JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Assessment of the Effect of Silicon on Antioxidant Enzymes in Cotton Plants by Multivariate Analysis

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ABSTRACT: Silicon has been extensively researched in relation to the response of plants to biotic and abiotic stress, as an element triggering defense mechanisms which activate the antioxidant system. Furthermore, in some species, adding silicon to unstressed plants modifies the activity of certain antioxidant enzymes participating in detoxifying processes. Thus, in this study, we analyzed the activity of antioxidant enzymes in leaves and roots of unstressed cotton plants fertilized with silicon (Si). Cotton plants were grown in hydroponic culture and added with increasing doses of potassium silicate; then, the enzymatic activity of catalase (CAT), guaiacol peroxidase (GPOX), ascorbate peroxidase (APX), and lipid peroxidation were determined. Using multivariate analysis, we found that silicon altered the activity of GPOX, APX, and CAT in roots and leaves of unstressed cotton plants, whereas lipid peroxidation was not affected. The analysis of these four variables in concert showed a clear differentiation among Si treatments. We observed that enzymatic activities in leaves and roots changed as silicon concentration increased, to stabilize at 100 and 200 mg Si L^{-1} treatments in leaves and roots, respectively. Those alterations would allow a new biochemical status that could be partially responsible for the beneficial effects of silicon. This study might contribute to adjust the silicon application doses for optimal fertilization, preventing potential toxic effects and unnecessary cost.

KEYWORDS: *silicon, antioxidant enzymes, cotton, oxidative stress, multivariate analysis*

INTRODUCTION

Essentiality and function of silicon in plant development has been largely discussed in plant nutrition literature.[1] Silicon is absorbed and accumulates in concentrations that range from 1% to 10% of dry matter. This variability among plant species has been attributed to the ability of the roots to absorb Si.[2,3] Si contributes to alleviate water stress because it reduces transpiration by developing a Si gel layer on the epidermal cell wall.[4] In addition, the presence of silicon cell walls in fibers makes them hard and resistant, not only to water stress but also to pest and pathogens' attacks, increasing their tolerance to diseases.[2] Therefore, the principal function of silicon in plants is to provide a "defense against environmental onslaughts, both biotic and abiotic".[5]

Multivariate calibration methods are widely used to explain data relationships that are difficult to observe otherwise, being useful to identify hidden differences and to establish groups of data.[6] For instance, cluster analysis (CA), discriminant analysis (DA), principal component analysis (PCA), factor analysis (FA), and partial-least-squares (PLS) modeling allowed the rapid analysis and interpretation of large complex data sets in environmental, chemical, biological, and ecotoxicological case studies.[7] These methods have been successfully used to determine denomination of origin for honey[8] and wines,[9] quality of edible oils,[10] genotype classification,[11,12] and varieties of fruits.[13] More recently, multivariate tools were successfully used for the classification of propolis samples[14] and South American herbs.[15]

A few reports were found, applying chemometrics to biological systems in order to analyze biochemical parameters in plants. In iron-exposed plants, chemometric approaches revealed a pattern of variation in the biochemical responses that could not be observed through univariate statistics.[7] Diaz-Jaramillo et al.[16] studied GST, GSH activity, and total antioxidant capacity in *Perinereis gualpensis*. Using multivariate analysis, they grouped different sites exposed to contamination in Chile, suggesting their potential use as biomarkers; Larrigaudière et al.[17] analyzed the role of antioxidant and fermentative metabolisms to determine if core browning and brown heart in pears (*Pirus communis*) are associated with the same disorder. Using principal component analysis, they studied antioxidant enzymes (i.e., catalase, ascorbate peroxidase, glutathione reductase, and superoxide dismutase activity),

Received: May 23, 2013
Revised: October 31, 2013
Accepted: November 4, 2013
Published: November 4, 2013

fermentative variables (pyruvate decarboxylase and alcohol dehydrogenase activity), and lipoxigenases activity, and they found that both postharvest disorders involve different metabolic pathways.

Cotton (*Gossypium hirsutum*) is a nonsilicon accumulator[18] and, as such, may be useful to study the biochemical effect of stress alleviation by silicon application. It has been reported that Si helped to mitigate aluminum toxicity[19] and to control the damage produced by insects in cotton;[20,21] however, the activity of antioxidant enzymes under silicon treatment has not been reported.

Si has been proved to trigger plant defense mechanisms as an activator of strategic signaling proteins.[22] Addition of Si to plants under stress decreases the oxidative damage by diminishing the levels of hydrogen peroxide, lipid peroxidation, or electrolytic leakage in several species such as soybean,[23] Chinese cabbage,[24] grapevine,[25] maize,[26] cucumber,[27,28] wheat,[29,30] tomato,[31] and barley.[32] In these studies, several authors reported that the activity of several enzymes such as superoxide dismutase, catalase, and peroxidases were divergent.

In addition, Si application affects the gene expression profile in plants. Under stress, addition of silicon induces a significant increase in the transcription of genes involved in the defense response of several plant species such as rice,[33,34] *Arabidopsis thaliana*,[22] tomato,[35] and wheat.[36] In the same studies, addition of silicon to plants under unstressed conditions has no significant modification in the transcriptomic profile. This fact suggests that Silicon has a latent role in nonstressed plants, and its effect is not evident until a stressful situation occurs.

On the other hand, at a transductional level, silicon treatments modified the activity of antioxidant enzymes in unstressed plants.[24,28−31] Several studies investigating the effect of Si on stressed plants also revealed that Si alters the metabolism in antioxidant activities. In the case of *Brassica chinensis*, it was observed that SOD, CAT, and APX increased in controls added with silicon.[24] In cucumber, an increase of APX was observed after silicon addition to the control plants, but no alteration was observed in CAT and GR.[28] Besides, a decrease in GPOX activity took place in tomato plants supplemented with silicon.[31]

Since the induction/inhibition of antioxidant enzymes is one of the first defense responses under stress, it is important to analyze whether silicon intervenes in the antioxidant metabolism in order to adjust the plant fitness for a potential defense response. In this regard, the use of multivariate analysis provides a complementary approach to understand the effect of Si on plants, since a variable reduction could offer hidden information, which is not noticeable using traditional univariate statistical analysis.

On this basis, the aim of this study was to evaluate the effect of silicon on antioxidant enzyme activities (i.e., catalase, ascorbate peroxidase, and guaiacol peroxidase) of unstressed cotton plants using multivariate analysis with the purpose of establishing whether there are patterns of antioxidant enzyme behavior that may help understand the benefits of silicon on plants.

MATERIALS AND METHODS

Cotton seeds of "CEDRO" cultivar were acquired from Agencia Paulista de Tecnologia dos Agronegócios, Instituto Agronômico de Campinas. The seeds were superficially sterilized with hypochlorite solution (2%), rinsed with water, and placed in mini spots with a sand−vermiculite (2:1) mixture. Seven to ten days after, germination seedlings with similar heights were selected and transferred to 2 L plastic pots containing a 5-fold diluted Hoagland and Arnon[37] nutrient solution at pH 7. The seedlings were adapted during 7 days to liquid nutrient media. Then, diluted nutrient media were replaced by a complete nutrient solution. Two seedlings per pot were grown in a glasshouse at 25 °C and 45% humidity. When plants reached 90 days in the nutrient solution, a soluble silicate such as K_2SiO_3 (Unaprosil, Rio de Janeiro) was added in concentrations ranging from 0, 25, 50, 100, to 200 mg Si L^{-1}. Potassium concentration was maintained at the level of the Hoagland & Arnon nutrient solution (0.003 M), proportionally decreasing the concentration of potassium nitrate with each Si treatment. On the other hand, nitrogen concentration was compensated by proportionally adding ammonium nitrate. Treatment solutions were made at pH 7 and replaced weekly. Samples of leaves and roots from 120 day plants were transplanted and later collected in liquid nitrogen and stored in the ultrafreezer at −80 °C. Experiments were carried out in a completely randomized design with five replications for each silicon treatment.

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content, as previously described.[38] Leaves and roots (250 mg fresh weight) were crushed and homogenized in a mortar with 20% (w/v) insoluble polyvinylpyrrolidone (PVPP) and 1.3 mL of 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10000 rpm for 5 min, and 250 μL of the supernatant was added to 1 mL of 0.5% 2-thiobarbituric acid (TBA) and 20% TCA solution, and incubated in a water bath at 95 °C for 20 min. The concentration of MDA was calculated from the absorbance at 532 nm, using the absorbance coefficient 155 mM^{-1} cm^{-1}, following a correction for unspecific turbidity determined by the absorbance at 600 nm.

Enzyme extracts were obtained from cotton leaves and roots crushed and homogenized in a mortar with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylene diaminetetracetic acid (EDTA), 3 mM DL-dithiothreitol, and 5% (w/v) insoluble PVPP. The homogenate was centrifuged at 10000 rpm for 30 min, and the supernatant was stored in separate aliquots at −80 °C for the determination of enzyme activity.[38]

Catalase (CAT-EC 1.11.1.6) activity was studied at 25 °C in a 1 mL eppendorff tube containing 100 mM potassium phosphate buffer (pH 7.5) and 0.0075% H_2O_2. The reaction was initiated by the addition of 20−40 μL of plant extract and determined by monitoring H_2O_2 degradation in the spectrophotometer at 240 nm over 1 min.[39] CAT activity was calculated using an extinction coefficient of 39.4 mM^{-1} cm^{-1} for H_2O_2, and it was expressed in nmol H_2O_2 min^{-1} mg $protein^{-1}$.

Guaiacol peroxidase (GPOX, EC 1.11.1.7) activity was measured in a 1 mL mixture reaction containing 0.2 M potassium phosphate−0.1 M sodium citrate buffer (pH 5.0), 0.01% guaiacol, and 0.06% H_2O_2. The reaction was initiated by the addition of 5−10 μL of plant extract and incubated at 30 °C for 15 min. The reaction was stopped by rapid cooling in an ice water bath and the addition of 20 μL of sodium metabisulphite 0.2% (w/v). After vortex, the reaction was kept for 10 min and the GPOX activity was determined by absorbance at 450 nm, and quantified according to Gomes−Junior et al.[39] GPOX activity was expressed in per milligram of protein.

Ascorbate peroxidase activity (APX, EC 1.11.1.11) was determined by monitoring the rate of ascorbate oxidation at 290 nm at 30 °C. The reaction was initiated by the addition of 40 μL of plant extract to 1 mL of a medium containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM H_2O_2. APX activity, expressed as nanomoles of ascorbate per minute milligrams of protein, was calculated using the extinction coefficient 2.8 mM^{-1} cm^{-1} for ascorbate.[40]

The protein concentration was determined by the method of Bradford[41] using bovine serum albumin (BSA) as a standard.

ANOVA (significant differences of $p < 0.05$) and linear regression analysis (significant regression of $p < 0.05$) was performed by SAS statistical program (SAS Institute Inc., Cary, NC, 1999), and principal component analysis (PCA) for multivariate analysis was performed using the Unscrumbler 6.11 package (CAMO AS, Norway).

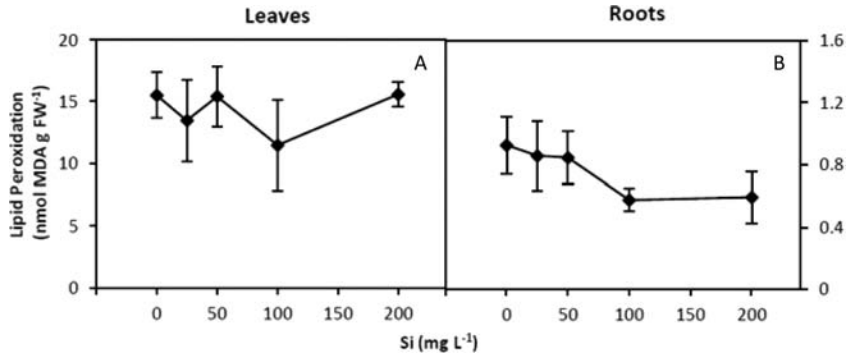

Figure 1. Effect of increasing silicon concentration on lipid peroxidation in leaves and roots of cotton plants. Bars represent the standard deviation of each mean, $n = 5$.

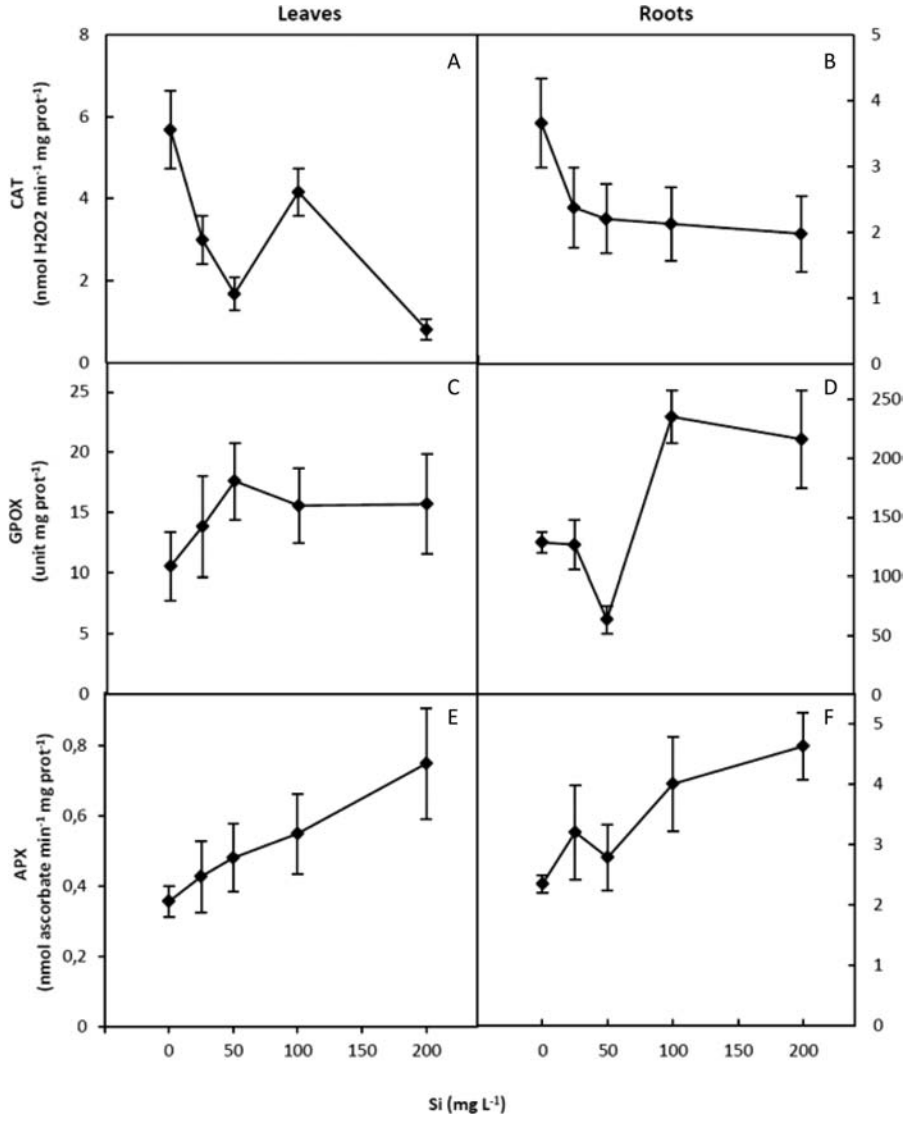

Figure 2. Effect of increasing silicon concentration on CAT, GPOX, and APX specific activity in leaves and roots of cotton plants. Bars represent the standard deviation of each mean, $n = 5$.

RESULTS

Lipid Peroxidation Analysis and CAT, APX, and GPOX Enzymatic Activity. Lipid peroxidation was measured by malondialdehyde contents in leaves and roots' extracts and analyzed by ANOVA and linear regression. Addition of silicon in the range of 0 to 200 mg L^{-1} did not alter lipid peroxidation in leaves (Figure 1A; ANOVA: $p = 0.0891$; Linear regression: $r = 0.0100$, $p = 0.9548$); however, in roots, lipid peroxidation decreased in treatments of 100 and 200 mg L^{-1}, showing a decrease trend as silicon treatment increased (Figure 1B; ANOVA: $p = 0.0089$; linear regression: $r = -0.6016$, $p =$

dx.doi.org/10.1021/jf4039088 | J. Agric. Food Chem. 2013, 61, 11243−11249

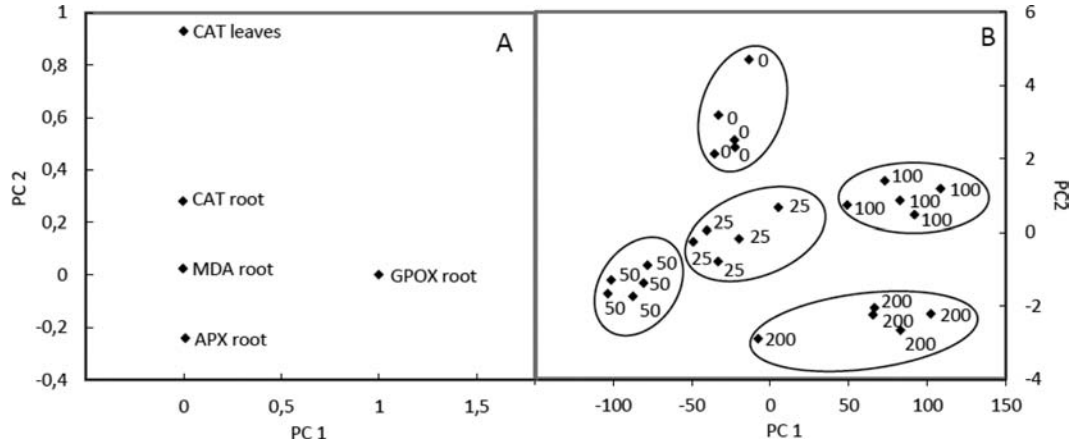

Figure 3. (A) Loading plot showing the influence of lipid peroxidation (MDA), CAT, APX, and GPOX variables measured in roots and the CAT variable measured in leaves, in the PCA model. (B) Scores plot shows the discrimination of Si treatments.

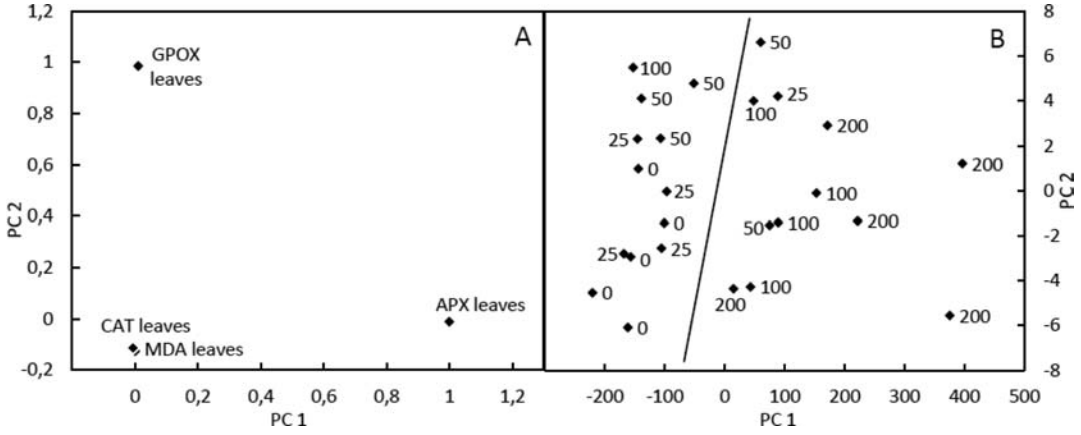

Figure 4. Loading plot showing the influence of the leaves variables in the PCA model. Discrimination obtained in the (B) Scores plot for every Si treatment.

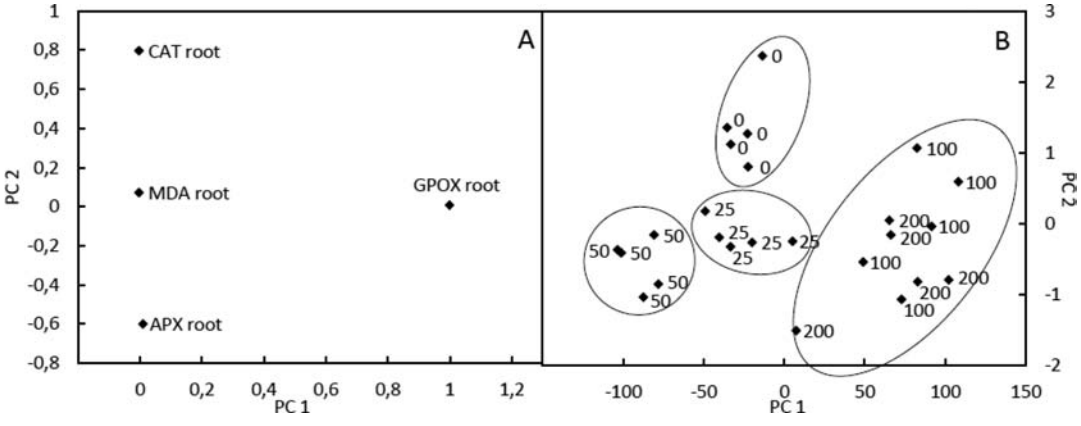

Figure 5. (A) Loading plot showing the influence of the roots variables for discrimination of Si treatments expressed in the (B) Score plot.

0.0015). Catalase activity diminished in leaves as well as roots when Si increased in the nutrient solution (Figure 2A; ANOVA: $p < 0.0001$; linear regression: $r = -0.6427$, $p = 0.0005$; and Figure 2B; ANOVA: $p = 0.0014$; linear regression: $r = -0.5201$, $p = 0.0077$). GPOX in leaves have no significant differences among treatments (Figure 2C; ANOVA: $p = 0.0551$; linear regression: $r = 0.3044$, $p = 0.1389$), while GPOX activity in roots (Figure 2D; ANOVA: $p < 0.0001$; linear regression: $r = 0.6486$, $p = 0.0005$) and APX activity in both leaves and roots (Figure 2E; ANOVA: $p = 0.0002$; linear regression: $r = 0.8064$, $p < 0.0001$, and Figure 2F; ANOVA: $p < 0.0001$; linear regression: $r = 0.7822$, $p < 0.0001$) show an opposite tendency compared to that of CAT in both tissues.

Chemometrics. Multivariate analysis was performed in order to find a pattern describing changes in metabolism of cotton plants after addition of Si. The MDA content and CAT, APX and GPOX activity of roots, and CAT of leaves were analyzed by principal component analysis (PCA) to evaluate

the grouping of samples in five Si concentrations (0, 25, 50, 100, and 200 mg L^{-1}). In the loading plot analysis, Figure 3A shows that GPOX root and CAT leaves (onto PC1 and PC 2, respectively) were the most important variables for grouping Si treatments. The scores plot (Figure 3B) indicates that principal component 2 (PC2) better discriminates the silicon treatments of 0, 50, and 200 mg L^{-1} application. On the other hand, principal component 1 (PC1) adjusts better to treatments of 25, 50, and 100 mg L^{-1} of Si. This model, based on four variables of roots and one of leaves, suggests that the parameters measured in roots are more precise to evaluate changes induced by silicon. For this reason, multivariate analysis of variables measured in roots and leaves was performed separately.

The loading plot for leaves variables (Figure 4A) indicated that APX leaves and GPOX leaves were the most significant variables in the first and second principal components, respectively. Treatments with high Si concentration were discriminated by influence of the APX leaves variable. Although the scores plot (Figure 4B) showed an important dispersion among the samples, a partition between the treatments 100 and 200 mg L^{-1} of Si and the rest of the treatments was still observed.

The loading plot for root variables (Figure 5A) indicates that the GPOX root variable in the first group and CAT root and APX root in the second one were three principal variables determining each group. The 0 mg L^{-1} Si treatment was discriminated from the rest of the treatments due to the influence of the CAT root and APX root variables, while the GPOX root grouped high silicon concentrations (100 and 200 mg L^{-1} Si). The scores plot (Figure 5B) showed an accurate division among treatments, except for 100 and 200 mg L^{-1} Si, which share the same group. These analyses provide a better grouping of treatments in roots in comparison with those of leaves.

DISCUSSION

In this study using multivariate analysis, we found that silicon alters the activity of GPOX, APX, and CAT in roots and leaves of unstressed cotton plants, while lipid peroxidation is not affected in leaves and decrease slightly in roots. The analysis of these variables in concert showed a clear differentiation among Si treatments, especially in roots. The studied variables showed a pattern of distribution in which four groups were detected (Figure 5B). However, these results were more difficult to observe using univariate statistics (Figures 1 and 2). Sinha et al.[7] underlined the difficulty in the analysis and interpretation of induced oxidative stress and biochemical changes due to the complex nature of biochemical responses and their interrelationships.

Antioxidant response to silicon application varies with plant species and often with cultivars of the same species.[24,42,43] If we compare the Si treatment with untreated controls, the antioxidant response does not always correlate with an increase of antioxidant enzyme activities. Results in our study showed a decrease in CAT activity both in leaves and roots. Wang et al.[42] report that silicon decreases GPOX activity in shoots and SOD activity in leaves of alfalfa. In leaves of soybean, SOD, CAT, and GPOX have no significant differences after silicon treatments.[23] Similar results were obtained in leaves of cucumber[28] and tomato.[31]

On the other hand, our work showed an increase in APX and GPOX activity, both in leaves and roots. Similar results were found by Liu et al.[44] showing significant increase of SOD, CAT, GPOX, and APX in leaves' extracts of cucumber. Feng et al.[45] found an increase in APX of cucumber leaves, while the same trend was reported by Song et al.[24] in leaves of two cultivars of *Brassica chinensis* treated with silicon in hydroponic solution. In *Seashore paspalum* turfgrass, silicon clearly induced the CAT activity in leaves.[46] Two peanut cultivars showed an increase in SOD, CAT, and GPOX activities in leaves and roots.[43] Overall, there is abundant literature reporting major alterations in antioxidant response when plants are exposed to silicon.

The multivariate analysis performed in this study allowed us to observe changes in enzymatic activities of leaves and roots as the silicon concentration increased to stabilize at 100 and 200 mg Si L^{-1} treatments. Gunes et al.[47] report a decrease of SOD and CAT activity and the increase of APX in barley shoots when plants were grown in a hydroponic solution added with silicon. They also found significant changes in antioxidant enzyme activity between 0 and 70 mg Si kg^{-1}. Activities in 70, 140, and 280 mg Si kg^{-1} were similar, resembling a plateau in concentrations over 70 mg Si kg^{-1}. Thus, results using multivariate statistical analysis clearly showed changes in the antioxidant metabolism that run parallel to increasing silicon doses, reaching a point where additional Si has no effect on the antioxidant activity (Figures 4 and 5).

Studies based on broad spectrum transcriptomic analysis (microarrays) have reported that few genes are differentially expressed when Si is added to unstressed plants. Fauteux et al.[22] studied the *Arabidopsis*-powdery mildew pathosystem at the transcriptional level and found only two genes (out of 40000 transcripts present on the microarray chip) differentially expressed in healthy plants exposed to silicon. Khandekar and Leisner[48] in their study of *Arabidopsis thaliana* did not observe any significant alteration in transcripts CSD1, CSD2 (copper/zinc superoxide dismutases) and GST24 (glutathione-*S*-transferase) in silicon treatments compared to nontreated controls. They concluded that silicon has no noticeable effect, inducing gene expression in unstressed plants growing in the presence of silicon. Similar results were obtained in rice by Watanabe et al., and [33] Brunnings et al.,[34] on their part, observed that silicon treatment in rice induces the upregulation of two transcripts of peroxidase precursors but downregulated the expression of a peroxidase gene. In plants of wheat supplemented with Si, Chain et al.[36] observed that four stress-response genes and one defense response gene were differentially expressed.

Even though some species do not seem to respond to silicon at a transcriptional level, there is some evidence showing a regulation at the level of protein activity, such as the alteration of antioxidant activity discussed earlier in this text. Besides, silicon effects could involve changes at other biochemical levels such as protein transduction, protein structure, or transport across membranes.[49,50] Studies about the destiny and transformation of soluble silicates into the plant could provide answers on the benefits of silicon nutrition.

Summarizing, our study showed that the activity of several antioxidant enzymes can be altered after Si addition to unstressed plants. The analysis of antioxidant parameters using multivariate tools showed that silicon, in the absence of stress, induced alterations in antioxidant activities in leaves and roots of cotton plants. These alterations, in turn, induced a new biochemical status which, applying multivariate analysis became noticeable; however, these differences were less evident when classical univariate statistical methods where applied. Such effects are likely due to the unknown influence of silicon on

biochemical and/or physiological processes. In addition, chemometrics of antioxidant enzymes activity could be a promising tool for the adjustment of silicon application doses for optimal fertilization, preventing excessive doses, unnecessary economic costs, and potentially eco-toxic effects.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was funded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Grant no. 309760/2003-8), Brazil. We would like to thank Prof. Roberto Olsina (Universidad Nacional de San Luis, Argentina) for providing The Unscrambler 6.11 software. We are also grateful for research grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) and Universidad Nacional de La Pampa, Argentina that supported this research.

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