nutritional deficiency, not presenting, in terms of multivariate analysis, sufficient differences to distinguish the other treatments (Fig. 8 and 9).

On the other hand, SMIF 212-3 genotype showed a considerable sensitivity in biochemical parameters, which resulted on the

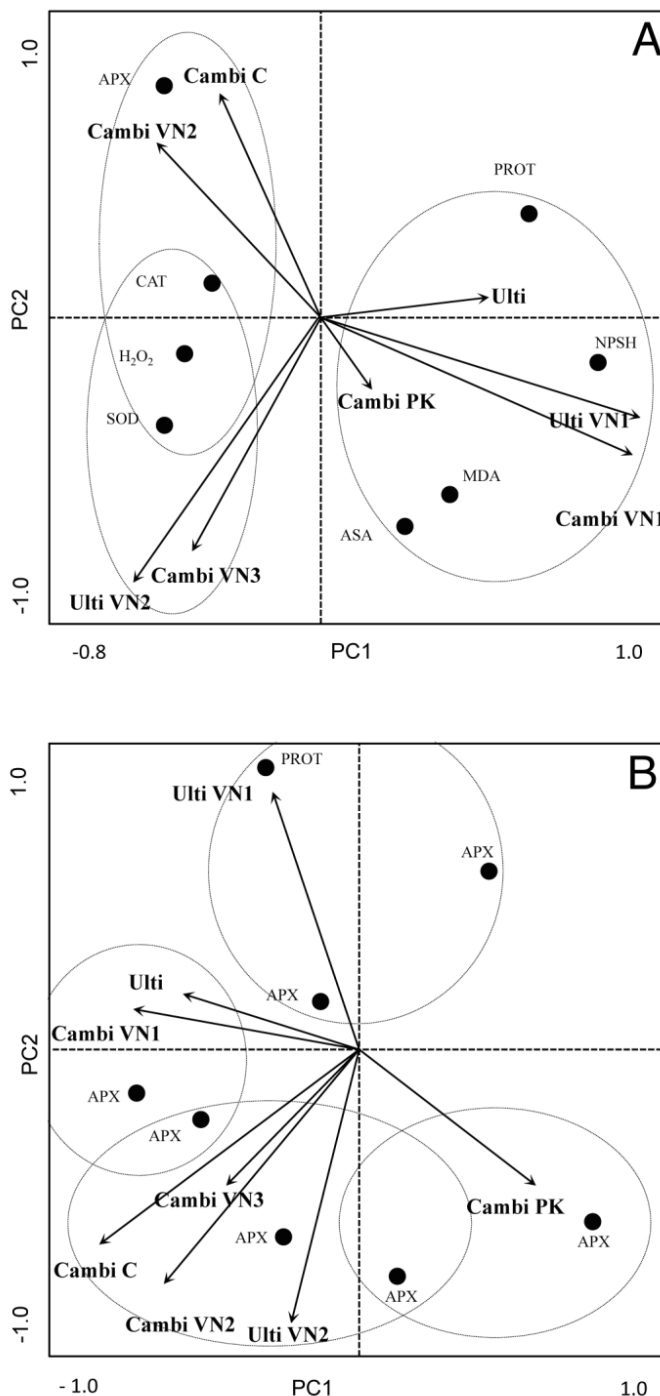


Fig. 8: Biplot graphic of scores and weights (loadings) for the first two principal components (PC1 and PC2) for biochemical parameters and tested soils at tuber initiation for SMINIA793101-3 (A) and SMIE (B) genotypes. MDA (lipid peroxidation); H₂O₂ (hydrogen peroxide concentration); SOD (Superoxide dismutase activity); CAT (catalase activity); APX (ascorbato peroxidase activity); PROT (protein concentration); AsA (ascorbic acid concentration); NPSH (non-protein thiol groups concentration).

formation of four clusters in both harvests. For this genotype, MDA concentration, NPSH and SOD activity were the main parameters related to Cu toxicity and P deficiency at the first harvest.

At the second harvest, not only the soil treatments relations changed, but also the relation among the biochemical parameters. In this view, for the SMINIA 79101-3 genotype at the initial development

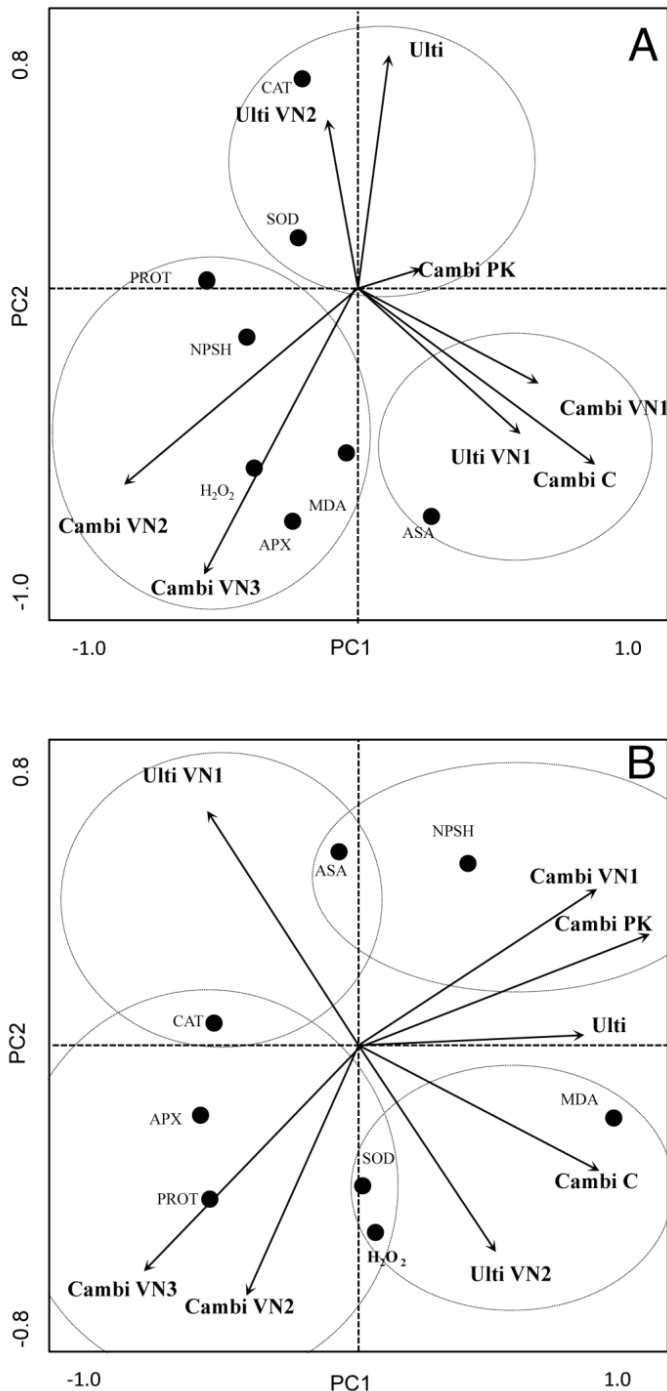


Fig. 9: Biplot graphic of scores and weights (loadings) for the first two principal components (PC1 and PC2) for biochemical parameters and tested soils 15 days before harvesting for SMINIA793101-3 (A) and SMIF212-3 (B) genotypes. MDA (lipid peroxidation); H₂O₂ (hydrogen peroxide concentration); SOD (Superoxide dismutase activity); CAT (catalase activity); APX (ascorbate peroxidase activity); PROT (protein concentration); AsA (ascorbic acid concentration); NPSH (non-protein thiol groups concentration).

stage, H₂O₂ concentration was not clustered with MDA, but near to the end of plant cycle these parameters were correlated. This pattern may be due to H₂O₂ function of signaling (TAMÁS et al., 2004) and the insufficient action of antioxidant system to prevent biological damage, which resulted in high MDA concentrations at the end of the plant cycle.

Other interesting data change was observed in relation to total protein concentration, which turned to be more related to treatment with high concentrations of Cu in matured plants. This change in protein concentration is probably due to the stage of plant development, which in plants grown in soils with no or low toxicity of Cu was ahead in relation to the other treatments during the first harvest, with plants in full growth and development, and gradually decreased during the plant cycle and the beginning of senescence. However, in plants grown under Cu toxicity, the initial growth was delayed in some cases even with reducing leaf expansion and shoot growth, which resulted in a higher protein concentration during the second harvest since these plants showed no signs of senescence or even maturation.

Conclusion

Vineyard soils with a long history of cupric fungicide application present toxic Cu levels to potato plants, resulting in oxidative damage, as evidenced by increased lipid peroxidation and high H₂O₂ concentration.

The enzymatic and non-enzymatic antioxidants as well as the Cu sensitivity of the growth parameters greatly varied among the genotypes with the P efficient genotypes being less sensitive to Cu. Furthermore, the data suggests a similar response to P deficiency and Cu toxicity in terms of oxidative stress. In contrast, plant nutrition with suitable levels of P and K preserves plants from ROS damage. This study also provides evidence that antioxidants are not sufficient to prevent biological damage mediated by ROS at the highest Cu concentrations, which still result in deleterious effects and that potato cultivation in areas with high Cu contamination is not recommended due to low production and potential risk to human health.

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