Leaf structural changes associated with iron deficiency chlorosis in field-grown pear and peach: physiological implications

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Abstract Plants grown in calcareous, high pH soils develop Fe deficiency chlorosis. While the physiological parameters of Fe-deficient leaves have been often investigated, there is a lack of information regarding structural leaf changes associated with such abiotic stress. Iron-sufficient and Fe-deficient pear and peach leaves have been studied, and differences concerning leaf epidermal and internal structure were found. Iron deficiency caused differences in the aspect of the leaf surface, which appeared less smooth in Fe-deficient than in Fe-sufficient leaves. Iron deficiency reduced the amount of soluble cuticular lipids in peach leaves, whereas it reduced the weight of the abaxial cuticle in pear leaves. In both plant species, epidermal cells were enlarged as compared to healthy leaves, whereas the size of guard cells was reduced. In chlorotic leaves, bundle sheaths were enlarged and appeared disorganized, while the mesophyll were more compacted and less porous than in green leaves. In contrast to healthy leaves, chlorotic leaves of both species showed a significant transient opening of stomata after leaf abscission (Iwanoff effect), which can be ascribed to changes found in epidermal and guard cells. Results indicate that Fe-deficiency may alter the barrier properties of the leaf surface, which can significantly affect leaf water relations, solute permeability and pest and disease resistance.

Introduction

Iron (Fe) deficiency chlorosis is a common abiotic stress affecting plants in many areas of the world. This physiological disorder is mainly found in crops grown in calcareous and/or alkaline soils and occurs as a result of several causes acting simultaneously (Rombolà and Tagliavini 2006). Although Fe is very abundant in the earth's crust, its availability to plants is often restricted by the very low solubility of Fe(III)-oxides under aerobic conditions (Schmidt 2003). Iron is a vital element for living organisms, since it is essential for the proper functioning of multiple metabolic and enzymatic processes related to electron transport, nitrogen fixation, DNA and hormone synthesis, etc. (Conrad and Umbreit 2000; Briat 2007). Plant growth under conditions of restricted Fe availability is a problem of economic significance for the fruit agricultural industry, since it reduces crop yield and quality (Álvarez-Fernández et al. 2006), and its control involves significant costs, chiefly related to treatment with synthetic Fe chelates (Lucena 2006).

Iron deficiency deeply alters the morphology and physiology of plants (Briat 2007). Typical iron chlorosis symptoms include leaf interveinal chlorosis, starting from the shoot apex, development of leaf necrotic spots and shoot defoliation during the growing season (Rombolà and Tagliavini 2006). Apart from leaf chlorophyll (Chl) and carotenoid concentration decreases, reductions in leaf size, fresh and dry weight have been found associated with lime-induced chlorosis (Hutchinson 1970; Anderson 1984; Morales et al. 1998; Larbi et al. 2006). Severe leaf Fe deficiency chlorosis has been shown to markedly reduce the photosynthetic rate of several plant species under controlled and field conditions, with light absorption, photosystem II and Rubisco carboxylation efficiencies being down-regulated (see Larbi et al. 2006 and references therein).

Early ecological studies carried out with detached leaves of several plant species grown in calcareous soils indicated that chlorotic leaves lost water more rapidly than healthy ones (Hutchinson 1970; Anderson 1984). Hutchinson (1970) hypothesised that the larger leaf water deficits of detached chlorotic leaves may be due to differences in stomatal behaviour or alternatively to a high cuticular transpiration rate. Anderson (1984) noted that despite lime-induced chlorosis may affect stomatal behaviour, cuticular rather than stomatal factors could be responsible for the more pronounced

water loss. According to Shimshi (1967), in several species chlorosis was accompanied by a lower degree of stomatal opening, and not by a decrease in stomatal density. Gas exchange and Chl fluorescence measurements carried out on severely Fe-deficient peach, pear and sugar beet leaves showed that Fe-deficiency led to decreases in stomatal opening, transpiration rates and water use efficiency (Larbi et al. 2006).

Working with Fe-sufficient and Fe-deficient Mexican lime (*Citrus aurantifolia*) leaves, Maldonado-Torres et al. (2006) observed that chlorosis led to morphological changes at the leaf, cellular, and ultracellular levels. Chlorotic leaves were thicker than green ones, due to increases in palisade and spongy parenchyma cell length and thickness (Maldonado-Torres et al. 2006). In contrast, no significant differences regarding leaf thickness were found between Fe-sufficient and Fe-deficient leaves of pear and peach grown in calcareous soils in Spain (Morales et al. 1998).

The effects of Fe deficiency on the leaf epidermis have not been investigated so far, despite the fact that it is the limiting barrier for the exchange of water and solutes between the leaf and the environment. Most epidermal cells of the aerial parts of higher plants, such as leaves, fruits and non-woody stems, are covered by a continuous extra-cellular membrane of soluble and polymerized lipids called cuticle or cuticular membrane (Heredia 2003). The structure and composition of the cuticle varies substantially among plants, organs and growth stages, but is basically composed by a cutin matrix with waxes embedded in (intracuticular waxes) and deposited on the surface (epicuticular waxes) (Heredia 2003; Jeffree 2006). Based on their constituents, the cuticle can be defined as a hydrophobic and non-reactive polyester with associated waxes (Heredia 2003). Cuticles have been shown to be permeable to water and ions, and also to polar compounds (Kerstiens 2006; Schreiber 2006).

The aim of this investigation was to study changes occurring in the surface and internal structure of peach and pear leaves affected by Fe chlorosis. Changes observed are discussed in the context of plant stress physiology, water relations and penetration of leaf applied-agrochemicals.

Materials and Methods

Plant Material

Green and chlorotic leaves were collected from 14 year-old peach (*Prunus persica* (L.)

103 Batsch, cv. Miraflores) and pear (*Pyrus communis* L. cv. Blanquilla) trees, grown in
104 commercial orchards located in the Jalón River Valley, in the Zaragoza province,
105 Spain. Soil was calcareous, with approximately 30% total $CaCO_3$, 10% active $CaCO_3$,
106 7 mg kg^{-1} DTPA-extractable Fe, 2.6% organic matter and pH 7.8 in water. The flood-
107 irrigated orchards were appropriately maintained in terms of pest and disease control.
108 The orchards had a frame of 5 × 4 m (peach) and 4 × 3 m (pear). Iron-chlorotic trees
109 did not receive any exogenous Fe input for two years prior to leaf analysis, and
110 developed Fe deficiency symptoms in springtime. Trees were Fe-deficient, since they
111 re-greened after Fe fertilization, either in the form of Fe foliar sprays (Álvarez-
112 Fernández et al. 2004; Fernández et al. 2006), branch Fe solid implants (Larbi et al
113 2003) or Fe-chelate treatments to the soil near the trunk (Álvarez-Fernández et al.
114 2003).

115 The experiment was designed as a completely randomized block. Trees with similar
116 chlorophyll levels were selected at the beginning of the trial, and monitored for Chl
117 levels for 2 years. Some trees were treated with Fe(III)-EDDHA (40 g per tree applied
118 in May; Sequestrene G 100, Syngenta Agro S.A., Spain) and remained fully green
119 throughout the experiment. Fully expended, non-damaged leaves were collected from
120 medium size shoots of Fe-sufficient and Fe-deficient trees, located at mid-crown
121 height, approximately 1.5 meters from the ground. Leaves were sampled during the
122 summer season of the years 2006 and 2007.

123 Leaf weight, area and SPAD value were determined prior to analysis. The Fe
124 concentration of leaves was analysed by Flame Atomic Absorption Spectroscopy by
125 using standard A.O.A.C. methods. Prior to processing, leaves were carefully washed
126 in a 0.1% detergent (Mistol, Henkel) solution and thoroughly rinsed, first in tap and
127 then in ultrapure water. Thirty samples per treatment, each composed of 10 leaves,
128 were taken throughout the whole experimental period.

129 Extraction of cuticular membranes and cuticular isolates

130 Cuticles from leaves of green and chlorotic peach and pear trees were isolated
131 enzymatically as described by Schönherr and Riederer (1986). Leaf discs 1.4 cm in
132 diameter, with the abaxial side labelled with a black felt-tip marker, were incubated in
133 citrate buffer (10 mM citric acid adjusted to pH 3.0 with KOH) containing 2% (v/v)
134 cellulase (Celluclast 1.5 L from Novozymes, Bagsvared, Denmark), 2% (v/v)

pectinase (Pectinex 100 L from Novozymes) and 1 mM NaN_3 (Sigma, St. Louis, Mo, USA), in an orbital shaker at low speed. Adaxial and abaxial leaf cuticles were separated after 1 week incubation. Isolated cuticular membranes were washed for 24 h in deionised water and then either dehydrated in an oven at 60ºC and directly weighed, or air-dried and stored at room temperature for further analysis.

Soluble cuticular lipids were extracted by immersion of 75 leaves in 300 ml of a 2:1 chloroform:methanol solution for 1 min, using 3 replicates per sample. Extracts were concentrated under a flow of N_2 and then evaporated until dryness in a watch glass in a laboratory fume cupboard. The amount of soluble cuticular lipids was expressed on a leaf surface area basis.

Microscopic examination

Leaf pieces were fixed in FAA (90% ethanol:water, 5% formol and 5% acetic acid), dehydrated, embedded in Historesin (Leica, Heidelberg, Germany) and transversal sections were cut with a microtome. Sections were stained with toluidine blue, berberine or auramine O and observed with a light microscope (Nikon E 800, Japan; only toluidine blue micrographs are presented). Fresh leaf transversal sections and pieces (for internal structure and surface studies, respectively), were frozen in liquid N, gold sputtered and observed with a low temperature scanning electron microscope (LTSEM, DSM 960 Zeiss, Germany, acceleration potential 15 kV, working distance 10 mm and probe current 5-10 nA). Scanning electron micrographs of fresh and dried leaf surfaces were also obtained after gold coating, with other SEM microscopes (Hitachi S-3400 N and Zeiss DSM 940 A). Stomatal densities and apertures were measured on SEM micrographs and also in nail-polish leaf fingerprints, using image analysis (software packages NIS-Elements D, Nikon Corporation, Japan and Carnoy v. 2.1, University of Leuven, Belgium).

Leaf transpiration

Transpiration rates of green and chlorotic leaves of recently flood-irrigated trees were measured with a portable steady-state porometer (LI-1600, LI-COR Inc., Lincoln, NE). First, leaves were measured in their natural orientation on the trees. Then, leaves were detached, the measuring cuvette with the clamped leaf was transferred to the shade, and transpiration rates were further recorded for 16 min after detachment. The time course of water loss for detached leaves was also measured gravimetrically for 4

days (Anderson 1984).

Results

General leaf characteristics and internal structure

Severely Fe-deficient, chlorotic leaves had Chl and Fe concentrations lower than those found in healthy leaves. Reductions in Chl were 70 and 84%, whereas decreases in Fe were 34 and 39% in peach and pear, respectively (Table 1). Leaf fresh weight (FW) and size were also significantly reduced by Fe-chlorosis as compared to the values measured for Fe-sufficient peach and pear leaves (Table 1). Decreases in FW and total leaf surface (in peach/pear) were 23/24% and 24/26%, respectively.

In both species, stomata were found only in the abaxial leaf side. While green and chlorotic leaves had similar stomatal densities, Fe deficiency appeared to decrease significantly the average size of stomatal pores in both plant species. Stomatal length decreases with Fe deficiency were 24% in peach and 17% in pear (Table 1).

Iron deficiency also affected the internal leaf structure of peach and pear leaves (Figs. 1 and 2). While no significant differences regarding leaf thickness were observed (data not shown), peach leaf transversal sections show that vascular bundle and palisade parenchyma cells were better organised and defined in green than in chlorotic leaves (Fig.1). Also, the spongy parenchyma was also more porous, with larger empty intracellular spaces, in green than in chlorotic leaves. Another remarkable feature observed in chlorotic peach leaves was the larger size of epidermal cells, especially in the adaxial side, as compared to Fe-sufficient leaves. In peach, adaxial epidermal cell length was increased by 23% by Fe deficiency (average length of approximately 23 and 18 μm in chlorotic and green leaves). In pear, an enlargement of leaf epidermal cells with Fe deficiency was also observed, but it was less pronounced than in peach (Fig. 2A,B versus F,G). Regarding the cell wall, both the toluidine blue staining (Figs. 1A,E and 2A,F) and autofluorescence (Fig.2E and J) intensities were markedly different in chlorotic and green leaves, suggesting changes in composition. Whereas cell walls in green leaves were thick and homogeneous, walls surrounding leaf cells in chlorotic leaves appeared as thin, discontinuous and apparently heterogeneous (see close up in Fig. 2J).

Leaf epidermis

198 Iron deficiency affected the morphology of the abaxial and adaxial leaf surface (Figs.
199 1C,D,G,H and 2C,D,H,I, for green and chlorotic peach and pear leaves). In peach,
200 both the adaxial and abaxial surfaces of Fe-sufficient leaves appear to have more
201 epicuticular waxes (Fig. 1C,D) when compared to Fe-deficient leaves (Fig. 1G,H), as
202 indicated by a smoother, glazed-like surface. In pear, the surfaces of Fe-sufficient and
203 Fe-deficient leaves also had a distinct appearance, although differences were much
204 less remarkable than in the case of peach.

205 In light of the above observations, both the cuticle weight and the amount of
206 soluble cuticular lipids per unit surface were quantified (Table 2). Iron chlorosis led to
207 different effects in the two plant species investigated, since in pear only the lower
208 cuticle of chlorotic leaves experienced remarkable changes, whereas in peach the
209 amount of soluble lipids was significantly reduced. In pear, the lower cuticular
210 membrane underwent a highly significant weight per unit surface reduction with Fe
211 chlorosis (35% when compared to control values), while the upper cuticle was not
212 significantly affected. In this species, soluble cuticular lipids accounted for 10 and
213 13% of the total leaf cuticle weight in green and chlorotic leaves. In peach, however,
214 Fe-deficiency caused a marked decrease (41%) in the amount of soluble cuticular
215 lipids, but the weight per unit surface of abaxial and adaxial cuticles was not affected
216 by the Fe status. In this plant species, soluble cuticular lipids accounted for 48 and
217 30% of the total cuticle weight in green and chlorotic leaves, respectively.

218 Stomata

219 As noted above, stomatal frequency was not significantly affected by Fe chlorosis, but
220 stomata in chlorotic leaves had significantly shorter (17 and 24% in pear and peach,
221 respectively) pore lengths as compared to green leaves (Table 1). An estimation of the
222 actual pore area using nail-polish leaf fingerprints indicated a lower degree of stomatal
223 opening (31 and 49% lower in pear and peach) in chlorotic than in green stomata.

224 Similar low transpiration rates were determined on adaxial (astomatous) surfaces of
225 Fe-deficient and Fe-sufficient attached peach and pear leaves (Table 3). Abaxial side
226 transpiration rates, however, were markedly reduced by Fe deficiency, the decrease
227 being 45 and 75% for pear and peach. A different response was observed between
228 green and chlorotic leaves for both plant species by assessing transpiration rates
229 immediately after detaching leaves from the tree (Fig. 3). Once detached, transpiration

rates of Fe-sufficient pear and peach leaves decreased over time, regardless the prevailing irradiation conditions. In contrast, the transpiration rate of detached chlorotic leaves increased markedly in the case of pear (by 40 and 20% in approximately 3-4 min, under high and low irradiation conditions). In the case of peach, transpiration rates decreased slightly shortly after detachment (in 1-2 min) but increased thereafter, within 7-10 min, to reach values similar to the ones measured prior to leaf detachment. This indicates an effect of Fe deficiency on the performance of stomata, which may be associated either with the mechanical properties of the leaf epidermis or to a disruption of normal stomatal functioning as a result of Fe chlorosis.

Gravimetric estimation of leaf water losses for a 4-day period provided evidence that chlorotic leaves lost water more rapidly than green leaves in both plant species, differences being remarkable after 2 days.

Discussion

Iron chlorosis induced changes in the epidermis and internal structure of peach and pear leaves at various levels, thereby influencing the two-way diffusion of gases and solutes between the leaf and the surrounding environment. While a higher dehydration rate of chlorotic versus green leaves has been described for several plant species (Hutchinson 1970; Anderson 1984) and Fe-deficient leaves have been suggested to be less water efficient (Larbi et al. 2006), this is the first study in which the possible causes relating to such impaired water relations have been directly tackled. Cuticular characteristics of leaves in Fe-sufficient trees are similar to those found in previous studies, both for pear (Norris and Bukovac 1968) and peach (Bukovac et al. 1979). The results obtained in this study provide evidence for changes occurring at the cuticular membrane level as a result of Fe chlorosis. Also, the morphology and mechanical properties of the epidermis and the structure of the cell wall and vascular bundle appeared to be altered by Fe deficiency.

Iron chlorotic leaves had reductions in size and FW as compared to Fe-sufficient leaves. While stomatal densities were not significantly affected by chlorosis, as also noted by Shimshi (1967), stomatal pore lengths decreased, possibly as a result of the reduction in leaf growth and expansion processes due to Fe shortage. In dicotyledonous plants such as peach and pear, leaves are enclosed in buds or folded up at earlier developmental stages, and the leaf surface expands *via* longitudinal and

lateral cell enlargement (Richardson et al. 2005), with stomata differentiating during development. This process, which continues until the leaf has reached 10-50% of its final size (Tichá 1982), is sensitive to environmental conditions, including the nutritional status of the plant (Weyers and Meidner 1990). When stomatal differentiation is completed, stomatal density reaches a maximum and declines thereafter in the course of leaf expansion. As a consequence, final stomatal densities can be affected by disturbances both in differentiation and expansion processes. The fact that in Fe chlorotic leaves leaf expansion and the absolute number of stomata per leaf was reduced, whereas stomatal density was not changed significantly, may suggest that Fe shortage affects stomatal differentiation. The observed reduction of the length of stomatal pores in Fe chlorotic leaves could also be associated with the reduction of leaf expansion at the epidermal and guard cell level.

The hypothesis that Fe chlorosis may hinder or stop leaf development processes was further supported by changes observed in the leaf cuticle and cell wall with Fe deficiency, including a decrease in soluble cuticular lipids in peach and a decrease in abaxial cuticle weight per unit surface in pear. The cuticle covers abaxial and adaxial leaf surfaces, lines stomatal apertures and the free inner epidermal cell spaces of the sub-stomatal cavity (Jeffree 2006). The cuticle appears on aerial plant organs very early during epidermal cell development, for instance in still unexpanded leaves in buds (Jeffree 2006). In parallel to leaf expansion, cuticular waxes must be deposited over epidermal cells to avoid desiccation (Richardson et al. 2005). Lipidic materials are required for adequate leaf growth and their synthesis may be affected by Fe deficiency. Indeed, it is plausible that Fe shortage affects cuticle formation *via* a limited production of lipidic material, as it was suggested to occur in pea and peach thylakoids (Abadía et al. 1988; Abadía 1992; Monge et al. 1993).

There was a significant enlargement of the upper epidermal peach leaf cells and bundle sheath cells in both plant species with Fe deficiency. Similar morphological variations in association with Fe chlorosis have been also described for Mexican lime (Maldonado-Torres et al. 2006). However, and in agreement with the results obtained for sugar beet by Terry (1980) we did not appreciate any significant variation regarding the number of mesophyll cells and average cell volume of Fe-deficient versus Fe-sufficient leaves.

Transpiration rates of attached chlorotic leaves were kept at low levels due to the lower degree of stomatal opening as compared to green leaves, in agreement with Larbi et al. (2006). However, results obtained provide evidence for a different behaviour of leaf stomata upon loss of turgor with Fe deficiency, since chlorotic leaves lost high amounts of water immediately after detachment (stomatal phase) and also over time (cuticular phase). Thereby, and in agreement with Anderson (1984), water loss through the cuticle was higher in Fe-deficient leaves than in Fe-sufficient controls, and therefore cuticular factors could be important in considering leaf water status of chlorotic trees. Iron chlorosis normally occurs in arid and semiarid areas of the world were high summer temperature, water shortage and low RH regimes prevail. We have shown that chlorotic leaves are more prone to desiccation due to their epidermal characteristics, which poses a further physiological disadvantage for survival on calcareous, high pH soils. The reason for the partial stomatal closure is unknown and research is in progress to elucidate the phenomenon.

A transient opening of stomata immediately after detachment, known as Iwanoff effect (Iwanoff 1928), was found to occur in chlorotic leaves. After interrupting xylem water supply to the leaf, stomatal opening could be explained by a rapid loss of turgor pressure, either of the surrounding epidermal cells (Raschke 1970a,b) or both the epidermal and guard cells (Kaiser and Legner, 2007). The mechanical advantage of epidermal cells over guard cells (DeMichele and Sharpe 1973) results in a hydro-passive stomatal opening phase, followed by an active stomatal closure phase. The Iwanoff effect was only observed in chlorotic leaves, and it was found to be independent of species, daytime, degree of stomatal aperture before detachment, and irradiation conditions. Healthy leaves of both species never showed this effect, even when transpiration rates were low and comparable to those of chlorotic leaves, discarding the possibility that it could be caused by differences in initial stomatal apertures (Lange et al. 1986). The differential opening of stomata in green and chlorotic leaves may not be attributed to differences in zeaxanthin contents, because the time courses of both processes are totally different (Larbi et al. 2006; Powles et al. 2006), with zeaxanthin reverting to violaxanthin only after several hours. Therefore, the stomatal behaviour of chlorotic leaves could be likely attributed to changes in mechanical properties related to constitutive morphological features of the epidermis. Larger surrounding epidermal cells with thinner walls could exert, upon sudden loss of

turgor, a stronger force on the smaller guard cells in the case of Fe-deficient leaves. Alternatively, guard cells in Fe-deficient leaves may loose temporarily control as a consequence of the many physiological changes (e.g., K concentration increases) brought about by Fe deficiency. The eco-physiological consequences of the morphological changes associated with Fe chlorosis in terms of the functionality of stomata *in vivo* and thus on plant water relations are not yet clear. Possibly the softness of the epidermal tissue could cause a disturbance of the fine tuning of stomatal aperture, especially under conditions requiring fast adaptation to changing ambient conditions.

The reduction of abaxial cuticular weight per unit surface observed in pear leaves will also have some physiological implications. The abaxial cuticle of a green leaf was indeed observed to be thicker than the one of a chlorotic leaf, but this does not imply directly a higher resistance to water loss (Norris 1974). It is remarkable that the reduction in cuticular weight was only observed in the abaxial leaf side, the upper cuticle being similar irrespective of Fe status in both plant species investigated. Our data stress the key role of the lower epidermis, a leaf side which has been traditionally neglected in cuticular studies. The cuticular lipid and cuticle reduction associated with Fe-chlorosis will also render the leaves more susceptible to pest and disease attack.

Since leaf water repellence is chiefly related to epicuticular waxes, while intra-cuticular waxes are important in water resistance (Holloway 1969), the decrease in soluble cuticular lipids observed in chlorotic peach leaves will have consequences in terms of leaf wettability and resistance to water loss. The observed epidermal changes in association with Fe chlorosis will have implications for the permeability of gases and polar and apolar solutes which should be studied. Concerning infiltration processes, lower stomatal apertures may imply higher capillary forces for penetration as suggested for citrus leaves with stomatal plugs (Turrell 1947). However, uptake across stomata has been recently shown to occur *via* diffusion (Eichert and Goldbach 2008), and generally a lower stomatal aperture also causes lower uptake rates (Eichert et al. 1998; Eichert and Burkhardt 2001). The occurrence of lower amounts of cuticular waxes may apparently facilitate leaf wetting and increase permeability. However, since chlorotic leaves exhibit a higher cuticular transpiration once they are detached from the tree, this may imply a higher water loss and possibly a lower degree of cuticular hydration, which in turn may cause a lower permeability to ions and polar

360 molecules. Research is in progress to assess the significance of Fe chlorosis in terms
361 of leaf permeability to water and ions.

362 In summary, Fe-chlorosis was found to induce structural changes in peach and pear
363 leaves and also to affect stomatal functioning. The observed reductions in soluble
364 cuticular lipids (peach leaves) and cuticle weight (pear leaves) in association with Fe
365 chlorosis, will yield leaves more prone to water loss and more susceptible to pest and
366 disease attack. Iron deficient leaves were found to be Iwanoff-responsive versus the
367 standard behaviour of healthy leaves, which may be due to stomatal malfunctioning or
368 differences in leaf water control. Research is in progress to better clarify the
369 detrimental effect of Fe-deficiency chlorosis at the leaf level.

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488 **Table 1** Leaf Chl (μmol m^{-2}; n=200) and Fe (μg g^{-1} DW; n=30) concentration, fresh weight (FW in g per leaf, n=200), leaf area (adaxial plus
489 abaxial leaf surfaces, cm^2; n=200), stomatal density (stomata mm^{-2}; n=50) and stomatal pore length (μm; n=300) of green and chlorotic pear and
490 peach leaves. Data shown are means ± SE
491

Species	Leaf type	Leaf [Fe] (μg g^{-1} DW)	[Chl] (μmol m^{-2})	FW (g per leaf)	Total leaf surface (cm^2)	Stomatal density (stomata mm^{-2})	Pore length (μm)
Peach	green	141.8±6.2***	300±4.3***	0.52±0.02***	62.8±2.2***	221±18 ns	26.1±0.4***
	chlorotic	92.8±4.1***	90±6.7***	0.40±0.02***	47.0±1.6***	233±11 ns	19.9±0.4***
Pear	green	143.8±5.6***	250±4.4***	0.72±0.04***	60.4±3.2***	160±9 ns	24.4±0.3***
	chlorotic	87.2±3.9***	40±3.2***	0.55±0.02***	44.6±2.6***	156±12 ns	20.3±0.3***

492 *** Significant at $P \leq 0.001$; ns, not significant

Table 2 Weight per leaf unit surface of abaxial and adaxial cuticles (n=20; each with 25 cuticles) and of total solvent-extractable (soluble) cuticular lipids (n=6) from chlorotic and green peach and pear leaves. Data are means ± SE. The level of significance according to Student's t test is indicated in different columns ($p \leq 0.05$)

Cuticle weight (μg cm^{-2})			
Species	Leaf surface	Green leaves	Chlorotic leaves
Peach	adaxial	191.3 ± 21.4 ns	164.9 ± 16.9 ns
	abaxial	179.7 ± 14.1 ns	175.2 ± 12.2 ns
Pear	adaxial	344.2 ± 22.6 ns	292.9 ± 31.4 ns
	abaxial	513.6 ± 20.8 ***	332.9 ± 21.2***
Soluble cuticular lipids (μg cm^{-2})			
Species		Green leaves	Chlorotic leaves
Peach	-	176.5 ± 13.3***	103.6 ± 7.9***
Pear	-	85.3 ± 7.2 ns	81.2 ± 5.1 ns

*** Significant at $P \leq 0.001$; ns, not significant

Table 3 Stomatal pore area (n=300), relative pore area on abaxial area basis (%; n=50) and transpiration rate (mmol m^{-2} s^{-1}; n=50) of chlorotic and green peach and pear leaves. Transpiration rates were measured in attached leaves at 1,400 µmol quanta m^{-2} s^{-1}. Data are means ± SE. The level of significance according to Student's t test is indicated in different columns ($p \leq 0.05$)

Species	Leaf type	Leaf surface	Stomatal pore area (μm^2)	Pore area as % of abaxial surface	Transpiration rate (mmol m^{-2} s^{-1})
Peach	green	adaxial	-	-	0.09±0.03 ns
	chlorotic	adaxial			0.04±0.02 ns
	green	abaxial	141.6±15.7***	0.983 ***	4.0±0.6***
	chlorotic	abaxial	71.6±5.9 ***	0.392 ***	1.0±0.2***
Pear	green	adaxial			0.09±0.02 ns
	chlorotic	abaxial			0.06±0.01 ns
	green	adaxial	45.8±5.3 *	0.221 ***	6.0±0.7***
	chlorotic	abaxial	31.5±3.8 *	0.110 ***	3.3±0.4***

*** Significant at $P \leq 0.001$; ** Significant at $P \leq 0.01$; * Significant at $P \leq 0.05$; ns, not significant

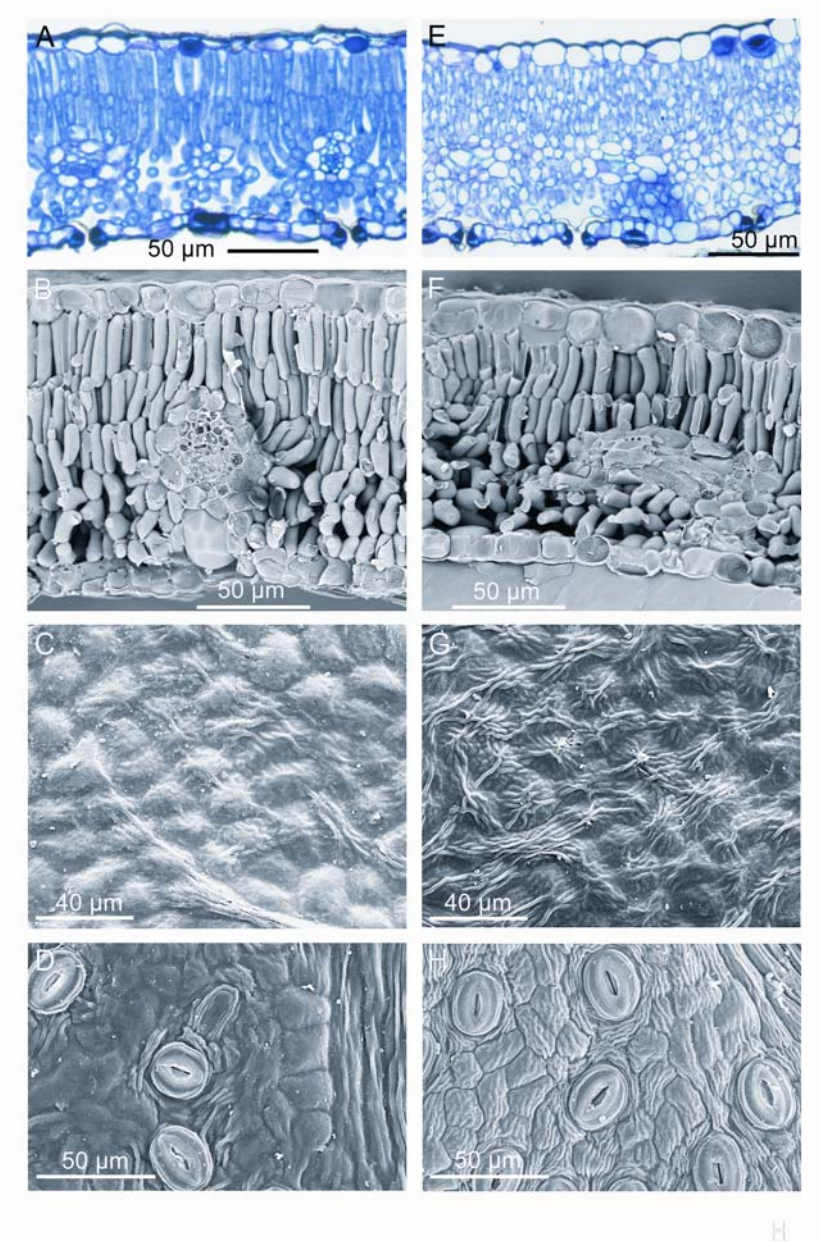
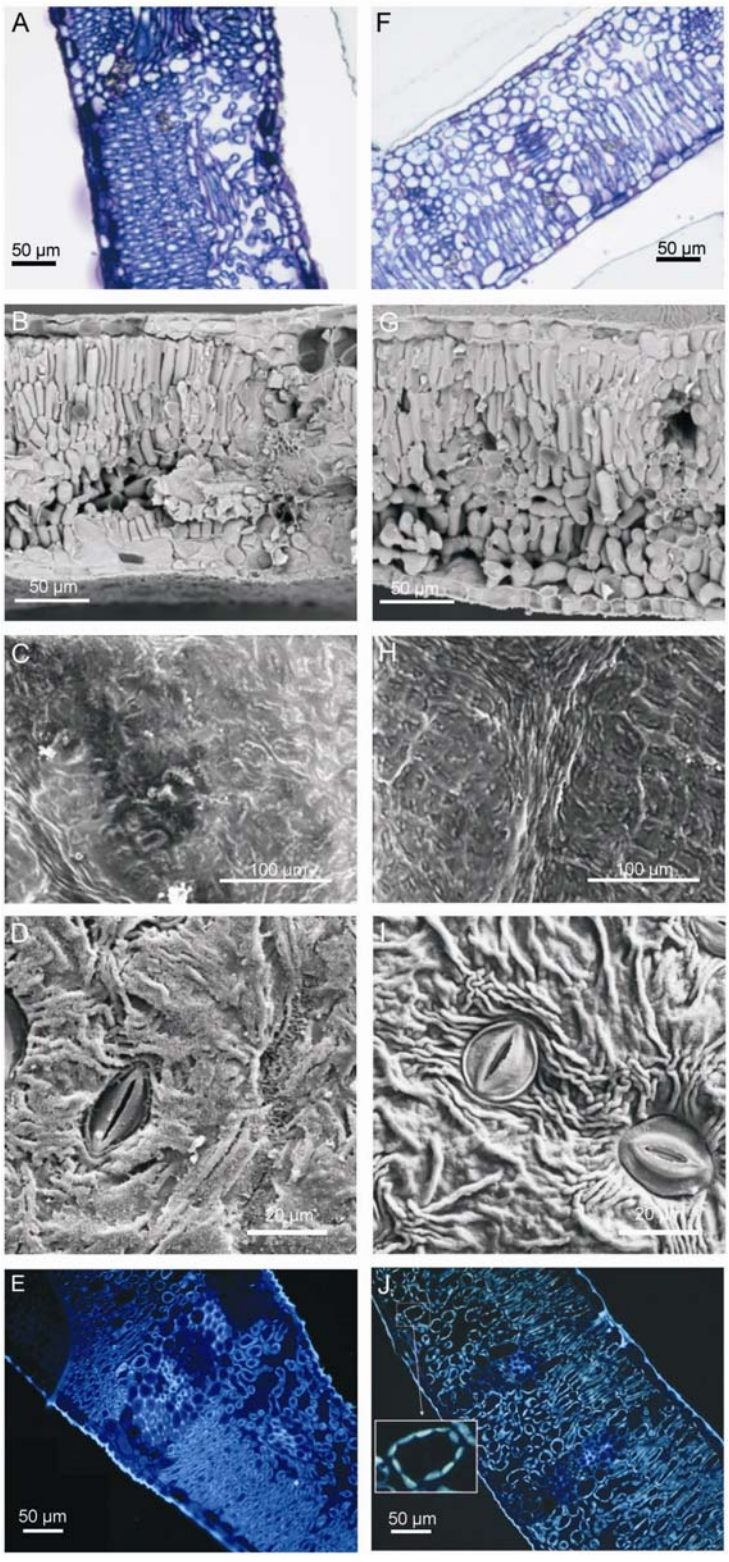


Fig. 1. Transversal section and leaf surface appearance of green (A,B,C,D) and chlorotic (E,F,G,H) peach leaves. (A,E) green and chlorotic embedded tissue samples stained with toluidine blue, observed by light microscopy; (B,F) LT-SEM micrographs of a green (B) and a chlorotic (F) leaf; (C,G) SEM micrographs of the adaxial leaf surface of a green (C) and a chlorotic (G) leaf; (D,H) SEM micrographs of the abaxial leaf surface of a green (D) and a chlorotic (H) leaf



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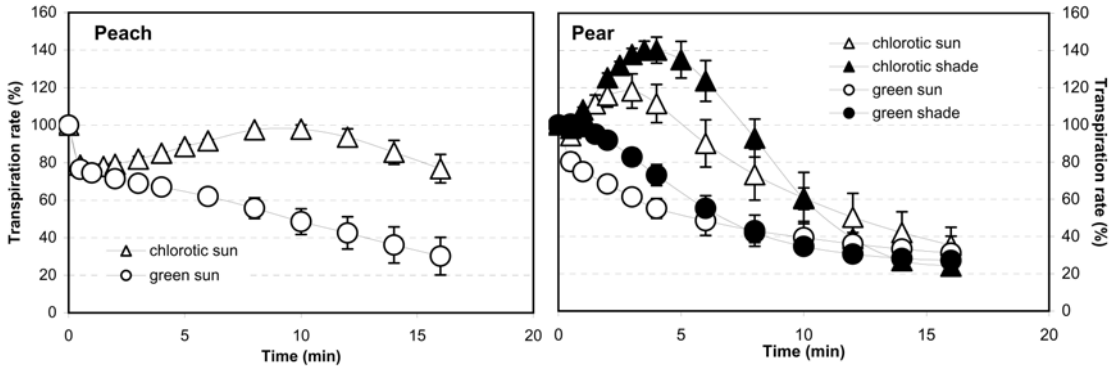
575 **Fig. 2.** Transversal section and leaf surface appearance of green (A,B,C,D,E) and
576 chlorotic (F,G,H,I,J) pear leaves. (A,F) green and chlorotic embedded tissue samples
577 stained with toluidine blue, observed by light microscopy; (B,G) LT-SEM
578 micrographs of a green (B) and a chlorotic (G) leaf; (C,H) SEM micrographs of the
579 adaxial leaf surface of a green (C) and a chlorotic (H) leaf; (D,I) SEM micrographs of
580 the abaxial leaf surface of a green (D) and a chlorotic (I) leaf; (E,J) autofluorescence
581 of a green (E) and chlorotic (J) leaf



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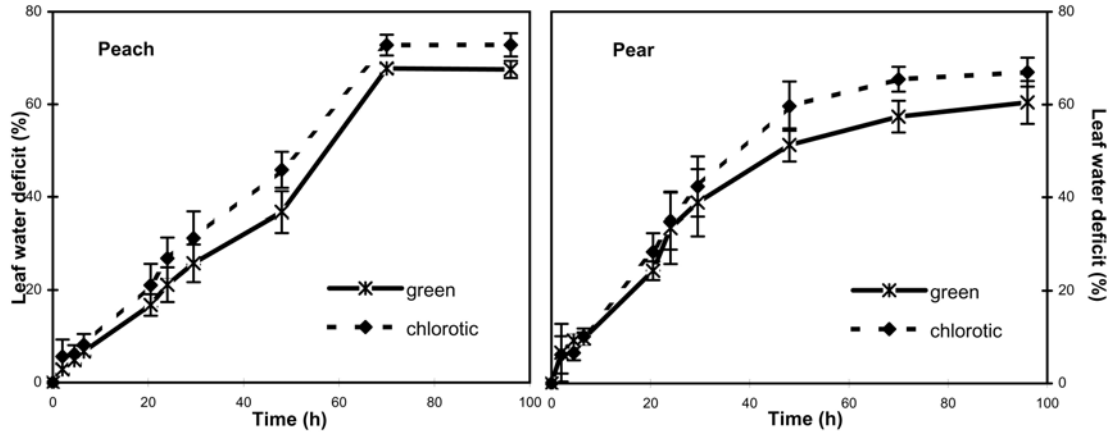
584 **Fig. 3.** Transpiration rates of detached green and chlorotic peach and pear leaves.
585 Leaves were first measured while still attached to the tree (t = 0) under high (PAR
586 1200–1900 µmol quanta m^{-2} s^{-1}) or low (in the case of pear leaves, PAR 70–180 µmol
587 quanta m^{-2} s^{-1}) irradiation levels. Transpiration rates of detached leaves were
588 subsequently assessed for 16 min, keeping the leaves in the shade (PAR 70–180 µmol
589 quanta m^{-2} s^{-1}). Leaf temperatures ranged from 24 to 34 °C in the sun and 22 to 24 °C
590 in the shade. Relative humidity was between 20 and 37%. Transpiration rates, given as
591 means and standard errors (n=3-5), are expressed as percentage of the value measured
592 at t = 0



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594 **Fig. 4.** Time course of leaf water loss in green and chlorotic peach and pear leaves.
595 Leaves were detached, immediately weighed and then placed in a dark room with the
596 lower side lying against a filter paper (T=24ºC, 40% RH). Leaf weight was monitored
597 for 4 days and water loss was expressed as a percentage of the initial FW

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