

## Changes in the ultrastructure of soybean cultivars in response to manganese supply in solution culture

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**ABSTRACT:** The deleterious effects of Mn stress on many species have been studied, mainly concerning biochemical, physiological and growth parameters of plants. However, there are few studies relating the anatomical and ultrastructural changes in response to manganese (Mn) nutritional disorders. This study examined the leaf ultrastructure of Mn-inefficient (IAC-15, Santa Rosa) and Mn-efficient (IAC-Foscarin 31) soybean (*Glycine max* L.) genotypes in response to three rates of Mn (0.5, 2 and 200  $\mu\text{mol L}^{-1}$ ) in the nutrient solution. Symptoms of Mn deficiency developed 12 days after transplanting in IAC-15 and Santa Rosa, followed by IAC-Foscarin 31 on the 15<sup>th</sup> day. Only IAC-15 and Santa Rosa leaves showed symptoms of Mn toxicity. The Mn concentration in leaves ranged from 8.6 (deficiency) to 886.3  $\text{mg kg}^{-1}$  d.w. (toxicity). There were no changes either in stomata length or stomata number per unit of leaf surface. Cytoplasm disorganization was observed in IAC-15 under Mn-excess. In this case, the cytoplasm was amorphous, densely stained and extensively disorganized, with increased vacuolation. Mn effects were not found in mitochondria and nucleus in any of the genotypes tested. Under all Mn concentrations, many lipid globules were observed in the IAC-15 chloroplasts. There was an increase in the number of plastids as well as in the size of starch grains within IAC-Foscarin 31 chloroplasts as Mn concentration in the nutrient solution increased. Genotypes had marked differences in the ultrastructure organization, mainly in leaf chloroplasts grown under conditions of both Mn deficiency and toxicity (the most sensitive genotype was IAC-15).

Key words: chloroplast, deficiency, plant nutrition, toxicity

## Alterações na ultra-estrutura de genótipos de soja em resposta ao fornecimento de manganês em solução nutritiva

**RESUMO:** Os efeitos negativos provocados tanto pela deficiência quanto pela toxidez de manganês (Mn) no desenvolvimento das plantas têm sido avaliados, considerando-se os aspectos bioquímicos e produtivos da parte aérea, particularmente, onde os sintomas visuais são manifestados. Entretanto, há poucas informações na literatura abordando as alterações anatômicas e de ultra-estrutura, em relação ao suprimento de Mn. Os objetivos do presente estudo foram avaliar os efeitos do fornecimento de três doses de Mn (0,5; 2 e 200  $\mu\text{mol L}^{-1}$ ), em solução nutritiva, nas ultra-estruturas de folhas de cultivares de soja *Glycine max* (L.): Santa Rosa, IAC-15 e IAC-Foscarin 31, contrastantes quanto à aquisição e ao uso do Mn. Os sintomas visuais de deficiência foram observados primeiramente em Santa Rosa e IAC-15 (ineficientes), os únicos a exibirem sintomas de toxidez. As concentrações de Mn nas folhas com sintomas variaram de 8,6 (deficiência) a 886,3  $\text{mg kg}^{-1}$  (toxidez). Não houve alterações no comprimento e no número de estômatos nos limbos foliares. Em condição de toxidez, constatou-se no IAC-15, citoplasma desorganizado, vacuolado em excesso e denso evidenciando alterações nas membranas dos tilacóides. Não ocorreram alterações ultra-estruturais nas mitocôndrias e no núcleo das células dos três genótipos. Constatou-se presença de glóbulos de lipídios nos cloroplastos do cultivar IAC-15, em todas as condições de fornecimento de Mn. Houve aumento no número de plastídeos e grãos de amido, bem como no tamanho destes no IAC-Foscarin 31 com o suprimento de Mn. Os genótipos, tanto na condição de deficiência quanto de excesso, exibiram distintos graus de organização das ultra-estruturas, notadamente, os cloroplastos. O IAC-15 exibiu maiores alterações das ultra-estruturas em função das desordens nutricionais em manganês.

Palavras-chave: cloroplasto, deficiência, nutrição mineral de plantas, toxidez

### Introduction

Although manganese (Mn) toxicity can be a common problem in tropical regions with acid soils, Mn defi-

ciency in soybean genotypes (*Glycine max* L.) grown in Brazilian Cerrado (savannah) has been recognized as a nutritional disorder frequently related to excessive liming (Tanaka et al., 1992). Variations in the concentrations

of Mn in plants have been attributed either to genetic factors or to soil fertility conditions (Fageria, 2001). Plant species and genotypes within the same species can differ widely in the tolerance to high Mn (Foy et al., 1988) as well as in susceptibility to the deficiency when grown under conditions of low Mn availability (Graham, 1988).

Mn plays important roles in plant metabolism, such as participation in photosystem II and chlorophyll biosynthesis (Malavolta, 2006). However, there are few studies in the literature reporting the anatomical and ultrastructural changes of soybean leaves in response to the Mn supply. The deleterious effects of deficiency and excess of Mn in many species have been studied, mainly concerning the biochemical, physiological and growth parameters of plants (Husted et al., 2009; Luz-Mora et al., 2009; Shi et al., 2009).

Recently, a few of the genes responsible for transport of mineral nutrients in plants have been identified, allowing some of the  $Mn^{2+}$  transport pathways to be identified at the molecular level. These include transporters responsible for Mn accumulation in the cell and release from various organelles as well as for active sequestration in endomembrane compartments, particularly in the vacuole and endoplasmic reticulum (Broadley and White, 2005; Pittman, 2005). These mechanisms could also be important for Mn leaf-tissue tolerance in crop species grown under conditions of either limited or excessive Mn availability (Pittman, 2005; Martinoia et al., 2007).

The present work was carried out to study the effect of both Mn deficiency and toxicity on leaf cell structures and ultrastructure as well as on Mn concentration in leaves of three soybean cultivars, two of them Mn-inefficient and one Mn-efficient.

## Material and Methods

### Plant material and cultivation of soybean plants

The experiment was carried out under greenhouse conditions, in Piracicaba, São Paulo state, between May 17<sup>th</sup> to June 27<sup>th</sup> 2006. Santa Rosa (Mn-inefficient), IAC-15 (Mn-inefficient) and IAC-Foscarin 31 (Mn-efficient) cultivars – as formerly classified by Mascarenhas et al. (1984, 1990) – of *Glycine max* (L.) Merrill were grown under three Mn rates (0.5, 2.0 and 200.0  $\mu\text{mol L}^{-1}$ ) in the nutrient solution. The solutions were prepared as recommended by Johnson et al. (1957) and adapted by Epstein and Bloom (2005), diluted to 1/5 of the usual concentration, and with initial pH of  $4.97 \pm 0.03$ . The experiment setup was a completely randomized block design with three replications.

Seeds were placed to germinate in a tray with vermiculite, moistened with calcium sulphate ( $\text{CaSO}_4$ ,  $10^{-4} \text{ mol L}^{-1}$ ). Plants reaching about 5 cm of height (phenologic stage V1, about five days post emergence) were transplanted to individual plastic pots, 20.0 cm in diameter and with a 2.5 L capacity. The solutions containing the desired Mn concentrations were supplied the third day after

transplanting, and were renewed every seven days. The pots were rearranged within each block every three days.

### Plant analysis

Plant samples were collected when visible symptoms of deficiency (in the 0.5  $\mu\text{mol L}^{-1}$  Mn treatment), and toxicity (200.0  $\mu\text{mol L}^{-1}$ ) occurred (V3 and V4 phenologic stages, respectively). All plants grown under 2.0  $\mu\text{mol L}^{-1}$  Mn were healthy. The remaining plant parts were collected, washed and dried at 65°C for 48 hours in a forced-air oven before being ground in a stainless steel mill. The material was digested with 4 mL  $\text{HNO}_3$  and 2 mL  $\text{HClO}_4$  of concentrated acids on a digestion block heated gradually to 203°C. Manganese was determined by atomic emission spectroscopy.

### Scanning electron microscopy and transmission electron microscopy analysis

For the scanning electron microscopy, leaf samples were fixed, and after post fixation in 1%  $\text{OsO}_4$  the samples were dehydrated in a graded ethanol series (30-100%) and processed in a critical point dryer through  $\text{CO}_2$ . The dried samples were mounted on metal stubs, sputter coated for 260 s with gold and examined under a scanning electron microscope at 20 kV. The images were digitalized.

For the transmission electron microscopy, small pieces of leaf tissues were fixed for 2 h in a modified solution (Karnovsky, 1965) composed of 2% glutaraldehyde, 2% formaldehyde in 0.05  $\text{mol L}^{-1}$  sodium cacodylate buffer at pH 7.2, followed by 1 h post fixation in 1%  $\text{OsO}_4$ , and then dehydrated gradually using acetone (25-100%). Later, the segments were embedded in resin. Blocks were trimmed and ultrathin sections were cut in an MT2 ultramicrotome equipped with a diamond knife. Sections were placed on Formvar-coated cooper grids, and then stained with 2.5% aqueous uranyl acetate for 15 min, followed by lead citrate solution for 8 min (Reynolds, 1963). Finally, the sections were examined under a transmission electron microscope at an accelerating voltage of 50 kV and the images were then digitalized. The morphometric assessments of leaf cross sections were obtained through optical microscopy, followed through by processing of the images, regularly registered, using the Photoshop Adobe® version 6.0 (San Jose, CA, USA), and calculated using the 3<sup>rd</sup> version of the software Integrated System for Analysis and Covering of Soil (Embrapa, São Carlos, Brazil). The same procedures were applied to measure various ultrastructural parameters.

### Statistical analysis

The experimental setup was completely randomized blocks, with the treatments arranged in a  $3 \times 3$  factorial scheme (three Mn rates x three soybean cultivars), with three replications. The data were submitted to statistical analyses by the SAS System for Windows 6.11 statistical program (SAS Institute, 1996). In cases when the F-test showed significant differences of means for the Mn rates, the Tukey test was applied to compare the means between the cultivars and Mn rates ( $p < 0.05$ ).

## Results and Discussion

The Santa Rosa and IAC-15 cultivars showed visual symptoms of Mn deficiency 12 d days after the beginning of the treatments at  $0.5 \mu\text{mol L}^{-1}$  Mn, whereas IAC-Foscarin 31 showed similar symptoms three days later. Symptoms of toxicity were observed only in Santa Rosa and IAC-15 plants treated with  $200 \mu\text{mol L}^{-1}$  on the sixth day (Figure 1). Chlorosis of interveinal areas first appeared in younger leaves (Mn deficiency symptoms), whereas toxicity symptoms were observed both in the young (markedly) and old leaves.

In all three genotypes, leaf Mn concentration increased as Mn concentration in the nutrient solution increased (Table 1). In the  $200 \mu\text{mol L}^{-1}$  Mn treatment, higher Mn concentrations were found in leaves of IAC-15 followed by Santa Rosa and IAC-Foscarin 31 cultivars, respectively. However, there were no differences between the three cultivars with regard to leaf Mn concentration, for any of the three Mn treatments. In general, the critical deficiency range in fully expanded leaves is quite narrow, varying between 10 and  $20 \text{ mg kg}^{-1}$  dry mass. On the other hand, critical leaf concentration for toxicity can vary within a very wide range, depending on plant species and genotypes within species,

and on environmental conditions, such as temperature and mineral nutritional status (Fageria, 2001). Fageria (2001) attributed Mn concentrations in soybean leaves, respectively, of 67 and  $720 \text{ mg kg}^{-1}$ , as adequate and toxic contents. Lima et al. (2004) pointed out concentrations of Mn of  $1,800 \text{ mg kg}^{-1}$  as being toxic to shoots of soybean cultivar Emgopa 316, grown in Oxisols from the Brazilian Cerrado.

The scanning electron microscopy images of sections from Mn-deficient leaves, in all three genotypes, showed a certain degree of tissue disorganization and minor alteration in the epidermis (abaxial and adaxial face), and no stomata reduction in relation of those observed in adequate Mn-supply ( $2 \mu\text{mol L}^{-1}$ ). At the highest Mn rate ( $200.0 \mu\text{mol L}^{-1}$ ), there were alterations in the epidermis and tissue agglomeration, resulting in epidermal hypertrophy – in other words, a huge disorganization in cell arrangement. This can also be confirmed by the morphometric assessments shown in Table 2. Under all Mn concentrations, there were no changes either in stomata length or stomata number both in the adaxial and abaxial face (Figure 2), a finding that agrees with the observations of Weiland et al. (1975) and Baldissotto et al. (2004). However, under severe Mn toxicity conditions, there was high disorganization of the

Table 1 – Mn concentration in the deficient leaf (rate of  $0.5 \mu\text{mol L}^{-1}$  Mn), Mn-normal ( $2.0 \mu\text{mol L}^{-1}$  Mn), and high Mn leaf ( $200.0 \mu\text{mol L}^{-1}$ ) in three-soybean cultivars: Santa Rosa, IAC-15 and IAC-Foscarin 31, as related to Mn rates in the nutrient solution.

Cultivars	Mn rates ( $\mu\text{mol L}^{-1}$ )		
	0.5	2.0	200.0
	----- mg kg <sup>-1</sup> -----		
Santa Rosa	12.5 a	30.6 a	786.3 a
IAC-15	8.6 a	25.9 a	886.3 a
IAC-Foscarin 31	9.8 a	24.0 a	744.3 a
MSD	4.8	24.0	254.9
CV (%)	16	30	11

Lower case letters on the same column do not differ (Tukey test,  $p > 0.05$ ).

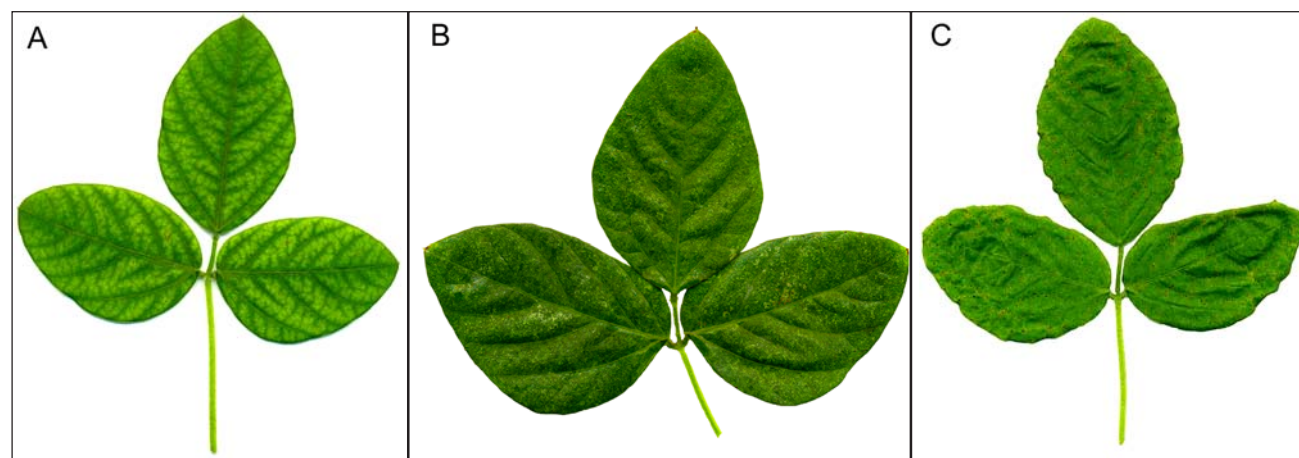


Figure 1 – Soybean leaf showing deficiency symptoms (A), Mn-normal leaf (B) and high-Mn concentration (C).

Table 2 – Morphometric assessments of leaves (cross sections) and mesophyll chloroplasts of Santa Rosa, IAC-15 and IAC-Foscarin 31, as related to Mn rates in the nutrient solution.

Cultivars	Mn rates ( $\mu\text{mol L}^{-1}$ )		
	0.5	2.0	200.0
	Leaf layer thickness ( $\mu\text{m}$ ) palisade parenchyma		
Santa Rosa	103.8 aC	124.8 aB	416.7 aA
IAC-15	101.8 aB	72.0 bC	318.8 bA
IAC-Foscarin 31	77.2 bB	77.1 bB	332.2 abA
CV%	9.0	13.7	12.9
	Leaf layer thickness ( $\mu\text{m}$ ) spongy parenchyma		
Santa Rosa	63.1 aC	97.0 aB	285.7 aA
IAC-15	12.4 bC	21.9 bB	115.3 bA
IAC-Foscarin 31	42.7 aC	63.4 aB	191.9 aA
CV%	16.7	10.5	20.1
	Area of chloroplast ( $\mu\text{m}^2$ )		
Santa Rosa	7.8 aB	9.3 aA	9.9 aA
IAC-15	7.2 aB	9.0 aA	5.4 bC
IAC-Foscarin 31	6.9 aC	8.7 aB	11.5 aA
CV%	9.9	14.3	10.2
	Area of starch grain ( $\mu\text{m}^2$ )		
Santa Rosa	–	0.6 aA	0.6 bA
IAC-15	–	0.5 a	–
IAC-Foscarin 31	–	0.4 aB	4.2 aA
CV%	–	9.4	11.8

Lower case letters on the same column and upper case letters on the same row do not differ (Tukey test,  $p > 0.05$ ).

tissue, characterized by hidden stomata throughout the leaf area as well. Lidon (2002) observed, in rice plants grown in nutrient solutions containing 2.4, 145.0 and 582.0  $\mu\text{mol L}^{-1}$  of Mn, alteration in stomata length, which decreased by about 50% under the supply of the highest rate. He concluded that the reduction was associated with the physiological control of the rice plants, as the mechanism responsible for the low Mn transport from roots to shoots, i.e., as a consequence of low xylem flow, which might be altered by the variation of transpiration rates.

External manifestation of an abnormality (visible symptom) caused by toxicity of any mineral element is the result of a chain of events starting with an alteration, with the final result being a modification at the subcellular level, which in turn leads to a cellular alteration that finally results in modification of the mesophyll (Malavolta, 2006). The deficiency of any nutrient can unleash this series of events until the appearance of a visible symptom. Several studies, more specifically dealing with the deficiency of Mn in plants, have demonstrated the role of Mn in the maintenance of chloroplast ultrastructures (Weiland et al., 1975), since Mn deficiency resulted in a reduction in the rate of PS II electron transport (Papadakis et al., 2007a,b,c). Conversely, marked alterations in the Golgi apparatus and the

endoplasmic reticulum were observed (Izaguirre-Mayoral and Sinclair, 2005), as well as in the mitochondria under Mn toxicity conditions (Santandrea et al., 1998). However, none of these effects were observed in the present study.

Transmission electron microscopy revealed ultrastructural alterations in the mesophyll of all the three genotypes (Figure 3). Since the leaves were sampled when the first symptoms appeared (deficiency and toxicity), the anomalies did not progress to complete disorganization of the epidermis (abaxial and adaxial face). Furthermore, increased Mn concentration in the nutrient solution caused an increase in leaf lamina thickness, primarily due to increased length of palisade parenchyma cells (Table 2). The spongy parenchyma thickness also increased with higher rates of Mn in the nutrient solution, ranging from 63.1 to 285.7  $\mu\text{m}$ , 12.4 to 115.3  $\mu\text{m}$  and 42.7 to 191.9  $\mu\text{m}$  for Santa Rosa, IAC-15 and IAC-Foscarin 31, respectively.

The palisade and spongy parenchymas of the three soybean cultivars grown in the solution with 2  $\mu\text{mol L}^{-1}$  Mn were regularly distributed. Two cell layers could be identified in the palisade parenchyma of the upper leaf surface and a single layer of spongy cells on the abaxial face. The mesophyll cells had normal size and shape, with easily distinguishable chloroplasts distrib-

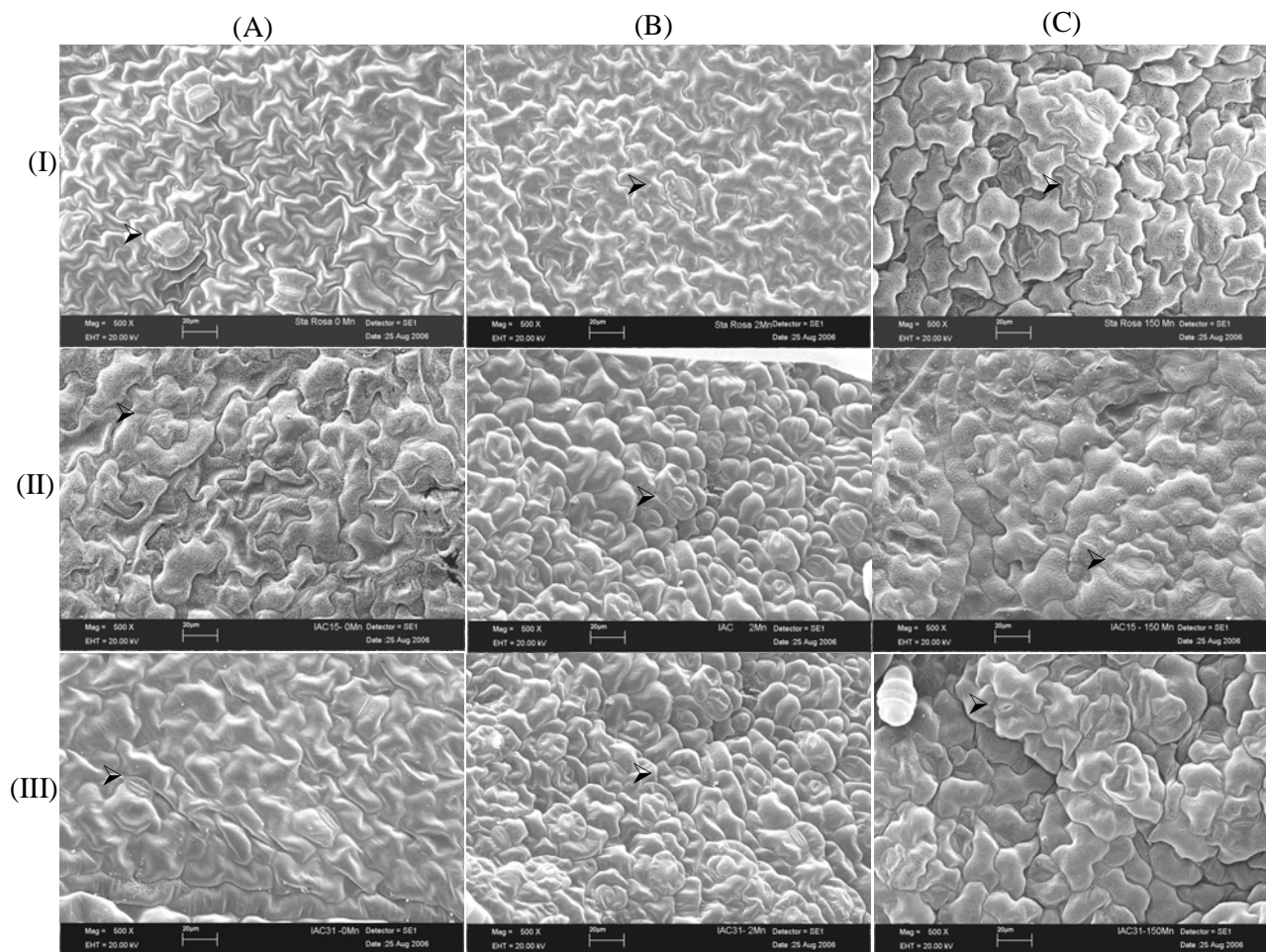


Figure 2 – Scanning electron micrographs showing stomata leaf of soybean Santa Rosa (I), IAC-15 (II) and IAC-Foscarin 31 (III) cultivars, as related to Mn rates of 0.5 (A), 2.0 (B) and 200.0  $\mu\text{mol L}^{-1}$  (C) in the nutrient solution. (➤) Detail of the stomata. The scale of each picture has a value of 20  $\mu\text{m}$ .

uted throughout the cells. Most of these details can be observed in Lavres Jr. et al. (2009). These results are the same as pointed out by Papadakis et al. (2007c), who reported that increased Mn concentration in the nutrient solution caused an increase in leaf lamina thickness of orange plants, primarily due to increased length of palisade parenchyma cells. Furthermore, at a Mn concentration of 686  $\mu\text{mol L}^{-1}$ , spongy parenchyma thickness also increased.

Transmission electron microscopy of transversely cut leaves from all genotypes grown at 0.5  $\mu\text{mol L}^{-1}$  Mn revealed small chloroplasts with round aspect (markedly in Santa Rosa and IAC-Foscarin 31) and scarce starch granules. Stacked thylakoids (*granum*) around the stroma and a higher number of vesicles in the cytoplasm were also observed. However, extensive cytoplasmic disorganization, increased vacuolation and amorphous cytoplasm were more evident in IAC-15. In this case, alterations in the thylakoid membranes were evident (Figure 3-IIA). For all genotypes, Mn-deficient chloroplasts were smaller than those of normal and Mn-toxicity (2.0 and 200.0  $\mu\text{mol L}^{-1}$  of Mn, respectively) (Table 2), where chloroplasts occupied larger part of the cell volume.

Within each chloroplast, the grana were arranged parallel to the envelope. Each granum was organized with two or three other grana into a stack characterized by a close association with adjacent granum surfaces. This effect was more evident in the Santa Rosa. Mn deficiency did not affect the ultrastructure of other cellular organelles, like the mitochondria and nucleus. The symptoms of Mn deficiency observed in soybeans in this study were similar to those described by Weiland et al. (1975).

Chloroplasts at the highest Mn supply, especially with regard to the IAC-15 leaves, had an elongated shape, with thylakoids piled in a disorderly manner, underdeveloped grana, scarce starch granules in comparison with those cultivars, and hole-like folds in the thylakoid membrane. In short, there was a general disorganization within the chloroplast. An incomplete structure of the plastid was seldom observed. The cytoplasm had an amorphous and dense aspect (spotted), widely disorganized and with great number of vesicles. In a few cells the protoplast was separated from the wall towards the inner part of the cell (Figure 3-IIC). High levels of Mn damage the structure and function of cell membranes (Santandrea et al., 1998). Furthermore, separation of the



of photosynthates from the leaf to other organs. Papadakis et al. (2007c) reported that Mn affected the size and shape of chloroplasts in seedlings of *Citrus volkameriana* (L.), which were shorter and thinner under  $0 \mu\text{mol L}^{-1}$  Mn compared to the treatments with 2 to  $686 \mu\text{mol L}^{-1}$ . Besides this, the percentage of starch grains per chloroplast was fivefold under  $686 \mu\text{mol L}^{-1}$  in comparison with the treatments with 0 to  $98 \mu\text{mol L}^{-1}$  Mn, as was also observed in IAC-Foscarin 31 in the present study.

In conclusion, the effects of Mn deficiency and toxicity on leaf cell ultrastructure, such as number, size and distribution of grana, starch grains and lipid globules per chloroplast, differed among the tested genotypes (IAC-15 and Santa Rosa were affected more). According to the ultrastructural alterations as well as the periods of the appearance of visual symptoms of Mn deficiency and toxicity, the existence in Mn-tolerant IAC-Foscarin 31 of several mechanisms that act jointly to maintain the structural and biochemical apparatus of the plants resulting can be supposed. This response of IAC-Foscarin 31 to both Mn deficiency and toxicity could be ascribed to higher levels of antioxidant enzyme activities and thus to lower oxidative stress.

The existence of a cell adaptation mechanism to excessive Mn availability ( $200.0 \mu\text{mol L}^{-1}$  Mn), by increasing the size of chloroplasts as well as their number per cell area was observed in Santa Rosa and markedly in IAC-Foscarin 31 cultivars (Table 2). Furthermore, according to the anatomical changes and timing of appearance of the symptoms of Mn deficiency and toxicity observed here, we believe there are various mechanisms that act together to maintain the biochemical processes and structural plant apparatuses, resulting in different degrees of tissue organization. We also suggest that Mn detoxification of cells by activation of the antioxidant defense system (enzymatic or not), binding of Mn to complexing agents in the root system (phytochelatin, metallothioneins, histidine and nicotianamine) and subsequent deposition of Mn in the apoplast, reduction in long-distance transport of the metal-ligand complex (e.g., citrate or malate), and finally, storage inside the vacuoles (Ducic and Polle, 2005; Haydon and Cobbett, 2007), may all be more effective in the tolerant genotype, IAC-Foscarin 31, as pointed out by Lavres Jr. et al. (2008, 2009).

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