



## RESEARCH ARTICLE OPEN ACCESS

# Leaf Anatomical Adaptation and Chloroplast Ultrastructure Changes Upon Magnesium Foliar Application of Faba Bean (*Vicia faba* L.) Grown Under Drought Stress

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## ABSTRACT

**Background:** Drought stress (DS) impedes plant growth and development by impairing the uptake of nutrients, such as magnesium, which is central to many physiological processes, particularly photosynthesis. Leaf application was proposed to be an effective strategy to compensate for inadequate  $Mg^{2+}$  supply from the nutrient solution.

**Aim:** The present study is designed to investigate the role of  $Mg^{2+}$  leaf application in ameliorating leaf anatomy and chloroplast ultrastructure changes in faba beans grown under DS.

**Methods:** Hydroponically grown plants were subjected to DS under various levels of  $Mg^{2+}$ , that is, sufficient (0.5 mM), deficient (0 mM), and leaf-application (250 mM). Light and transmission electron microscopy (TEM) were conducted to examine leaf anatomy and ultrastructural changes.

**Results:**  $Mg^{2+}$  deficiency alone and under DS significantly affected plant biomass and photosynthesis. Additionally, sucrose concentration, oxidative stress, and lipid peroxidation were increased. Accordingly, the excessive deposition of photoassimilates in source organs due to the inhibition of phloem loading results in a disruption of the thylakoid structures leading to chloroplast damage. In the current study leaf application of  $Mg^{2+}$  partially ameliorated physiological functions, most notably chlorophyll concentration, photosynthesis and transpiration rate, plant biomass, and preservation of ultrastructure of the chloroplast.

**Conclusion:** Although the Mg application via roots enhanced drought tolerance, compared to  $Mg^{2+}$  leaf application. However,  $Mg^{2+}$  leaf application was proven to be an efficient strategy in mitigating DS in field trials. Therefore,  $Mg^{2+}$  foliar application should be prioritized for further investigation under relevant environmental stress conditions.

## 1 | Introduction

Faba bean (*Vicia faba* L.), a cool-season annual legume, is globally cultivated for its value in food, fodder, and medicinal purposes. Beyond its agricultural uses, faba bean improves soil fertility

through atmospheric nitrogen fixation, phosphorus solubilization, and enhancing plant–microbe interactions. Asia and Africa lead in production, with an annual global production exceeding 5.5 million tons. In Germany, grain legumes cover about 1.4% of the arable land, whereas in the European Union, this figure

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risers to approximately 2.1%, with faba beans accounting for nearly 30%. However, a global decline in faba bean production has been observed, attributed in part to environmental challenges such as drought stress (DS) (Krenz et al. 2023).

DS is one of the most detrimental abiotic factors affecting crop yield and productivity. During water scarcity, the mass flow of nutrients is disrupted due to reduced plant transpiration. Consequently, plants struggle to absorb the essential nutrients to sustain physiological processes and recovery mechanisms. Among these, macronutrients, particularly magnesium ( $Mg^{2+}$ ), plays a critical role in helping plants adapt to stress conditions.  $Mg^{2+}$  is involved in various physiological and biochemical processes crucial for plant growth and development. Plants deficient in  $Mg^{2+}$  are more susceptible to DS, and a sufficient supply of  $Mg^{2+}$  is essential for optimal yield formation under these conditions (Senbayram et al. 2015).

Abiotic stress conditions coupled with  $Mg^{2+}$  deficiency result in dynamic changes in ions like potassium ( $K^+$ ) and manganese ( $Mn^{2+}$ ) showing synergistic or antagonistic effects. The antagonistic effect of  $K^+$ , which impacts the uptake of calcium (Ca) and  $Mg^{2+}$ , suggests that  $Mg^{2+}$  foliar application can influence leaf cation composition (Geilfus, Wendler, and Mühling 2016). Earlier studies by Jezek et al. (2015) reported  $Mn^{2+}$  antagonism in *V. faba* plants under  $Mg^{2+}$  deficiency. Excessive accumulation of  $Mn^{2+}$  may result in chloroplast lamellae disorganization (Lidon & Teixeira, 2009). Under  $Mg^{2+}$  deficiency, sugar accumulation in the source leaves causes feedback inhibition of Rubisco and lowers the rate of photosynthesis. Inhibition of photosynthesis by DS results in the misallocation of electrons to oxygen, producing reactive oxygen species (ROS), which causes oxidative stress.  $Mg^{2+}$  not only enhances the efficiency of photosynthesis but also plays a central role in ROS scavenging, protecting the plant from oxidative stress.  $Mg^{2+}$  is directly involved in the activation of enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), all of which mitigate the harmful effects of ROS (Uzilday et al. 2017). Enzymes like SOD convert superoxide radicals into hydrogen peroxide ( $H_2O_2$ ), which is then neutralized by CAT and APX, preventing the accumulation of ROS that can damage cell membranes, proteins, and DNA.

Chloroplasts are the sites of photosynthesis and the first organelles of mesophyll cells to be affected by DS.  $Mg^{2+}$  availability affects chloroplast's size, structure, and function, including electron transfer in PSII (Chaudhry et al. 2021). In DS plants, thylakoid membranes appear deformed and disorganized, and depending on the severity of the dehydration and susceptibility of species/cultivars, it may lead to impairment of grana formation (Zhang et al. 2015). Earlier research studies by Rumak et al. (2010) reported that in vitro application of  $Mg^{2+}$  improves the stacking of grana and stroma thylakoids in peas and bean chloroplasts, thereby affecting the structural changes of a chloroplast, however, there are no reports of  $Mg^{2+}$  foliar application conferring protection to photosynthetic apparatus under DS.

Therefore, it is hypothesized that the foliar application of  $Mg^{2+}$  aids in recovery capacity and structural reorganization of the leaf anatomy and chloroplast ultrastructure in terms of well-organized grana and stroma lamellae thereby conferring

protection to photosynthetic apparatus with the ability to preserve functional photochemical activity apart from increasing the physiological processes during plant growth and development under DS. Hence, it can be inferred that the foliar application of  $Mg^{2+}$  is of relevance to cope with the DS-induced structural anomalies in the leaf.

## 2 | Materials and Methods

### 2.1 | Plant Material and Growing Conditions

The hydroponic experiment was conducted at the Institute of Plant Nutrition and Soil Science, Kiel University, Kiel, Germany. *V. faba* L. cultivar, namely, Mallory (NPZ, Hohenlieth, Germany), was chosen for the present study due to its high yield potential, seed size and quality, and resistance to diseases, such as chocolate spot (*Botrytis fabae*) and rust (*Uromyces viciae-fabae*). The seeds were spread into sandwich blots and placed in a container moistened with 1:5 diluted 10 mM  $CaSO_4$  solution. After 10 days, the seedlings were transferred to 5-L plastic pots (four plants per pot) containing one quarter-strength nutrient solution raised to total concentration stepwise. The pots were placed in a climate chamber under controlled conditions (20/16°C day/night; 250  $\mu mol/m^2/s$  photosynthetically active radiation). The total nutrient solution had the following composition: 2.0 mM  $Ca(NO_3)_2$ , 1.0 mM  $K_2SO_4$ , 0.1 mM  $KH_2PO_4$ , 0.2 mM KCl, 0.5 mM  $MgSO_4$ , 10  $\mu M$   $H_3BO_3$ , 2.0  $\mu M$   $MnSO_4$ , 0.5  $\mu M$   $ZnSO_4$ , 0.2  $\mu M$   $CuSO_4$ , 0.05  $\mu M$   $(NH_4)_6Mo_7O_{24}$ , and 60  $\mu M$  Fe-EDTA. Plants were subjected to five different treatments as follows: positive control (+Mg @ 0.5 mM), negative control (−Mg @ 0 mM), sufficient Mg and DS (+Mg @ 0.5 mM), deficient Mg and DS (−Mg @ 0 mM), and leaf-application of  $MgSO_4$  with DS (+Mg @ 250 mM). Control plants were supplied with 0.5 mM  $MgSO_4$  to the roots. The experimental treatments had four biological replicates arranged in a completely randomized design. The foliar application was conducted four times, starting at 21 days after transplanting (DAT) on every alternate day (21, 23, 25, and 27 DAT). The application was conducted using a brush by gently rubbing over the adaxial and abaxial surfaces of the leaf. The plants were harvested at 35 DAT. The plants were subjected to DS for about one week before the harvest. Subsequently, the fresh weight (FW) was recorded immediately after the plants were harvested. Dry weight (DW) was determined after placing the plant shoots into a drying oven for 72 h at 65°C.  $Mg^{2+}$  is a macronutrient; however, a low supply would also be sufficient to meet the plant's physiological needs. The research utilized a hydroponic system and induced DS during 28 DAT by polyethylene glycol (PEG). PEG, with a molecular weight of 6000, is a natural, water-soluble, and nonionic polymer. This method mimics DS, which reduces the plant's water potential due to osmotic stress (Muscolo et al. 2014).

### 2.2 | Quantification of $Mg^{2+}$ , $K^+$ , and $Mn^{2+}$

For the quantification of  $Mg^{2+}$ ,  $K^+$ , and  $Mn^{2+}$  accumulation, the dried plant samples obtained from the top four fully grown active leaves were ground into a fine powder. Around 200 mg was digested with 10 mL of 69%  $HNO_3$  (ROTIPURAN Supra

for ICP, 69%) in an 1800 W microwave oven (MARS 6 Xpress; CEM, Matthews, MC, USA) with the following program: 2 min at 100°C, 1 min at 120°C, 20 min at 180°C, and 20 min cooling time. Samples were diluted with deionized sterile water (18.2 MΩ cm conductivity) to 100 mL afterward and kept at 4°C until analysis. Mg<sup>2+</sup>, K<sup>+</sup>, and Mn<sup>2+</sup> concentrations were quantified using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent Technologies 7700 Series, Böblingen, Germany). Blank digestions were performed in the same way as for the samples. For every 20 samples, internal mineral standards and reference material samples were analyzed as an internal control.

### 2.3 | SPAD and Gas Exchange Measurements

A fully expanded fourth leaf was monitored in each pot to measure relative chlorophyll content, CO<sub>2</sub> and H<sub>2</sub>O exchange, and chlorophyll fluorescence. The relative leaf chlorophyll content was determined with a Konica-Minolta SPAD-502 meter (Minolta Camera Co. Ltd., Japan). Measurements were made on each leaf at 20, 25, 30, and 35 DAT. Leaf gas exchange was recorded from 9:00 a.m. to 2:00 p.m. by using an infrared gas analyzer (IRGA) (model LI-6400, Li-COR Biosciences, Lincoln, NE, USA). The LI-6400 was operated as an open system. The leaf temperature, flow rate, and CO<sub>2</sub> (Ref CO<sub>2</sub>) concentration were set at 20°C, 500 μmol/s, and 400 mL/L, respectively. The leaves attached to the stem were inserted into the chamber (2 cm × 3 cm) and held horizontally to receive enough light within the chamber. The temperature and CO<sub>2</sub> within the chamber were controlled by the LI-6400 portable photosynthesis system.

### 2.4 | Lipid Peroxidation Assay

Malondialdehyde (MDA) content is regarded as an indicator of the level of DS-induced peroxidation of the lipids. The concentration of MDA was determined according to the procedure of Cui and Wang (2006) with modifications. Samples obtained from primary leaves weighing 500 mg were homogenized in 10 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 rpm for 5 min. The supernatant of around 1 mL was collected and used for the assay. The reaction mixture containing 1.0 mL of aliquot and 4 mL of 0.5% TBA in 20% TCA was heated at 95°C for 1 h, cooled to room temperature, and centrifuged at 10,000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm and corrected for specific turbidity by subtracting the absorbance at 600 nm. The amount of MDA was calculated by using an extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup> and expressed in nmol mL<sup>-1</sup> FW.

### 2.5 | Determination of Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>)

The concentration of H<sub>2</sub>O<sub>2</sub> was determined using the method described by Hussain et al. (2023). The primary leaves weighing 500 mg were homogenized in 1.6 mL of 0.1% TCA in an ice bath for 30 min. The resulting mixture was centrifuged at 12,000 rpm at 4°C for 20 min. To 0.5 mL of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (K-P buffer; pH 7.8) and 1 mL of 1 M potassium iodide (KI) were added. The mixture was then placed

in the dark for approximately 1 h. The concentration of H<sub>2</sub>O<sub>2</sub> was determined by measuring the absorbance of the mixture at 390 nm using a standard curve. The final concentration was expressed as μmol g<sup>-1</sup> FW.

### 2.6 | Determination of Soluble Sugars

Approximately, 0.02 g of finely ground samples collected from fully grown top four leaves were extracted in 1 mL of deionized water at 100°C for 5 min to quantify soluble sugars following a modified protocol described by Cataldi et al. (2000). Briefly, the extracts were placed on ice for 30 min, and consequently, the mixture was centrifuged at 12,000 rpm for 10 min. Subsequently, the supernatants were diluted 10-fold with deionized water and then vortexed. A few drops of chloroform were added, and the mixture was centrifuged at 4°C for 5 min at 12,000 rpm. Subsequently, the collected supernatants were purified by C18 columns (Strata 8B-S001-DAK, Phenomenex, Torrance, CA, USA). Soluble sugars (glucose [Glu], fructose [Fru], and sucrose [Suc]) were separated using Dionex Carbo Pac PA 100 IC column (Thermo Scientific) as described by Abdalla, Nisa, and Mühling (2023).

### 2.7 | Determination of Proline

About 0.5 g of primary leaves were ground in 10 mL of 3% aqueous sulfosalicylic acid homogenized and filtrated via Whatman No.2. Subsequently, 2 mL of extract was taken and mixed with 2 mL of acid ninhydrin and 2 mL of glacial acetic acid and heated at 100°C for 1 h. Later, samples were allowed to cool in an ice bath to stop the reaction. The reaction mixture was then extracted with 4 mL toluene mixed vigorously using a magnetic stirrer for 15–20 s. Chromophore containing toluene was aspirated from the aqueous phase, the solution was warmed to room temperature, and absorbance was recorded at 520 nm with toluene as blank. Proline content was calculated using the formula (Bates, Waldren, and Teare 1973):

$$\text{Proline } (\mu\text{mole g}^{-1} \text{ FW}) = (\mu\text{g proline mL}^{-1} \times \text{toluene in mL}) / (115.5 \mu\text{g}/\mu\text{mol}) / (\text{sample [g]} / 5)$$

### 2.8 | Determination of CAT, SOD, and APX Enzyme Activity Assays

CAT, SOD, and APX enzyme activity assays were determined as described by Alici and Arabaci (2016). For CAT activity, 3 mL of the reaction mixture was prepared in a test tube by dissolving 100 mM phosphate buffer with pH 7.0 (1.9 mL), 30 mM H<sub>2</sub>O<sub>2</sub> (1.0 mL), and 100 μL of primary leaf sample extract. Just before the spectrophotometric readings, the sample was added to the reaction mixture. A test tube containing 100 mM potassium phosphate buffer with pH 7.0 (2 mL) and 30 mM H<sub>2</sub>O<sub>2</sub> (1.0 mL) without sample extract was served as blank. The absorbance was measured at 240 nm. The sample was mixed thoroughly into the reaction mixture, reading the absorbance immediately at 0, 30, 60, 90, 120, 150, and 180 s. The CAT activity was determined spectrophotometrically at room temperature by monitoring the decrease in absorbance resulting from the decomposition of H<sub>2</sub>O<sub>2</sub>

at 240 nm. The enzyme activity was expressed in  $\mu\text{M H}_2\text{O}_2$  ( $\epsilon = 39.4 \text{ mM}^{-1}\text{cm}^{-1}$ ) oxidized  $\text{min}^{-1} \text{mg}^{-1}$  protein. SOD activity was determined by the inhibition in the photoreduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are generated by the autooxidation of hydroxylamine hydrochloride. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 50 mM sodium carbonate buffer (pH 10.2), 96  $\mu\text{M}$  NBT, 20 mM hydroxylamine chloride (pH 6.0), Triton X-100 (0.6%), and 70  $\mu\text{L}$  of crude extract in a final volume of 3.0 mL. A control reaction was performed without crude extract. The SOD reaction was carried out by exposing the reaction mixture to white light for 15 min at room temperature. After 15 min incubation, absorbance was recorded at 560 nm using a spectrophotometer. One unit (U) of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT. APX was determined by the decrease in absorbance at 290 nm following  $\text{H}_2\text{O}_2$ -dependent oxidation of ascorbate (Nakano and Asada 1981). The assay mixture contained 50 mM K-P buffer (pH 7.0), 0.1 mM EDTA and 0.1 mM  $\text{H}_2\text{O}_2$ . The activity of APX was calculated using the extinction coefficient of  $2.8 \text{ mM}^{-1} \text{cm}^{-1}$ , and one unit of APX was defined as  $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{mg}^{-1}$  protein.

## 2.9 | Morphological Analyses

A mid part of a photosynthetically active fourth leaf was harvested, immediately cut up into segments with 1–2 mm long sides, and fixed with 1% glutaraldehyde in 200 mM Hepes, pH 7.4, initially under moderate vacuum pressure. Next, segments were postfixed with 1% osmium tetroxide supplemented with 1.5% aqueous potassium ferricyanide for 1 h on ice, followed by incubation with 2% aqueous uranyl acetate for 1.5 h at room temperature. Samples were dehydrated using a graded ethanol series (50%–70%–80%–90%–96%–100%–100%), each step for 15–30 min, followed by acetone (100%),  $2 \times 30$  min, and then gradually infiltrated with epoxy resin diluted with acetone (33%–66%–100%), each step for minimum 12 h. Finally, tissue was embedded in silicone molds, and resin was heat polymerized at  $60^\circ\text{C}$  for 48 h. A Leica UC7 ultramicrotome and Diatome diamond knives were used to make sections. Semithin, 1  $\mu\text{m}$  thick epoxy sections were stained with Richardson's solution and imaged with a Zeiss Primostar upright microscope equipped with an Axiocam 105 color camera and ZEN 3.2 software (Zeiss, Oberkochen, Germany), using 10 $\times$  and 40 $\times$  Plan-Achromat objectives. Ultrathin sections (70–80 nm) were transferred onto formvar-coated slot grids. The sections were then contrasted with saturated aqueous 2% uranyl acetate for 10 min, followed by Reynolds' lead citrate solution for 3 min. Subsequently, the sections were examined using a CM10 transmission electron microscope (Philips) operated at 80 kV and equipped with a LaB6 filament. Images were taken with a MegaView III camera (Olympus Soft Imaging Solutions).

## 2.10 | Statistical Analysis

Statistical analysis was performed by using the STATISTICS 10 software. Analysis of variance (ANOVA) was used to determine the significance of the effects of treatments. The means were compared by Tukey's honestly significant difference (HSD) TEST ( $p \leq 0.05$ ), where ANOVA indicated a significant impact.

## 3 | Results and Discussion

### 3.1 | Plant Biomass

Growth data of the faba bean plants grown in control conditions or subjected to drought at various levels of  $\text{Mg}^{2+}$  are presented in Figure 1. For each group, the results represent the mean of four plants. Compared to  $\text{Mg}^{2+}$  sufficient control plants (+Mg), in  $\text{Mg}^{2+}$  deficient plants grown in the control condition (−Mg) or subjected to DS (−Mg  $\times$  DS), the shoot FW decreased by about 51% and 57%, respectively. On the other hand, in plants supplemented with sufficient  $\text{Mg}^{2+}$  under DS (+Mg  $\times$  DS), the shoot FW was reduced by only 7%. Similarly, (−Mg) and (−Mg  $\times$  DS) showed a reduction of root FW of around 71% and 84%, respectively. Shoot DW of (−Mg) and (−Mg  $\times$  DS) was reduced by 42% and 52%. A similar trend was observed in (−Mg) and (−Mg  $\times$  DS) with a significant reduction in root DW to 73% and 87%, respectively. The plants supplemented with leaf application of  $\text{Mg}^{2+}$  under DS (+Mg LA  $\times$  DS) showed significantly increased shoot and root FW when compared to (−Mg  $\times$  DS) (Figure 1).

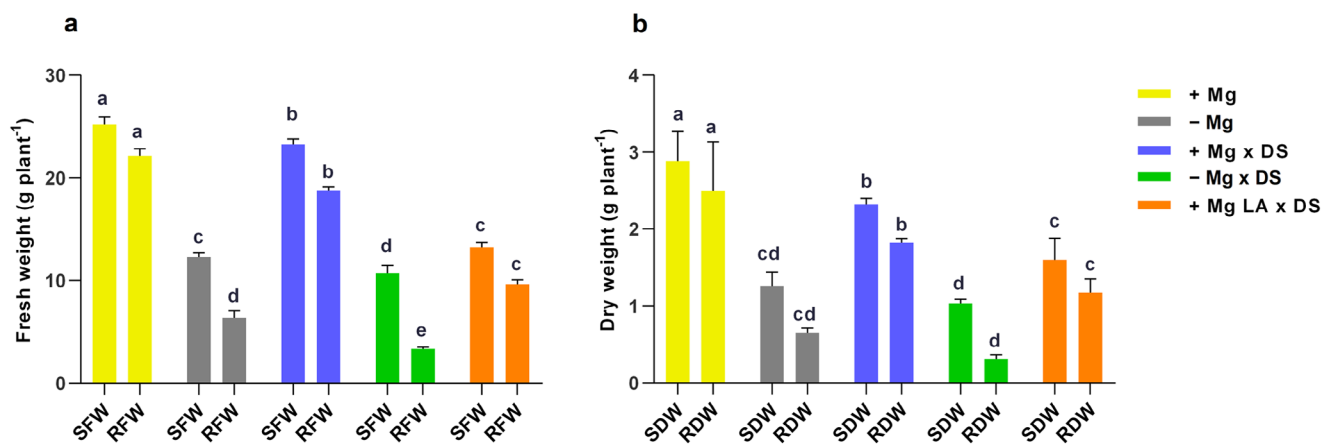
It was reported earlier that  $\text{Mg}^{2+}$  deficiency results in a lower yield of the faba bean plants than the positive control as  $\text{Mg}^{2+}$  is involved in carbohydrate and protein biosynthesis (Neuhaus, Geilfus, and Mühling 2014). Moreover, a severe decrease in root biomass has been reported in bean plantlets after 12 days of complete  $\text{Mg}^{2+}$  starvation (Cakmak et al. 1994).  $\text{Mg}^{2+}$  limitation has affected leaf area increment of all leaves and shoot biomass, which could have also readily restricted assimilate flux to the root. The reduction in the growth rate due to water stress in the shoot and the root of the faba bean could be ascertained as the loss of turgidity, decrease in relative water content, reduction in cell division and elongation, the decline of net  $\text{CO}_2$  assimilation rate, stomatal conductance, and transpiration rate (Abid et al. 2017). In an earlier study, the foliar application of  $\text{Mg}^{2+}$  in faba bean plants demonstrated a remarkable strategy for improving growth and yield under conditions of  $\text{Mg}^{2+}$  deficiency (Neuhaus et al. 2014; Wolf et al. 2019). Additionally,  $\text{Mg}^{2+}$  application ameliorated adverse effects under water deficit conditions (Santos et al. 2023).

### 3.2 | $\text{K}^+$ , $\text{Mg}^{2+}$ , and $\text{Mn}^{2+}$ Concentrations in Leaves

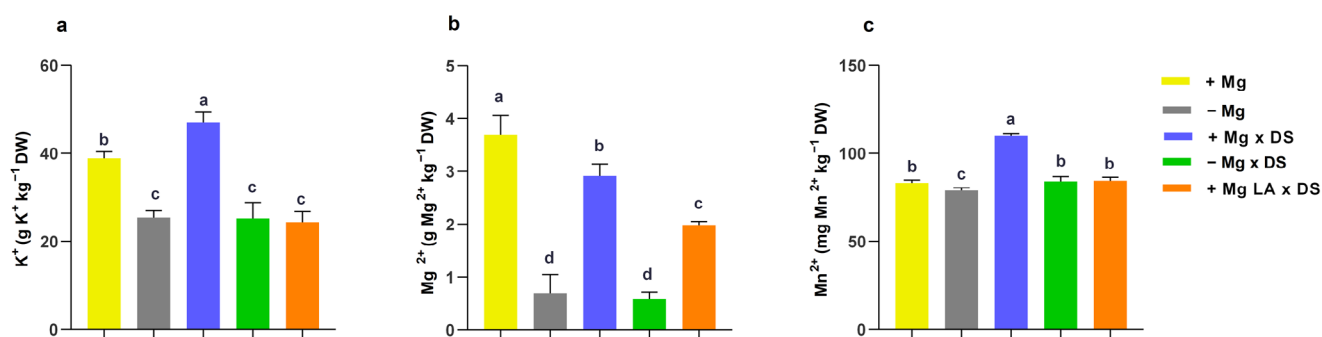
Higher  $\text{K}^+$  concentration (by 1.2-fold) was observed in leaves in (+Mg  $\times$  DS) conditions compared to the positive control (+Mg). Meanwhile,  $\text{K}^+$  concentration diminished (80%) in all groups without root supply of  $\text{Mg}^{2+}$ , and leaf application of  $\text{Mg}^{2+}$  had no effect (Figure 2a). Regarding  $\text{Mg}^{2+}$  accumulation under varied treatments,  $\text{Mg}^{2+}$  application via roots under DS (+Mg  $\times$  DS) displayed higher  $\text{Mg}^{2+}$  levels than foliar application (+Mg LA  $\times$  DS) (Figure 2b). Compared to (+Mg),  $\text{Mn}^{2+}$  concentration was increased (by 1.3-fold) only in (+Mg  $\times$  DS), whereas no changes were observed in −Mg  $\times$  DS and +Mg LA  $\times$  DS (Figure 2c).

In our study,  $\text{Mg}^{2+}$  deficiency led to reduced  $\text{K}^+$  and  $\text{Mn}^{2+}$  concentration in leaves, and  $\text{Mg}^{2+}$  foliar application could not reverse it. Under sufficient  $\text{Mg}^{2+}$  root supply,  $\text{K}^+$  concentration under DS increased, which suggests that the uptake of  $\text{K}^+$  is likely associated with an extensive and dense root system, which





**FIGURE 1** | (a) Fresh weight and (b) dry weight of shoots and roots of 35 days old faba bean plants subjected to DS at 28 DAT. +Mg (0.5 mM MgSO<sub>4</sub>), -Mg (0 mM MgSO<sub>4</sub>), +Mg × DS (0.5 mM MgSO<sub>4</sub>), -Mg × DS (0 mM MgSO<sub>4</sub>), +Mg LA × DS (leaf-application of 250 mM MgSO<sub>4</sub> for four times). Each value is the mean of four replicates; the bars represent ± standard error. Small letters indicate significant differences between treatments (ANOVA with Tukey's HSD,  $p \leq 0.05$ ). ANOVA, analysis of variance; DAT, days after transplanting; DS, drought stress; HSD, honestly significant difference; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight; RDW, root dry weight.



**FIGURE 2** | The effect of Mg supply on (a) [K<sup>+</sup>], (b) [Mg<sup>2+</sup>], and (c) [Mn<sup>2+</sup>] in leaves analyzed at harvest (35 DAT). +Mg (0.5 mM MgSO<sub>4</sub>), -Mg (0 mM MgSO<sub>4</sub>), +Mg × DS (0.5 mM MgSO<sub>4</sub>), -Mg × DS (0 mM MgSO<sub>4</sub>), +Mg LA × DS (leaf-application of 250 mM MgSO<sub>4</sub> for four times). Each value is the mean of four replicates; the bars represent ± standard error. Small letters indicate significant differences between treatments (ANOVA with Tukey's HSD,  $p \leq 0.05$ ). ANOVA, analysis of variance; DAT, days after transplanting; DS, drought stress.

most likely optimizes water uptake coupled with ion uptake. K<sup>+</sup> increases the plant's drought tolerance by osmoregulation, maintains turgor pressure, and reduces transpiration under DS. Similar results were observed by Morgan (1992) in wheat lines displaying high osmotic adjustments through the accumulation of K<sup>+</sup> in their tissues. K<sup>+</sup> concentrations in leaves diminished to the level of negative control plants (-Mg) upon resupply of Mg<sup>2+</sup> via leaf under DS, illustrating the antagonistic interaction between these cations (Jezek et al. 2015). Although Mg<sup>2+</sup> was enhanced under Mg<sup>2+</sup> leaf application and DS compared to the negative control, Mg<sup>2+</sup> was still higher under root application and DS. This might be attributed to limited Mg<sup>2+</sup> translocation within faba bean plants from the application site to the sites of excessive nutrient demand as Mg<sup>2+</sup> is a tremendously phloem-mobile cation (White and Broadley 2009).

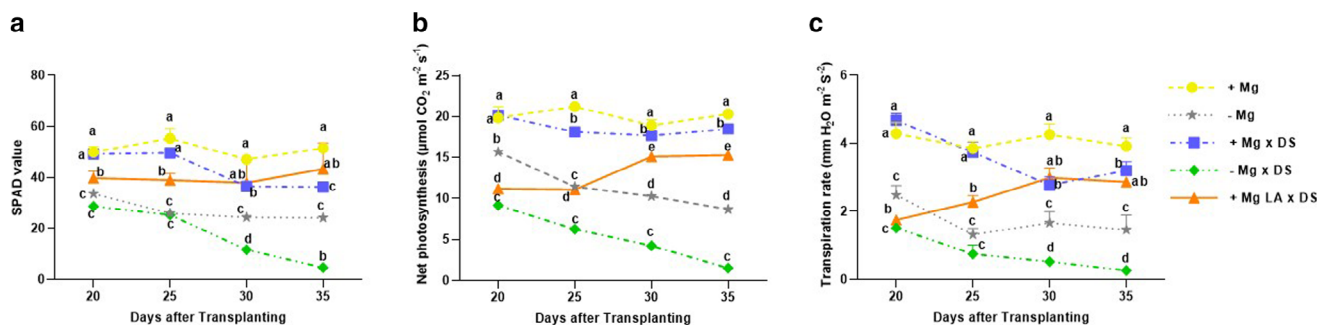
Mn<sup>2+</sup> is necessary for plant growth. However, excessive amounts of it in the growth medium can be harmful. Even concentrations as low as 1 mg Mn/L in solution culture can be toxic to plants like tobacco, potato, or bush clover (Wang et al. 2002). Elevated levels of Mn<sup>2+</sup> in the growth medium can interfere with the absorption,

translocation, and utilization of other essential mineral elements like calcium, magnesium, iron, and phosphorus (Clark 1982). High Mn<sup>2+</sup> in plant tissues can also alter the activities of enzymes and hormones, making other Mn-requiring processes less active or nonfunctional (Horst 1988).

A higher accumulation of Mn<sup>2+</sup> in leaves of Mg-sufficient plants under DS was observed in the current study. Plants grown under Mg<sup>2+</sup> foliar application and DS have a slightly lower Mn<sup>2+</sup> than plants resupplied with Mg<sup>2+</sup> via root application and DS. Such antagonistic interactions between Mg<sup>2+</sup> and Mn<sup>2+</sup> were also reported in wheat and tomato plants (Husted et al. 2009).

### 3.3 | Chlorophyll Concentration and Gas Exchange Parameters

Mg<sup>2+</sup> deficiency symptoms (interveinal chlorosis) on the older leaves tended to start after 3 weeks from transplanting faba bean plants grown under Mg<sup>2+</sup> limitation. Consequently, DS aggravated the severity of these symptoms in Mg<sup>2+</sup> deficient plants.



**FIGURE 3** | (a) Course of SPAD values of the sixth leaf over time, (b) net CO<sub>2</sub> assimilation rate (μmol CO<sub>2</sub>/m<sup>2</sup>/s) of the sixth leaf and (c) transpiration rate mm H<sub>2</sub>O/(m s)<sup>2</sup> of the sixth leaf. +Mg (0.5 mM MgSO<sub>4</sub>), -Mg (0 mM MgSO<sub>4</sub>), +Mg x DS (0.5 mM MgSO<sub>4</sub>), -Mg x DS (0 mM MgSO<sub>4</sub>), +Mg LA x DS (leaf-application of 250 mM MgSO<sub>4</sub> for four times). Each value is the mean of four replicates; the bars represent ± standard error. Small letters indicate significant differences between treatments (ANOVA with Tukey's HSD,  $p \leq 0.05$ ). ANOVA, analysis of variance; DS, drought stress.

As expected, the chlorophyll concentration values measured by SPAD were significantly reduced in (-Mg) and (-Mg x DS). On the other hand, the resupply of Mg<sup>2+</sup> via leaf application of MgSO<sub>4</sub> on Day 21 immediately increased chlorophyll content. However, after 7 days of drought treatment, no further increments in chlorophyll concentration (SPAD values) were observed under both conditions, including sufficient Mg<sup>2+</sup> and DS (+Mg x DS) and leaf application of Mg<sup>2+</sup> and DS (+Mg LA x DS). Meanwhile, Mg<sup>2+</sup> deficient and DS plants (-Mg x DS) recorded the lowest SPAD values (Figure 3a).

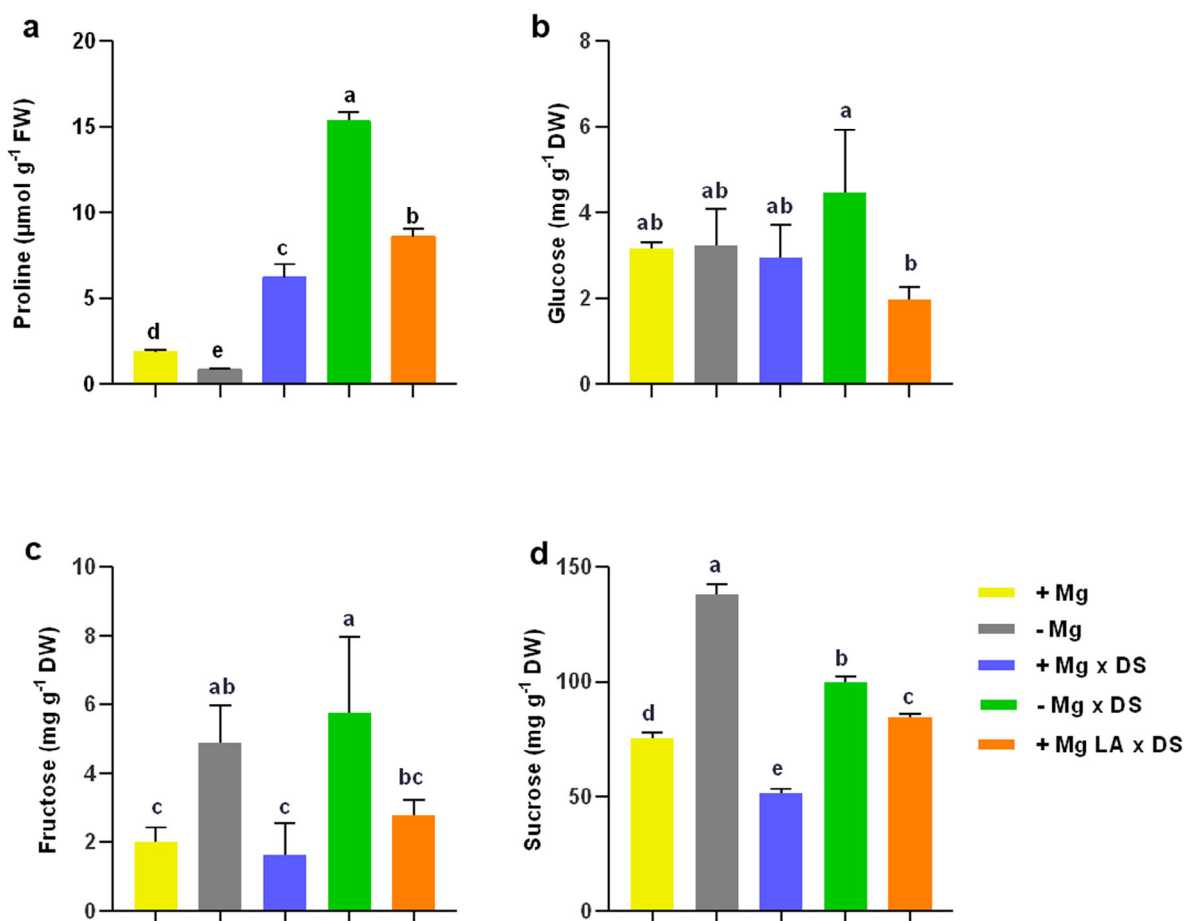
Net CO<sub>2</sub> assimilation and transpiration rates of both positive (+Mg) and negative control (-Mg) plants demonstrated a significant ( $p < 0.05$ ) difference as revealed by the gas exchange measurements (Figure 3b). Positive control (+Mg) plants exhibited more or less stable assimilation rates of around 20 μmol CO<sub>2</sub>/m<sup>2</sup>s<sup>-1</sup>. Moreover, (-Mg) and (-Mg x DS) had a severe negative impact on the rate of assimilation, which reached close to zero as the plants started withering and wilting 5 days after drought initiation. Negative control plants (-Mg) have also recorded a declining trend in net assimilation rates by 5 WAT. On the other hand, (+Mg x DS) displayed a higher net assimilation rate, which is on par with the positive control (+Mg) plants. Surprisingly, the relative contribution of Mg<sup>2+</sup> leaf application to an increase in the net assimilation rate was recorded, which remained stable during the last week of DS (Figure 3b). Similar trends in the transpiration rate were observed in positive control plants (+Mg), which recorded the highest rates of transpiration. However, (-Mg x DS) treatment exhibited the lowest transpiration rates by the end of 5 weeks (Figure 3c).

A decrease in chlorophyll levels and net photosynthesis during DS has been reported in various studies, depending on the duration and severity of drought (Kyparissis, Petropoulou, and Manetas 1995). In this study, Mg deficiency under DS led to a significant reduction in chlorophyll content, as evident from the SPAD values (Figure 3a). This led to a complete inhibition in photosynthetic activity (Figure 3b,c). As Mg<sup>2+</sup> is a crucial constituent of chlorophyll molecules, it is associated with inter-veinal chlorosis. These symptoms indicate the high involvement of Mg<sup>2+</sup> in chlorophyll synthesis, the main pigment responsible for plant light harvesting. Hence, Mg<sup>2+</sup> deficiency inhibited photosynthesis, carbohydrate accumulation in source leaves, and

reduced root growth. The importance of Mg<sup>2+</sup> in Suc phloem loading was related to its interaction with nucleotide triphosphate (Mg-ATP), fueling the H<sup>+</sup> ATPases in the plasma membranes of sieve tube cells. Lack of Mg<sup>2+</sup> reduced H<sup>+</sup> ATPase activity in sieve elements results in an abnormal buildup of nonstructural carbohydrates in Mg<sup>2+</sup> deficient leaves (Tanoi and Kobayashi 2015). On the other hand, excessive accumulation of carbohydrates is also detrimental in terms of irreversible structural anomalies leading to the destruction of the organelles. Excessive deposition of the starch granules led to the chloroplast's swelling and the breakdown of the organelle membrane surrounding the chloroplast. The underlying mechanism for so-called autophagy or cell necrosis is due to the DS-regulated metabolic breakdown of chloroplasts to provide access to stored reserves that may sustain the survival of plants under unfavorable conditions. A decrease in total chlorophyll under DS implies a lowered capacity for light harvesting. As the production of ROS is mainly driven by the absorption of excess energy in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments (Herbinger et al. 2002).

### 3.4 | The Osmolyte Accumulation

DS significantly ( $p \leq 0.05$ ) enhanced proline levels in all plants subjected to Mg<sup>2+</sup> limitation treatment compared to a positive control (Figure 4a). The highest proline concentration was measured in (+Mg x DS), it was increased by 233% compared to (+Mg x DS). Next, the three major soluble sugars, Fru, Glu, and Suc in the leaves were quantified (Figure 4b-d). In control conditions, Mg<sup>2+</sup> deficiency (-Mg) resulted in a significant increase in the concentration of soluble Suc (1.8fold) compared to (+Mg). Under DS, Suc concentration decreased compared to control conditions irrespective of Mg supply. In (+Mg x DS), the Suc concentration decreased by 31% with respect to (+Mg). Suc concentration in (-Mg x DA) and (+Mg LA x DS) was significantly higher than in (+Mg x DS). Absolute values for Glu and Fru concentration were significantly lower, resulting in higher variation of data. Concentrations of these two sugars were increased in (-Mg x DS) compared to (+Mg x DS). In (+Mg LA x DS), the increase in Fru concentration was smaller, whereas the Glu concentration decreased also in comparison to (+Mg x DS).

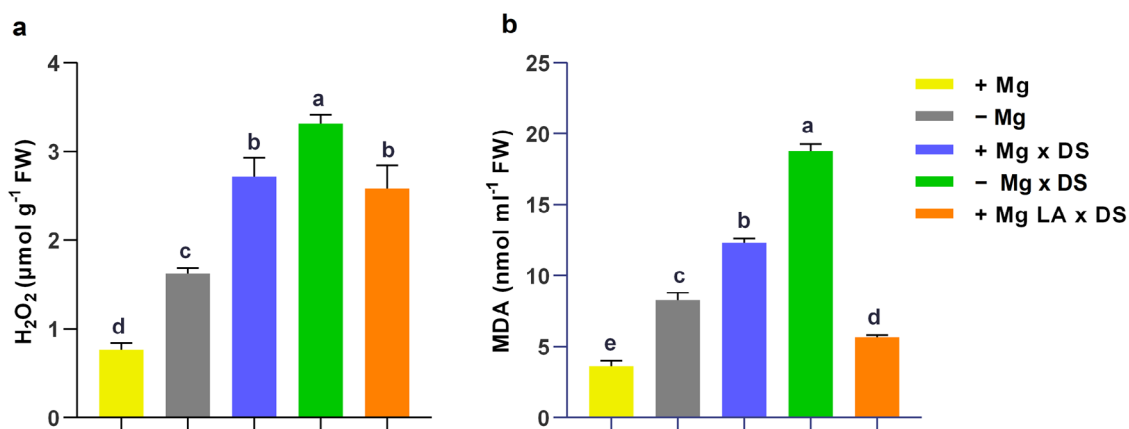


**FIGURE 4** | Accumulation of osmolytes: (a) proline ( $\mu\text{mol g}^{-1}\text{ FW}$ ), (b) glucose, (c) fructose, and (d) sucrose ( $\text{mg g}^{-1}\text{ DW}$ ) in leaves harvested at 35 DAT. +Mg (0.5 mM  $\text{MgSO}_4$ ), -Mg (0 mM  $\text{MgSO}_4$ ), +Mg x DS (0.5 mM  $\text{MgSO}_4$ ), -Mg x DS (0 mM  $\text{MgSO}_4$ ), +Mg LA x DS (leaf-application of 250 mM  $\text{MgSO}_4$  for four times). Each value is the mean of four replicates; the bars represent  $\pm$  standard error. Small letters indicate significant differences between treatments (ANOVA with Tukey's HSD,  $p \leq 0.05$ ). ANOVA, analysis of variance; DAT, days after transplanting; DS, drought stress; DW, dry weight; FW, fresh weight; HSD, honestly significant difference.

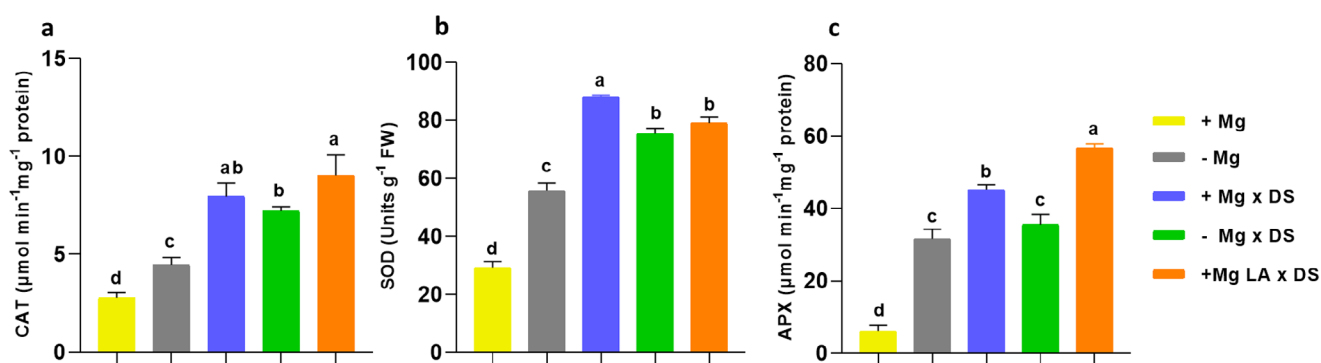
Induction of adaptive morpho-physiological and biochemical responses like the accumulation of large quantities of osmolytes, including organic solutes like proline, in response to DS is the major adaptive mechanism in plants (Ma et al. 2020). Moreover, the accumulation of osmoprotectants mitigates the ROS levels, prevents cellular damage, provides energy to manage stressful conditions, and enhances growth and development (Fàbregas and Fernie 2019).

Suc is the main photosynthetic product in higher plants and is the principal carbohydrate translocated from source leaves to sink organs such as developing roots. Hence, different environmental factors, particularly macronutrient (N, P,  $\text{K}^+$ , and  $\text{Mg}^{2+}$ ) deficiencies, were reported to affect the partitioning of Suc and starch. Our data showed that the highest concentration of Suc was reported in (-Mg). Suc is not just a passive molecule for long-distance transport. A negative correlation was observed between Suc level on one side, and photosynthetic activities, chlorophyll content, and expression of photosynthetic genes on the other (Hermans et al. 2004). In plants grown under  $\text{Mg}^{2+}$  deficiency and DS, Suc concentration was reduced compared to the negative control. It is known that DS causes changes in the source-sink relationship, as well as in the partitioning between Suc and starch in leaves,

to favor the export of Suc toward the sink organs, thus leading to a decline in Suc and starch accumulation in source leaves and an increase of Suc in various sinks (Basu, Ali, and Chaturvedi 2007). On the other hand, our results show that Glu and Fru concentrations increased under DS coupled with  $\text{Mg}^{2+}$  deficiency. Similar results were observed in pigeon pea (*Cajanus cajan* (L.) Millsp) by Keller and Ludlow (1993), who reported that drought caused a moderate increase in Glu and Fru concentrations as well as a pronounced increase in the activities of enzymes hydrolyzing soluble starch (amylases) and Suc (invertase and Suc synthase) so that the Suc content of leaves became diminished. Elevated concentrations of Glu and Fru due to more significant acid invertase activity in leaves could lead to maintaining leaf turgor pressure, thus enhancing the probability of survival during a considerable period of DS (Liu, Jensen, and Andersen 2004). DS stimulates the mobilization of soluble sugars to high-demand organs, as Nerd and Neumann (2004) demonstrated in *Hylocereus undatus*.  $\text{Mg}^{2+}$  application rapidly enhanced Suc export to phloem from source leaves (Hermans and Verbruggen 2005). Such fast recovery of Suc export is achieved during dark and light circumstances, confirming that increased Suc export after  $\text{Mg}^{2+}$  supplementation is not related to photosynthesis but only to the availability of  $\text{Mg}^{2+}$  (Guo et al. 2016).



**FIGURE 5** | The effect of Mg under DS on (a) H<sub>2</sub>O<sub>2</sub> production and (b) lipid peroxidation (MDA). +Mg (0.5 mM MgSO<sub>4</sub>), -Mg (0 mM MgSO<sub>4</sub>), +Mg x DS (0.5 mM MgSO<sub>4</sub>), -Mg x DS (0 mM MgSO<sub>4</sub>), +Mg LA x DS (leaf-application of 250 mM MgSO<sub>4</sub> for four times). Each value is the mean of four replicates; the bars represent  $\pm$  standard error. Small letters indicate significant differences between treatments (ANOVA with Tukey's HSD,  $p \leq 0.05$ ). ANOVA, analysis of variance; DS, drought stress; HSD, honestly significant difference; MDA, malondialdehyde.



**FIGURE 6** | The effect of Mg under DS on (a) CAT, (b) SOD, and (c) APX activity. +Mg (0.5 mM MgSO<sub>4</sub>), -Mg (0 mM MgSO<sub>4</sub>), +Mg x DS (0.5 mM MgSO<sub>4</sub>), -Mg x DS (0 mM MgSO<sub>4</sub>), +Mg LA x DS (leaf-application of 250 mM MgSO<sub>4</sub> for four times). Each value is the mean of four replicates; the bars represent  $\pm$  standard error. Small letters indicate significant differences between treatments (ANOVA with Tukey's HSD,  $p \leq 0.05$ ). ANOVA, analysis of variance; APX, ascorbate peroxidase; CAT, catalase; DS, drought stress; SOD, superoxide dismutase.

### 3.5 | ROS Production, Lipid Peroxidation, and Antioxidant Enzyme Activity

To measure the amount of lipid peroxidation, MDA, a product generated during the oxidation of membrane lipids induced by ROS like H<sub>2</sub>O<sub>2</sub>, was monitored (Figure 5). The amounts of MDA in (-Mg x DS) were significantly higher with an increase of about 416% compared to the positive control (+Mg). On the other hand, (+Mg LA x DS) resulted in a significantly lower amount of MDA content with an increase of around 55% compared to the positive control (+Mg).

The effect of Mg on the activity of antioxidant enzymes CAT, APX, and SOD under drought-stressed faba bean is presented in Figure 6. Activities of the enzymes were increased by Mg<sup>2+</sup> limitation and/or DS. Compared to (+Mg), CAT activity increased 1.68fold, 5.17fold, 4.43fold, and 6.29fold in (-Mg), (+Mg x DS), (-Mg x DS), and (+Mg LA x DS), respectively (Figure 6a). SOD activity was enhanced the most (58-fold) in (+Mg x DS) followed by (-Mg x DS) (46-fold), (+Mg x LA DS) (49-fold), and (-Mg) (26-fold) (Figure 6b). Similarly, the highest increase in APX activity compared to (+Mg) was reported in (+Mg LA x DS) (7.4-

fold) followed by (+Mg x DS) (5.84-fold) and (-Mg) (4.98-fold) (Figure 6c).

We observed that Mg<sup>2+</sup> deficiency or/and DS increased both the oxidative stress markers as well as the activity of the antioxidant defense enzymes. The two systems are linked, and a higher accumulation of H<sub>2</sub>O<sub>2</sub> was observed in Mg<sup>2+</sup> deficient DS plants thus indicating that the antioxidant defense system cannot completely buffer the increased production of ROS. It has been reported previously that Mg-deficiency-induced up-regulation in the leaf antioxidant system does not provide enough protection to Mg-deficient leaves against oxidative damage (Yang et al. 2012). It was observed that an elevated lipid peroxidation can alter membrane permeability and ultimately result in the degeneration of chloroplasts coupled with a disturbance in their ultrastructure by modifying grana and stroma stacking (Shao et al. 2016). DS leads to oxidative stress in cells due to intensified production of ROS like alkoxy radicals, superoxide anion, H<sub>2</sub>O<sub>2</sub>, and hydroxyl radical (OH•) (Sies et al. 2020). Chloroplasts, mitochondria, and peroxisomes are the damage sites where DNA denaturation restricts normal cell function (Corpas et al. 2015).



**TABLE 1** | The effect of Mg application on leaves anatomical characters of faba bean grown under DS.

Parameters	Control + Mg	Control – Mg	DS + Mg	DS – Mg	DS + Mg LA
<b>A (Based on fresh leaves; <math>n = 4</math>)</b>					
Leaflet length (cm)	10.20 $\pm$ 0.39 a	7.24 $\pm$ 0.25 c	9.75 $\pm$ 0.18 ab	4.20 $\pm$ 0.38 d	8.80 $\pm$ 0.22 b
Leaflet width (cm)	4.64 $\pm$ 0.09 a	3.30 $\pm$ 0.01 b	4.47 $\pm$ 0.11 a	2.57 $\pm$ 0.15 c	4.17 $\pm$ 0.22 a
<b>B (Based on Technovit resin sections; <math>n \geq 3</math>)</b>					
Leaf thickness ( $\mu\text{m}$ )	528 $\pm$ 62	428 $\pm$ 32	472 $\pm$ 30	465 $\pm$ 57	431 $\pm$ 59
The thickness of palisade mesophyll (PM) ( $\mu\text{m}$ )	131 $\pm$ 7	121 $\pm$ 13	126 $\pm$ 6	123 $\pm$ 19	114 $\pm$ 11
The thickness of spongy mesophyll (SM) ( $\mu\text{m}$ )	339 $\pm$ 52	258 $\pm$ 11	291 $\pm$ 27	286 $\pm$ 36	265 $\pm$ 51
Ratio of PM and SM (%)	0.40 $\pm$ 0.05	0.47 $\pm$ 0.04	0.43 $\pm$ 0.02	0.44 $\pm$ 0.06	0.44 $\pm$ 0.04

Note: Small letters indicate significant differences between treatments (ANOVA with Tukey's HSD,  $p \leq 0.05$ ).

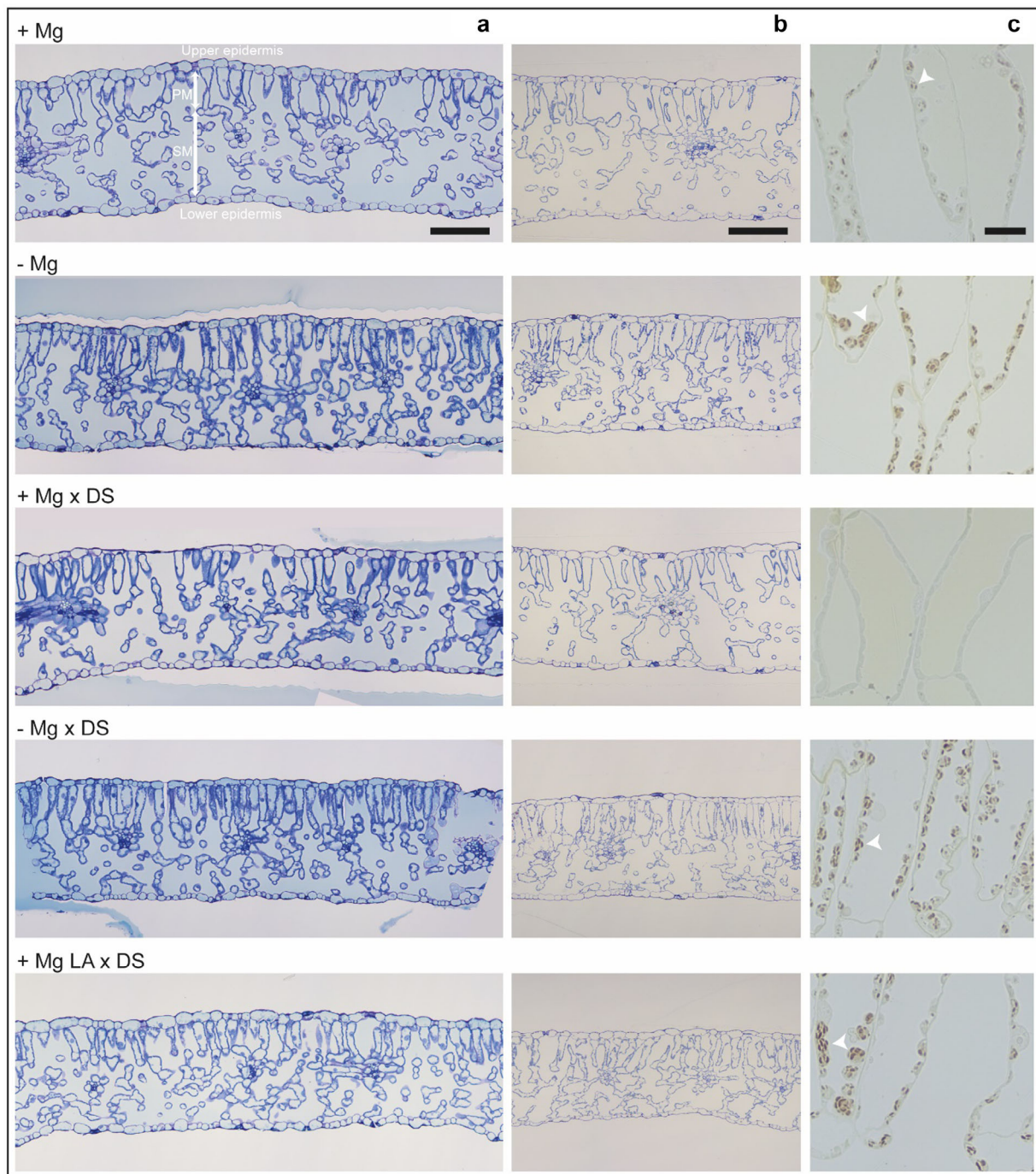
In our study, DS increased the up-regulation of enzymatic antioxidant defense systems to reduce  $\text{H}_2\text{O}_2$  and MDA accumulation. Higher accumulation of  $\text{H}_2\text{O}_2$  was observed in  $\text{Mg}^{2+}$  deficient drought-stressed plants, either due to enhancement in  $\text{H}_2\text{O}_2$  generation or a decline in the activity of catalyzing enzymes. CAT activity was tested to identify the ability of cells to metabolize ROS production. The results showed that the CAT activity was higher in all the drought-stressed plants. Previous studies presented evidence that ROS and cytosolic  $\text{Ca}^{2+}$  are signaling factors responding to  $\text{Mg}^{2+}$  deficiency, in agreement with a previous hypothesis that antioxidation is an early response to  $\text{Mg}^{2+}$  deficiency induced by ROS (Guo et al. 2016). The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and, subsequently, on the up-regulation of other downstream antioxidant enzymes (Hasanuzzaman et al. 2020). In our experiments, the results showed significantly enhanced SOD activity in seedlings exposed to DS (Figure 6). According to our results, the maximum increase in SOD activity was observed in +Mg  $\times$  DS. The induction of SOD in response to elevated ROS levels likely has a significant role in strengthening the plant's antioxidant defense system and balancing the cell's redox status. Our results are consistent with other studies reporting increased SOD activity in response to DS in sunflower (Gunes et al. 2008), poplar (Xiao et al. 2008), cowpea (Manivannan et al. 2007), liquorice (Pan et al. 2006), and oilseed rape (Abedi and Pakniyat 2010). Moreover, a recent study indicated higher enzyme activities of SOD and CAT after Mg foliar application (ur Rehman et al. 2018).

APX enzymes are a group of essential proteins that perform a vital function in plant cells. They act as catalysts in converting harmful  $\text{H}_2\text{O}_2$  into harmless  $\text{H}_2\text{O}$  by using ascorbate as an electron donor. This process helps to prevent oxidative damage and maintain cellular homeostasis. APX enzymes are found in various subcellular compartments, including chloroplasts, mitochondria, peroxisomes, and cytosol. The APX responses are directly involved in protecting plant cells against adverse environmental conditions. Plants experience a variety of negative impacts when subjected to DS, including stunted growth, altered gene expression, and an increase in enzymes responsible for ROS production. Recent research on two separate cowpea cultivars

revealed that the drought-resistant cultivar exhibited 60% higher APX activity than its counterpart under standard conditions (Li 2023).

### 3.6 | Leaf Anatomy and Ultrastructure of Chloroplasts

Mg deficiency had a significant impact on the leaf length and leaf width, which were additionally decreased upon DS (Table 1). However, the effect of drought on the leaf size of Mg leaf application (+Mg LA  $\times$  DS) was significantly minimal (Table 1). In contrast, the mesophyll morphology evaluated on Technovit or epoxy resin cross sections was not considerably changed by  $\text{Mg}^{2+}$  deficiency or DS.  $\text{Mg}^{2+}$  sufficient plants seemed to have larger intercellular spaces both in control and DS conditions. Compared to the positive control (+Mg), in all other conditions, leaf thickness was reduced and the ratio of the thickness of the palisade to the spongy mesophyll increased (Table 1, Figure 7a,b). More notable was the increased accumulation of starch in (–Mg) and (–Mg  $\times$  DS), as revealed by Lugol's iodine solution staining for light microscopy evaluation, and by the ultrastructural analysis. Under DS the amount of starch decreased compared to the control condition (Figures 7c and 8a,b). Chloroplasts in positive control (+Mg) had electron-dense stroma and a dense thylakoid network. On most chloroplast profiles, several plastoglobules associated with stromal thylakoids were observed. Under DS, the thylakoid organization in Mg-sufficient plants has not notably changed, except for a slight increase in the abundance of plastoglobules and a reduced amount of starch. Leaves of the negative control (–Mg) had the largest chloroplasts with large and abundant starch grains. The Thylakoid network was comprised granal and stromal thylakoids, but they were squeezed in between large starch grains. Plastoglobules were enlarged compared to  $\text{Mg}^{2+}$  sufficient plants. Plants grown under  $\text{Mg}^{2+}$  limitation have chloroplasts with parts of stroma devoid of the thylakoid network (Figure 9). The remaining thylakoid network was very dense, organized in granal and stromal thylakoids, and associated with plastoglobules. Compared to chloroplasts with normal ultrastructure in the same or adjacent cells, the content of starch seemed reduced, which could indicate that these



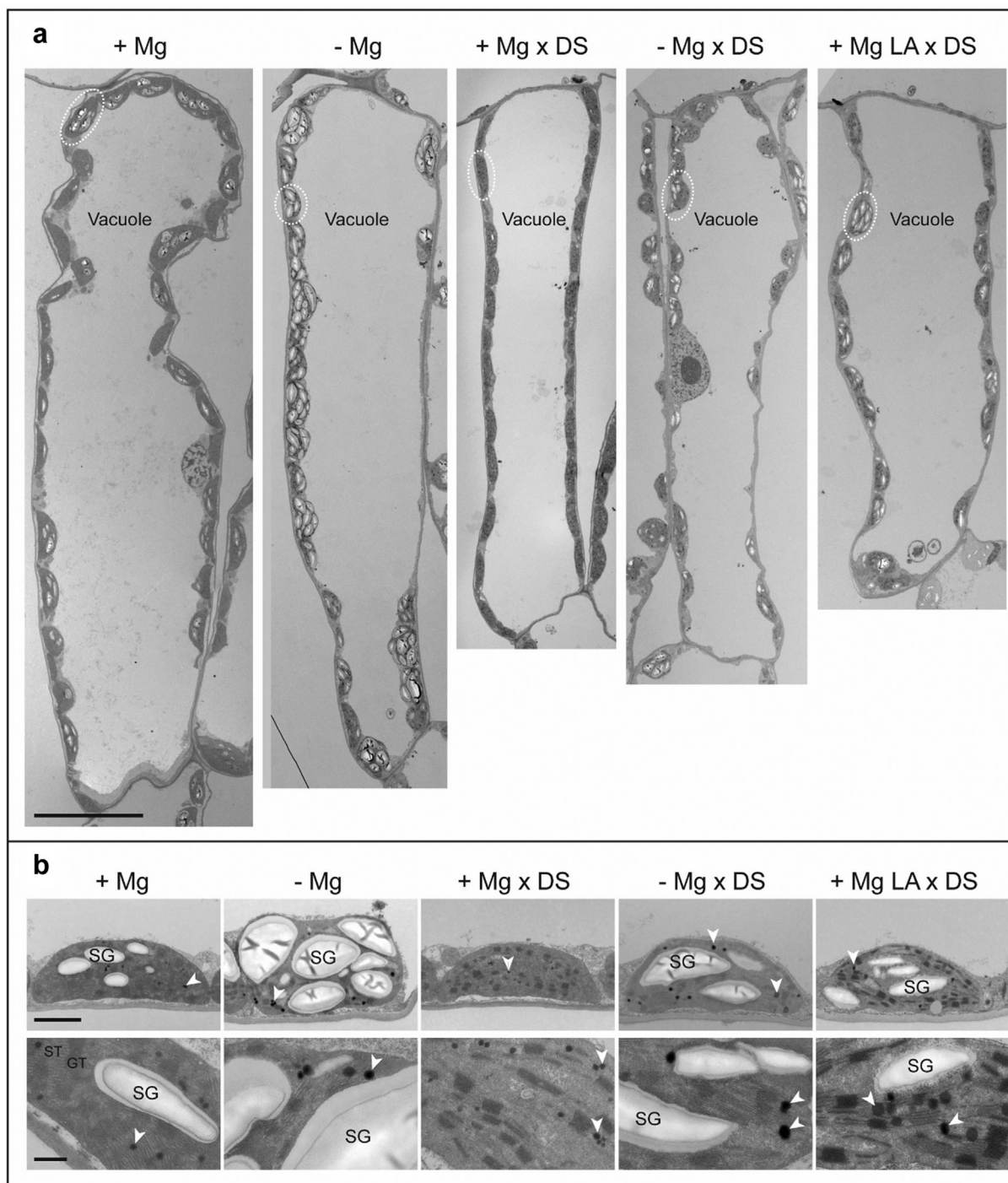
**FIGURE 7** | The effect of  $\text{Mg}^{2+}$  supply on the faba bean leaf anatomy and the starch content. (a) Technovit resin sections (8  $\mu\text{m}$ ) stained with the Richardson's solution. (b) Epoxy resin sections (1  $\mu\text{m}$ ) stained with the Richardson's solution. (c) Epoxy resin sections stained with Lugol's iodine solution to visualize starch grains (white arrowheads point to starch grains in one chloroplast). +Mg (0.5 mM  $\text{MgSO}_4$ ), -Mg (0 mM  $\text{MgSO}_4$ ), +Mg  $\times$  DS (0.5 mM  $\text{MgSO}_4$ ), -Mg  $\times$  DS (0 mM  $\text{MgSO}_4$ ), +Mg LA  $\times$  DS (leaf-application of 250 mM  $\text{MgSO}_4$  for four times), PM (palisade mesophyll), SM (spongy mesophyll). Scalebar: (a, b) 200  $\mu\text{m}$ , (c) 20  $\mu\text{m}$ . DS, drought stress

chloroplasts had reduced photosynthetic capacity. Such abnormal chloroplasts were more common in the spongy mesophyll than in the palisade mesophyll.

Structural changes in the leaf mesophyll upon  $\text{Mg}^{2+}$  deficiency or DS seemed to be minor. Mesophyll of  $\text{Mg}^{2+}$  deficient leaves had less intercellular space, and in both conditions, the leaves were a bit thinner, whereas the spongy mesophyll became relatively

thicker. Changes at the ultrastructural level were more prominent.  $\text{Mg}^{2+}$  deficiency in control conditions was characterized by extreme overaccumulation of starch inside chloroplasts, which can be explained by impaired Mg-dependent phloem loading of Suc through a Suc transporter (Hermans and Verbruggen 2005). The starch content decreased under DS irrespective of the  $\text{Mg}^{2+}$  status, which is likely related to a decreased photoassimilation rate. The other notable feature of  $\text{Mg}^{2+}$  deficiency was enlarged

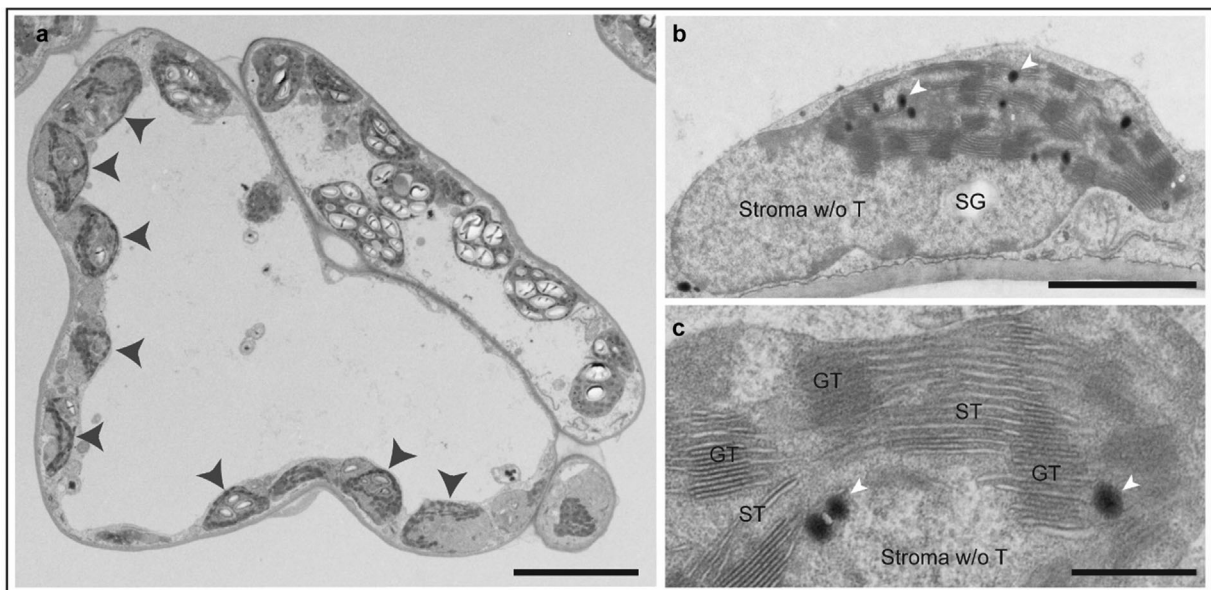




**FIGURE 8** | The effect of Mg supply on the ultrastructure of chloroplasts in the palisade mesophyll of faba bean leaves. (a) One cell of the palisade mesophyll. Chloroplasts are aligned along the cell wall; a single chloroplast is indicated with a white dotted circle. (b) Higher resolution images of single chloroplasts (upper row) and of a part of chloroplasts (lower row). +Mg (0.5 mM  $\text{MgSO}_4$ ), -Mg (0 mM  $\text{MgSO}_4$ ), +Mg x DS (0.5 mM  $\text{MgSO}_4$ ), -Mg x DS (0 mM  $\text{MgSO}_4$ ), +Mg LA x DS (leaf-application of 250 mM  $\text{MgSO}_4$  for four times), SG (starch grain), white arrowheads (plastoglobules), grana thylakoids (GT) and stromal thylakoids (ST). Scalebar: (a) 20  $\mu\text{m}$ , (b, upper row) 2  $\mu\text{m}$ , (b, lower row) 500 nm. DS, drought stress

plastoglobules. Their composition varies depending on the stress, and some metabolites, like prenol lipids, have a protective role under oxidative stress (Arzac et al. 2022), which was present in our  $\text{Mg}^{2+}$  deficient or/and DS plants as evidenced by biochemical analyses. The third common ultrastructural characteristic of plants grown under  $\text{Mg}^{2+}$  deficient root supply were chloroplasts, in which thylakoids were not spread throughout the stroma but

were confined to about half of a chloroplast profile. Notably, this kind of chloroplasts had a relatively low amount of starch, and relatively sparse plastoglobules localized only along thylakoids. They were not present in plants with sufficient  $\text{Mg}^{2+}$  root supply. It is tempting to speculate that in this kind of chloroplasts, thylakoid-devoid stroma was formerly occupied by enlarged starch grains, or that they represent organelles most affected



**FIGURE 9** | Chloroplasts with part of stroma devoid of thylakoid membranes observed in plants grown under a limited supply of  $Mg^{2+}$ . (a) Two cells of the spongy mesophyll. In the left cell, chloroplasts have areas of stroma without (w/o) thylakoid membranes (gray arrowheads). (b) Higher resolution images of a single chloroplast. (c) Part of a chloroplast showing the thylakoid network organized into grana thylakoids (GT) and stromal thylakoids (ST). SG (starch grain), white arrowheads (plastoglobules). Scalebar: (a) 10  $\mu m$ , (b) 2  $\mu m$ , and (c) 500 nm.

by the oxidative stress. Either way, these chloroplasts probably have a low photoassimilation activity. At the same time, foliar  $Mg^{2+}$  has resulted in a well-established network of grana and stroma. The reason for the respective stacking unstacking and reversible stacking is mainly attributed to  $Mg^{2+}$ . As a divalent cation,  $Mg^{2+}$  causes electrostatic screening on the surface of the thylakoids, which allows the thylakoids to approach each other in a parallel way. Similar results were reported by Izawa and Good (1966) in the case of spinach chloroplasts isolated in low salt media that lost their grana and were considerably swollen; upon addition of cations, grana reappeared and chloroplasts shrunk, which proved that  $Mg^{2+}$  ions greatly influence the self-assembly, and disassembly of thylakoids.

#### 4 | Conclusion

We investigated whether in faba bean plant foliar application of  $Mg^{2+}$  can reverse the symptoms of  $Mg^{2+}$  deficiency in the nutrient solution under DS. Foliar application of  $Mg^{2+}$  significantly increased the fresh and dry plant biomass, leaf area, chlorophyll concentration,  $CO_2$  assimilation rate, and transpiration rate. Increased activity of CAT and APX enzymes in plants supplemented with foliar application of  $Mg^{2+}$  depicts the role of  $Mg^{2+}$  in imparting defense mechanism toward DS. Moreover, foliar application of  $Mg^{2+}$  has lower lipid peroxidation evident from levels of MDA, which directly correlates with minimal damage to the ultrastructure of the chloroplasts. However, root application of  $Mg^{2+}$  helped in better preservation of the ultrastructure of the chloroplasts in terms of photoassimilate translocation from source to sink organs and reduced starch and sugar accumulation, whereas  $Mg^{2+}$  foliar application is not on par with the root supply but depicted a significant role in overcoming the DS. Although Mg root application exhibited better results, we recommend  $Mg^{2+}$  leaf application as a better practical approach under DS in

field experiments. Consequently, further studies are needed to investigate the efficiency of  $Mg^{2+}$  foliar application under relevant agricultural conditions.

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#### Data Availability Statement

The data that support the findings of this study are available from the authors upon reasonable request.

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