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Manganese toxicity in rice plants mitigated by silicon effects on leaf tissues

Toxidez de manganês na planta de arroz atenuada pela ação do silício nos tecidos foliares

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Highlights:
Si in the nutrient solution can mitigate the adverse effects of Mn toxicity.
The adequate dose of Mn seems to increase silicon absorption by the leaf tissues.
Si increased the thickness of rice foliar tissues and resistence to adverse conditions.

Abstract

Anatomical modifications of leaves and other organs associated with mineral nutrition have been observed in many plants. However, little is known about the quantitative effects of Si and Mn on the anatomy of plant leaves, especially in rice. This study aimed to quantify the tissue thickness of rice leaves and the density of silica bodies in rice leaves grown in a nutrient solution supplemented with Si and Mn and evaluate the possible effects of Si on Mn toxicity. Treatments were arranged in a 2×3 factorial scheme = six combinations of treatments, two doses of Si (0 and 2 mmol L^{-1}), and three doses of Mn (0.5, 2.5, and 10 μmol L^{-1}) in randomized complete block design with four replications. After 39 days in the nutrient solution with the respective treatments, anatomical and micromorphometric measures of the leaf blade were carried out to determine the thickness and area of leaf tissues. The data were submitted to analysis of variance (ANOVA) and Tukey's multiple comparisons test of means. The abaxial and adaxial epidermal thickness, as well as the density of silica bodies increased with the addition of Si to the nutrient solution. This study demonstrated that Si reduced the number of vascular bundles and Mn reduced the thickness of the chlorenchyma with increasing doses. Manganese doses of up to 10 μmol L^{-1} do not inhibit the uptake and deposition of silicon in rice leaf tissues. Higher Si concentration in the solution caused anatomical changes in the leaf, which was associated with a possible alleviation of Mn toxicity due to the higher concentration of Si in plants since this effect was observed mainly when Si was present in the nutrient solution.
Key words: *Oryza sativa*. Plant nutrition. Micronutrient. Foliar micromorphometry.

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Resumo

Modificações anatômicas foliares e de outros órgãos, relacionadas à nutrição mineral, têm sido observadas em muitos vegetais. No entanto, sabe-se pouco sobre os efeitos quantitativos do Si e do Mn na anatomia foliar das plantas, especialmente o arroz. Com este estudo foi quantificado a espessura dos tecidos e a densidade de corpos silicosos de folhas de arroz cultivado em solução nutritiva com doses de Si e Mn e avaliar a possível ação do Si como atenuante à toxidez de Mn. Os tratamentos foram dispostos em um esquema fatorial 2×3 = seis combinações de tratamentos, sendo duas doses de Si (0 e 2 mmol L^{-1}) e três doses de Mn (0,5; 2,5 e 10 µmol L^{-1}), em delineamento de blocos completos casualizados, com quatro repetições. Após 39 dias em solução nutritiva com os respectivos tratamentos, foi realizado o estudo anatômico micromorfométrico do limbo das folhas e determinadas as espessuras e áreas dos tecidos foliares. Os dados foram submetidos à análise de variância (ANOVA) e o teste de comparações múltiplas para as médias pelo teste de Tukey . As espessuras da epiderme das faces abaxial e adaxial das folhas de arroz aumentaram com a adição de Si à solução nutritiva bem como a densidade de corpos silicosos. Verificou-se que o Si reduziu a quantidade de feixes vasculares e o Mn reduziu a espessura do parênquima clorofiliano com o aumento da sua dose na solução nutritiva. Doses de Mn de até 10 µmol L^{-1} não inibem a absorção e deposição de silício em tecidos foliares de arroz. O aumento da concentração de Si na solução causou alterações anatômicas dos órgãos foliares do arroz, o que foi associado a uma possível atenuação da toxidez de Mn devido à maior concentração de Si nas plantas, uma vez que esse efeito foi observado, principalmente, quando o Si estava presente na solução nutritiva.

Palavras-chave: *Oryza sativa.* Nutrição de plantas. Micronutriente. Micromorfometria foliar.

Introduction

Silicon (Si) increases plant resistance to stress conditions such as diseases and manganese (Mn) toxicity. Leaves generally reflect how resistant the plant may be to these and other adverse environmental conditions. The study of plant leaf anatomy in response to Si and Mn can therefore help better understand the effects of these elements on plant morphology, generating data that are scarce in the literature.

Silicon is considered a '*quasi-essential*' or agronomically essential element because it has several positive effects on many plant species. Its direct ability to mitigate stress or improve the nutritional balance in the plant leads to better yields (EPSTEIN; BLOOM, 2005). Although not recognized as an essential element, there are reports of its indirect effects on plant development and yield in many crops, including increased tolerance to abiotic agents such as Mn excess and biotic agents such as diseases and pests (MA, 2004; MA; YAMAJI, 2006). For rice, Si brings both increased yield and improved resistance to stress conditions like diseases, pests, drought, salinity, lodging, and toxicity caused by Al, Fe, and Mn (EPSTEIN, 2009; REYNOLDS et al., 2009, MEENA et al., 2014). Enhanced rice resistance to brown spot was reported by Zanão Júnior et al. (2009a) with the application of Si via soil. The authors also observed that this enhancement was associated with a higher foliar content of Si absorbed by the roots when the application was via soil, not via foliar.

After Si uptake, transport through the xylem and deposition, silicification takes place through the polymerization of monosilicic acid and the formation of hydrated amorphous silica ($SiO_2.nH_2O$), also called biogenic silica, silica gel or opal, rendering Si immobile. This silica can be deposited intra- or extra-cellularly, mainly in epidermal cells, and many of the benefits of Si to plants are attributed to this silica form. In the Gramineae, such as rice, Si is deposited in silica cells or silica bodies, bulliform cells, stomata and vascular bundles (CURRIE; PERRY, 2007).

Manganese is highly correlated with physiological processes that improve plant resistance to diseases because it participates in the composition of several enzymes as the activator or

a co-factor. However, high levels of Mn may cause plant toxicity (SANTOS et al., 2017), which can be diminished by the proven interaction of Mn with Si as observed when Si is used during the cultivation process (LIANG et al., 2007).

Nutrients and beneficial elements, such as Si, can alter the anatomy of plants and their reproductive organs. In the present study, we found that the modifications of the plant anatomy were related to mineral nutrition (SHANE et al., 2004; PAPADAKIS et al., 2004). Consequently, quantitative anatomical leaf assessments were applied to better understand the effects of Si and Mn on plants.

Therefore, the objective of this study was to quantify the thickness of the leaf tissues and the density of silica bodies in rice in response to Mn doses added to a nutrient solution, in the presence and absence of Si, and to evaluate the possible effect of Si on mitigating Mn toxicity through changes in leaf tissues.

Material and Methods

The experiment was conducted using a nutrient solution in a greenhouse of the Soil Science Department at the Federal University of Viçosa, MG. The treatments were arranged in a 2×3 factorial scheme (0 or 2 mmol L^{-1} Si; 0.5; 2.5 and 10 µmol L^{-1} Mn) with four replications in a randomized complete block design. Mn doses of 0.5, 2.5 and 10 µmol L^{-1} were defined to create the conditions of insufficiency, adequacy and toxicity, respectively, according to Zanão Júnior et al. (2010).

The experimental unit was composed of a 4 L plastic vessel containing six plants. The 'Metica-1' rice cultivar was used. Seeds were germinated on sheets of germitest paper, moistened with distilled water and kept for six days in a germinator at 25ºC. Removed from the germinator, seedlings were grown for three days in the base nutrient solution diluted to ½ strength using $MnCl_2.4H_2O$ and H_4SiO_4 as the sources of Mn and Si. The basic nutrient solution was composed of the following: 1.25 mmol L^{-1} K (KNO_3); 0.25 mmol L^{-1} P ($NH_4H_2PO_4$); 3.75 mmol L^{-1} N [KNO_3, NH_4Cl, $Ca(NO_3)_2.4H_2O$ and $NH_4H_2PO_4$); 1.0 mmol L^{-1} Ca [$Ca(NO_3)_2$]; 0.5 mmol L^{-1} Mg ($MgSO_4$); 0.5 mmol L^{-1} S ($MgSO_4$); 20 µmol L^{-1} Fe (Fe-EDTA); 0.3 µmol L^{-1} Cu ($CuSO_4.5H_2O$); 0.33 µmol L^{-1} Zn ($ZnSO_4.7H_2O$); 11.5 µmol L^{-1} B (H_3BO_3) and 0.1 µmol L^{-1} Mo ($Na_2MoO_4.2H_2O$), according to Zanão Júnior et al. (2009b). H_4SiO_4 was obtained by passing potassium silicate solution through a cation exchange resin column (MA et al., 2001). The nutrient solution was changed every four days, and the volume of the solution in the pots was maintained by adding distilled water. The level of pH was monitored daily and kept close to 5.5 by adding NaOH or HCl solutions (1 mol L^{-1}).

To implement the treatments, after a period in the base solution diluted to ½ strength, six uniform seedlings were selected and placed in pots containing a nutrient solution supplemented with Si and Mn.

For anatomical studies and micromorphometric assessments, samples of 2.0 cm^2 from the median part of the blade of developed leaves (YOSHIDA, 1981) were collected from two plants in each pot after 39 days in the nutrient solution. The samples were fixed in $FAA_{50\%}$, dehydrated in a graded ethylic series and embedded in methacrylate (Historesina, Leica Instruments, Heidelberg, Germany). The embedded material was sectioned using an automated rotary microtome into cross sections of 5 µm thickness and stained with toluidine blue pH 4.0 (O'BRIEN; MCCULLY, 1981). The sections were mounted with Permount (SP15-500, Fisher Scientific, New Jersey, EUA). In order to determine the silica veinal and interveinal bodies, the diaphanization technique was used (KRAUS; ARDUIM, 1997).

Samples for scanning electron microscopy (SEM) were fixed in $FAA_{50\%}$ and dehydrated in a graded ethylic series. Drying to the critical point was performed with liquid CO_2 in a critical point dryer (model CPD 020, Bal-Tec, Balzers, Liechtenstein).

After covering with gold in a sputter coater (model FDU010, Bal-Tec, Balzers, Liechtenstein), the plant material was documented using scanning electron microscope with an attached digital camera (LEO, model 1430 - VP, England) at the Nucleus of Microscopy and Microanalysis of the Federal University of Viçosa (UFV).

From the same material used for SEM, samples were taken and prepared for energy dispersive spectroscopy (EDS) analysis, which was carried out at the Microscopy and Microanalysis Laboratory of the State University of Londrina (UEL). Ten fragments were cut and mounted on aluminum supports, half with the leaf surface up and the other half down. These specimens were first dried for 24 h in a desiccator containing silica gel and then sprayed (MED 010 Balzer) with a thin layer of C for analysis with SEM-EDS equipment model FEI Quanta 200 at 25 kV and a working distance of 12 mm.

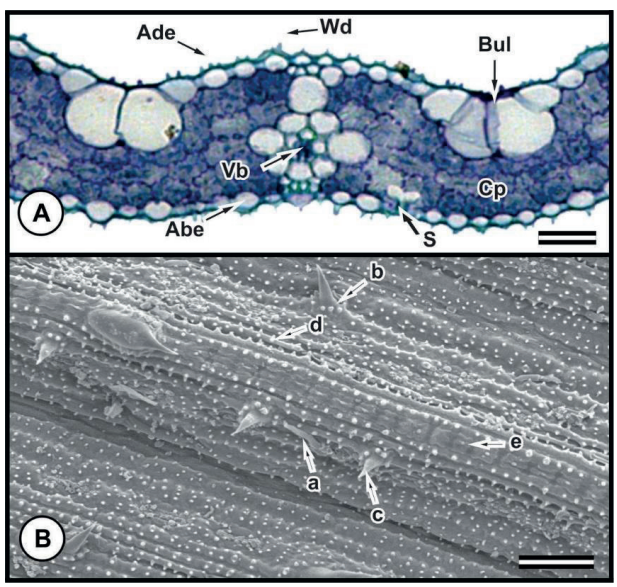
Digitization of the images was carried out under a photo-microscope (Olympus AX70TRF, Olympus Optical, Tokyo, Japan) with a U-photo system at the Plant Anatomy Laboratory of the Federal University of Viçosa (MG). Thickness and tissue area data (45 measurements per treatment) were obtained with the aid of Image Pro-Plus 4.5 software (Media Cybernetics, Inc., USA) and the density of silica bodies was assessed using the Anati-Quanti software (AGUIAR et al., 2007).

The data were submitted to the ANOVA multiple comparisons test for the means and to Tukey's test of additivity of the model . Analyses were performed using software R, followed by the Shapiro-Wilk test for normality of residuals), and Bartlett's test for homoscedasticity).

Results and Discussion

The rice leaf is amphistomatic, with unstratified epidermis, homogeneous mesophyll and vascular bundles enclosed by bundle sheaths of parenchyma cells. Like in other Gramineae, it has costal and intercostal zones (Figure 1) The costal zone is the one that covers veins and the intercostal zone is located between the veins (METCALFE, 1960).

Figure 1. Rice leaf anatomy, *Oriza sativa* (Poaceae). A: Light microscopy (cross-section). B: Scanning electron microscopy of the abaxial surface. a = micro-hair, Abe: abaxial epidermis, Ade: adaxial epidermis, b = macro-hair, Bul: bulliform cells, c = prickle hair, cp: parenchyma cells, d = papillae, e = silica bodies, m: mesophyll, s: stomata, vb: vascular bundles, wd: wax deposition. Scale bar = 100 μm.



The epidermis has been observed to have a thick cuticle with prominent wax deposition and bulliform cells placed at a lower level than the other cells in the adaxial epidermis. Presence of macro-hairs, micro-hairs, prickle hairs, papillae and silica bodies was also evidenced. Prickle hairs were found mostly in the adaxial epidermis (Figure 1) The stomata are paracytic, concentrated in rows in the intercostal zones, restricted to ridges of the leaf.

Macro- and micro-hairs are defined by their structural features not by their size. Macro-hairs are unicellular, pointed, and very similar to prickle hairs, differing only by the bulge found at the base of the hair. The macro-hairs are distributed along the blade, mostly concentrated in the intercostal zones. A micro-hair generally consist of two cells and is narrower and longer in relation to a macro-hair.

A prickle hair has a bulgy base with basal cells surrounded by papillae. They are spiky and mostly occur in the intercostal zones of the leaf blade. Two types of papillae have been observed, one "inflated" or swollen and the other elongated. They are arranged in rows in both the intercostal zones, in greater quantity, and in the costal zones. According to Kumar et al. (2017) these papillae are natural to the rice leaf and are not Si-accumulation sites.

In rice leaves, Mauad et al. (2013) found higher levels of silica in epidermis cells after silicate fertilization. Melo (2005) observed deposition of silica in epidermis cells of *Brachiaria brizantha*, including bulliform cells, which are considered to be easily degraded due to their thin cell walls and a thin cuticle cover, resembling parenchyma cells (WILSON, 1976).

The deposition of silica in the epidermal cells forms a mechanical barrier called the double silica-cuticle layer, which decreases transpiration, improves the use of water and increases the resistance of plants to pathogens (FAUTEUX et al., 2005; LIANG et al., 2007; DEBONA et al., 2017).

Silica bodies are present in the epidermal cells of the costal zones of rice leaves. These cells are called silica cells. They are short and dumb-bell shaped (Figure 1). The mesophyll is homogeneous and has three to five cell layers. The bundle sheath is composed of thin-walled parenchyma cells and has an extension of sclerified cells that attach to the epidermis. Vascular tissues, in front view, are located in rows (Figure 1).

It was found that the parenchyma cells occupy around 60% of the total area of a rice leaf, while the epidermis on the adaxial side occupies around 20% and the abaxial side and vascular bundles approximately 10% each, thus totaling 100% of the composition of the leaf (Table 1). The addition of Si to the solution did not modify the area of the evaluated tissues, except for the area of vascular bundles that presented a significant reduction (Table 1). This reduction was probably caused by the rearrangement of the cells since epidermis expansion was observed (Table 2), although they remained proportionally equivalent.

Doses of Mn above 0.5 μmol L^{-1} decreased the proportional area of the parenchyma in both the presence and absence of Si in the solution. For the other evaluated tissues, Mn doses did not influence their proportional area in the leaves. Research has shown that leaf Mn has more significant effects on mesophyll tissues, such as the chlorenchyma, but insignificant effects on epidermal layers (FERNANDO et al., 2006).

The density of silica bodies increased significantly both in the veinal and interveinal epidermis with the addition of 2 mmol L^{-1} Si to the nutrient solution (Table 3). The different doses of Mn did not influence the density of silica bodies in the absence of silicon. In the presence of silicon, the dose of 2.5 μmol L^{-1} of Mn significantly increased the density of silica bodies in the veinal part of rice leaves (Table 3). This result indicates that the adequate dose of Mn seems to increase silicon absorption by the leaf tissues. Freitas, Fernandes and Maia (2015) also observed interactions between Mn and Si in rice plants.

Table 1. Area (%) of the leaf tissues of rice grown with three doses of Mn, in the presence and absence of Si in the nutrient solution.

Silicon	Mn	Epidermis		Parenchyma	Vascular bundles
		Adaxial	Abaxial		
mmol L^{-1}	µmol L^{-1}	---------- % ----------			
0	0.5	19.42a	10.82a	58.63 a*	11.02a
	2.5	21.19a	11.50a	57.04 b	10.50a
	10.0	20.43a	11.78a	57.29 b	10.27a
Mean		20.35 A	11.36 A	57.65 A	10.59 A
2	0.5	18.18a	11.92a	60.56 a	10.03a
	2.5	20.67a	13.10a	56.32 b	9.86a
	10.00	20.28a	10.81a	58.87 b	9.33a
Mean		19.71 A	11.94 A	58.59 A	9.74 B
CV (%)		11.98	18.75	4.14	8.89

* Means followed by the same letters in columns differ significantly by Tukey's test (P < 0.05). Lowercase letters compare Mn doses within Si dose and uppercase letters the doses.

Table 2. The density of silica bodies in rice leaves cultivated with three doses of Mn, with and without the addition of Si to the nutrient solution.

Silicon	Mn	Silica bodies		
		Veinal	Interveinal	Total
mmol L^{-1}	µmol L^{-1}	----------n° cm^{-2} ----------		
0	0.5	33.50 a	0 a	33.50 a
	2.5	28.67 a	0 a	28.67 a
	10.0	19.17 a	0 a	19.17 a
Mean		27.11 B	0.0 B	27.11 B
2	0.5	449.50 b	84.00 a	533.50 b
	2.5	485.42 a	129.00 a	614.42 a
	10.00	379.00 b	105.67 a	484.67 b
Mean		437.97 A	106.22 A	544.19 A
CV (%)		19.09	42.45	15.02

* Means followed by the same letters in columns differ significantly by Tukey's test (P < 0.05). Lowercase letters compare Mn doses within each Si dose and uppercase letters the doses.

Table 3. The thickness of the abaxial and adaxial epidermis and the parenchyma of rice leaf blade cultivated with three doses of Mn, with and without the addition of Si to the nutrient solution.

Silicon	Mn	Espessura			
		Abaxial epidermis	Adaxial epidermis	Chlorenchyma	Total
mmol L^{-1}	µmol L^{-1}	----------µm ----------			
0.0	0.5	0.532 a*	0.596 a	3.758 a	4.886 a
	2.5	0.471 b	0.553 a	3.632 a	4.656 a
	10.0	0.535 a	0.583 a	3.469 a	4.587 a
Mean		0.513 B	0.577 B	3.619 A	4.709 A
2.0	0.5	0.576 a	0.607 a	3.558 a	4.741 a
	2.5	0.604 a	0.642 a	3.441 a	4.687 a
	10.00	0.556 a	0.598 a	3.502 a	4.656 a
Mean		0.579 A	0.616 A	3.501 B	4.696 A
CV (%)		12.49	12.21	7.84	6.03

* Means followed by distinct letters in columns differ significantly by Tukey's test ($P < 0.05$). Lowercase letters compare Mn doses within each Si dose and uppercase letters the doses.

Plants treated with and without Si showed significant differences in silica deposition and the formation of silica bodies. The silica content in the leaves increases with the supply of Si and was strongly correlated with the number of silica bodies per unit of leaf area (AGARIE et al., 1996).

The EDS analysis demonstrated a high concentration of Si in foliar tissues in the treatments that received Si in comparison to the samples that did not receive (Figure 2). This analysis confirms the increase of veinal and interveinal silica bodies in the front view of the epidermis. Andrade, Andrade and Miglioranza (2012) and Domiciano et al. (2010) detected a large concentration of silica in silicate-fertilized wheat leaves, about 90% higher than in plants that did not receive this fertilizer.

A higher number of silica cells and papillae were observed distributed on the leaf surface and around the stomata with the addition of Si (Figure 3A). Silica cells are dumb-bell shaped and distributed in rows along the veinal zones of the leaves, as evidenced by the X-ray microanalysis (Figure 3B-C).

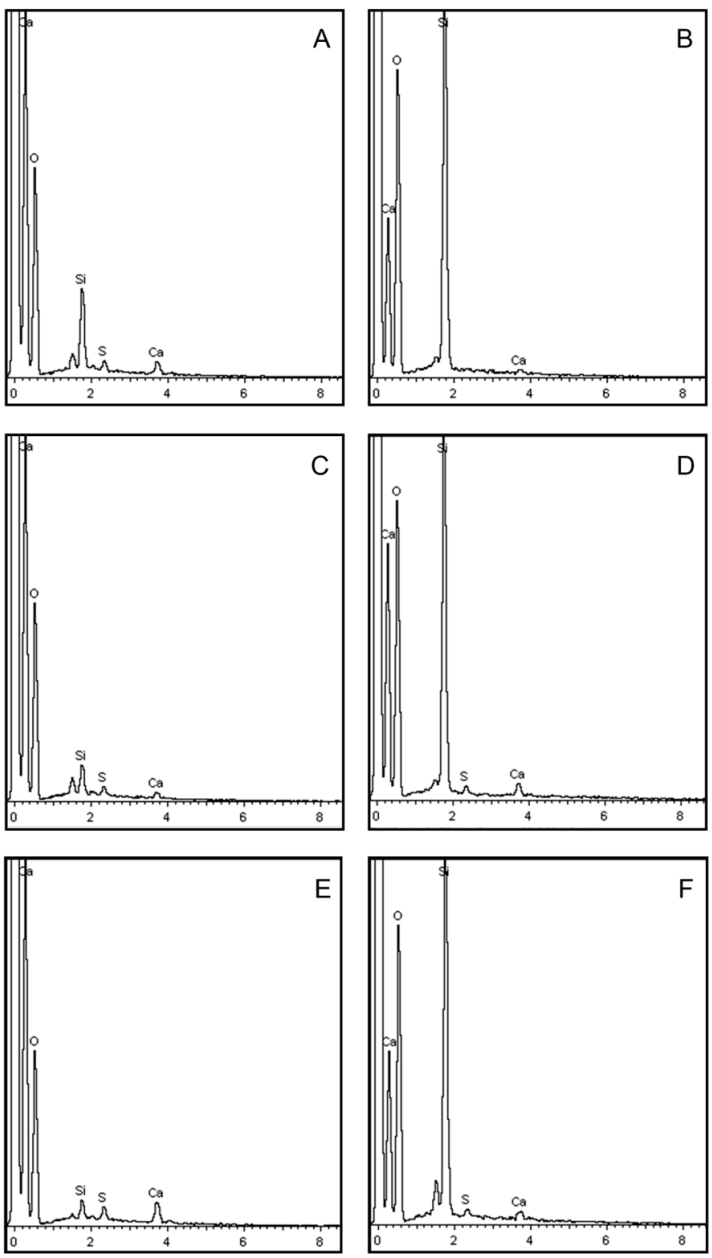
In other Gramineae, such as sorghum, silica cells are also distributed in regular rows over leaf veins (LUX et al., 2002). The addition of Si in rice cultivation leads to more pronounced cell silicification with a higher number of siliceous cells and papillae (ZHANG et al., 2013; NING et al., 2014). The epidermal thickness of the abaxial and adaxial sides of the leaves increased with the addition of Si to the nutrient solution (Table 2). It was observed that the epidermis on the abaxial side thickened proportionally more (12.86%) than the epidermis on the adaxial side (6.75%). Gong et al. (2005) also observed such thickening of wheat leaves grown with Si. It was also evidenced that in maize leaves Si promoted thickening of the adaxial epidermis (CUNHA; NASCIMENTO, 2008) but not the abaxial epidermis, as in this work.

The increased thickening of the epidermis possibly occurred due to the deposition of Si in the form of amorphous or hydrated silica ($SiO_2.nH_2O$) in the epidermal cells. Ma and Takahashi (2002) state that more than 90% of Si in rice shoots is found in this form. According to Motomura et al. (2000), this silica can be deposited extracellularly, mainly in these cells under the cuticle. Kumar et al. (2017) also report other forms of silica deposition in the Gramineae species, impregnating both the wall of the common epidermal cells and the micro-

and macro-hairs. In addition, metabolic control of silica deposition does not interfere in the cell-to-cell diffusion process.

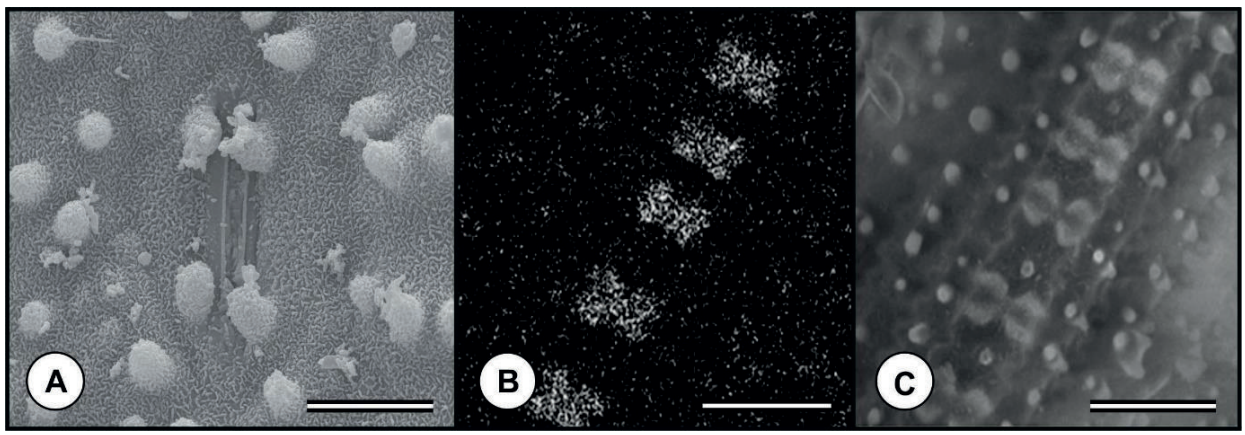
Manganese doses altered the thickness of the abaxial epidermis but only in the absence of Si in the nutrient solution (Table 2). It shows that the presence of Si in the nutrient solution can mitigate the adverse effects of Mn through variations in the thickness of the foliar tissues. According to Williams and Vlamis (1957), the presence of silicon provides a more uniform distribution of Mn in the leaf blade, thus preventing it from accumulating high concentrations in certain parts, which would prompt the death of the cells in that region.

Figure 2. The spectrum of the elements present in the abaxial epidermis in a rice leaf detected by EDS. A, C and E: spectrum of the elements in rice leaves grown with three doses of Mn and without the addition of Si to the nutrient solution. B, D and F: spectrum of the elements in rice leaves grown with the addition of Si to the nutrient solution.



Although there is a strong link between the presence of Si and the reduction of Mn accumulation in leaf tissues (ROGALLA; RÖMHELD, 2002), studies show that the alleviation of Mn toxicity in plants by Si occurs mainly in non-photosynthetic tissues through enzymatic and non-enzymatic antioxidant reactions (LI et al., 2012; DONCHEVA et al., 2009; SHI et al., 2005). In this study, the total mean thickness was higher in the presence of Si, regardless of the applied Mn doses. However, since the high concentration of Mn in rice plants considerably reduces shoot dry matter production (LI et al., 2012), Si, which increases the thickness of leaf tissues, ends up as a mitigator of the adverse effects of Mn toxicity as thicker leaf tissues generate higher dry matter yield.

Figure 3. A. Abaxial epidermis of a rice leaf, *Oriza sativa* (Poaceae) (Scanning electron microscopy. B. Silicon in the veinal zone on the abaxial surface of a rice leaf. C: Corresponding mapping of Si by X-ray microanalysis. Bar scale: a = 10 μm, B and C: 40 μm.



The presence of Si also reduced the thickness of the chlorenchyma (Table 2). According to Agrios (1988), plants that are more resistant to stress conditions have thicker epidermis and more compact parenchyma. Therefore, the effect of silicon on features associated with higher physical resistance of foliar tissues in rice was verified in this study, which makes the plant less susceptible to metal toxicity and disease infection (FLECK et al., 2010).

In the present study, the uptake of Si by rice from the nutrient solution was similar to its uptake directly from the soil solution as observed by Zanão Júnior et al. (2009a), who reported increased leaf Si content with the consequent induction of resistance to the incidence of brown spot disease. The alleviation of Mn toxicity by Si may be similar to the induction of disease resistance, according to the above-mentioned authors. Also, in ornamental plants, in rose bush cultivation, higher doses of Si applied to the substrate in pots lead to improved root uptake, increased leaf Si content, higher yield, and better flower quality (ZANÃO JÚNIOR et al., 2013).

Conclusions

Manganese doses of up to 10 μmol L^{-1} in rice do not inhibit the uptake of Si by the roots and its deposition in leaf tissues. A possible alleviation of Mn toxicity at higher Si concentrations in the leaves by means of anatomical alterations of leaf organs was observed since these were mainly observed when Si was present in the nutrient solution. The presence of Si increased the thickness of rice foliar tissues, which can be associated with higher physical resistance and tolerance of the plant to adverse conditions.

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