
Title: Physiological profile of CAX1a TILLING mutants of *Brassica rapa* exposed to different calcium doses

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**Title Page**

**Title:** Physiological profile of CAX1a TILLING mutants of *Brassica rapa* exposed to different calcium doses

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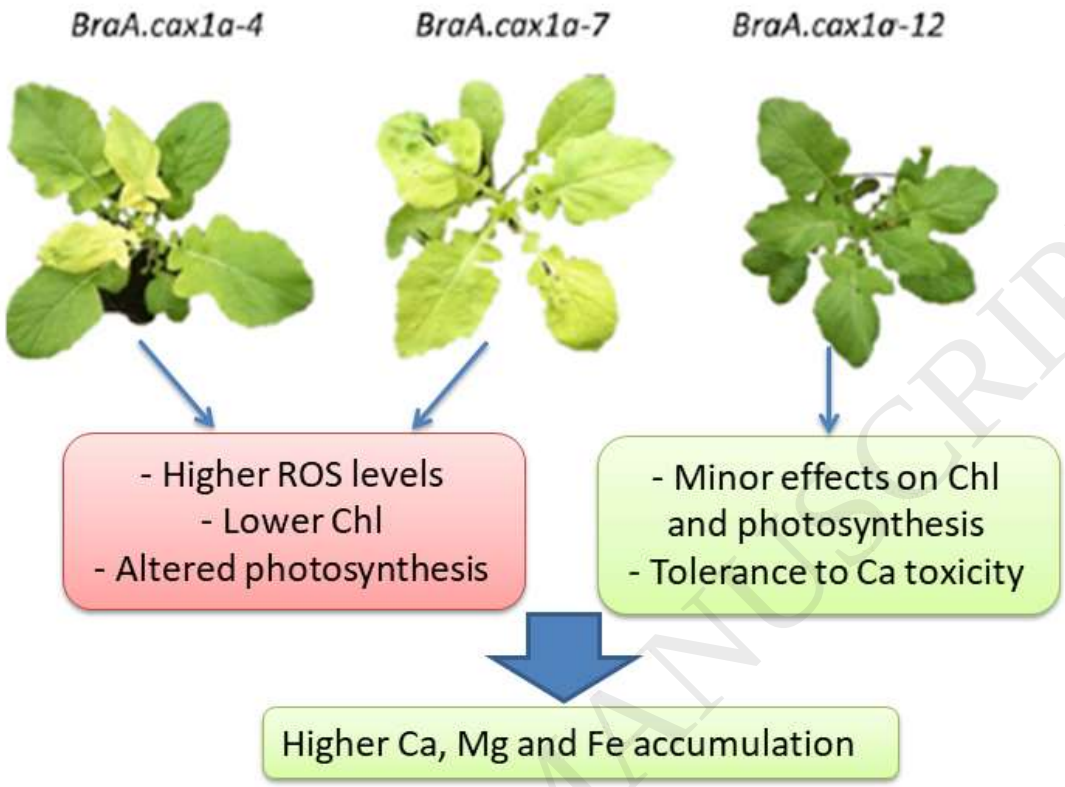
**Graphical abstract**




**Highlights**

- *BraA.cax1a* mutants appear to be unhelpful to better Ca-deficiency tolerance
- *BraA.cax1a-4* and *BraA.cax1a-7* showed stress symptoms in comparison to R-o-18
- Mutants are useful for biofortification as accumulated more Ca, Mg and Fe in leaves
- *BraA.cax1a-12* mutation allows a better growth under high Ca conditions
- *BraA.cax1a-12* could be useful in biofortification and phytoremediation programs

**Abstract**

Calcium (Ca) is an essential macronutrient for plants and its homeostasis is basic for many processes in plants. Therefore, both Ca deficiency and toxicity constitute potential issues for crops. CAX1 transporter is a potential target to obtain plants with better Ca

homeostasis and higher Ca concentration in edible parts. Three *Brassica rapa* mutants for CAX1 were obtained through TILLING. The objective of this work is to evaluate the growth, physiological state and nutrients concentration of these mutants grown with different Ca doses. The mutants and the parental line were grown under low, control and high Ca doses and parameters related to their oxidative stress, photosynthetic performance and nutrients concentration were determined. *BraA.cax1a-4* and *BraA.cax1a-7* mutants presented lower total Chl, an altered photosynthesis performance and higher ROS levels. *BraA.cax1a-12* mutant grew better under high Ca conditions. All mutants accumulated more Ca and Mg in leaves under control and high Ca doses and accumulated more Fe regardless the Ca dose. The results obtained point to *BraA.cax1a-12* as a potential candidate for biofortification with Fe, Ca and Mg since it accumulate higher concentrations of these elements, do not present an altered growth and is able to tolerate higher Ca doses.

*Abbreviations:* APX, ascorbate peroxidase; CaUpE, Ca uptake efficiency; CaUtE, Ca utilisation efficiency; CAX, cation/$H^+$ exchanger; Chl, chlorophyll; DC, distribution coefficient; GMOs, genetically modified organisms; LOX, lipoxygenase; MDA, malondialdehyde; ROS, reactive oxygen species; TILLING, targeting induced local lesions in genomes.

*Key words: Brassica rapa*, calcium, CAX1, Chl fluorescence, ionome, oxidative stress, physiological profile, TILLING.

## 1. Introduction

Calcium (Ca) is an essential macronutrient for plants that is present in membranes and cell walls playing a basic structure role, and in the cytosol being crucial in cell signalling

processes [1]. For this reason, Ca deficiency produces serious alterations in plants and it may cost losses in crop productions. Ca deficiency can occur even whether there is an adequate supply due to low redistribution or limitations in its transport [1, 2]. On the other side, Ca toxicity also reduces the plant growth rate and produce damages due to the formation of Ca oxalate crystals. Cytosolic Ca concentration must be maintained at submicromolar levels in the resting cell in order to allow rapid increases for cell signalling, which can be jeopardized by Ca toxicity [1]. Ca fluxes are also necessary in cell guards for stomata closure, so an elevated Ca concentration may promote this closure and thereby a reduction in internal $CO_2$ concentration and a lower photosynthesis rate [2]. Both Ca deficiency and toxicity, constitute abiotic stresses that interfere with photosynthesis impairing electron transport, decreasing photosystems efficiency, reducing photosynthetic pigments, and promoting the formation of reactive oxygen species (ROS) [3]. In turn, ROS damage the photosynthetic apparatus through the disruption of thylakoid structures, inhibition of chloroplastic enzymes, and blocking PSII repair process [4].

Plants prevent Ca disorders through the regulation of plant cation/$H^+$ exchangers (CAXs) [2]. CAXs are a family of Ca/H antiporters located on plasma and organelle membranes including vacuoles. Together with Ca-ATPases, CAXs are responsible of Ca homeostasis and Ca removal from the cytosol to generate different Ca profiles to respond environmental cues or in signalling processes [5]. CAXs are involved in several important aspects of plant growth and development playing a role in stomatal conductance and in pH regulation [6]. There is a strong correlation between CAXs expression and Ca accumulation. Thus, *Brassica rapa* plants have an enhanced Ca accumulation in palisade mesophyll cells [7] where CAX expression is higher [5]. Among CAXs, CAX1 is one of

the antiporters with greater Ca/H activity [6]. *CAX1* was identified as an expression quantitative trait loci that is affected by external Ca concentration in *B. rapa* [8]. Therefore, CAX1 is a potential target to obtain plants with better Ca homeostasis or with higher Ca concentrations in edible parts [6]. This fact could be useful since Ca, as well as iron (Fe) and magnesium (Mg) are essential elements for human diet. Stein [9] reported that two-thirds of the human population have a deficient diet of at least one of these elements, increasing the risk of certain diseases. Crop nutrients can be improved through two ways: providing an adequate nutrient supply in the culture medium, considering interaction between nutrients, and the other way is through crop breeding (traditional breeding and by the use of genetically modified organisms (GMOs)) [10]. Experiments have already been carried out in this regard through the expression of *Arabidopsis thaliana CAX1* (*AtCAX1*) that increased Ca concentration in vegetables such as carrots [11], and lettuce [12]. These vegetables are GMOs, i.e. they were obtained by genetic engineering techniques. A possible alternative to the use of GMOs would be the generation of mutants with a modification in CAX1 activity affecting its self-regulation or activity.

A successful approach to obtain CAX1 variants is TILLING (Targeting Induced Local Lesions In Genomes). TILLING make possible the generation and the study of allelic series of mutations in order to evaluate their effects on gene expression and in protein structure and function [8]. TILLING was used to generate and identify three missense mutations in *B. rapa* ssp. trilocularis 'R-o-18' Ca transporter; *BraA.CAX1a*: *BraA.cax1a-4* (A-to-T change at amino acid 77), *BraA.cax1a-7* (R-to-K change at amino acid 44), and *BraA.cax1a-12* (P-to-S change at amino acid 56) [13]. These mutations affect AAs upstream of the N-terminal autoinhibitory domain, but that could change protein

conformation and thereby affecting CAX1 function or activity [8]. The genotyping and characterization of these mutants has been started. *BraA.cax1a-4* and *BraA.cax1a-7* lines presented paler/yellow leaves than parental line R-o-18 and in *BraA.cax1a-7* and *BraA.cax1a-12* lines was detected a variation in Ca concentration with respect their segregant wild types [8]. The species chosen for this study presents a rapid cycle, is self-compatible and include vegetable crops such as Chinese cabbage, turnip and some oil-seed crops [13]. Therefore, the working hypothesis to test is that CAX1a mutations will cause changes in growth, physiological state and nutrients accumulation and these changes will be influenced by Ca dose applied. The results could be useful to make an initial evaluation in order to improve *B. rapa* and other related crop species.

## 2. Material and methods

### *2.1. Plant material, growth conditions, and treatments*

Three *B. rapa* ssp. trilocularis 'R-o-18' mutants (*BraA.CAX1a*: *BraA.cax1a-4*, *BraA.cax1a-7*, and *BraA.cax1a-12*) and the parent line R-o-18 were employed as plant material for the experiment [13]. Seeds were sown on filter paper moistened with milli-Q water (18.2 MV cm) in 9 cm Petri dishes. The dishes were sealed with plastic film, and incubated in the dark for 1 d at 4ºC before transferring to pots filled with vermiculite. These pots where placed in a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 22/18ºC (day/night) and 14/10-h photoperiod at a photosynthetic photon flux density of 350 µmol $m^{-2}$ $s^{-1}$ (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Throughout the experiment the plants received a growth solution composed of 4 mM $KNO_3$, 3 mM $NH_4NO_3$, 2 mM $MgSO_4 \cdot 7\ H_2O$, 6 mM $KH_2PO_4$, 1 mM $NaH_2PO_4 \cdot 2\ H_2O$, 2 µM $MnCl_2 \cdot 4\ H_2O$, 0.25 µM $CuSO_4 \cdot 5\ H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2\ H_2O$, 5 µM Fe-

chelate (Sequestrene; 138FeG100) and 10 µM $H_3BO_3$. This solution, with a pH of 5.5–6.0, was renewed every three days.

*2.2. Experimental design and treatments*

Treatments were started 30 days after germination and were kept for 21 days. Plants were grown with different Ca doses: 0.4 mM of $CaCl_2$ as low Ca dose, 4 mM of $CaCl_2$ as control Ca dose, and 40 mM of $CaCl_2$ as high Ca dose. The two factors involved in the experiment were the Ca dose applied (D) and the mutant employed (M). The experimental design consisted of randomized complete block with 12 treatments, arranged in individual benches with eight plants per treatment and three replications each.

*2.3. Plant sampling*

Fully expanded leaves were washed with distilled water, dried on filter paper, and weighed for fresh weight (FW). Half of the leaves from each treatment were frozen at −30ºC for later biochemical assays and the other half of the plant material was lyophilized to measure dry weight (DW) and nutrient concentrations.

*2.4. Analysis of mineral nutrients*

Sulphur (S), phosphorus (P), potassium (K), Ca, Mg, Fe, copper (Cu), manganese (Mn), zinc (Zn) and boron (B) were determined after a sample of 150 mg dry material was subjected to a process of mineralization by wet digestion [14]. To carry out this assay, dry leaves were ground and mineralized with a mixture of nitric acid ($HNO_3$)/perchloric acid ($HClO_4$) (v/v) and $H_2O_2$ at 30%. From the resulting mineralization, and after the addition of 20 ml of mili-Q $H_2O$, elements concentrations were determined by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA). Internal standards

included Sc (50 ng $ml^{-1}$) and Ir (5 ng $ml^{-1}$) in 2% TAG $HNO_3$. External multi-element calibration standards (Claritas-PPT grade CLMS-2, SPEX Certi-Prep Ltd, Stanmore, Middlesex, UK) included Al, As, Ba, Bi, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr,U, V, and Zn, in the range 0–100 µg $l^{-1}$, and Ca, Mg, K, and Na in the range 0–100 mg $l^{-1}$.

Total nitrogen (N) concentration was calculated as the sum of $NO_3^-$ and total reduced N. $NO_3^-$ was analysed from an aqueous extraction of 0.1 g of DW in 10 ml of Millipore-filtered water. A 100 µl aliquot was taken and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the $NO_3^-$ concentration by spectrophotometry as performed by Cataldo et al. [15]. For total reduced N determination, a sample of 0.1 g DW was digested with sulfuric acid and $H_2O_2$ [14]. After dilution with deionized water, a 1-ml aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 µM sodium phosphate, and 5.4% w/v sodium hydroxide), 15%/0.03% (w/v) sodium silicate/sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37ºC for 15 min, and total reduced N was measured by spectrophotometry using spectrophotometer (Infinite 200 Nanoquant, Tecan, Switzerland) according Baethgen and Alley [16].

*2.5. Ca efficiency parameters (CaUE) and distribution coefficient (DC)*

CaUE parameters were calculated as follow:

Ca uptake efficiency (CaUpE) was calculated as total Ca accumulation divided by root DW (mg Ca $g^{-1}$ RDW) [17].

Ca utilisation efficiency (CaUtE) was calculated as leaf tissue DW divided by Ca concentration ($g^2$ LDW $mg^{-1}$ Ca) [17].

Distribution coefficient (DC) was calculated as the quotient between Ca concentration in leaves and Ca concentration in roots [18].

*2.6. Pigment concentrations and SPAD value*

Total chlorophyll (Chl) and carotenoid were extracted in methanol and centrifuged at 5000 × *g* for 5 min. Thereafter, the absorbance of the supernatant was measured at 664, 648, and 470 nm. The chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids were estimated by using the equation of Lichtenthaler [19]. Total Chl was calculated as the sum of Chl a and Chl b.

SPAD value was measured using meter SPAD-502 (Konica Minolta Sensing Inc., Japan). Three measurements were made in each leaf and average was calculated.

*2.7. Chl a fluorescence analysis*

Plants were adapted to dark for 30 min before measurements using a leaf clip holder that was allocated in each fully expanded leaf. Chl a fluorescence kinetics was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK); the OJIP transients were induced by red light (650 nm) with 3000 µmol photons $m^{-2}s^{-1}$ light intensity and recorded by the instrument. OJIP transients data were analysed using the JIP-test [20]. Measurements were conducted with six plants of fully expanded leaves at midstem position. Parameters employed to study the energy flow and photosynthetic activities by JIP-test were: initial fluorescence (Fo), maximum

fluorescence (Fm), variable fluorescence (Fv = Fm – Fo), maximum quantum yield for primary photochemistry ($\Phi_{Po}$ = Fv/Fm), performance index ($PI_{ABS}$), proportion of active reaction centres (RCs) (RC/ABS), efficiency/probability with which a PSII trapped electron is transferred from $Q_A$ to $Q_B$ ($\Psi_o$), maximum quantum yield of electron transport ($\Phi_{Eo}$ = ETo/ABS) and fluorescence value at 300 µs (K step) [20].

*2.8. Malondialdehyde (MDA), $O_2^{\cdot -}$, and $H_2O_2$ concentrations*

Determination of $O_2^{\cdot -}$ in leaf extracts was based on the ability to reduce nitroblue tetrazolium (NBT) [21]. Absorbance was measured at 580 nm and the $O_2^{\cdot -}$ concentration was expressed as µg $g^{-1}$ DW.

For $H_2O_2$ determination leaf samples were extracted with cold acetone and the intensity of yellow colour of the supernatant was measured at 415 nm. The result of $H_2O_2$ concentration was expressed as µg $g^{-1}$ DW [21].

For MDA assay, 0.1 g of leaves was homogenized in 1 ml of a 0.25% thiobarbituric acid solution in 10% trichloroacetic acid. The mixture was heated at 95 °C for 30 min and then cooled in an ice bath. Subsequently samples were centrifuged at 9500 rpm for 10 min. MDA concentration in the supernatant was quantified by measuring absorbance at 532 nm. The non-specific absorbance value at 600 nm was obtained to correct the turbidity. MDA concentration was calculated using 155 $mM^{-1}$ $cm^{-1}$ as extinction coefficient [22].

*2.9. Lipoxygenase (LOX) and ascorbate peroxidase (APX) activities*

LOX activity in leaf extracts was measured according to Minguez-Mosquera et al. [23] using 50 mM K–phosphate buffer (pH 6.0) containing 5 mM EDTA and 1% PVP for

extraction. LOX activity was calculated following the rise in the extinction at $A_{234}$ using an extinction coefficient of 25,000 $M^{-1}cm^{-1}$.

APX activity in leaf extracts was determined by registering the absorbance change at 290 nm for 3 min of a reaction mixture containing 100 mM K-phosphate buffer (pH 7.5), 0.5 mM ascorbic acid, and 0.2 mM $H_2O_2$ [24].

The protein concentration of extracts was determined according to the method of Bradford [25], using bovine-serum albumin as the standard.

All spectrophotometry determinations were carried out employing spectrophotometer (Infinite 200 Nanoquant, Tecan, Switzerland).

*2.10. Statistical analysis*

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics Centurion XVI program. A two-tailed ANOVA was applied to ascertain whether the doses of Zn, the species, or the interaction (D * M) significantly affected the results. Means were compared by Fisher's least significant differences (LSD). The significance levels for both analyses were expressed as * $P<0.05$, ** $P<0.01$, *** $P<0.001$, or NS (not significant).

## 3. Results and discussion

*3.1. Plant biomass and Ca concentration*

The cause-effect link between plant biomass and Ca homeostasis may be explained through modifications in CAX1 activity. For instance, studies with *A. thaliana cax1*

knockout mutants showed an altered plant growth and a lower foliar Ca concentration [5, 6]. In comparison, although the experiments employing plants with higher CAX1 activity expressing *AtCAX1* showed more shoot Ca, biomass can be reduced e.g. in tobacco [26] or not altered e.g. in carrot [11] and lettuce [12]. The results in the present work showed that *BraA.cax1a* mutations did not negatively affect to foliar biomass regardless of the Ca dose applied. However, mutants roots biomass were lower at low Ca dose and in *BraA.cax1a-4* and *BraA.cax1a-7* grown under high Ca dose (Table 1), suggesting that mutant roots are more sensitive to Ca concentration changes in the medium. Focusing on high Ca dose, *BraA.cax1a-12* mutation allowed a better growth with an 82% more leaf DW than R-o-18 plants. In the rest of lines, the application of 40 mM $CaCl_2$ caused Ca toxicity symptoms since plants showed lower foliar biomass than in control conditions (Table 1). These results coincides with the results observed by Blasco et al. [27] in which R-o-18 *B. rapa* plants also presented lower DW under high Ca dose than under a low dose. The biomass reduction may be due to Ca toxicity produces damages by the formation of Ca-oxalate crystals and interfere with Ca fluxes needed for stomata closure, causing a lower photosynthesis rate, and thereby reducing plant growth [1, 2].

All *BraA.cax1a* mutations allowed a higher Ca accumulation in leaves but only when plants were grown under control and high Ca doses (Table 1). Graham et al. [8] grew *BraA.cax1a* mutants with compost in a glasshouse and also observed higher Ca concentration in *BraA.cax1a-12* mutants but not in *BraA.cax1a-4* and in *BraA.cax1a-7* mutants. These differences may be due to the different culture conditions employed. In the present experiment, under low Ca dose, mutants showed higher Ca accumulation in roots (Table 1) and a better Ca uptake capacity (Fig. 1A), although this did not result in a higher foliar Ca concentration (Table 1) since they presented a lower DC value (Fig. 1C).

Therefore, mutations could enhance Ca uptake in roots but this extra Ca is not efficiently transported to the shoot. *BraA.cax1a-7* mutation reduced foliar Ca concentration but it did not decrease its foliar DW (Table 1) because it presented a higher CaUtE (Fig. 1B), i.e. this mutant may be able to improve CaUE under low Ca supply. Under control Ca supply mutant plants increased shoot Ca concentration from 8% to 16% in comparison to R-o-18 plants (Table 1) and this is due to the higher CaUpE (Fig. 1A) and higher DC values (Fig. 1C). On the other hand, *BraA.cax1a* mutations enhanced Ca uptake when high Ca dose was applied (Fig. 1A) and a great increase in its DC and foliar Ca concentration was observed (Fig. 1C; Table 1). Nevertheless, this increase in Ca concentration was moderate in *BraA.cax1a-12* (Table 1) due to a lower CaUpE (Fig. 1A) and a better CaUtE (94% higher than R-o-18; Fig. 1B) in comparison with the other mutants. This could mean that *BraA.cax1a-12* may limit Ca uptake and accumulation in order to prevent Ca toxicity which resulted in an increased leaf biomass (Table 1). Besides, *BraA.cax1a-12* CAX1 might store the Ca excess more efficiently than other mutants as it was proved that CAX1 can help to avoid Ca toxicity by storing it in vacuoles [6].

*3.2. Pigments concentration and photosynthetic performance*

Under stress, chlorophyll content usually decreases and one method to indirectly estimate it is through SPAD meters that determine the greenness and interaction of thylakoid chlorophyll with incident light [28]. In this work, *BraA.cax1a-4* and especially *BraA.cax1a-7* mutants presented lower SPAD and total Chls values in all Ca treatments in comparison to R-o-18 plants. However, total Chls concentration was slightly affected in *BraA.cax1a-12* (Table 2) but SPAD value was not altered (table 2). In agreement with our results, Graham et al. [8] observed leaf chlorosis in *BraA.cax1a-4* and *BraA.cax1a-7*

mutants and Hirschi [26] in tobacco plant expressing *AtCAX1*. Therefore, it appears that *BraA.cax1a* mutations caused alterations in Ca homeostasis that could affect to Chls biosynthesis, chloroplast ultrastructure or photo deterioration [29]. Meanwhile Chl a is mainly associated with RCs and core antenna proteins of PSII, Chl b is mainly present in light-harvesting complexes (LHCII). Thus, Chl a/b ratio is a suitable indicator of RC/ LHCII proportion. When Chl a/b is higher, this might indicate a conversion of Chl b to Chl a in order to maintain Chl a levels and thereby active RCs [30]. This could be occurring in mutants because they presented higher Chl a/b ratios (Table 2) suggesting a role of Ca homeostasis in Chl a/b ratio adjustment. Another important type of chloroplastic pigments are carotenoids. They are components of thylakoids that play a role as accessory light-harvesting pigments helping in the dissipation of excess energy [31]. Our results showed that *BraA.cax1a-7* mutation reduced carotenoids content especially under low and control Ca dose (Table 2), which might affect to photosynthetic process in this mutant.

Chl a fluorescence reflects the photosynthesis status and the effects on this due to stress. When plant metabolism is disturbed, fluorescence dissipates redundant energy in order to avoid damage. Fluorescence can be quantified using the JIP test (data provided by fluorimeter) that offers parameters indicating the in vivo PS II performance [20]. According JIP test results, under all Ca doses *BraA.cax1a-12* and R-o-18 plants presented similar values of fluorescence parameters except for $PI_{ABS}$ and $\Psi o$, $\Phi_{Eo}$ under control and high Ca conditions that were higher in R-o-18 plants (Table 3). These results indicated that *BraA.cax1a-12* mutation only caused slight effects on photosynthesis. On the other hand, under low and control Ca dose *BraA.cax1a-4* and *BraA.cax1a-7* presented similar parameters and their values indicate an altered photosynthetic performance (Table 3). Under high Ca dose *BraA.cax1a-7* showed lower Fv/Fm, $PI_{ABS}$, RC/ABS, and $\Phi_{Eo}$ and

higher Fo, Fm, and K step values. $PI_{ABS}$ shows the plant capacity to resist external pressures and is a combined index that depends on RC/ABS, Fv/Fm, and $\Psi_o$ [20]. Among these factors, RC/ABS was significantly reduced in *BraA.cax1a-4* and *BraA.cax1a-7* mutants (Fig. 2). RC/ABS determines how much energy is emitted as fluorescence and how much is usable for photosynthesis [20]. Therefore, the results for Fo, Fv/Fm and RC/ABS in *BraA.cax1a-4* and *BraA.cax1a-7* suggest that an impairment in Ca homeostasis caused a loss of active RCs which reduce the energy that reach to the PS II [20]. Regarding $\Psi_o$ and $\Phi_{Eo}$ parameters in *BraA.cax1a* mutants, their reduced values indicate a decrease in the electron flux through the PSII that could be caused by a decrease in quinones receptors or an accumulation of reduced quinones as suggested also by the higher Fo values (Fig. 2) [32]. Another parameter that usually increases under stress is K step. The higher value observed in *BraA.cax1a-4* and *BraA.cax1a-7* (Fig. 2) could indicate an uncoupling between oxygen-evolving complex (OEC) and the rest of FSII [20]. Ca is part of $Mn_4$-Ca cluster in OEC, [33], so in these mutants, an altered Ca homeostasis might lead to the lack of Ca for OEC and thereby affecting water oxidation and increasing K step (Table 3).

Lang et al. [34] observed that the reduction in Chl a and carotenoids concentration affected negatively photosynthesis and promoted a Chl fluorescence rise. This was observed especially in *BraA.cax1a-7* grown under high Ca conditions (Table 2 and Fig. 2C). In this case, light energy is emitted as fluorescence instead of being absorbed in RCs and light-harvesting complexes could be reorganized in order to maximize the dissipation of excess energy produced by stress [34]. Tan et al. [32] observed that exogenous Ca application can protect photosynthesis against stress improving $\Psi_o$ and $PI_{ABS}$ parameters. Ca helps to repair the PSII complex, maintain photosynthesis and activates antioxidant

enzymes. The latter is important since ROS cause photodamage to PSII and inhibits its reparation [4]. Besides, Ca can directly active antioxidant enzymes to eliminate ROS dissipating excess energy and preventing photodamage [32].

*3.3. Oxidative stress*

In the present study MDA, $O_2^-$, $H_2O_2$ concentrations and LOX activity were analysed as oxidative-stress indicators. The higher MDA and $O_2^-$ concentrations and LOX activity in *BraA.cax1a-4* and *BraA.cax1a-7* plants indicated that they were the mutants that showed higher oxidative stress (Table 4). In these mutant plants, ROS could react especially with unsaturated fatty acids producing MDA as a subproduct of lipid peroxidation. Likewise, this could be strengthened by the increase in LOX activity that, in turn, promotes $O_2^-$ formation [35]. Pokotilo et al. [36] showed that an altered *CAX1* expression can lead to a higher oxidative stress as observed in *cax1* tobacco mutants that presented higher MDA levels and lower activities of antioxidant enzymes. Therefore the higher oxidative stress in *BraA.cax1a-4* and *BraA.cax1a-7* probably was caused by alterations in Ca homeostasis and because Ca affects ROS concentration since both are involved in cell signalling to stress [37]. On the other hand, Gururani et al. [4] observed that ROS adversely affect the photosynthetic system and total Chl reduction can be an indicator of damage caused by ROS. Accordingly, the higher oxidative stress observed in *BraA.cax1a-4* and *BraA.cax1a-7* mutants (Table 4) might be responsible for their altered photosynthetic performance (Fig. 2). To prevent the oxidative damage, antioxidant compounds in chloroplasts such as carotenoids prevent and eliminate ROS accumulation [31]. Thus, in our study, the results for $O_2^-$ (Table 4), total Chls and carotenoids (Table 2) suggested that *BraA.cax1a-7* mutant presented the higher oxidative stress and lower antioxidant capacity under low and control conditions. In contrast, under high Ca conditions, *BraA.cax1a-7*

registered lower MDA and LOX levels (Table 4). Hence, in this mutant high Ca dose might not affect to the antioxidant machinery that protect against MDA formation.

Plants possess mechanisms to eliminate ROS through antioxidant enzymes such as APX activity that is an efficient $H_2O_2$ scavenger [38]. In the present work, under low and control Ca doses mutants registered lower $H_2O_2$ values and higher APX activity in comparison to R-o-18. Blasco et al. [27] in *B. rapa* plants submitted to Ca toxicity observed also observed an increase in APX activity. These results suggest that APX is an important enzyme in ROS elimination in this species. On the other hand, some studies proved that Ca applied at the proper dose can be beneficial to reduce oxidative stress [32]. Likewise, in the present study *BraA.cax1a* mutations produced an improved $H_2O_2$ detoxification through APX enzyme. One exception is *BraA.cax1a-7* plants submitted to high Ca dose where the higher APX activity was not efficient enough to reduce $H_2O_2$ concentration because its concentration was higher than in R-o-18 plants. Nevertheless *BraA.cax1a-4* and *BraA.cax1a-7* presented higher $O_2^-$ levels than R-o-18 (Table 4) and this could explain the altered photosynthesis performance (Fig. 2) since $O_2^-$ is the ROS with the highest oxidant power and thereby more dangerous than $H_2O_2$ [35].

*3.4. Nutrient concentrations*

Several studies carried out on plants with modified *CAX1* expression reported altered accumulations of mineral nutrients [5, 26]. In the present study, *BraA.cax1a-12* mutation caused a higher N and S accumulations regardless the Ca dose applied (Fig. 3A-B). N has a direct relationship with plant biomass [39] and S is an important component of glutathione, hormones, and certain amino acids [40]. Therefore, this higher N and S concentrations may help *BraA.cax1a-12* to maintain growth, especially when high Ca

doses are applied (Table 1). In addition, S deficiency limits yield in crops all over the world [40], so *BraA.cax1a-12* mutation could be useful under S deficiency conditions (Fig. 3C). With respect P concentration, only slight differences were found between mutants and R-o-18 plants, highlighting a higher P accumulation in mutants under control Ca dose (Fig. 3C). This may be due to the relationship between CAX1 activity and P absorption observed by Liu et al. [41]. Regarding Mg, its concentration increased when both low and high Ca dose were applied in mutant plants in comparison to control Ca dose; whereas in comparison to R-o-18 all mutants accumulated more Mg under control and high Ca doses (Fig. 3E). In previous experiments in *B. rapa* plants authors observed the well-known negative interaction between Ca and Mg [7, 27]. Nevertheless, in the present work this relation was not observed in *BraA.cax1a* mutants when high Ca dose was applied (Fig. 3E). One possibility is that the extra Ca is being stored in vacuoles. The higher Mg accumulation may be beneficial to counteract the higher Ca concentration that could be toxic especially in high Ca treatment.

Micronutrients are required in lower concentrations but play a key role in numerous processes in plants [42]. In the present study, *BraA.cax1a* mutations enhanced Fe accumulation disregarding the Ca dose applied (Fig. 3F) while the accumulation of Cu, Mn and B generally decreased in comparison to R-o-18 plants (Fig. 3G-J). A higher Fe accumulation might explain the higher APX activity in *BraA.cax1a* mutants (Table 4) since Vansuyt et al. [43] proved that this element is necessary for APX activation. On the other hand, the antagonistic relationship between Ca and B [44] might be boosted in *BraA.cax1a* mutants and reduce B accumulation in leaves (Fig. 3J). Finally, regarding Mn, *BraA.cax1a-4* and *BraA.cax1a-12* reduced its accumulation in leaves (Fig. 3H). Conn et al. [5] observed that besides Ca, CAX1 can also transport Mn. Therefore, the

CAX1 transporter of *BraA.cax1a-4* and *BraA.cax1a-12* mutants might have a lower Mn transport capacity that may limit its accumulation in leaves.

In addition to the importance of nutrients for the normal plant growth and development, it is interesting to study its accumulation from the human nutrition point of view [10]. Thus, the results suggest that *BraA.cax1a* mutants could be employed in Fe biofortification programs regardless the Ca dose applied, especially *BraA.cax1a-12* that reached increases in its Fe concentration around double of R-o-18 (Fig. 3F). However, in the case of other nutrients, their accumulation depends on the Ca dose applied, so biofortification programs efficiency could be enhanced through Ca managing. Thereby, Ca and Mg only are accumulated under control and high Ca doses (Table 1 and Fig. 3E), whereas Zn only is accumulated under low Ca dose (Fig. 3I). Therefore, the Ca dose supplied to the plant is a key factor to be considered in biofortification program with *BraA.cax1a* mutants.

## 4. Conclusions

The results obtained proved the working hypothesis because CAX1a mutations caused changes in growth, physiological state and nutrients accumulation and these changes were influenced by Ca dose applied. *BraA.cax1a* mutants appear to be unhelpful to better Ca-deficiency tolerance since they did not improve plant growth neither foliar Ca accumulation under low Ca application. In addition, regardless the Ca dose applied, *BraA.cax1a-4* and *BraA.cax1a-7* showed stress symptoms such as lower total Chl, an altered photosynthesis performance and higher ROS levels. However, *BraA.cax1a-12* did not affect negatively to plant vitality. Indeed, this mutation allows a better growth under high Ca conditions. All mutants accumulated more Ca and Mg in leaves when Ca is in

adequate concentration in the medium and accumulated more Fe regardless the Ca dose. These results obtained point to *BraA.cax1a-12* as a potential candidate for biofortification with Fe, Ca and Mg since it accumulates these elements to a greater extent than R-o-18, does not present an altered growth and is able to tolerate higher Ca doses. Besides this mutant might be employed for phytoremediation purposes in soils with toxic concentrations of these elements, although specific studies are required.

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## Tables

**Table 1** Leaf and root biomass and Ca concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

| | | Leaf biomass (g DW plant$^{-1}$) | Root biomass (g DW plant$^{-1}$) | Foliar Ca concentration (mg Ca g$^{-1}$ DW) | Root Ca concentration (mg Ca g$^{-1}$ DW) |
|---|---|---|---|---|---|
| 0.4 mM | R-o-18 | 0.63$^{ab}$ | 0.20$^{a}$ | 10.05$^{a}$ | 7.71$^{b}$ |
| | *BraA.cax1a-4* | 0.61$^{b}$ | 0.14$^{b}$ | 9.37$^{ab}$ | 15.05$^{a}$ |
| | *BraA.cax1a-7* | 0.76$^{ab}$ | 0.11$^{c}$ | 8.98$^{b}$ | 8.22$^{b}$ |
| | *BraA.cax1a-12* | 0.77$^{a}$ | 0.14$^{b}$ | 10.03$^{a}$ | 14.71$^{a}$ |
| | p-value | NS | *** | NS | *** |
| | $LSD_{0.05}$ | 0.15 | 0.03 | 0.94 | 1.11 |
| 4 mM | R-o-18 | 0.87$^{a}$ | 0.21$^{a}$ | 15.20$^{c}$ | 20.91$^{a}$ |
| | *BraA.cax1a-4* | 0.69$^{b}$ | 0.22$^{a}$ | 16.44$^{b}$ | 25.24$^{a}$ |
| | *BraA.cax1a-7* | 0.87$^{a}$ | 0.17$^{b}$ | 17.71$^{a}$ | 15.34$^{b}$ |
| | *BraA.cax1a-12* | 0.85$^{ab}$ | 0.18$^{b}$ | 17.29$^{a}$ | 14.84$^{b}$ |
| | p-value | NS | ** | *** | ** |
| | $LSD_{0.05}$ | 0.17 | 0.03 | 0.79 | 4.72 |
| 40 mM | R-o-18 | 0.34$^{b}$ | 0.18$^{a}$ | 28.69$^{c}$ | 24.38$^{a}$ |
| | *BraA.cax1a-4* | 0.40$^{b}$ | 0.07$^{b}$ | 40.30$^{a}$ | 15.34$^{b}$ |
| | *BraA.cax1a-7* | 0.37$^{b}$ | 0.06$^{b}$ | 43.53$^{a}$ | 10. 05$^{c}$ |
| | *BraA.cax1a-12* | 0.62$^{a}$ | 0.16$^{a}$ | 35.37$^{b}$ | 13.48$^{b}$ |
| | p-value | *** | *** | *** | *** |
| | $LSD_{0.05}$ | 0.12 | 0.05 | 4.30 | 2.65 |
| Analysis of variance | | | | | |
| Doses (D) | | *** | *** | *** | *** |
| Mutation (M) | | *** | *** | *** | *** |
| D x M | | * | *** | *** | *** |
| $LSD_{0.05}$ | | 0.08 | 0.02 | 1.33 | 1.65 |

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

**Table 2** SPAD chlorophyll, total Chls concentration, Chl a/b ratio, and carotenoids concentration in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

| | | SPAD value | Total Chls mg $g^{-1}$ FW | Chl a/b | Carotenoids µg $g^{-1}$ FW |
|---|---|---|---|---|---|
| 0.4 mM | R-o-18 | 39.45$^{a}$ | 0.41$^{a}$ | 1.85$^{c}$ | 34.47$^{a}$ |
| | *BraA.cax1a-4* | 34.51$^{b}$ | 0.30$^{c}$ | 2.39$^{a}$ | 32.34$^{b}$ |
| | *BraA.cax1a-7* | 25.57$^{c}$ | 0.22$^{d}$ | 2.32$^{b}$ | 23.23$^{c}$ |
| | *BraA.cax1a-12* | 39.79$^{a}$ | 0.37$^{b}$ | 2.30$^{b}$ | 33.52$^{ab}$ |
| | p-value | *** | *** | *** | *** |
| | $LSD_{0.05}$ | 3.60 | 0.01 | 0.06 | 1.19 |
| 4 mM | R-o-18 | 44.77$^{a}$ | 0.41$^{a}$ | 2.01$^{c}$ | 36.25$^{a}$ |
| | *BraA.cax1a-4* | 39.43$^{b}$ | 0.34$^{b}$ | 2.24$^{b}$ | 29.71$^{ab}$ |
| | *BraA.cax1a-7* | 37.16$^{b}$ | 0.27$^{c}$ | 2.44$^{a}$ | 29.35$^{b}$ |
| | *BraA.cax1a-12* | 45.27$^{a}$ | 0.34$^{b}$ | 2.29$^{b}$ | 33.15$^{ab}$ |
| | p-value | *** | *** | *** | ** |
| | $LSD_{0.05}$ | 4.30 | 0.02 | 0.09 | 3.87 |
| 40 mM | R-o-18 | 54.07$^{a}$ | 0.41$^{a}$ | 1.93$^{c}$ | 32.32$^{a}$ |
| | *BraA.cax1a-4* | 45.32$^{b}$ | 0.34$^{b}$ | 2.24$^{b}$ | 31.24$^{ab}$ |
| | *BraA.cax1a-7* | 44.77$^{b}$ | 0.27$^{c}$ | 2.38$^{a}$ | 29.43$^{b}$ |
| | *BraA.cax1a-12* | 52.21$^{a}$ | 0.33$^{b}$ | 2.32$^{b}$ | 30.90$^{ab}$ |
| | p-value | ** | *** | *** | NS |
| | $LSD_{0.05}$ | 5.73 | 0.02 | 0.15 | 2.40 |
| Analysis of variance | | | | | |
| Doses (D) | | *** | *** | * | NS |
| Mutation (M) | | *** | *** | *** | *** |
| D x M | | NS | *** | *** | *** |
| $LSD_{0.05}$ | | 2.60 | 0.01 | 0.05 | 1.53 |

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

**Table 3** Values of Chl a fluorescence parameters derived from the JIP test in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

| | | Fo | Fm | Fv | Fv/Fm | $PI_{ABS}$ | RC/ABS | Ψo | $\Phi_{Eo}$ | K step |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.4 mM | R-o-18 | $249^{b}$ | $1615^{ab}$ | $1366^{a}$ | 0.85a | $8.68^{a}$ | $0.85^{a}$ | $0.66^{a}$ | $0.56^{a}$ | $394^{c}$ |
| | *BraA.cax1a-4* | $299^{a}$ | $1690^{a}$ | $1390^{a}$ | 0.82b | $5.11^{c}$ | $0.67^{b}$ | $0.60^{b}$ | $0.50^{bc}$ | $499^{a}$ |
| | *BraA.cax1a-7* | $273^{ab}$ | $1473^{b}$ | $1200^{b}$ | 0.82b | $4.27^{c}$ | $0.64^{b}$ | $0.60^{b}$ | $0.49^{c}$ | $453^{ab}$ |
| | *BraA.cax1a-12* | $252^{b}$ | $1582^{ab}$ | $1330^{a}$ | 0.84a | $7.15^{b}$ | $0.78^{a}$ | $0.63^{ab}$ | $0.53^{ab}$ | $408^{bc}$ |
| | p-value | NS | * | ** | ** | *** | *** | ** | *** | *** |
| | $LSD_{0.05}$ | 43 | 140 | 104 | 0.01 | 1.46 | 0.08 | 0.03 | 0.03 | 48 |
| 4 mM | R-o-18 | $252^{b}$ | $1660^{b}$ | $1408^{b}$ | $0.85^{a}$ | $10.30^{a}$ | $0.89^{a}$ | $0.69^{a}$ | $0.59^{a}$ | $382^{b}$ |
| | *BraA.cax1a-4* | $329^{a}$ | $1800^{a}$ | $1471^{a}$ | $0.82^{b}$ | $6.27^{c}$ | $0.73^{b}$ | $0.62^{bc}$ | $0.51^{bc}$ | $517^{a}$ |
| | *BraA.cax1a-7* | $343^{a}$ | $1841^{a}$ | $1498^{a}$ | $0.81^{b}$ | $5.49^{c}$ | $0.73^{b}$ | $0.59^{c}$ | $0.48^{c}$ | $558^{a}$ |
| | *BraA.cax1a-12* | $262^{b}$ | $1683^{b}$ | $1421^{b}$ | $0.84^{a}$ | $8.77^{b}$ | $0.84^{a}$ | $0.64^{b}$ | $0.54^{b}$ | $416^{b}$ |
| | p-value | ** | ** | ** | *** | *** | ** | *** | *** | ** |
| | $LSD_{0.05}$ | 52.46 | 92 | 50 | 0.01 | 1.76 | 0.09 | 0.04 | 0.04 | 94 |
| 40 mM | R-o-18 | $250^{b}$ | $1668^{b}$ | $1418^{ab}$ | $0.85^{a}$ | $15.72^{a}$ | $1.02^{a}$ | $0.73^{a}$ | $0.63^{a}$ | $344^{c}$ |
| | *BraA.cax1a-4* | $294^{b}$ | $1675^{b}$ | $1381^{b}$ | $0.82^{b}$ | $7.27^{c}$ | $0.85^{b}$ | $0.65^{c}$ | $0.53^{c}$ | $452^{b}$ |
| | *BraA.cax1a-7* | $422^{a}$ | $1965^{a}$ | $1544^{a}$ | $0.79^{c}$ | $5.33^{d}$ | $0.77^{c}$ | $0.63^{c}$ | $0.49^{d}$ | $607^{a}$ |
| | *BraA.cax1a-12* | $264^{b}$ | $1702^{b}$ | $1438^{ab}$ | $0.85^{a}$ | $12.53^{b}$ | $1.06^{a}$ | $0.68^{b}$ | $0.57^{b}$ | $365^{c}$ |
| | p-value | *** | ** | NS | *** | *** | *** | *** | *** | *** |
| | $LSD_{0.05}$ | 55 | 182 | 131 | 0.01 | 1.50 | 0.07 | 0.02 | 0.02 | 51 |
| Analysis of variance | | | | | | | | | | |
| Doses (D) | | ** | ** | ** | NS | *** | *** | *** | *** | NS |
| Mutation (M) | | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| D x M | | *** | *** | ** | *** | *** | * | NS | NS | *** |
| $LSD_{0.05}$ | | 28 | 76 | 56 | 0.01 | 0.87 | 0.05 | 0.02 | 0.02 | 37 |

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

**Table 4** Values of $O_2^-$, $H_2O_2$, and MDA concentrations, and APX and LOX activities in *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses

| | | MDA ($\mu M\ g^{-1}$ FW) | LOX ($\Delta Abs\ mg\ prot^{-1}min^{-1}$) | $O_2^-$ ($\mu g\ g^{-1}$ FW) | $H_2O_2$ ($\mu g\ g^{-1}$ FW) | APX ($\Delta Abs\ mg\ prot^{-1}min^{-1}$) |
|---|---|---|---|---|---|---|
| 0.4 mM | R-o-18 | $10.64^{b}$ | $7.74^{b}$ | $6.08^{bc}$ | $0.41^{a}$ | $0.23^{c}$ |
| | *BraA.cax1a-4* | $12.49^{a}$ | $7.66^{ab}$ | $6.30^{b}$ | $0.34^{b}$ | $0.37^{b}$ |
| | *BraA.cax1a-7* | $12.65^{a}$ | $8.36^{a}$ | $7.68^{a}$ | $0.29^{c}$ | $0.50^{a}$ |
| | *BraA.cax1a-12* | $11.94^{ab}$ | $6.18^{b}$ | $5.56^{c}$ | $0.29^{c}$ | $0.55^{a}$ |
| | p-value | NS | * | *** | *** | *** |
| | $LSD_{0.05}$ | 1.63 | 1.62 | 0.66 | 0.03 | 0.09 |
| 4 mM | R-o-18 | $19.14^{c}$ | $6.07^{b}$ | $3.67^{c}$ | $0.34^{a}$ | $0.20^{c}$ |
| | *BraA.cax1a-4* | $28.64^{a}$ | $10.85^{a}$ | $4.66^{b}$ | $0.28^{b}$ | $0.36^{b}$ |
| | *BraA.cax1a-7* | $22.77^{b}$ | $10.90^{a}$ | $6.67^{a}$ | $0.26^{b}$ | $0.48^{a}$ |
| | *BraA.cax1a-12* | $18.92^{c}$ | $7.82^{b}$ | $3.76^{c}$ | $0.27^{b}$ | $0.41^{ab}$ |
| | p-value | *** | *** | *** | * | *** |
| | $LSD_{0.05}$ | 1.36 | 1.89 | 0.42 | 0.06 | 0.10 |
| 40 mM | R-o-18 | $19.59^{b}$ | $10.43^{b}$ | $3.78^{b}$ | $0.37^{b}$ | $0.35^{c}$ |
| | *BraA.cax1a-4* | $32.85^{a}$ | $13.41^{a}$ | $4.80^{a}$ | $0.32^{c}$ | $0.60^{a}$ |
| | *BraA.cax1a-7* | $10.49^{d}$ | $7.08^{c}$ | $4.72^{a}$ | $0.42^{a}$ | $0.57^{a}$ |
| | *BraA.cax1a-12* | $12.24^{c}$ | $8.18^{c}$ | $3.88^{b}$ | $0.28^{d}$ | $0.49^{b}$ |
| | p-value | *** | *** | *** | *** | *** |
| | $LSD_{0.05}$ | 1.22 | 1.87 | 0.33 | 0.03 | 0.08 |
| Analysis of variance | | | | | | |
| Doses (D) | | *** | *** | ** | *** | *** |
| Mutation (M) | | *** | *** | *** | *** | *** |
| D x M | | *** | *** | *** | *** | *** |
| $LSD_{0.05}$ | | 0.79 | 1.05 | 0.28 | 0.02 | 0.05 |

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**) and p<0.001 (***).

**Figure legends**

**Fig. 1**. Ca efficiency parameters (A and B) and DC (C) in BraA.cax1a mutants and R-o-18 plants submitted to three Ca doses. Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

**Fig. 2**. Values of Chl a fluorescence parameters derived from the JIP test in *BraA.cax1a* mutants and R-o-18 plants submitted to 0.4 mM $CaCl_2$ (A), 4 mM $CaCl_2$ (B), and 40 mM $CaCl_2$ (C). Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

**Fig. 3**. Nutrient concentrations of *BraA.cax1a* mutants and R-o-18 plants submitted to three Ca doses. Values are expressed as means ± standard error (n=9). Column marked with the same letters were not significantly different based on the LSD test ($P < 0.05$).

**Fig. 1.**

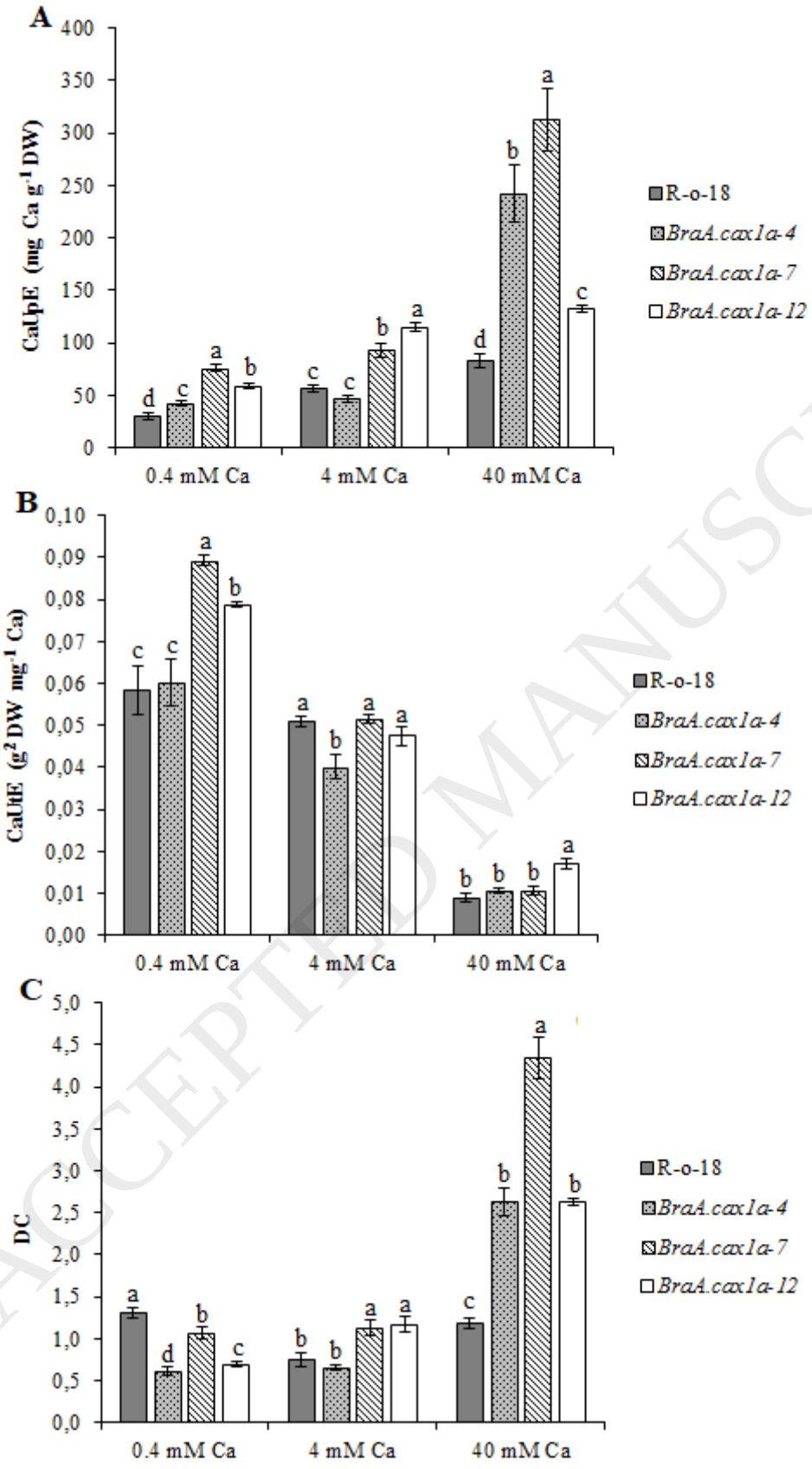

**Fig. 2.**

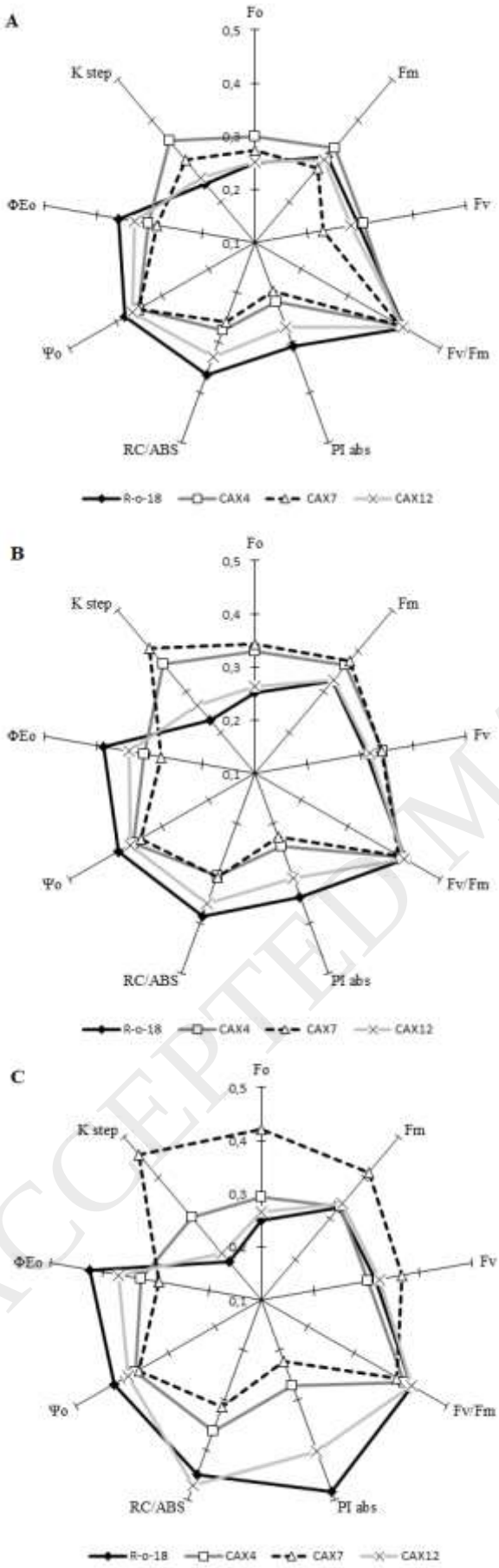

**Fig 3.**