



# Silicon nanoparticles confer hypoxia tolerance in citrus rootstocks by modulating antioxidant activities and carbohydrate metabolism

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## ABSTRACT

Citrus is a remarkable fruit crop, extremely sensitive to flooding conditions, which frequently trigger hypoxia stress and cause severe damage to citrus plants. Silicon nanoparticles (SiNPs) are beneficial and have the potential to overcome this problem. Therefore, the present study aimed to investigate the effect of silicon nanoparticles to overcome hypoxia stress through modulating antioxidant enzyme activity and carbohydrate metabolism. Three citrus rootstocks (Carrizo citrange, Roubidoux, and Rich 16–6) were exposed to flooding (with and without oxygen) through different SiNP treatments via foliar and root zone. SiNPs applied treatment plants showed a significant increase in photosynthesis, leaf greenness, antioxidant enzymes, and carbohydrate metabolic activities, besides the higher accumulation of proline and glycine betaine. The rate of lipid peroxidation was drastically higher in flooded plants; however, SiNPs application reduced it significantly, ultimately reducing oxidative damage. Overall, Rich16-6 rootstock showed good performance via root zone application compared to other rootstocks, possibly due to genotypical variation in silicon uptake. Our outcomes demonstrate that SiNPs significantly affect plant growth during hypoxia stress conditions, and their use is an optimal strategy to overcome this issue. This study laid the foundation for future research to use at the commercial level to overcome hypoxia stress and a potential platform for future research.

## 1. Introduction

Citrus represents the most commercially significant evergreen fruit trees around the globe and often faces environmental challenges such as drought, cold, salt, and hypoxia [1]. Hypoxia is the most concerning among these stresses because of various physiological problems such as chlorosis, root rot, growth retardation, and fruit dieback [2]. Due to climate change and excessive use of chemical fertilizers, waterlogging and soil hardening are becoming increasingly severe, signifying that hypoxia is one of the factors that can't be

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ignored and affecting citrus production [3]. Floods can cause waterlogging or submergence, the most common cause of hypoxia in plants [4]. Citrus is classified as flood-sensitive due to its inability to adapt to various environmental stresses, such as aerenchyma development and lenticel hypertrophy [1,5]. Hypoxia severely damages the root, the first organ to detect and respond to hypoxia [6]. Under hypoxia stress, the roots exhibit a decrease in both respiration and adenosine triphosphate (ATP) generation [7], as well as metabolic processes such as photosynthesis and transpiration are also reduced [8]. In addition, hypoxia stress increases the production of reactive oxygen species (ROS) [9], which restricts root function and plant development and ultimately leads to cell and tissue death [10].

Under hypoxic stress, plants adapt to several complex cellular, physiological, molecular, and biochemical mechanisms. This stress may cause a severe interruption in plant cell development processes and antioxidant defense systems [11,12]. Plants produce abundant reactive oxygen species (ROS) during stress, reducing chloroplast activity, photochemical efficiency, and photosynthetic rate [13]. Plants have an antioxidant system, including enzymes such as SOD, POD, CAT, APX, and GPX, as well as non-enzymatic components such as glutathione and ascorbate, each of which can jointly substitute ROS to prevent cells from oxidative damage [14]. Osmolytes reduce oxidative damage caused by ROS and protect proteins from ROS damage [15]. It has been found that proline, glycinebetaine, total soluble sugars, and total free amino acids can protect the cell wall structure and therefore are involved in abiotic stress resistance [16]. Proline is an essential amino acid that regulates primary metabolism and protects cellular structures from oxidative damage throughout growth and development [17,18]. Glycine betaine is another key osmolyte that enriches enzyme and membrane integrity, which is related to enhancing tolerance to various abiotic stresses [19].

Nanotechnology is an attractive and dynamic field of study that has resulted in several innovations and gained considerable attention in recent years due to its diverse range of applications [20–22]. Nanoparticles and nano fertilizers are the main applications of nanotechnology in agriculture. Nanoparticles (NPs) are small molecules with unique physicochemical properties and a size range of 1–100 nm [23,24]. Modern agriculture is gaining more popularity due to its ability to increase growth and production and resist various abiotic stresses and diseases [25]. NPs can also be used as macro and micro-nano fertilizers in plants to alleviate nutritional deficiencies and substitute essential elements [26]. NPs can enter the cell through plant organs such as the cuticle, epidermis, stomata, root tips, cortex, lateral root, or wounds and change various plant processes such as germination, antioxidant activity, nutrition, chlorophyll content, and photosynthesis [27,28]. During hypoxia stress in soybeans, NP application reduced the synthesis of cytotoxic glycolysis byproducts and increased the number of stress-related proteins and seedling development [29]. When applied to the flooded saffron, it promotes the development of the root system and enhances leaf diameter and root length [30].

Silicon dioxide (SiO<sub>2</sub>) is important in alleviating the adverse effects of abiotic stresses [31]. Silicon nanoparticles (SiNP) are widely used in agriculture as nano-fertilizers [32], which have been proven to promote plant development, primarily by increasing photosynthetic activity [33], nutrient uptake [34], and reducing ROS-induced oxidative stress [35]. Silicon (SiO<sub>2</sub>) is a prominent active element that interacts with various plant hormones and regulates antioxidant and enzymatic activity in stressed plants [36,37]. Therefore, the current research aims to evaluate the response of silicon nanoparticles (SiNPs) to hypoxic stress in increasing physiological and biochemical attributes, including the antioxidant defense system in citrus plants.

## 2. Material and methods

### 2.1. Plant material and treatment procedures

Two-year-old plants of three citrus rootstocks, i.e., Rubidoux (*Poncirus trifoliata* L.), Carrizo citrange (*Poncirus trifoliata* L.), and Rich 16–6, were selected for study and grown in a greenhouse facility at the Department of Horticulture, University of Florida, Gainesville. The plants were grown in Citri pots (4 × 4 × 32") filled with compost, peat moss, and vermiculite (1:1:1 v/v). Plants were subjected to grow at temperatures of 26 ± 2 and 22 ± 2 °C day and night, respectively, with 90–95 % relative humidity, and a photoperiod of 16-h through fluorescent lamps (490 μmol m<sup>-2</sup> s<sup>-1</sup>) for seven days to fully acclimated. Afterward, place the plants into individual 50 L plastic containers filled with water. Typically, plants are grafted at a height of 12 cm, so we immersed the plants up to 8 cm (4 cm below the expected grafted union height). Flooding was created by immersing plants in water up to 8 cm tall. Flooded plants were divided into two groups: aerated and non-aerated. Aeration was provided by connecting each container through an air pump (Deluxe LGPUMPAIR38). The pumps continued to run throughout the experiment. Both aerated and non-aerated plants were treated with 250 mg/L of silicon nanoparticles (SiNP) as foliar and root zone applications. The experiment was performed in a randomized complete block design (RCBD) with six treatments. Each treatment consisted of four replicates, with ten plants per replicate. The six treatments were as follows: (T<sub>1</sub>) flooded + aerated (FA); control plants connected with air pumps to supply oxygen, (T<sub>2</sub>) flooded (F); plants without air/oxygen supply, (T<sub>3</sub>) FA + SiNP (foliar); FA plants treated with SiNP by foliar spray, (T<sub>4</sub>) F + SiNP (foliar); F plants treated with SiNP by foliar spray, (T<sub>5</sub>) FA + SiNP (root); FA plants treated with SiNP by root application added via nutrient solution, (T<sub>6</sub>) F + SiNP (root); F plants treated with SiNP by root application added via nutrient solution.

### 2.2. Silicon nanoparticles (SiNPs) application strategy

SiNP with sizes ranging from 20 to 30 nm was purchased from US Research Nanomaterials Inc., Houston, TX, USA. For foliar applications, handheld sprayers were used. Each plant was treated with 30 mL of SiNP (250 mg/L). During spray, the containers were covered with aluminum foil to prevent foliar solution from entering the pots. However, root zone application was carried out by adding 30 mL of SiNP solution into the water containers. The foliar applications were applied twice with an interval of three days, while the root application was applied just one time. Root zone application led to an increase in pH, which was adjusted to 6.7 ± 2. All

containers' pH values were monitored twice daily with a pH meter (HI9124, Hanna Instruments, Smithfield, RI, USA), and any fluctuation was adjusted to 6.7. The oxygen levels in both aerated and non-aerated plants were tracked using an oxygen meter (HI98193, Hanna Instruments, Smithfield, RI, USA). The average oxygen content of aerated plants was 7.89 mg/L, while, in non-aerated plants, it was 1.19 mg/L. One week after SiNP treatment, plant tissues (leaves and roots) were collected for biochemical analysis.

### 2.3. Dry matter contents

To measure the dry biomass of the leaf, stem, and root, plants were carefully removed and rinsed twice with distilled water to eliminate debris. After that, the plants were dried with filter paper to remove any remaining water from the leaves, stems, and roots. The plant tissues were oven-dried for four days at 72 °C (Memmert-110, Schwabach, Germany), and the average dry weight was measured.

### 2.4. Measurement of photosynthetic activity and greenness index

The net photosynthesis rate and stomatal conductance of fully expanded developed leaves were measured from 9:00 to 11:30 a.m. using a portable photosynthesis system (LI-6400XT, Licor, Inc., Lincoln, NE, USA). All observations were taken under the following environmental conditions:  $25 \pm 2$  °C leaf temperature,  $65 \pm 5$  % relative humidity, and  $1200 \mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux. Three newly fully expanded and mature leaves from each plant in each replication were selected to measure an average soil plant analysis development (SPAD) index value (SPAD-502; Konica Minolta Sensing Inc., Osaka, Japan) to evaluate leaf greenness.

### 2.5. Proline, lipid peroxidation, antioxidant enzyme activity

To measure antioxidant enzyme activity, leaves and roots (0.5 g) were ground in a cold tissue grinder with 5 mL of 50 mM cooled phosphate buffer (pH 7.8). The homogenate was centrifuged at  $15,000 \times g$  for 20 min at 4 °C. The supernatant was used to determine the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), Ascorbate peroxidase (APX) and Glutathione peroxidase (GPX).

The Tiwari et al. [38] method was used to determine proline content. 0.5 g of fresh leaf sample was immersed in 10 mL of aqueous sulfosalicylic acid (3 %) and centrifuged at  $10,000 g$  for 15 min. The extract was combined with 2 mL of glacial acetic acid and 4 mL of toluene. A UV-spectrophotometer was used to measure absorbance at 520 nm. The amounts of malondialdehyde (MDA) and thiobarbituric acid (TBA) were measured to determine lipid peroxidation, as the method described by Ref. [39]. After, equal amounts of the leaf or root tissue extract were mixed with 0.5 % (w/v) TBA solution containing 20 % (w/v) trichloroacetic acid (TCA). The mixture was kept at 95 °C for 30 min, then immediately cooled in a cooling bath and centrifuged at  $3000 \times g$  for 10 min. The absorbance of the supernatant was measured at 532 and 600 nm. The concentration of MDA was calculated using its molar extinction coefficient and presented as  $\mu\text{mol MDA mL}^{-1} g^{-1} \text{ DW}$ .  $\text{MDA (nmol)} = \Delta (A_{532 \text{ nm}} - A_{600 \text{ nm}}) / 1.56 \times 105$ . For glycinebetaine content, dried leaf powder at 0.5 g was ground in 10 mL DDW, and the remaining procedure was followed as previously described by Grieve and Grattan, (1983).

### 2.6. Sugars, starch, and enzyme activities

The sugar content was measured using the Buisse and Merckx technique [40]. The glucose content was assessed using the method followed by Jones et al. [41]. 0.5 g of frozen leaves tissues were taken to determine the enzyme activities. Leaves were homogenized with 50 mM Tris-hydrochloric acid added with magnesium chloride (5 mM), mercaptoethanol (5 mM), ethylenediaminetetraacetic acid (1 mM), ethylene glycol-bis- $\beta$ -aminoethyl ether (1 mM), 2-hydroxyl-1-ethanethiol (0.1 mM), and glycerol (15 %). The homogeneous mixture was centrifuged at  $17,000 g$  for 15 min at 4 °C. The supernatant was used to determine the different enzymatic activities. For total acid invertase (AI), the reaction mixture (1 mL) contained 0.2 mL extract, sodium acetate (0.1 M, pH 4.8), and sucrose (0.1 M). For neutral invertase (NI), the reaction mixture comprised dipotassium phosphate (0.1 M), sodium citrate (0.1 M), and sucrose (0.1 M). For both AI and NI, the reaction mixture was added with 0.2 mL of enzyme extract, incubated at 37 °C for 40 min, and then stopped by adding dinitro salicylic acid (1 mL). Sucrose phosphate synthase (SPS) and sucrose synthase (SS) enzymes were obtained by the methods used by Ruffy and Huber [42]. The SPS activity for SS and SS activity for sucrose cleavage were evaluated by the method of Bird et al., [43]. Fructokinase was examined by the method of Zrenner et al. [44]. The reaction mixture contained magnesium chloride (5 mM), Tris-hydrochloric acid (0.1 M, pH 8.5), nicotinamide adenine dinucleotide (0.5 M), fructose (10 mM), glucose-6-phosphate dehydrogenase (2 U) and adenosine triphosphate (2 mM). Hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) activities were determined using enzyme kits (Sigma-Aldrich, USA) by following the manufacturer's protocols.

### 2.7. Statistical analysis

The data were analyzed through Statistix 8 (v 8.1, USA) using ANOVA and Tukey's test for significant differences. The graphs were generated using Sigmaplot (v 15.0) software. Each treatment consisted of four replicates with 10 plants per replicate. The lower-case letters on graphs indicate significant differences among treatments, and the vertical bars indicate the standard error of the mean value.

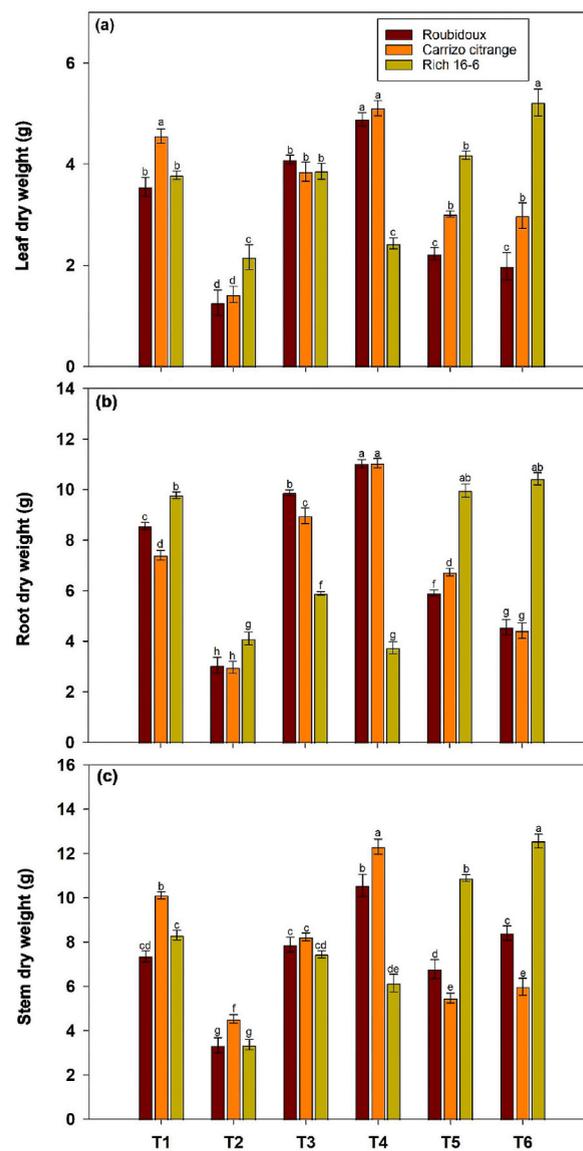
### 3. Results

#### 3.1. Biomass contents

The application of SiNP improved the overall growth of plants both under normal oxygen conditions and flooding stress. A significant difference was observed between the plants with hypoxia stress and oxygen supply. However, Rich-16-6 plants with F + SiNP root application show improving plant biomass in leaf, root, and stem (5.22, 10.43, and 12.56 g), followed by Rubidoux (4.89, 11.03, and 10.56 g) and Carrizo Citrange (5.11, 11.05 and 12.31 g) with F + SiNP foliar application (Fig. 1A–C).

#### 3.2. Photosynthesis rate and leaf greenness

A significant decrease in net photosynthesis rate, stomatal conductance, and leaf greenness was recorded in flooded plants. Overall, SiNP application, either foliar or root zone, improved the photosynthesis rate. The photosynthesis rate in flood-aerated plants with SiNP foliar application showed maximum rate in Rubidoux, carrizo citrange, and Rich 16–6, i.e., 21.50, 21.85, and 18.38  $\mu\text{mol}$ , respectively. However, Rich16-6 showed a maximum photosynthesis rate in FA + SiNP plants (22.98  $\mu\text{mol}$ ) and F + SiNP (19.51  $\mu\text{mol}$ ) with root zone application. In the case of stomatal conductance, the same trend as photosynthesis was achieved. The flooded plants

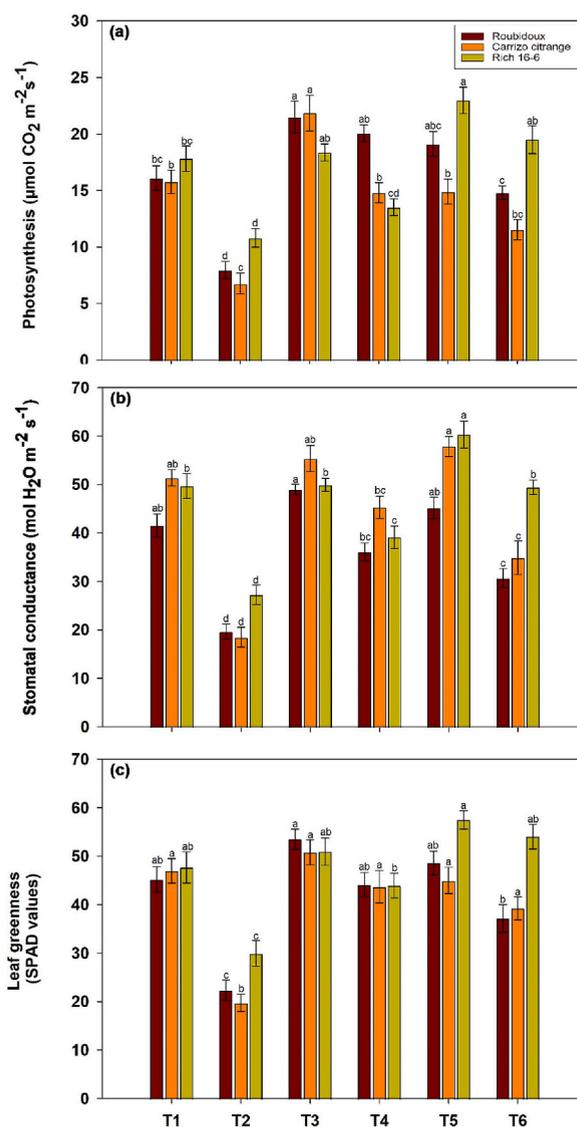


**Fig. 1.** Effect of silicon nanoparticles (SiNPs) on dry biomass of leaf (a), root (b), and stem (c) of three different citrus rootstocks exposed to hypoxia stress. The lower-case letters on graphs represent significant differences, while vertical bars represent the mean standard error.

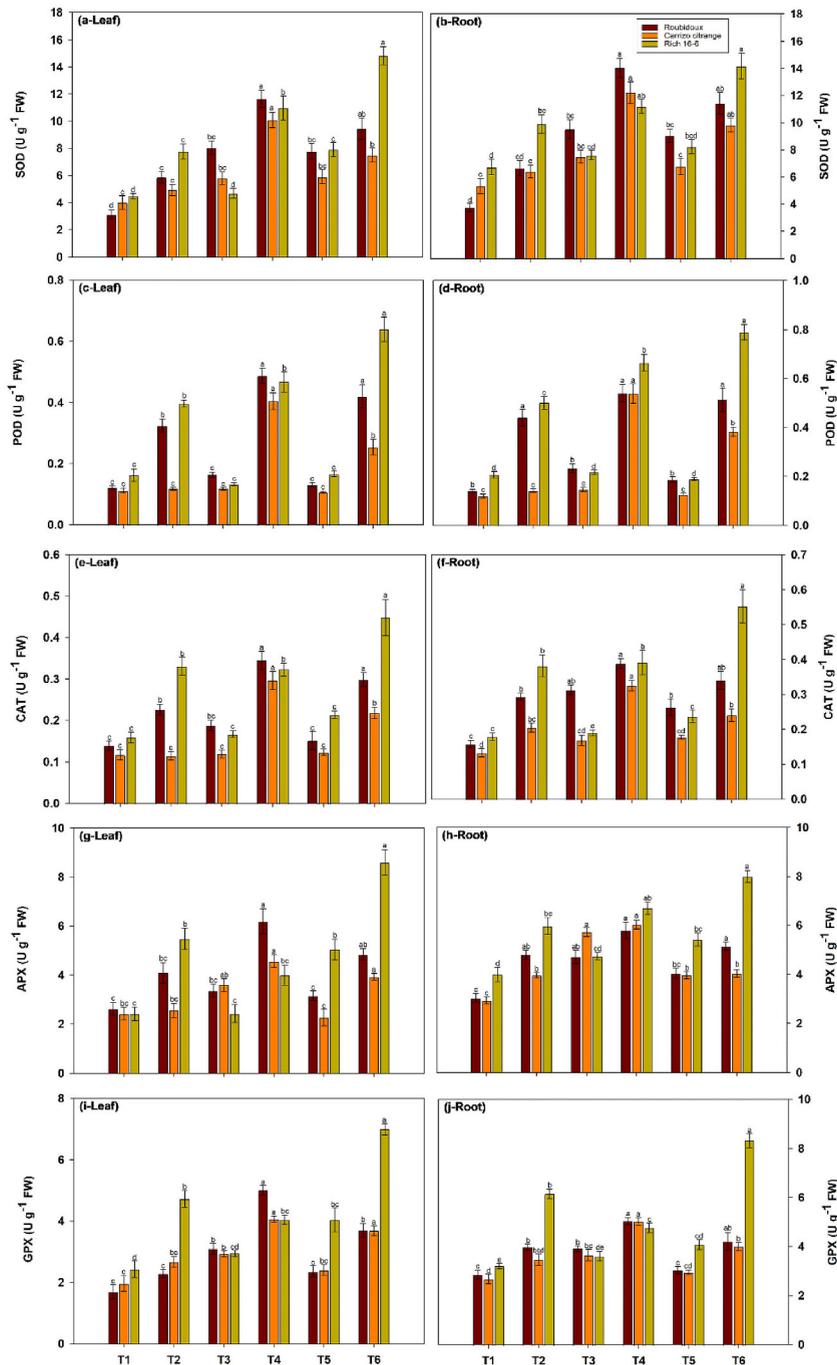
showed less stomatal conductance in Roubidoux, carrizo citrange, and Rich 16–6, i.e., 19.66, 18.47, and 27.24 mol, respectively. The maximum stomatal conductance was recorded in Rich16-6 plants (60.32 mol) treated with FA + SiNP root zone application. (Fig. 2A–B). Leaf greenness was almost similar to control plants, but flooded plants without oxygen showed a significant decrease in leaf greenness of Roubidoux, carrizo citrange, and Rich 16–6, i.e., 22.33, 19.75, and 29.94 SPAD index, respectively. Rich 16-6 showed the highest leaf greenness in all treatments and rootstocks, i.e., 57.52 SPAD index (Fig. 2C).

### 3.3. Antioxidant enzyme activity

The leaves and roots had similar antioxidant enzyme activities (SOD, POD, CAT, APX, and GPX). The flood-aerated controlled plants showed the lowest SOD, POD, CAT, APX, and GPX values compared to other treatments. The highest value of SOD was observed in the leaves and roots of Rich 16-6 plants (14.81 and 14.17  $\text{U g}^{-1}$ ) with F + SiNP root zone application followed by Roubidoux (11.65 and 14.03  $\text{U g}^{-1}$ ) and carrizo citrange (10.08 and 12.02  $\text{U g}^{-1}$ ) with F + SiNP foliar application (Fig. 3A–B). For POD activity, peak value was observed in both leaf and root of Rich 16-6 plants (0.63 and 0.78  $\text{U g}^{-1}$ ) with F + SiNP root zone application followed by Roubidoux (0.48 and 0.54  $\text{U g}^{-1}$ ) and carrizo citrange (0.40 and 0.53  $\text{U g}^{-1}$ ) with F + SiNP foliar application (Fig. 3C–D). CAT activity was similar to POD, as the maximum value was recorded in both leaf and root of Rich 16-6 plants (0.44 and 0.55  $\text{U g}^{-1}$ ) with F + SiNP root zone application followed by Roubidoux (0.34 and 0.38  $\text{U g}^{-1}$ ) and carrizo citrange (0.29 and 0.32  $\text{U g}^{-1}$ ) with F + SiNP foliar



**Fig. 2.** Effect of silicon nanoparticles (SiNPs) on photosynthesis (a), stomatal conductance (b), and leaf greenness of three different citrus rootstocks exposed to hypoxia stress. The lower-case letters on graphs represent significant differences, while vertical bars represent the mean standard error.



**Fig. 3.** Effect of silicon nanoparticles (SiNPs) on antioxidant enzyme activities of SOD (a-leaf, b-root), POD (c-leaf, d-root), CAT (e-leaf, f-root), APX (g-leaf, h-root), and GPX (i-leaf, j-root) of three different citrus rootstocks exposed to hypoxia stress. The lower-case letters on graphs represent significant differences, while vertical bars represent the mean standard error.

application (Fig. 3E–F). For APX and GPX, maximum enzyme activity was noted in the leaves (8.59 and 6.99 U g<sup>-1</sup>) and roots (7.99 and 8.31 U g<sup>-1</sup>) of Rich 16-6 plants with F + SiNP root zone application followed by Roubidoux leaves (6.18 and 5.01 U g<sup>-1</sup>) and roots (5.79 and 5.03 U g<sup>-1</sup>), and carrizo citrange leaves (4.56 and 4.05 U g<sup>-1</sup>) and roots (6.03 and 5.02 U g<sup>-1</sup>) with F + SiNP foliar application (Fig. 3G–H). Generally, SiNP improved enzymatic activities both as foliar and root zone application.

### 3.4. Lipid peroxidation, proline, and glycine betaine contents

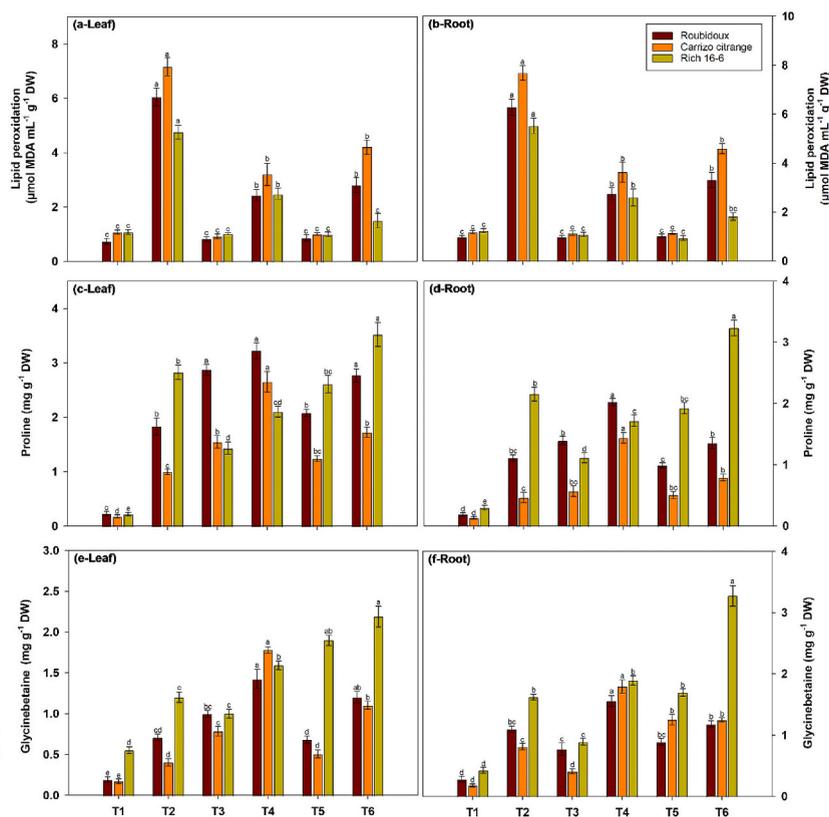
Lipid peroxidation was measured as MDA content and was similar in leaves and roots. Among all treatments, flooded plants without oxygen showed higher lipid peroxidation rate levels than others. In leaves of flooded plants, the lipid oxidation rate in Roubidou, carrizo citrange, and Rich 16–6 was 6.05, 7.15, and 4.75  $\mu\text{mol}$ , respectively. In roots of flooded plants, the lipid oxidation rate in Roubidou, carrizo citrange, and Rich 16–6 was 6.27, 7.68, and 5.52  $\mu\text{mol}$ , respectively. In contrast, the lower rate of lipid peroxidation was recorded in flood-aerated plants with SiNP (both foliar and root) (Fig. 4A–B).

The proline and glycine betaine contents exhibited the lowest level in both leaves and roots of flood-aerated control plants. The highest level of proline was recorded in Rich 16-6 leaves and roots (3.52 and 3.23  $\text{mg g}^{-1}$ ) with F + SiNP root zone application, followed by Roubidou (3.22 and 2.02  $\text{mg g}^{-1}$ ) and carrizo citrange (2.65 and 1.44  $\text{mg g}^{-1}$ ) with F + SiNP foliar application (Fig. 4C–D). Similarly, glycine betaine (GB) contents in both leaves and roots were higher in the plants treated with SiNP through foliar or root zone application. The maximum GB contents were recorded in Rich 16-6 leaves and roots (2.19 and 3.27  $\text{mg g}^{-1}$ ) with F + SiNP root zone application, followed by Roubidou (1.42 and 1.55  $\text{mg g}^{-1}$ ) and carrizo citrange (1.78 and 1.79  $\text{mg g}^{-1}$ ) with F + SiNP foliar application (Fig. 4E–F).

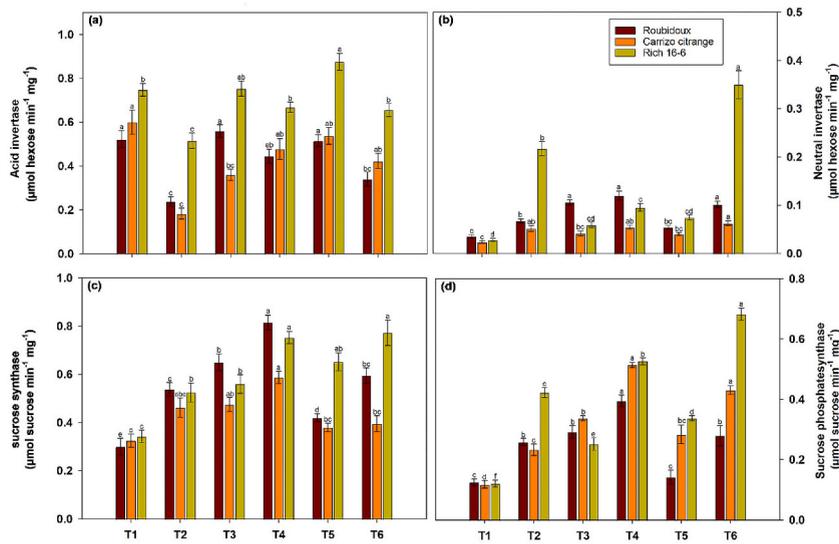
### 3.5. Glycolysis and sucrose metabolic enzymes

The enzymatic activities of acid invertase (AI), neutral invertase (NI), sucrose synthase (SS), and sucrose phosphate synthase (SPS) were slightly lower in flood-aerated control plants as compared to other treatments (Fig. 5A–D). The neutral invertase (NI) enzymatic activity showed lower expression than others. From all three rootstocks, Rich 16-6 plants showed higher enzymatic activities of AI, NI, SS, and SPS (0.875, 0.35, 0.77, and 0.68  $\text{min}^{-1} \text{mg}^{-1}$ ) with F + SiNP root zone application compared to Roubidou (0.56, 0.12, 0.81 and 0.39  $\text{min}^{-1} \text{mg}^{-1}$ ) and carrizo citrange (0.36, 0.05, 0.58 and 0.51  $\text{min}^{-1} \text{mg}^{-1}$ ), respectively.

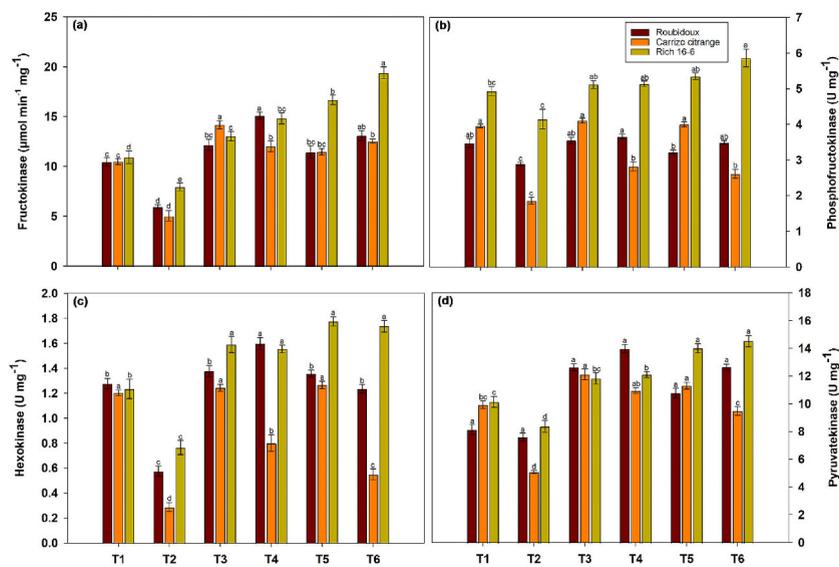
A significant increase was observed in the activities of fructokinase (FK), phosphofructokinase (PFK), hexokinase (HK), and pyruvate kinase (PK). In all treatments, flooded plants without oxygen showed lower values in all treatments (Fig. 6A–D). From all rootstocks, Rich 16-6 showed the highest activity in FK, PFK, HK, and PK (19.39, 5.86, 1.77, and 14.5  $\text{U mg}^{-1}$ ) with F + SiNP root zone application followed by Roubidou (15.09, 3.66, 1.59, 13.97  $\text{U mg}^{-1}$ ) with F + SiNP foliar application and carrizo citrange (14.17, 4.10, 1.24 and 12.12  $\text{U mg}^{-1}$ ) FA + SiNP foliar application, respectively. Overall, SiNP application, either foliar or root zone, increased



**Fig. 4.** Effect of silicon nanoparticles (SiNPs) on lipid peroxidation (a), proline (b), and glycine betaine (c) of three different citrus rootstocks exposed to hypoxia stress. The lower-case letters on graphs represent significant differences, while vertical bars represent the mean standard error.



**Fig. 5.** Effect of silicon nanoparticles (SiNPs) on acid invertase (a), neutral invertase (b), sucrose synthase (c), and sucrose phosphatesynthase (d) of three different citrus rootstocks exposed to hypoxia stress. The lower-case letters on graphs represent significant differences, while vertical bars represent the mean standard error.



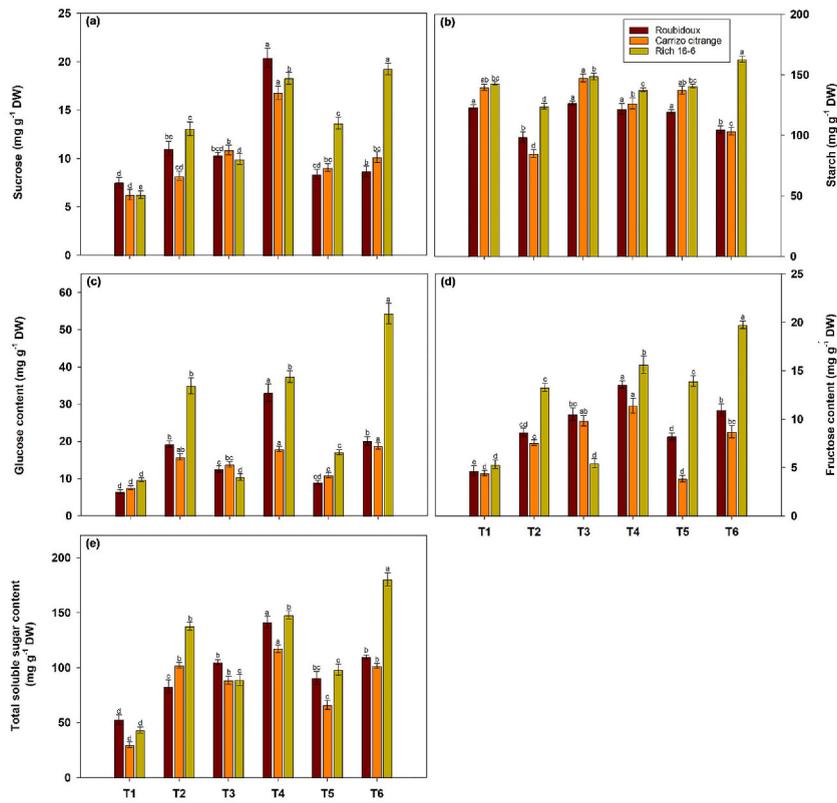
**Fig. 6.** Effect of silicon nanoparticles (SiNPs) on fructokinase (a), phosphofructokinase (b), hexokinase (c), and pyruvatekinase activity (d) of three different citrus rootstocks exposed to hypoxia stress. The lower-case letters on graphs represent significant differences, while vertical bars represent the mean standard error.

enzymatic activity.

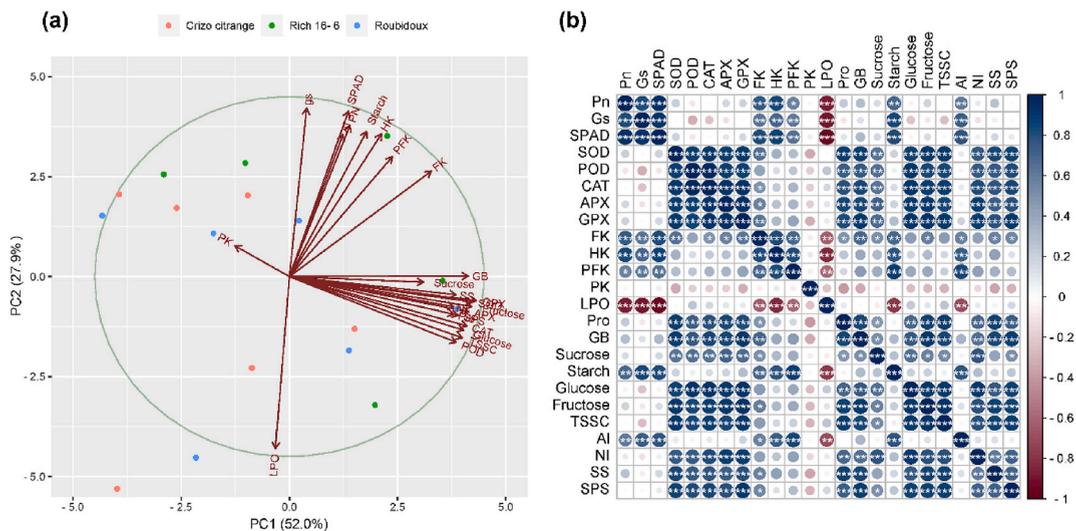
### 3.6. Soluble sugars and starch contents

The soluble sugar contents showed a slight difference in control compared to the other treatments. The highest rate of sucrose, glucose, fructose, and total soluble sugars was recorded in Rich 16-6 plants with F + SiNP root zone application, i.e., 19.26, 54.32, 19.76, and 180.25  $\text{mg g}^{-1}$ , respectively, followed by Roubidoux (20.40, 33.10, 13.59, and 141.25  $\text{mg g}^{-1}$ ), and carrizo citrange (16.78, 17.98, 11.40 and 117.25  $\text{mg g}^{-1}$ ). Overall, SiNP application through foliar or root zone improved the soluble sugar contents (Fig. 7A, C-E).

The plants of all three rootstocks didn't show any significant difference in starch content concerning all treatments. However, flooded plants without oxygen have a lower starch concentration among all treatments. Maximum starch contents were noted in Rich



**Fig. 7.** Effect of silicon nanoparticles (SiNPs) on sucrose (a), starch (b), glucose (c), fructose (d), and total soluble sugars (e) of three different citrus rootstocks exposed to hypoxia stress. The lower-case letters on graphs represent significant differences, while vertical bars represent the mean standard error.



**Fig. 8.** Principal component analysis (PCA) (a) and correlation analysis (b) of all parameters used in this study of three different citrus rootstocks exposed to hypoxia stress. Dot colors in the PCA graph indicate the rootstock type. In the correlation graph, the positive and negative correlation is shown by the color strip given on the right side of the graph, and the asterisk (\*) showed the significance level as  $p < 0.05$  (\* significant),  $p < 0.01$  (\*\* more significant), and  $p < 0.001$  (\*\*\*) highly significant).

16-6 plants ( $163.12 \text{ mg g}^{-1}$ ), followed by Roubidoux ( $121.75 \text{ mg g}^{-1}$ ) and carrizo citrange ( $126.5 \text{ mg g}^{-1}$ ) (Fig. 7B).

### 3.7. Correlation and principal component analysis

Principal component analysis (PCA) was performed to understand the relationship among different variables. The PC1 component represents 52 % of the explained variables, while PC2 represents 27.9 %. The variables close to each other have a group and similarity between each other's, that they are correlated, whereas far from these are dissimilar (Fig. 8A). PC1 represents most of the variable's effects together, while only two variables in PC2 (LPO and PK) are different from the others. On the other hand, the correlation plot also showed the positive or negative relationship between variables (Fig. 8B). The correlation analysis showed the degree of correlation between variables and differentiated based on Pearson's value. The correlation between variables is represented by color and the mode of significance as  $p < 0.05$  (\* significant),  $p < 0.01$  (\*\* more significant), and  $p < 0.001$  (\*\*\*) highly significant).

## 4. Discussion

The present study showed significant differences in SiNP applications on different rootstocks. This could be due to the higher efficiency of silicon nanoparticle transportation within plant parts and increased biochemical activities [45]. Hypoxia, known as flooding, adversely affects plant biomass and overall plant health due to lower oxygen than the aerated one, ultimately affecting plant growth and development [17,46]. The plant biomass of all citrus rootstocks is reduced under hypoxia stress. However, our results showed a remarkable improvement in dry biomass and growth of citrus rootstock using SiNP applications. Earlier studies in banana and mint (*Mentha × piperita*) showed a significant impact of SiNPs application in plant growth, development, physiological, biochemical, morphological attributes, and overall plant survival [47,48]. Silicon is involved in various protective roles in plants' growth, specifically, the preservation of photosynthesis (*Pn*) under stress conditions [49,50], that allow the plants to grow well under this condition. Hypoxia occurs due to the unavailability of  $O_2$  that eventually affects the root water uptake capacity, reduction in photosynthesis, and stomatal closure [51]. Thus, silicon is the one that mitigates the harmful effect on photosynthesis and pigments [52,53], and is reported in various species under different stress conditions like in tomatoes [54], strawberries [53], soybean [55], maize [52], and rice [56]. In the present study, SiNPs application, either foliar or root zone under flooded conditions (hypoxia), didn't reinstate the photosynthesis to an optimum level compared to control and other applications. The root zone application showed more efficiency in photosynthesis and stomatal conductance than foliar because the foliar application might have a detrimental effect due to changes in light interceptions or leaves pore blockage that subsequently delayed the photosynthesis process [57], as previously reported in grapes [58]. However, SiNP-treated plants showed higher levels of greenness than hypoxic plants, while control plants didn't show a substantial difference compared to SiNP-treated plants.

Silicon nanoparticles (SiNPs) can increase the activity of antioxidant enzymes like SOD, POD, CAT, and APX [59,60]. Stimulating antioxidant enzymes is considered an essential Si-mediated mechanism to alleviate the abiotic stress in plants [61]. Previous studies in sweet pepper and mung bean reported that the exogenous application (foliar or root zone) of SiNPs improved the antioxidant activity of SOD, POD, CAT, APX, and GR [62,63]. Our study showed a considerable increase in antioxidant enzyme activity in SiNP-treated plants compared to control and flooding plants. Subsequently, the stimulation of antioxidant enzymes in the capacity of silicon to reduce oxidative damage in plants has been reported under various stress conditions [64] and in several crops such as mango [65], strawberry [66], tomato [67], wheat [68], and rice [69].

Numerous studies have discussed that silicon protects against oxidative stress due to decreased lipid peroxidation and increased antioxidant enzyme activity [70,71]. The present study also showed a significant decrease in lipid peroxidation in SiNP-treated plants compared to flooded ones. Similarly, SiNP-treated seedlings showed a distinct increase in the proline contents in citrus rootstock seedlings compared to control ones. The results of our current study are consistent with the previous reports stated in peach [72,73], olive [74], mango [64], and lentils [75]. Furthermore, the current study observed increased glycine betaine (GB) contents under the flooding (hypoxia) condition. We also observed that the SiNP application further improves the GB contents, which agrees with previous studies [63,76,77]. Thus, proline and glycine betaine help sustain cell membrane integrity and stimulate antioxidant enzyme activity, which further assists in reducing oxidative damage [78]. It also contributes to osmotic regulation, which allows the plant to sustain water uptake during hypoxia stress. Si was observed to enhance its root hydraulic conductance in sorghum by increasing water uptake [79]. So, improved root capacity for water uptake using SiNP applications might help citrus seedlings for proper nutrient uptake and are reported in previous studies in beans [80], tomatoes [67], and rice [81].

Similarly, the activity of acid invertase (AI), neutral invertase (NI), sucrose synthase (SS), and sucrose phosphate synthase (SPS) showed a positive increase in their activity because of SiNPs application. Thus, elevated sucrose accumulation is directly related to the higher sucrose phosphate synthase activity, and higher glucose and sucrose activity is directly associated with higher sucrose synthase and neutral invertase activity. Our results align with the previous study by Chen et al. [82], and Michalak et al. [83], which described that increased related metabolic activity could enhance their stress resistance and reduce oxidative damage in plants. Glycolysis is also an essential metabolic activity that provides energy to different plant parts and is mediated by other enzymes like fructokinase, hexokinase, phosphofructokinase, and pyruvate kinase. In the present study, we found an active increase of these enzymatic activities under SiNP application compared to hypoxic stress plants. Previous studies by Vega et al. [82], and Gupta and Kaur [83] reported that fructokinase (FK) and hexokinase (HK) perform an important function in sugar metabolism in plants, which helps to regulate its activity and proper growth. Thus, glycolysis is considered an important enzymatic activity for appropriate plant growth.

The improvement in the photosynthetic process by applying SiNPs is directly reflected by the production of sugars, their derivatives, and consumption behavior [77]. Glucose is the primary product stored in starch, significantly involving proper plant growth

and carbon assimilation [84]. In our study, we found an increase in the starch contents of the SiNPs-treated plants, possibly through improved photosynthesis. The same phenomenon was reported in cucumber, which described the increase in the starch level of Si-treated cucumber plants under stress conditions, increasing its energy storage for appropriate growth [85]. Starch is mainly reactivated by the enzymatic activity of amylase and release soluble sugars, which act as a signaling molecule to support plant growth and osmolyte production [84]. Moreover, SiNP application further increases the total soluble sugars (TSS) and fructose levels that agree with the previous study reported in peas [86]. A mechanism by which SiNP application mediates hypoxia stress in plants is shown in Fig. 9.

## 5. Conclusion

The present study provides an innovative insight into silicon nanoparticles (SiNPs) role in overcoming hypoxia stress via modulating antioxidant enzymatic activity and carbohydrate metabolism. This critical action of SiNPs improved the photosynthetic activity that subsequently helps produce sugar enzymes to provide energy to plants for tremendous growth. This improvement also helps to maintain higher antioxidant activity to prevent plants from under hypoxia stress. Moreover, SiNPs also help reduce the lipid peroxidation rate by enhancing proline and glycine betaine activity, finally enabling the plant to prevent oxidative damage. As a result, the present study provides immense value in growing citrus plants under hypoxia stress as the best management strategy to overcome this problem. However, its application at the commercial level is our next concern to optimize these findings.

## Funding statement

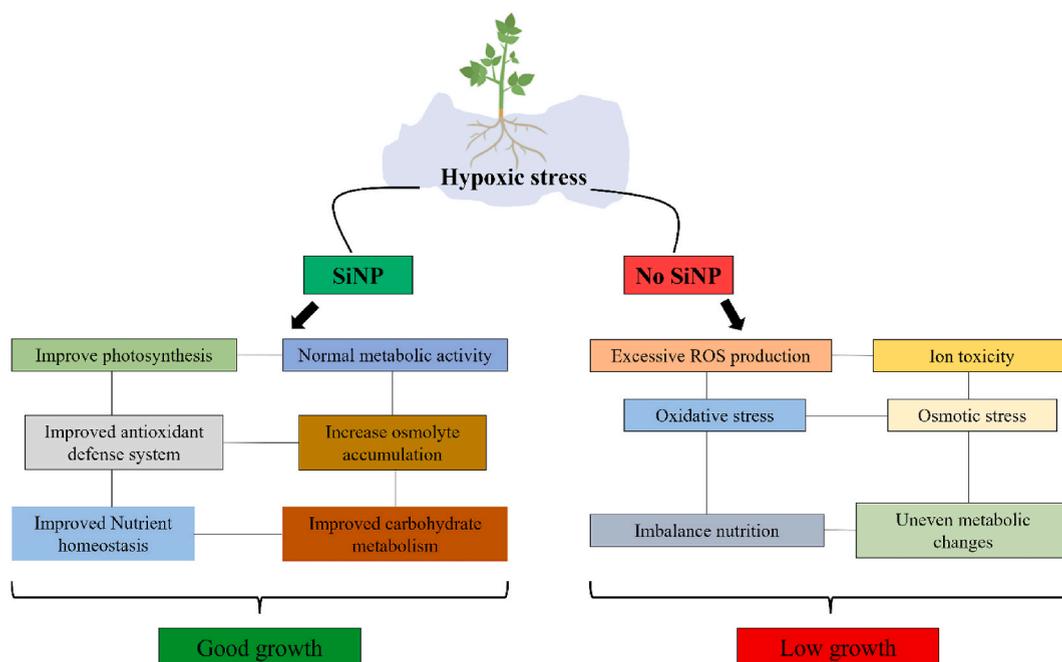
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## Data availability statement

Data included in article/supp. Material/referenced in article.

## CRediT authorship contribution statement

**Shahid Iqbal:** Formal analysis, Methodology, Software, Writing – original draft. **Mujahid Hussain:** Formal analysis, Methodology. **Saleha Sadiq:** Formal analysis, Software. **Mahmoud F. Seleiman:** Formal analysis, Software. **Ali Sarkhosh:** Resources, Writing – review & editing. **John M. Chater:** Resources, Writing – review & editing. **Muhammad Adnan Shahid:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – review & editing.



**Fig. 9.** Mechanism of SiNPs application mediates hypoxia stress in plants. This figure represents the strategies of plant growth with and without silicon nanoparticle application.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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