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Effects of changes in leaf properties mediated by methyl jasmonate (MeJA) on foliar absorption of Zn, Mn and Fe

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• **Background and Aims** Foliar fertilization to overcome nutritional deficiencies is becoming increasingly widespread. However, the processes of foliar nutrient absorption and translocation are poorly understood. The present study aimed to investigate how cuticular leaf properties affect the absorption of foliar-applied nutrients in leaf tissues.
• **Methods** Given that methyl jasmonate (MeJA) can cause alterations in leaf properties, we applied 1 mM MeJA to sunflower (*Helianthus annuus*), tomato (*Solanum lycopersicum*) and soybean (*Glycine max*) to assess changes in leaf properties. Using traditionally analytical approaches and synchrotron-based X-ray fluorescence microscopy, the effects of these changes on the absorption and translocation of foliar-applied Zn, Mn and Fe were examined.
• **Key Results** The changes in leaf properties caused by the application of MeJA increased foliar absorption of Zn, Mn and Fe up to 3- to 5-fold in sunflower but decreased it by 0·5- to 0·9-fold in tomato, with no effect in soybean. These changes in the foliar absorption of nutrients could not be explained by changes in overall trichome density, which increased in both sunflower (86 %) and tomato (76 %) (with no change in soybean). Similarly, the changes could be not attributed to changes in stomatal density or cuticle composition, given that these properties remained constant. Rather, the changes in the foliar absorption of Zn, Mn and Fe were related to the thickness of the cuticle and epidermal cell wall. Finally, the subsequent translocation of the absorbed nutrients within the leaf tissues was limited (<1·3 mm) irrespective of treatment.
• **Conclusions** The present study highlights the potential importance of the combined thickness of the cuticle and epidermal cell wall in the absorption of foliar-applied nutrients. This information will assist in increasing the efficacy of foliar fertilization.

Key words: Biofortification, cuticle, foliar absorption, methyl jasmonate, nutrients, trichomes, sunflower, soybean, tomato.

INTRODUCTION

Foliar fertilization is becoming increasingly widely used, with this approach potentially being more environmentally friendly and more efficient compared with soil-based applications of fertilizers (Fernández and Eichert, 2009). For example, Ram *et al.* (2016) found that foliar application of Zn significantly increased both grain Zn concentrations and grain yield for wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) when examined at 31 sites across seven countries. However, the processes of foliar absorption and the subsequent translocation of absorbed nutrients are not well understood. As a consequence, foliar fertilizers are generally not designed on a physiological basis, and hence potentially suffer from various limitations. For instance, the use of soluble Zn salts for foliar fertilization can result in leaf scorching (Drissi *et al.*, 2015), while chelated forms of Zn, such as Zn-EDTA, are under increased scrutiny due to associated environmental concerns (Oviedo and Rodríguez, 2003).

The penetration of nutrients through the leaf surface has been demonstrated in both living plants and isolated cuticle membranes (Koontz and Biddulph, 1957; Schlegel and Schönherr, 2001; Riederer and Friedmann, 2006; Fernández and Eichert, 2009; Du *et al.*, 2015). The process by which nutrients are absorbed through aerial tissues differs from uptake through roots, with the cell walls of leaves covered by a cuticle. Given that the cuticle is hydrophobic, it has been proposed that the cuticle has two separate pathways responsible for the transport of lipophilic and hydrophilic substances (Buchholz, 2006; Schönherr, 2006). The process by which lipid-insoluble (hydrophilic) ions penetrate through the cuticle has been studied extensively (Koontz and Biddulph, 1957; Schönherr, 1976; Buchholz *et al.*, 1998; Schreiber, 2001; Schlegel *et al.*, 2005, 2006; Schreiber, 2005; Fernández *et al.*, 2014). It is generally assumed that the cuticle has small aqueous pores that can allow hydrated ions to pass. Although this hypothesis has been proven theoretically (Schönherr, 2006), the chemical composition, structure and existence of such pores have never been directly confirmed. Through the use of fluorescent dyes and silver nitrate to localize the foliar absorption pathway, it has also been proposed that the aqueous pores preferentially occur

in guard cells, trichome bases (especially glandular trichomes) and cuticular anticlinal walls (Schlegel *et al.*, 2005; Schönherr, 2006). Therefore, it is possible that the absorption of nutrients across leaf surfaces may occur via (1) the cuticle, (2) cuticular cracks and imperfections or (3) stomata, trichomes and lenticels (Fernández *et al.*, 2013).

It is known that methyl jasmonate (MeJA), as a phytohormone, is involved in plant defence systems and can cause alterations in leaf properties, such as trichome density (Tian *et al.*, 2012). In the present study, using sunflower (*Helianthus annuus*), tomato (*Solanum lycopersicum*) and soybean (*Glycine max*), we utilized 1 mM MeJA to alter leaf properties and examined whether changes in leaf properties influence foliar absorption and the subsequent translocation of Zn, Fe and Mn. First, the effects of 1 mM MeJA on leaf properties were assessed by examining changes in thickness of the cuticle (measured as the combined thickness of the cuticle and epidermal cell wall; see later), cuticle composition, trichome density and stomatal density. Next, the absorption of foliar-applied nutrients was assessed by examining changes in bulk nutrient concentrations of the leaf tissues. Finally, synchrotron-based X-ray florescence microscopy (μ-XRF) was used to provide *in situ* analyses of the distribution of nutrients within hydrated and fresh leaf tissues. The results of the present study will assist in understanding the process by which nutrients move across the leaf surface, and thereby provide underlying information required for improving the efficacy of foliar fertilizers.

MATERIALS AND METHODS

Experimental design and plant growth

The plant growth study was conducted at The University of Queensland (St Lucia, Australia), in a growth room at 25 °C with high-pressure sodium lamps providing light (photon flux density of 1500 μmol m^{-2} s^{-1}) for 14 h d^{-1}. Seeds of soybean ('Bunya'), sunflower ('Hyoleic 41') and tomato ('Red Luck') were germinated in rolled paper towel moistened with tap water for either 3 (soybean and sunflower) or 4 d (tomato) before being transferred to 11 L black buckets. Each bucket had four holes in the lid with a total of either eight (tomato and soybean) or four (sunflower) plants, with each bucket forming one experimental unit. The buckets were filled with a basal nutrient solution which contained (μM): 910 N (94 % NO_3^- and 6 % NH_4^+), 475 K, 20 P, 1126 Ca, 227 Mg, 1251 Cl, 556 S, 25 Fe(III) EDTA, 3 B, 0·5 Mn, 0·5 Zn, 0·2 Cu and 0·01 Mo (Blamey *et al.*, 2015). Nutrient solutions were changed after the first week, and then after every 4 d. After the plants had been growing for 10 d, 5 mL of 44 mM KH_2PO_4 was added to each 11 L bucket every second day thereafter progressively to replace P which had been taken up by the plants.

The experiment consisted of three plant species and two concentrations of MeJA [0 (control) and 1 mM] with four replicates, yielding a total of 24 experimental units. Application of the MeJA occurred after the plants had been growing for 7 (sunflower), 8 (soybean) or 10 d (tomato). The MeJA (0 or 1 mM in 0·8 % ethanol) was sprayed onto the foliage until the leaves were fully saturated (approx. 300 mL per container) (Boughton *et al.*, 2005; Tian *et al.*, 2012), with the growth lamps switched off for 4 h following the application of the MeJA. Thereafter, plants were grown until the youngest leaves were fully expanded (approx. 10 d after application of MeJA). The youngest fully expanded leaves (YFELs) were then used for examining leaf morphology (stomatal and trichome density), the thickness of the cuticle and epidermal cell wall, and cuticle composition.

Assessment of leaf characteristics

Using the YFELs, leaf (adaxial) stomatal density and trichome density were examined using scanning electron microscopy (SEM). Fresh materials (approx. 3 × 3 mm) were collected from the YFELs of each treatment and immediately fixed in 3 % glutaraldehyde in 0·1 M sodium cacodylate, followed by a microwave processing which was performed using a Pelco BioWave (Ted Pella Inc., CA, USA) with a ColdSpot water recirculating device. Then the samples were post-fixed with 1 % osmium tetroxide, subjected to a dehydration series using ethanol (30, 50, 70, 90, 100 and 100 %) and processed using the BioWave. The tissues were then processed using a critical point dryer (Autosamdri-815 CPD, USA) before being coated with Au. Finally, samples were examined using a scanning electron microscope (JEOL NEOSCOPE, Japan) at 10 kV accelerating voltage, with the density of trichomes and stomata determined.

Cuticle composition was examined using Fourier transform infrared spectroscopy (FTIR). Briefly, the abaxial side of each YFEL was scraped as clean as possible using a scalpel, and the remaining adaxial cuticles were placed into an enzyme solution containing 2 % pectinase and 0·1 % cellulase in 50 mM sodium acetate buffer to remove the residual cell debris from the cuticle. This enzymatic digestion was performed overnight at room temperature (25 °C) before the cuticles were washed twice using deionized water. The samples were examined using light microscopy to ensure that the underlying tissues were completely removed before being air-dried and analysed by FTIR (the obtained spectra were then normalized) (Guzmán *et al.*, 2014).

Although the cuticle has traditionally been thought to be independent of the underlying epidermal cell wall, it has recently been proposed that the cuticle is an extension of the epidermal cell wall (Guzmán *et al.*, 2014; Fernández *et al.*, 2016). Therefore, in the present study, we measured the combined thickness of the cuticle and epidermal cell wall using light microscopy by examining sections cut from resin-embedded leaf samples. Briefly, the fresh YFELs of each treatment were cut into pieces approx. 1 × 4 mm. The segments were fixed in 3 % glutaraldehyde in 0·1 M sodium cacodylate at 4 °C overnight, rinsed twice with 0·1 M sodium cacodylate, post-fixed with 1 % OsO_4 in 0·1 M sodium cacodylate for 4 h and dehydrated in a graded ethanol series of 20, 30, 40, 50 (10 min each), 70, 90, 100 (30 min each) and 100 % (overnight). A series of graded Epon mixtures (Epon in ethanol) were used to infiltrate the tissues: 10, 20, 30, 40 (90 min), 50 (overnight), 65, 80 (4 h) and 100 % (overnight). The tissues were then embedded in 100 % Epon blocks and polymerized at 60 °C overnight. Sections (1 μm thick) were cut using an ultramicrotome (LEICA EM UC6), stained by Toluidine blue (0·5 % in 1 % borax) and observed by light microscopy (Obrien, 1995).

Foliar application of Zn, Mn and Fe

Bulk leaves (six replicates) were analysed by inductively coupled plasma mass spectrometry (ICP-MS) in order to obtain total elemental concentrations. A half-leaf loading method was used (Vu *et al.*, 2013), with half of each leaf receiving nutrients and the other half receiving deionized water. To increase contact with the leaf surface, 0·05 % Tween-20 was used as a mild non-ionic surfactant (Reuveni *et al.*, 1994). Specifically, to one half of each leaf, 20 droplets (5 µL per droplet) of 1000 mg L^{-1} Zn (15·4 mM, pH 5·2, supplied as $ZnSO_4 \cdot 7H_2O$), Mn (18·2 mM, pH 4·1, supplied as $MnSO_4 \cdot 4H_2O$) and Fe (17·9 mM, pH 3·8, supplied as $FeSO_4 \cdot 7H_2O$) were applied with 0·05 % Tween-20, while the other half of each leaf received 60 droplets (5 µL per droplet) of deionized water with 0·05 % Tween-20 (pH 5·3). The leaves were incubated in Petri dishes for 6 h before being cut from the plants and then blotted dry with filter paper. Thereafter, the leaves were cut in half along the mid-vein, rinsed separately using 2 % HNO_3, followed by 3 % ethanol, and deionized water (Du *et al.*, 2015). The samples were oven dried, digested using a 5:1 mixture of nitric acid and perchloric acid, and analysed using ICP-MS. Blanks and reference materials were included to ensure accuracy.

Finally, to examine the *in situ* distribution of foliar absorbed Zn, Mn and Fe in leaf tissues, the same three plant species were grown at La Trobe University (Bundoora, Australia) and exposed to either 0 or 1 mM MeJA as described earlier. Two droplets (5 µL each) of each nutrient (1000 mg L^{-1} Zn, Mn or Fe with 0·05 % Tween-20) were applied to the adaxial YFELs of each of the three plant species. For sunflower and soybean, six droplets were applied near the tip of the leaf, with the three nutrients applied on one side of the mid-rib and three replicate droplets on the other side of the mid-rib. For tomato, the six droplets were applied on the base, middle or tip of a leaflet of a compound leaf with three nutrients on each side (see later). Following application of the droplets, the leaves were sealed inside Petri dishes containing moist filter paper for 6 h, with lights on. For this entire incubation period, the leaves remained attached to the plants, with the petiole passing through a small hole in the side of the Petri dish (Supplementary Data Fig. S1). After incubation for 6 h, leaves were cut and rinsed thoroughly (as described earlier) before analysis using µ-XRF at the XFM beamline at the Australian Synchrotron (Melbourne, Australia).

Details of the XFM beamline as used to analyse plant tissues have been provided previously (Kopittke *et al.*, 2011; Paterson *et al.*, 2011). Briefly, X-rays were selected by a Si (111) monochromator and focused (approx. 2 × 2 µm) on the specimen by a pair of Kirkpatrick–Baez mirrors. The X-ray fluorescence emitted by the specimen was collected using the 384-element Maia detector system in a backscatter position at an excitation energy of 12 900 eV. Washed leaves were carefully blotted dry and mounted between two pieces of 4 µm thick Ultralene film, forming a tight seal around the leaf to limit dehydration. There was generally <5 min between excision of the leaf and commencement of the µ-XRF analysis. For each sample, two scans were performed. The first scan ('survey scan') was comparatively rapid and aimed to examine the entire sample and identify the portion of the leaf surface to which the Zn, Mn and Fe had been applied. The second scan ('detailed scan') was performed on a smaller area of the tissues surrounding the portion of the leaf to which the nutrients had been applied. This detailed scan was conducted with a smaller step size to increase spatial resolution (see details below).

Elemental mapping of each specimen involved continuous 'on the fly' analysis in the horizontal direction and discrete steps in the vertical direction. For the survey scan, the step size (i.e. pixel size) was 200 µm with a horizontal stage velocity of 18 mm s^{-1} (resulting in a pixel transit time of 11 ms). For the detailed scan, the step size was 20 µm with a horizontal stage velocity of 3 mm s^{-1} (resulting in a pixel transit time of 6·7 ms). The full X-ray fluorescence spectra were analysed using the CSIRO Dynamic Analysis method in GeoPIXE (http://www.nmp.csiro.au/dynamic.html) which enables quantitative, true-elemental images (Ryan and Jamieson, 1993; Ryan, 2000). All images were corrected for variations in leaf thickness through the use of Compton scattering as an internal standard.

Statistical analyses

Data were analysed using IBM SPSS Statistics version 24 (IBM Corporation, NY, USA). Comparisons between means were made using the *t*-test (95 %).

RESULTS

Effects of methyl jasmonate on leaf properties

Application of 1 mM MeJA resulted in changes in leaf properties, with these changes varying among the three plant species. First, in regard to trichome density, it was observed that 1 mM MeJA significantly increased non-glandular trichome density in sunflower by 56 % and glandular trichome density in tomato by 134 %, but had no significant effect on trichome density in soybean (Fig. 1). Secondly, in contrast to trichome density, 1 mM MeJA had no significant effect on stomatal density in any of the three plant species (Fig. 2). Next, consideration was given to the thickness of the cuticle and epidermal cell wall. For sunflower, application of 1 mM MeJA significantly decreased thickness by 35 %, but in direct contrast, the thickness of the cuticle and epidermal cell walls in tomato actually increased by 30 %, while no significant differences were observed for soybean (Fig. 3). Finally, FTIR was used to examine changes in cuticle composition, but no marked differences were observed following the application of 1 mM MeJA for any of the three plant species (Fig. 4).

Comparison of foliar absorption of Zn, Mn and Fe

Upon detailed quantitative examination of bulk tissue concentrations using ICP-MS (Table 1), it was found that foliar absorption occurred in all treatments, although differences were observed depending upon both the plant species and the MeJA treatment (control or 1 mM MeJA). Specifically, for soybean, the application of 1 mM MeJA did not have a significant influence on foliar absorption for any of the three nutrients (Table 1). In contrast, for sunflower, treatment with 1 mM MeJA resulted in marked increases (approx. 3- to 5- fold) compared with the controls (Table 1) while, for tomato, treatment with 1 mM MeJA actually decreased absorption of the three nutrients by 50–90 % (Table 1). Across the control treatments of the three

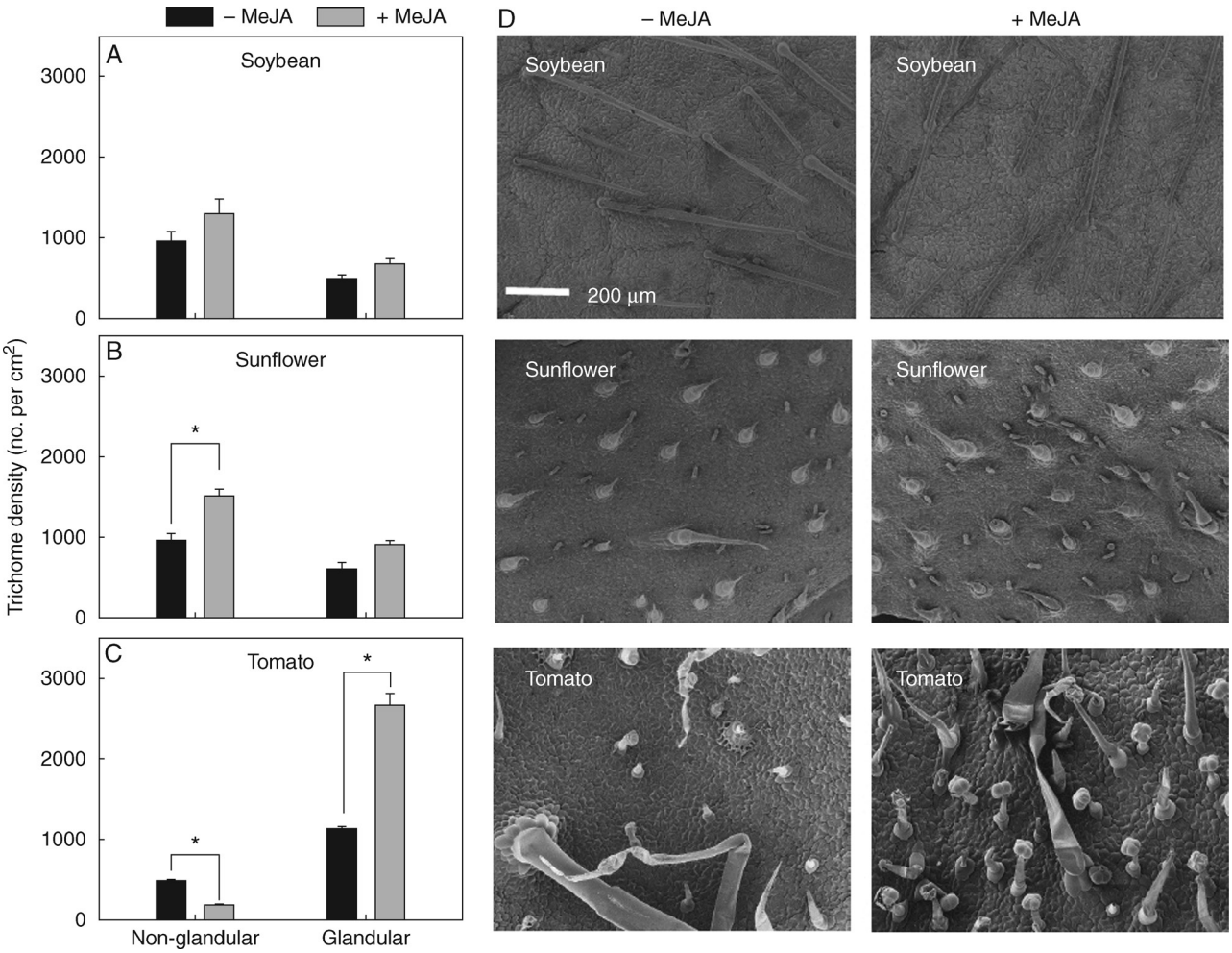

FIG. 1. Effects of methyl jasmonate (MeJA) on leaf trichome densities. (A–C) Density of non-glandular and glandular trichomes following the application of 1 mM MeJA in soybean (A), sunflower (B) and tomato (C). Values are means with the SD ($n = 4$). Within each plant species, $P < 0{\cdot}05$ between 0 and 1 mM MeJA is indicated with an asterisk (*). (D) Scanning electron micrographs showing leaf trichome densities of 0 and 1 mM MeJA of soybean, sunflower and tomato. The scale bar applies to all images.

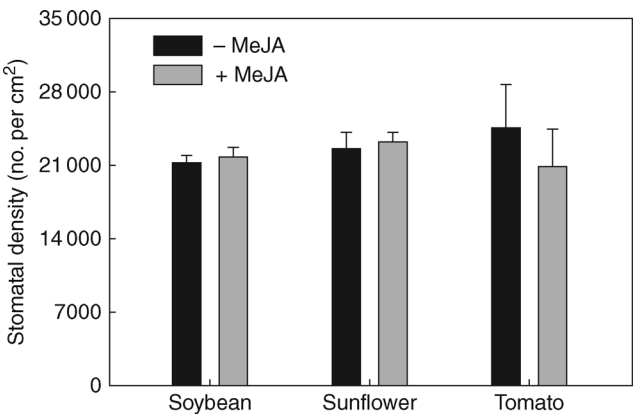

FIG. 2. Comparison of leaf stomatal density of 0 and 1 mM methyl jasmonate (MeJA)-treated leaves of soybean, sunflower, and tomato.

plant species, soybean (<1 µg per leaf) had the lowest levels of absorption for all the three nutrients, followed by sunflower (approx. 4–6 µg per leaf) and tomato (approx. 5–12 µg per leaf) (Table 1).

Using µ-XRF analysis, it was found that foliar application of Zn, Mn and Fe resulted in substantial increases in their concentrations in the underlying leaf tissues, indicating that after 6 h, the three nutrients had crossed the leaf cuticle and penetrated into leaf tissues of soybean (Fig. 5A), sunflower (Fig. 5B) and tomato (Fig. 5C). In these tri-colour images (Fig. 5), red (Zn), green (Mn) and blue (Fe) indicate the concentrations of these nutrients. It was noted that, below the site of application, the concentration of the nutrients in the veins was higher than in the surrounding interveinal tissues. Indeed, for all three nutrients in both sunflower and tomato, projected concentrations in the veins were 2- to 4-fold higher than in the corresponding interveinal tissues (Supplementary Data Figs S2 and S3).

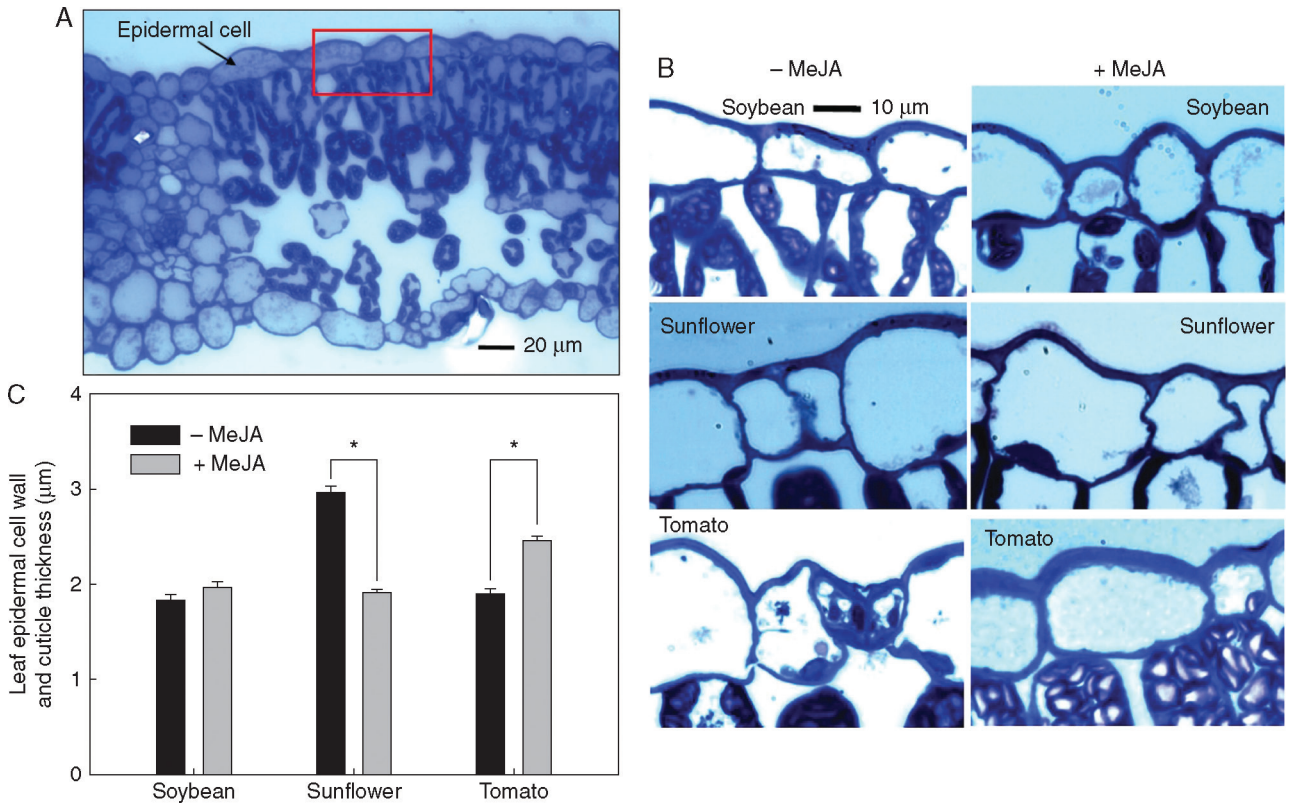

FIG. 3. Effects of methyl jasmonate (MeJA) on the thickness of the cuticle and epidermal cell wall. (A) A light micrograph showing a cross-section of soybean leaf, with the red rectangle showing the area in (B), (B) Light micrographs showing the cuticle and epidermal cell wall thickness (the blue layer above the epidermal cells) of 0 and 1 mM MeJA-treated leaves of soybean, sunflower and tomato (the scale bar applies to all images), (C) Comparison of leaf cuticle and epidermal cell wall thickness of 0 and 1 mM MeJA-treated leaves of soybean, sunflower and tomato. Values are the mean with the SD ($n = 4$). Within each plant species, P-values < 0·05 between 0 and 1 mM MeJA are indicated with an asterisk (*).

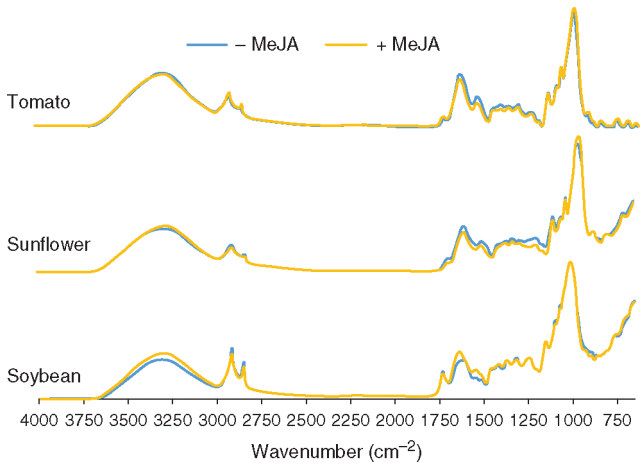

FIG. 4. Fourier transform infrared spectroscopy (FTIR) spectra obtained from isolated cuticles from the adaxial leaf surfaces of tomato, soybean and sunflower, either from 0 or 1 mM methyl jasmonate (MeJA)-treated leaves.

TABLE 1. *Compa rison of foliar absorption of Mn, Zn and Fe in soybean, tomato and sunflower after 6 h of foliar application*

Treatment	Nutrient absorption (μg per leaf)		
	Mn	Zn	Fe
Soybean			
– MeJA	0·11 ± 0·05^a	0·11 ± 0·07^a	0·69 ± 0·27^a
+ MeJA	0·18 ± 0·06^a	0·26 ± 0·15^a	0·82 ± 0·03^a
Sunflower			
– MeJA	6·45 ± 0·40^a	4·15 ± 0·35^a	5·77 ± 1·16^a
+ MeJA	25·6 ± 3·66^b	18·4 ± 3·22^b	36·5 ± 6·85^b
Tomato			
– MeJA	7·44 ± 1·41^a	5·32 ± 0·93^a	11·5 ± 4·52^a
+ MeJA	3·69 ± 0·66^b	0·88 ± 0·12^b	1·47 ± 0·63^b

Plants were treated with either 0 mM (control) or 1 mM methyl jasmonate (MeJA).

Elemental absorption (μg per leaf) = (elemental concentration in the treated half – elemental concentration in the control half) × dry weight of the treated half of the leaf.

Values are the mean ± SD ($n = 6$).

Where $P < 0·05$ occurred between control and 1 mM MeJA for each species is indicated with different lower case letters.

Effects of MeJA on subsequent movement of nutrients following their absorption

Using μ-XRF, it was possible to examine the movement of Zn, Fe and Mn by comparing their concentrations at increasing distance from the surface-applied droplets (Fig. 6). The distance that nutrients moved in the interveinal tissues and veins was calculated based upon measurements of droplet size from light microscopy and from maps of elemental distribution obtained from μ-XRF

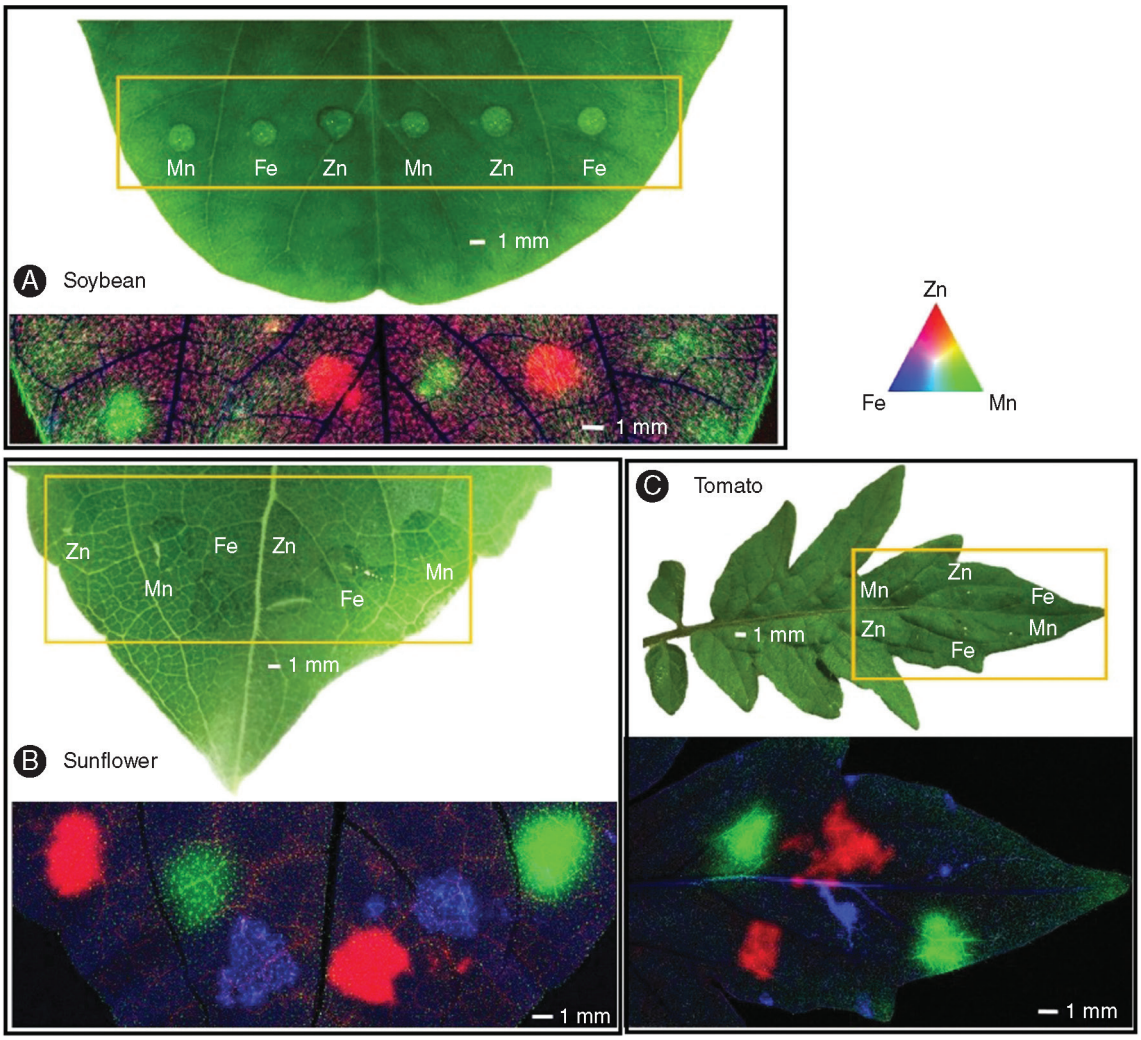

FIG. 5. Zn, Mn, and Fe distribution (after 6 h of foliar application) in control leaf of soybean (A) and tomato (C), and 1 mM methyl jasmonate (MeJA)-treated leaf of sunflower (B). In each image, the upper images are light microscopy before μ-XRF analysis, with the orange rectangle indicating the area examined by μ-XRF. The images below are tricolour μ-XRF maps of Zn (red), Mn (green) and Fe (blue) distribution.

analyses (Fig. 6). As shown in Table 2, except for a significant reduction in the movement of Fe in the veins of sunflower, MeJA had no significant effect on the movement of Zn, Fe or Mn in either the interveinal tissues or the veins from the site of application. In all instances, the extent to which Zn, Fe and Mn moved from the site of their application was small, ranging from 0·22 to 1·32 mm regardless of the plant species or the nutrient, with the movement distance in veins being slightly greater than in the interveinal tissues. For both sunflower and tomato, it was noted that Mn had a similar movement distance within the vein and the interveinal tissues, whereas Zn had a greater movement distance within the vein (approx. 2-fold longer) than the interveinal tissues (Table 2; Fig. 7). However, of the three nutrients, Mn had a slightly greater movement in interveinal tissues (mean 0·76 mm) than Zn (mean 0·45 mm) and Fe (mean 0·32 mm) (Table 2).

DISCUSSION

Given that the leaf surface is covered with a cuticle, the underlying process whereby foliar-applied nutrients are absorbed through the leaf surface has been debated for many years. In the present study, we have utilized MeJA to alter leaf properties in order to examine the effects of leaf properties on foliar nutrient absorption. We found that foliar nutrient absorption was not related to overall trichome density – the application of 1 mM MeJA increased trichome density in both sunflower (86 %) and tomato (76 %) (Fig. 1), but the foliar absorption of Zn, Mn and Fe increased for sunflower but actually decreased in tomato (Table 1). Similarly, changes in foliar absorption could not be attributed to changes in either stomatal density or cuticle composition (Figs 2 and 4), given that these leaf properties remained constant. Rather, we suggest that the foliar absorption was related to the combined thickness of the cuticle and epidermal cell wall. Indeed, in sunflower, foliar absorption of the three nutrients increased by 300–530 %, corresponding to a decrease in the cuticle and epidermal thickness from 3·0 to 1·9 μm (Table 1; Fig. 3). Similarly, an increase in the thickness of the cuticle and epidermal cell wall from 1·9 to 2·5 μm was accompanied by a decrease (50–90 %) in the absorption of the three nutrients in tomato (Table 1; Fig. 3). Finally, in

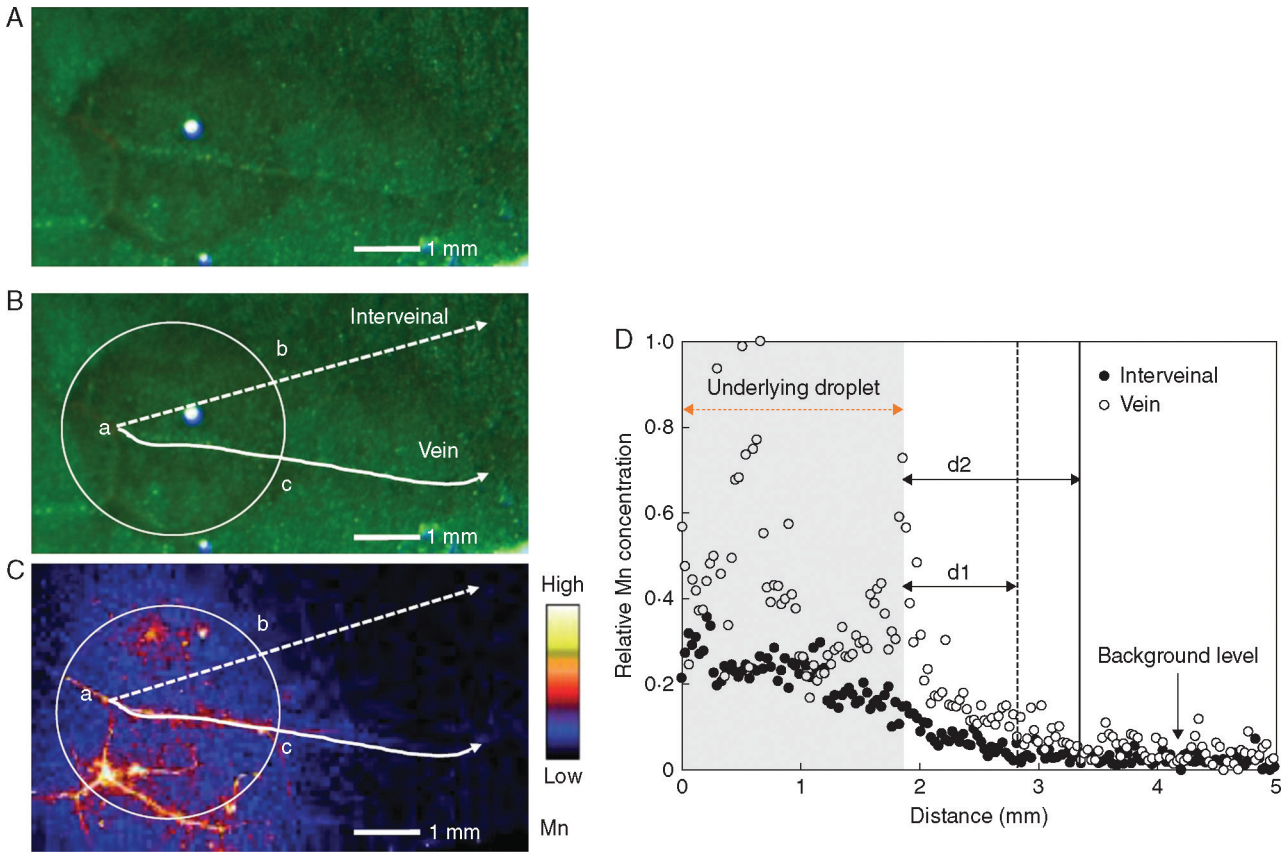

FIG. 6. Comparison of Mn movement through the vein and interveinal tissues of a tomato control leaf after 6 h of foliar application. (A) Light micrograph showing the Mn-containing droplet. (B) Same image as (A), showing the corresponding analysed area using μ-XRF in (C). White circles indicate the edge of the Mn droplet, while dashed lines represent a position of interveinal tissue and solid lines indicate the position of is of the same length in the droplet. (C) Compton-corrected Mn concentration of the area of (A) and (B). Brighter colour corresponds to higher Mn concentrations. The projected Mn concentrations underlying the solid (vein) and dashed (interveinal) line are shown in (D). (D) Compton-corrected Mn concentration in vein and interveinal tissue of tomato control leaf with the shaded part (shaded distance = ab = ac) representing the section under the Mn droplet; According to the Mn concentration of the background level, d1 is the Mn movement distance in the interveinal tissues, while d2 is the Mn movement distance in the vein.

soybean, we observed no changes in either nutrient absorption or the thickness of the cuticle and epidermal cell wall. We also found that following their movement across the leaf surface, the absorbed Zn, Fe and Mn moved only a very limited distance (0·22–1·32 mm) from the site of application before concentrations decreased to background levels, with MeJA (and the concomitant changes in leaf properties) having no effect on this translocation regardless of the plant species (Table 2).

The role of the cuticle in the foliar absorption of nutrients

Given that the cuticle is generally defined as a lipid-rich layer with its outer compartment dominated by waxes (Koch and Ensikat, 2008; Fernández *et al.*, 2013), it is generally assumed that the cuticle is a layer distinct from the underlying epidermal cell wall. As a result, almost all studies examining the foliar absorption of nutrients have utilized isolated cuticles, with the underlying epidermal cell wall not taken into account (Baur *et al.*, 1997; Riederer and Schreiber, 2001; Schreiber, 2005; Buchholz, 2006; Riederer and Friedmann, 2006; Schönherr, 2006; Jetter and Riederer, 2016). However, given that it has recently been reported that the cuticle and epidermal cell wall have similar chemical constitution and function, it has been proposed that the cuticle was actually an extension of the epidermal cell wall region (Guzmán *et al.*, 2014; Fernández *et al.*, 2016). To the best of our knowledge, the present study is the first to relate changes in foliar absorption of nutrients (Zn, Mn and Fe) to the combined thickness of the epidermal cell wall and thickness of cuticle.

We found that the absorption of these three nutrients, in all the three plant species, was related to changes in cuticle and epidermal cell wall thickness (Fig. 3; Table 1). Nevertheless, in contrast to these findings, some previous studies have suggested that cuticle thickness is not related to the cuticular permeability, although it must be noted that these previous studies have utilized isolated cuticles without also considering the underlying epidermal cell wall (Riederer and Schreiber, 2001; Jetter and Riederer, 2016).

In this present study, we also found differences in absorption of foliar nutrients among the three plant species. Overall, soybean (approx. 0·1–0·7 μg per leaf) had a markedly lower absorption of the three nutrients than sunflower or tomato (approx. 4–12 μg per leaf) (Table 1). We suggest that this is potentially because the soybean cuticle has a higher wax content than sunflower and tomato, as evidenced by the FTIR

TABLE 2. *Movement distance of Zn, Fe and Mn in leaves of sunflower, tomato and soybean after 6 h of foliar application*

Treatments	Movement distance (mm)					
	Mn		Zn		Fe	
	Interveinal	Vein	Interveinal	Vein	Interveinal	Vein
Sunflower						
– MeJA	0·72 ± 0·25[a]	0·95 ± 0·19[a]	0·53 ± 0·03[a]	0·96 ± 0·26[a]	0·41 ± 0·08[a]	1·31 ± 0·19[a]
+ MeJA	0·62 ± 0·02[a]	0·79 ± 0·10[a]	0·40 ± 0·22[a]	0·83 ± 0·03[a]	0·22 ± 0·02[a]	0·27 ± 0·05[b]
Tomato						
– MeJA	1·32 ± 0·11[a]	1·27 ± 0·14[a]	0·43 ± 0·05[a]	0·88 ± 0·13[a]	NA	NA
+ MeJA	0·76 ± 0·23[a]	1·01 ± 0·13[a]	0·57 ± 0·10[a]	0·95 ± 0·13[a]	NA	NA
Soybean						
– MeJA	0·44 ± 0·08[a]	NA	0·30 ± 0·04[a]	NA	NA	NA
+ MeJA	0·69 ± 0·12[a]	NA	0·46 ± 0·06[a]	NA	NA	NA

Values are the mean ± SD (n = 4).

Where $P < 0·05$ occurred between control and 1 mM MeJA for each species is indicated with different lower case letters.

NA, data not available due to the comparatively low concentrations under the droplet.

analyses (Supplementary Data Fig. S4), with a relatively high content of waxes (two bands at approx. 2900 cm^{-1}) and a low content of polysaccharides (the band at 3400 cm^{-1}) which have been reported previously in soybean compared with sunflower and tomato (Guzmán *et al.*, 2014; Heredia-Guerrero *et al.*, 2014). Also, we noted that it was substantially more difficult to apply the droplets to the leaves of soybean than it was to either sunflower or tomato because of higher surface tension (Fig. 5A), consistent with the analyses using SEM revealing a waxy layer covering leaves of soybean (Supplementary Data Fig. S5). This is in agreement with the previous studies that have reported the role of cuticular waxes in determining the permeability of plant cuticles (Riederer and Schreiber, 1995; Zhang *et al.*, 2005; Bondada *et al.*, 2006). In addition, by using μ-XRF, we found that the nutrient concentrations in the tissues underlying the droplets were higher in veins compared with the interveinal tissues (Figs 6 and 7). Given the low mobility of nutrients in the leaf tissues, this could be due to nutrient absorption being higher in veins than in the interveinal leaf tissues.

Leaf cuticle composition is expected to be important for the foliar absorption of nutrients. Indeed, it has been reported that MeJA increased polysaccharide content in the callus of *Rhodiola sachalinensis* (Li *et al.*, 2014) and *Dendrobium officinale* (Yuan *et al.*, 2016) through upregulating the metabolism enzymes and biosynthetic genes of sucrose. Similarly, MeJA can influence lipid metabolism, especially for fatty acyl metabolism (Cao *et al.*, 2016). In the present study, however, FTIR analysis did not identify any marked changes in cuticle composition among the three plant species upon treatment with MeJA (Fig. 4). The effects of MeJA on leaf properties varied among different species. For example, MeJA increased the trichome density of sunflower and tomato, but did not change the leaf trichome for soybean (Fig. 1). Therefore, the effects of MeJA on leaf cuticle composition are probably species dependent.

The role of trichomes in the foliar absorption of nutrients

Trichomes are appendages that originate from epidermal cells and develop outwards on the surface of various plant organs, classified either as non-glandular or glandular trichomes, with one of the well-accepted functions being protecting the plant from herbivories (Werker, 2000). In the present study, the foliar absorption was not related to the overall trichome density – although application of 1 mM MeJA increased the overall trichome density in both sunflower (86 %) and tomato (76 %) (Fig. 1) – foliar absorption of Zn, Mn and Fe increased for sunflower but actually decreased in tomato (Table 1). However, the changes in nutrient absorption were consistent with changes in the densities of non-glandular trichomes. Specifically, 1 mM MeJA increased non-glandular trichome density of sunflower by 56 % while it decreased non-glandular trichome density of tomato by 62 %, and no changes occurred in non-glandular trichome density of soybean under the application of MeJA. We contend, however, that it is unlikely that these non-glandular trichomes play an important role in the foliar absorption of nutrients based on the fact that non-glandular trichomes are just appendages at the leaf surface which have no connections to the inside leaf tissues. However, it has been suggested that trichomes can change the surface roughness and in turn influence leaf wettability (Bickford, 2016). This could potentially impact the foliar absorption of nutrients, with leaf wettability decreasing with increasing trichome density (Brewer *et al.*, 1991). In the present study, the non-glandular trichomes of both sunflower and tomato were larger than the glandular trichomes (Supplementary Data Fig. S6) and therefore non-glandular trichomes would be expected to have a larger impact on leaf wettability than glandular trichomes. Regardless, the change in leaf wettability induced by trichome density could not explain the differences in foliar absorption of nutrients in the present study, due to the fact that although leaf wettability decreased in sunflower (and increased in tomato), the foliar absorption of Zn, Mn and Fe increased for sunflower (and decreased for tomato).

As trichomes vary markedly in structure, morphology and function, it is difficult to generalize regarding the functions of all trichomes. Interestingly, several studies have examined foliar absorption by trichomes in epiphytic Bromeliaceae (Benzing *et al.*, 1976, 1985; Winkler and Zotz, 2010). These studies have, for example, reported that foliar absorption of water, Ca and Zn was associated with leaf trichome densities of Bromeliaceae

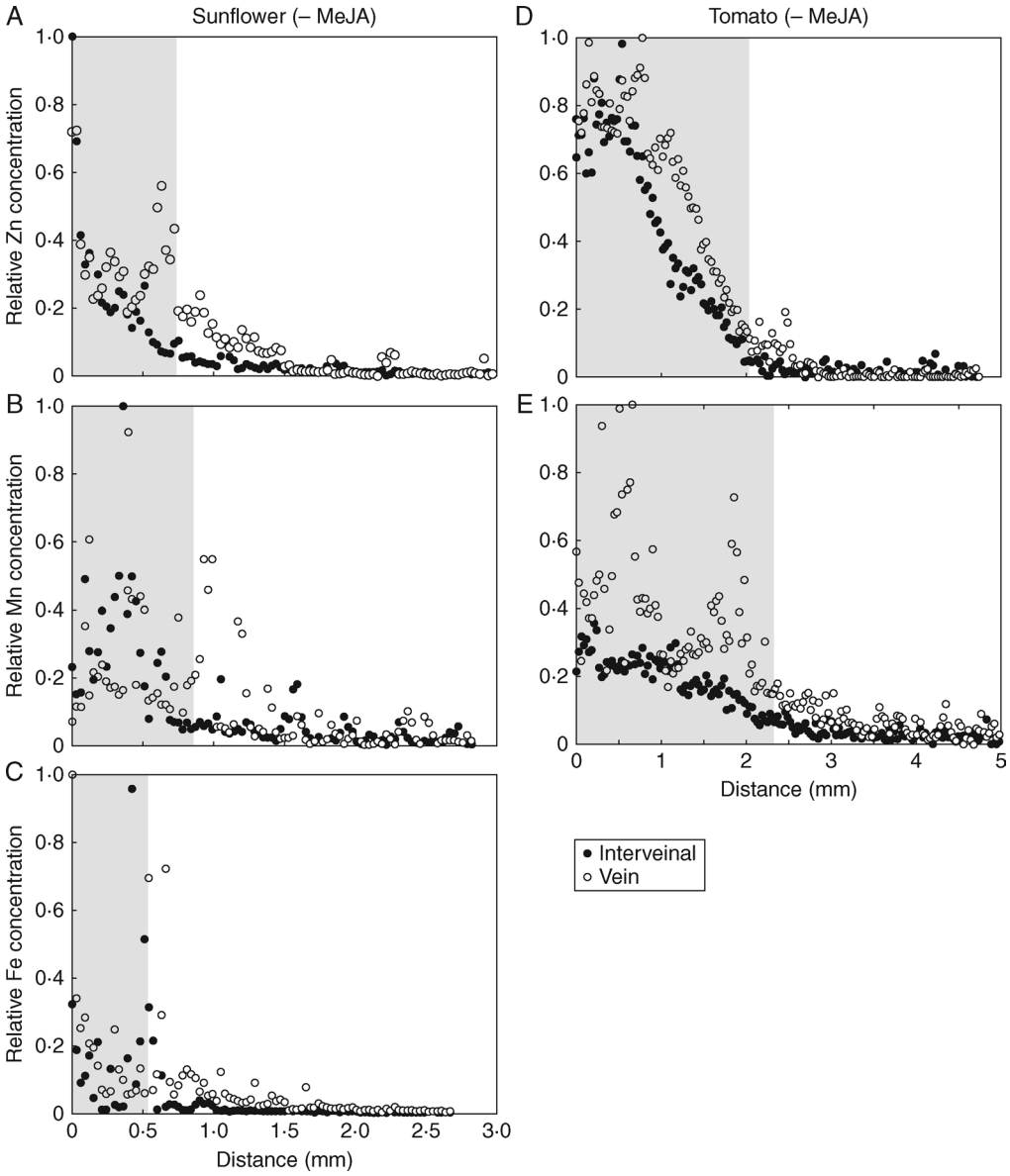

FIG. 7. Comparison of nutrient diffusion in vein and interveinal tissues of sunflower control (A–C) and tomato control (D–E) leaves after 6 h of foliar application. The shaded area represents the portion of the leaf under the droplet. Soybean control and Fe for tomato control are not shown due to the comparatively low absorption or no detectable veins under the droplets.

(Benzing and Burt, 1970; Ohrui *et al.*, 2007; Winkler and Zotz, 2010). However, given that these epiphytic Bromeliaceae are generally highly drought resistant and do not have absorbent roots, the leaf trichomes of Bromeliaceae could potentially be highly specialized, with the peltate trichomes different from trichomes of other species (Ohrui *et al.*, 2007).

Translocation of Zn, Fe and Mn away from the site of application

The efficacy of foliar nutrient application not only depends upon the rate at which the nutrients move through the leaf surface, but also the mobility of nutrients from the application site of the treated leaves to other parts of the plant. The mobility of these nutrients depends upon three factors: (1) the ability of the nutrients to enter the phloem; (2) the ability of the nutrients to move within the phloem; and (3) the ability of the nutrients to move out of the phloem into the sink tissues (Fernández *et al.*, 2013). In the present study, we examined the distribution of Zn, Fe and Mn 6 h after their application. It was observed that these three nutrients moved only a limited distance from their site of application, regardless of the plant species. This result is in agreement with previous studies which have reported that Zn, Mn or Fe have only a low mobility in plants (Eddings and Brown, 1967; Kannan and Charnel, 1986; Ferrandon and Chamel, 1988; Zhang and Brown, 1999; Marešová *et al.*, 2012; Du *et al.*, 2015). The reason for the low mobility of these nutrients remains unclear, although Fernández and Brown (2013)

suggested that the low mobility of Zn may be due to the binding of Zn to the negative charge of the apoplastic space. Further studies are required to examine the location and movement of foliar-applied nutrients at a cellular and sub-cellular level.

In conclusion, using three plant species (soybean, sunflower and tomato), we utilized 1 mM MeJA to induce changes in leaf properties in order to examine the effects of these changes on absorption of foliar-applied nutrients. For all three plant species, we found that the foliar absorption of Zn, Mn and Fe was related to the thickness of the cuticle and epidermal cell wall. In contrast, foliar absorption of the nutrients was not related to changes in the total trichome density. In addition, using μ-XRF to provide *in situ* data from hydrated and fresh leaves, we found that the translocation of Zn, Mn and Fe away from the sites of application was limited (0·22–1·32 mm) either in the vein or in the interveinal tissues, although the concentration of three nutrients within the underlying leaf tissues were higher in the veins than in the surrounding interveinal tissues.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Fig. S1: Foliar application of Zn, Mn and Fe droplets on a sunflower leaf for X-ray fluorescence microscopy (μ-XRF) analysis. Fig. S2: Compton-corrected μ-XRF images showing the distribution of Mn, Zn and Fe in a control leaf of tomato after 6 h of foliar application. Fig. S3: Compton-corrected μ-XRF images showing the distribution of Mn, Zn and Fe in a control leaf of sunflower after 6 h of foliar application. Fig. S4: Fourier transform infrared spectroscopy (FTIR) spectra obtained from isolated cuticles from the adaxial leaf surfaces of tomato, soybean and sunflower (all controls). Fig. S5: Scanning electron micrographs showing the surfaces of the leaves of soybean, tomato and sunflower. Fig. S6: Scanning electron micrographs showing non-glandular and glandular trichomes on the leaf surface of tomato and sunflower.

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