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# Rice responses to silicon addition at different Fe status and growth pH. Evaluation of ploidy changes.

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**Abstract:** It has been described in rice that Si only plays a physical barrier that does not allow Fe to enter cell apoplast, causing Fe deficiency responses even under Fe sufficiency growth conditions. Most of the conclusions were attained at acidic pH, but rice is also grown at calcareous conditions, which especially induce Fe deficiency in the plants. In this study, we assay the effect of Si in rice suffering both Fe deficiency and sufficiency in hydroponics at two pHs (5.5 and 7.5). Plant biometric parameters, ROS concentration, enzymatic activities, and total phenolic compounds, as well as ploidy levels, have been determined. In general, both pHs promoted similar rice responses under Fe sufficiency and deficiency status, but at pH 7.5, stress was favored. Flow cytometry studies revealed that Fe deficiency increased the percentage of cells in higher ploidy levels. Moreover, under this Fe status, Si addition enhanced this effect. This increase contributed to maintaining chloroplast structure which may have preserved antioxidant activities, and fortified cell walls, diminishing Fe uptake. The first is considered a beneficial effect as plants presented acceptable SPAD values, well chloroplast structure, and qualitatively high fluorescence observed by confocal microscopy, even under Fe deficiency. But contributes to intensify the Fe shortage, by decreasing apoplast Fe pools. In summary, Si addition to rice plants may not only behave as an apoplastic barrier but may also protect plant chloroplast and alter the plant endoreplication cycle, giving a memory effect to cope with present and future stresses.

**Keywords:** Rice; Silicon; Iron deficiency; endoreplication; Antioxidant System

## 1. Introduction

Rice (*Oryza sativa*) is one of the three most important crops in the world, providing 23% of consumed calories (Khush, 2003). The International Grain Council estimated an annual world production of 482 Mt of rice (MAPA, 2017) and the European Union estimated for the 2016/2017 season, that 442000 ha, will be dedicated to this crop, with 2,86 million tons of rice harvested. For the same season, Spain dedicated 109411 ha (55% japonica and 45% indica), of which 105910 ha were in wetland and 3501 ha in dryland (SSO, 2016). It is widely reported that rice is one of the major crops that positively respond to silicon fertilization in the field (Liang et al., 2015). But despite silicon is a beneficial element to many crops, it is not been listed among essential elements for plants yet. Takahasi et al. (1990) categorized plant species based on the mechanisms of silicon uptake. The plants that depend on active, passive, or rejective mechanisms are classified as high-, intermediate- or non-accumulators, respectively. In this classification, cucumber is classified as intermediate and rice as Si-accumulator. Likewise, iron is an essential element for most living organisms and rice is especially susceptible to Fe deficiency in the early stages of its development (Mori et al., 1991). In plants, its deficiency can cause severe yield losses. Rice is also a characteristic crop, which contains a low concentration of Fe in polished seeds, thus is disadvantageous as a major Fe supply in the human diet. Iron is the most abundant transition metal in the organism, and it has a vital role in a

variety of physiological and metabolic processes like chlorophyll synthesis. Despite being one of the most abundant elements in the Earth's crust, Fe is only slightly soluble at calcareous soil conditions. This type of soils cover approximately 30% of the Earth's surface and contain high amounts of calcium carbonate, keeping soil solution pH at 7.5–8.5, due to a high bicarbonate concentration in the soil solution. In general, iron availability is controlled by Fe oxides, which is limited to around  $10^{-10}$  M in calcareous soils, which is not enough for a plant optimal growth ( $10^{-8}$  M) (Römheld and Marschner, 1986). In contrast, a low soil pH and anaerobic conditions, such as in a paddy field, lead to the reduction of Fe(III) to Fe(II), when present. In these soils, lixiviation and intensive crop production controlled the Fe availability and may cause also Fe deficiency. Fe deficiency in rice causes chlorosis, yellowing the new leaves, and decreasing grain yield (Mori et al., 1991). Rice can prevent this deficiency by chelating Fe(III) with biosynthesized phytosiderophores before absorbing (Strategy II) and taking Fe(II) directly by the OsIRT1 transporter (Strategy I mechanism) (Ishimaru et al., 2006).

Previous studies have demonstrated a beneficial effect of Si on plants alleviating multiple environmental stresses as plant diseases (Datnoff and Rodrigues, 2015), salt and drought stresses (Zhu and Gong, 2014), metal toxicity (Wu et al., 2013; Maksimović et al., 2012; Song et al., 2011) or micronutrient (Fe, Zn) deficiency (Gonzalo et al., 2013; Pavlovic et al., 2013; Pascual et al., 2016; Hernandez-Apaolaza, 2014; Bityutskii et al., 2014, Nikolic et al., 2019, Peris-Felipo et al., 2020; Hernandez-Apaolaza et al., 2020). Related to the role of Si alleviating nutrient deficiency; it has been reported that Si may alleviate Fe deficiency in cucumber plants in hydroponics (pH 6.0) (Bityutskii et al., 2014). Silicon enhances Fe distribution towards apical shoot parts due to Si induced accumulation of Fe mobilizing compounds such as citrate or catechins. Pavlovic et al. (2013) by growing cucumber plants with and without 1.5 mM Si and with Fe(III)-EDTA as Fe source at pH 6.0 found no differences in Fe concentration in leaves of Fe sufficient plants, but they reported an increase in Fe concentration in leaves of Si treated plants under Fe deficiency. On the contrary, Gonzalo et al., (2013) when studied soybean and cucumber plants grown in optimal condition for a week before inducing Fe-deficiency at nutrient solution pH of 7.5, reported a significant decrease in Fe concentration in shoot in Fe sufficient plants with the presence of 1.0 mM Si in the nutrient solution and no differences in Fe concentration due to Si application in plants suffering Fe deficiency. Carrasco-Gil et al. (2018) concluded that the Si effect might vary depending on the plant Fe status, decreasing Fe concentration in Fe-sufficient plants due to the iron plaque formation, and increasing Fe concentration in Fe-deficient plants due to iron remobilization from the plaque that could be acting as a Fe source. Their study has been done at calcareous pH. In summary, knowing that the Fe plaque was favored by Si addition to the media (Wu et al., 2016), Carrasco-Gil et al. (2018) proposed that the Fe plaque formation induces Fe chlorosis symptoms in plants despite a normal Fe nutrition. When Fe was added as a highly stable Fe chelate (Fe(III)-HBED) (Gonzalo et al., 2013) the formation of the Fe plaque was avoided at any pH so Fe concentration in shoot remained lower for Si treated plants under Fe sufficiency and deficiency status. Recently, Becker et al. (2020) reported a similar Fe uptake reduction and activation of Fe deficiency responses in rice at pH 6.0 of the nutrient solution due to Si addition to the growth media. These authors observed that Si reduced shoot Fe concentration independently of the Fe status of the plants (Fe deficient, optimal or high levels) and the Fe source tested (FeSO<sub>4</sub>, Fe-EDTA, and Fe-EDDHA). They observed that Si addition enhanced the Casparian band formation in root exodermis decreasing the apoplastic Fe concentrations in roots. As a reaction to the Fe deficiency promoted by Si, the root Fe-homeostasis-related genes were upregulated, and the plant strategy to overcome the chlorosis was set up. This fact was in

1 accordance with the Coskun model that hypothesized that all the Si beneficial effects  
2 previously described could be attributed to the apoplastic obstruction promoted by Si  
3 addition to the roots (Coskun et al., 2019).

4  
5 Every biotic and abiotic stress, including Fe deficiency, produces an accumulation of  
6 reactive oxygen species (ROS), but living cells have very efficient antioxidant systems,  
7 including enzymatic and non-enzymatic mechanisms to control ROS levels (Foyer and  
8 Noctor, 2005). There has been reported that the exogenous addition of Si could improve  
9 the ability to eliminate ROS by regulating the enzymatic activity of antioxidants (Torabi  
10 et al., 2015; Kim et al., 2016; Tripathi et al., 2017). Song and collaborators (2011)  
11 reported that Si may ameliorate Zn toxicity symptoms by diminishing Zn availability and  
12 by altering superoxide dismutase (SOD) and catalase (CAT) activities, two of the mayor  
13 antioxidative system enzymes. It has been reported too that Si prevents the grana  
14 degradation by drought stress, decreasing ROS production and antioxidant enzyme  
15 activity (Cao et al., 2015). As previously reported for different plant species (Ranieri et  
16 al., 2001; Ramírez et al., 2013; Nikolic et al., 2019) iron deficiency decreased the activity  
17 of the heme-containing enzymes ascorbate peroxidase and catalase, as both are strongly  
18 dependent on Fe status. Silicon addition has been proven to restore both activities to the  
19 levels found under iron-sufficiency (Nikolic et al., 2019). SOD, unlike the previous ones,  
20 exists in three isoforms containing Fe, Mn, or Cu/Zn, this could probably explain that no  
21 significant changes have been detected due to Fe deficiency or Si supply in barley  
22 (Nikolic et al., 2019). Concerning the defensive antioxidant responses of a non-enzymatic  
23 nature, phenolic compound accumulation is one of the most important ones. Hernandez-  
24 Apaolaza et al. (2020) observed that cucumber plants grown at pH 7.5 increased phenolics  
25 concentration in leaves when Si was added to the roots of Fe sufficient and deficient  
26 plants; concluding that at both Fe status Fe deficiency has occurred.

27  
28 Another effect of any stress in plants is growth reduction that occurs rapidly after  
29 stress beginning (Skirycz and Inzé, 2010). In leaves, the final size depends on, among  
30 other factors, cell proliferation and cell expansion (Beemster et al., 2005). Cell  
31 proliferation relies on the mitotic cycle that includes DNA replication and cell division,  
32 to obtain two identical cells. Cell expansion is generally correlated with nuclei size; if the  
33 nucleus is not big enough, the cell will stop its expansion. Cells can inhibit their transition  
34 to mitosis once they have completed chromosome replication. Then, the cells switch from  
35 the cell division cycle to an alternative cycle, the endoreplication cycle, whereby  
36 successive rounds of full genome replication occur in the absence of intervening mitosis.  
37 The skipping of nuclear and cell division leads to an exponential increase in genome  
38 ploidy level (from 2C to 4C, 8C, 16C, and so forth). Ploidy levels are the number of  
39 complete sets of chromosomes in a cell. In the 2C level, there are two copies, 4C four  
40 copies, and so on. This endoreplication cycle produces polyploid cells, very common in  
41 plants, and are very often associated with cell differentiation and increases in cell size  
42 (Melaragno et al., 1993; Traas et al., 1998). The ability to initiate endocycle is crucial  
43 during the development of many plants (Inzé and De Veylder, 2006). External factors  
44 causing stress to the plant affect this developmental process. Water deficiency and  
45 osmotic stress reduce both cell number and size of leaves (Skirycz and Inzé, 2010). Under  
46 mild drought stress, leaf area in *A. thaliana* can be maintained by ploidy-mediated cell  
47 expansion, showing increased endoreduplication (Cookson et al., 2006). Metal toxicity  
48 seems to affect ploidy as proved by Fusconi et al., (2006) that showed higher levels of  
49 ploidy in *Pisum sativum* roots exposed to cadmium. Tolerant varieties of *Sorghum bicolor*  
50 endoreduplication in response to NaCl exposure while non-tolerant do not (Ceccarelli et

al., 2006). These authors suggest that endoreduplication could be an evolutive mechanism for tolerating or contend stress conditions. Amzallag et al. (1990) observed that in efficient genotypes of sorghum a previous exposure to sublethal salinity induced its capacity to grow and set seeds at a lethal concentration for non-pretreated plants. Genome rearrangements and chromosome duplication have been shown to accompany the variations observed, especially those related to reproductive development (Peschke and Phillips, 1992). Therefore, genomic changes may be a component of the salt adaptation response. This fact was also supported by findings observed in other plant species, such as sunflower (Cavallini et al., 1996), or *Festuca arundinacea* (Ceccarelli et al., 2002), which have shown that genomic rearrangements may be part of the adaptive response of plant genomes to environmental stresses. Ceccarelli et al., (2006) showed that in salt-treated plants, the percentages of 8C, 16C, and 32C nuclei in roots in the primary state of growth were 21.9%, 13.3%, and 4.3%, respectively. By contrast, in non-salinized plants, only 3.5% of the nuclei had an 8C content. Concluding that the salt treatment induced chromosome endoreduplication during the differentiation of cells in the root cortex. Hernandez-Apaolaza et al. (2020) reported that root Si application to cucumber plants significantly increased the percentages of 16C and 32C nuclei in new leaves either at sufficient or deficient Fe status. Although the increase in Fe deficient plants was lower than under Fe sufficiency.

As Si seems to diminish Fe uptake through the apoplastic obstruction, and this effect may differ with the Fe nutritional status of the plant and the growth pHs; knowing that Fe status alters endoreplication, does Si influence endoreplication? As far as we know this objective has not been elucidated yet, so it becomes necessary to deepen the investigation with new data in this direction. Moreover, the influence of growth pH on the beneficial effect of Si needs to be elucidated. The aim of this work is to determine the Si effect at early stages of growth of rice seedlings under Fe sufficient and deficient conditions at optimal and calcareous pH and study a possible implication of silicon on mitigating Fe deficiency symptoms through its contribution to changing ploidy levels.

## 2. Materials and Methods

### 2.1 Plant Culture and experimental design

Rice seeds (*Oryza sativa* L. var. Japonica cv. Marisma) were surface sterilized in 20% sodium hypochlorite and 0.05% Tween80 for 10 minutes, rinsed with electrodeionized type III water (Direct-Q 3, Merck Millipore, Germany) several times and immersed in type III water for 24 hours in darkness. Then seeds were placed on moist filter paper at 27°C also in darkness to germinate for 10 days. After germination, seeds were transferred to nutrient solution containing: (mM) 1  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 0.9  $\text{KNO}_3$ , 0.3  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1  $\text{KH}_2\text{PO}_4$ , 0.035  $\text{NaCl}$ , 0.01  $\text{H}_3\text{BO}_3$ , 0.00005  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.001  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.0005  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.0005  $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ , 0.0001  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.001  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ . Half of the plants were cultivated at pH 5.5, fixed with 1 mM MES and Fe-EDTA 40  $\mu\text{M}$ . The other half were cultivated at pH 7.5, fixed with 0.3 mM HEPES and Fe-EDTA 60  $\mu\text{M}$ . The difference in Fe concentration at different pHs was calculated using Visual MINTEQ v3.1 to predict possible precipitation during the experiments. For each pH, half of the plants were cultivated with silicon (+Si treatment) added as silicic acid ( $\text{H}_4\text{SiO}_4$ ) 1.5 mM or 0 mM (-Si treatment). Silicic acid was freshly prepared as described by (Nikolic et al., 2007) passing  $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$  (Sigma-Aldrich, Germany) throughout a column containing cation-exchange resin in its  $\text{H}^+$  form (Amberlite IR 120<sup>+</sup>, Sigma-Aldrich, Germany). Type II water was used ( $<7.1 \cdot 10^{-4}$  mM of Si, determined by ICP-

OES (iCAP PRO, Themofisher Scientific)) for the preparation of the nutrient solutions. The pH was adjusted with HCl at 5.5 and with KOH at 7.5 every two days and the solution was renewed twice a week. After 15 days, for each treatment (+Si/-Si) Fe was removed from the solution for half of the plants obtaining 8 different treatments, four for each pH: pH 5.5: +Fe-Si, +Fe+Si, -Fe-Si, -Fe+Si; and the same for pH 7.5. Plants were grown under controlled conditions for 15 days more in a Dycometal type CCK growth chamber provided with a photosynthetic photon flux density at the leaf of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation and a photoperiod 16/8 h, temperature 25/20°C with 40/60% humidity. SPAD values were periodically recorded during the Fe deficiency period with a Minolta Co. (Osaka, Japan) model 502 SPAD-meter.

Samples were collected at days 0, 1, 4, 7, and 15 days after Fe removal from the solution (days 14, 15, 18, 21, and 28 for plants grown with Fe the complete experiment). Part of rice seedlings was separated into roots and shoots and cleaned with 1% HCl and 0.1% Tween80 for 15 seconds, rinsed with distilled water and type III water, and dried. Another part was separated in roots and shoots and frozen in liquid nitrogen.

To evaluate if it is better to add Si at the beginning of plant culture or when Fe deficiency started, a second set of experiments has been designed. The same procedure was followed for this second set of experiments in which different Si timing has been studied during the Fe deficiency period. For that purpose, plants were grown for two weeks at pH 5.5-6.0 as described before with (+Si) or without (-Si) 1.5 mM in the nutrient solution. Then Fe was eliminated from the solution for 15 days more and during this period half of the plants of the +Si set were submitted to a nutrient solution without Si (+Si/-Fe-Si) and the other half continued with the Si supply (+Si/-Fe+Si). Likewise, half of the plants which did not receive Si during preculture with Fe were supplied with Si (-Si/-Fe+Si) and the other half remained without Si addition (-Si/-Fe-Si). Sampling dates were on days 10 and 17 after Fe shortage and plant preparation was similar to for the previous experiment.

## 2.2 Micronutrient analysis and apoplastic Fe content determination

For the micronutrient concentration assessment, 0.1 g of dry and grounded material were digested in a microwave (CEM Corporation MARS 240/50, Mathews, NC, USA) with 8 ml HNO<sub>3</sub> 65% Suprapur (Merck, Germany), 2 ml H<sub>2</sub>O<sub>2</sub> 30% and 1 ml HF 40% (Panreac). The digestion program consisted of 15 minutes temperature ramp-up to 200 °C and 40 additional minutes at 200 °C. The solutions were made up to 20 g with type III water, and Fe concentration was determined by atomic absorption spectrophotometry (AAs, Perkin-Elmer Analyst 800). Three plant replicates were used in this determination. Free space Fe concentration was measured at 1, 4, 7, and 15 days of deficiency following the procedure described by (Bienfait et al., 1985) with minor modifications: Roots of 10 fresh rice seedlings were washed in a solution containing 0.5 mM CaSO<sub>4</sub> (Panreac) for 10 min. Then they were placed in a 50 ml falcon tube containing 21 ml of 1.5 mM 2,2'-bipyridyl (99%, Sigma-Aldrich, Germany), 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub> (Panreac), and 10 mM MES (Sigma-Aldrich, Germany) under continuous N<sub>2</sub> bubbling. Before the initial reading, 0.15 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> (Merck, Germany) was added. Optical density at 250 nm (OD<sub>520</sub>) was measured to determine Fe(II)-(bipyridyl)<sub>3</sub> complex concentration using  $\epsilon=8.65 \text{ mM}^{-1}$ . The procedure was repeated three times with 10 plants per treatment for each repetition.

## 2.3 Oxidative stress parameters

For reactive oxygen species (ROS) analysis, fresh plant material (0.2 g) was chopped in 2 mL 50 mM HEPES at pH 7. The extract (50  $\mu$ L) was mixed with 150  $\mu$ L 50 mM HEPES and 4  $\mu$ L 5  $\mu$ M H<sub>2</sub>DCFDA (diacetate of 2',7'-diclorodihydrofluorescein) (Molecular Probes, Invitrogen, Carlsbad, CA, USA), and incubated 30 min at 37°C in agitation (100 rpm). Then the extract was centrifuged at 145 xg for 10 min and the pellet was resuspended in 0.2 ml HEPES and incubated for 10 min more at 37 °C. The fluorescence intensity of DCF was measured with a Fluorescence Spectrophotometer (Cary Eclipse Fluorescence, Varian, Australia) at room temperature, with an excitation wavelength of 488 nm and emission filter between 500 and 600 nm (the excitation and emission slits width 5 nm). The fluorescence intensity was used to determine relative ROS production. Nine replicates were measured for each treatment.

To determine the protein content and the activities of the enzymes, approximately 300 mg of frozen tissue were ground in a cold mortar in a solution containing 50 mM phosphate buffer (pH 7.8), 2 mM Na<sub>2</sub>EDTA, 10 mM dithiothreitol (DTT)(Sigma), 0.2 mM ascorbic acid (Sigma), protease inhibitor cocktail (Sigma P2714) and 0.006% g/ml PVPP (Poly(vinylpolypyrrolidone), CAS Number 9003-39-8)(Merck). Homogenates were centrifuged at 28486 xg for 15 min at 4°C and the supernatants were collected. Protein content was determined by the Bradford method using TriReagent (Sigma). CAT (catalase, E.C.1.11.1.6) activity was determined by following the consumption of H<sub>2</sub>O<sub>2</sub> at 240 nm according to the method of Aebi, (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM of Na<sub>2</sub>EDTA, 15 mM H<sub>2</sub>O<sub>2</sub> and leaf extract. One unit of CAT activity was defined as the amount of enzyme that catalyzes the decomposition of 1 mmol of H<sub>2</sub>O<sub>2</sub> per minute and the specific activity was expressed as units per mg protein (extinction coefficient 0.0436 mM<sup>-1</sup> cm<sup>-1</sup>). Five biological replicates were used for this determination. SOD (Superoxide dismutase, E.C. 1.15.1.1) activity was determined using the method described by Giannopolitis and Ries (1977). The 300  $\mu$ l reaction mixture comprising 214  $\mu$ l of 50 mM phosphate buffer pH 7.8, 30  $\mu$ l of 0.75 mM nitrobluetetrazolium chloride (NBT, Sigma-Aldrich, Germany), 30  $\mu$ l of 1 mM Na<sub>2</sub>EDTA (Merck, Germany), 26  $\mu$ l of 150 mM methionine, 2  $\mu$ l of 0.3 mM riboflavin (Panreac) and 10  $\mu$ l of plant extract. The reaction was started by the addition of riboflavin and exposure to light by illuminating the mixtures for 30 minutes. Optical density at 560nm (OD<sub>560</sub>) was monitored at minutes 0 and 30. One unit of SOD activity is defined as the amount of enzyme required to result in a 50% inhibition of NBT reduction rate after 30 minutes. Activity is expressed as a specific activity. Five biological replicates were used for this determination.

Total phenolic concentration has been determined only at pH 7.5 plants following the Ainsworth and Gillespie (2007) protocol by using Folin-Ciocalteu reagent. For that purpose, 0.15 g of frozen plant material was extracted with 3 ml of methanol solution 95% and the after 48h in dark, were centrifuged 15 min at 9000 g. The F-C assay relies on the transfer of electrons in an alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which are determined spectroscopically at 760 nm. Three biological replicates were used for this determination.

For malondialdehyde (MDA) concentration determination 0.1 g of N<sub>2</sub> cold mortar ground frozen tissue were placed in a 1.5 ml centrifuge tube with 1 ml of a solution containing 15% trichloroacetic acid (99%, Sigma-Aldrich, Germany), 0.37% thiobarbituric acid (98%, Sigma-Aldrich, Germany), 0.25M HCl (30%) Suprapur, Merck, Germany) and 0.01% butylhydroxytoluene (BHT). Tubes were incubated at 90°C for 30 min and then centrifuged at 28486 xg for 15 min. The supernatant was collected and optical density at

600 nm and 535 nm was measured. MDA concentration was calculated. Nine biological replicates were used for this determination.

#### 2.4 *Flow cytometry*

Plant samples of rice grown at pH 7.5 under Fe deficiency or sufficiency were studied. Sample preparation generally followed the procedure from Doležal et al. (2007). The material of three biological replicates was used. Fresh leave tissue was homogenized by hand with a new razor blade in a Petri dish while imbibed in 1ml Otto I solution (0.1 M citric acid, 0.5% (vol/vol) Tween 20, filtered through a 0.22-mm filter). The resulting homogenized was mixed gently with a cut pipette tip and filtered through a 40 µm nylon filter. Then 2ml of Otto II solution (0.4 M Na<sub>2</sub>HPO<sub>4</sub> · 12 H<sub>2</sub>O, filtered through a 0.22-mm filter) was added. Propidium Iodine (Sigma) was added for a 50 µg/ml final concentration. The suspension was analyzed in a BD FACSCanto™ II flow cytometer (BD Bioscience, USA).

#### 2.5 *Statistical analysis*

The IBM SPSS Statistics Version 26.0 Software was used to analyze the obtained data. The means were compared using the Duncan test with  $p < 0.05$ .

### 3. Results

Silicon has been found to show a different effect when plants were ongoing Fe deficiency or when plants were under optimal nourishing conditions, so results are displayed separately for its easier understanding.

#### 3.1 *Silicon effect over optimally nourished plants*

Shoot dry weight (DW) was measured at days 14, 15, 18, 21, and 28 of plant growth in plants grown at pH 5.5 and 7.5. Results indicated that Si addition did not increase dry weight at any pH when Fe was present in the media (Figure 1 A, B). SPAD data reflects that at pH 5.5 it remained unchanged at pH 5.5, but at pH 7.5 were lower when Si was not present in the media (Table 1). This fact was also shown in the picture of Figure 1B, in which plants grown at pH 7.5 are presented. As no significant differences were observed in DW or SPAD index related to Si addition at pH 5.5, a picture of these plants at harvest was not provided.



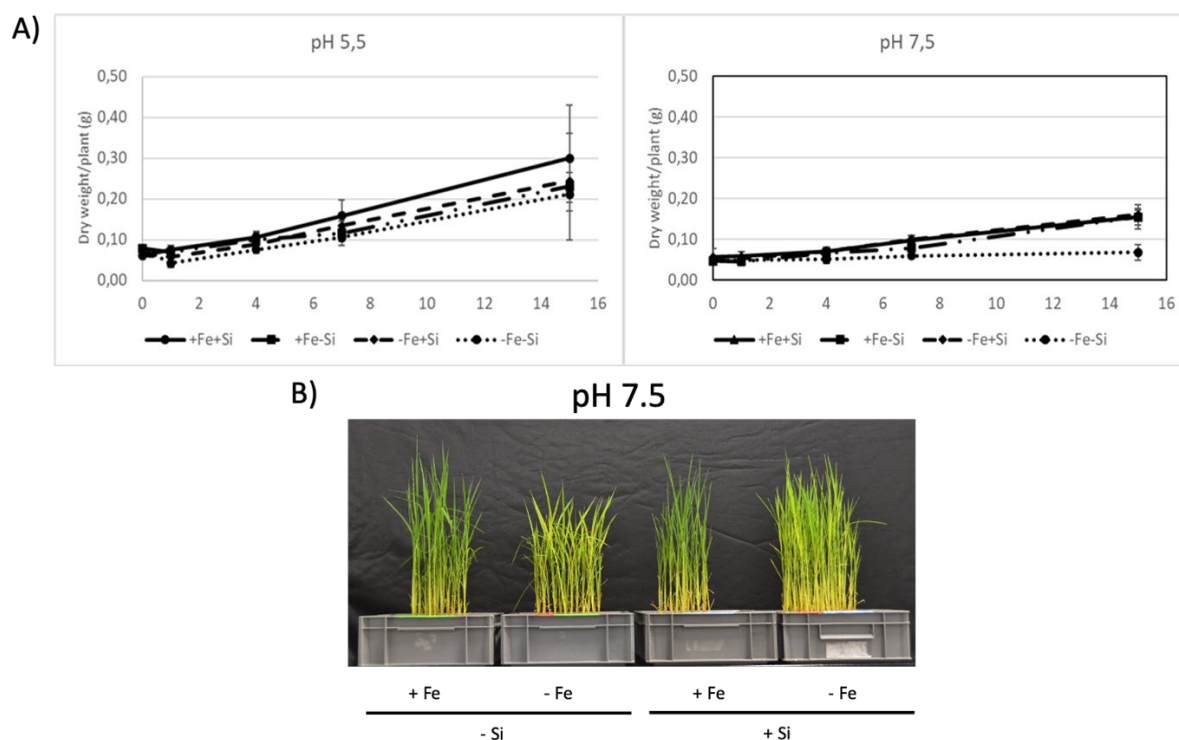


Figure 1: A) Effect of Si addition in shoot dry weight (g)/plant of rice plants under Fe sufficiency (+Fe) at days 14, 15, 18, 21, and 28 of plant growth (data corresponded to days 0, 1, 4, 7 and 15 in the figure) and Fe deficiency (-Fe) plants at days 0, 1, 4, 7 and 15 after starting Fe deficiency at pH 5.5 and 7.5. Data are means  $\pm$  SD (n=3). B) Photography of rice plants grown at pH 7.5 at the last sampling date.

Table 1. SPAD values of rice leaves cultured with (+Fe) or without (-Fe) iron at two different pH (5.5 and 7.5) after 21 days of plant growth (7 days of Fe deficiency in -Fe treatments). Data are means  $\pm$  SD (n=6). Different letters denote significant differences according to the Duncan test ( $p < 0.05$ ).

SPAD Values		
	pH 5.5	pH 7.5
+Fe -Si	36.76 $\pm$ 1.69 a	37.74 $\pm$ 2.00 b
+Fe +Si	36.56 $\pm$ 1.34 a	41.25 $\pm$ 1.59 a
-Fe -Si	12.67 $\pm$ 4.22 b	10.65 $\pm$ 1.24 d
-Fe +Si	17.43 $\pm$ 6.14 b	17.43 $\pm$ 4.17 c

When determined the Fe concentration in shoot after 21 days of growth (Figure 2), it has been observed that Si addition significantly decreased Fe concentration in shoots at both pHs. In the case of pH 7.5, Fe uptake was, in general, lower than at pH 5.5.

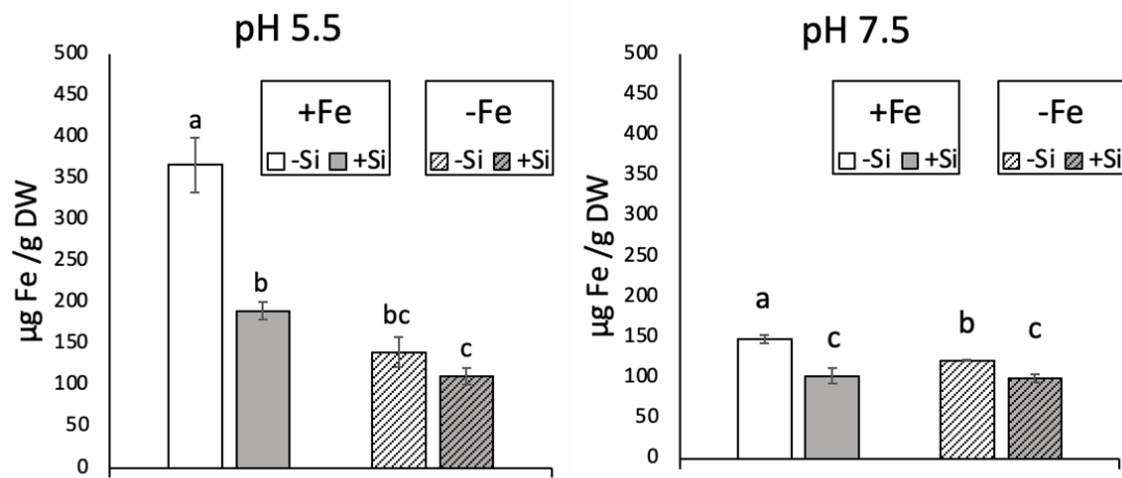


Figure 2: Effect of Si addition in shoot Fe concentration ( $\mu\text{g/g}$ ) in sufficient (+Fe) or deficient (-Fe) rice plants after 21 days of plant growth (7 days of Fe deficiency in -Fe treatments) at two different nutrient solution pHs (5.5 and 7.5). Data are means  $\pm$  SD ( $n=3$ ). Different letters denote significant differences according to the Duncan test ( $p < 0.05$ ).

Iron translocation rate (ratio between Fe concentration in shoot and root) were measured to evaluate the Si effect on Fe mobilization from roots to shoots (Figure 3A). This parameter showed that Si addition leads to a significant decrease in Fe translocation rate at pH 7.5 but no changes at pH 5.5 were observed due to Si supply.

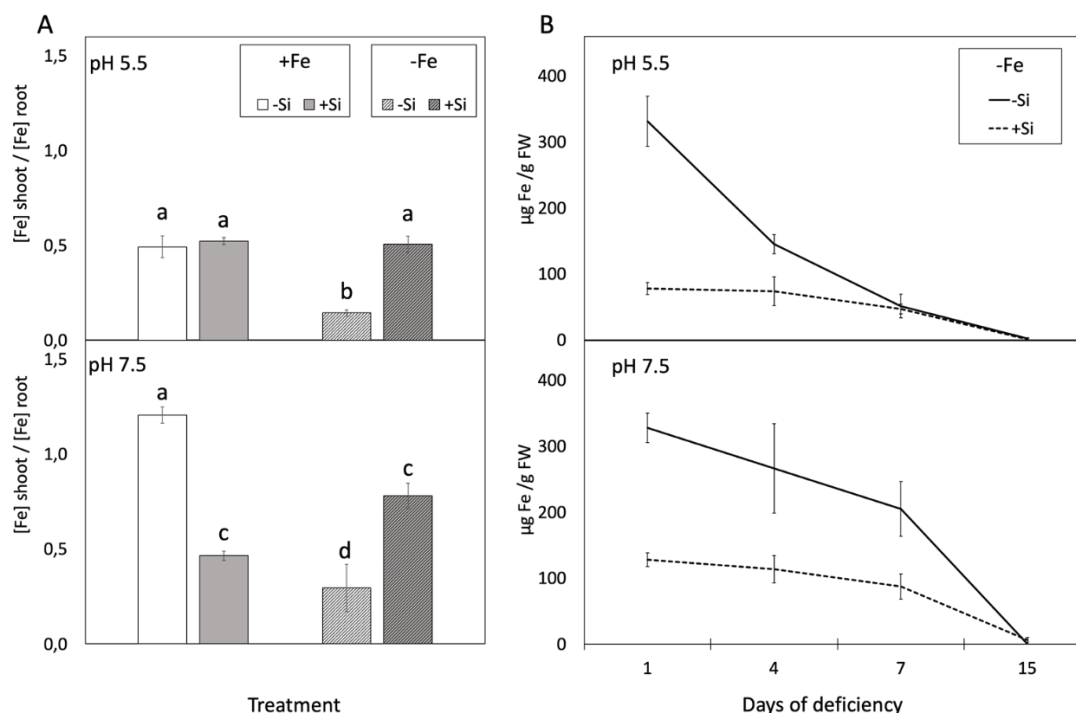


Figure 3: A) Effect of Si addition in Fe translocation factor (Fe in shoot/Fe in root) in sufficient (+Fe) or deficient (-Fe) rice plants after 15 days of Fe deficiency (28 days after transplanting for Fe sufficient plants) at two different pH (5.5 and 7.5). B) Effect of Si treatment on Fe concentration in the apoplast of rice plants at days 1, 4, 7, and 15 of Fe deficiency at two different pHs (5.5 and 7.5). Data are means  $\pm$  SD (n=3). Different letters denote significant differences according to the Duncan test ( $p < 0.05$ )

To check the activation of the antioxidant responses to a possible Fe deficiency induced by the Si addition to the media, catalase, superoxide dismutase, and phenolic compounds production were studied. Catalase (CAT) and superoxide dismutase (SOD) activities were measured after 18 days of plant growth (Table 2) both at pH 5.5 and 7.5. CAT activity significantly decreased with Si addition at both pHs. No differences related to Si supply have been obtained for SOD activity, but Fe deficiency significantly increased SOD at pH 5.5 in the absence of Si. This was not applicable at pH 7.5. Total phenolic compounds were only tested at pH 7.5 (Figure 4) and data obtained showed significantly higher amounts of these compounds in shoot along with the four samplings in plants without Si treatment.

Table 2: Evaluation of catalase (CAT) and superoxide dismutase (SOD), both in U/mg protein, in response to silicon treatments in shoots of iron sufficient (+Fe) or deficient (-Fe) rice plants at two pHs (5.5 and 7.5) after 18 days of plant growth (4 days of Fe deficiency). Data are means  $\pm$  SD (n=5). Different letters in the same row denote significant differences according to the Duncan test ( $p < 0.05$ ).

	Catalase		SOD	
	(U/mg protein)		(U/mg protein)	
	pH 5.5	pH 7.5	pH 5.5	pH 7.5
+Fe -Si	22,89 $\pm$ 0.42 <i>a</i>	14,89 $\pm$ 0.00 <i>a</i>	12,69 $\pm$ 3.65 <i>b</i>	21,34 $\pm$ 5.78 <i>a</i>
+Fe +Si	17,34 $\pm$ 1.39 <i>b</i>	9,84 $\pm$ 0.44 <i>c</i>	14,78 $\pm$ 3.90 <i>ab</i>	21,01 $\pm$ 6.42 <i>a</i>
-Fe -Si	11,21 $\pm$ 0.57 <i>c</i>	11,26 $\pm$ 0.73 <i>b</i>	18,20 $\pm$ 4.25 <i>a</i>	20,06 $\pm$ 3.06 <i>a</i>
-Fe +Si	16,79 $\pm$ 1.82 <i>b</i>	11,08 $\pm$ 0.67 <i>b</i>	15,90 $\pm$ 7.06 <i>ab</i>	21,45 $\pm$ 6.12 <i>a</i>

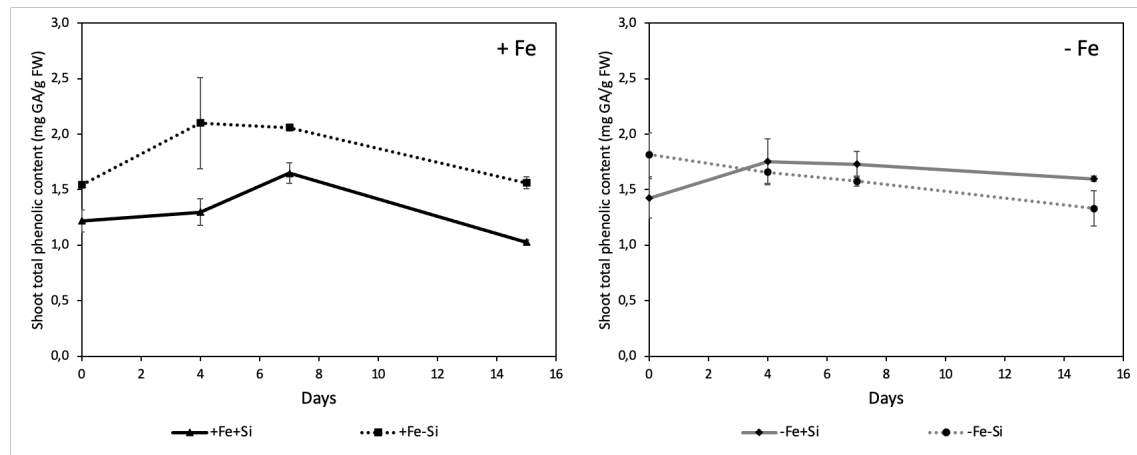


Figure 4. Effect of Si addition in total phenolic compounds (mg of Gallic acid/g fresh weight) of rice plants grown at pH 7.5 under Fe sufficiency (+Fe) at days 14, 18, 21, and 28 of plant growth (data corresponded to days 0, 4, 7 and 15 in the figure) and Fe deficiency (-Fe) plants at days 0, 4, 7 and 15 after starting Fe deficiency. Data are means  $\pm$  SD (n=3).

To test if cell damages have occurred in the plants, MDA concentration was determined at every sampling date. Data recorded at 21 days of plant growth are shown in Figure 5. At both pHs 5.5 and 7.5, MDA concentration in shoots did not show any difference related to Si supply under +Fe conditions.

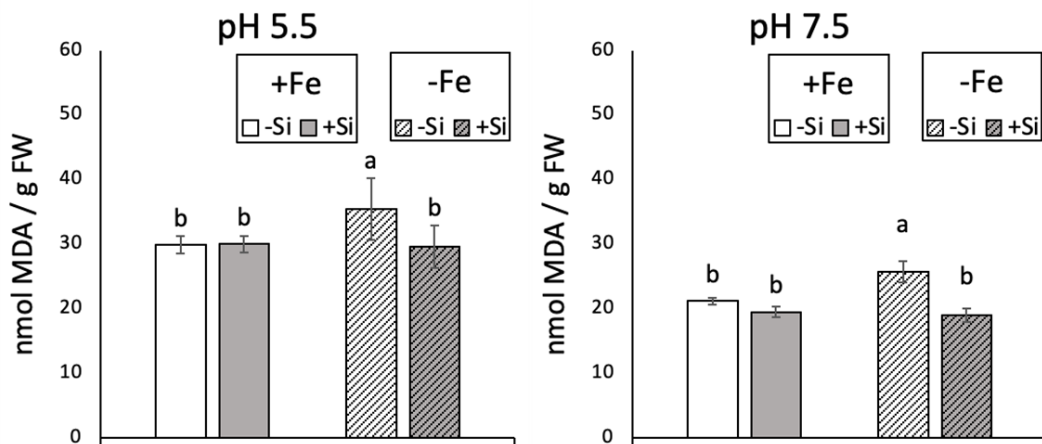


Figure 5: Effect of Si addition in MDA concentration in shoots (nmol/g FW) in sufficient (+Fe) or deficient (-Fe) rice plants after 21 days of plant growth (7 days of Fe deficiency plants) at two different pHs (5.5 and 7.5). Data are means  $\pm$  SD (n=9). Different letters denote significant differences according to the Duncan test ( $p < 0.05$ ).

Ploidy levels at 21 days of growth with Fe and with or without Si were analyzed in leaves of plants grown at pH 7.5 to determine a possible Si effect over ploidy at calcareous pH (Figure 6). Flow cytometry did not show statistically significant changes in ploidy levels distribution in response to Si when Fe was present in the media. Comparing +Fe and -Fe plants an increase in 4C and 8C ploidy levels was shown related to Fe deficiency.

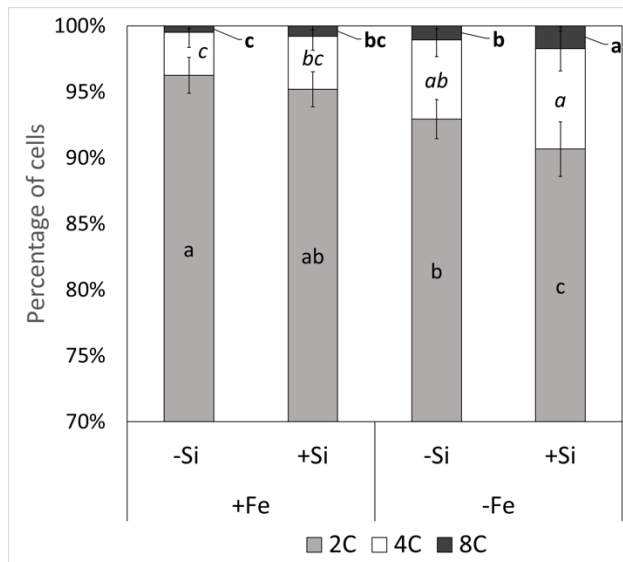


Figure 6: Silicon effect on ploidy levels distribution in the percentage of cells of rice leaves grown in sufficient (+Fe) or deficient (-Fe) conditions after 21 days of plant growth (7 days of Fe deficiency for -Fe plants) at pH 7.5. Data are means  $\pm$  SD (n=4). Different letters denote significant differences according to the Duncan test ( $p < 0.05$ ).

### 3.2 *Silicon effect over Fe-deficient plants*

At pH 5.5, no differences were found in shoot dry weight, but at pH 7.5, plants of the -Fe -Si treatment showed significantly lower DW than the -Fe +Si treatment (Figure 1A, B). Furthermore, Fe-deficient plants treated with Si had a similar weight than the Fe-sufficient ones at both pHs (Figure 1A, B). After 7 days of deficiency, no beneficial effect of Si was observed at pH 5.5 in SPAD results. On the contrary, at pH 7.5, the Si treated plants showed higher SPAD levels (Table 1). Iron concentration in the shoot (Figure 2), were similar for +Si and -Si plants at pH 5.5, but at pH 7.5 Si addition decreased Fe concentration. When Fe concentration in root apoplast was measured at days 1, 4, 7, and 15 of Fe-deficiency (Figure 3B) several differences were obtained. It was remarkable that the Fe apoplastic concentration in non-Si treated plants was significantly higher than the Fe pool of plants with added Si at both pHs. At pH 5.5 in -Fe-Si plants, Fe concentration in the apoplast was reduced more than half (61%) between days 1 and 4 of Fe deficiency, although at pH 7.5 this reduction was slower. Moreover, at both pH tested (pH 5.5 and 7.5) when Si was added to media, the translocation rate significantly increased (Fig. 4A)

Considering +Fe -Si as the control plants, catalase activity (Table 2) decreased under Fe deficiency at pH 5.5 and 7.5 when Si was not added, but with Si supply, this enzymatic activity reached values of Fe sufficient plants in the case of pH 5.5 plants, although at pH 7.5 no effect was detected. Regarding SOD activity (Table 2) also no consistent differences were obtained.

Total phenolic compounds data at pH 7.5 did not show any increase related to the Si addition to the media (Figure 4). Strikingly, MDA concentration (Figure 5) showed that Si supply diminished lipid peroxidation of the shoot cells, either at pH 5.5 or 7.5.

Flow cytometry (Figure 6) showed that the percentage of cells in higher ploidy levels were significantly higher in response to Si addition to the nutrient solution. The 2C ploidy level percentage (basal ploidy level) decreased in Si treated plants. The 4C remained unchanged but the 8C presented a statistically significant increase in response to Si. Iron removal from the nutrient solution contributed to a decreasing percentage of cells with a 2C ploidy level and increase the 4C, regardless of Si presence. But the 8C percentage only increased when Si was supplied. In summary, Fe deficiency increased the percentage of cells in higher ploidy levels; and Si enhanced this effect.

#### 3.2.1. *Timing of Si addition under Fe deficiency*

Another experiment at pH 5.5 was performed in which Si was added or not during the 2 weeks at the preculture period of the rice plants (+Fe). Following that a -Fe period was assessed, in which half of the plants treated with Si, were submitted to -Si conditions (+Si/-Fe-Si) and the other half remained with +Si (+Si/-Fe+Si). The same for the initial -Si plants. In that way, Si application at the beginning of plant growth or the initiation of the Fe deficiency could be evaluated. After 10 days of -Fe, no differences in dry weight were obtained (Table 3). In respect to Fe concentration in the shoot (Table 3) the higher values were observed for plants that did not have Si during the last 10 days (Fe deficiency period) in the NS (+Si/-Fe-Si and -Si/-Fe-Si), but the Si presence during the 2 weeks preculture with Fe did not affect Fe concentration in shoots. Plants that did have Si at both the +Fe preculture and the -Fe periods (+Si/-Fe+Si) showed higher Fe concentration levels than plants that only had Si at the deficiency phase (-Si/-Fe+Si). SPAD index (Table 3) at day 10 and 17 reflected that Si addition did not contribute to increasing this index. The higher SPAD index values were observed for plants without Si addition during the entire experiment (-Si/-Fe-Si) or without Si at the Fe deficiency period (+Si/-Fe-Si). Rice plants with Si addition only at the Fe deficiency period (-Si/-Fe+Si) showed higher

SPAD data than plants grown all the time with Si in the NS. Strikingly, plants without Si addition at the Fe deficiency period (+Si/-Fe-Si and -Si/-Fe-Si) presented higher ROS levels, independently of the Si addition or not during the preculture with Fe period, than plants treated with Si at the Fe deficiency period (Table 3).

Table 3. Shoot dry weight (DW, g), Fe concentration ( $\mu\text{g/g}$ ), and SPAD index at day 10 after Fe deficiency and SPAD index and ROS concentration (Fluorescence units) at day 17 of Fe deficiency at pH 5.5. Data are the mean  $\pm$  SE (n=3; except for ROS with n=9). Different letters in the same row showed significant differences according to Duncan's test ( $p < 0.05$ ).

	DW (g)	[Fe] ( $\mu\text{g/g}$ )	SPAD index		ROS (FU)
			Day 10	Day 17	
+Si/-Fe +Si	0,31 $\pm$ 0,10 a	59,5 $\pm$ 3,3 b	24,0 $\pm$ 1,1 d	28,6 $\pm$ 3,6 c	430 $\pm$ 74 bc
+Si/-Fe -Si	0,32 $\pm$ 0,04 a	65,5 $\pm$ 3,4 a	38,6 $\pm$ 1,2 b	38,2 $\pm$ 2,6 ab	619 $\pm$ 80 a
-Si/-Fe +Si	0,43 $\pm$ 0,07 a	49,3 $\pm$ 1,2 c	35,1 $\pm$ 1,2 c	36,6 $\pm$ 3,0 b	322 $\pm$ 108 c
-Si/-Fe -Si	0,44 $\pm$ 0,05 a	67,9 $\pm$ 2,5 a	40,6 $\pm$ 1,9 a	41,3 $\pm$ 0,9 a	532 $\pm$ 59 ab

## 4. Discussion

### 4.1 Si effect over optimally nourished plants

#### 4.1.1 Silicon effect on biometric parameters

It has been suggested before that Si has a greater effect over plants under stress situations, but not so evident on plants optimally grown (Hernández-Apaolaza, 2014). In higher plants, endoreplication is especially prominent, and the endocycle activity has been correlated with increased metabolic output, rapid cell growth, and cell differentiation (De Veylder et al., 2011; Edgar et al., 2014). A link between endoploidy and cell wall characteristics has been proposed (Bhosale et al., 2019). Bhosale and coworkers propose that ploidy may regulate the expression of cell wall modifications, presumably to prepare cells for massive cell enlargement following cell cycle exit, in the line with the idea that vacuolar expansion drives the cell growth, rather than a ploidy-driven increase in cellular volume. This mechanism relies upon a higher number of copies of genes in the cells with higher ploidy levels. The more copies, the faster cell wall biosynthesis genes can be expressed and fortify it. In this scenario, a higher ploidy could stimulate cell growth by regulating the strength of the cell wall by increasing the expression of both cell wall

loosening and fortifying genes. Therefore, the endocycle might be of particular importance for tissues containing extremely rapidly expanding cells, where an increase in the gene copy number through endoreplication might be a way to cope with the high demand for new cell wall materials (Bhosale et al., 2019). In our study, ploidy levels were studied in plants grown at pH 7.5 treated with or without Si (Figure 6), and no changes were found in response to Si when Fe was present in the media. If ploidy increases indicate an ability of cells to increase their size due to a vacuolar increase in volume, the ploidy results agree with our DW results (Figure 1A). Silicon addition did not affect DW (Figure 1 A, B) not at pH 5.5 or 7.5 grown with Fe. These results were in agreement with data obtained by Fleck et al. (2015) for rice plants grown at pH 6.0 with 1.07 mM of Si and by Goto et al. (2003) for rice grown in a paddy field with calcium silicate addition. This contrasts with Ma and Takahashi (1990, 2002) that demonstrated that 1.0 mM Si addition and at the same pH increased rice DW in optimally nourished plants. These differences could be due to the different rice varieties used by these authors or to the different plant stages (seedlings vs. completely developed plants). For other plant species, Pavlovic et al. (2013) and Hernandez-Apaolaza et al. (2020) reported no changes in DW in Fe sufficient cucumber when plants were treated with 1.5 mM Si at pH 6.0 and 7.5 respectively; as well as Gonzalo et al. (2013) in Fe sufficient soybean grown at pH 7.5 after 35 days of growth with Si (0.1 and 0.2 mM) and Peris-Felipo et al.(2020) in strawberries fruits grown with 1.5 mM Si addition.

#### 4.1.2 Silicon decreased Fe uptake and Fe translocation rate when Fe is present in the media

Calcareous conditions promote a situation in which Fe is not available in the media due to its precipitation as oxyhydroxides. Iron deficiency and toxicity are frequently found in dryland rice cultivation and during initial plant growth when the root system is not yet fully developed. This deficiency is also found in dryland nurseries. Under submerged conditions, iron deficiency occurs in soils with high pH (> 7.5). Measures to increase iron availability in dryland rice crops include: use of cultivars tolerant to iron deficiency, stimulation of anaerobic field conditions using furrows or ridges to keep as much water as possible or spray with iron chelates or iron sulfates; the iron sulfate should be pulverized with hydrated lime to prevent burning of the leaves (FAO 2003). Results obtained in this work showed that silicon addition significantly reduced shoot Fe concentration at both pHs tested (Figure 2). This fact was previously observed by several authors: Ma and Takahashi, (1990), Hinrichs et al., (2017) and Becker et al., (2020) for rice grown at pH 6.0, Carrasco-Gil et al. (2018) for rice grown at pH 7.5; and for other crops such as cucumber grown at calcareous condition Gonzalo et al., (2013) and Hernandez-Apaolaza et al. (2020). But this is the first time to our knowledge in which this effect has been compared at both calcareous and acidic pHs in the same experimental set with analogous growth conditions. Iron chelate stability is a major factor that could influence rice response to Fe. Carrasco-Gil et al. (2018) used Fe-EDTA and Becker et al. (2020) used Fe-EDTA, FeSO<sub>4</sub>, and Fe-EDDHA for testing different Fe status in rice growth, they have added 1.5 and 1.07 mM of Si respectively. Differences in the stability



of both chelates, Fe-EDTA and Fe-EDDHA are considerable, as Fe-EDDHA is even so stable ( $\log k = 33.28$  for the meso isomer and  $\log K = 35.54$  for the racemic isomer (Bannochie and Martell, 1989)), then rice plants could not be able to take Fe from this chelate. Moreover, Fe-EDDHA is stable at a wide range of NS pH, but Fe from Fe-EDTA ( $\log K = 26.5$ , (Norvell, 1991)) remains fully chelated below a pH around 6.5 (for that reason Fe-EDTA concentration has been increased in our experiments at pH 7.5). On the contrary, Mehrabanjoubani et al. (2015) did not find differences in Fe concentration in response to 1.5 mM of sodium silicate nor roots or shoots of an Indian cultivar of rice plants after 105 days of treatment at optimal growth conditions at fixed pH 6. Also, at pH 6, Bityutskii et al., (2014), found no differences in Fe concentration in the root or shoot in Fe-fed cucumber plants due to Si addition.

As mentioned before, in this research Fe(III)-EDTA has been used; this chelate is not stable at pHs above 6.5 allowing the Fe to precipitate at roots surfaces forming the iron plaque, which reduced Fe uptake, and consequently its translocation to shoot. According to our results, the Fe translocation rate decreased in the presence of Si under iron sufficiency conditions at pH 7.5, but no differences were observed at pH 5.5 (Figure 3A). Si presence increases this process (Wu et al., 2016). Carrasco-Gil et al., (2018) reported lower Fe root concentration in rice plants in the presence of 1.5 mM of silicic acid using Fe(III)-EDTA as a chelate at pH 7.5. They found that Fe distribution in root changed in the presence of Si, localizing Fe mainly in the epidermis when Si was present. They concluded that the increased Fe plaque formation due to Si presence can act as a barrier so the Fe uptake by Si-treated plants was decreased. In our experiments, the Fe source was also Fe(III)-EDTA so the same principle applies at pH 7.5, and when Fe and Si are present in the media, the Fe translocation rate is decreased (Figure 3A), but at pH 5.5, Fe(III)-EDTA is stable enough to not allow the formation of Fe plaque and in this case, translocation in presence of Si do not change. A possible physiological effect that may depend on Fe nutritional status on the plant may be occurring. Plants growing at pH 7.5 with Fe and Si produced a consistent iron plaque which reduced Fe uptake (Figure 3A). ROS production increase and regulate the corresponding responses including expression changes in Fe homeostasis-related genes. Iron uptake strategies were developed in consequence, and when the plant will suffer Fe deficiency, all the mechanisms are already activated. As Carrasco-Gil et al., (2018) indicated, this iron plaque pool will be used when necessary. In any case, the lower Fe concentration in shoots of pH 5.5 plants could not be explained through the Fe plaque formation, as this plaque will not be produced with Fe-EDTA as Fe source because this chelate is stable at this pH. Becker et al., (2020) explained this Fe uptake reduction by an enhancement of the Casparian band in the exodermis promoted by the presence of Si in the nutrient solution, which reduced the Fe flux into the apoplast. This is in agreement with the Fe accumulation in the epidermis described by Carrasco-Gil et al., (2018). Probably the initial effect of Si in the Casparian band enhancement could be complemented with the formation of the Fe plaque at pH 7.5, which reduced more the Fe uptake and redistribution within the plant. This explained the lower amount of Fe in shoots at pH 7.5 compared to pH 5.5 when Fe was available at sufficient amount. Consequently, the Fe accumulation in the apoplast decreased. In

1 general, this Fe pool systematically increased with increasing amounts of Fe. This  
2 hypothesis was in accordance with the "apoplastic obstruction" theory proposed by  
3 Coskun et al., (2019).

4 As mentioned before Bhosale et al., (2019) proposed that ploidy may regulate the  
5 expression of cell wall modifications, for example, maturing xylem cells develop a thick  
6 secondary cell wall to provide the mechanical strength needed for the transport of water  
7 and nutrients. These authors showed that xylem cells rapidly engaged into the endocycle,  
8 in contrast to the phloem and phloem companion cells that mainly remained diploid. This  
9 suggested a role of endocycle-driven gene expression in controlling cell wall  
10 biosynthesis. This fact could explain the differences in Fe uptake shown under Fe  
11 sufficiency, although only slight and no significant variations have been shown in the  
12 ploidy level of +Si and -Si plants (Figure 6). It is necessary to consider that Figure 6  
13 corresponded to ploidy levels of plants grown at pH 7.5, in which Fe shoot concentration  
14 presented shorter differences among Si treatments (Figure 2). Further studies should be  
15 done at pH 5.5 and different plant maturity stages. A link between endoploidy and cell  
16 wall modification might also explain the protection against pathogens attributed to Si  
17 addition to plants.

#### 19 4.1.3 Silicon effect over the antioxidant system

20 Reactive Oxygen Species (ROS) concentration produced under stress conditions may  
21 come directly from an electron chain damage caused by an alteration in chloroplast  
22 structure. Van Breusegem et al., (2001) indicated that an excessive level of ROS, which  
23 is related to a high level of MDA, could result in severe cellular damage and leaf chlorosis.  
24 Our SPAD index results (Table 1) showed that, although at pH 5.5 no changes were  
25 observed in response to Si presence in the media, its addition to plants at pH 7.5, that  
26 could be suffering some stress due to pH and the Fe plaque formation, can help to  
27 maintain chloroplast correct structure in cells, and showed an ameliorating effect in SPAD  
28 index loss (Table 1). Cao et al., (2015) reported that thylakoid ultrastructure damage in  
29 the tomato plant due to drought stress could be ameliorated by Si addition to the media.  
30 This agrees with our observations that Si can help to maintain SPAD values in plants  
31 grown at pH 7.5 which confirms its high effectiveness under stressed conditions.

32 The antioxidant activity was tested to evaluate the Si effect over plants without any stress  
33 ongoing at both pHs (Table 2). CAT activity at both pHs shown an important decrease  
34 when Si was added to the media (Table 2). CAT is a heme-containing enzyme so its  
35 activity is highly dependent on Fe status, and Fe concentration in shoots of +Fe+Si plants  
36 was lower than in +Fe-Si (Figure 2), so CAT and Fe concentration were positively  
37 correlated, as expected. Nikolic et al. (2019) also observed in barley that Fe deficiency  
38 reduced CAT activity, and as Fe concentration data obtained in this experiment shown  
39 (Figure 2), Si addition decreased Fe uptake at both pHs tested. So, a reduction in CAT  
40 activity was expected. These authors also observed that Si addition to the Fe deficient

plants restored CAT activity to those of plants grown under Fe sufficient levels. So Fe deficiency and Si addition presented opposite effects. SOD exists in three isoforms containing Fe, Mn, or Cu/Zn, allowing maintenance of enzyme activity in respect to metal availability. Si did not seem to alter this enzymatic activity at both pH tested (Table 2). Total phenolic compounds were only tested at pH 7.5 (Figure 4) and data obtained showed significantly higher amounts of these compounds in the shoot, along with the four samplings, in plants without Si. To take up apoplastic precipitated iron, plants secrete phenolics such as protocatechuic acid (PCA) and caffeic acid, which is the typical response of strategy I plants. Hernandez-Apaolaza et al. (2020) found a significantly higher amount of phenolic compounds in new leaves of +Si cucumber plants grown at pH 7.5 with enough Fe. These authors considered it as a detoxification procedure through non enzymatic antioxidants to minimized ROS produced by the Fe deficiency induced by Si. Ishimaru et al., (2011) also described a phenolics efflux transporter in rice, which may explain the higher Fe concentration in shoots (Figure 2) of plants grown without Si, as Si seemed to decrease phenolic compounds production under Fe sufficiency conditions, therefore diminishing apoplastic Fe remobilization. These decreased phenolics productions might be explained through the apoplastic obstruction by Si, which did not allow Fe to enter the apoplast and therefore no need for phenolics is required. Carrasco-Gil et al. (2018) observed at pH 7.5 an opposite behavior in rice roots, where phenolic compounds production was higher for +Si treated plants. This high production in roots may suggest a response to Fe deficiency caused by Si.

The peroxidation of lipids is considered the most damaging process known to occur in every living organism. Membrane damage is sometimes taken as a single parameter to determine the level of lipid destruction under various stresses. Peroxidation takes place when above-threshold ROS levels are reached, thereby not only directly affecting normal cellular functioning, but also worsening the oxidative stress through the production of lipid-derived radicals (Montillet et al., 2005). At both pHs, MDA concentration in shoots did not present any changes in response to Si under +Fe. Our results were not in agreement with Song et al., (2011), who reported a decrease in MDA and H<sub>2</sub>O<sub>2</sub> concentration in Si-treated plants under control optimal conditions in rice, but were in accordance with Hernandez-Apaolaza et al. (2020) in cucumber who did not show differences in this parameter due to Si at optimal growth conditions.

In general, no differences have been found due to pH in the effect of Si on well feed plants. In both pHs tested Si addition induced a reduction in the Fe concentration in the shoot. However, SPAD index indicated that chloroplast structure and fluorescence were better with Si addition at pH 7.5, but no differences were observed at pH 5.5. This fact could be related to changes in ploidy, but no clear differences were obtained under Fe sufficiency status for this parameter.

## 4.2 Si effect over Fe deficient plants

### 4.2.1 Silicon enhances endoreduplication in leaves of plants ongoing Fe-deficiency

One of the first and most common symptoms of stress in plants is DW loss. Rice is highly susceptible to Fe deficiency (Mori et al., 1991) and insufficient Fe causes a severe impact in shoot and root DW as well as in grain yield. Silicon application to the nutrient solution of rice plants affected the dry weight (DW) in a different manner depending on the pH of the media. Silicon addition prevented the DW loss due to Fe deficiency in rice shoots at pH 7.5 (Figure 1 A, B), while no significant differences have been observed at pH 5.5, although data seemed to follow the same tendency as at pH 7.5. As pH 5.5 is optimal for plant growth, DW loss could not be as high as it was at pH 7.5, which appears as an additional limiting factor, so the Si effect maybe not that evident at pH 5.5. Gonzalo et al. (2013) had tested the Si effect in soybean DW during Fe deficiency at calcareous pH obtaining that 0.5 mM Si contributed to maintaining DW losses due to Fe deficiency, but 1.0 mM did not provide the same beneficial effect. Since as has been said before, soybean is a Si low-accumulator species, the Si effect may be not as visible as it is in rice. At pH 6, when Fe was not present in the media (Bityutskii et al., 2014) or was supplied as Fe(OH)<sub>3</sub> (Pavlovic et al., 2013) had been shown an increase in total DW in Si-treated cucumber plants in comparison to -Si-Fe plants at acidic pH. This fact was not observed by Hernandez-Apaolaza et al. (2020) at Fe deficient cucumber or after plant Fe resupply when the experiments have been done at pH 7.5. It has been suggested that endoreduplication may be a mechanism helping the plants relieving symptoms of unfit soil conditions acting as an adaptive response of plant genomes to environmental stresses (Ceccarelli et al., 2006). In this research, Ceccarelli et al., (2006) showed that salt tolerant varieties of *Sorghum bicolor* had higher ploidy levels in response to NaCl stress. Also, Cookson et al., (2006) proved that in *A. thaliana* the leaf area, which is reduced under drought stress, can be maintained when the stress was mild through cell expansion and increased endoreduplication. Our results showed that during Fe deficiency at pH 7.5 the distribution of cells in different ploidy levels changed, significantly decreasing the lower levels (2C) and increasing the higher ones (4C and 8C) in comparison with the non-deficient plants (Figure 6). This agrees with the previous studies that pointed out how stress causes a decrease in the numbers of cells in leaves, but an increase in ploidy trying to balance and maintain the size. In Fe-deficient treatments, this effect is enhanced by Si, probably showing a higher level of stress when plants were treated with this element as well as/or an increase in the adaptive responses.

The fact that Si has been proven to deposit in the cell wall (He et al., 2013) helping plants to grow stronger may also be related to this ploidy increase. As has been said before, endoploidy onset mutants (where endocycle is repressed) present a weaker cell wall due to the minor number of DNA copies of genes expressing cell wall biosynthesis proteins. This results in a weaker cell wall that will not allow them to rapidly expand cell volume, in contrast with wild-type lines, that higher ploidy levels match a rapid cell's expansion (Bhosale et al., 2019). According to Bhosale et al., (2019), this might explain the observed altered pathogen response of endoreplication onset mutant lines. Modification of the cell wall at sites of pathogen attack is a common response to infection (Clay et al., 2009; Luna et al., 2011), and the inability to do so, or the presence of a weakened cell wall, might explain in part the pathogen susceptibility phenotypes of endocycle onset mutants

(Hamdoun et al., 2016). In brief, our results confirmed that Si addition prevented the DW loss due to Fe-deficiency (Figure 1 A, B) by increasing the percentage of cells in a higher ploidy level at pH 7.5 (Figure 6). Moreover, this increase in ploidy may contribute to fortifying the cell walls of the plant, which may explain the known beneficial effects of Si in erectness, and consequently an increase in the photosynthesis efficiency, as well as in the prevention of biotic stress through Si physical deposition in leaves (Ma et al., 2007). However, this cell wall fortification could play a negative role in Fe entrance into the apoplast (Becker et al., 2020).

#### 4.2.2 Silicon decreases apoplastic iron concentration and increases translocation rate both at pH 5.5 and 7.5

Plants were grown for 14 days with Fe before Fe deficiency implementation. Becker et al., (2020) observed that under Fe sufficiency, rice adventitious roots accumulated higher amounts of Fe when Si was not present in the media. This observation was corroborated in the present work, as it can be seen on day 1 of Fe deficiency, at the two pHs tested (Figure 3B). After 15 days of Fe deficiency, the Fe apoplast deposits were remobilized. Carrasco-Gil et al., (2018) reported similar results in iron concentration in apoplast in response to silicic acid at pH 7.5. However, Becker et al., (2020) did not obtain significant differences due to Si supply in the Fe concentration in the apoplast under Fe shortage. Moreover, these results did not agree with those obtained by Pavlovic et al., (2013) showing that Si addition first increases the apoplast deposits under Fe sufficiency, and after 1 day of Fe-deficiency Si may help to faster remobilization of the Fe in cucumber apoplast, which diminished the apoplastic Fe deposits. Moreover, Pavlovic et al., (2013) and Bityutskii et al., (2014) reported an increase in Fe leaves in cucumber plants after Fe deficiency. Knowing that after 1 day of Fe-deficiency, plants grown with Si in the media showed a 61% lower Fe concentration in apoplast compared with those grown without Si, Fe translocation was studied. In the present work with rice, the translocation rate increased in response to Si both at pH 5.5 and 7.5, which could be related to the lower Fe concentration found in the apoplast. As the plant did not find enough Fe, they onset the Fe deficiency responses to solubilize the available Fe, enhancing root to shoot transport (Hernandez-Apaolaza et al., 2020). Zhang et al., (1991) proved that a reduction in apoplastic Fe when Fe was removed from the media, resulted in an increase of Fe in shoots in wheat plants. Results obtained shown no differences in Fe concentration in shoots at pH 5.5 (Figure 2), which was in agreement with Becker et al., (2020) data. However, at pH 7.5 Fe concentration in -Fe-Si treatment was significantly higher than the ones with Si supply (-Fe+Si), with values near to those obtained for the +Fe+Si treatment (Figure 2). Likewise, phenolic compounds have been described as one of the Fe deficient plants responses to uptake Fe from apoplast in rice (Ishimaru et al., 2011), but our results at pH 7.5 (Figure 4) did not show significant differences due to Si supply to the media.

#### 4.2.3 Silicon prevented the increase in antioxidant system enzymes due to stress

It is well established that various environmental stresses often lead to the increased generation of ROS, where CAT and SOD have been proposed to be important in stress

tolerance and provide the first line of defense against the toxic effects of elevated ROS levels. The detoxification of this species is carried out by the enzymatic antioxidant system and non-enzymatic compounds such as phenolics. Considering +Fe-Si as the reference treatment, Fe deficiency reduced CAT activity either with or without Si supply. However, this reduction was lower when Si was added to the media at pH 5.5. This effect was not observed at pH 7.5 (Table 2). These data were not in accordance with the Fe concentration in shoots (Figure 2), as -Fe-Si plants presented higher Fe concentration than -Fe+Si, especially at pH 7.5. At pH 5.5 Si supply increased CAT activity, although this treatment had lower Fe in shoots than -Fe-Si. Similar findings were obtained by Nikolic et al., (2019) in barley. SOD activity was not significantly changed (Table 2). As mentioned before, CAT and SOD have Fe on their structure, but CAT activity directly depends on the Fe status of the plant, while SOD has three isoforms: Fe, Mn, or Cu/Zn, allowing maintenance of enzyme activity at Fe deficiency plant status. Moreover, total phenolic compounds did not allow to establish differences in the non-enzymatic antioxidant system of plants with or without Si (Figure 4). When Si was added to the media at Fe deficiency conditions (-Fe+Si) and both pHs (5.5 and 7.5), plants presented lower MDA concentration (Figure 5), compared to the Si untreated plants. Plants without Si (-Fe-Si) had higher Fe levels in shoots (Figure 2), but at least at pH 5.5, showed lower CAT activity (Table 2) than -Fe+Si plants, which may explain this higher MDA concentration. This fact suggested a different than a purely physical role of Si in plants. It was reported before that the addition of Si in tomato reduced the ROS production and the MDA accumulation (Cao et al., 2015), which was in accordance with results present in this work. But Hernandez-Apaolaza et al. (2020) did not observe MDA differences in old leaves (grown before Fe deficiency) of -Fe cucumber plants due to the Si supply, although new leaves (grown under Fe deficiency) observed higher MDA concentration in plants fed with Si. It has been said before that ROS production may come directly from an electron chain damage produced by alterations in chloroplast structure. This agreed with the higher SPAD values recorded at pH 7.5 with Si supply (Table 1). At pH 5.5 no differences in SPAD data were obtained due to Si addition to the media. This high degree of chloroplast organization can explain the decrease in MDA in Fe deficiency due to Si addition because as Liang (1998) described maintaining chloroplast ultrastructure grana will reduce ROS production. Moreover, Cao et al. (2015) reported that the addition of exogenous Si under drought stress reduced the H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup> production, and the MDA accumulation, which was in accordance with results present in this work, and also showed no changes in SOD, and other enzymatic activities in tomato chloroplast. The maintenance of chloroplast structure could closely be related to the higher ploidy level observed in -Fe+Si plants (Figure 6) which as discussed before could reinforce the chloroplast ultrastructure, contributing to a higher SPAD observed in +Si treated plants (Table 1). So, the Si effect could not be only physical, but the physical observed effects could be controlled by ploidy changes in the nuclei.

#### 4.2.4. Timing of Si addition under Fe deficiency

To evaluate if the timing in Si addition could have any influence on plant recovery from Fe deficiency, which will also mean significant savings in fertilizers and increases in crop value, four treatments under Fe deficiency were evaluated at pH 5.5. The four treatments were: two in which plants were grown +Si during the preculture, and then each of them grown with or without Si when transferred to -Fe (+Si/-Fe+Si and +Si/-Fe-Si) and the other two grown -Si in the preculture and with or without Si during -Fe (-Si/-Fe+Si and -Si/-Fe-Si). After 10 days of -Fe, no differences in dry weight were obtained (Table 3). In the previous experiment (Figure 1 A, B) DW differences were obtained after 7 days of Fe shortage, but day 10 maybe was too early to obtain significant differences. In respect to Fe concentration in the shoot (Table 3) the higher values were observed for plants that did not have Si during the Fe deficiency period (+Si/-Fe-Si and -Si/-Fe-Si), independently of the Si presence during +Fe preculture. Plants that did have Si at both the +Fe preculture and the -Fe periods (+Si/-Fe+Si) showed higher Fe concentration levels than plants that only had Si at the deficiency phase (-Si/-Fe+Si). These results supported the data obtained at the previous experiment in which -Fe-Si treatment showed higher Fe concentration values than -Fe+Si (Figure 2). In this case -Fe-Si treatment corresponded to -Si/-Fe-Si and -Fe+Si to +Si/-Fe+Si. SPAD index (Table 3) at day 10 and 17 reflected that Si addition did not contribute to increasing this index. The higher SPAD index values were observed for plants without Si addition during the entire experiment (-Si/-Fe-Si) or without Si at the Fe deficiency period (+Si/-Fe-Si). Rice plants with Si addition only at the Fe deficiency period (-Si/-Fe+Si) showed higher SPAD data than plants grown all the time with Si in the NS. These data were in accordance with Becker et al., (2020), who only observed SPAD differences in rice plants suffering from Fe deficiency, but not in plants under Fe sufficiency or at high concentration levels. Fe act as a co-factor for proteins involved in photosynthesis and respiration (Broadley et al., 2012), and any damage in these processes would cause the liberation of reactive oxygen species (ROS) including mainly non-radical molecules (da Silva et al., 2008). According to this, Hernandez-Apaolaza., (2020) found a positive correlation between Fe uptake reduction by the Si addition to the NS and an increased in ROS production in cucumber. Strikingly, in this work, plants without Si addition at the Fe deficiency period (+Si/-Fe-Si and -Si/-Fe-Si), which had higher Fe concentration levels in shoots, also presented higher ROS levels, independently of the Si addition or not during the +Fe preculture (Table 3). So Si seemed to reduce ROS production. Nikolic et al., (2019) obtained a similar ROS concentration attenuation in barley due to Si supply. Data obtained in this and other works, supported that Si addition reduced Fe in the apoplast and the shoot of rice plants, which will necessarily lead to an increment in ROS concentration, but this fact was not in accordance with results presented in Table 3, so Si addition may play a crucial role, especially in gramineous plants, which accumulated Si is much higher amounts than other plants such dicots. This could be related to the adaptative responses devoted to high ploidy levels in Si treated plants especially under stress (Fe deficiency) conditions (-Fe+Si) (Figure 6). As discussed before, Ceccarelli et al., (2006) and other authors recognized that endoreduplication could be an evolutive mechanism for tolerate or controlled stress conditions and that genomic rearrangement may be part of the adaptive response of plant genomes to environmental stresses. Our data showed that Si induced higher ploidy levels,

as well as the apoplastic Fe obstruction. The high ploidy levels are in accordance with cell fortification, which explains the higher chloroplast integrity and fluorescence with Si addition, clearly a beneficial effect. But also explain the Casparian band fortification that conducted to the Fe shortage caused by obstruction of apoplast, not beneficial at all. These ploidy levels rearrangements may contribute to a plant memory effect, that prepare plants to cope with future stresses, as demonstrated by Hernandez-Apaolaza., (2020). Fe deficiency itself could promote endoreplication (Figure 6), and Si, as increasing the Fe shortage, could reinforce this effect. But Si effect is not completely explained through the apoplastic Fe obstruction but also chloroplast structure maintenance should be addressed (and maybe other effects) to give a complete overview of the beneficial contribution of Si in plant nutrition.

## 5. Conclusions

Acidic and calcareous pH promoted a reduction in shoot Fe concentration in response to silicon addition under both Fe sufficiency and deficiency status, but, in general, at pH 7.5 stress was favored, as is not the optimal pH for rice growth. When Fe deficiency was imposed on plants grown at pH 7.5, the silicon beneficial effect became clearer by significantly increasing dry weight and SPAD index. Moreover, Fe deficiency increased the percentage of cells in the higher ploidy levels compared to Fe sufficient plants and, Si addition enhanced this endoreplication. This effect is often suggested as a strategy to maintain the size of the plant under mild stress and fortifying the cell walls, making the plant more resistant and contributing to plant erectness, which will promote photosynthesis and other antioxidant responses, either by diminishing ROS production or by activating the antioxidant system. Likewise, the fortification of the cell walls reinforced the apoplastic obstruction theory, supported by the lower Fe concentration levels in apoplast and shoot of rice plants at both pHs with the Si supply. The genome rearrangement could potentiate a memory effect to cope with future stresses. Under Fe sufficiency, no effect has been detected in the percentage of cells with higher ploidy levels.

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