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Title:
Elemental microanalysis in leaf transversal sections of peach by SEM/EDXA: Influence of iron nutritional status

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Abstract:
Immobilization and accumulation of Fe in physiologically inactive forms somewhere in the leaf has been suggested to be associated to chlorosis in Fe-deficient leaves. The localization of elements at tissue level is essential to understand the plant processes involved in inorganic ion homeostasis. In this study we have used LT-SEM-EDX microanalysis to investigate the spatial distribution of Fe at tissue level in peach leaves under Fe-sufficient and Fe-deficient status. Chlorotic leaves from field peach trees showed a preferential localization of Fe in spongy parenchyma.

Introduction

Immobilization of inactive Fe forms has been reported to occur in leaves of Fe-deficient, chlorotic field grown plant species. This is the so-called "chlorosis paradox", where Fe-deficient leaves have relatively high Fe concentrations, 80 μg Fe g^{-1} dry weight or even higher (Morales *et al.*, 1998; Römheld, 2000). The localization of Fe in leaves of Fe-sufficient and Fe-deficient leaves has been recently studied by two-dimensional (2-D) synchrotron radiation-induced X-ray fluorescence (μ-SRXF) imaging by Jiménez *et al.* (2009). In this study, authors showed that in chlorotic leaves Fe is preferentially located in midrib and vein leaf areas, with interveinal mesophyll areas having low concentrations of Fe. However, μ-SRXF data give 2-D images that integrate elemental concentrations across the full leaf depth, but provide no information about the internal distribution of Fe in the transversal leaf profile. The localization of elements at tissue level is essential to understand the plant processes involved in inorganic ion homeostasis. In the last years, scanning electron microscopy coupled to energy-dispersive x-ray microanalysis (SEM-EDX) has been successfully used to obtain transversal metal distribution in leaves of some hyper-accumulator species (Fernando *et al.*, 2008; Vogel-Mikus *et al.*, 2008). Most of these studies show that the epidermis is the principal site of heavy metal accumulation. Also, a differential accumulation of nutrients in epidermis and mesophyll cell layers has been reported in monocot species (Karley *et al.* 2000).

The aim of this work was to study the distribution of Fe in transversal sections of peach leaves, by using SEM-EDX, in plants grown under different Fe nutritional status.

Materials and methods

Field samples

Control (green Fe-sufficient, +Fe) and chlorotic (Fe-deficient, -Fe) leaves were collected from 14 year-old peach (*Prunus persica* (L.) Batsch, cv. Catherina) trees, grown in an orchard located near Zaragoza, Spain (Peñaflor 41º, 46', 47''N; 0º, 47', 34''W). Fully expanded leaves were collected from medium size shoots of Fe-sufficient and Fe-deficient trees, located at mid-crown height, approximately 1.5 m from the ground. Leaves were sampled during the summer season. Chlorophyll and total Fe concentrations were also measured.

Low temperature-scanning electron microscopy and microanalysis (LT-SEM-EDX)

Sections of fresh peach leaves were then cryo-fixed in slush nitrogen (-196 ºC), cryo-transferred to a vacuum chamber at -180 ºC and fractured using a stainless steel spike. Once inside the microscope, the samples underwent superficial etching under vacuum (-90 ºC, 120 s, 2 kV) and then overlaid with Au for observation and microanalysis with a Zeiss digital scanning microscope (DSM 960). Microanalysis was carried out in specific leaf areas (approximately 15 x 15 μm) using an energy dispersive X-ray microanalysis (EDX) Pentaflet apparatus (Pentaflet, Oxford, UK). Areas tested were adaxial epidermis, palisade parenchyma, xylem vessels, spongy parenchyma and abaxial epidermis (Fig. 1). Whenever possible, a single cell area was targeted. Microanalysis was carried out using a resolution of 133 eV, operating with a 35º take-off angle, an accelerating voltage of 15 kV, a working distance of 25 mm and a specimen current of 1-5 nA. Semi-quantitative element analysis was obtained using standard ZAF (atomic number, absorption and fluorescence) correction procedures, using Link Isis v. 3.2 software (Link Isis, Oxford, UK).

Statistics of LT-SEM-EDX analysis

One-way ANOVA was used to compare the results obtained in the different leaf tissues, followed by a post hoc, multiple comparison of means with the Duncan test ($P < 0.05$; $n = 8$). Results shown are means of 8 measurements per leaf in each area type tested. All calculations were made using SPSS v. 17.0 software.

Results and discussion

The cryo-fracture images of peach leaves by LT-SEM reveal the main plant tissues that form the leaf blade: the adaxial -upper- epidermis, the palisade parenchyma, the xylem vessels, the spongy parenchyma and the abaxial -lower- epidermis (Fig. 1a, b). Morphologically, Fe-deficient leaves are thinner and more compacted, with the size of the apoplastic spaces between spongy mesophyll cells being smaller.

Direct analysis of leaf tissues in field samples showed that there were no major changes in the Fe signal in the leaf transversal section in control leaves (Fig. 1c). In Fe-deficient leaves, a higher Fe signal was often found in spongy parenchyma when compared to the rest of tissues (Fig. 1d). Fe accumulation in the spongy parenchyma of chlorotic leaves is in accordance with data of Lambert *et al.* (2006) who found, using three-dimensional nuclear magnetic resonance imaging (MRI), that Fe is preferably located as Fe(III) in the apoplast of spongy cells close to veins in red stem dogwood. A preferential localization of Fe near leaf veins was observed by Jiménez *et al.* (2009) in chlorotic peach-almond hybrid leaves using 2-D μ-SRXF. All these findings support the hypothesis that in chlorotic, Fe-deficient leaves, Fe is accumulated in the apoplast of spongy parenchyma located close to leaf veins, possibly as inactive Fe(III) forms. This would be in agreement with the decrease found in the activity of the leaf mesophyll Fe(III)-chelate reductase in chlorotic leaves with respect to the Fe-sufficient controls (Brüggemann *et al.*1993; González-Vallejo *et al.*, 2000), that could therefore facilitate the accumulation of Fe in the apoplast.

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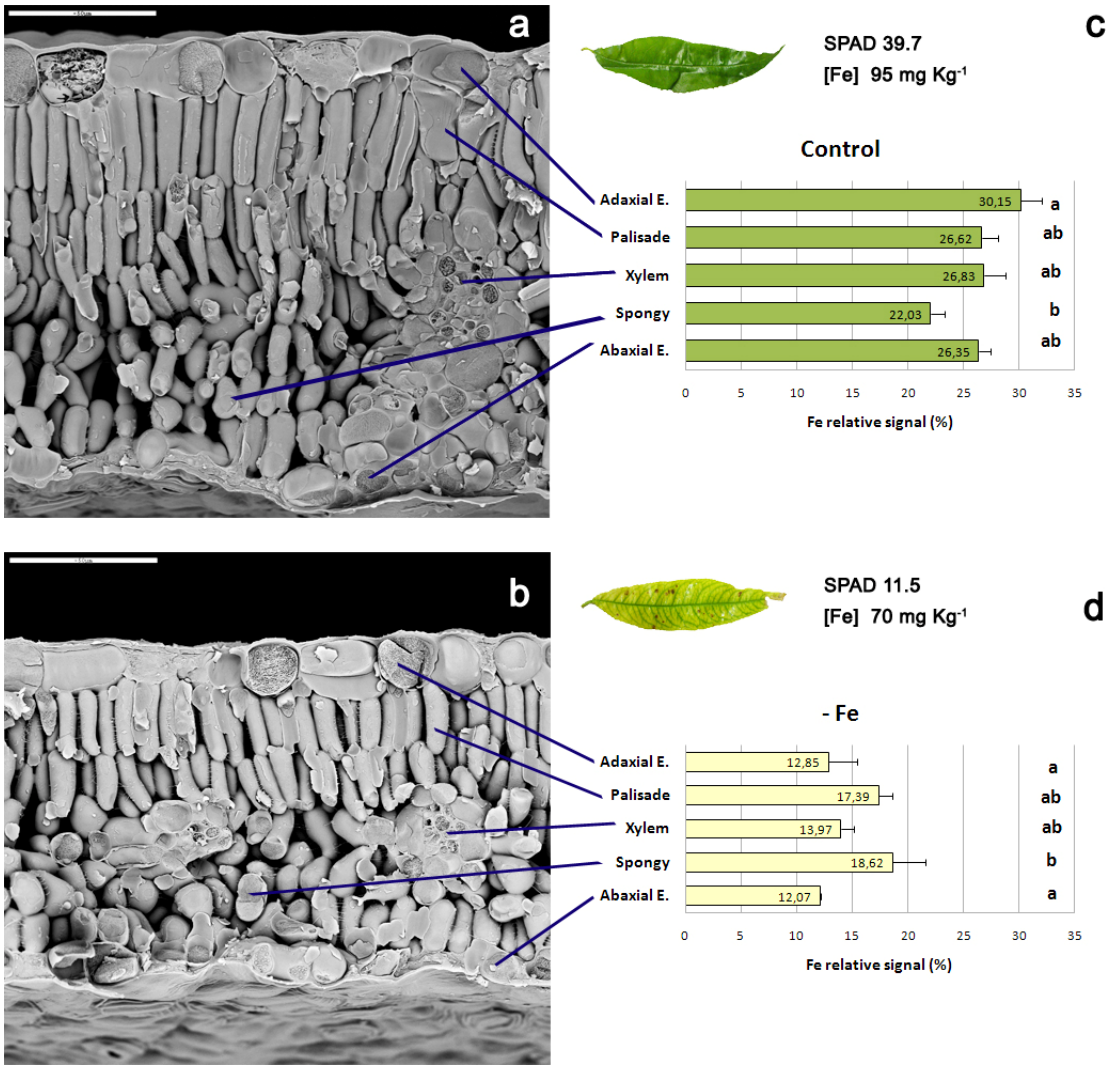

Fig. 1. Control (green Fe-sufficient, +Fe) and chlorotic (Fe-deficient, -Fe) LT-SEM micrographs (a, b) and Fe analysis by EDX (c, d) of leaf transversal sections from field peach trees. The measured values are presented as means (± SE). Significant differences among plant tissues are indicated by different characters ($P < 0.05$; $n = 8$).