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Silicon-enhanced non-enzymatic antioxidant defense mechanisms in young orange trees under glyphosate-induced stress

Inácio João Barbosa¹, Jonas Pereira de Souza Junior^{2*}, Milton Garcia Costa¹, José Clebson Barbosa Lúcio¹, Davie M. Kadyampakeni², Priscila Lupino Gratão¹, Leonardo Bianco de Carvalho¹, Renato de Mello Prado¹ and Silvano Bianco¹

Abstract

Background Glyphosate is widely used in citrus production, but its overuse can cause oxidative stress and reduced growth in young orange trees. Silicon (Si), a beneficial element, strengthens antioxidant defense pathways and attenuates oxidative damage. However, its role in alleviating glyphosate-induced stress, particularly through the non-enzymatic antioxidant systems, remains unclear. This study examined whether Si application can reduce oxidative stress in young “Valencia” orange trees by enhancing non-enzymatic defenses, reducing oxidative stress indicators, and improving plant growth.

Results In an 8-month greenhouse experiment using a 4 × 2 factorial design, four glyphosate rates (0, 576, 1008 and 1440 g acid equivalent (a.e.) ha⁻¹) and two Si treatments (0- and 2-mM Si), were applied to trees via fertigation and foliar sprays. Key parameters were measured nine and sixteen days after the fourth Si application in older and younger leaves, respectively. Trees treated with Si exhibited a 66% Si increase in young leaves and 44% in old leaves. Oxidative stress, measured by malondialdehyde (MDA) levels, was significantly lower in trees treated with Si across all glyphosate rates in old leaves, and in young leaves at higher glyphosate rates (1008 and 1440 g a.e. ha⁻¹). Proline levels were elevated in control trees exposed to glyphosate, whereas Si treatment increased carotenoid accumulation, particularly in old leaves. Phenolic compounds increased in old leaves where Si was applied across all glyphosate rates, while in young leaves, increases occurred only at lower glyphosate rates (0 and 576 g a.e. ha⁻¹). Trees treated with Si retained more leaves across most glyphosate rates and showed increased dry matter production, except at 1440 g a.e. ha⁻¹.

Conclusion Si application effectively mitigates glyphosate-induced oxidative stress in young orange trees by enhancing non-enzymatic antioxidant defenses, particularly carotenoids and phenolic compounds, while lowering MDA levels. These findings suggest Si as a sustainable strategy to improve herbicide tolerance and strengthen the citrus tree resilience.

*Correspondence:
Jonas Pereira de Souza Junior
jpereiradesouza@ufl.edu

Full list of author information is available at the end of the article



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Keywords Beneficial element, *Citrus sinensis* L., Herbicide contamination, Non-enzymatic antioxidant, Oxidative metabolism, Oxidative stress

Background

Orange (*Citrus sinensis* (L.) Osbeck) is one of the most economically and nutritionally significant citrus species worldwide [1, 2], contributing to both human health and global markets through the production of fresh fruit and processed products such as juice [3]. However, the sustainability and productivity of orange orchards are impacted by the unintended effects of herbicide use, particularly glyphosate [4, 5].

Glyphosate [*N*-(phosphonomethyl)-glycine], a systemic herbicide widely used for weed management in citrus production, is highly effective in controlling undesirable vegetation. However, excessive application has been shown to negatively impact orchard growth, fruit yield, and economic viability [4, 6]. Glyphosate exerts its herbicidal activity by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), a key enzyme in the shikimate pathway, thereby disrupting the biosynthesis of aromatic amino acids [7]. This inhibition leads to a reduction in the synthesis of phenolic compounds and other secondary metabolites that play critical roles in plant defense. As a result, glyphosate overuse induces oxidative stress, diminishing the plant's antioxidant capacity and weakening its physiological resilience [8].

Silicon (Si) has emerged as a beneficial element with potential to mitigate herbicide-induced stress in various plant species [9]. Several studies indicate that Si enhances both enzymatic and non-enzymatic antioxidant defense systems, reducing oxidative damage [10–12]. Despite these findings, research on Si-mediated attenuation of glyphosate stress in citrus remains limited, particularly in relation to its role in enhancing non-enzymatic antioxidant defenses such as proline, phenolic compounds, and carotenoids, which are key in scavenging reactive oxygen species (ROS) [13–15].

Although citrus trees are classified as low Si-accumulators [16], studies have demonstrated that foliar application of soluble or nanoparticulate Si can enhance Si accumulation, even in species with inherently Si absorption capacity [17–19]. Moreover, as a perennial crop, citrus may exhibit differential Si accumulation between older and younger leaves, which could influence the effectiveness of Si in mitigating oxidative stress. These age-related variations necessitate a more detailed investigation into Si's protective role across different leaf developmental stages, to optimize its application for citrus management.

While Si has been extensively studied for its role in various abiotic stresses, including salinity [20], hypoxia [21, 22], chilling [23], and nutritional disorders [24–26], its

effectiveness in counteracting glyphosate-induced stress in citrus remains largely unexplored. Furthermore, most research on Si focuses on its influence on enzymatic antioxidant responses, with relatively little information available on its role in regulating non-enzymatic antioxidants. Additionally, it remains unclear whether Si application differentially affects young and old leaves in citrus under herbicide-induced stress.

This study aims to address these gaps by evaluating whether Si supplementation can attenuate glyphosate-induced oxidative stress in young orange trees through the enhancement of non-enzymatic antioxidant defenses. Specifically, the research investigates the effects of Si on proline, carotenoids, and phenolic compounds accumulation in both young and mature leaves, providing insights into its potential role in improving citrus resilience under herbicide stress. It is hypothesized that glyphosate exposure impairs growth by inducing oxidative stress and that Si application mitigates these effects by reinforcing the plant's non-enzymatic antioxidant system. The findings in this study are expected to contribute to the development of optimized Si application strategies aimed at enhancing citrus tolerance to herbicide-induced stress, ultimately improving orchard sustainability and productivity.

Methods

Growth conditions

The experiment was conducted in a greenhouse at Sao Paulo State University “Julio de Mesquita Filho” (UNESP), Jaboticabal, Brazil. Temperature and humidity levels were continuously recorded using a KTJ Thermo meter® MSC Industrial Thermo-hydrometer, with an average relative humidity of $81 \pm 7\%$, a maximum temperature of $36 \pm 4^\circ\text{C}$, and a minimum temperature of $12 \pm 3^\circ\text{C}$.

Plant material and transplantation

Eight-month-old ‘Valencia’ orange young plants (*Citrus sinensis* (L.) Osbeck), grafted onto Swingle citrumelo rootstock, were sourced from a certified citrus nursery to ensure uniform age and physiological development. The nursery follows strict quality control protocols to maintain genetic and physiological consistency among seedlings. Upon arrival, the young orange plants were transplanted into 12 dm^3 polyethylene pots filled with a clayey soil substrate composed of 59% clay, 18% silt, and 23% sand.

Soil properties and fertilization

Soil chemical analysis [27] showed the following properties: pH (CaCl_2): 5.9, organic matter: 13 g dm^{-3} ,

phosphorus: 16 mg dm⁻³, sulfur: 16 mg dm⁻³, calcium: 22 mmol dm⁻³, magnesium: 7 mmol dm⁻³, potassium: 2.8 mmol dm⁻³, hydrogen + aluminum: 18 mmol dm⁻³, and silicon content: 8.1 mg dm⁻³.

Fertilization was conducted 15 days after transplanting, following Boaventura et al. [28], using 75 mg dm⁻³ urea and 0.3 mg dm⁻³ zinc sulfate to ensure adequate nutrient availability for young plant establishment.

Experimental design and treatment application

The study was conducted using a randomized block design with a 4 × 2 factorial arrangement, comprising four glyphosate application rates and two Si treatments. The glyphosate rates were 0 (Control), 576, 1008, and 1440 g of acid equivalent (g a.e.) ha⁻¹, while the Si treatments included 0 and 2 mM Si. Each treatment combination was replicated four times.

Glyphosate rates, supplied as an ammonium salt (Roundup WG®), were selected based on the commercial recommendation for Roundup WG® application in citrus orchards [4], with the full recommendation of 1440 g of a.e. ha⁻¹. The chosen rates represent 0%, 40%, 70%, and 100% of recommended field application rates.

Glyphosate was applied 41 days after transplanting to the aerial parts of young trees. A CO₂-pressurized backpack sprayer, fitted with flat-fan nozzles (TeeJet® 110.02, Brazil) spaced 0.5 m apart was used to deliver the herbicide at a pressure of 2 bar and a spray volume of 200 L ha⁻¹. The application was conducted at a walking speed of 4 km h⁻¹ under controlled environmental conditions (22.3 °C temperature, 74% relative air humidity, and 3.61 m s⁻¹ wind speed) to ensure uniform deposition and minimize drift.

Silicon was supplied as potassium silicate, a highly soluble source commonly used in agriculture [29]. A concentration of 2 mM was selected based on prior research indicating its efficacy in enhancing stress tolerance while avoiding polymerization at concentrations exceeding 3 mM [30]. Silicon was administered through both fertigation and foliar sprays to optimize plant uptake.

For fertigation, a 1000 mL potassium silicate solution (or water for control trees) was applied daily per pot using the mass replacement method, ensuring 80% soil water retention capacity [31]. Foliar applications commenced 8 days after transplanting and were applied five times at 8-day intervals. The sprays were applied early in the early morning (6:00–7:00 AM, temperature < 20 °C, humidity > 80%) to enhance leaf absorption. Each plant received 59 mL per application (0.118 mM of Si), totaling 295 mL (0.59 mM of Si) over five sprays.

Potassium chloride was applied to non-Si-treated plants via both fertigation and foliar methods, to maintain potassium balance and ensure that any observed

effects were attributed to Si rather than potassium application.

Oxidative stress and antioxidant analysis

Lipid peroxidation (MDA content)

Oxidative stress was assessed by quantifying malondialdehyde (MDA) levels, a biomarker of lipid peroxidation, following the methodology described by Gratão et al. [32]. Leaf samples, categorized as young and old, were collected between 7 and 8 AM, immediately frozen in liquid N, and stored at -80 °C until analysis. For MDA quantification, 0.2 g of leaf tissue was homogenized in 20% (w/v) polyvinylpyrrolidone (PVP) and 0.1% trichloroacetic acid (TCA). The homogenized mixture was then centrifuged at 11,000 rpm (4 °C) for 10 min, and the supernatant was incubated with a thiobarbituric acid (TBA) solution at 95 °C for 30 min. Absorbance was measured at 535 and 600 nm using a spectrophotometer, and MDA concentration was calculated accordingly [32].

Proline accumulation

Proline content was quantified following Bates et al. [33] to assess plant responses to stress. Fresh leaf tissue (0.5 g) was macerated in sulfosalicylic acid, filtered, and reacted with glacial acetic acid and ninhydrin acid in a 100 °C water bath for 1 h. The reaction mixture was extracted with toluene, and absorbance was recorded at 520 nm using a spectrophotometer.

Phenolic compound quantification

Total soluble phenolic compounds were measured using methanol extraction and a colorimetric Folin-Ciocalteu assay, following methods described by Singleton and Rossi [34]. Absorbance was recorded after incubation, and phenolic content was expressed according to a calibrated standard curve.

Carotenoid content analysis

Carotenoid concentration was determined using the acetone extraction method and spectrophotometric analysis, as described by Lichtenthaler [35]. Absorbance values were used to calculate total carotenoid content, providing insight into the antioxidant capacity of the treated plants.

Number and dry mass of leaves

Leaf number was recorded 30 days after glyphosate application. Leaves were carefully washed, oven-dried at 65 °C in a forced-air oven, and weighed to determine dry mass production.

Analysis of silicon concentration

Silicon concentration in leaf tissue was measured following Kondörfer et al. [36]. Dried samples were finely ground and subjected to alkaline digestion (H₂O₂ and

NaOH, 90 °C for 4 h). The extracted Si was quantified using a colorimeter reaction with an ammonium molybdate reagent in oxalic acid and HCl, with absorbance measured using a spectrophotometer.

Statistical analysis

Data analysis and graph construction were performed using the Python programming language (version 3.9.7; Python Software Foundation). Data normality [37] and homogeneity [38] was evaluated. Once the assumptions of normality and homogeneity were verified, an analysis of variance (ANOVA) was conducted to evaluate significant treatment effects. When significant differences were observed ($p < 0.05$), treatment means were compared using Tukey's test at a 5% significance level.

Hierarchical clustering analysis was performed using the Euclidean distance as the similarity measure and the single linkage method for grouping. Additionally, principal component analysis (PCA) was conducted on the correlation matrix to identify the primary variables contributing to variation and to reveal patterns within the dataset.

Results

The effects of silicon, glyphosate and their interaction

The results of the analysis of variance (ANOVA) (Table 1) demonstrated the effects of Si, glyphosate, and their interaction on physiological and biochemical parameters in old and new leaves of young orange plants. Silicon application significantly increased its concentration in both old and new leaves, while glyphosate and its interaction with Si showed no significant effect on Si levels. Malondialdehyde (MDA) was significantly influenced by

Si and glyphosate in both old and new leaves and was further affected by the interaction between these treatments, indicating a combined impact on oxidative stress. Proline accumulation was significantly influenced in old leaves by Si and glyphosate independently and with significant interaction in new leaves. However, the interaction of Si and glyphosate was significant only in new leaves, suggesting a synergistic effect under certain conditions. Carotenoid levels were significantly affected by Si and glyphosate in both leaf types, with a significant interaction observed only in old leaves. Similarly, phenolic compound levels were significantly influenced by Si and glyphosate in both old and new leaves, with their interaction being significant in old leaves but not in new leaves. Growth parameters such as the number of leaves and dry matter production, were significantly affected by Si, glyphosate, and their interaction.

Silicon concentration and oxidative stress in young orange trees under glyphosate stress and silicon supply

Silicon applied through fertigation and foliar sprays, significantly increased Si concentrations from 9.97 mg kg⁻¹ to 16.70 mg kg⁻¹ in old leaves, and from 9.68 mg kg⁻¹ to 14.52 mg kg⁻¹ in new leaves, corresponding to increases of 66% and 40%, respectively (Fig. 1a, b).

An interaction between Si and glyphosate was observed in oxidative stress, as indicated by MDA production in old (Fig. 1c) and new (Fig. 1d) leaves. In old leaves, glyphosate application in trees not treated with Si significantly increased MDA content across all rates compared to the control (0 g e.a. ha⁻¹). However, in trees treated with Si, MDA content increased only at the glyphosate rate of 1440 g e.a. ha⁻¹, with no significant difference compared to 1008 g e.a. ha⁻¹. Si application significantly reduced MDA content across all glyphosate concentrations, except the control (0 g e.a. ha⁻¹).

In new leaves (Fig. 1b), glyphosate application increased MDA content at the two highest rates (1008 and 1440 g e.a. ha⁻¹) in both Si-treated and Si-untreated plants. Silicon application significantly reduced MDA content at these two glyphosate concentrations but had no effect at the lower rates (0 and 576 g e.a. ha⁻¹).

Non-enzymatic mechanisms antioxidants on old and new leaves of young orange trees under glyphosate stress and silicon supply

In old leaves, no interaction between Si and glyphosate rates was observed for proline concentration (Table 1). In old leaves, proline content was consistently higher in Si-untreated plants compared to Si-treated plants (Fig. 2a). Glyphosate application significantly increased proline levels in old leaves, with no differences among the glyphosate rates. In new leaves, an interaction between Si and glyphosate rates was observed (Table 1). In control

Table 1 Analise of variance (ANOVA) for the effects of silicon, glyphosate, and their interaction (Silicon x Glyphosate) on physiological and biochemical variables in old and new leaves of young orange plants

Variable	CV (%)	Sum of squares		
		Si	Gly	Si x Gly
Si conc. (old leaves)	7.61	362.07**	1.44 ^{ns}	1.15 ^{ns}
Si conc. (new leaves)	9.93	187.21**	4.41 ^{ns}	2.57 ^{ns}
MDA (old leaves)	5.45	6.61**	14.13**	1.56**
MDA (new leaves)	8.79	3.61**	23.35**	2.06**
Pro (old leaves)	13.6	26,026.21**	160,593.24**	23,987.22 ^{ns}
Pro (new leaves)	8.14	42,184.23**	121,746.45**	16,659.37**
Car (old leaves)	9.49	0.0038**	0.0086**	0.0023**
Car (new leaves)	7.18	0.0008**	0.0020**	0.0004 ^{ns}
Phen (old leaves)	6.71	71.02*	183.03**	1,110.53**
Phen (new leaves)	5.80	54.73**	414.92**	24.88 ^{ns}
Leaf number	2.12	164.98**	177.29**	57.28**
DM	5.29	17.41**	134.98*	20.73**

Si Silicon, Gly Glyphosate, MDA Malondialdehyde, Pro Proline, Car Carotenoids, Phen Phenolic compounds, DM Dry matter

**, * and ns-significant a 1% and 5% and non-significant by the F-test

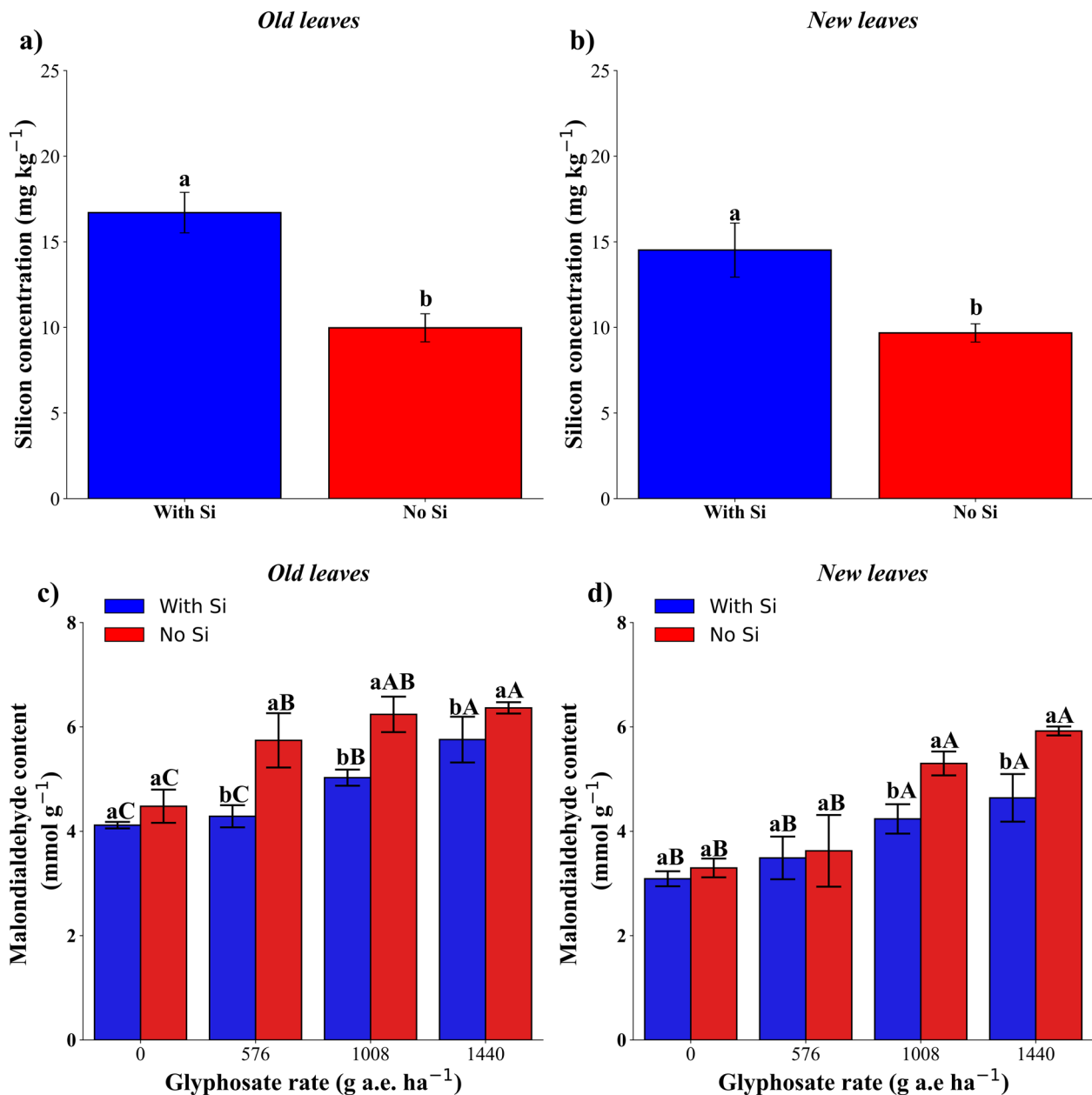


Fig. 1 Silicon (Si) concentration (**a, b**), and malondialdehyde (MDA) content (**c, d**) in old and new leaves of young orange trees subjected to different glyphosate rates (0; 576; 1,008; and 2,550 g of equivalent acid (g e.a.) per hectare) in the absence (No Si) and presence (with Si) of Si. **a, b**. Different letters indicate significant differences according to Tukey's test at a 5% probability level. **c, d**. Lowercase letters represent differences between the presence and absence of Si at the same glyphosate concentration, while uppercase letters indicate differences among glyphosate concentrations within the same Si condition, both determined by Tukey's test at a 5% probability level

trees (without Si), proline content increased at the two highest glyphosate rates (1008 and 1440 g e.a. ha⁻¹), with no significant difference between these concentrations (Fig. 2b). In trees treated with Si, the highest proline concentration was recorded at the highest glyphosate rate (1440 g e.a. ha⁻¹), with no significant difference compared to 1008 g e.a. ha⁻¹. Trees treated with Si showed lower proline concentration across all glyphosate rates studied, except for the control treatment (0 g e.a. ha⁻¹).

Carotenoid content exhibited a significant interaction between Si and glyphosate treatments, but this effect was observed exclusively in old leaves (Table 1). In old leaves, carotenoid content decreased at the highest glyphosate rate (1440 g e.a. ha⁻¹) in both Si-treated and untreated plants. However, at this rate, as well as at 576 g e.a. ha⁻¹, Si application resulted in a significant increase in carotenoid content compared to Si-untreated plants. At other glyphosate rates, Si treatment had no significant effect on

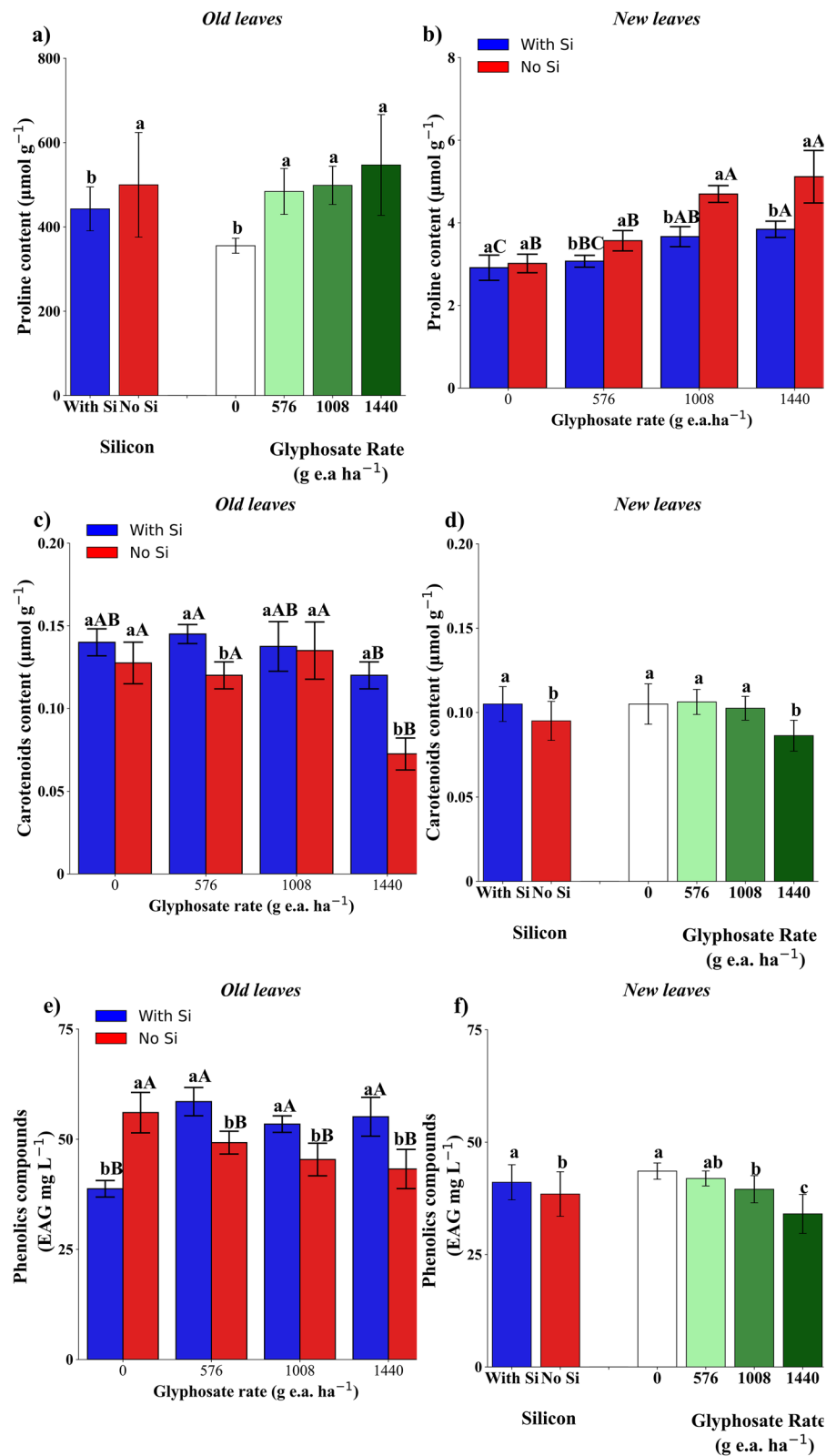


Fig. 2 Proline concentration (a, b), carotenoids content (c, d), and phenolics compounds content (e, f) in new and old leaves of young orange trees subjected to different glyphosate rates (0; 576; 1008; and 2,550 g of equivalent acid (g e.a.) per hectare) in the absence (No Si) and presence (with Si) of Si. a, d, f. Different letters indicate significant differences according to Tukey's test at a 5% probability level. b, c, e. Lowercase letters represent differences between the presence and absence of Si at the same glyphosate concentration, while uppercase letters indicate differences among glyphosate concentrations within the same Si condition, both determined by Tukey's test at a 5% probability level

carotenoid levels in old leaves (Fig. 2c). In new leaves, Si application increased carotenoid content, while a reduction in carotenoid levels was observed at the highest glyphosate rate (1440 g e.a. ha⁻¹) (Fig. 2d).

Concentration of phenolic compounds content also showed a significant interaction between Si and glyphosate treatment, but this effect was limited to old leaves (Table 1). In Si-untreated plants, the highest concentration of phenolic compounds was observed in the control (0 g a.e. ha ha⁻¹), with no significant differences among the other glyphosate rates. In contrast, for trees treated with Si, the lowest concentration of phenolic compounds was recorded in the control, while no significant differences were observed among the other glyphosate rates (Fig. 2e). Additionally, Si application increased phenolic compound content across all glyphosate rates except for the control (0 g e.a. ha⁻¹). In new leaves, plants treated with Si exhibited higher phenolic concentration compared to untreated plants. The highest concentration of phenolic compounds content in new leaves was found in the control treatment (0 g e.a. ha⁻¹), which was not significantly different from the 576 g e.a. ha⁻¹ glyphosate treatment (Fig. 2f).

Number of leaves and leaves dry matter of young orange trees under glyphosate stress and silicon supply

A significant interaction between Si and glyphosate treatments was observed for both the number of leaves and dry matter production (Table 1). In trees not treated with Si, the number of leaves is not significantly affected by glyphosate application. Conversely, in trees treated with Si, the number of leaves decreased with increasing glyphosate rates, with the lowest numbers recorded at 1008 and 1440 g a.e. ha⁻¹, and no significant difference between these two highest rates (Fig. 3a). In addition, Si

applications increased the number of leaves at the lowest glyphosate rates tested (0 and 576 g e.a. ha⁻¹).

For dry matter production (Fig. 3b), glyphosate application consistently reduced production across all rates in Si-untreated plants, with reductions of 10%, 18%, and 17%, for 576, 1008, and 1440 g e.a. ha⁻¹, respectively, relative to the control (0 g e.a. ha⁻¹). In Si-treated plants, Si supplementation effectively attenuated the negative effects of glyphosate on dry matter production. Although reductions of 10% and 16% were observed at 576 and 1008 g a.e. ha⁻¹, respectively, the decreases were more pronounced (31%) at the highest glyphosate rate of 1440 g a.e. ha⁻¹. Additionally, Si treatment increased the dry matter production in all glyphosate rate tested, except at the highest rate (1440 g a.e. ha⁻¹).

Correlation analysis, hierarchical clustering of the variables and principal component analysis

In old leaves (Fig. 4a), trees treated with Si exhibited significant positive correlation between Si concentration and carotenoids, number of leaves, and dry matter at 0 g e.a. ha⁻¹ of glyphosate, suggesting Si's role in promoting antioxidant accumulation and growth under low-stress conditions. At higher glyphosate rates, these correlations shifted, with Si concentration positively associated with the concentration of phenolic compounds at 576 g e.a. ha⁻¹ and with both phenolic compounds and MDA at 1,440 g e.a. ha⁻¹. This indicates Si's role in attenuating oxidative stress and stimulating phenolic synthesis under herbicide-induced stress. In contrast, correlations in trees not treated with Si were primarily associated with oxidative stress markers, such as MDA and proline, particularly at higher glyphosate rates (1,008 and 1,440 g e.a. ha⁻¹), reflecting a heightened stress response in the absence of Si.

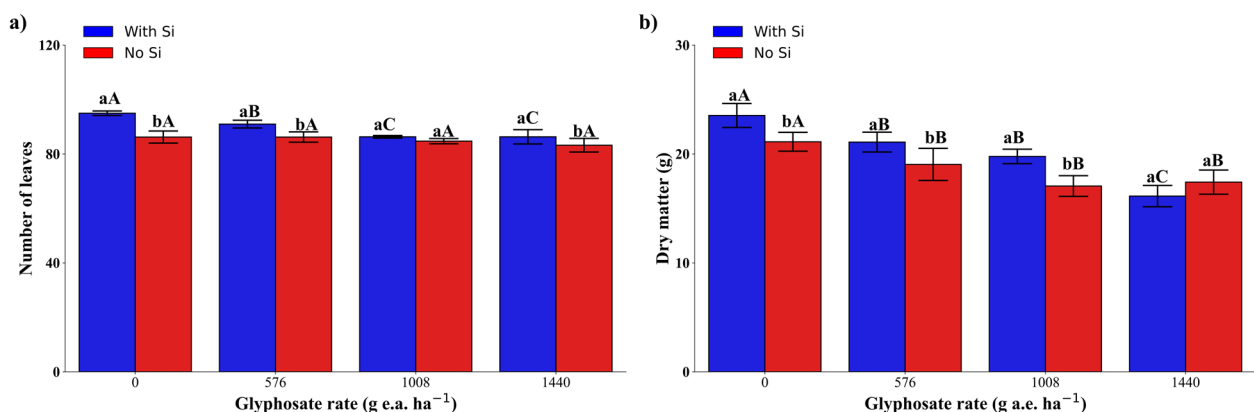


Fig. 3 Number of leaves (a) and dry matter production (b) of young orange trees subjected to different glyphosate rates (0; 576; 1008; and 2550 g of equivalent acid (g e.a.) per hectare) in the absence (No Si) and presence (with Si) of Si. Lowercase letters represent differences between the presence and absence of Si at the same glyphosate concentration, while uppercase letters indicate differences among glyphosate concentrations within the same Si condition, both determined by Tukey's test at a 5% probability level

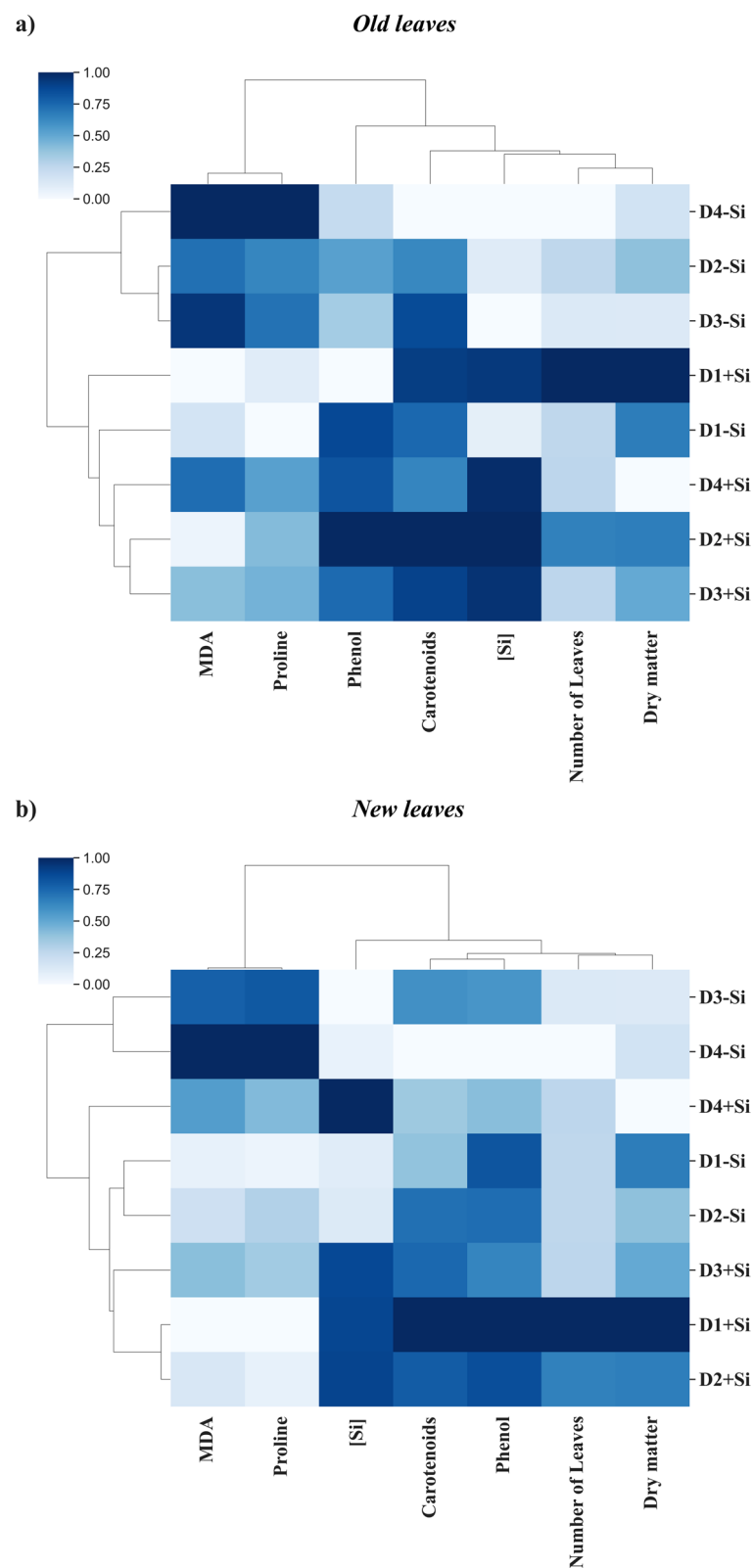


Fig. 4 Hierarchical clustering heatmap of response variables of old (a) and new (b) leaves of young citrus trees grown under different glyphosate rates (0 (D1); 576 (D2); 1,008 (D3); and 2,550 (D4) g of equivalent acid (g. a.e.) per hectare) in the absence (- Si) and presence (+ Si) of Si. Response variables: Malondialdehyde (MDA), proline, phenolic compounds (phenol), carotenoids, silicon content ([Si]), number of leaves and leaves dry matter

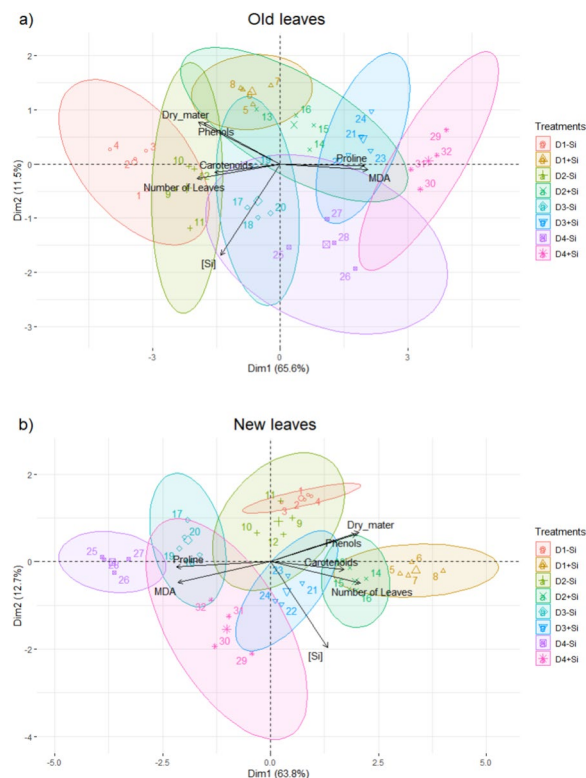


Fig. 5 Principal component analysis of response variables of old (a) and new (b) leaves of young citrus trees grown under different glyphosate (Gly) rates (0; 576; 1,008; and 2,550 g of equivalent acid (g. a.e.) per hectare) in the absence (- Si) and presence (+ Si) of Si. Response variables: Malondialdehyde (MDA), proline, phenol, carotenoids, silicon content ([Si]), number of leaves and leaves dry matter

In new leaves (Fig. 4b), trees treated with Si displayed strong correlations at lower glyphosate rates (0 g e.a. ha⁻¹ and 576 g e.a. ha⁻¹), where Si concentration was positively associated with carotenoids, phenolic compounds, number of leaves, and dry matter. However, at higher glyphosate rates (1008 and 1440 g e.a. ha⁻¹), these correlations weakened, suggesting that new leaves are less effective at Si accumulation and utilization for stress mitigation. Correlations were primarily associated with MDA and proline at higher glyphosate rates in trees not treated with Si, further emphasizing Si's critical role in modulating oxidative stress markers.

Hierarchical clustering analysis highlighted distinct groupings of variables based on their responses to glyphosate and Si treatments. In old leaves (Fig. 4a), MDA and proline clustered together, reflecting their shared role as oxidative stress markers. Growth-related parameters, such as dry matter and number of leaves, formed a separate cluster that also included Si concentration, carotenoids, and phenolic compounds, indicating a close association between Si uptake, antioxidant activity, and growth. A similar clustering pattern was observed in new leaves (Fig. 4b), where dry matter and number

of leaves formed one subgroup, while carotenoids and phenolic compounds formed another. Both subgroups merged with Si concentration, underscoring its central role in modulating stress responses across glyphosate rates.

Principal component analysis (PCA) provided a comprehensive overview of variable contributions to stress responses, explaining 73.8% and 76.5% of the variance through PC1 and PC2 in old and new leaves, respectively (Fig. 5a, b). In old leaves, PC1 was primarily driven by Si content, reflecting its influence on mitigating stress and promoting growth, while PC2 was strongly influenced by MDA, proline, and carotenoids, highlighting their roles in oxidative stress response. Si presence was associated with increased phenolic compounds and carotenoid content across glyphosate rates, whereas MDA and proline levels were higher in the absence of Si at higher glyphosate rates.

In new leaves (Fig. 5b), Si content similarly dominated PC1, while PC2 was influenced by MDA, proline, and carotenoids. Si presence was positively associated with increases in carotenoids, phenolic compounds, number of leaves, and dry matter, particularly at lower glyphosate rates (0, 576, and 1,008 g e.a. ha⁻¹). In contrast, MDA and proline were more strongly associated with the absence of Si at higher glyphosate rates, indicating reduced stress mitigation under these conditions.

The correlation, clustering, and PCA analyses (Figs. 4, 5), collectively underscore the pivotal role of Si in modulating stress responses in both old and new leaves. Old leaves, characterized by higher Si accumulation, exhibited stronger antioxidant responses and greater growth benefits under glyphosate stress, while new leaves displayed more pronounced stress indicators in the absence of Si. These findings highlight the age-dependent effects of Si and its critical importance in mitigating herbicide-induced oxidative stress and enhancing resilience in citrus plants.

Discussion

Glyphosate effects on old and new leaves of young orange trees

The negative impact of glyphosate spraying on young orange trees is evident in the reduction of leaf dry matter production (Fig. 3b). Glyphosate toxicity in citrus has been associated with rates exceeding 1,440 g a.e. ha⁻¹, leading to disruptions in the shikimate pathway, chlorophyll degradation, and oxidative stress [6]. Although commonly used for weed management, non-target exposure to high doses of glyphosate can significantly impair growth and development in citrus trees.

Pioneering studies have documented the detrimental effects of direct glyphosate application on citrus trees [4–6]. However, most of these investigations have focused

on mature plants, leaving a critical gap regarding the responses of young citrus plants to glyphosate-induced stress, particularly the differential responses of old and new leaves. Young plants, due to their limited physiological resilience and higher metabolic demands, may exhibit heightened sensitivity to herbicide stress [39]. Understanding this vulnerability is crucial, as losses at early developmental stages necessitate replanting, which can lead to plant heterogeneity and compromise the uniform development of the orchard.

Glyphosate spraying significantly increased oxidative stress in old and new leaves of young orange trees, as evidenced by the increase in MDA content (Fig. 1c, d). Glyphosate is known to target the enzyme 5-enolpyruvylshikimate-2-phosphate synthase [20], resulting in chlorophyll degradation and a reduction in the photosynthetic electron transfer rate in citrus [5]. This disruption leads to the overproduction of ROS, causing oxidative stress and elevating MDA content, a marker of lipid peroxidation [40].

Non-enzymatic defense mechanisms, such as proline accumulation, play a key role in mitigating oxidative stress [41, 42]. Proline levels increased in both old and new leaves with rising glyphosate concentration (Fig. 2a, b), indicating its critical function as a defense molecule against herbicide stress. This observation aligns with previous studies highlighting proline's role in mitigating paraquat-induced toxicity and other oxidative stress conditions [43]. Proline likely reduces oxidative damage through its function as a ROS scavenger, stabilizer of membrane integrity and redox buffer [41], as evidenced by the strong correlation between MDA and proline in glyphosate-stressed in Si-untreated plants (Fig. 5).

Carotenoid content decreased at the highest glyphosate rate (1,440 g e.a. ha⁻¹) in both old and new leaves (Fig. 2c, d). At lower glyphosate concentrations, carotenoid levels remained stable in both leaf types, suggesting a protective role under moderate stress. Carotenoids can contribute to stress attenuation by stabilizing membrane lipid bilayers, regulating light harvesting, and scavenging ROS [44]. They neutralize ROS by oxidizing their conjugated double bonds, thereby minimizing oxidative damage to cell membranes [45]. However, at higher glyphosate concentrations, this protective capacity diminishes [46]. This reduction may be attributed to the formation of carotenoid aggregates under severe stress, which lack antioxidant properties [46]. These findings suggest that carotenoid-mediated protection is effective only under low to moderate glyphosate stress, beyond which the capacity of plants to attenuate oxidative damage declines.

Phenolic compounds, another class of essential non-enzymatic antioxidants, decreased in both old and new leaves of young orange trees with increasing glyphosate concentration (Fig. 2e, f). Glyphosate's inhibition of the

shikimic acid pathway disrupts the synthesis of chorismate and phenylalanine, critical precursors for phenolic biosynthesis [46, 47]. This mechanism likely explains the observed reductions in phenolic compounds under glyphosate stress, as has been reported in other crops exposed to glyphosate [10, 48–50].

The negative impact of glyphosate spraying on young orange trees is evident in the reduction of leaf dry matter production (Fig. 3b). Symptoms of glyphosate toxicity, such as reduction in dry matter production, have been previously reported in citrus tree [5, 47]. Although proline concentration increases (Fig. 2a, b), as a defense mechanism against oxidative stress, this response appears insufficient to counteract the damage caused by glyphosate. Elevated oxidative stress, as indicated by higher MDA levels (Fig. 1c, d), and reductions in non-enzymatic antioxidants, such as carotenoids (Fig. 2c, d) and phenolic compounds (Fig. 2e, f), result in impaired plant development. Consequently, as glyphosate levels increase, young orange trees exhibit lower leaf dry matter, emphasizing the adverse effects of glyphosate on overall plant health and growth.

These findings highlight the detrimental effect of glyphosate sprays on young citrus trees, underscoring the importance of precise herbicide management practices to minimize unintentional exposure and protect plant health and productivity. However, this study is subjected to certain limitations, including its relatively short duration and the controlled greenhouse conditions, which may not fully capture field dynamics or the long-term effects of glyphosate exposure. Future research should investigate the impacts of prolonged glyphosate exposure under field conditions, where environmental variables such as fluctuating temperatures, diverse soil conditions, and microbial activity may influence plant responses. Additionally, examining the combined effects of glyphosate and other abiotic stressors, such as drought or nutrient deficiencies, would provide a more comprehensive understanding of plant resilience mechanisms [9, 10].

Silicon effects attenuating glyphosate toxicity in old and new leaves of young orange trees

Silicon is unique among chemical elements in its capacity to attenuate a wide range of biotic and abiotic stress in plants [48]. Its benefits include enhancing plant growth, supporting the flowering phase, and improving fruit quality, particularly under stress conditions [25, 49–52]. Recent studies suggest that Si may also improve plant tolerance to herbicide-induced stress [9]. However, rigorous investigations are required to validate this potential and elucidate the precise mechanism involved. A comprehensive understanding of the role of Si in attenuating herbicide stress is crucial for advancing sustainable agricultural practices and enhancing crop resilience.

Citrus trees are classified as Si non-accumulators [16]. However, the application of stabilized soluble Si through root supplementation, combined with foliar application, effectively increased Si concentration in both old and new leaves of young orange trees (Fig. 1a, b). This increase demonstrates that the use of foliar applications are effective strategies for enhancing the concentration of this beneficial element in plants, particularly Si non-accumulators, as reported in previous studies [17, 18].

The application of Si in citrus plants has been shown to facilitate the absorption and translocation of Si to the leaves (Fig. 1). However, a critical question remains whether the leaf Si concentration are sufficient to enhance the tolerance of young citrus trees to herbicides. Silicon application significantly reduced MDA in glyphosate-stressed leaves highlighting its protective role in attenuating oxidative stress. The beneficial effects of Si in reducing oxidative stress and, consequently, MDA content have been well-documented in the literature, including in citrus trees subjected to various environmental stresses such as hypoxia [21, 22], low temperature [53], salinity [20], and iron deficiency [24]. Notably, this study is the first to demonstrate the protective effect of Si in both old and new leaves of young orange trees under glyphosate toxicity.

The beneficial effect of Si in reducing oxidative stress in citrus has been attributed to the enhancement of the enzymatic antioxidant system [22, 24]. However, Si application also stimulated the non-enzymatic antioxidant system (Fig. 2). Silicon did not increase proline levels (Fig. 2a, b). This indicates that the primary defense mechanism in new and old orange leaves under glyphosate toxicity changes when Si is supplied.

Notably, phenolic compounds were higher across all concentrations of glyphosate treatments, except for the control (Fig. 2e, f). Furthermore, the PCA analysis revealed that phenolic compounds and leaf dry matter were aligned in the same direction, particularly in trees treated with Si (Fig. 5), suggesting that phenolic compounds play a crucial role as a non-enzymatic response to glyphosate stress in the presence of Si. It is well-documented that Si can enhance the concentration of phenolic in crops, especially under environmental stress [10, 14, 26], including in citrus [54]. However, limited information is available regarding the effect of Si on the concentration of phenolic compounds under glyphosate toxicity. Glyphosate is known to negatively impact the concentration of phenolic compounds by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, thereby reducing phenylalanine [55], however further investigation is needed to elucidate the mechanisms underlying Si's influence on phenolic content in citrus trees under glyphosate stress [10].

The presence of Si appears to contribute to the maintenance rather than the augmentation of carotenoid content (Fig. 2c, d). This effect is likely an indirect consequence of the reduced oxidative stress facilitated by Si, rather than a direct enhancement of this antioxidant defense mechanisms.

The effectiveness of Si in attenuating glyphosate-induced stress was demonstrated by its ability to minimize the decline in leaf dry matter production at all glyphosate concentrations, except the highest (1440 g a.e. ha⁻¹), compared to the trees not treated with Si.

The hierarchical clustering and PCA analyses provide a broader understanding of how Si mitigates glyphosate-induced stress by influencing key physiological and biochemical responses (Fig. 5). Clustering patterns indicate that oxidative stress markers, such as MDA and proline, are strongly associated in glyphosate-stress plants without Si (Fig. 4), suggesting that these parameters play a central role in the plant's response to herbicide toxicity. However, in trees treated with Si, this association is weaker, implying that Si reduces the oxidative burden, thereby diminishing the reliance on proline as a defense molecule.

Conversely, in trees treated with Si, growth-related parameters such as dry matter production, leaf number, carotenoid content, and phenolic compounds cluster together, suggesting a coordinated enhancement of plant health and resilience. The alignment of Si concentration with antioxidant compounds in the PCA analysis underscores the role of Si in reinforcing non-enzymatic defense pathways. These findings indicate that Si application not only reduces oxidative stress but also improves physiological performance, contributing to plant growth even under herbicide stress.

The findings of this study make a significant contribution to the sustainable management of citrus, demonstrating that Si application can enhance tolerance to glyphosate. This advancement holds global significance, given the extensive cultivation of citrus species worldwide. Incorporating Si as a stress attenuator in citrus production has the potential to improve resilience and productivity, thereby supporting sustainable agricultural practices on a global scale. Future research should explore the effects of Si in combination with other herbicides, both in citrus and other crop species, to broaden the understanding of its applicability.

Although this study provides valuable insights into Si's role in enhancing non-enzymatic antioxidant defenses in citrus, additional antioxidants such as ascorbate, glutathione, and flavonoids could further elucidate Si-mediated oxidative stress mitigation mechanisms. Future studies should explore a broader range of non-enzymatic antioxidants to comprehensively assess Si's contribution to plant stress responses under herbicide-induced stress.

Conclusion

In a short-term greenhouse experiment with citrus plants, proline concentration increased in trees not treated with Si as a defense mechanism against glyphosate toxicity. However, this response was insufficient to attenuate the damage caused by herbicide stress. In contrast, the application of soluble Si effectively attenuated these negative impacts by reducing MDA content and enhancing phenolic compound production. Silicon application improved the resilience of plants to glyphosate toxicity, preserving dry matter production. These findings emphasize the importance of non-enzymatic antioxidant mechanisms in mitigating oxidative stress and highlight the potential of Si as a valuable tool for protecting citrus trees from herbicide-induced stress.

Text edition

During the preparation of this work the authors used Chat GPT® in order to check scientific writing English style. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Authors' contributions

IJB conducted the experiment and collected data. JPSJ and MGC analyzed the data and wrote the initial draft of the manuscript. JCBL and PLG contributed to the analysis of oxidative stress parameters. DMK, RMP, SB, and LBC were responsible for the study's conceptualization and provided critical revisions to the manuscript. All authors read and approved the final version of the manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

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Consent for publication

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Competing interests

The authors declare no competing interests.

Author details

¹School of Agricultural and Veterinary Sciences, São Paulo State University (UNESP), Via de Acesso Prof. Paulo Donato Castellane, N.O.S., Jaboticabal 14884900, Brazil

²Citrus Research and Education Center, University of Florida, 700 Experiment Station Rd, Lake Alfred, FL 33850, USA

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