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REGULAR ARTICLE

Anatomical and ultrastructural damage to citrus leaves from phosphite spray depends on phosphorus supply to roots

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Abstract
Background and aims Although phosphite (PO_3, Phi) cannot replace phosphate (PO_4, Pi) as a nutritional source of phosphorus (P) for plants, decisions about using foliar Phi application in citrus groves scarcely take into account P availability and plant nutritional status. Accordingly, we studied the interactive effects of P supply in the rooting medium and foliar sprays of Pi or Phi on citrus tree performance and leaf anatomy and ultrastructure.
Methods Young sweet orange trees were grown hydroponically using P-deficient (PD) or P-sufficient (PS) nutrient solutions for 15 weeks in combination with 3 foliar spray treatments: 0.16 M P in the form of Pi (KH_2PO_4) or Phi (KH_2PO_3) and a control spray of 0.16 M potassium chloride (KCl). Six foliar sprays were applied to the drip point at 15-day intervals during the experimental period. Before the trees were harvested to estimate growth, mature leaves were sampled to study the anatomical and ultrastructural organization and to evaluate P concentration.
Results Under PD, the Pi spray stimulated tree growth compared with the control, whereas Phi sprays impaired tree performance, suggesting toxic effects. Both Pi and Phi applications increased the leaf P concentration relative to that of the control. Leaves of PD trees exposed to Phi exhibited pronounced damage to the epidermis, and stomata showed deformed ostioles and surface flaking. Moreover, phloem and xylem vessels were disorganized, cell wall presented sinuosity, cell membranes were plasmolyzed, and chloroplast thylakoids were disrupted, with accumulation of starch and plastoglobuli. However, no similar changes were observed either in PD trees under Pi spray or in PS trees independent of foliar treatment. PD trees receiving KCl exhibited intermediate responses between those of Pi and Phi applications.
Conclusions This was the first study to demonstrate that Phi spray disrupts the anatomical and ultrastructural organization of citrus leaves under P deficiency. Our results provide insights into the mechanisms explaining the poor nutritional value of Phi and support information to guide the use of Phi-based products.

Keywords Cell wall · Chloroplast · Nutritional stress · Plastoglobuli · Starch · Stomata

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Introduction

The productivity of citrus groves in tropical soils is frequently constrained by phosphorus (P) deficiency (Mattos

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et al. 2006), denoted by visible leaf bronzing and leaf abscission at the stem base during the growth of new flushes in the spring and summer. Since the remobilization of the internal pool of P rather than the current root uptake is the major source of the nutrient sustaining new growth (Zambrosi et al. 2012), this deficiency is likely due to an insufficient accumulation of P by the trees during the previous growing seasons. Therefore, in-season soil P fertilization is not a suitable strategy to ameliorate current P deficiency stress, raising the interest of growers in using foliar P sprays to rapidly supply P in fruit-bearing citrus trees grown in low-P soils. Indeed, the effectiveness of adopting foliar P application in fertilization management is supported by a number results demonstrating that foliar spray of phosphate (PO_4, Pi) improves the performance of fruit trees and other agricultural crops (Reddy and Majmudar 1983; Hossain and Ryu 2009; Noack et al. 2010) under the condition in which root uptake does not meet the current plant demand for P (Marschner 1995; Fernández and Brown 2013).

In addition to the traditional formulations of foliar P fertilizers containing Pi, phosphite (PO_3, Phi)-based products are also registered as P fertilizers, and Phi-based products have been recommended as a foliar spray in the field (McDonald et al. 2001; Calvo et al. 2014; Gómez-Merino and Trejo-Téllez 2015) based on results showing increases in production and quality of agricultural products (Albrigo 1999; Moor et al. 2009). This may be related to the ability of Phi in inducing resistance to biotic and abiotic stresses and acting as an antifungal agent (Orbovic et al. 2008; Thao and Yamakawa 2009; Eshraghi et al. 2014). Despite such claims supporting the beneficial effects of Phi usage, other studies have consistently shown that Phi does not replace Pi as a direct source of P for the plants and can result in toxic effects and reduced growth (Carswell et al. 1996, 1997; Varadarajan et al. 2002; Jost et al. 2015). This could also explain the poor performance of P-deficient plants receiving Phi application to their foliage (Thao et al. 2008; Ratjen and Gerendás 2009). Accordingly, the use of foliar Phi spray in agricultural crops remains controversial, and further studies are needed to clarify the use of Phi for field-grown plants.

Additionally, impairment on growth of P-deficient plants might be attributed, at least in part, to malfunctioning of leaves caused by damages to their anatomical and ultrastructural organization. For instance, compromised energy production in P-deficient leaves of pine needles is explained by a swelling in mitochondrial cristae (Palomäki and Holopainen 1994). Moreover, P starvation leads to reduction on stomatal density (Fernández et al. 2014) and disorganization of chloroplast ultrastructure (Whatley 1971; Sutinen et al. 1998; Hernández and Munné-Bosch 2015). Given these results and the well-documented inability of Phi in replacing Pi in plant metabolism, we hypothesized that: foliar P applications in the forms of Phi and Pi would have contrasting effects on the anatomy and ultrastructural integrity of leaf tissues and subsequently on the performance of citrus trees growing under P-deficient but not under P-sufficient supply to roots. To test this hypothesis, we examined the growth responses and leaf anatomy under light microscopy as well the leaf ultrastructure by electron microscopy of young citrus trees subjected to varying P availability in the rooting medium receiving either Pi or Phi applications to the foliage.

Materials and methods

Plant material and growth conditions

The experiment was carried out in unshaded greenhouse conditions, where the average minimum and maximum air temperatures were 21 and 38 °C, respectively, and the relative humidity varied from 45 to 99%. Young trees (approximately 18 months old) of sweet orange [*Citrus sinensis* (L.) Osbeck cv. Valência] grafted on Swingle citrumelo [*C. paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.] were obtained from a commercial nursery; the trees were bare rooted and supported in non-draining aerated pots containing 8 L of nutrient solution (NS). Acclimation of the trees to the rooting medium was achieved by growing them for 7 days in a NS without P and diluted to $^1/_4$ of the ionic strength (Zambrosi et al. 2013). The $^1/_4$-strength NS was replaced with $^1/_2$-strength solution for 7 days and then with full-strength NS [in mM: 9.6 N (11% as ammonium), 3.0 K, 4.5 Ca, 1.2 Mg, and 1.2 S; and, in μM: 41.6 B, 54.0 Fe, 8.2 Mn, 2.5 Zn, 1.0 Cu and 1.0 Mo] with varying P concentrations (see below). Each pot contained one citrus tree and was equipped with a tube extending to the bottom through which air was continuously bubbled for aeration of the NS. Water lost through evapotranspiration was replaced every day with distilled water, and the hydroponic solutions were replaced every 15 days. The pH of NS was monitored and

maintained between 5.0 and 6.0 during the 15-week experimental period.

Treatments were set up in a completely randomized design with 4 replications, combining 2 levels of P supply in the NS [P deficient (PD), 0.01 mM P and P sufficient (PS), 0.5 mM P] (Zambrosi et al. 2013) and 3 foliar spray treatments. Foliar spray solutions consisted of 0.16 M P prepared from stock solutions (1 M) of potassium phosphate (KH_2PO_4, Pi) or potassium phosphite (KH_2PO_3, Phi) and a control spray treatment of potassium chloride without P (KCl, 0.16 M). Accordingly, there were 6 treatments: PD + Pi, PD + Phi, PD + KCl, PS + Pi, PS + Phi and PS + KCl. The pH of spray solutions corresponded to 4.1, 5.2 and 6.8 for Phi, Pi and KCl treatments, respectively, and the selected P concentration was based on our field investigations and previous studies concerning foliar P application in the form of Pi and Phi (Jackson et al. 2000; Ratjen and Gerendás 2009; Zambrosi and Mesquita 2016). There were 6 foliar sprays, each containing 0.5% mineral oil as a surfactant and applied until the drip point. Foliar treatments started 15 days after the onset of P treatments in the NS and they were repeated at 15-day intervals throughout the experiment. To avoid contamination of the rooting medium with the spray solution, the pots were covered with tissue paper, and the NS was replaced the same day the foliar P application was made.

Light microscopy analyses

The anatomical characteristics of the leaf tissue were evaluated in samples obtained from mature leaves that had developed by the end of the experimental period. Samples of approximately 20 mm^2 were collected 5 days after the last foliar P spray between 9:00 and 11:00 a.m. from the middle third of the leaves from 4 different trees per treatment. They were fixed in Karnovsky solution (Karnovsky 1965) and dehydrated in an increasing ethanol series [30%, 50%, 70%, 90%, and 100% (3 times)]. Tissue samples were infiltrated with acrylic resin glycolmethacrylate (Leica 1) and 100% ethanol at a ratio of 1:1 for polymerization and then were submerged in pure resin. The blocks were cut using a microtome (Spencer Lens, USA) with a C-type stainless steel blade (Leica, Wetzlar, Germany). Sections were stained with 0.05% toluidine blue at pH 2.6 (Feder and O'Brien 1968) and mounted on glass slides after drying. Viewing occurred under a microscope (Zeiss Axioskop 2, Zeiss, Berlin, Germany), and images were recorded as digital files.

Scanning electron microscopy analyses

After fixation for light microscopy, the leaf samples were post-fixed for 1 h with 1% osmium tetroxide in 0.05 M cacodylate buffer. Samples were dehydrated in an increasing acetone concentration series [30%, 50%, 70%, 90%, 100% (3 times)] and dried until the critical point. Further, the samples were attached to stubs and both sides of the leaf surface were exposed for metallization with gold plating (mod. MC 50, Balzers, Germany). A scanning electron microscope (LEO 435 VP, Cambridge, England) was then used for image analysis.

Transmission electron microscopy analyses

Leaf samples that were fixed in Karnovsky solution were post-fixed for 1 h with 1% osmium tetroxide, dehydrated in an increasing acetone concentration series [30%, 50%, 70%, 90%, and 100% (3 times)], infiltrated and polymerized into Spurr epoxy resin (14,300, Electron Microscopy Sciences). The blocks (4 per treatment) were cut into 70-nm-thick sections with an ultramicrotome (LeicaUC6, Leica Microsystems Inc., Buffalo Grove IL, USA), and sections were contrasted using uranyl acetate and lead citrate (Reynolds 1963). Analyses were performed using a transmission electron microscope (EM900, Zeiss, Jena, Germany) equipped with a digital camera.

From the blocks prepared for each treatment, 20 chloroplasts were randomly observed in order to count the number of plastoglobuli per chloroplast. Moreover, starch grain and plastoglobuli areas were calculated based on the area of an ellipse (area = π*a*b, where a is the distance from the center to a vertex, and b is the distance from the center to a co-vertex) (Mesquita et al. 2011).

SPAD readings, tree growth and leaf P concentration

Immediately after tissue sampling for anatomical and ultrastructural analyses, the leaf greenness (chlorophyll index) was evaluated in the mature leaves using a SPAD-502 m (Minolta Corp., Ramsey, NJ, USA). Furthermore, the stem diameter was measured with a digital caliper approximately 3 cm above the intersection between the scion and rootstock. Trees were harvested and separated into leaves, branches and roots, and the leaf area was determined using a digital planimeter (LI-3000, LI-COR Inc., Lincoln, NE, USA). The different tree parts were washed in tap water, rinsed in deionized

water and dried at 60 °C for at least 3 days to obtain the dry mass (DM) production. The mature leaves were ground, and total concentration of P was determined using plasm emission spectrometry (ICP-OES) following nitroperchloric digestion (Bataglia et al. 1983).

Data analyses

The results were subjected to a two-way analysis of variance (ANOVA). When a significant ($p < 0.05$) interaction between the factors (P supply in the NS versus foliar treatment) was found, the effects of foliar treatments were compared by using Duncan's multiple range test ($p < 0.05$) within a given P concentration in the NS. The effect of P supply in the rooting medium was compared using the F test ($p < 0.05$) within the same foliar treatment. Light, scanning and transmission electron microscopy images of citrus leaves were interpreted with respect to the treatments.

Results

Tree growth and visual symptoms of Phi toxicity

Tree growth parameters including leaf area, stem diameter, and shoot and root DM showed significant ($p < 0.05$) interactions regarding the supply of P versus foliar treatments. Although the PD condition impaired the citrus growth compared with PS, this effect depended on foliar treatment. For instance, the relative ranking of reductions in leaf area, stem diameter and shoot DM of PD trees was Phi > KCl > Pi (Figs 1 and 2). In contrast, growth was not affected by foliar spray treatments under PS.

The Pi spray in PD trees increased the leaf area (31%, Fig. 1a), stem diameter (9%, Fig. 1b) and shoot DM (28%, Fig. 2a, e) more than the PD + KCl treatment, but there was no difference in root DM (Fig. 2d, f). On the other hand, Phi spray reduced the shoot DM by 24%, stem diameter by 39% and root DM by 41% relative to those of PD + KCl trees. There was no difference in leaf area between PD + KCl and PD + Phi trees.

The visible symptoms of Phi toxicity appeared after approximately 4 foliar applications (~55 days after the first foliar Phi spray) and were exclusively detected in PD trees (Fig. 2b).We observed leaves with chlorotic spots that became necrotic in combination with leaf folding. Leaf drop also occurred by the end of the experiment in PD + Phi trees.

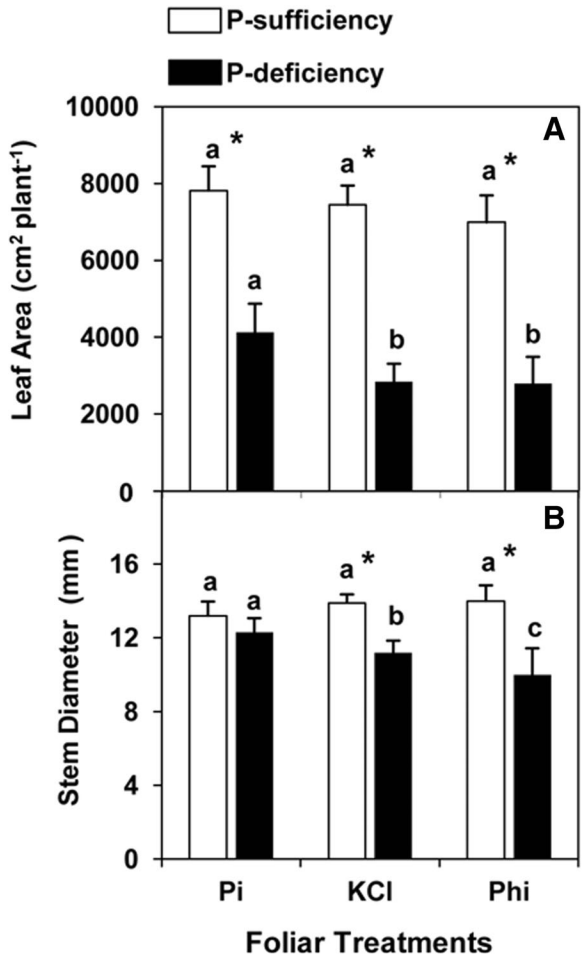


Fig. 1 Leaf area (in **a**) and stem diameter (in **b**) of citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS. Foliar treatment comparison: columns followed by different letters within the same P concentration in the NS are significantly different by Duncan's multiple range test ($p < 0.05$). P availability in the NS comparison: the presence of the asterisk for each foliar treatment indicates that the effect of P supply in the rooting medium is significantly different by the F test ($p < 0.05$). The bars indicate the standard-error ($n = 4$)

Leaf P concentration and SPAD readings

There were significant ($p < 0.05$) interactions between foliar treatments and P availability for leaf P concentration and SPAD readings. In the PD supply, the sprays with Pi and Phi increased the leaf P concentration by 30% and 63%, respectively, compared with the KCl application, while no increases were observed in PS

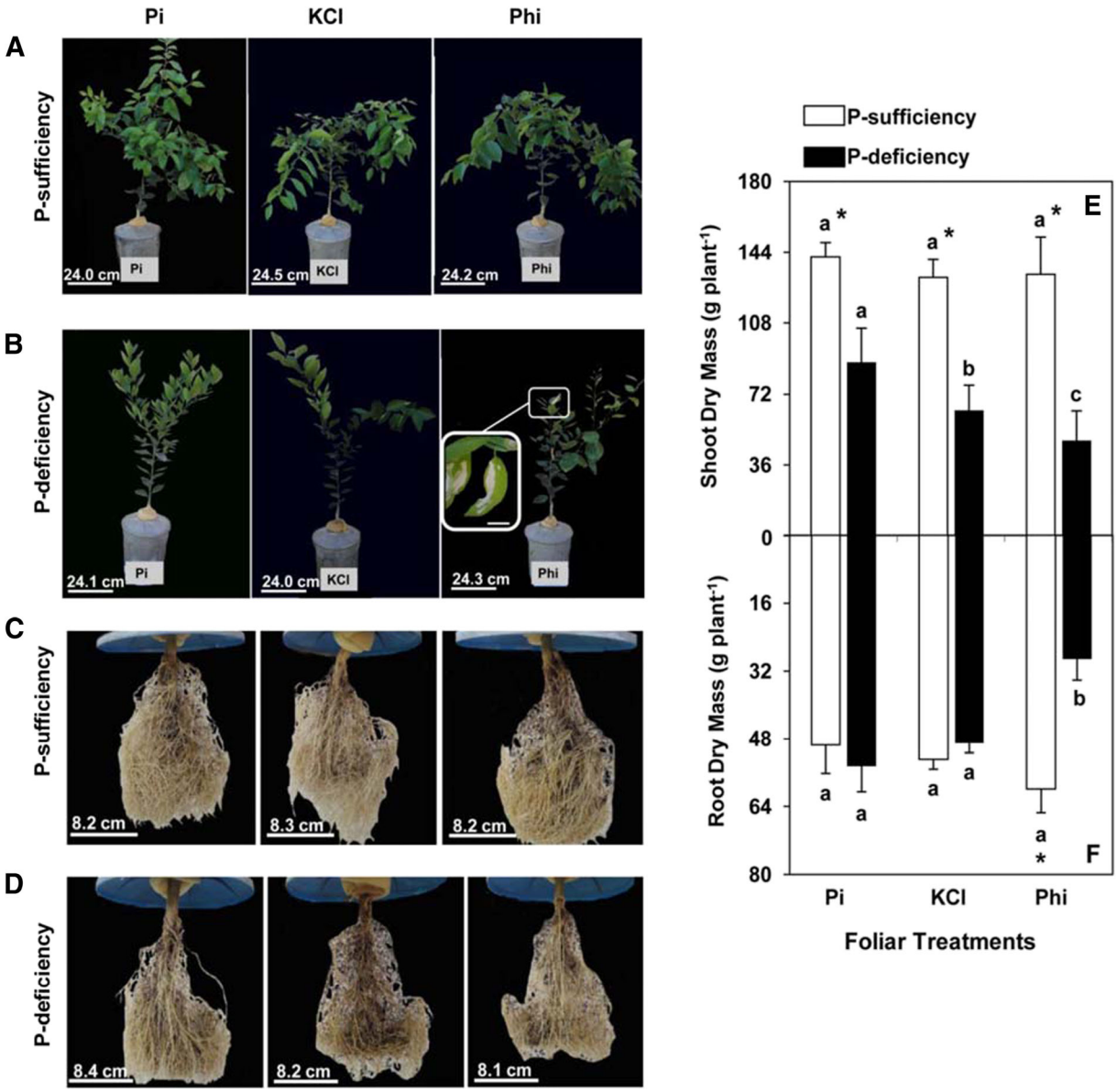


Fig. 2 Overview of the shoots (in **a** and **b**) and roots (in **c** and **d**) and their respective values of shoot (in **e**) and root (in **f**) dry mass productions of citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Under P-deficiency, the visual symptoms of Phi toxicity (chlorosis and necrosis) in the leaves are highlighted in the white contour. Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS. Foliar treatment comparison: columns followed by different letters within the same P concentration in the NS are significantly different by Duncan's multiple range test ($p < 0.05$). P availability in the NS comparison: the presence of the asterisk for each foliar treatment indicates that the effect of P supply in the rooting medium is significantly different by the F test ($p < 0.05$). The bars indicate the standard-error ($n = 4$)

trees (Fig. 3a). SPAD readings in PD + Phi trees were 35% lower than those of both PD + KCl and PD + Pi trees (Fig. 3b). Under PS, however, there was no effect of foliar treatments on leaf chlorophyll concentration.

Anatomical changes in citrus leaves

Light microscopy revealed that the leaves of all PS trees were well-structured regardless of the foliar treatments

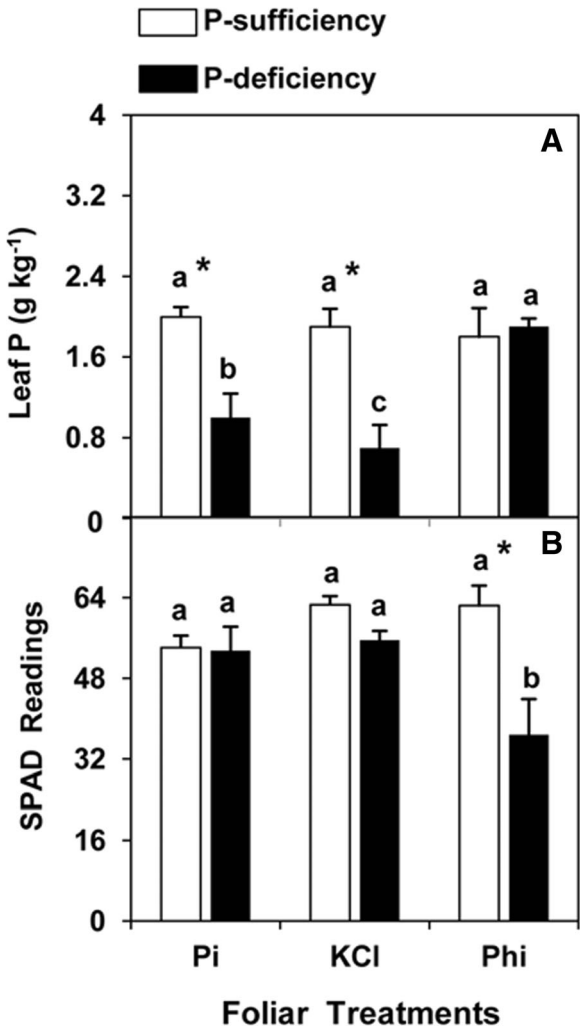


Fig. 3 Concentration of phosphorus (P) (in **a**) and SPAD readings (in **b**) of mature leaves from citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS. Foliar treatment comparison: columns followed by different letters within the same P concentration in the NS are significantly different by Duncan's multiple range test ($p < 0.05$). P availability in the NS comparison: the presence of the asterisk for each foliar treatment indicates that the effect of P supply in the rooting medium is significantly different by the F test ($p < 0.05$). The bars indicate the standard-error ($n = 4$)

(Fig. 4a-c). The mesophyll exhibited palisade parenchyma composed of two interconnected cell layers with regular shape, spongy parenchyma with visible intercellular spaces and presence of well-defined substomatal cavities in the abaxial epidermis. Moreover, the vascular bundles were composed of well-structured sclerenchyma fibers and phloem and xylem vessels (Fig. 4a-c). Externally, there were no visible leaf alterations in the scanning microscopy images among foliar treatments in PS trees, being found complete integrity of the leaf epidermal surface and stomata (Fig. 5a-c, g-i).

There were profound modifications to the leaf surface and internal structure of PD trees, however, and the effects depended on the foliar treatment (Figs 4d-f and 5d-f, j-l). For instance, the PD + KCl trees showed leaves with palisade parenchyma having degenerate cells (arrow, Fig. 4e), deformed substomatal cavities in the abaxial epidermis and stomata exhibiting surface with minor flaking (arrow, Fig. 5k). In contrast, the leaves of PD + Pi trees exhibited complete epidermal integrity and no damage to the stomata (Fig. 5d, j). The leaves of PD + Phi trees showed xylem and phloem vessels with irregular shapes and ruptured sclerenchyma fibers compared with PD + Pi trees (arrow, Fig. 4f). Furthermore, it was observed in these leaves the presence of epicuticular waxes with compromised integrity (Fig. 5f, l), as well stomata showing severe surface flaking, irregular contour and presence of deformed ostioles, all suggesting stomata degeneration (arrow, Fig. 5l).

Ultrastructural changes in citrus leaves

Ultrastructural analysis of leaves of PS trees revealed a well-organized ultrastructure regardless of foliar treatment, as there were no alterations of the cell wall, middle lamella or plasma membrane (Fig. 6a-c). The PS leaves also had typical chloroplasts with thylakoids arranged in well-structured grana, and only small starch grains were present (Fig. 7a-c).

On the other hand, modifications occurred to the leaf ultrastructure of PD trees, and they were dependent on the foliar treatment (Figs 6 and 7). For instance, PD trees subjected to KCl spray had deformed cell walls and apparent fragmentation of the middle lamella (thin arrow, Fig. 6e). In addition, the chloroplasts of these trees exhibited irregular shape, an increased number of plastoglobuli and large starch grains (Figs 6e and 7e). However, there were few such alterations in the PD trees under Pi spray, since the organization of their cell wall and middle lamella were comparable to the PS ones (Fig. 6a, d). Moreover, there were fewer starch grains and plastoglobuli in PD leaves under Pi spray compared with PD leaves under KCl spray (Figs 6d, e and 7d, e).

The mesophyll cells of PD + Phi leaves had cell walls with marked sinuosities, disorganization of middle

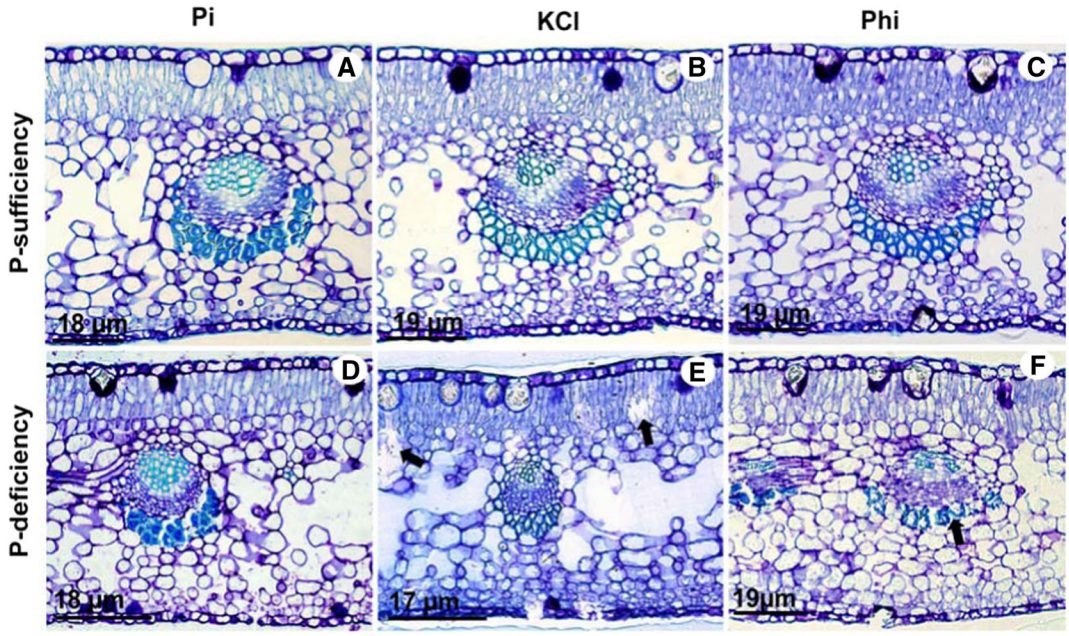


Fig. 4 Cross sections of leaves under light microscopy from citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Arrows indicate disruption in the palisade parenchyma cells (in **e**) and disruption in the connection between the sclerenchyma fibers of the vascular bundle (in **f**). Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS

lamella and plasma membrane plasmolysis (thin arrow, Fig. 6f). There were also great numbers of plastoglobuli and starch grains occupying large spaces inside the chloroplasts, with profound thylakoid degeneration (Fig. 7f).

The PD treatment increased the starch grain area of those trees subjected to KCl and Phi sprays (Fig. 8a). These two foliar treatments also increased the starch grain area by 89% and 211%, respectively, in relation to PD + Pi trees. In all foliar treatments, the PD trees showed more plastoglobuli area and number than PS trees (Fig. 8b, c). Moreover, Phi spray in PD trees increased these two parameters compared with application of Pi and KCl to the foliage of these trees. However, in PS trees, the starch grain area, plastoglobuli area and plastoglobuli number were not influenced by foliar treatments (Fig. 8).

Discussion

The results of the present study consistently revealed that the performance of the citrus trees exposed to foliar Phi spray depends on the regime of P supply to the root system, since (i) no effect of the spray foliar treatments was observed on the growth of the trees grown under PS condition and (ii) the PD + Phi trees, despite presenting a higher leaf P concentration, had less growth than both the PD + Pi and PD + KCl trees (Figs 1, 2 and 3a). In other studies, an impaired performance of plants exposed to deficient P supplies following foliar Phi spray has also been documented, even with significant increases in leaf P concentration (Thao et al. 2008; Ratjen and Gerendás 2009). These results are in accordance with the notion that whereas Phi is taken up by Pi transporters leading to both increased total tissue P concentration and suppressed Pi starvation-induced responses, it cannot replace Pi as a source of P for the plants (Ticconi et al. 2001; Varadarajan et al. 2002). Nevertheless, the mechanistic basis governing the lower efficiency of foliar-applied Phi in promoting growth of P-deficient plants as compared with Pi spray remains to be completely revealed and merits further investigation, as Phi spray is a widespread management practice for field grown plants. Indeed, the recognized poor nutritional value of Phi has been mostly determined in studies with P-deficient plants taking up Phi applied in the rooting medium, which could indirectly affect leaf metabolism because of direct toxic effects of Phi on growth and physiology of the root system (Berkowitz et al.

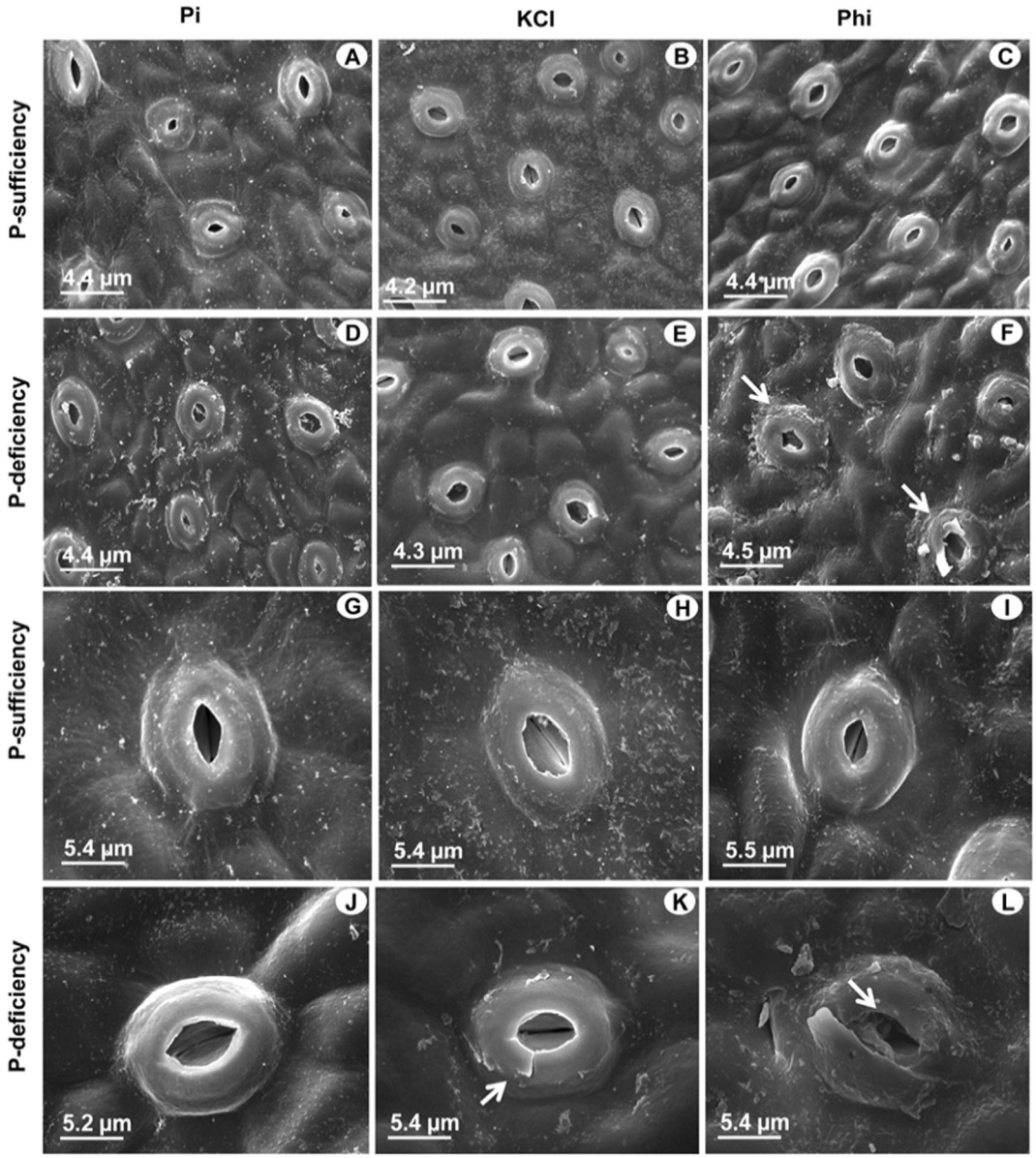


Fig. 5 Scanning electron microscopy of abaxial surface of leaves from citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Overview of leaf epidermis (in **a**-**f**) and detailed visualization of injuries to the stomata (in **g**-**l**). Arrows indicate surface flaking (in **k** and **l**) and deformed ostioles (in **l**). Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS

2013; Jost et al. 2015; Zambrosi 2016). In this sense, we aimed to shed light on the interaction between P availability and foliar Phi spray on the functioning of citrus leaves by analyzing the anatomical and ultrastructural integrity of samples from trees receiving Phi application to their foliage and grown under both P sufficiency and deficiency in the rooting medium.

Herein, scanning microscopy images revealed that the abaxial leaf surface of PD + Phi trees showed, comparatively to PD + Pi and PD + KCl trees, compromised integrity of epicuticular waxes and stomata injury, which was associated to marked surface flaking and presence of deformed ostioles (Fig. 5f, l). Similar changes found on the integrity of stomata have been associated with its

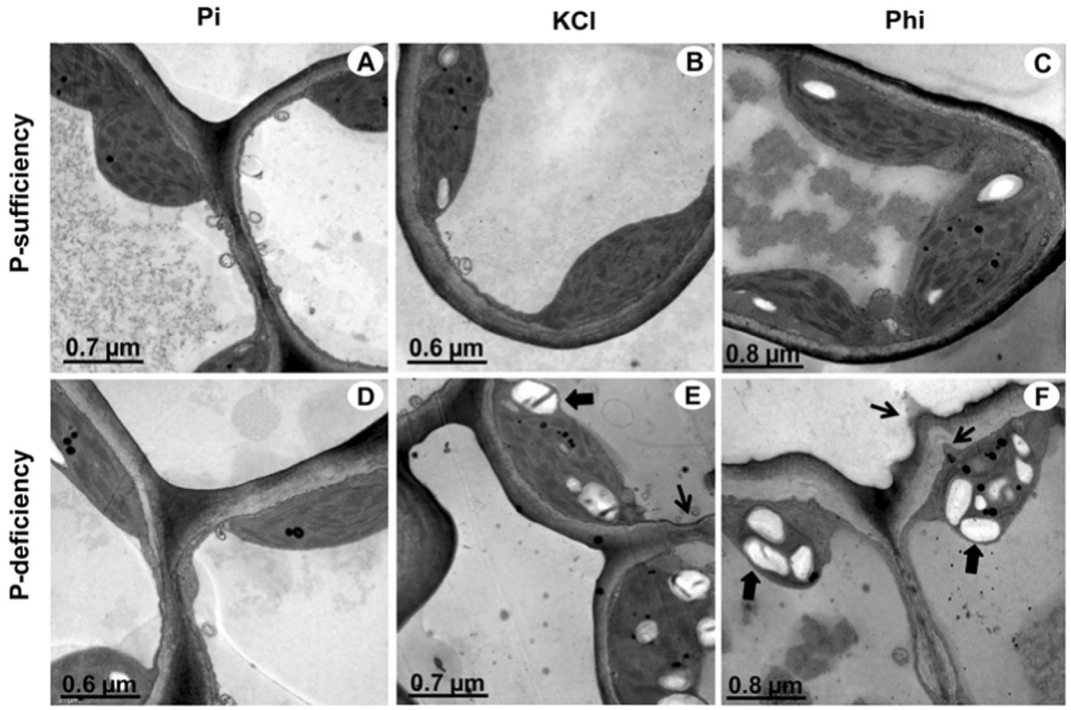


Fig. 6 Mesophyll cells from citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Large and thin arrows indicate the presence of starch grains and cell wall with sinuosities, respectively (in **e** and **f**). Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS

malfunctioning (i.e., misregulation of stomatal closure) and, thus, an increased water loss due to higher transpiration rates (Neighbour et al. 1988; Mesquita et al. 2016a). Furthermore, it was detected internally via light microscopy investigations that leaves of PD + Phi trees exhibited the rupture of sclerenchyma fibers and irregularly shaped xylem vessels (Fig. 4f), which could lead to hindered water transport in leaf tissues (Fernández et al. 2008; Eichert et al. 2010; Mesquita et al. 2016b). Although leaf water relations were not evaluated, such described alterations in the structure of the leaves of PD trees exposed to Phi application suggest that whereas the damage to the stomata would contribute to increased water loss through the leaf surface, the impaired structure of the xylem could not be able to sustain this higher water demand, making the leaves more prone to water stress. Accordingly, additional studies are still necessary to investigate how water relations in PD trees respond to foliar Phi spray and their further implications for overall plant performance in the field.

It was also evident from the images of light microscopy that PD trees receiving Phi spray to their foliage exhibited a severe disarrangement of phloem vessels compared with other foliar treatments (Fig. 4d-f). Such damage could cause an impaired transport of carbohydrates from source leaves to sink tissues (Forschner et al. 1989; Thompson 2006) and thus contribute to explain the greater abundance of starch grains occupying large spaces inside the chloroplasts in the mesophyll cells of PD + Phi trees (Figs 6f, 7f and 8a). Based on these findings, we might further propose that the malfunctioning of phloem and reduction in carbohydrate supply to sink tissues would be an important mechanism by which foliar Phi spray compromises the growth of PD trees. However, rather than impairments in carbohydrate transportation, one could argue that the reason for starch accumulation in source leaves of PD + Phi trees was a decreasing in photoassimilate demand at the whole-plant level due to direct effects of Pi deficiency and/or Phi toxicity in the activity of sink tissues (Pieters et al. 2001). Moreover, starch accumulation is a common response in P-deficient leaves because of limitations in triose-phosphate translocator function and a decreasing in starch mobilization (Heber and Heldt 1981; Usuda and Shimogawara 1991; Qiu and

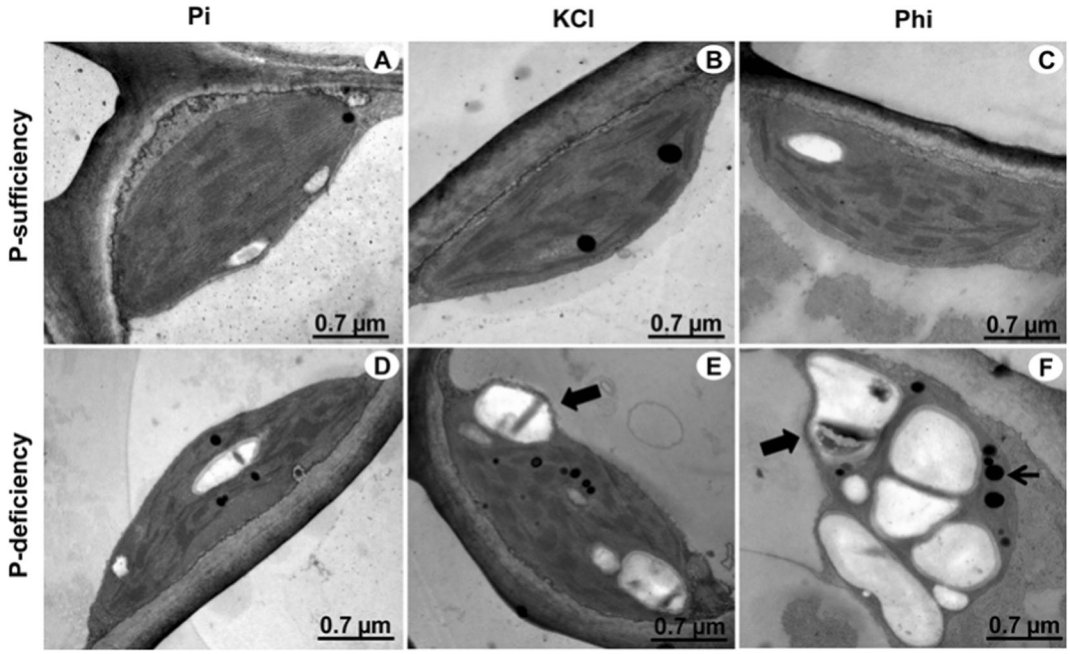
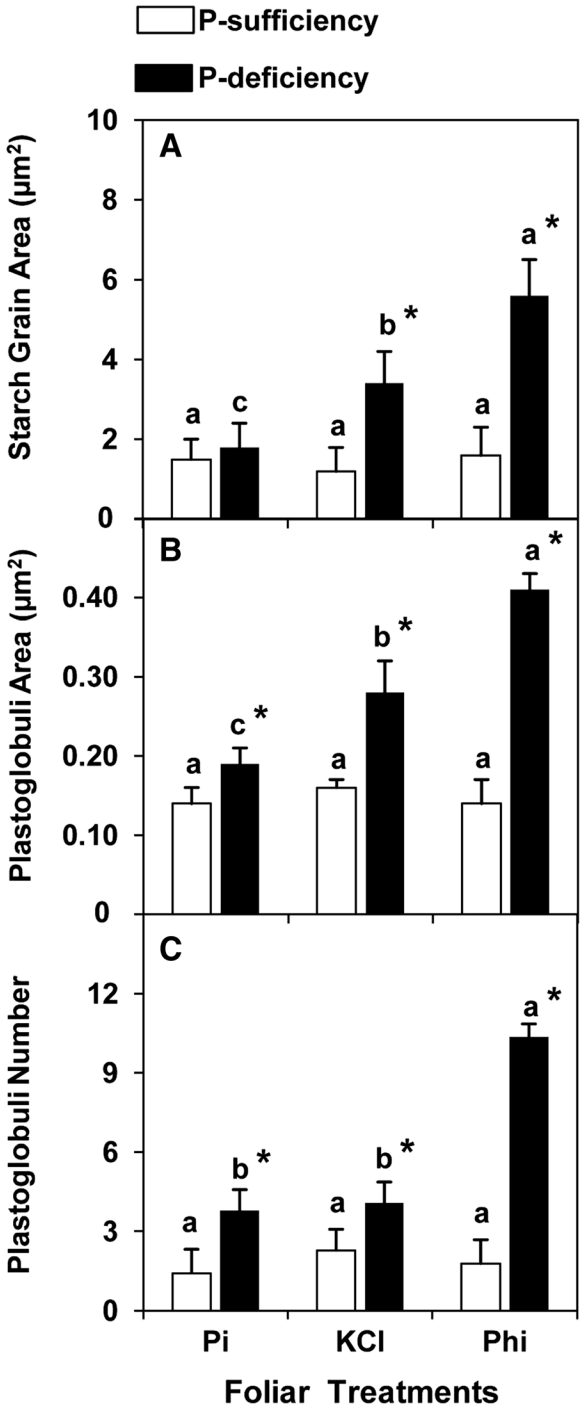


Fig. 7 Detailed view of chloroplasts in mesophyll cells from citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Large and thin arrows indicate starch grains and plastoglobuli, respectively (in **e** and **f**). Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS

Israel 1992). Accordingly, it is likely that such an observed alteration in photoassimilate partitioning in the mesophyll cells of PD + Phi trees was associated with the inability of Phi to replace Pi in carbohydrate metabolism and/or competition between these two forms of P in the metabolic reactions (Danova-Alt et al. 2008; Berkowitz et al. 2013). Indeed, this idea is supported by results revealing that Phi application increased leaf P concentration compared with that of PD trees sprayed with both Pi and KCl (Fig. 3a) and citrus plants experiencing Phi toxicity showed impaired photosynthetic P-use efficiency (Zambrosi et al. 2011).

While the major process leading to starch accumulation in the mesophyll cells of Pi-starved plants under Phi sprays remains to be determined, it is clear from ultrastructural analysis of the leaves of PD + Phi trees that an important consequence of this phenomenon corresponds to damages to the internal membrane system of the chloroplasts (i.e., thylakoid degeneration) (Fig. 7f). Such a disruption in the ultrastructure of this organelle is linked to chlorophyll molecule degradation and subsequent manifestation of the symptoms of leaf chlorosis (Schaffer et al. 1986; Stettler et al. 2009; Hernández and Munné-Bosch 2015). Herein, our data revealed also that PD + Phi trees had the lowest values of SPAD readings (Fig. 3b) and the development of necrosis (Fig. 2b), typical symptoms of toxicity of Phi spray observed in field conditions (Scott et al. 2016). The disarrangement of the photosynthetic apparatus and reduction in chlorophyll concentration coupled with a higher abundance of plastoglobuli have been considered clear indication of leaf senescence (Matile et al. 1996; Lim et al. 2007) as well the occurrence of nutritional stresses in citrus leaves (Bondada and Syvertsen 2005; Papadakis et al. 2007). In view of this evidence, the presence of chloroplasts exhibiting an increased size and number of plastoglobuli was also found in mesophyll cells of PD + Phi trees (Figs 7f and 8b, c). The occurrence of a more pronounced leaf senescence in PD + Phi trees agrees with previous results demonstrating that Phi supply accelerates programmed cell death of Pi-starved cells of rape (*Brassica napus*) (Singh et al. 2003). In addition to the effects at chloroplast level, PD + Phi trees exhibited a marked damage to the cell wall structure (i.e.,



◀ **Fig. 8** Starch grain area (in **a**), plastoglobuli area (in **b**) and number of plastoglobuli per chloroplast (in **c**) in the mesophyll cell of mature leaves from citrus trees treated with foliar sprays of Pi, KCl and Phi and grown under a sufficient and deficient supply of P in the NS. Foliar treatments: 6 applications of a solution containing 0.16 M P as potassium phosphate (Pi) or potassium phosphite (Phi) and a control without P (KCl) at 15-day intervals. P-sufficiency = 0.5 mM P in the NS; P-deficiency = 0.01 mM P in the NS. Foliar treatment comparison: columns followed by different letters within the same P concentration in the NS are significantly different by Duncan's multiple range test ($p < 0.05$). P availability in the NS comparison: the presence of the asterisk for each foliar treatment indicates that the effect of P supply in the rooting medium is significantly different by the F test ($p < 0.05$). The bars indicate the standard-error ($n = 4$)

presence of pronounced sinuosity and fragmented middle lamella) and plasmolysis of the plasma membrane (Figs 6f and 7f), which might have lead to a complete collapse of the cells and an impaired leaf functioning (Gao et al. 2016; Zambrosi et al. 2016).

In conclusion, we were able to provide evidences that the impaired growth of PD citrus trees under Phi spray was associated with severe damage to the anatomical and ultrastructural organization of the leaf tissue, which, in turn, induced early leaf senescence, compromised leaf growth and the functioning of the photosynthetic apparatus. Accordingly, at the whole-canopy level, we could argue that Phi spray will exacerbate the reduction in the productivity of PD trees because of the diminished photosynthetic capacity (i.e., lower leaf area formation and leaf photosynthesis). Our results also support the view that while Phi-based products might be toxic and not correct the P deficiency, Pi spray could be used a supplementary management strategy to supply the nutrient to trees grown in problem soils. Moreover, the possible use of Phi as an inducer of plant tolerance to biotic and abiotic stresses or even in the control of diseases in groves under high disease pressure should be combined with a sufficient P nutritional status of the trees to avoid Phi toxicity and damage to growth. Indeed, this view is supported by the fact that PS trees did not show any growth response or alterations to the anatomical and ultrastructural organization of the leaf tissue to foliar Phi spray, most likely because such condition favours the vacuolar uptake of Phi and reduces its toxic potential (Danova-Alt et al. 2008).

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