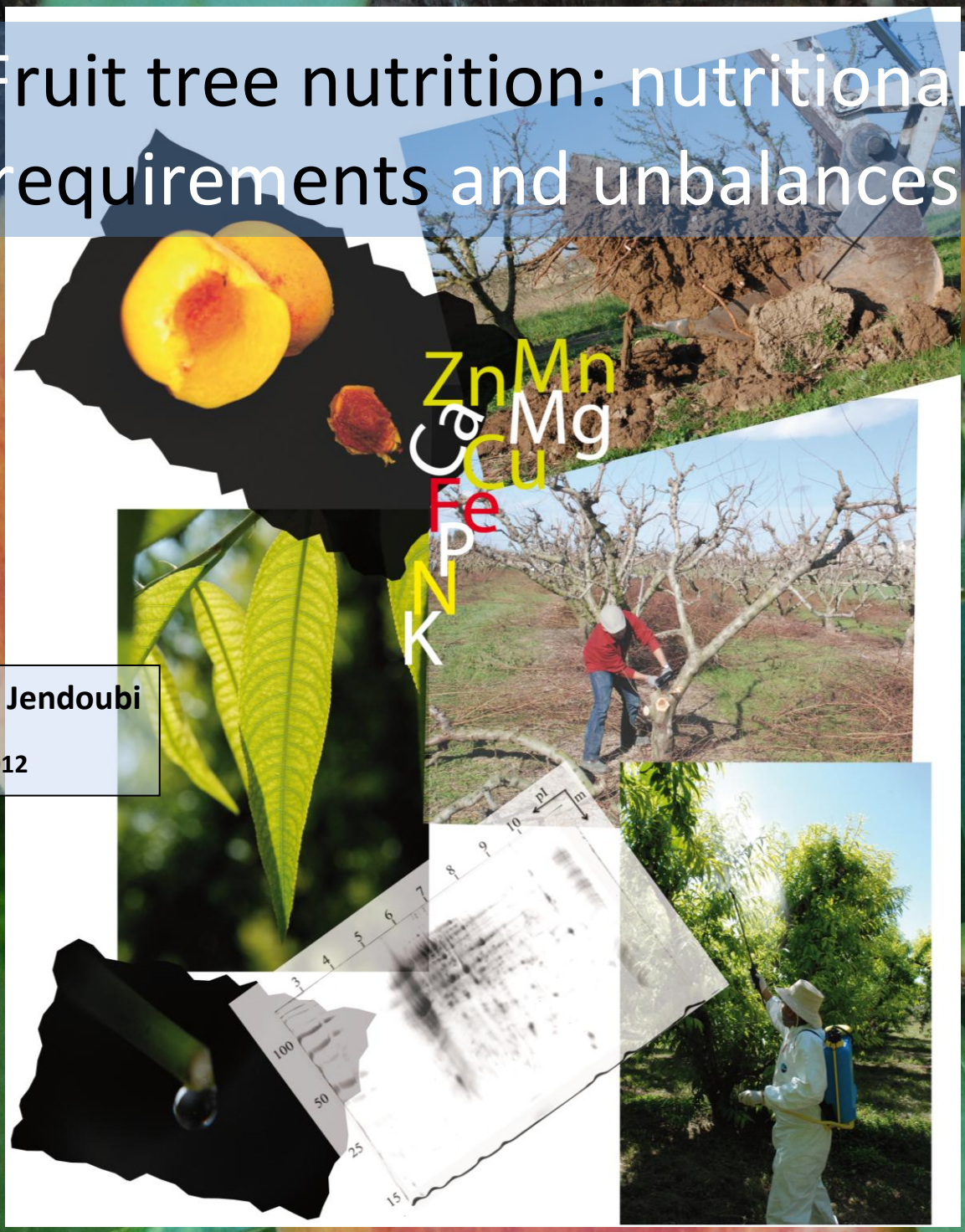
TESIS DOCTORAL

Fruit tree nutrition: nutritional requirements and unbalances



Hamdi El Jendoubi

Zaragoza 2012

Universidad de Lérida – Consejo Superior de Investigaciones Científicas

AUTORIZACIÓN DE LOS DIRECTORES PARA LA PRESENTACIÓN DE TESIS DOCTORAL

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La presentación de la siguiente memoria de Tesis Doctoral, titulada "NUTRICIÓN DE ARBOLES FRUTALES: NECESIDADES Y DESEQUILIBRIOS NUTRICIONALES" presentada por D. HAMDI EL-JENDOUBI para optar al grado de Doctor por la Universidad de Lérida.

Y para que conste a los efectos oportunos expide la presente autorización

En Zaragoza, 17 de Febrero de 2012

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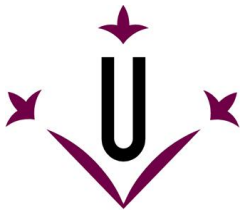

Universitat de Lleida

Memoria presentada por Hamdi El-Jendoubi, Ingeniero Agrónomo, para optar al grado de Doctor Ingeniero Agrónomo por la Universidad de Lérida.

El Doctorando:

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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS

ESTACION EXPERIMENTAL DE AULA DEI

Departamento de Nutrición Vegetal

Laboratorio de Estrés Abiótico en Plantas

Nutrición de frutales: Necesidades y desequilibrios nutricionales

TESIS DOCTORAL

Hamdi El-Jendoubi

Lleida 2012

INDEX

Abstract/Resumen/Resum

Abstract

This work deals with fundamental aspects of fruit tree nutrition, including the following: (i) estimation of total nutrient requirements; (ii) nutritional diagnostics; (iii) remediation for nutritional disorders; and (iv) understanding of nutrient transport.

Field studies were carried out in the Ebro river basin, Zaragoza, Northern Spain, were peach tree was taken as an example of fruit tree and Fe chlorosis as an example of nutritional disorder. In some studies, model plants grown in controlled environments have also been used.

In the first chapter of Results part, whole tree analysis was carried out by quantifying the amounts of nutrients removed at each event of the peach tree annual cycle, as well as the amounts stored in the permanent tree structures, in three different peach tree cultivars. In the second chapter, Fe chlorosis was taken as a typical nutrient disorder in the region, and we show advances in its diagnosis by studying the possibility of using tree materials in early tree phonological stages. Results found indicate that it is possible to carry out the prognosis of Fe chlorosis using early materials such as buds and flowers. The third chapter deals with the correction of iron chlorosis, in an attempt to improve the scientific background for foliar fertilizer practices. We evaluated the success of treatments with a Fe compound by studying the capacity for penetration and re-greening. In the fourth chapter, studies on the transport of Fe into the xylem tissue were carried out by metabolomic and proteomic analysis, opening the way for advancing the understanding of nutrient transport in this fruit tree compartment. The fifth chapter discusses advices and aspects that researchers should take in consideration when assessing the effect of Fe fertilizers, including the following: i) design of Fe-fertilization experiments; ii) assessment of chlorosis recovery upon Fe-fertilization by monitoring leaf chlorophyll; and iii) analysis of the plant responses upon Fe-fertilization. The phases of leaf chlorosis recovery and the control of other leaf nutritional parameters were discussed.

Resumen

El presente trabajo trata sobre nociones fundamentales en la nutrición de árboles frutales: (i) estimación de las pérdidas totales de nutrientes (ii) diagnostico nutricional (iii) soluciones para desordenes nutricionales (iv) estudio del transporte de nutrientes.

Los estudios se han realizado en la zona del Ebro, Zaragoza, en el norte de España dónde el melocotonero se escoge como ejemplo de árbol frutal, y la clorosis férrica como ejemplo de desorden nutricional. En algunos estudios, se han usado plantas modelo crecidas en condiciones controladas.

En el primer capítulo de los resultados, se realiza un análisis del árbol entero mediante la cuantificación de las pérdidas de nutrientes en cada evento del ciclo anual del melocotonero, y de las cantidades almacenadas en las estructuras permanentes de tres cultivares de melocotonero: Calanda, Catherina y Babygold 5. En el segundo capítulo, se considera la clorosis férrica como el típico desorden nutricional de la zona, y se presentan avances en su diagnostico mediante el estudio de materiales del árbol en épocas fenológicas avanzadas (precoces), tal como yemas en dormancia y flores. Los resultados adquiridos indican que es posible predecir la clorosis férrica usando los materiales vegetales indicados. El tercer capítulo, trata sobre del uso de fertilizantes foliares para la corrección de la clorosis férrica, mejorando el conocimiento científico sobre el uso de dichos fertilizantes. Se evalúa la eficacia de un tratamiento foliar de un compuesto de hierro estudiando su capacidad de penetración y reverdecimiento. En el cuarto capítulo, se realizan estudios sobre el transporte de hierro en el tejido de xilema a través de análisis de proteomica y metabolómica, aportando avances en la comprensión de dicho tejido, responsable de transporte de nutrientes en plantas. El quinto capítulo trata sobre consejos y aspectos a considerar por parte de los investigadores a la hora de realizar un seguimiento del efecto de un fertilizante de hierro, y que incluyen: i) el diseño del experimento; ii) el seguimiento de la evolución de la corrección de la clorosis después de una fertilización con hierro, controlando la concentración de clorofila en la hoja; y iii) el análisis de la respuesta de la planta después de una fertilización con hierro. Asimismo, también se analizan las fases de la desaparición de la clorosis en la hoja, y la observación de otros parámetros nutricionales a nivel de hoja.

Resum

El present treball tracta de nocions fonamentals en la nutrició d'arbres fruiters: (i) estimació de les pèrdues totals de nutrients (ii) diagnòstic nutricional (iii) solucions per desordres nutricionals (iv) estudi de transport de nutrients.

Els estudis s'han realitzat a la zona de l'Ebre, Saragossa, al nord d'Espanya on el presseguer s'escull com a exemple d'arbre fruiter i la clorosi fèrrica com exemple de desordre nutricional. En alguns estudis, s'han fet servir plantes model crescudes en condicions controlades.

En el primer capítol dels resultats, es fa una anàlisi de l'arbre sencer a mitjançant la quantificació de les pèrdues de nutrients en cada esdeveniment del cicle anual del presseguer, i de les quantitats emmagatzemades a les estructures permanents dels tres cultivars de presseguer: Calanda, Catherina y Babygold 5. Al segon capítol, es considera la clorosi fèrrica com el típic desordre nutricional de la zona, i es presenten avenços al seu diagnòstic a través de l'estudi de materials de l'arbre en èpoques fenològiques avançades (precoces), com gemmes en dormància i flors. Els resultats obtinguts indiquen que és possible predir la clorosi fèrrica utilitzant els materials vegetals indicats. El tercer capítol, tracta sobre la utilització de fertilitzants foliars per la correcció de la clorosi fèrrica, millorant el coneixement científic en l'ús d'aquests fertilitzants foliars. S'avalua la eficàcia d'un tractament foliar d'un compost de ferro estudiant la seva capacitat de penetració i reverdiment. En el quart capítol, es realitzen estudis sobre el transport de ferro en el teixit del xilema, a través d'anàlisis de proteòmica i metabolòmica, aportant avenços en la comprensió d'aquest teixit, responsable del transport de nutrients en plantes. El cinquè capítol tracta sobre consells i aspectes a considerar per part dels investigadors a l'hora de realitzar un seguiment de l'efecte d'un fertilitzant de ferro, i que inclouen: (i) el disseny experimental (ii) el seguiment de l'evolució de la correcció de la clorosi després d'una fertilització amb ferro, controlant la concentració de clorofil·la a la fulla, i (iii) l'anàlisi de la resposta de la planta després d'una fertilització amb ferro. A més, també s'analitzen les fases de la desaparició de la clorosi a la fulla, i l'observació d'altres paràmetres nutricionals a nivell de fulla.

Introduction

Introduction

Fruit tree nutritional requirements

The world population has increased from less than 2 billion people in 1900 to 5.7 billion in 1995, and it is expected to reach 8.5 billion in 2025 (Byrnes and Bumb 1998). This unprecedented growth in population will create tremendous pressures on the natural resource base to produce enough food and fiber to meet human needs and wants (Cakmak 2002, Grusak et al. 1999).

In order to meet the food demands of the rising population, farmers must manage nutrients and soil fertility with an adequate, balanced supply of nutrients. This balance will not be achieved unless "nutrient cycles" are better understood (Gruhn et al. 2000). The nutrient cycle is defined as the continuous recycling of nutrients into and out of the soil (NRC 1993), and it involves complex biological and chemical interactions, some of which are not yet fully understood. A simplified version of this nutrient cycle during plant growth was proposed by Stoorvogel et al. (1993) (Fig. I.1). The cycle has two parts: "inputs" that add plant nutrients to the soil, and include mineral fertilizers, organic manures, atmospheric deposition, biological nitrogen fixation and sedimentation, and "outputs" that export nutrients largely in the form of agricultural products (crop harvest and residues), and also due to leaching, gaseous losses and water erosion.

The difference between inputs and outputs constitutes the nutrient balance. Positive nutrient balances in the soils, often associated to over-application of fertilizers which makes nutrient additions to the soil greater than those removed from the soil (Conway and Barbie 1988), could indicate that farming systems are inefficient and, in the extreme, that they may be polluting the environment. Negative balances, such as in case of under-application of fertilizers, could well indicate that soils are being mined and that farming systems will be unsustainable over the long term. Therefore, nutrients should be supplied in order to sustain agriculture in the long term, increase crop productivity and maintain soil fertility (Gruhn et al. 2000). The nutrient rates applied should meet the demand of the crop, but should not exceed the demand to any major extent (Mengel 1982). In the case of fruit trees the nutrient demand is secured in the beginning of the season by the remobilization of nutrients already stored during the previous winter in perennial parts of the trees (Millard 1995, Muñoz et al. 1993, Quartieri et al. 2002,

Tagliavini et al. 1998). The most studied case is N, where the remobilization is well studied and quantified and seems to be unaffected by the current N supply in the spring (Millard 1995). During the rest of the season, nutrient uptake must occur from the soil.

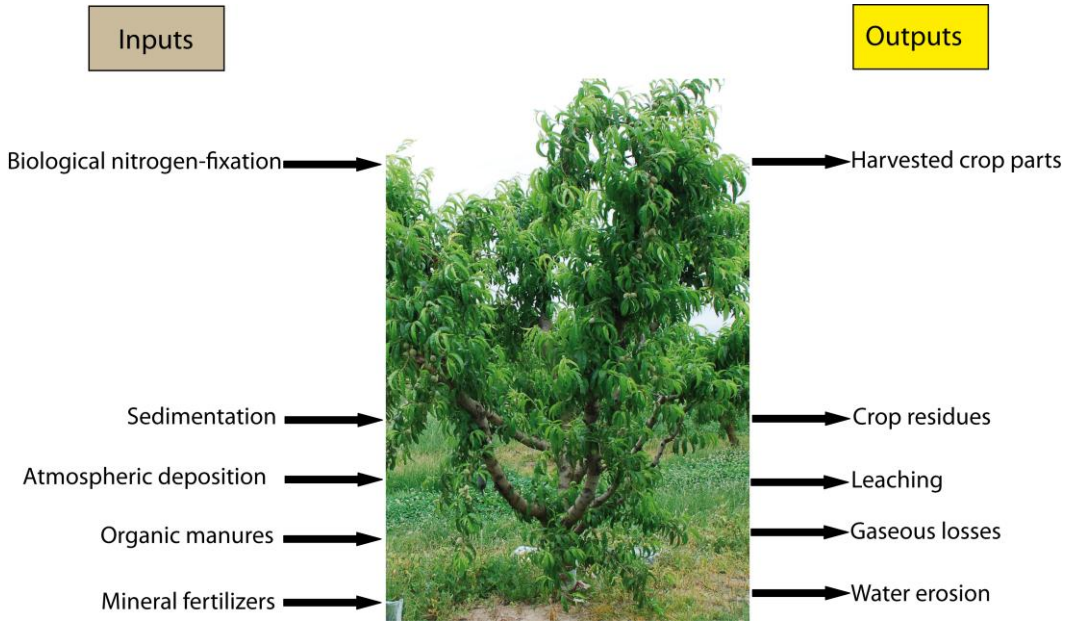


Figure I.1 *Plant nutrient balance system* (Stoorvogel et al. 1993).

Although many important functions fulfilled by macro- and microelements are well known (Clarkson and Hanson 1980, Mengel and Kirkby 1982, Neilsen and Neilsen 2003), the specific elemental requirements for optimum growth, production and fruit quality for each fruit plant species and cultivar need to be determined, especially when using high planting densities. A relatively simple approach for determining tree nutrient requirements is based on the mineral analysis of whole trees. Different such studies have been carried out in apple (Batjer et al. 1952, Haynes and Goh 1980), peach (Stassen 1987), mango (Stassen et al. 1997a, b), avocado (Stassen et al. 1997c) and pear trees (Stassen and North 2005), as well as in vines (Conradie 1981). This approach takes into account mineral nutrient losses due to the removal of fruits and pruned wood from the orchard, losses of leaves at fall and nutrient fixation in permanent parts of the tree (old wood and roots) relative to tree age (Stassen 1987). However, the studies carried out so far mainly focused on macroelements, even though some microelement deficiencies can be very important (Rashid et al. 2008). For instance, N deficiency led to small fruits, shorter shoots and lower yields (Johnson 2008), and similar symptoms were also described in the case of Fe-deficiency chlorosis (Álvarez-Fernández et al.

2006, Álvarez-Fernández et al. 2011, Álvarez-Fernández et al. 2003) and (Rombolá and Tagliavini 2006).

Iron chlorosis as a typical fruit tree nutritional disorder in the Mediterranean region

Fruit tree requirements for Fe are relatively small. However, plants grown on alkaline and calcareous soils can be inefficient in absorbing and using Fe and therefore become deficient. In fact, Fe deficiency is the most prevalent nutritional disorder in fruit tree crops growing in calcareous soils (Abadía et al. 2004). This widespread nutritional disorder (Chen and Barak 1982, Marschner 1995, Mengel et al. 2001, Wallace and Lunt 1960) can affect several woody plants and grapevine in particular (Dell'Orto et al. 2000, Romheld 2000).

In fruit tree crops, Fe deficiency is considered as the main constraint for successful cultivation in many production areas worldwide, causing decreases in tree vegetative growth, a shortening of the orchard life span as well as losses in both fruit yield (Rombolá and Tagliavini 2006) and quality (Álvarez-Fernández et al. 2006, Álvarez-Fernández et al. 2011). The incidence of Fe chlorosis is widespread in the Mediterranean basin (Abadía et al. 2004, Sanz et al. 1992) (Fig. I.2). It was reported in Northern Greece (Tagliavini et al. 2000), France (Ollat et al. 2003), Italy (Rombolá and Tagliavini 2006, Tagliavini et al. 2000), Turkey (Tekin et al. 1998), Morocco (El Houssine et al. 2003), Tunisia (Ksouri et al. 2001, Ksouri et al. 2005), Lebanon, Syria, Libya (Rashid et al. 2008) and Portugal (Pestana et al. 2002).

In Spain, Fe chlorosis in fruit trees has been reported in the Ebro Valley (Sanz et al. 1992), Andalusia (Pastor et al. 2002) and the Valencia Community (Legaz et al. 1995). In the Ebro Valley it was reported that crops affected by Fe chlorosis are mainly fruit tree species, such as pear, peach, apple, apricot, plum, cherry and almond, with the most affected one being peach. The incidence of Fe chlorosis is so heavy that most (90%) of the peach orchards (23,400 ha) are treated with Fe compounds during their productive lifetime (Sanz et al. 2002). Iron fertilizers, either applied to the soil or delivered to the foliage, are provided to these crops every year to control Fe deficiency, and the use of Fe fertilization is increasing (Abadía et al. 2011). Approximately 45,000 ha of orchards are affected, and the cost of Fe fertilizers is more than 20 million US$ per year (Sanz et al. 1992).

Figure I.2 *Distribution of iron chlorosis in the Mediterranean basin, based in the different studies reported from each country (Google earth image). Data were reported in Abadía et al. 2004, Sanz et al., 1992, Tagliavini et al. 2000, Ollat et al. 2003, Tagliavini et al. 2000, Fichera 1968, Rusco and Quaglino 2001, Tekin et al. 1998, El Houssine et al. 2003, Ksouri et al. 2001, Ksouri et al. 2005, Rashid et al. 2008, Pestana et al. 2003.*

The general belief is that Fe deficiency decreases fruit yield in tree crops (Pestana et al. 2003, Tagliavini and Rombolà 2001, Tagliavini and Rombolà 2001). Fruit yield losses can be due to decreases in the number of fruits per tree, decreases in fruit size or a combination of both factors (Rombolá and Tagliavini 2006). Even in moderately Fe chlorotic trees the loss in potential yield could be as high as 50%. Total yields of Fe-deficient and Fe-sufficient trees, growing side by side in the field, are indeed different. Furthermore, in severely chlorotic trees the decrease in fruit number per tree could be higher than 80% as compared to Fe-sufficient, control trees. Decreases in fruit size may be as large as 30% in severely deficient trees. Consequently, fruit yield (in fruit fresh mass per tree) was considerably decreased by Fe deficiency (Álvarez-Fernández et al. 2006, Álvarez-Fernández et al. 2011).

Diagnostics of fruit tree iron chlorosis

Diagnosis of nutrient deficiencies and toxicities, especially considering micronutrients, can contribute to a better nutrition of crops and greater productivity (Rashid et al. 2008). The mineral concentration of plant tissues is generally used by farmers to diagnose nutrient deficiencies, excesses or imbalances in crops (Bould et al. 1983, Chapman 1966, Marschner 1995). Also, changes in mineral nutrient concentrations are commonly accepted as a reliable guide for evaluating the success of orchard fertilization programs

(Basar 2006, Brown and Kiyoto 1996, Zuo and Zhang 2011). A renewed interest in new ways to diagnose and monitor plant nutrient status has arisen, based on the farmer's need to have an optimal crop nutrient supply in order to increase not only crop yield but also fruit quality (Brown and Kiyoto 1996, Gruhn et al. 2000, Cakmak 2002, Abadía et al. 2004, Zuo and Zhang 2011).

The material used more often for plant nutrient status monitoring is the leaf tissue. This is because the leaf nutrient composition integrates many factors, from soil nutrient availability to plant uptake and distribution, and therefore reflects very often in an adequate manner the nutritional balance of the plant at the time of sampling (Pestana et al. 2003). When used in some fruit tree species, however, the leaf analysis approach may have a major problem, since recommended times for leaf sampling are too late in the season for any subsequent corrective measure to improve fruit yield and quality (Abadía et al. 2004, El-Jendoubi et al. 2011).

The diagnosis of Fe deficiency in fruit tree species, conversely to what happens with other nutrient disorders, cannot be adequately carried out using leaf elemental composition, because Fe-deficient field-grown leaves often have Fe concentrations as high as those of Fe-sufficient leaves (this has been described as the "chorosis paradox"; Morales et al. 1998). This is likely associated to the preferential distribution of Fe in leaf areas close to the vascular system (Jiménez et al. 2009, Tomasi et al. 2009). Therefore, methods alternative to leaf analysis have been proposed to prognosis (diagnose in advance) Fe deficiency in fruit trees. For instance, the mineral composition of flowers has been used with this purpose in pear (Sanz et al. 1993), peach (Belkhodja et al. 1998, Igartua et al. 2000, Sanz and Montañés 1995, Sanz et al. 1997), apple (Sanz et al. 1998), nectarine (Toselli et al. 2000), olive (Bouranis et al. 1999), almond (Bouranis et al. 2001) and orange (Pestana et al. 2004) trees. Also, bark analysis has been used for Fe deficiency prognosis in peach trees (Karagiannidis et al. 2008). Other studies have proposed to use additional parameters such as nutrient ratios to assess the tree Fe nutrition status. For instance, the ratios K/Ca and P/Fe in leaves (Abadía et al. 1985, Belkhodja et al. 1998, Köseoğlu 1995) and K/Zn and Mg/Zn in flowers (Igartua et al. 2000, Pestana et al. 2004) have been used with this aim.

Use of fertilizers for the correction of iron chlorosis in fruit tree crops

Iron fertilizers are grouped into three main classes: inorganic Fe compounds, synthetic Fe-chelates and natural Fe-complexes (Abadia et al. 2011). In calcareous soils, the correction of Fe chlorosis in trees is normally achieved by the application of Fe(III)-chelates such as Fe(III)-EDDHA to the soil (Legaz et al. 1992, Papastylianou 1993). This practice has to be repeated every year because Fe is rapidly immobilized in the soil (Pestana et al. 2001).

Fertilizers based on inorganic Fe-compounds include soluble ones such as Fe salts (e.g., $Fe_2(SO_4).7H_2O$) and insoluble compounds such as Fe oxide-hydroxides and other cheap Fe minerals and industrial by-products (Hansen et al. 2006, Shenker and Chen 2005). Soluble inorganic Fe salt applications to the soil are quite inefficient, especially in high pH (i.e., calcareous) soils, due to the rapid transformation of most of the Fe applied into highly insoluble compounds such as Fe(III)-hydroxides similar to that already present in the soil in large amounts (Lucena 2006). This occurs even when very high doses of these low cost Fe-fertilizers are applied (Abadía et al. 2011). Insoluble inorganic Fe-compounds have a similar prospect, and also present additional problems, such as the occurrence in many of them of other potentially toxic metals and the difficulties in matching the rates of Fe-release (from the fertilizer to the soil solution) and plant Fe uptake. Therefore, these fertilizers have a limited value as plant Fe sources, even when having low particle size and using local acidification and band application, and may cause environmental concerns (Hansen et al. 2006, Shenker and Chen 2005).

Synthetic Fe(III)-chelate fertilizers are derived from polyaminocarboxylic acids which have high affinity for Fe(III), such as ethylenediamine tetraacetic acid (EDTA) (Lucena 2006, Shenker and Chen 2005). These chelates are obtained by carrying out first the synthesis of the chelating agents and then incorporating Fe(III) from inorganic salts. Synthetic Fe(III)-chelates are remarkably effective as soil fertilizers, even in calcareous soils, because Fe is bound to the chelating agent over a wide range of pH values and therefore remains soluble (Andreu et al. 1991). In the particular case of calcareous soils, synthetic Fe(III)-chelates from chelating agents with phenolic groups (e.g., the ethylenediamine- *N*-*N*´bis (*o*-hydroxyphenylacetic) acid; *o*,*o*EDDHA) are very effective Fe fertilizers (Abadía et al. 2011, Lucena 2006). Due to the high price, only with cash crops, synthetic Fe chelates are used to correct Fe deficiency (Chen and Barak 1982). In orchards with drip irrigation Fe can be applied by fertirrigation, but in others the application of Fe-chelates is time consuming since they are placed around each

individual tree, normally in the spring between the beginning of flowering and full bloom (Abadia et al. 1992). Polyaminocarboxylate chelating agents used in Fe-fertilization are also under scrutiny due to their influence on metal availability and mobility, especially because of their persistence in the environment (Nowack 2002). Natural Fe-complex fertilizers include a large number of substances (e.g., humates, lignosulfonates, amino acids, gluconate, citrate, etc.) (Lucena 2006). They are less stable in the soil than synthetic Fe(III) chelates, and are easily involved in reactions of metal- and ligand-exchange and/or adsorption on soil solid phase, (Cesco et al. 2000, Lucena et al. 2010) thus reducing the plant-availability of the Fe delivered with the fertilizer. That's why these Fe compounds could be useful for foliar application or in nutrient solution, in conditions where the chlorosis is not severe (Lucena 2006).

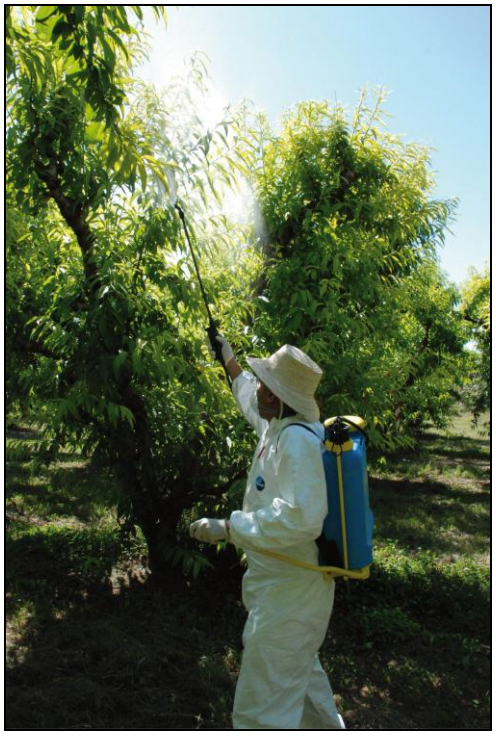


Figure I.3 *Foliar fertilization of a Fe-deficient peach tree with a Fe-containing formulation.*

Applying Fe treatments to the foliage instead of soil application can avoid the inhibitory effects of soil bicarbonate on Fe uptake and transport to the shoot (Wallace 1995, Mengel 1995) and can be a cheaper, environmentally friendly alternative to soil treatments for the control of Fe chlorosis (Wojcik 2004). An example of this type of fertilization is shown in Fig. I.3. Foliar fertilization is usually effective in alleviating chlorosis, and is generally used in countries where farmers cannot afford the costs of synthetic chelates. Of course, the commercial interests of companies producing and

selling synthetic chelates have amplified the problems that may occur when using foliar sprays. Current environmental concerns on the fate of the synthetic Fe (III)-chelates used to control Fe chlorosis have also triggered a new interest in foliar fertilization techniques (Pestana et al. 2003). The success of treatments with Fe compounds depends on their capacity to penetrate the cuticle, travel through the apoplastic free space and cross the plasma membrane of leaf cells to reach the cytoplasm and then the chloroplast (Abadía et al. 2011, Rombolà et al. 2000).

Several authors tested foliar applications of Fe chelates to plants such as orange (El-Kassa 1984, Pestana et al. 2001, Pestana et al. 2002), tangerine (Pestana et al. 1999) and kiwi (Rombolà et al. 2000, Tagliavini et al. 2000). The foliar application of chelates can be less efficient than soil application, due to limited uptake by aerial parts, but the results obtained by Rombolà et al. (2000) suggest that leaves of field-grown kiwi were able to take up Fe(III) from foliar-applied Fe(III)-diethylenetriaminepentaacetic acid (DTPA). This is also true for citrus (orange and tangerine) since a recovery from Fe chlorosis symptoms was obtained after frequent foliar sprays with Fe(III)-EDDHA (Pestana et al. 2002, Pestana et al. 1999). Other treatments that can be applied directly to trees are products that promote the activity of the Fe-chelate reductase present in the plasma membrane of leaf mesophyll cells. Examples are dilute solutions of mineral or organic acids, hormones, alcohols and urea (Pestana et al. 2003).

Iron(II)sulphate was tested as foliar fertilizer in many previous studies. It was reported to increase leaf chlorophyll content in kiwi (Rombolá et al. 2000), citrus (Pestana et al. 2001, Amri and Shahsavar 2009), pear (Álvarez-Fernández et al. 2004) and peach (Fernández et al. 2006, Fernández et al. 2008). This treatment can improve fruit size and quality, as observed in orange (El-Kassa 1984, Pestana et al. 2001, Pestana et al. 1999). The positive effects obtained on leaf chlorophyll content did not always translate into increased yield, because the translocation of the applied Fe into developing new leaves or fruits can be small. A study on the effectiveness of foliar fertilization with acids, $FeSO_4$ with and without acids and Fe-DTPA to re-green chlorotic pear trees was carried out, and it was concluded that foliar fertilization cannot offer a good alternative for the full control of Fe chlorosis (Álvarez-Fernández et al. 2004). These authors proposed that this could be a management technique complementary to soil Fe(III)-chelate applications. This is also a normal practice in crops where the use of chelates is too expensive. On the other hand, Abadia et al. (1992)

indicated that there are not enough SCI references dealing with the foliar treatments for the correction of Fe chlorosis.

Physiological effects of iron chlorosis and iron resupply in fruit trees: xylem as case study

The movement of solutes from roots to the aerial parts of the plant is accomplished by the tracheary elements of the xylem, which was traditionally considered as the main conduit for water and minerals (Evert 2006). However, xylem sap contains also organic solutes, including carbohydrates, amino acids, organic acids, hormones and proteins (Satoh 2006).

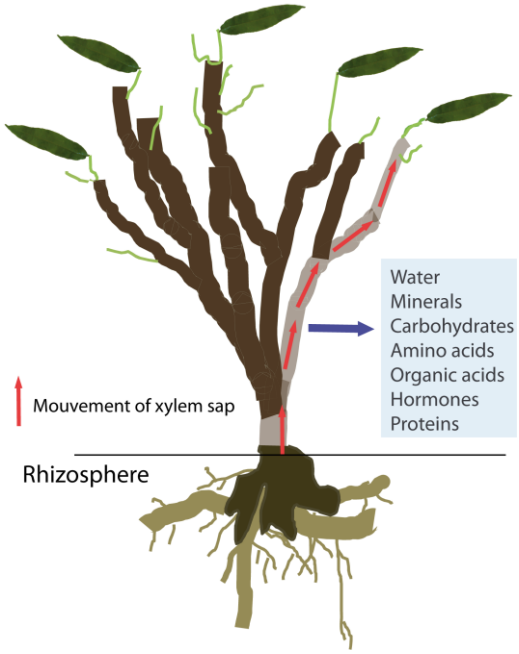


Figure I.4 *Xylem sap movement in the plant.*

Because plants are immobile and have to cope with changes in their environment, interaction of different organs is essential to coordinate growth, development and defense reactions also between the most distant plant parts (Oda et al. 2003). This interaction is mediated by signal molecules that are supplied from the root system *via* xylem (Dodd 2005) and whose concentration change in case of biotic stress or abiotic stress (Cánovas et al. 2004, Kehr et al. 2005) (Fig. I.4). Samples of xylem sap can be obtained using different methods (Fig. I.5).

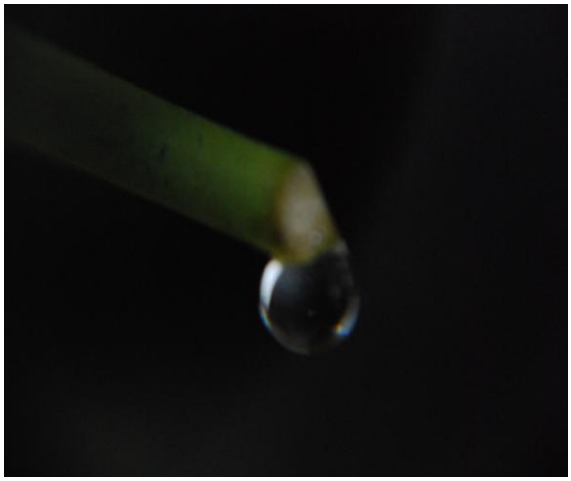


Figure I.5 *A xylem sap drop going out from a peach tree current year-old shoot after application of a pressure of about 10 bars.*

In the case of Fe chlorosis, the most prevalent abiotic stress in the Mediterranean region (Abadía et al. 2011, El-Jendoubi et al. 2011), plant adaptation also involves different metabolic changes occurring at the root, xylem, leaf and fruit levels. In roots, there are increases in the activities of phospho*enol*pyruvate carboxylase (PEPC, (Andaluz et al. 2002) and several enzymes of the glycolytic pathway and the tricarboxylic acid (TCA) cycle (Brumbarova et al. 2008, Herbik et al. 1996, Li et al. 2008). The increased anaplerotic C fixation mediated by PEPC leads to an accumulation of organic acids (Abadía et al. 2002), which may play important roles in the transport of Fe and C (López-Millán et al. 2000) *via* xylem to the leaf. Organic acid concentrations in xylem sap and leaf apoplastic fluid are markedly increased in several Strategy I plant species with Fe deficiency (Jiménez et al. 2007, Larbi et al. 2003, López-Millán et al. 2001b, López-Millán et al. 2009, López-Millán et al. 2000). At the leaf level, the most characteristic Fe-deficiency symptom is the yellow color of young leaves, caused by a relative enrichment in carotenoids (Abadía 1992), associated to changes in the light-harvesting pigment-protein complex composition (Abadía 1992, Larbi et al. 2004, Timperio et al. 2007). Iron deficiency-induced leaf chlorosis leads to reduced photosynthetic efficiency and electron transport, with less C being fixed via photosynthesis (Abadía 1992, Larbi et al. 2006).

Changes in plant metabolism occurring shortly after Fe resupply have been only partially characterized. Whereas Fe resupply leads to rapid (within 3-6 h) increases in the concentration of Fe in the xylem sap (Orera et al. 2010, Rellán-Álvarez et al. 2010a), significant increases in leaf chlorophyll concentrations and photosynthetic rates

only occur after one or two days in controlled environments or one week in the field (Larbi et al. 2004, Larbi et al. 2003, Timperio et al. 2007). Also, Fe-resupply, either to leaves or to roots, leads to the rapid (within 24 h) de-activation of transcripts associated to root Fe acquisition mechanisms, including *FRO* and *IRT*, whereas the activities of FRO and PEPC decrease more slowly (Abadía et al. 2011, Enomoto et al. 2007, López-Millán et al. 2001c). Xylem sap and leaf apoplastic carboxylate concentrations decrease progressively after Fe resupply in Fe-deficient sugar beet plants (Larbi et al. 2010). In roots, organic acid concentrations and metabolite profiles reach control levels only within a few days after Fe-resupply (Abadía et al. 2011, Rellán-Álvarez et al. 2010b). Also, Fe resupply leads to progressive decreases in the concentration of organic acids in the whole plant (López-Millán et al. 2001a, López-Millán et al. 2001c). Only few studies have been made on the interaction between Fe fertilization and Fe long-distance transport (Abadía et al. 2011b).

Effects of iron chlorosis on photosynthetic parameters

A possible physiological explanation for the decrease in productivity is the decrease of the photosynthetic activity of the chlorotic leaves, because Fe is involved in major plant functions, including respiration, nitrate reduction and photosynthesis (Terry 1980). Leaves from Fe-deficient plants have a reduced number of granal and stromal lamellae per chloroplast (Spiller and Terry 1980). This is accompanied by decreases in all thylakoid membrane components, including light-harvesting chlorophylls (Chls) and carotenoids (Abadía and Abadía 1993, Morales et al. 1990) and photosynthetic electron transport carriers (Spiller and Terry 1980). It has been proposed that the Fe deficiency-mediated decreases in light harvesting, electron transport and carbon fixation are well coordinated (Winder and Nishio 1995). Because of their low photosynthetic rates, Fe-deficient plants are prone to be exposed to an excess of photosynthetic photon flux density (PPFD) under natural conditions (Abadía et al. 1999). Iron deficiency does not decrease to the same extent all photosynthetic pigments, carotenoids being less affected than Chls; Chl *b* is more affected than Chl *a*, whereas lutein and xanthophyll cycle carotenoids (zeaxanthin, Z; antheraxanthin, A and violaxanthin, V) are less affected than the other carotenoids (Morales et al. 1994, Morales et al. 2000).

The xanthophyll cycle in higher plants, green (Chlorophyta), and brown algae (Phaeophyceae) consists of the pH-dependent conversion from V, a xanthophyll with two epoxide groups, first to A (one epoxide group) and then to Z (no epoxide group).

Diatoms and most other eukaryotic algae have a different xanthophyll cycle (the diadinoxanthin cycle) that involves a conversion from diadinoxanthin (one epoxide group) to diatoxanthin (no epoxide group) (Lohr and Wilhelm 1999) (Fig. I.6). In plants, the de-epoxidation reaction is catalyzed by the enzyme violaxanthin de-epoxidase (VDE), a 43-kD nuclear-encoded protein localized in the thylakoid lumen (Bugos and Yamamoto 1996). A different enzyme, zeaxanthin epoxidase (ZE), catalyzes the epoxidation reactions that complete the violaxanthin cycle (Bouvier et al. 1996).

An important mechanism to avoid the deleterious effects of excess PPFD is thermal dissipation within the PS II antenna (Abadía et al. 1999). This dissipation process involves the de-epoxidized xanthophylls Z and A (Demmig-Adams et al. 2004, Gilmore and Yamamoto 1993). In dark-adapted Fe-deficient plants, most of the xanthophyll cycle pigment pool is in the epoxidized form V, but in response to light the de-epoxidized forms A and Z are formed rapidly at the expense of V (Morales et al. 1990). A good measure of the status of the VAZ cycle pigments is the epoxidation index, defined as the relative number of epoxide groups over the maximum (Abadía and Abadía 1993). When Fe is resupplied to Fe-deficient plants, the Fe deficiency effects recover progressively, and Chl and other components of light harvesting and photosynthetic electron transport chain are gradually synthesized *de novo*. This has been documented for several species, including sugar beet (Nishio et al. 1985), soybean (Hecht-Buchholz and Ortmann 1986) and tobacco (Pushnik and Miller 1989).

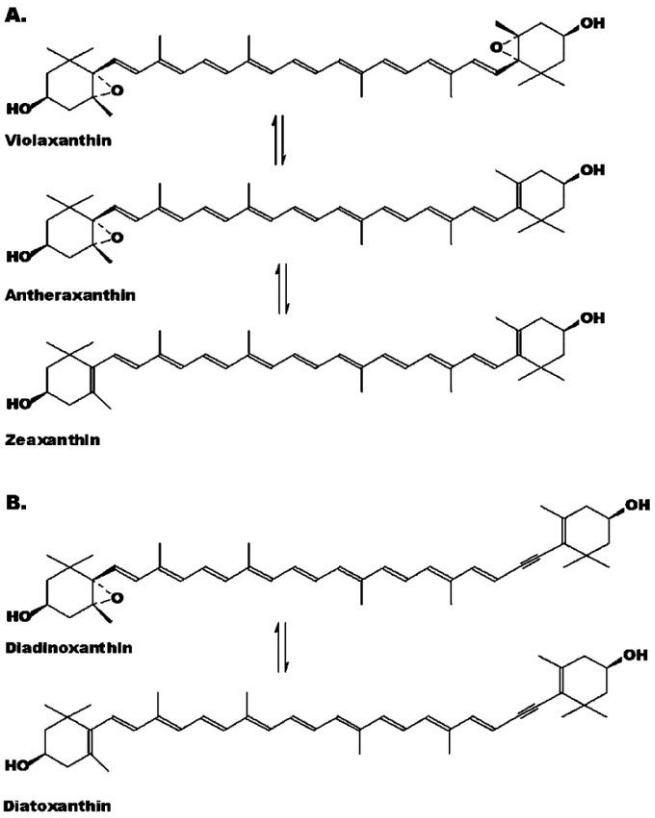


Figure I.6 *Xanthophyll cycles. (A): the violaxanthin cycle consists in the de-epoxidation of violaxanthin in high light to first antheraxanthin and then zeaxanthin, catalyzed by VDE; ZE catalyzes the reverse reaction. (B): The diadinoxanthin cycle consists in the conversion of diadinoxanthin to diatoxanthin by diadinoxanthin de-epoxidase in high light and the reverse reaction in low light.*

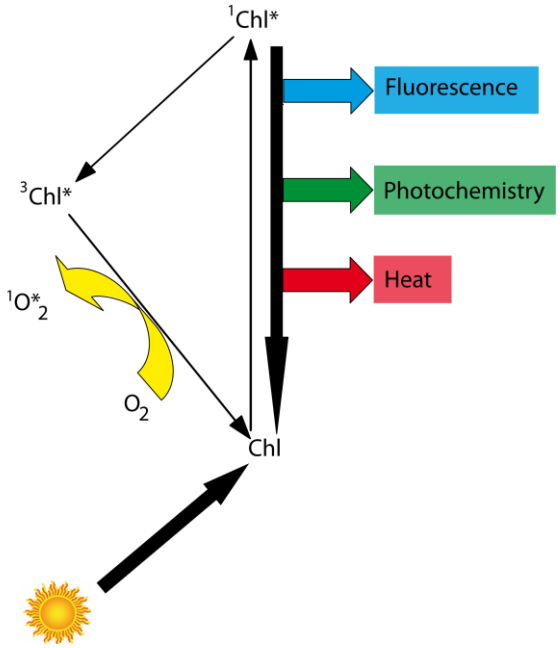
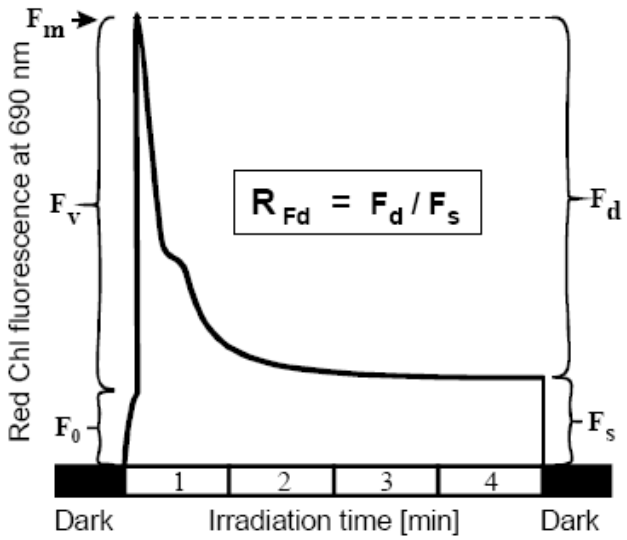


Figure I.7. *Possible fates of excited Chl. When Chl absorbs light it is excited from its ground state to the singlet excited state, 1Chl*. From there it has several ways to relax*

back to the ground state: by emitting light, seen as fluorescence (1), to fuel photosynthetic reactions (2) or de-excite by dissipating heat (3); all these mechanisms reduce the rate of fluorescence. Also, $^1Chl^*$ can produce $^3Chl^*$ (4), which in turn is able to produce $^1O_2^*$, a very reactive oxygen species.

On the other hand, light absorption results in singlet-state excitation of a Chl *a* molecule (^{1}Chl*), which can return to the ground state *via* one of several pathways (Fig. I.7). The excitation energy can be re-emitted as Chl fluorescence, transferred to reaction centers and used to drive photochemistry, de-excited as heat by thermal dissipation processes (NPQ), or decay via the triplet state (^{3}Chl*) (Muller et al. 2001).

Fluorescence represents radiation emitted during the de-excitation of pigments that have been excited by absorption of visible (PAR) or UV-radiation. The Chl fluorescence of intact leaves varies with time, being inversely related to the photosynthetic activity (Krause and Weis 1991, Lichtenthaler et al. 2005) (Fig. I.8)



F_0 = initial fluorescence
F_v = variable fluorescence
F_m = maximum fluorescence
F_d = fluorescence decrease
F_s = steady state fluorescence
R_{Fd} = fluorescence decrease ratio

Figure I.8. *Light-induced chlorophyll (Chl) fluorescence induction kinetics (Kautsky effect) in 20 min pre-darkened green, photosynthetically active leaves measured at saturation irradiance >1,500 µmol photon m^{-2} s^{-1}. Upon irradiation, the Chl fluorescence rises via F_0 to the maximum F_m (within 100– 200 ms) and then declines with the onset of photosynthetic CO_2 fixation, within 3–5 min, to a low steady state fluorescence, F_s, which in fully photosynthetically active leaves is slightly above the*

level of F_0. F_d is the Chl fluorescence decrease from F_m to F_s. The Chl fluorescence decrease ratio R_{Fd}, defined as ratio F_d/F_s, when measured at saturation irradiance, correlates with the potential CO_2 fixation rate PN of leaves as shown for several plants as well as sun and shade leaves. The ratio R_{Fd} can be expressed either by F_d/F_s or by $(F_m/F_s) – 1$. The 'state 1' of the dark-adapted photosynthetic apparatus, where F_m is reached after a few hundred ms of irradiation, is in the light gradually turned into the functional 'state 2' of the light-adapted photosynthetic apparatus. (Lichtenthaler et al. 2005).

Although the triplet pathway can be a significant valve for excess excitation (4-25% of absorbed photons (Foyer et al. 2004), ^{3}Chl* can transfer energy to ground-state O_2 to generate singlet oxygen ($^1O_2$*), an extremely damaging reactive oxygen species. At room temperature, Chl fluorescence mainly originates from photosystem (PS) II, and the yield of fluorescence is generally low (0.6-3%; Krause and Weis 1991). The high quantum efficiency of photochemistry in limiting light, results in a decrease or quenching of fluorescence that is termed photochemical quenching (qP). Non-photochemical processes that dissipate excitation energy also quench Chl fluorescence and are collectively called NPQ (or qN) (Muller et al. 2001). These Chl fluorescence parameters were reported to change in the case of Fe-deficiency and after Fe-resupply to Fe-deficient plants (Morales et al. 1994, Larbi et al. 1996).

Concluding remarks

The information indicated previously shows the importance of Fe chlorosis as a nutritional disorder in our region of study. Through the present work, we want to improve major essential aspects related to this nutritional disorder by carrying out studies that in principle are considered as difficult to achieve. First, the nutrient requirements of Fe were characterized in peach trees, the most affected fruit tree crop in the region, using a whole tree analysis approach. This study was also carried out for the rest of nutrients to uncover a complete "nutritional profile" of the fruit tree. Since nutritional diagnostic is important for an adequate correction of this nutritional disorder, an advance in the prognosis of Fe deficiency using early plant materials such as buds was also developed. We also tried to explore the most adequate statistical approach for studying the Fe chlorosis-nutrient concentrations relationships. In a further step, we decided to assess the relationships between scientific background and agronomic practices such as the correction of Fe chlorosis. Since foliar treatments is an agronomical management practice not thoroughly studied, we used using various

approaches to study the effects of foliar Fe-compounds. Many works have reported the effect of Fe chlorosis in different parts of the plant, including leaf, roots and flowers, but its effects in the fruit tree xylem sap composition was not well studied because of the difficulties of xylem sap extraction. Therefore, we optimized the xylem sap extraction process and started studies on xylem sap characterization. Being conscious of the importance of methodology and experimental designs in the field, the studies were oriented to obtain scientific background, as well as advices and comments that could be important for people working in the field.

Objectives

General objective

The general objective of the present work consists in the improvement of the agronomic correction practices of iron chlorosis in fruit trees, by making advances in several aspects considered closely related to such a typical nutritional disorder in the Mediterranean region.

Specific objectives

1. To study the annual requirements of Fe and other macro- and microelements in peach trees by means of a whole tree analysis approach, assessing the amounts of nutrients removed in the different events during the year as well as the amounts stored in permanent tree parts.
2. To study the possibility of carrying out the prognosis of Fe chlorosis in peach and pear trees using the mineral concentrations in early plant materials such as flowers and buds.
3. To advance the scientific background on foliar iron treatments using new approaches for the evaluation of effects in treated and untreated leaf surfaces.
4. To set up the basis to study the changes caused by iron deficiency in the composition of the xylem sap of peach trees, using metabolomics and proteomics approaches.
5. To summarize the current knowledge on how to make a sound assessment of the effects of Fe-fertilizers in fruit trees.

Assessment of nutrient removal in bearing peach trees (*Prunus persica* L. Batsch) based on whole tree analysis

Abstract

Background and Aims

In this study, the amounts of macro- (N, P, K, Ca and Mg) and microelements (Fe, Mn, Cu and Zn) lost by peach trees (*Prunus persica* L. Batsch) in all the nutrient removal events (pruning, flower abscission, fruit thinning, fruit harvest and leaf fall), as well as those stored in the permanent structures of the tree, have been quantified in three fruit tree bearing cultivars.

Methods

Peach trees were selected in two orchards, a commercial, highly productive one (20 trees of the 'Calanda' cv.) and a local grower owned, low productive one (9 trees of the 'Catherina' cv. and 11 trees of the 'Babygold5' cv.). The experiment lasted three years. The biomass lost by trees during winter pruning, flower abscission, fruit thinning, summer pruning, fruit harvest and leaf fall were recorded, and all tissues were analyzed. The biomass of permanent structures (roots, trunk and main branches) was also measured after full tree excavation in two trees per cv. and year, and these materials were also analyzed.

Results

The major biomass losses occurred… Winter pruning and leaf fall were the events where most nutrients were removed. Nutrient losses and requirements are given as amounts of nutrients needed per tree and also as amounts necessary to produce a t of fresh fruit. Yearly peach tree nutrient losses were (in g $tree^{-1}$, for 'Calanda/Catherina/Babygold5') 340/103/98, 53/10/9, 518/21/21, 74/104/89 and 425/149/141 for N, P, Ca, Mg and K, respectively, and (in mg $tree^{-1}$) 4074/1126/933, 821/233/217, 824/724/216 and 875/169/155 for Fe, Mn, Cu and Zn, respectively.

Conclusions

The allocation of all nutrients analyzed in the different plant parts was similar in different types of peach trees, with each element having a typical "fingerprint" allocation pattern. This indicates that the nutrient allocations found could be used as a guide for the estimation of nutrient requirements in other cultivars. Peach tree materials removed at tree pruning and leaf fall include substantial amounts of nutrients that could

be recycled to improve soil fertility and tree nutrition. Poorly known tree materials such as flowers and fruit stones contain measurable amounts of nutrients.

Introduction

The world population has increased from less than 2 billion people in 1900 to 5.7 billion in 1995, and it is expected to reach 8.5 billion in 2025 (Byrnes and Bumb 1998). This unprecedented growth in population will create tremendous pressures on the natural resources to produce enough food and fiber to meet human needs (Byrnes and Bumb 1998, Cakmak 2002, Grusak et al. 1999).

In order to meet the food demands of the rising population, farmers must manage nutrients and soil fertility with an adequate and balanced supply of nutrients. This balance will not be achieved until "nutrient cycles" are better understood (Gruhn et al. 2000). The nutrient cycle is defined as the continuous recycling of nutrients into and out of the soil (NRC 1993), and it involves complex biological and chemical interactions, some of which are not yet fully understood. A simplified version of this type of cycles in plant growth has been proposed (Stoorvogel et al. 1993). The cycle has two parts: "inputs" that add plant nutrients to the soil and include mineral fertilizers, organic manures, atmospheric deposition, biological nitrogen fixation and sedimentation, and "outputs" which include the harvested crop parts and crop residues, as well as nutrients lost by leaching, gaseous losses and water erosion. The difference between inputs and outputs constitutes the nutrient balance. Positive nutrient balances in the soils (e.g., in the case of over-application of fertilizers which makes nutrient additions to the soil greater than the removals; Bumb and Baanante 1996, Conway and Barbie 1988) could indicate that farming systems are inefficient and, in the extreme, that they will pollute the environment. Negative balances (in case of under-application of fertilizers) could indicate that soils are being mined and that farming systems will be unsustainable over the long term. In the latter case, enough nutrients should be supplied in order to sustain agriculture in the long term, to increase crop productivity and to maintain soil fertility (Gruhn et al. 2000). The nutrient rates applied should meet the demand of the crop, but should not exceed the demand in large excess (Mengel 1982).

In the case of fruit trees, the nutrient demand is fulfilled at the beginning of the season by the remobilization of nutrients already stored in the perennial parts of the trees during the previous season (Millard 1995, Muñoz et al. 1993, Quartieri et al. 2002, Tagliavini et al. 1998). This nutrient remobilization is well studied and quantified in the case of N, and seems to be unaffected by N supply in the spring (Millard 1995). For the

rest of the season, nutrient uptake depends on soil supply. Although the important functions fulfilled by macro- and micro- elements are well known (Clarkson and Hanson 1980, Mengel and Kirby 1982, Neilsen and Neilsen 2003), the specific elemental requirements for optimum growth, fruit yield and quality needs to be determined for each fruit species and cultivar. This is especially important when using high planting densities.

A relatively simple approach to assess tree nutrient requirements is based on whole tree mineral analysis. Several studies of this kind have been carried out in apple (Batjer et al. 1952, Haynes and Goh 1980), peach (Stassen 1987), avocado (Stassen et al. 1997c) and mango trees (Stassen et al. 1997a, b, Stassen et al. 1999), as well as in grapevine (Conradie 1981). This approach usually takes into account mineral nutrient losses due to removal of fruit and pruned wood from the orchard, as well as also those associated to leaf fall. The method also considers the nutrient contents of permanent parts of the tree; including old wood and roots, taking into account the tree age (Stassen 1987). Most of these studies have focused on macroelements, even though some microelement deficiencies can have also major effects. For instance, N deficiency led to smaller fruits, shorter shoots and low yields than those found in the control trees (Johnson 2008), and similar effects were described to occur in the case of the Fe deficiency (Alvarez-Fernandez et al. 2006, Rombolá and Tagliavini 2006).

In this study, the annual nutrient requirements of macro- and microelements (N, P, K, Ca, Mg, Fe, Cu, Zn and Mn) has been estimated in two peach tree commercial orchards with very different management, plantation density and fruit yields. Nutrient outputs from the trees at different removal events, including winter pruning, flower loss, fruit thinning, summer pruning, fruit harvest and leaf fall, were estimated. Also, an estimation of the amounts of macro and micro-nutrients stored in perennial tree parts (both underground and aboveground) was carried out.

Material and Methods

The study was made during three consecutive seasons (from 2007 to 2010) using peach trees planted in two commercial orchards grown in clay loam soils in the Ebro river basin area, Northeastern Spain. An orchard included the cultivars ‘Babygold5’ and ‘Catherina’ and was located in Peñaflor (41º 46’ 42.65’’N and 0º 47’ 38.70’’ O). This orchard had a 6 x 2.5 m frame (670 trees ha^{-1}), was managed by a local farmer and had a

low yield (approximately 15 kg fruits FW tree^{-1}). A second orchard included the cultivar 'Calanda' and was located in Utebo (41º 42' 54.99''N and 0º 58' 38.02'' O). This orchard had a 5 x 4 m frame (500 trees/ha), was managed by a commercial farmer and had an average yield (approximately 60 kg fruits FW tree^{-1}). All trees were grafted on GF677 rootstock. 'Babygold5' and 'Catherina' are early season cultivars with fruits being harvested in July and August, respectively, and 'Calanda' is a late season cultivar with fruits being harvested in October. All trees were 14 year-old. A total of 20, 9 and 11 'Calanda, 'Catherina' and 'Babygold5' trees, respectively, were selected, although data from some trees could not be used because of tree failure or uncontrolled fruit removal. Orchards were managed according to commercial practices for pest and weed control and were watered using flood irrigation.

Samples were taken from the trees at different events along the season to determine the tree nutrient requirements. Samples included wood at winter pruning, flowers at full bloom, fruits at thinning, wood at summer pruning, fruits at harvest and leaves at fall. Also, permanent tree structures (root, trunk and main branches) were sampled by full tree excavation (two trees per cultivar and year).

Flower sampling

In the case of flowers, the total number was counted on each tree at full bloom in March (when 50% or more of the flowers of each shoot were open; Fig. 1.1A). Sixty whole flowers per tree (including petals, sepals, reproductive parts, bracts and peduncles) were also taken at full bloom from the central part of the shoots around the tree crown (30 in the upper part and 30 in the lower part of the crown; Belkhodja et al. 1998, Igartua et al. 2000, El-Jendoubi et al., 2012). Flowers were dried in an oven at 60 ºC for mineral analysis. The number of abscised flowers was estimated from the total number of flowers and those resulting in fruits (including fruits removed at thinning, harvested and dropped to the soil). Data shown are means ± SD (n = 36, 21 and 25 for 'Calanda, 'Catherina' and 'Babygold5'; approximately one third of the samples was obtained each year).

Fruit sampling

Fruit thinning was carried out manually in May, at phenological state 73 according to Meier (2001) when fruits had a diameter of approximately 31 mm (Fig. 1.1B), recording the number of fruits and total fresh mass removed from each tree. At the end of July,

beginning of August and October, fruits were harvested in accordance with commercial picking standards for 'Babygold5', 'Catherina' and 'Calanda', respectively (Fig. 1.1C). Fruit number and fresh mass per tree were recorded at harvest, and a subsample of the harvested fruits was oven-dried and weighed to calculate a fresh mass to dry mass conversion factor. Dried fruit materials (fruit endocarp and stones) were ground separately and stored for mineral analysis. Also, the number of the fruits which had dropped naturally to the soil at harvest time was recorded (Fig. 1.1D). Data shown are means ± SD (n = 18, 21 and 25 for 'Calanda, 'Catherina' and 'Babygold5'; approximately one third of the samples was obtained each year, and values were low in the case of 'Calanda' because uncontrolled fruit removal).

Leaf sampling

Leaf samples were taken at two different times along the season to assess the orchard nutrient status. 30-50 leaves per tree were sampled (fully developed leaves, 4^{th}-6^{th} from the top in the distal third of the current year's growth; Belkhodja et al. 1998) 60 and 120 days after full bloom (DAFB) in May and July (El-Jendoubi et al. 2012, Sanz et al. 1991). Data are means ± SD (n = 54, 21 and 27 for 'Calanda, 'Catherina' and 'Babygold5'; one third of the samples was obtained each year).

In September ('Babygold5' and 'Catherina') or October ('Calanda'), four trees from each cultivar were completely covered by a net to recover abscissed leaves (Fig. 1.1G). When leaf fall was complete (Fig. 1.1H; in October-November) the total weight of fallen leaves was calculated and subsamples of 100 leaves per tree were used to estimate the total leaf number per tree. Leaf samples were ground and stored for mineral analysis. Data are means ± SD (n = 12 for each of the cultivars; one third of the samples was obtained each year).

Wood sampling

Summer pruning was made in July (in 'Babygold5' and 'Catherina'), and August ('Calanda'; only in 2008), by removing some shoots to control vegetative growth, improve fruit growth and increase light penetration. Pruned shoots were separated in leaves and one year-old wood samples, and a subsample from each part was taken, dried and stored for mineral analysis (Fig. 1.1E and F). Winter pruning was carried out in December-January (Fig. 1.1I) and the wood mass removed per tree was recorded (Fig. 1.1J), and a subsample was taken, dried and stored for mineral analysis. Data are means

± SD (n = 54, 21 and 27 for ‘Calanda, ‘Catherina’ and ‘Babygold5’; one third of the samples was obtained each year).

In December each year, two trees per cultivar were excavated (Fig. 1.2C). The aboveground part was separated using a chain saw (Fig. 1.2A) in trunk wood, old wood and one year-old wood. The underground part was divided into roots and rootstock part (Fig. 1.2D). From each part, a subsample was taken, cut into small parts using a vertical saw (Fig. 1.2B) and then ground and stored for mineral analysis. Data are means ± SD (n = 12; one third of the samples was obtained each year).

Mineral analysis

Samples were washed, mineralized and analyzed using standard procedures (Abadía et al. 1985, Igartua et al. 2000). Nitrogen and P were analyzed by the Dumas method and spectrophotometrically, respectively. Potassium was measured by flame emission spectroscopy, and Ca, Mg, Fe, Mn, Cu and Zn were measured by atomic absorption spectrophotometry. Concentrations were expressed as % dry weight (DW) for macronutrients (N, P, K, Mg and Ca) and as mg kg^{-1} DW for micronutrients (Fe, Mn, Cu and Zn).

Statistical analysis

All data shown are means ± SD.


A
B
C
D
E
F
G
H
I
J

Figure 1.1 *Nutrient removal events in a peach tree orchard: **A**, Shoot in full bloom stage (end of March); **B**, tree at fruit thinning time (May); **C**, harvested fruits (July-October); **D**, fruits dropped to the soil at harvest time; **E**, One-year old wood from shoots removed at summer pruning; **F**, Leaves from shoots removed at summer pruning; **G**, net structure covering the tree to recover fallen leaves in autumn; **H**, fallen leaves accumulated in the net; **I**, winter pruning; **J**, wood from shoots removed at winter pruning.*

Figure 1.2 *Peach tree excavation: A, cutting of the aboveground part using a chain saw; B, cutting of the wood samples taken using a vertical saw; C, Excavation of the underground peach tree part and D, classification of the underground part into rootstock and roots.*

Results

Average production in the orchards was 30.2, 8.7 and 8.9 t/ha for the 'Calanda', 'Catherina' and 'Babygold5' cultivars, respectively (corresponding to 60.4, 13.0 and 13.3 kg tree^{-1} respectively).

Nutrient composition of removed and permanent peach tree materials

The concentrations of macro and micro-elements in all tree materials, including flowers at full bloom, leaves at leaf fall, fruits during thinning and at harvest times, wood at summer and winter pruning, and tree samples obtained by excavating full trees (root, trunk and main branches) are shown in Tables 1, 2 and 3 for the cultivars 'Calanda', 'Catherina' and 'Babygold5', respectively.

Nutrient concentrations in 'Calanda' peach cultivar

This cultivar was grown by a commercial grower with moderate tree density and had an average yield in the area. Concerning biomass, fruit harvesting (including fruits and stones) was the event where more biomass was removed (11103 g DW tree^{-1}, from which 2836 were in the stones). Large amounts of biomass were also removed at winter pruning, leaf fall and summer pruning (9174, 6539 and 2964 g DW tree^{-1}, respectively). Fruit thinning and flower abscission were the events where less biomass was lost (443 and 45 g DW tree^{-1}, respectively).

Regarding nutrient concentrations, the values found in each material were compared to those found in leaves at 60 and 120 DAFB. Flowers were richer in Fe, Cu and Zn, and relatively low in N, Ca and Mg. Thinned fruits were relatively low in N, Ca, Mg and Mn. The endocarp of the harvested fruits had low concentrations of all elements with the exception of Fe, whereas stones were low in all elements; most mineral concentrations on a DW basis were quite similar in fruit endocarp and stones, with the exception of P and Ca that were lower and higher, respectively, in the stones than in the endocarp. Leaves of summer pruning material were relatively high in Ca, Mg and Cu and low in N and P, whereas the wood was low in all elements excepting Zn. Fallen leaves were rich in Ca, Mg and Fe and low in N, P and Zn. Winter pruning material had low concentrations of most elements, with the exception of Ca, Fe and Zn.

Regarding immobilized tissues obtained through tree excavation, roots generally had higher element concentrations than wood, with the exception of Ca and K, which were similar. The concentrations in the rootstock and scion parts of the trunk were similar.

Nutrient concentrations in 'Catherina' and 'Babygold5' peach cultivars

These cultivars were grown in conditions where tree density was higher and management was different than in the case of the 'Calanda' cultivar, resulting in a lower yield. Winter pruning (including one-year old wood and old wood) was (in

'Catherina'/'Babygold5') the event where more biomass was removed (2731/2379 g DW tree^{-1} wood, from which 491/715 g tree^{-1} were old wood). Large amounts of biomass were removed at leaf fall and fruit harvest (1769/2138 and 1755/1900 g DW tree^{-1}, respectively). Summer pruning, fruit thinning and flower abscission were the events where less biomass was lost (940/643, 172/167 and 31/21 g DW tree^{-1}, respectively).

Table 1. *Total dry weight and mineral composition of the vegetative material removed at the different events and of the permanent structures of peach trees (in % of DW for N, P, Ca, Mg and K and in mg kg $^{-1}$ DW for Fe, Mn, Cu and Zn). The mineral composition of the 60 and 120 DAFB leaves is also shown as a reference. A: 'Calanda' cultivar: data are means ± SD (n = 36, 54, 18, 18, 12, 54, 6, 54 and 54 for flowers, thinning, fruit harvest, summer pruning, fallen leaves, winter pruning, excavated trees, leaves at 60 and leaves at 120 DAFB, respectively; approximately one third of the samples was obtained each year).*

Event	Material	Total dry weight	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn
Flower abscission	Flowers	45 ±18	2.68±0.36	0.45±0.03	2.05±0.38	0.70±0.16	0.21±0.04	206.2±68.3	54.4±65.4	806.6±189.4	62.6±9.1
Fruit thinning	Fruits	443±307	2.21±0.47	0.29±0.03	1.92±0.35	0.27±0.14	0.12±0.02	101.3±39.8	11.5±2.8	33.1±14.4	30.0±5.4
Fruit harvest	Fruits	8267±3662	0.64±0.29	0.18±0.03	1.38±0.33	0.11±0.06	0.06±0.01	79.2±16.1	5.6±1.3	9.5±1.7	8.1±1.6
	Stones	2836±1030	0.54±0.29	0.06±0.03	0.36±0.09	0.17±0.08	0.11±0.01	42.3±25.8	7.8±1.0	5.4±2.1	9.6±3.1
Summer pruning	Leaves	1916±820	2.85±0.16	0.27±0.04	2.30±0.27	3.05±0.54	0.52±0.06	158.0±24.2	24.4±2.4	124.9±55.0	36.6±8.5
	One-year old wood	1048±491	0.55±0.19	0.24±0.02	0.70±0.23	0.97±0.29	0.07±0.01	36.4±13.8	6.0±0.4	17.0±2.2	58.2±15.1
Leaf fall	Leaves	6539±1945	1.29±0.22	0.15±0.03	2.32±0.37	4.66±0.21	0.61±0.09	299.5±51.7	78.0±33.0	29.9±9.9	24.0±2.7
Winter pruning	One-year old wood	9174±2957	1.22±0.60	0.18±0.06	0.84±0.53	1.50±0.53	0.19±0.10	98.4±43.6	13.8±3.2	22.7±8.0	51.3±30.9
Tree removal	One-year old wood	2476±1435	0.14±0.03	0.11±0.01	0.40±0.04	0.61±0.03	0.05±0.01	88.2±25.1	9.6±0.3	9.5±0.7	167.6±76.3
	Older wood	39587±12596	0.15±0.11	0.07±0.4	0.13±0.07	0.83±0.28	0.04±0.02	144.1±28.2	6.9±1.1	36.3±20.0	41.5±37.9
	Scion trunk	7139±2024	0.31±0.18	0.05±0.02	0.17±0.04	1.32±0.15	0.05±0.03	277.1±86.4	12.1±5.2	63.1±36.8	65.8±51.1
	Rootstock trunk	7857.5±3313.2	0.29±0.07	0.08±0.04	0.20±0.14	1.08±0.22	0.05±0.02	216.0±63.3	12.6±7.0	29.4±23.7	11.9±3.7
	Excavated Roots	7678±3412	0.73±0.16	0.24±0.04	0.19±0.06	1.17±0.35	0.11±0.04	393.9±82.3	21.1±21.8	13.5±5.0	20.1±11.0
60 DAFB leaves		- - -	4.81±0.55	0.47±0.08	2.20±0.19	1.47±0.44	0.42±0.05	127.8±38.5	29.1±3.1	40.6±17.9	57.3±11.6
120 DAFB leaves		- - -	3.95±0.50	0.30±0.07	2.39±0.73	1.55±0.51	0.42±0.10	87.7±26.6	88.2±59.6	30.0±12.2	32.4±5-4

Table 1 (Contn.). *B: 'Catherina' cultivar: data are means ± SD (n = 21, 21, 21, 21, 12, 21, 5, 21 and 21 for flowers, thinning, fruit harvest, summer pruning, fallen leaves, winter pruning, excavated trees, leaves at 60 and leaves at 120 DAFB, respectively; approximately one third of the samples was obtained each year).*

Event	Material	Total dry weight	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn
Flower abscission	Flowers	31±26	2.83±.36	0.45±0.08	2.19±0.49	0.76±0.20	0.25±0.06	245.6±69.4	43.2±12.4	150.9±94.7	52.6±5.1
Fruit thinning	Fruits	172±140	2.42±0.54	0.29±0.06	1.96±0.32	0.24±0.06	0.12±0.02	76.7±21.2	14.0±2.8	19.5±3.7	28.1±4.8
Fruit harvest	Fruits	1579±1075	1.01±0.48	0.16±0.07	1.74±0.36	0.08±0.05	0.09±0.02	71.7±30.6	7.1±1.5	11.0±2.1	9.71±2.4
	Stones	176±119	0.86±0.16	0.07±0.02	0.80±0.13	0.16±0.24	0.10±0.02	77.0±19.1	9.1±0.8	8.9±1.4	9.9±1.3
Summer pruning	Leaves	375±252	2.97±0.41	0.17±0.03	2.22±0.30	2.37±0.55	0.54±0.10	131.4±37.9	56.8±10.4	10.7±2.2	189±3.4
	One-year old wood	565±816	0.90±0.43	0.10±0.03	0.62±0.16	1.94±0.65	0.13±0.03	67.1±21.7	15.4±4.7	19.2±6.7	31.9±7.3
Leaf fall	Leaves	1769±1619	1.75±0.21	0.11±0.02	1.37±0.36	3.69±0.75	0.62±0.10	265.6±75.0	72.9±16.3	18.6±2.6	15.5±2.8
Winter pruning	One-year old wood	2240±1742	1.36±0.25	0.13±0.02	1.05±0.31	2.37±0.18	0.16±0.01	120.7±27.2	18.9±2.2	55.0±24.2	36.2±8.4
	Old Wood	491±323	0.28±0.17	0.06±0.02	0.29±0.15	0.68±0.43	0.06±0.01	42.6±21.7	10.2±3.3	33.3±14.5	22.6±11.8
Tree removal	One-year old wood	361±308	0.67±0.17	0.09±0.00	0.61±0.03	1.27±0.04	0.10±0.02	288.2±56.9	14.4±2.2	27.3±9.2	86.0±9.5
	Older wood	16165±5310	0.22±0.22	0.05±0.05	0.15±0.08	0.82±0.28	0.04±0.02	173.0±102.7	10.7±2.9	46.6±36.0	39.0±35.5
	Scion trunk	3095±764	0.16±0.09	0.03±0.02	0.17±0.10	1.08±0.55	0.05±0.03	481.3±359.5	18.1±14.5	27.5±12.5	30.8±39.8
	Rootstock trunk	4928±2622	0.41±0.32	0.07±0.07	0.32±0.24	1.38±0.67	0.07±0.07	449.1±279.1	19.1±12.2	21.2±8.1	13.4±7.1
	Excavated Roots	2451±1141	0.96±0.30	0.09±0.02	0.36±0.06	1.23±0.08	0.13±0.04	295.0±46.7	16.8±5.9	37.3±34.1	21.1±17.5
60 DAFB leaves		- - -	4.47±0.44	0.35±0.08	2.03±0.33	1.20±0.22	0.51±0.20	64.0±10.5	41.5±7.1	19.6±1.6	39.5±6.6
120 DAFB leaves		- - -	3.46±0.50	0.22±0.04	2.18±0.47	1.74±0.56	0.65±0.19	81.5±11.1	52.0±17.8	19.5±32.5	26.6±5.3

Table 1 (Contn.). *C: 'Babygold5' cultivar: data are means ± SD (n = 25, 27, 25, 27, 12, 27, 5, 27 and 27 for flowers, thinning, fruit harvest, summer pruning, fallen leaves, winter pruning, excavated trees, leaves at 60 and leaves at 120 DAFB, respectively; approximately one third of the samples was obtained each year).*

Event	Material	Total dry weight	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn
Flower abscission	Flowers	21±17	2.79±0.42	0.40±0.08	2.27±0.59	0.90±0.26	0.30±0.07	278.7±88.4	50.2±20.9	175.0±53.9	57.4±9.8
Fruit thinning	Fruits	167±150	2.15±0.55	0.50±1.44	1.78±0.33	0.20±0.04	0.11±0.02	74.7±30.8	11.4±2.8	19.5±4.4	28.3±7.6
Fruit harvest	Fruits	1368±992	0.83±0.33	0.12±0.05	1.64±0.32	0.09±0.03	0.09±0.03	53.2±16.2	6.1±1.4	10.9±2.9	9.1±2.4
	Stones	535±383	0.55±0.16	0.04±0.02	0.61±0.13	0.26±0.18	0.07±0.02	61.2±19.0	8.6±4.2	7.0±1.6	10.0±1.5
Summer pruning	Leaves	361±128	2.81±0.29	0.16±0.02	1.99±0.28	2.23±0.49	0.53±0.06	120.5±31.6	44.9±9.2	9.0±1.2	18.5±2.9
	One-year old wood	283±171	0.68±0.25	0.09±0.03	0.52±0.12	1.57±0.31	0.12±0.02	63.1±13.4	11.5±3.0	15.3±3.8	30.9±8.8
Winter pruning	One-year old wood	1664±1406	1.43±0.30	0.13±0.03	1.02±0.34	2.50±0.42	0.17±0.03	120.3±26.4	18.1±5.1	67.0±45.8	39.8±8.8
	Old wood	715±421	0.39±0.22	0.05±0.01	0.18±0.07	0.74±0.24	0.02±0.0	33.9±7.4	7.5±1.8	18.6±6.3	22.8±8.1
Leaf fall	Leaves	2138±1120	1.83±0.22	0.11±0.02	1.27±0.25	3.74±0.42	0.60±0.10	227.7±38.4	70.0±21.5	23.3±14.2	14.9±2.6
Tree removal	One-year old wood	1046±311	0.63±0.11	0.06±0.01	0.50±0.08	1.32±0.50	0.09±0.01	398.6±224.5	11.8±2.9	41.0±17.3	158.9±99.3
	Older wood	19569±7945	0.18±0.10	0.02±0.01	0.20±0.03	0.91±0.18	0.03±0.01	801.1±315.9	10.8±7.4	52.2±24.6	66.7±65.3
	Scion trunk	2637±279	0.14±0.16	0.02±0.01	0.13±0.06	0.96±0.51	0.02±0.01	304.2±88.5	8.5±3.6	18.4±5.2	129.9±141.2
	Rootstock trunk	3246±1282	0.17±0.12	0.01±0.01	0.16±0.08	0.99±0.39	0.04±0.02	379.6±354.4	16.5±10.6	20.4±19.2	13.3±8.9
	Excavated Roots	3213±994	0.77±0.22	0.04±0.01	0.27±0.15	1.18±0.19	0.06±0.01	314.3±119.0	16.7±2.9	10.0±1.0	12.3±2.1
60 DAFB leaves		- - -	4.46±0.49	0.40±0.10	2.05±0.19	1.06±0.22	0.50±0.16	70.2±13.8	34.4±7.4	21.5±6.2	47.0±8.0
120 DAFB leaves		- - -	3.44±0.25	0.23±0.03	2.21±0.25	1.54±0.41	0.63±0.17	83.5±12.7	43.7±7.7	10.4±2.8	28.4±4.9

Regarding nutrient concentrations, the values found in each material were compared to those found in leaves at 60 and 120 DAFB. The nutrient concentrations were quite similar in both cultivars. Flowers were rich in Fe, Cu and Zn, and relatively low in N, Ca and Mg. Thinned fruits were relatively low in N, Ca, Mg and Mn. The endocarp of the harvested fruits had low concentrations of all elements with the exception of K and Fe, whereas stones were low in all elements excepting Fe; mineral concentrations on a DW basis were quite similar in fruit endocarp and stones, with the exception of P and Ca that were lower and higher, respectively, in the stones than in the endocarp. Leaves of summer pruning material were relatively high in Ca and Fe (and Zn only in 'Catherina') and low in P and Cu (and Zn in 'Babygold5'), whereas the wood was low in N, P, K, Mg and Mn. Fallen leaves were rich in Ca, Fe and Mn and low in N, P, K and Zn. The one-year tissue in winter pruning has relatively high concentrations of Ca, Fe and Cu and low concentrations in N, P, K, Mg and Mn. The old wood from the winter pruning had lower nutrient concentrations than those found in the one-year old tissues.

In the immobilized tissues obtained through tree excavation, roots generally had similar element concentrations than those found in the rootstock part of the trunk and the rest of the permanent tree materials.

Amounts of nutrients in the removal events and permanent peach tree parts

Nutrient outputs in 'Calanda' peach cultivar

When considering the events where the largest amounts of nutrients were removed, in the case of 'Calanda' the largest amounts of N, P and Zn were removed at winter pruning (114, 17 and 0.5 g $tree^{-1}$, respectively) (Table 2A). The largest amounts of K, Ca, Mg (152, 302 and 38 g $tree^{-1}$), Fe and Mn (2 and 0.6 g $tree^{-1}$) were lost at leaf fall, and that of Cu (0.2 g $tree^{-1}$) was lost at summer pruning.

When considering the most abundant element at each removal event, N was the most abundant one in flowers and fruit thinning materials (1 and 9 g $tree^{-1}$, respectively), whereas K was the more abundant in the harvested fruits (132 g $tree^{-1}$), and Ca was the more abundant in summer pruning, fallen leaves and winter pruning materials (66, 302 and 134 g $tree^{-1}$ respectively) (Table 2A). In the case of microelements, the most abundant one was Fe in fruit thinning, harvested fruits, summer pruning, fallen leaves and winter pruning materials (37, 752, 332, 2011 and 932 mg $tree^{-1}$), whereas Cu was

the more abundant in flowers (40 mg DW/tree). The less abundant macroelement was Mg in flowers, fruit thinning, harvested fruits and winter pruning materials (<1, <1, 9 and 17 g tree^{-1}), whereas P was the less abundant in summer pruning and fallen leaves materials (8 and 9 g tree^{-1}, respectively). Manganese was the less abundant microelement in all tissues (5, 67, 52 and 125 g tree^{-1} in fruit thinning, harvested fruits, summer pruning and winter pruning materials, respectively), except in flowers and fallen leaves, where Zn was the less abundant (3 and 159 mg tree^{-1}).

Regarding total annual nutrient outputs, 'Calanda' peach trees lose 340, 53, 425, 518 and 74 g tree^{-1} of N, P, K, Ca and Mg, respectively (corresponding to 5.6, 0.9, 7.0, 8.6 and 1.2 g kg^{-1} fruit, respectively) (Table 2A). Also, annual nutrient outputs include Fe, Mn, Cu and Zn losses of 4.1, 0.8, 0.8 and 0.9 g tree^{-1}, respectively (corresponding to 67, 14, 14 and 15 mg kg fruit^{-1}, respectively).

The results of the tree excavation indicate that branches contain the largest part of most elements in the tree in winter (Table 2A). Nitrogen, however, is an exception and the largest amount is in the root system. When considering the amount of nutrients lost and those stored in permanent parts (taking into account the age of the trees), the annual nutrient requirements will be 354, 59, 441, 575 and 78 g tree^{-1} for N, P, K, Ca and Mg, respectively (corresponding to 5.9, 1.0, 7.3, 9.5 and 1.3 g kg fruit^{-1}, respectively) (Table 2). Also, micronutrient requirements will be 5.2, 0.9, 1.0 and 1.5 g tree^{-1} of Fe, Mn, Cu and Zn, respectively (corresponding to 86, 15, 17 and 24 mg kg fruit^{-1}, respectively).

Nutrient outputs in 'Catherina' and 'Babygold5' peach cultivars

When considering the events where the largest amounts of nutrients were removed, the largest amounts of P and Zn were removed (in 'Catherina'/'Babygold5') at winter pruning (3/3 g tree^{-1} and 86/82 mg tree^{-1}, respectively) (Table 2B-C). The largest amounts of Ca and Mg (73/80, 12/13 g tree^{-1}), Fe and Mn (573/494 and 136/142 mg tree^{-1}) were lost at leaf fall. Differences between 'Catherina' and 'Babygold5' include: N, where that the largest amount was removed in winter pruning (31 g tree^{-1}) in 'Catherina' and in fallen leaves (39 g tree^{-1}) in 'Babygold5'; Cu, where the largest amount was removed in fallen leaves (0.6 g tree^{-1}) in 'Catherina' and in winter pruning (0.1 g tree^{-1}) in 'Babygold5'; and K, where the largest amount was removed in fruit harvest (32 g tree^{-1}) in 'Catherina' and in leaf fall (29 mg tree^{-1}) in 'Babygold5'.

When considering the most abundant element at each removal event, N was the more abundant one in flowers and fruit thinning materials (1/1 and 4/4 g tree^{-1} respectively, in 'Catherina'/'Babygold5'), whereas K was the more abundant in the harvested fruits (32/28 g tree^{-1}), and Ca was the more abundant in summer pruning, fallen leaves and winter pruning materials (19/13, 73/80 and 55/45 g tree^{-1} respectively) (Table 2B-C). In the case of microelements, the most abundant one was Fe in all events (7/5, 12/11, 112/124, 93/61, 573/494 and 298/241 mg tree^{-1} in flowers, fruit thinning, harvested fruits, summer pruning, fallen leaves and winter pruning materials, respectively). The less abundant macroelement was Mg in flowers and fruit thinning (in all these cases <1 g tree^{-1}), whereas P was the less abundant in summer pruning, fallen leaves and winter pruning materials (1/1, 2/2 and 3/3 g tree^{-1}, respectively). The only difference is in fruit harvest, where Ca was the less abundant in 'Catherina' (2 g tree^{-1}), whereas in 'Babygold5' the less abundant was Mg (2 g tree^{-1}). Manganese was the less abundant microelement in all tissues (1/1, 2/2, 16/16 and 48/37 mg tree^{-1} in flowers, fruit thinning, harvested fruits and winter pruning materials, respectively), except in summer pruning, where the less abundant was Cu (16/8 mg tree^{-1}) and fallen leaves, where the less abundant was Zn (30/31 mg tree^{-1}).

Regarding total annual nutrient outputs, 'Catherina' and 'Babygold5' peach trees lose 103/98, 10/9, 104/89, 149/141 and 21/21 g tree^{-1} of N, P, K, Ca and Mg, respectively, respectively (corresponding to 7.9/7.3, 0.8/0.7, 7.8/6.7, 11.5/10.6 and 1.6/1.5 g kg fruit^{-1}, respectively) (Table 2B-C). Also, nutrient outputs include Fe, Mn, Cu and Zn losses of 1.1/0.9, 0.2/0.2, 0.2/0.2 and 0.2/0.2 g tree^{-1}, respectively (corresponding to 87/70, 18/16, 14/16 and 13/11 mg kg fruit^{-1}, respectively).

The results of the tree excavation indicate that branches contain the largest part of all elements in the tree in winter (Table 2B-C). When considering the amount of nutrients lost and those stored in permanent parts (taking into account the age of the trees), the annual nutrient requirements will be 110/108, 12/10, 109/99, 170/172 and 22/22 g tree^{-1} for N, P, K, Ca and Mg, respectively (corresponding to 8.5/8.1, 0.9/0.7, 8.4/7.4, 13.1/12.9 and 1.7/1.7 g kg fruit^{-1}, respectively) (Table 2B-C). Also, micronutrient requirements will be 1.7/2.6, 0.3/0.3, 0.3/0.3 and 0.3/0.4 g tree^{-1} of Fe, Mn, Cu and Zn, respectively (corresponding to 133/194, 20/19, 20/25 and 21/30 mg kg fruit^{-1}, respectively).

Nutrient requirements breakdown considering the different events during the season

A detailed event-associated breakdown of the total nutrient requirements is shown in Figs. 1.3 and 1.4 for macro- and micronutrients, respectively (these Figures do not include nutrients stored in permanent tree parts).

Relatively mobile macronutrients such as N, P and K were mainly lost (in % of the total, for N/P/K) in leaf fall (25-40/18-26/27-36), fruit harvest (16-21/24-33/31-32) and winter pruning (28-33/30-32/19-26), with summer pruning accounting for a smaller portion (12-18/9-15/10-12). On the other hand, relatively immobile macronutrients such as Ca and Mg were mainly lost (in % of the total, for Ca/Mg) in fallen leaves (49-58/51-64), with winter and summer pruning accounting for smaller portions (26-37/16-22 and 9-13/11-14, respectively). Fruit harvest accounted for 8-12% of the total Mg and only for 1-3% of the total Ca. Fruit thinning and flower abscission accounted for small but still measurable portions of the total (1-10 and 1%, respectively).

The relatively immobile micronutrients Fe and Mn were mainly lost (in % of the total, for Fe and Mn) in leaf fall (37-53/58-69) and winter pruning (25-33/15-20), with fruit harvest and summer pruning accounting for smaller portions (13-19/7-8 and 7-9/7-13, respectively). In the case of the relatively mobile metals Cu and Zn, the largest loss event (in % of the total, for Cu and Zn) was winter pruning (26-64/51-54), with the second one being leaf fall (12-26/18-20). Fruit harvest and summer pruning accounted for smaller portions (10-12/11-14 and 4-29/10-15, respectively). Fruit thinning and flower abscission accounted for small but still measurable portions of the total (1-4 and 1-5%, respectively). It should be taken into account that both Cu and Zn are usually added as agrochemicals in the area.

Prediction of the biomass dry weight removed in different events in 'Calanda' peach cultivar from the assessment of trunk circumference area

Previous studies have reported the possibility of using the trunk circumference area to build models for estimating tree size, growth or potential yield (Kim et al. 2003, Lakso and Johnson 1990, Miranda and Royo 2003a, b, 2004, Miranda et al. 2008, Santesteban et al. 2008). We studied the relationships between a parameter that can be measured easily (and non destructively) at the beginning of the active period, such as trunk sectional area (TCA), and the biomass lost in the different nutrient removal events, using the stepwise multiple regression method described in El-Jendoubi et al. (2011) (Neter et al. 1996; SAS Institute 1989). Using this approach, we found

statistically significant relationships only in two cases, fruit thinning and pruning wood. The best-fit regression equations obtained for the prediction of biomass DW were:

Biomass DW = 3.39 - 145.83*(1/TCA) (R^2 0.466; $p < 0.001$; $n = 54$) (Fruit thinning)

Biomass DW = 7950.81 + (7.83*10-6)*$(TCA)^3$ (R^2 0.178; $p < 0.003$; $n = 54$) (pruning wood)

The amounts of nutrient removal in these events may be estimated by multiplying the predicted biomass removal amount by the correspondent nutrient concentrations in the plant material in question. However, more work is necessary to further substantiate this approach.

Table 2. Amounts of macro- (in g tree^{-1} for N, P, Ca, Mg and K) and microelements (in mg tree^{-1} for Fe, Mn, Cu and Zn) lost in the removing events or fixed in the permanent structure. A: 'Calanda' cultivar peach trees.

Event	Material	N	P	K	Ca	Mg	Fe	Mn	Cu	Zn
Flower abscission	Flowers	1.4±0.9	0.2±0.1	1.1±0.8	0.4±0.2	0.1±0.1	10.8±6.9	3.9±6.7	40.4±19.9	3.4±2.2
Fruit thinning	Fruits	8.9±5.8	1.3±0.9	7.8±78.1	1.1±1.0	0.5±0.3	37.3±297.3	4.5±20.9	15.2±49.3	13.1±43.8
Fruit harvest	Fruits	53.3±32.4	15.5±8.7	122.4±4.0	10.5±9.7	5.6±3.1	642.8±50.0	45.2±8.4	82.5±9.0	70.5±15.6
	Stones	16.4±12.4	1.9±1.2	9.2±5.1	4.5±1.3	3.1±1.1	109.0±25.3	22.0±2.9	15.7±16.4	27.9±9.9
Summer pruning	Leaves	54.4±23.1	5.2±2.2	44.4±20.3	56.5±23.4	9.7±3.9	296.8±128.6	46.0±18.5	227.1±137.5	67.3±28.3
	One-year old wood	5.5±2.8	2.5±1.1	7.4±4.8	9.5±3.8	0.7±0.3	34.9±12.6	6.1±2.7	17.6±8.1	57.2±23.0
Fallen leaves	Leaves	86.2±34.1	9.4±2.6	152.0±52.4	302.2±84.3	38.1±7.4	2011.1±740.3	567.7±367.1	211.5±116.5	159.2±55.0
Winter pruning	One-year old wood	114.2±79.2	17.3±9.8	80.4±69.1	133.6±56.8	16.7±9.7	931.6±564.7	125.4±50.4	213.8±120.0	476.8±346.4
Tree removal	Old wood	55.6±49.1	21.7±11.7	54.1±30.3	346.7±167.0	15.2±3.8	5989.5±2619.1	274.7±92.8	1521.3±1075.5	1681.9±1603.3
	Trunk old wood	21.6±16.1	3.7±1.5	12.6±6.1	92.4±20.0	3.4±1.2	1888.8±561.8	77.6±15.3	387.3±121.9	570.5±522.7
	One-year old wood	3.4±2.3	2.6±1.5	10.0±6.0	15.2±8.7	1.3±0.7	216.9±152.4	23.7±13.7	23.5±14.0	410.9±342.8
	Rootstock trunk	23.4±13.3	6.5±5.8	10.8±9.0	81.9±29.7	4.0±2.7	1782.5±1152.2	117.2±122.4	281.8±310.4	95.0±49.0
	Excavated roots	59.3±30.9	18.3±8.1	15.8±10.8	96.6±64.9	9.2±6.4	3579.4±3692.6	209.3±279.0	119.1±80.9	183.2±158.2
Total output in events [E]		340.2	53.3	424.6	518.2	74.4	4074.4	820.9	823.7	875.2
Immobilized [I]		14.0 (*4%)*	6.0 (*10%)*	56.4 (*10%*)	3.4 (*4%*)	16.2 (*4%*)	1099.6 (*21%*)	69.0 (*8%*)	177.5 (*18%*)	579.6 (*40%*)
I + E		354.2	59.3	440.8	574.5	77.8	5174.0	889.8	1001.2	1454.8
E /kg DW fruit		5.6	0.9	7.03	8.6	1.2	67.4	13.6	13.6	14.5
E+I /kg DW fruit		5.9	1.0	7.3	9.5	1.3	85.6	14.7	16.6	24.1

Table 2 (Contn.). B: ‘Catherina’ cultivar peach trees.

Event	Material	N	P	Ca	Mg	K	Fe	Mn	Cu	Zn
Flower abscission	Flowers	0.9±0.7	0.1±0.1	0.2±0.2	0.1±0.1	0.6±0.5	7.4±6.4	1.2±0.9	3.9±2.7	1.7±1.5
Fruit thinning	Fruits	4.1±3.2	0.5±0.4	0.4±0.5	0.2±0.1	3.2±2.5	12.4±9.8	2.2±1.6	3.3±2.7	4.7±3.4
Fruit harvest	Fruits	16.6±16.1	2.7±2.6	1.3±1.2	1.5±1.2	28.1±22.3	107.5±84.1	11.7±9.7	17.7±12.6	16.0±13.5
	Stones	4.2±3.6	0.3±0.3	0.4±0.4	0.5±0.4	3.9±3.3	4.2±29.5	4.2±3.5	4.4±3.8	4.7±4.0
Summer pruning	Leaves	11.5±8.4	0.7±0.5	8.5±5.5	1.9±1.2	8.8±6.9	47.1±30.7	20.8±14.2	4.0±2.6	7.2±5.2
	One-year old wood	6.2±11.1	0.6±0.9	10.8±14.8	0.7±1.1	3.8±5.2	46.0±82.4	9.8±15.9	12.1±20.0	18.9±29.8
Fallen leaves	Leaves	28.5±21.2	2.1±2.1	73.1±84.6	11.8±13.0	28.2±34.0	572.8±744.7	135.8±150.8	560.2±13.6	30.0±35.0
Winter pruning	Old wood	1.0±0.6	0.3±0.3	2.3±1.0	0.3±0.2	1.3±1.1	15.1±6.5	4.5±3.2	12.3±5.9	8.0±3.8
	One-year old wood	29.9±24.2	2.9±2.2	52.3±40.4	3.6±2.8	25.7±22.8	282.6±243.0	43.2±34.8	106.4±93.4	78.3±60.8
Tree removal	Old wood	29.3±24.6	10.7±10.7	127.1±44.5	5.8±1.5	23.8±8.1	3595.2±2248.3	154.6±36.6	675.0±361.0	853.0±706.0
	Trunk old wood	4.6±2.6	1.0±0.9	31.1±14.3	1.2±0.4	5.0±2.8	1343.6±895.1	47.7±22.9	78.1±22.2	92.6±122.4
	One-year old wood	2.5±2.5	0.3±0.3	4.6±3.9	0.4±0.3	2.2±1.9	101.9±81.6	5.1±4.1	10.2±11.0	31.4±28.7
	Rootstock trunk	15.3±10.5	2.4±2.5	56.5±19.0	2.8±2.4	12.1±8.1	1792.0±1166.8	76.0±45.7	88.5±20.6	54.7±21.2
	Excavated roots	21.8±7.6	2.0±0.7	30.6±15.5	2.9±1.0	8.4±2.9	714.2±329.7	38.7±15.7	78.9±61.1	43.3±30.7
Total output in events [E]		102.8	10.1	149.3	20.5	103.5	1126.4	233.4	185.4	169.4
Immobilized [I]		7.2 *(7%)*	1.4 *(12%)*	20.9 *(12%)*	1.2 *(6%)*	5.5 *(5%)*	598.2 *(35%)*	26.2 *(10%)*	71.6 (28%)	101.0 *(37%)*
I + E		110.0	11.5	170.2	21.7	109.0	1724.6	259.6	256.9	270.4
E /kg DW fruit		7.9	0.8	11.5	1.6	78.0	86.9	18.0	14.3	13.1
E+I /kg DW fruit		8.5	0.9	13.1	1.7	8.4	133.1	20.0	19.8	20.9

Table 2 (Contn.). C: 'Babygold5' cultivar peach trees.

Event	Material	N	P	Ca	Mg	K	Fe	Mn	Cu	Zn
Flower abscission	Flowers	0.6±0.4	0.1±0.1	0.2±0.2	0.1±0.1	0.4±0.3	5.0±3.7	0.8±0.6	3.5±2.9	1.1±0.8
Fruit thinnig	Fruits	3.5±2.9	0.9±2.9	0.3±0.3	0.2±0.2	3.0±2.6	10.7±9.1	1.8±1.5	3.0±2.6	4.5±3.7
Fruit harvest	Fruits	10.7±8.6	1.9±1.9	1.4±1.3	1.2±1.0	22.7±18.8	73.6±61.5	8.6±7.5	16.2±15.5	13.0±11.9
	Stones	4.6±3.1	0.2±0.1	1.7±1.3	0.6±0.4	5.0±3.2	50.2±37.0	7.5±9.1	5.7±3.7	7.9±5.1
Summer pruning	Leaves	10.0±3.4	0.6±0.2	8.2±4.0	1.9±0.8	7.4±3.3	44.0±23.6	16.1±6.8	3.3±1.3	6.5±2.1
	One-year old wood	2.0±1.4	0.3±0.2	4.5±3.0	0.4±0.3	1.6±1.3	17.3±9.3	3.4±2.2	4.3±2.4	8.6±5.2
Fallen leaves	Leaves	39.0±19.9	2.3±1.4	80.2±43.1	13.1±7.5	28.6±19.0	494.0±283.2	142.0±73.1	55.4±55.0	31.4±16.0
Winter pruning	Old wood	3.0±2.7	0.4±0.3	4.9±2.7	0.2±0.1	1.4±1.1	23.7±13.6	5.0±2.7	13.2±8.7	14.5±7.5
	One-year old wood	24.2±23.4	2.3±2.5	40.0±31.7	3.1±2.9	19.1±19.5	214.3±200.4	32.1±31.2	111.6±156.4	67.1±62.1
Tree removal	Old wood	36.0±29.8	3.5±2.1	172.4±66.2	3.9±2.0	40.6±17.3	16164.5±28532.4	217.6±173.3	1089.7±792.0	1274.9±1176.6
	Trunk old wood	3.3±3.7	0.4±0.1	23.9±11.4	0.6±0.9	3.3±1.2	790.9±200.6	21.6±7.5	47.9±11.9	333.5±348.4
	One-year old wood	6.3±0.8	0.5±0.1	12.3±1.1	0.9±0.1	6.0±1.1	347.1±110.9	11.4±0.6	37.5±5.3	135.4±54.5
	Rootstock trunk	6.0±5.6	0.3±0.4	33.7±21.3	1.3±0.9	5.5±4.0	1433.1±1570.7	60.2±50.6	74.5±84.7	47.4±42.0
	Excavated roots	22.8±3.4	1.3±0.3	39.1±17.1	1.9±0.3	7.6±3.7	1124.8±771.4	56.4±27.4	32.3±11.2	38.3±8.9
Total output in events [E]		97.5	9.0	141.3	20.5	89.1	932.7	217.3	216.1	154.6
Immobilized [I]		10.8*(10%)*	0.9 *(9%)*	30.2 *(18%)*	1.4 *(6%)*	9.8 *(10%)*	1648.0 *(64%)*	35.1 *(14%)*	120.4 *(36%)*	248.3 *(62%)*
I + E		108.3	9.9	171.5	21.9	98.9	2580.7	252.4	336.6	402.9
E /kg DW fruit		7.3	0.7	10.6	1.5	6.7	70.1	16.3	16.3	11.3
(E+I) /kg DW fruit		8.1	0.7	12.9	1.7	7.4	194.0	19.0	25.3	30.3

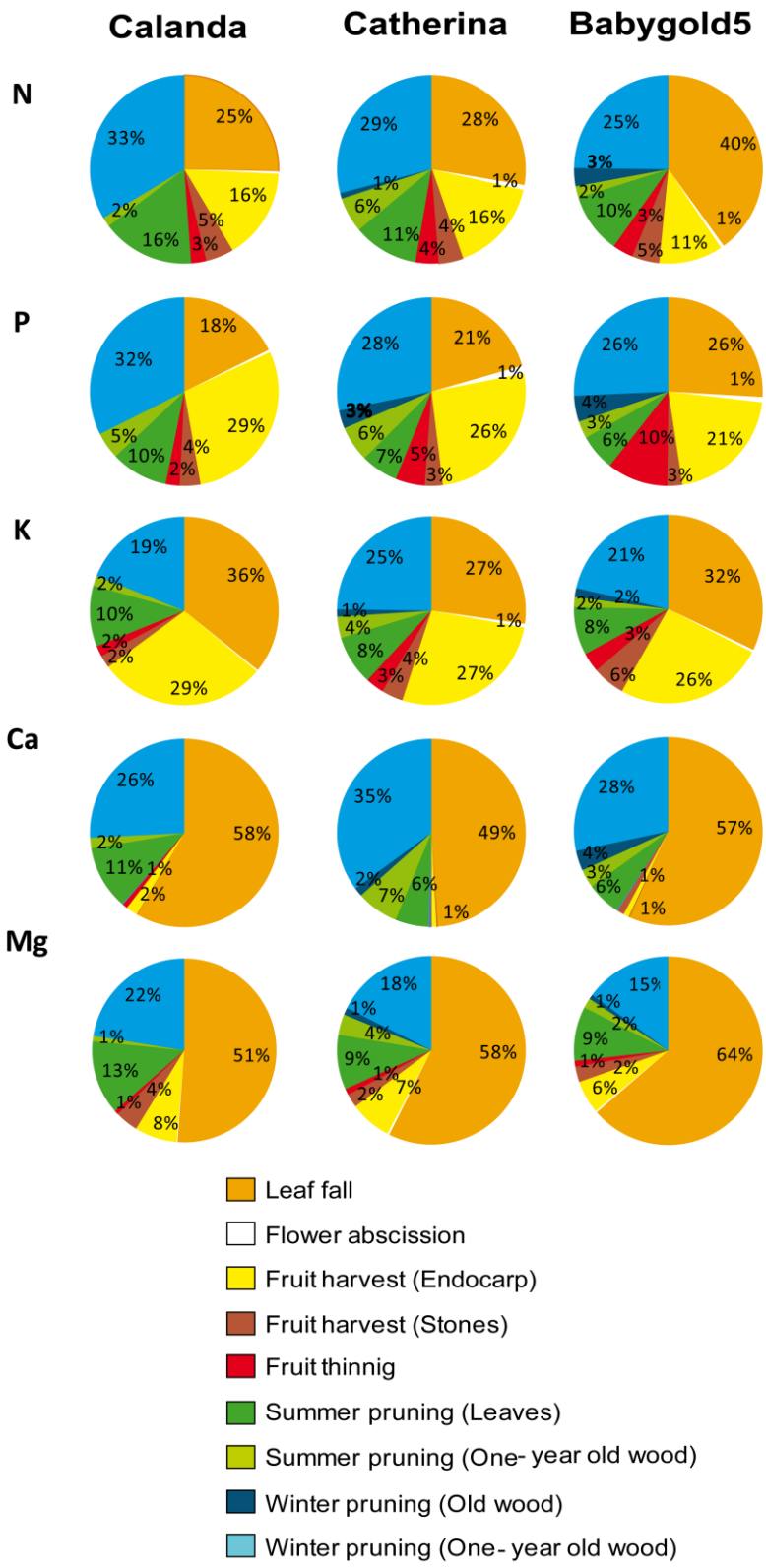


Figure 1.3. *Contribution of the different events along the season to the total macronutrient requirements.*

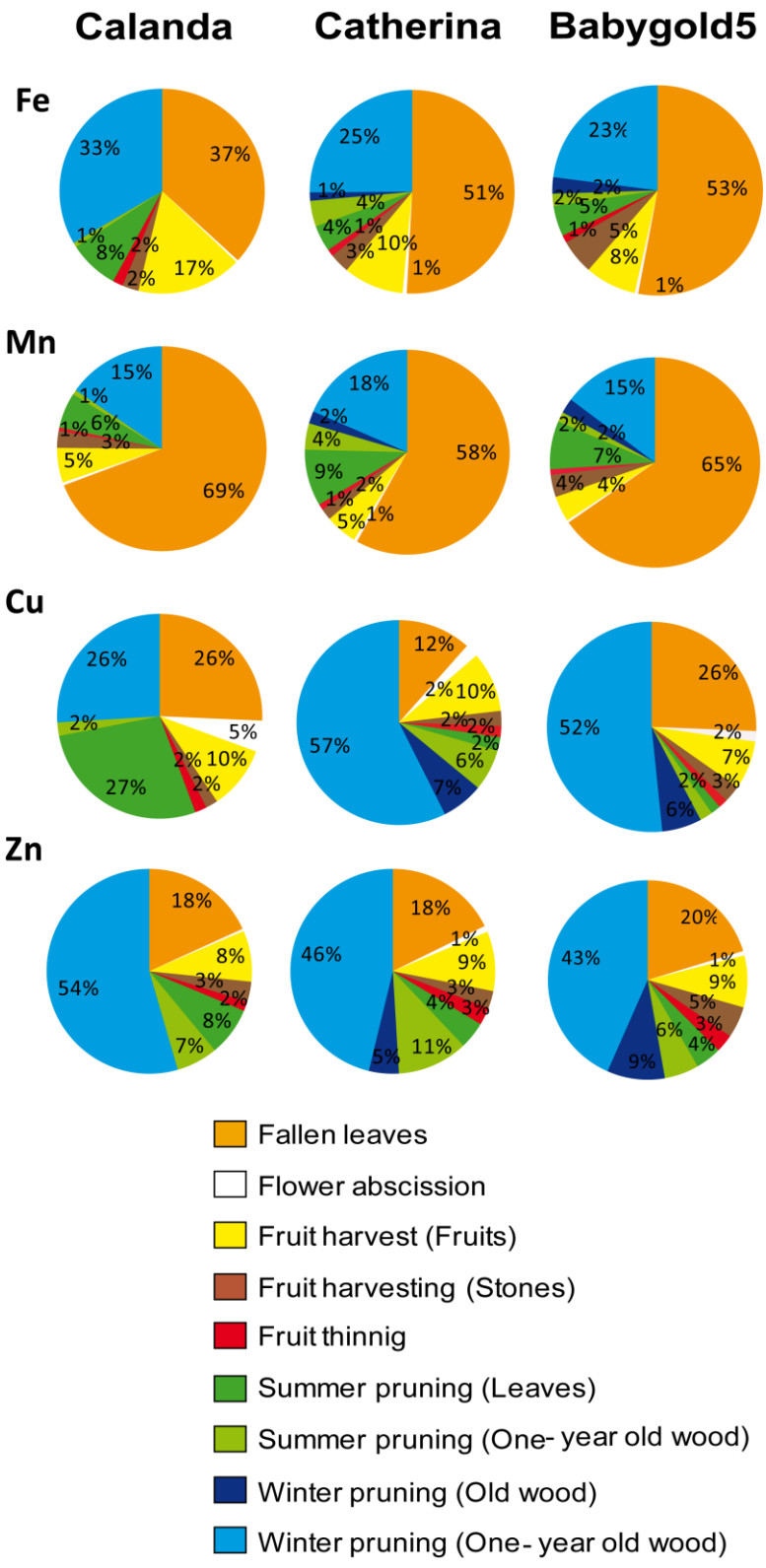


Figure 1.4. *Contribution of the different events along the season to the total micronutrient requirements.*

Discussion

This study provides a complete profile of the macro- and microelement requirements in bearing peach trees. The study covered three years of data obtained from three cultivars grown in two different orchards differing in tree density, management and yield. The weight of all materials lost by the trees during winter pruning, flower abscission, fruit thinning, summer pruning, fruit harvest and leaf fall were recorded, and the weight of permanent structures (roots, trunk and main branches) were also measured after full tree excavation. All tree tissues were analyzed for N, P, K, Ca, Mg, Fe, Mn, Cu and Zn, and the nutrient losses were calculated from the tissue weight and the corresponding elemental concentrations. As described in the Results, tissues sampled at the different natural and management events had peculiar nutrient compositions. There was little published information until now on the mineral composition of some of them, such as thinned fruits (relatively rich in Ca and Mg), stones in fruits, rootstock wood, etc., and data shown here could serve as a basis for further studies. On the other hand, the mineral concentrations of flowers and leaves were within the nutrient ranges reported by (Sanz et al. 1998, Belkhodja et al, 1998, Iguartua et al, 2000, El-Jendoubi et al, 2012). Also, macronutrient concentrations in 60 and 120 DAFB peach tree leaves are within the ranges proposed by Sanz et al, 1991 as reference values for the "Calanda" cv. nutritional diagnosis.

It is remarkable that the breakdown of the nutrient requirements was quite similar in the three peach tree cultivars used, in spite of the large differences in orchard yield and management (Figs. 1.3 and 1.4). Furthermore, each nutrient exhibited a characteristic "fingerprint" breakdown allocation pattern. Among macronutrients, the most striking differences were found for the relative contribution of fruits, which was largest for K, followed by P and N, being very small for Mg and especially for Ca. Another major component was the relative contribution of leaf fall, which was much larger for Mg and Ca than for K, N and P. Regarding micronutrients, fingerprint allocation patterns were also observed, with Mn, and to a lesser extent Fe, being largely lost in leaf fall. In the case of Fe, losses at winter pruning were also large, whereas fruits (including stones) also accounted for a significant part of the losses. Data for Cu and Zn are more difficult to interpret due to the possible presence of agrochemicals, but winter pruning was clearly an event where major losses occur.

Management events, including winter pruning, fruit thinning and summer pruning, accounted for a large part of the total nutrient requirements: approximately 43-54, 49, 33-40, 40-50, 28-37, 33-44, 23-34, 57-74 and 65-71% of the total in the cases of N, P, K, Ca, Mg, Fe, Mn, Cu and Zn, respectively. On the other hand, natural events, including flower abscission, fruit harvest, and leaf fall, accounted for 46-57, 51, 60-67, 50-60, 63-72, 56-67, 66-77, 26-43 and 29-35% of the total in the case of N, P, K, Ca, Mg, Fe, Cu and Zn, respectively. The large amounts of nutrients removed in management events underline how important is to use plant materials removed during management events for nutrient recycling and the improvement of soil fertility. Although pruning is a beneficial event for peach tree orchard management, it also leads to major nutrient losses and it could be useful to re-assess current pruning strategies under this viewpoint.

Most of the nutrients studied had similar time-course concentration patterns during the vegetative cycle for the three cultivars studied. The concentrations of N, P and Zn decreased continuously from the values found in 60 DAFB leaves to those of the leaves at fall, whereas those of Mg, Ca, Fe and Mn showed increases in the same period. For K and Cu the pattern was not common in the three cultivars studied. The decreases found in N, P and Zn concentrations can be attributed to their reallocation to the permanent tree structures at the end of the season. Approximately 70, 60 and 30-50% of the leaf P, K and N content in peach trees has been found to be transported to the permanent structures prior to leaf fall, whereas Ca and Mg were largely lost (Terblanche 1972, Stassen 1981, Stassen et al. 1981, Taylor and May 1967, Taylor and van DE 1970, (Carpena and Casero 1987, Heras et al. 1976, Montañes et al. 1990). The retranslocation of Fe induced by the natural leaf senescence in oak and beech plants was reported (Abadía et al. 1996). Also, the retranslocation of Fe has been shown to change depending on the plant species (Abadía et al. 1996, Rongli et al. 2011).

The estimation of the requirements on a tree basis is very useful for fertilization purposes. When calculated on a per tree basis, nutrient requirements are (in g tree^{-1}, for 'Calanda'/'Catherina'/'Babygold5') 340/103/98, 53/10/9/, 425/149/141, 518/21/21 and 74/104/89 and for N, P, K, Ca, and Mg, respectively. Concerning micronutrients, requirements are (in g tree^{-1}, for 'Calanda'/'Catherina'/'Babygold5'): 4.1/1.1/0.9, 0.8/0.2/0.2, 0.8/0.7/0.2, 0.9/0.1/0.2 for Fe, Mn, Cu and Zn, respectively. In previous studies, it has been assumed that Fe needs for peach tree are in the range 1-2 g tree^{-1}

(Abadía et al. 2004). In the case of soil-applied Fe chelates, a dose of approximately 50 g tree^{-1} of commercial product, equivalent to 3 g Fe tree^{-1} is quite common in developed peach orchards (Abadía et al. 2004).

The amounts of nutrients needed for fruit production (in kg t^{-1} of fruits) shown in this study are more accurate than those presented in previous studies where less loss-associated events had been considered. Requirements found were (in kg, for 'Calanda'/'Catherina'/'Babygold5'): 5.9/8.5/8.0 N, 1.0/0.9/0.7 P, 7.3/8.4/7.4 K, 9.5/13.1/12.9 Ca and 1.3/1.7/1.7 Mg (including nutrients stored in permanent tree structures), whereas previous studies indicated that requirements will be 3.8-5.6 N, 0.3-0.4 P, 3.2-4.4 K, 2.0-3.0 Ca and 0.7-0.8 Mg (in kg, for the cv. 'Kakamas'; Stassen et al. 2010). In these previous studies, losses associated to thinning, flower abscission and stones in fruits were not taken into account. Actually, the actual needs for the crop may be quite higher, and for instance it is normally accepted that ca. 30% of the applied N would not be available to the plant roots, due to leaching, volatilization and ineffective fertilizer placement (Stassen et al. 2010). Concerning micronutrients, requirements to produce 1 t of fruits are (in g, for 'Calanda'/'Catherina'/'Babygold5'): 86/133/194 Fe, 15/20/19 Mn, 17/61/25 Cu and 24/27/30 Zn. These values are also higher than those reported in the only studies reporting overall macronutrient requirements for peach trees, that indicate macronutrient requirements of 37 Fe, 8 Mn, 3 Cu and 14 Zn (in kg, for the cv. 'Kakamas'; Stassen et al. 2010).

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Prognosis of iron chlorosis in pear (*Pyrus communis* L.) and peach (*Prunus persica* L. Batsch) trees using bud, flower and leaf mineral concentrations

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REGULAR ARTICLE

Prognosis of iron chlorosis in pear (*Pyrus communis* L.) and peach (*Prunus persica* L. Batsch) trees using bud, flower and leaf mineral concentrations

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Abstract
Background and Aims The possibility of using tree materials in early phenological stages, such as dormant buds and flowers, for the prognosis of Fe deficiency occurring later in the year has been studied in peach and pear trees.
Methods Thirty-two peach trees and thirty pear trees with different Fe chlorosis degrees were sampled in different commercial orchards. In peach, samples included flower buds, vegetative buds, bud wood, flowers and leaves at 60 and 120 days after full bloom (DAFB). In pear, samples included buds, bud wood, flowers and leaves at 60 and 120 days DAFB. Leaf chlorophyll was assessed (SPAD) at 60 and 120 DAFB. Sampling was repeated for 3–5 years depending on the materials. Mineral nutrients measured were N, P, K, Ca, Mg, Fe, Mn, Zn and Cu.
Results The relationships between the nutrient concentrations in the different materials and leaf SPAD were assessed using four different statistical approaches: i) comparison of means depending on the chlorosis level, ii) correlation analysis, iii) principal component analysis, and iv) stepwise multiple regression. In all cases, significant associations between nutrients and SPAD were found. The best-fit multiple regression curves obtained for the multi-year data set provided good prediction in individual years.
Conclusions Results found indicate that it is possible to carry out the prognosis of Fe chlorosis using early materials such as buds and flowers. The relationships obtained were different from those obtained in previous studies using a single orchard. The different methods of analysis used provided complementary data.

Keywords Buds · Diagnosis · Flowers · Iron chlorosis · Nutrient concentrations · Prognosis

Abbreviations
SPAD Soil and plant analyzer development
DAFB Days after full bloom

Introduction

The mineral concentration of plant tissues is generally used by farmers to diagnose nutrient deficiencies,

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excesses or imbalances in crops (Chapman 1966; Bould et al. 1983; Marschner 1995). Also, changes in mineral nutrient concentrations are commonly accepted as a reliable guide for assessing the success of orchard fertilization programs (Brown and Kiyoto 1996; Basar 2006; Zuo and Zhang 2011). A renewed interest in new ways to diagnose and monitor plant nutrient status has arisen, based on the farmer's need to have an optimal crop nutrient supply in order to increase not only crop yield but also fruit quality (Brown and Kiyoto 1996; Gruhn et al. 2000; Cakmak 2002; Abadía et al. 2004; Zuo and Zhang 2011).

Iron deficiency is the most prevalent nutritional disorder in fruit tree crops growing in calcareous soils (Abadía et al. 2004), causing decreases in tree vegetative growth, a shortening of the orchard life span as well as losses in both fruit yield (Rombolà and Tagliavini 2006) and quality (Álvarez-Fernández et al. 2006, 2011). The material used more often for plant nutrient status monitoring is the leaf tissue. This is because leaf nutrient composition integrates many factors, from soil nutrient availability to plant uptake and distribution, and therefore reflects very often in an adequate manner the nutritional balance of the plant at the time of sampling (Pestana et al. 2003). The diagnosis of Fe deficiency in fruit tree species, conversely to what happens with other nutrient disorders, cannot be adequately carried out using leaf elemental composition, because Fe-deficient field-grown leaves often have Fe concentrations as high as those of Fe-sufficient leaves (this has been described as the "chorosis paradox"; Morales et al. 1998; Römheld 2000). This is likely associated to a preferential distribution of Fe in leaf areas close to the vascular system (Jiménez et al. 2009; Tomasi et al. 2009). Also, the leaf analysis approach may have a major problem when used in some fruit tree species, because recommended times for sampling are too late in the season for any subsequent corrective measure that can improve fruit yield and quality (Abadía et al. 2004; El-Jendoubi et al. 2011).

Therefore, methods alternative to leaf analysis have been proposed to prognose (diagnose in advance) Fe deficiency in fruit tree crops. For instance, the mineral composition of flowers has been used with this purpose in pear (Sanz et al. 1993), peach (Sanz and Montañés 1995; Sanz et al. 1997; Belkhodja et al. 1998; Igartua et al. 2000), apple (Sanz et al. 1998), nectarine (Toselli et al. 2000), olive (Bouranis et al. 1999), almond (Bouranis et al. 2001) and orange (Pestana et al. 2004) trees. Also, bark analysis has been used for Fe deficiency prognosis in peach trees (Karagiannidis et al. 2008). Other studies have proposed to use additional parameters such as nutrient ratios to assess the tree Fe nutrition status. For instance, the ratios K/Ca and P/Fe in leaves (Abadía et al. 1985, 1989; Köseoğlu 1995; Belkhodja et al. 1998) and K/Zn and Mg/Zn in flowers (Igartua et al. 2000; Pestana et al. 2004) have been used with this aim.

In this work, we have tested the hypothesis that tree materials occurring early in the season, such as dormant buds (in winter) and flowers (in late winter or early spring), could be used for the prognosis of Fe deficiency that occurs later in the growth season. With this aim we obtained a multi-year database of nutrient concentrations in dormant buds, flowers and leaves, from 32 peach trees and 30 pear trees growing in commercial orchards in the field and affected to different extents by Fe chlorosis. To assess Fe chlorosis, leaf chlorophyll was measured each year at two different dates during the season. The consistency across years of the relationships between nutrient concentrations and leaf chlorosis was assessed using four different statistical approaches: i) comparison of means depending on the chlorosis level, ii) correlation analysis, iii) principal component analysis, and iv) stepwise multiple regression.

Material and methods

Plant material

Forty-five peach trees (*Prunus persica* L. Batsch) and 45 pear (*Pyrus communis* L.) trees were selected in 2001 in 30 different commercial fruit orchards located in the Ebro river basin area, Northeastern Spain (see Online Resource 1 for the location of the orchards; no more than two trees per orchard were selected). This is a calcareous soils area where Fe chlorosis is widespread (Sanz et al. 1992). The only criterion for the selection of orchards was the presence of leaf Fe chlorosis symptoms in the summer of 2001. Since the orchards were privately owned, they were not experimentally controlled and both the orchard characteristics and management techniques were decided by the grower and were very diverse. At the

time of selection, trees ranged from fully green to severely Fe-deficient (Fe-chlorotic). Trees were then tagged and monitored over a period of up to 5 years. Results in this study are shown only for 32 and 30 trees in the case of peach and pear trees, respectively, because the rest of the trees did not survive, due to different causes, at the end of the multi-year experiment.

Materials sampled in each of the trees included different bud materials, as well as flowers and leaves. Several samples were taken per tissue from a given tree (leaves, flowers or bud materials), and then were mixed and homogenized to get one sample (approximately 1 g DW) per tissue type and tree. First, 100–150 bud samples per tree were taken in 1 year-old dormant shoots in winter (in mid December-mid January, around the tree crown and in the same position used for flower sampling, see below). In the case of peach trees, bud materials sampled were flower buds, vegetative buds and an adjacent bud wood sample that included the bud support (Fig. 1). In the case of pear trees, materials sampled were buds and also an adjacent wood sample that included the bud support. All bud materials were sampled at the same time and from the same shoots. In the case of peach trees, bud and bud wood samples were first collected separately from the apical and central parts of the shoot, to explore the possibility that the localization within the shoot may have an effect in mineral composition. Since no significant mineral concentration differences were found in materials taken in the apical and central parts of the shoot (data not shown), data are presented on a whole shoot basis. Afterwards, 60 whole flowers per tree (including petals, sepals, reproductive parts, bracts and peduncles) were taken at full bloom (in early March for peach and late March-early April for pear). Flowers were sampled from the central part of the shoots around the tree crown (30 in the upper part and 30 in the lower part of the crown; Belkhodja et al. 1998; Igartua et al. 2000). Finally, 30–50 leaves per tree were sampled (fully developed leaves, 4th–6th from the top in the distal third of the current year's growth; Belkhodja et al. 1998) 60 and 120 days after full bloom (DAFB), in May and July. Wood, flower and leaf samples were taken during five consecutive growth seasons (2001–2002 to 2005–2006), with the exception of pear tree bud wood samples, which were taken only in three consecutive growth seasons (2003–2004 to 2005–2006). Bud samples were taken for three consecutive growth seasons in peach trees (2001–2002 to 2003–2004), and only in the 2003–2004 growth season in pear trees.

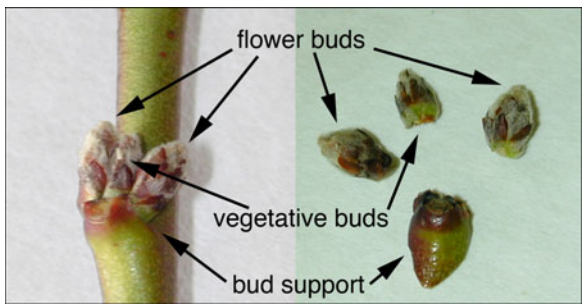

Fig. 1 Peach tree buds, showing the vegetative and flower buds as well as the adjacent bud wood sample that included the bud support

Leaf chlorophyll estimation

The leaf chlorophyll concentration per area was estimated in the field by using a SPAD 502 meter (Minolta Co., Osaka, Japan). Measurements were made at 60 and 120 DAFB in 30 leaves per tree all around the crown, and average values are referred to as SPAD60 and SPAD120, respectively. Leaves sampled were young, fully developed ones located in the position 4th–6th from the top (El-Jendoubi et al. 2011).

Mineral analysis

Samples were washed, mineralized and analyzed using standard procedures (Abadía et al. 1985; Igartua et al. 2000). Nitrogen and P were analyzed by the Dumas method and spectrophotometrically, respectively. Potassium was measured by flame emission spectroscopy, and Ca, Mg, Fe, Mn, Cu and Zn were measured by atomic absorption spectrophotometry. Results were expressed as % dry weight (DW) for macronutrients (N, P, K, Mg and Ca) and as mg kg^{-1} DW for micronutrients (Fe, Mn, Cu and Zn).

Statistical analysis

The relationships between nutrient concentrations in the different materials and SPAD were assessed using four different statistical approaches: i) comparison of means depending on the chlorosis level, ii) correlation analysis, iii) principal component analysis, and iv)

stepwise multiple regression. Differences in nutrient concentrations over the years were examined using analysis of variance, using PROC GLM of the SAS package (SAS Institute 1989). Duncan Multiple Range Test was used at $P \leq 0.05$ for the multiple mean comparison. For the evaluation of the possible relationships between nutrient concentrations and SPAD indexes, correlation and principal component analyses were carried out using PROC CORR and PROC PRINCOMP (SAS Institute 1989). To distinguish which nutrients contribute more than others to the explanation of the SPAD variance, multiple regression analyses were performed using PROC REG (SAS Institute 1989).

Results

Leaf chlorophyll concentrations in peach and pear trees

The trees included in the study had different leaf chlorosis levels due to the presence of Fe deficiency in the area. Iron deficiency is known to affect differently trees growing in the same orchard, leading to tree chlorosis heterogeneity (El-Jendoubi et al. 2011). Leaf SPAD values were measured at 60 (SPAD60) and 120 DAFB (SPAD120) in all trees, and SPAD values of those trees having the maximal (Fe-sufficient, green trees) and minimal values (Fe-deficient, chlorotic trees) in each of the 5 years of study are shown in Table 1. Maximal and minimal values were dependent on the year. In peach trees, minimal SPAD60 and SPAD120 values were in the ranges 12–18 and 9–15, respectively, whereas the corresponding values in pear trees were 12–19 in both sampling times. In peach trees, maximal SPAD60 and SPAD120 values were in the ranges 36–45 and 41–45, respectively, whereas the corresponding values in pear trees were 44–51 and 48–53, respectively.

For the nutrient mean comparison (see below) trees were assigned to three different chlorosis categories, i.e., markedly chlorotic, moderately chlorotic and green (each category composed of 10–11 trees), using the 5-year averaged SPAD120 value for each tree (Fig. 2). These three categories are representative of the range of values found in the region for these crops. During the 5-year study period, the average SPAD value for each of the three tree categories established was quite stable both in peach and in pear trees (Fig. 2).

Table 1 Maximal and minimal SPAD values observed in the peach and pear tree orchards during the 5 years of study

Year	SPAD	Peach		Pear	
		Min	Max	Min	Max
2002	SPAD60	12.8	42.8	15.4	43.6
	SPAD120	8.5	40.8	15.9	48.0
2003	SPAD60	12.6	35.8	18.6	46.6
	SPAD120	9.6	41.8	18.8	47.7
2004	SPAD60	17.5	45.4	13.6	46.2
	SPAD120	15.4	41.1	19.0	50.2
2005	SPAD60	14.3	38.4	11.8	48.6
	SPAD120	15.2	41.6	11.8	53.2
2006	SPAD60	11.6	44.6	12.7	51.4
	SPAD120	12.8	45.0	11.5	52.4

Nutrient concentration ranges in peach tree buds, flowers and leaves

The overall mineral composition of the different peach tissues in the multi-year study is shown in Table 2. Values shown include maximal and minimal nutrient concentrations found in individual trees each

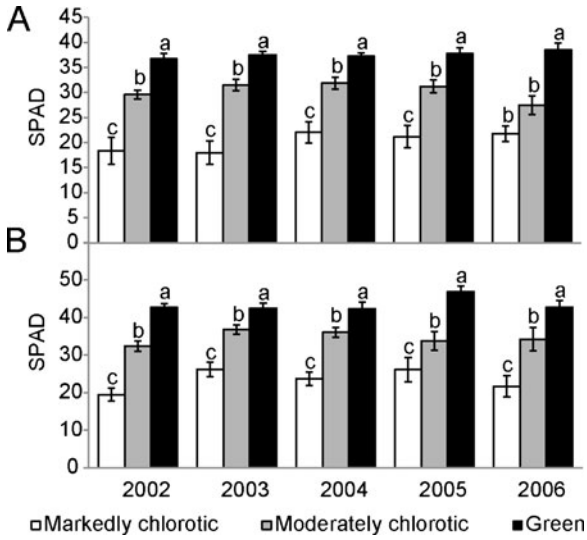

Fig. 2 SPAD values of three different peach (**a**) and pear tree (**b**) chlorosis categories in the different years of study (mean± SE; n=10–11). Trees were selected using the average SPAD120 value in all years

Table 2 Macro- (in % DW) and micronutrient (in mg kg^{-1} DW) concentrations in peach tree flower buds and vegetative buds (three growth seasons), bud wood, flowers, leaves at 60DAFB and leaves at 120DAFB (five growth seasons). Maximal and minimal concentrations found in individual trees each year, as well as average concentration values for all trees each year (in parenthesis; n=32 trees) are shown. Also, multi-year mean maximal and minimal concentrations and (in parenthesis) means±SD are shown in italics

Peach		N	P	K	Ca	Mg	Fe	Mn	Zn	Cu
Flower buds	2001	0.9–2.2 (1.4)	0.1–0.3 (0.2)	0.2–1.0 (0.7)	0.6–2.7 (1.6)	0.1–0.6 (0.3)	55.6–373.6 (197.1)	7.8–29.5 (17.9)	7.5–59.6 (29.6)	9.3–347.1(49.6)
	2002	1.0–2.6 (1.7)	0.1–0.3 (0.2)	0.2–0.8 (0.5)	0.9–2.3 (1.4)	0.2–0.4 (0.3)	71.6–197.0 (115.1)	3.0–38.4 (21.2)	14.7–59.3 (29.2)	21–310.7 (39.7)
	2003	1.1–2.7 (1.7)	0.1–0.3 (0.2)	0.6–0.9 (0.8)	1.1–6.5 (2.1)	0.3–0.5 (0.3)	81.2–490.9 (210.8)	7.5–39.9 (24.1)	17.9–71.7 (30.0)	8.8–882.4 (140.5)
	Means	*1.0–2.5 (1.6±0.3)*	*01.–0.3 (0.2±0.1)*	*0.3–0.9 (0.6±0.2)*	*0.9–3.8 (1.7±0.6)*	*0.2–0.5 (0.3±0.1)*	*69.5–353.9 (174.2±76.6)*	*6.1–35.9 (21.1±7.3)*	*13.3–63.5 (29.6±9.9)*	*13.3–513.4(76.6±143.2)*
Vegetative buds	2001	0.7–1.5 (1.1)	0.1–0.2 (0.1)	0.3–1.0 (0.6)	1.6–4.4 (2.7)	0.1–0.5 (0.3)	123.4–504.5 (276.3)	8.7–30.7 (18.2)	14.3–59.7 (29.4)	10.9–402.4 (47.0)
	2002	1.0–2.0 (1.4)	0.1–0.3 (0.1)	0.2–0.8 (0.4)	1.5–4.1 (2.3)	0.1–0.5 (0.3)	96.8–321.4 (169.5)	2.1–41.4 (22.7)	9.7–70.0 (26.2)	2.5–353.6 (38.3)
	2003	0.8–1.9 (1.2)	0.1–0.3 (0.2)	0.1–0.9 (0.6)	2.3–4.8 (3.4)	0.2–0.5 (0.3)	142.4–638.2 (296.6)	8.9–178.4 (31.2)	11.5–66.9 (30.3)	7.7–876.0 (125.0)
	Means	*0.8–1.8 (1.3±0.3)*	*0.1–0.3 (0.1±0.1)*	*0.2–0.9 (0.5±0.2)*	*1.8–4.4 (2.8±0.7)*	*0.1–0.5 (0.3±0.1)*	*120.9–488.0 (247.4±102.4)*	*6.6–83.5 (24.1±16.1)*	*11.8–65.5 (28.6±12.8)*	*7.0–544.0 (70.1±133.7)*
Bud wood	2001	1.2–2.5 (1.7)	0.1–0.3 (0.2)	0.6–1.7 (1.0)	2.9–6.5 (4.8)	0.1–1.0 (0.4)	116.2–307.7 (211.2)	11.1–30.0 (18.4)	13.8–158.4 (59.3)	7.7–212.2 (36.5)
	2002	1.4–3.0 (2.1)	0.1–0.4 (0.2)	0.2–1.1 (0.7)	2.5–6.3 (4.4)	0.2–0.7 (0.4)	90.1–209.0 (147.1)	7.6–32.0 (20.9)	12.9–130.3 (60.2)	2.8–126.6 (25.9)
	2003	1.4–2.9 (2.0)	0.1–0.3 (0.2)	0.6–1.5 (1.0)	3.4–7.2 (5.5)	0.3–0.7 (0.4)	107.6–368.3 (187.4)	8.1–37.5 (24.3)	12.2–126.1 (55.0)	7.9–687.4 (98.2)
	2004	1.6–3.1 (2.2)	0.10.3 (0.2)	0.6–1.4 (0.9)	2.6–5.6 (3.7)	0.2–0.6 (0.3)	96.9–227.7 (145.5)	7.5–31.5 (17.8)	22.6–86.31 (51.3)	10.2–473.6 (80.7)
	2005	1.4–2.7 (1.9)	0.1–0.2 (0.2)	0.7–1.3 (0.9)	2.5–6.6 (3.9)	0.1–0.5 (0.2)	30.3–913.3 (83.8)	3.2–24.8 (13.0)	18.7–80.1 (40.9)	6.0–108.6 (26.8)
	Means	*1.4–2.8 (2.0±0.4)*	*0.1–0.3 (0.2±0.1)*	*0.5–1.4 (0.9±0.2)*	*2.8–6.4 (4.4±1.1)*	*0.2–0.7 (0.4±0.1)*	*88.2–405.2 (155.0±82.9)*	*7.5–31.2 (18.8±6.9)*	*16.0–116.2 (53.3±29.0)*	*6.9–321.7 (53.6±98.3)*
Flowers	2002	2.1–3.1 (2.6)	0.1–0.4 (0.4)	0.5–2.6 (2.3)	0.2–0.9 (0.6)	0.1–0.2 (0.2)	75.8–436.4 (261.5)	7.3–42.4 (23.6)	16.5–69.6 (43.3)	19.8–846.4 (300.3)
	2003	2.1–3.3 (2.6)	0.3–0.5 (0.4)	1.4–2.3 (1.8)	0.4–0.9 (0.6)	0.2–0.4 (0.3)	138.1–427.2 (233.6)	3.7–47.5 (22.2)	31.6–65.2 (49.2)	21.9–596.8 (215.2)
	2004	2.0–2.8 (2.5)	0.3–0.5 (0.4)	1.6–3.3 (2.3)	0.6–1.2 (0.8)	0.2–0.4 (0.2)	80.7–265.7 (143.8)	16.9–42.1 (26.8)	26.0–176.7 (49.7)	10.1–712.2 (253.2)
	2005	2.3–3.2 (2.7)	0.3–0.5 (0.4)	1.3–2.3 (2.0)	0.5–0.8 (0.6)	0.2–0.3 (0.2)	83.5–233.5 (151.5)	9.1–41.9 (23.7)	23.2–170.5 (55.0)	25.9–657.2 (288.6)
	2006	2.3–3.5 (3.0)	0.3–0.5 (0.4)	1.4–2.2 (1.9)	0.4–0.8 (0.6)	0.2–0.3 (0.2)	45.2–222.5 (123.3)	6.3–40.6 (24.5)	31.8–48.4 (42.6)	17.2–777.2 (303.3)
	Means	*2.2–3.2 (2.7±0.3)*	*0.3–0.5 (0.4±0.1)*	*1.3–2.5 (2.0±0.3)*	*0.4–0.9 (0.7±0.1)*	*0.1–0.3 (0.2±0.1)*	*84.6–317.1 (182.7±80.1)*	*8.7–42.9 (24.2±7.5)*	*25.8–106.1 (47.9±22.0)*	*19.0–717.9(272.1±218.8)*
60 DAFB	2002	2.8–5.3 (3.8)	0.2–0.3 (0.3)	1.8–4.2 (2.7)	0.9–2.3 (1.5)	0.2–0.3 (0.3)	39.3–118.1 (70.5)	21.5–74.0 (41.1)	18.8–130.5 (45.4)	9.0–40.5 (18.2)
	2003	3.2–5.7 (4.2)	0.2–0.4 (0.3)	2.1–3.6 (2.6)	0.9–1.9 (1.3)	0.4–0.9 (0.5)	36.8–106.2 (55.7)	4.0–77.4 (28.5)	27.4–111.7 (44.9)	12.1–46.8 (23.1)
	2004	2.4–4.7 (3.7)	0.2–0.6 (0.4)	1.7–4.1 (2.7)	0.9–2.6 (1.7)	0.3–0.7 (0.4)	37.0–134.6 (72.9)	12.1–93.3 (41.8)	23.5–96.6 (55.5)	6.6–27.5 (14.0)
	2005	3.8–5.8 (4.5)	0.3–0.3 (0.3)	1.7–4.0 (2.7)	1.1–2.7 (1.8)	0.3–0.7 (0.5)	49.4–160.9 (94.6)	9.7–74.0 (37.9)	22.0–120.6 (41.7)	8.2–17.0 (12.6)
	2006	3.2–5.2 (4.1)	0.2–0.4 (0.3)	1.3–4.3 (2.5)	1.0–2.6 (1.9)	0.4–0.7 (0.5)	51.1–170.1 (85.8)	21.6–72.7 (40.0)	21.6–123.3 (45.6)	10.8–39.6 (16.1)
	Means	*3.1–5.3 (4.1±0.1)*	*0.2–0.4 (0.3±0.1)*	*1.7–4.0 (2.6±0.5)*	*1.0–2.4 (1.6±0.5)*	*0.3–0.7 (0.5±0.1)*	*42.7–138.0 (75.9±26.0)*	*13.8–78.3 (37.9±16.9)*	*22.7–116.5 (46.6±22.5)*	*9.3–34.3 (16.8±7.4)*
120 DAFB	2002	2.6–4.9 (3.7)	0.1–0.4 (0.2)	1.5–4.3 (2.5)	0.8–2.5 (1.6)	0.4–1.1 (0.6)	23.7–298.9 (89.4)	11.7–87.5 (34.2)	20.2–91.2 (35.0)	8.1–28.7 (15.6)
	2003	2.8–4.6 (3.5)	0.2–0.3 (0.2)	1.6–3.2 (2.2)	0.6–1.7 (1.1)	0.6–1.7 (1.1)	42.2–110.5 (73)	5.6–74.2 (30.7)	17.9–44.9 (29.3)	7.7–14.5 (10.8)
	2004	2.6–4.7 (3.5)	0.1–0.3 (0.2)	1.9–5.0 (3.3)	1.3–3.4 (2.0)	0.4–1.2 (0.7)	48.9–124.9 (86.5)	12.4–107.8 (46.7)	16.3–63.0 (30.0)	8.5–22.0 (15.1)
	2005	2.7–4.1 (3.3)	0.2–0.2 (0.2)	1.2–4.3 (2.4)	1.2–3.1 (1.8)	0.4–1.0 (0.6)	82.1–347.6 (126.3)	7.7–205.1 (47.9)	14.5–148.1 (30.5)	1.4–10.4 (3.2)
	2006	3.3–4.4 (3.9)	0.2–0.3 (0.2)	1.9–3.0 (2.3)	1.3–3.1 (2.0)	0.4–0.9 (0.6)	56.9–197.1 (110.1)	13.4–88.3 (39.7)	17.9–115.2 (30.7)	7.2–21.5 (11.1)
	Means	*2.8–4.5 (3.6±0.5)*	*0.2–0.3 (0.2±0.1)*	*1.6–4.0 (2.5±0.6)*	*1.1–2.8 (1.7±0.5)*	*0.4–1.2 (0.7±0.3)*	*50.8–215.8 (97.1±43.0)*	*10.2–112.6 (39.8±27.1)*	*17.4–92.5 (31.1±19.1)*	*6.6–19.4 (11.1±5.4)*

year as well as (in parenthesis) average values for all trees each year. Also, multi-year mean maximal and minimal values and (in parenthesis) means±SD are shown for each material (in italics in Table 2). Generally, differences between years were larger in the case of microelements than in the case of macroelements.

In peach tree flower buds, N, P, K, Ca and Mg concentration mean values were 1.6, 0.2, 0.6, 1.7 and 0.3%, respectively. Concentration averages for Fe, Mn, Zn and Cu were 174, 21, 30 and 77 mg kg^{-1}, respectively. In peach tree vegetative buds, N, P, K, Ca and Mg concentration averages were 1.3, 0.1, 0.5, 2.8 and 0.3%, respectively. Mean concentrations for Fe, Mn, Zn and Cu were 247, 24, 29 and 70 mg kg^{-1}, respectively. Therefore, vegetative buds had higher concentrations of Ca and Fe and lower concentrations of N than flower buds. In bud wood samples, N, P, K, Ca and Mg average concentrations were 2.0, 0.2, 0.9, 4.4 and 0.4%, respectively, whereas those for Fe, Mn, Zn and Cu were 155, 19, 53 and 54 mg kg^{-1}, respectively. Bud wood had the highest Ca and Zn concentrations found within the buds. The concentrations of Cu were high in some samples possibly because of common Cu-containing agrochemical treatments in the area.

In peach tree flowers, N, P, K, Ca and Mg concentration averages were 2.7, 0.4, 2.0, 0.7 and 0.2%, respectively. Concentration averages for Fe, Mn, Zn and Cu were 183, 24, 48 and 272 mg kg^{-1}, respectively. Flowers were, when compared to flower buds, markedly enriched in N, P, K, Mn and Cu, and had less Ca. Flower concentrations of Cu were also very high, possibly because of agrochemical treatments with Cu.

In peach tree leaves sampled at 60 DAFB, N, P, K, Ca and Mg concentration averages were 4.1, 0.3, 2.6, 1.6 and 0.5%, respectively. Concentration averages for Fe, Mn, Zn and Cu were 76, 38, 47 and 17 mg kg^{-1}, respectively. In the case of peach tree leaves at 120 DAFB, N, P, K, Ca and Mg concentration means were 3.6, 0.2, 2.5, 1.7 and 0.7%, respectively, whereas those for Fe, Mn, Zn and Cu were 97, 40, 31 and 11 mg kg^{-1}, respectively. Leaves at 60 DAFB had, when compared to vegetative buds, higher concentrations of N, K and Zn, and lower concentrations of Ca and Fe. Data show that marked decreases in N, P and Zn, and marked increases in Mg and Fe occurred in leaves from 60 to 120 DAFB.

Effects of the leaf chlorosis level in the nutrient concentrations of peach tree buds, flowers and leaves

The mineral composition of peach tree tissues affected by different degrees of chlorosis is shown in Online Resource 2. As indicated above, trees were separated into three categories, i.e., markedly chlorotic, moderately chlorotic and green (each composed of 10–11 trees), using the 5-year averaged SPAD120 value for each tree. Significant changes (at $P \leq 0.05$) between nutrient concentrations in peach trees with different degrees of chlorosis were found for several nutrients: chlorosis led to decreases in P (flowers and leaves at 60 DAFB), Cu (bud wood and flowers) and Zn (bud wood, flowers and leaves at 60 DAFB), and to increases in Mg (bud wood, flower and vegetative buds and flowers).

Nutrient concentration ranges in pear trees: buds, flowers and leaves

The mineral composition of pear tissues in the different years is shown in Table 3. In pear bud wood samples, N, P, K, Ca and Mg concentration averages were 1.3, 0.2, 1.1, 2.2 and 0.3%, respectively. Concentration averages for Fe, Mn, Zn and Cu were 106, 26, 36 and 65 mg kg^{-1}, respectively. In pear buds (data were obtained only in the season 2003–2004), N, P, K, Ca and Mg concentration averages were 1.0, 0.2, 0.7, 2.8 and 0.2%, whereas those for Fe, Mn, Zn and Cu were 161, 26, 43 and 155 mg kg^{-1}.

In pear flowers, N, P, K, Ca and Mg concentration averages were 3.2, 0.6, 2.4, 0.5 and 0.3%, respectively. Concentration averages for Fe, Mn, Zn and Cu were 116, 31, 56 and 123 mg kg^{-1}, respectively. Flowers had higher concentrations of N, P, K and Zn and lower concentrations of Ca when compared to buds.

In pear leaves sampled at 60 DAFB, N, P, K, Ca and Mg concentration averages were 2.4, 0.2, 1.6, 1.3 and 0.5%, respectively. Concentration averages for Fe, Mn, Zn and Cu were 89, 30, 33 and 46 mg kg^{-1}, respectively. At 120 DAFB, N, P, K, Ca and Mg concentration averages were 2.2, 0.2, 1.4, 1.7 and 0.6%, respectively, whereas those for Fe, Mn, Zn and Cu were 115, 34, 38 and 36 mg kg^{-1}, respectively. Data show no marked differences in nutrient concentrations between leaves sampled at 60 and 120 DAFB.

Table 3 Macro-(in % DW) and micronutrient (in mg kg^{-1} DW) concentrations in pear tree buds (one growth season), bud wood (four growth seasons), flowers, leaves at 60DAFB and leaves at 120DAFB (five growth seasons). Maximal and minimal concentrations found in individual trees each year, as well as average concentrations for all trees each year (in parenthesis; $n=30$ trees) are shown. Also, multi-year mean maximal and minimal concentrations and (in parenthesis) means±SD are shown in italics

		N	P	K	Ca	Mg	Fe	Mn	Zn	Cu
Bud wood	2003	0.1–1.6 (1.3)	0.1–0.3 (0.2)	0.4–1.4 (1.2)	1.2–3.8 (2.9)	0.1–0.5 (0.3)	57.2–200.7 (136.0)	10.4–55.1 (25.2)	13.681.7 (43.0)	17.1–418.2 (118.1)
	2004	0.9–1.7 (1.4)	0.1–0.2 (0.2)	0.4–1.7 (1.1)	1.4–2.9 (2.1)	0.2–0.5 (0.3)	78.8–240.8 (129.1)	18.8–60.4 (30.5)	11.1–98.3 (39.5)	19.2–347.1 (73.6)
	2005	1.1–1.5 (1.3)	0.1–0.2 (0.1)	0.8–2.0 (1.0)	1.1–3.2 (1.8)	0.1–0.4 (0.3)	21.6–128.2 (54.2)	8.5–28.1 (19.0)	18.7–44.5 (30.6)	13.2–141.0 (28.6)
	2006	0.7–1.3 (1.0)	0.2–0.2 (0.2)	0.8–1.4 (1.0)	1.3–2.5 (1.8)	0.2–0.5 (0.3)	44.5–171.2 (104.7)	17.2–47.2 (28.6)	20.1–49.4 (31.3)	15.0–178.5 (40.7)
	Means	*0.7–1.5 (1.3±0.2)*	*0.1–0.2 (0.2±0.2)*	*0.6–1.6 (1.1±0.2)*	*1.2–3.1 (2.2±0.6)*	*0.2–0.5 (0.3±0.1)*	*50.6–185.2 (106.0±45.9)*	*13.7–47.7 (25.8±9.3)*	*15.9–68.5 (36.1±16.5)*	*16.1–271.2 (65.3±86.0)*
Buds	2003	0.7–1.3 (1.0)	0.1–0.3 (0.2)	0.4–1.0 (0.7)	1.8–4.0 (2.8)	0.1–0.4 (0.2)	71.7–231.1 (160.7)	12.7–49.1 (26.2)	13.6–81.7 (42.6)	19.8–495.9 (155.1)
	Means	*0.7–1.3 (1.0±0.2)*	*0.1–0.3 (0.2±0.2)*	*0.4–1.0 (0.7±0.2)*	*1.8–4.0 (2.8±0.5)*	*0.1–0.4 (0.2±0.1)*	*71.7–231.1 (160.7±38.8)*	*12.7–49.1 (26.2±9.5)*	*13.6–81.7 (42.6±18.9)*	*19.8–495.9 (155.1±163.9)*
Flowers	2002	2.3–3.9 (3.1)	0.2–0.6 (0.5)	0.6–3.1 (2.5)	0.1–0.5 (0.3)	0.0–0.2 (0.2)	35.0–242.9 (112.5)	9.2–48.3 (30.0)	13.4–88.7 (51.7)	14.8–208.4 (70.3)
	2003	2.9–3.8 (3.3)	0.6–0.8 (0.7)	1.9–2.5 (2.2)	0.2–0.8 (0.4)	0.3–0.4 (0.3)	85.7–262.0 (160.5)	16.3–50.1 (32.1)	42.6–89.2 (61.7)	27.3–531.7 (131.7)
	2004	2.3–3.7 (2.8)	0.5–0.6 (0.6)	2.1–3.0 (2.8)	0.3–1.0 (0.5)	0.3–0.4 (0.3)	80.7–265.7 (143.8)	21.4–49.2 (30.1)	30.1–85.1 (53.7)	22.3–1107.8 (202.6)
	2005	2.8–4.0 (3.4)	0.5–0.7 (0.6)	2.0–2.6 (2.3)	0.3–0.9 (0.4)	0.3–0.4 (0.3)	40.6–170.2 (88.1)	19.3–51.6 (30.1)	38.1–79.1 (54.7)	21.9–206.8 (95.5)
	2006	2.3–4.2 (3.3)	0.5–0.7 (0.6)	1.9–2.6 (2.2)	0.3–1.1 (0.6)	0.2–0.4 (0.3)	36.2–205.4 (74.8)	21.9–46.4 (32.3)	43.8–85.5 (57.4)	21.5–781.7 (116.6)
	Means	*2.5–3.9 (3.2±0.4)*	*0.4–0.7 (0.6±0.6)*	*1.7–2.8 (2.4±0.3)*	*0.2–0.8 (0.5±0.2)*	*0.2–0.4 (0.3±0.1)*	*55.6–229.2 (116.0±52.8)*	*17.6–49.1 (30.8±7.4)*	*33.6–85.5 (55.8±13.5)*	*21.5–567.3 (123.3±167.7)*
60 DAFB	2002	1.5–3.0 (2.3)	0.1–0.3 (0.2)	1.2–2.8 (1.8)	0.6–1.8 (1.2)	0.2–0.3 (0.3)	29.7–142.9 (57.1)	5.4–78.8 (24.5)	16.8–135.8 (39.2)	5.1–493.5 (38.9)
	2003	1.2–3.2 (2.3)	0.1–0.2 (0.2)	1.1–2.2 (1.4)	0.8–2.0 (1.4)	0.4–0.7 (0.5)	51.0–161.1 (97.3)	4.7–77.4 (29.4)	13.5–126.1 (30.4)	5.7–147.5 (26.9)
	2004	1.4–3.2 (2.3)	0.1–0.2 (0.2)	1.0–2.2 (1.5)	0.7–1.7 (1.1)	0.3–0.6 (0.5)	42.8–97.7 (67.7)	13.3–45.0 (26.4)	16.6–85. (33.3)	7.0–25.7 (15.2)
	2005	1.3–3.6 (2.8)	0.1–0.3 (0.2)	1.1–2.3 (1.6)	0.8–2.3 (1.6)	0.4–0.6 (0.5)	60.6–166.8 (95.5)	17.8–102.8 (34.8)	16.4–139.9 (33.4)	9.5–309.7 (60.9)
	2006	1.4–3.2 (2.5)	0.20.3 (0.2)	1.0–2.2 (1.5)	0.7–2.4 (1.4)	0.2–0.7 (0.5)	65.3–361.7 (129.5)	9.8–58.9 (34.9)	17.9–45.3 (27.8)	9.0–626.1 (88.5)
	Means	*1.4–3.3 (2.4±0.4)*	*0.1–0.2 (0.2±0.2)*	*1.1–2.3 (1.6±0.3)*	*0.7–2.0 (1.3±0.4)*	*0.3–0.6 (0.5±0.1)*	*49.9–186.0 (89.4±42.6)*	*10.2–72.6 (30±15.8)*	*16.2–106.5(32.8±18.7)*	*7.3–320.5 (46.1±81.6)*
120 DAFB	2002	1.6–2.9 (2.4)	0.1–0.2 (0.2)	0.7–2.5 (1.3)	0.8–2.7 (1.2)	0.3–0.8 (0.6)	67.5–197.7 (104.3)	9.9–101.9 (35.1)	14.3–190.0 (47.5)	7.8–298.3 (34.6)
	2003	1.0–3.1 (2.3)	0.1–0.2 (0.2)	1.0–1.9 (1.4)	1.0–2.3 (1.5)	0.3–0.8 (0.5)	65.7–163.8 (110.2)	3.3–83.2 (28.5)	14.4–140.2 (34.1)	7.4–198.4 (25.3)
	2004	1.1–2.9 (2.1)	0.1–0.3 (0.2)	0.9–2.0 (1.5)	1.2–2.9 (1.7)	0.3–0.8 (0.6)	47.2–132.3 (84.5)	4.7–83.7 (32.6)	17.2–230.1 (55.6)	3.7–373.4 (27.1)
	2005	1.2–3.0 (2.3)	0.1–0.2 (0.2)	0.9–2.0 (1.4)	1.0–2.4 (1.8)	0.4–0.8 (0.5)	76.9–192.0 (131.0)	12.2–83.6 (31.2)	13.7–106.4 (27.8)	8.4–381.6 (44.2)
	2006	1.0–2.7 (2.1)	0.1–0.3 (0.2)	0.9–1.8 (1.2)	1.2–2.2 (1.6)	0.3–0.7 (0.5)	71.5–359.2 (143.9)	12.4–80.5 (41.5)	13.6–96.5 (23.8)	9.0–332.3 (50.9)
	Means	*1.2–2.5 (2.2±0.4)*	*0.1–0.4 (0.2±0.2)*	*0.9–1.9 (1.4±0.3)*	*1.0–2.5 (1.7±0.4)*	*0.3–0.5 (0.6±0.1)*	*65.8–207.9 (114.5±36.8)*	*8.5–86.6 (33.8±19.6)*	*14.6–85.6 (37.8±35.3)*	*7.3–316.8 (36.4±60.2)*

Effects of the leaf chlorosis level in the nutrient concentrations of pear tree buds, flowers and leaves

The mineral composition of pear tree tissues affected by different degrees of chlorosis is shown in Online Resource 3. As in the case of peach, trees were separated into three categories, i.e., markedly chlorotic, moderately chlorotic and green (each composed of 10 trees), using the 5-year averaged SPAD120 value for each tree. In pear trees with different degrees of chlorosis, significant changes (at $P \leq 0.05$) between nutrient concentrations were found for several nutrients: chlorosis led to decreases in N (leaves at 60 and 120 DAFB), P (leaves at 120 DAFB), Mg (flowers), Fe (flowers and leaves at 60 DAFB), Mn (buds, flowers and both types of leaves) and Zn (flowers), and to increases in Mg (leaves at 120 DAFB).

Correlations between nutrient concentrations and SPAD values

Correlation analysis was used as a preliminary exploration tool to assess the consistency across years of the relationships between nutrient concentrations and leaf SPAD values at both measuring dates. The coefficients of correlation (r values) and the corresponding statistical significances are shown for peach and pear, respectively, in Tables 4 and 5. In these Tables, combinations of nutrient concentrations and SPAD that have consistent relationships across years (significant correlations with the same sign and more than 50% of the years) are shaded in grey.

In peach, the relationships between the concentrations of some elements and leaf SPAD values were quite consistent for all materials (Table 4). The correlations between Mg and SPAD were generally negative and significant (at $P \leq 0.05$) in many cases, including early materials such as flower and vegetative buds, bud wood and flowers, and also in leaves at 120 DAFB. The correlations between Zn and SPAD values were generally positive and occur in many cases, including materials such as flower buds, bud wood, flowers and leaves. In the case of K, negative significant correlations occurred with SPAD, but mostly in late materials such as leaves at 60 and 120 DAFB. Positive correlations occurred between Ca concentrations and SPAD (bud wood and 60 DAFB leaves) and between P and SPAD (flower buds, bud wood and flowers). Iron and Cu were correlated with SPAD only in the case of leaves.

In pear, the correlation analysis revealed that Zn concentration was consistently and positively correlated with SPAD values in the case of bud wood and flowers (Table 5). Nitrogen concentration was correlated with SPAD positively in the case of both types of leaves. Manganese concentration, conversely to what occurs in peach, was positively correlated with SPAD in buds, flowers and both types of leaves. Also, Mg showed a negative correlation just in the case of 120 DAFB leaves. Calcium was positively correlated with SPAD in flowers. Finally, SPAD values were positively correlated with Fe only in the case of flowers and 60 DAFB leaves.

Principal component analysis of the mineral nutrient peach and pear databases

The correlation analysis revealed a consistent behavior of the two sets of trees across years (Tables 4 and 5). This was confirmed by the analysis of variance for each element analyzed, which confirmed in most cases rather small differences among years (not shown). Therefore, to obtain a more general perspective of the relationships of mineral nutrients and SPAD values, we made a principal component analysis per tissue and species, using the multi-year peach and pear tree datasets (Figs. 3 and 4, respectively). Nutrient concentrations, measured in the different plant parts each year, and SPAD indexes were included as variables. Since nutrient concentrations are generally not fully independent and show some degree of correlation among them, a principal component analysis is a practical way of extracting information from a large dataset. Principal components are newly derived variables, which account for the main dimensions of variability existing in the original database (Igartua et al. 2000).

In the case of the peach tree 5-year database, the first component explained between 24 and 29% of the total variance in the different plant materials (Fig. 3). The second component explained between 14 and 17% of the variance, with further components explaining less than 11%. Some patterns were consistent for all plant materials. Magnesium had always a negative load on the first component (X-axis in Fig. 3), conversely to SPAD120, SPAD60, Ca and Zn, which always had positive loads. Iron had a

Table 4 Correlations between peach tree nutrient concentrations in different tissues and SPAD values at 60 and 120 DAFB

	Year	SPAD	N	P	Ca	Mg	K	Fe	Mn	Cu	Zn
Flower buds	2001	SPAD60	-0.49**	0.20	0.25**	-0.39**	-0.15	0.04	0.01	0.06	0.01
		SPAD120	-0.47**	0.30*	0.26	-0.31**	-0.05	0.08	0.02	-0.10	0.04
	2002	SPAD60	0.02	0.67**	0.64**	-0.34**	0.16	0.01	0.09	0.34**	0.48**
		SPAD120	-0.09	0.50**	0.63**	-0.45**	-0.03	0.10	-0.06	0.26*	0.39**
	2003	SPAD60	0.47**	0.52**	0.21	-0.59**	0.07	-0.10	0.42**	0.31**	0.48**
		SPAD120	0.33**	0.13	0.24	-0.54**	-0.10	-0.08	0.27*	0.19	0.52**
Vegetative buds	2001	SPAD60	-0.24	0.30*	0.24	-0.35**	0.15	-0.26	-0.13	0.02	0.21
		SPAD120	-0.15	0.31*	0.27*	-0.35**	0.12	-0.15	-0.13	-0.15	0.24
	2002	SPAD60	0.09	0.24	0.07	-0.17	0.04	-0.31*	0.03	0.06	0.20
		SPAD120	0.22	0.27*	0.07	-0.17	0.09	-0.28*	-0.14	-0.10	0.07
	2003	SPAD60	0.34**	0.16	0.52**	-0.55**	-0.06	-0.26*	-0.01	0.26*	0.63**
		SPAD120	0.22	0.10	0.39**	-0.52**	-0.29*	-0.07	0.14	0.16	0.53**
Bud wood	2001	SPAD60	-0.32**	0.53**	0.35**	-0.32**	0.27*	-0.08	-0.04	0.09	0.39**
		SPAD120	-0.28*	0.42**	0.26*	-0.40**	0.14	0.01	-0.09	-0.09	0.46**
	2002	SPAD60	0.02	0.44**	0.44**	-0.37**	0.03	0.15	0.02	0.40**	0.61**
		SPAD120	0.21	0.41**	0.51**	-0.43**	-0.14	0.19	-0.19	0.33**	0.57**
	2003	SPAD60	0.16	0.12	0.60**	-0.54**	-0.36**	-0.22	0.31**	0.29*	0.67**
		SPAD120	0.03	0.04	0.41**	-0.36**	-0.33**	-0.22	0.24	0.18	0.53**
	2004	SPAD60	0.01	0.22	0.27	-0.40**	0.02	0.38**	0.00	0.14	0.11
		SPAD120	0.03	0.39**	0.24	-0.52**	0.23	0.46**	0.11	0.29	0.25
	2005	SPAD60	0.27	0.08	0.09	-0.54**	-0.03	0.12	0.06	0.23	-0.06
		SPAD120	0.41**	0.26	0.11	-0.32	0.13	0.33	0.04	0.28	0.06
Flowers	2002	SPAD60	0.14	0.35**	0.14	-0.29	-0.20	0.03	-0.19	-0.12	0.29
		SPAD120	0.25	0.35**	0.22	-0.38**	-0.21	0.01	-0.19	-0.04	0.47**
	2003	SPAD60	0.07	0.30	0.27	-0.47**	-0.55**	-0.20	-0.15	0.21	0.50**
		SPAD120	0.15	0.48**	0.47**	-0.50**	-0.44**	0.11	-0.06	0.24	0.63**
	2004	SPAD60	0.38**	0.64**	0.29	-0.31*	-0.13	0.18	0.19	0.15	0.25
		SPAD120	0.47**	0.53**	0.46**	-0.23	-0.03	0.34	0.17	0.20	0.36**
	2005	SPAD60	0.28	0.39**	0.31	-0.45**	-0.30	0.37	0.02	0.27	0.31
		SPAD120	0.37*	0.29	0.28	-0.47**	-0.30	0.18	0.02	0.40**	0.35*
	2006	SPAD60	0.10	0.31	0.64**	-0.42**	-0.36*	0.53**	0.12	0.51**	0.45**
		SPAD120	0.09	0.46**	0.34*	-0.43**	-0.11	-0.05	0.00	0.41**	0.49**
60 DAFB Leaves	2002	SPAD60	-0.40**	-0.19	0.64**	-0.10	-0.44**	0.58**	-0.22	0.44**	0.51**
		SPAD120	-0.47**	-0.18	0.54**	-0.28*	-0.33**	0.66**	-0.25	0.41**	0.49**
	2003	SPAD60	-0.30*	-0.09	0.43**	-0.56**	-0.41**	0.44**	-0.11	-0.05	0.02
		SPAD120	-0.10	0.12	0.20	-0.70**	-0.40**	0.35*	-0.08	-0.07	0.12
	2004	SPAD60	-0.03	0.16	0.64**	0.15	-0.20	0.36	0.44**	0.46**	0.65**
		SPAD120	-0.08	0.30	0.48**	0.07	-0.05	0.53**	0.29	0.51**	0.63**
	2005	SPAD60	-0.25	0.21	0.36**	0.00	-0.47**	0.39**	0.07	0.27	0.38**
		SPAD120	-0.18	0.24	0.18	-0.29	-0.10	0.39**	0.09	0.51**	0.45**
	2006	SPAD60	-0.42**	-0.41**	0.68**	-0.16	-0.29	0.58**	0.19	-0.03	0.34
		SPAD120	-0.21	-0.20	0.51**	-0.38*	0.10	0.36*	0.09	-0.12	0.53**
120 DAFB Leaves	2002	SPAD60	-0.25	0.00	0.20	-0.58**	-0.33**	0.21	-0.17	0.07	0.29*
		SPAD120	-0.42**	-0.26	0.58**	-0.43**	-0.55**	0.14	0.00	-0.12	0.25
	2003	SPAD60	-0.29	-0.01	0.33*	0.33*	-0.41**	0.32*	-0.06	0.14	0.45**
		SPAD120	-0.05	0.12	0.28	0.28	-0.47**	0.34*	-0.13	0.15	0.59**
	2004	SPAD60	0.05	0.23	0.66**	-0.21	-0.07	0.09	0.12	0.34*	0.38**
		SPAD120	-0.11	-0.16	0.23	-0.57**	-0.39**	0.34*	0.11	0.28	0.46**
	2005	SPAD60	-0.17	-0.14	0.19	-0.18	-0.59**	-0.02	0.33**	0.05	0.32
		SPAD120	-0.22	-0.30	0.19	-0.27	-0.45**	-0.21	0.44**	0.12	0.38**
	2006	SPAD60	0.35	0.13	0.23	-0.63**	-0.27	0.44**	0.34	0.12	0.30
		SPAD120	-0.17	-0.33	0.48**	-0.50**	-0.02	0.59**	0.41**	0.28	0.42**

**, * Significant at $P \leq 0.05$ and $P \leq 0.10$, respectively

positive load on this component except in the case of flowers. Potassium had low negative loads in the case of flowers and both types of leaves, whereas N had negative loads in the case of bud wood, vegetative buds and both leaf types. Phosphorus and Cu loads were negative only in the case of 120 DAFB leaves. Regarding the second component (*Y*-axis in Fig. 3), both SPAD120 and SPAD60 had negative loads, excepting the case of SPAD60 in 120 DAFB leaves. Most nutrients were generally in the positive part of the axis, excepting N in the case of bud wood, vegetative buds and flowers, Mg and Fe in leaves at 120 DAFB, Zn in vegetative buds, Cu in flower buds and leaves at 60 DAFB, and Mn in flower buds.

In the case of the multi-year pear database (five growth seasons for flowers and leaves, three seasons for bud wood and one for buds), the first component explained between 30 and 35% of the total variance, with a second component explaining between 15 and 28% of the variance (Fig. 4). In the case of pear, the pattern seems to be dependent on the plant material. For instance, both types of SPAD have low positive loads on the first component (*X*-axis in Fig. 4) for buds and bud wood, and higher positive loads in the case of flowers and both types of leaves. For other nutrients the loads were generally positive, except for N, Ca and Fe in the case of buds, K in the case of flowers and both types of leaves and Mg in the case of 120 DAFB leaves. Considering the second component (*Y*-axis in Fig. 4), the SPAD indexes had high positive loads in the case of bud wood and buds and low loads for the other materials. Magnesium had positive loads in the case of both leaf types and flowers, and K and P had positive loads in most materials, with the exception of bud wood and flowers (P). Nitrogen had positive loads also for all materials excepting flowers.

Regression analysis of the mineral nutrient peach and pear database

The correlation and principal component analysis described above revealed the existence of significant relationships between SPAD and mineral nutrients. Then, we used a stepwise multiple regression method to find the main nutrients responsible for changes in SPAD. In this stepwise method, all the variables already included in the model are re-assessed after a new variable is added, and any variable that is not statistically significant (using F values; at the SLSTAY=level) is removed. Only after this check is made and the necessary deletions are accomplished can a new variable be added. This stepwise process ends when none of the variables outside the model is statistically significant and every variable is statistically significant, or when the variable to be added is the one just deleted from it (Neter et al. 1996).

In peach trees, the contribution of every nutrient to the explanation of the variability of the SPAD values was assessed in the different tissues from the corresponding average partial determination coefficients (R^2) across years (*Y*-axis, Fig. 5). The average global determination coefficients (R^2) across years found by each stepwise regression is also shown in the insets in Fig. 5. In many cases, the same set of elements contributed to the explanation of SPAD values at both sampling dates. However, when comparing tissues, differences occurred both in the number of elements included in the stepwise regression and in the maximal R^2 values. The only common elements in the final regression step for all plant materials were Mg and Zn. The tissues that included more nutrients in the model were leaves at 60 and 120 DAFB; these plant materials also had the highest partial R^2 values, 0.26 for Ca in 60 DAFB leaves and 0.20 for Mg in 120 DAFB leaves. In vegetative buds, the only elements included in the model were Mg and Zn, with partial R^2 values of 0.078 and 0.134 in the case of SPAD60 and 0.082 and 0.091 in the case of SPAD120, respectively. On the other hand, Fe was found to contribute to the model only in the case of leaves at 60 and 120 DAFB, conversely to the correlations found previously between flower Fe and SPAD (Belkhodja et al. 1998; Igartua et al. 2000). Potassium was included in the model in the cases of leaves at 60 DAFB and flowers (with SPAD60), bud wood (with SPAD120) and in the case of leaves at 120 DAFB (with SPAD60 and SPAD120). In a previous study with a single orchard, a regression model including K and Zn explained approximately 28% of the changes in leaf chlorophyll concentration, and this relationship was quite constant across years (Igartua et al. 2000).

In peach trees, the average global coefficients of determination (R^2 in insets in Fig. 5) values were higher when using SPAD60 than when using SPAD120 in all materials, excepting in leaves at 120

Table 5 Correlations between pear tree nutrient concentrations in different tissues and SPAD values at 60 and 120 DAFB

	Year	SPAD	N	P	Ca	Mg	K	Fe	Mn	Cu	Zn
Buds		SPAD60	0.17	0.31	0.50**	-0.37	-0.02	-0.01	0.41**	0.31	0.12
		SPAD120	0.08	0.47**	0.39	-0.24	0.12	-0.13	0.50**	0.38*	0.18
Bud wood	2003	SPAD60	-0.10	0.22	0.15	-0.05	-0.05	-0.06	0.32	0.29	0.34*
		SPAD120	-0.22	0.27	0.15	-0.09	-0.11	-0.05	0.39	0.38	0.38*
	2004	SPAD60	0.39**	0.03	-0.01	0.07	-0.03	-0.05	-0.13	0.23	0.47**
		SPAD120	0.31	0.02	0.04	0.03	-0.17	-0.06	-0.08	0.05	0.50**
	2005	SPAD60	0.29	-0.06	0.08	-0.15	0.03	0.21	0.18	0.22	0.19
		SPAD120	0.53**	0.15	0.21	-0.01	0.05	0.34*	0.11	0.30	0.30
Flowers	2002	SPAD60	-0.11	0.40**	0.02	0.07	0.26	0.57**	0.57**	0.04	0.39**
		SPAD120	0.00	0.44**	0.10	0.09	0.26	0.50**	0.65**	-0.09	0.50**
	2003	SPAD60	0.18	0.29	0.25	0.13	-0.24	0.46**	0.36**	0.17	0.39**
		SPAD120	0.11	-0.19	0.41**	0.61**	-0.20	0.22	0.03	-0.10	0.11
	2004	SPAD60	0.38**	0.50**	0.38**	0.29	-0.03	0.43**	0.46**	0.01	0.71**
		SPAD120	0.23	0.33*	0.35*	0.27	-0.10	0.48**	0.34*	-0.01	0.61**
	2005	SPAD60	0.38**	0.05	0.43**	0.43**	-0.27	0.76*	0.38**	0.34*	0.47**
		SPAD120	0.49**	0.01	0.41**	0.35*	-0.39**	0.74*	0.44**	0.42**	0.38**
	2006	SPAD60	0.11	0.16	-0.01	-0.35*	-0.22	0.39	0.45**	0.21	0.53**
		SPAD120	0.07	0.13	0.01	-0.35*	-0.21	0.35	0.08	0.39*	0.37*
60 DAFB leaves	2002	SPAD60	0.32**	-0.03	0.16	-0.46**	-0.38**	0.43**	0.56**	0.15	0.24
		SPAD120	0.35**	0.04	0.26	-0.35**	-0.29*	0.47**	0.54**	0.22	0.23
	2003	SPAD60	0.63**	0.38**	0.19	-0.33*	-0.10	0.48**	0.51**	0.11	0.38**
		SPAD120	0.58**	0.36*	0.13	-0.41**	-0.05	0.25	0.53**	0.17	0.32*
	2004	SPAD60	0.48**	0.08	0.45**	-0.21	0.02	0.38**	0.44**	0.45**	0.21
		SPAD120	0.36*	-0.03	0.39**	-0.18	0.01	0.46**	0.41**	0.37**	0.20
	2005	SPAD60	0.57**	0.17	0.30	-0.23	-0.45**	0.59**	0.47**	0.30	0.18
		SPAD120	0.51**	0.23	0.18	-0.27	-0.41**	0.60**	0.50**	0.36*	0.21
	2006	SPAD60	0.45**	0.08	0.09	-0.40**	-0.40**	0.22	0.41**	0.41**	0.32
		SPAD120	0.42**	-0.03	0.01	-0.29	-0.43**	0.31	0.09	0.32	-0.02
120 DAFB leaves	2002	SPAD60	0.46**	0.14	0.05	-0.57**	-0.37**	0.40*	0.54**	0.14	0.18
		SPAD120	0.48**	0.14	0.21	-0.41**	-0.37**	0.41**	0.56**	0.21	0.31*
	2003	SPAD60	0.49**	0.43**	-0.10	-0.50**	-0.03	0.06	0.46**	0.18	0.12
		SPAD120	0.57**	0.35*	0.04	-0.46**	-0.06	0.12	0.54**	0.23	0.16
	2004	SPAD60	0.66**	0.37**	0.18	-0.61**	0.27	0.35*	0.39**	0.31*	0.34*
		SPAD120	0.57**	0.38**	0.15	-0.61**	0.12	0.47**	0.37**	0.30	0.36*
	2005	SPAD60	0.48**	0.15	0.20	-0.50**	-0.54**	0.09	0.33*	0.18	0.18
		SPAD120	0.54**	0.05	0.19	-0.48**	-0.56**	0.20	0.43**	0.28	0.19
	2006	SPAD60	0.32	0.31	-0.33*	-0.75**	-0.42**	0.07	0.36	0.46**	-0.19
		SPAD120	0.37*	0.10	-0.26	-0.62**	-0.62**	0.23	0.20	0.35*	-0.21

**, * Significant at $P \leq 0.05$ and $P \leq 0.10$, respectively

DAFB. The global R^2 values were (SPAD60/SPAD120): 0.555/0.468, 0.212/0.174, 0.368/0.274, 0.441/0.364, 0.658/0.466 and 0.572/0.628 for flower buds, vegetative buds, bud wood, flowers, leaves at 60 DAFB and leaves at 120 DAFB, respectively. Therefore, R^2 values were (in decreasing order): for SPAD60, leaves at 60 DAFB>leaves at 120 DAFB>flower buds>flowers>bud wood>vegetative buds, and for SPAD120, leaves at 120 DAFB>flower buds>leaves at 60 DAFB>flowers>bud wood>vegetative buds.

In the case of pear trees, the coefficients of determination across years are shown in Fig. 6 (partial R^2 for single nutrients in the *Y*-axes and global R^2 in the insets, respectively). The tissues in which more nutrients contributed to the explanation of the SPAD values were both types of leaves. In bud wood, the only elements showing a relationship with SPAD

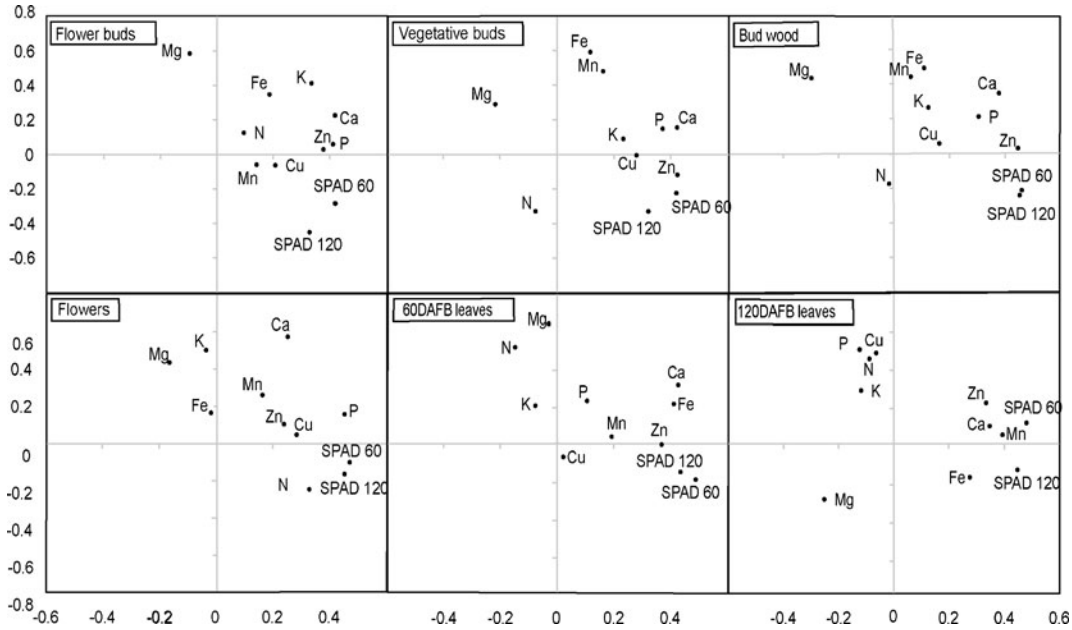

Fig. 3 Principal component analysis of the peach tree nutrient database. Nutrient concentrations measured in different years in bud wood, flower buds, vegetative buds, flowers and leaves at 60 and 120 DAFB leaves, as well as SPAD values at 60 and 120 DAFB were included as variables in the analysis. The first component explained 29, 28, 28, 25, 25 and 24% of the total variance for flower buds, vegetative buds, 60 DAFB leaves, flowers, bud wood and 120 DAFB leaves respectively. The second component explained 17, 17, 16, 16, 16 and 14% of the total variance for 120 DAFB leaves, flowers, bud wood, flower buds, vegetative buds, 60 DAFB leaves, respectively

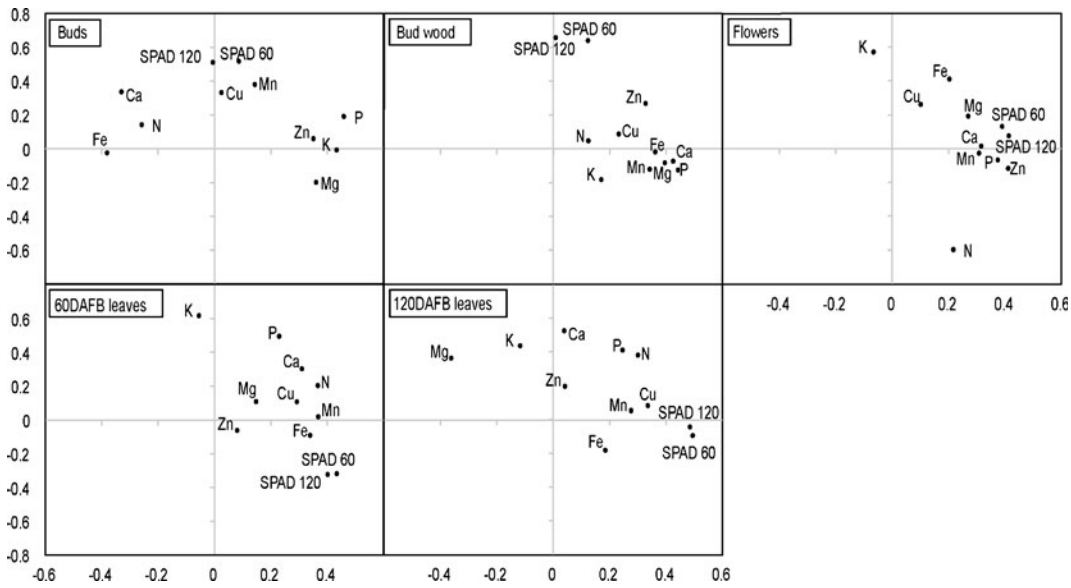

Fig. 4 Principal component analysis of the pear tree nutrient database. Nutrient concentrations measured in different years in bud wood, buds, flowers and leaves at 60 and 120 DAFB leaves, as well as SPAD values at 60 and 120 DAFB were included as variables in the analysis. The first principal component explained 35, 33, 33, 32 and 30% of the total variance for buds, bud wood, 60 DAFB leaves, 120 DAFB leaves and flowers, respectively. The second principal component explained 28, 19, 18, 18 and 15% of the total variance for flowers, bud wood, buds, 60 DAFB leaves, and 120 DAFB leaves, respectively

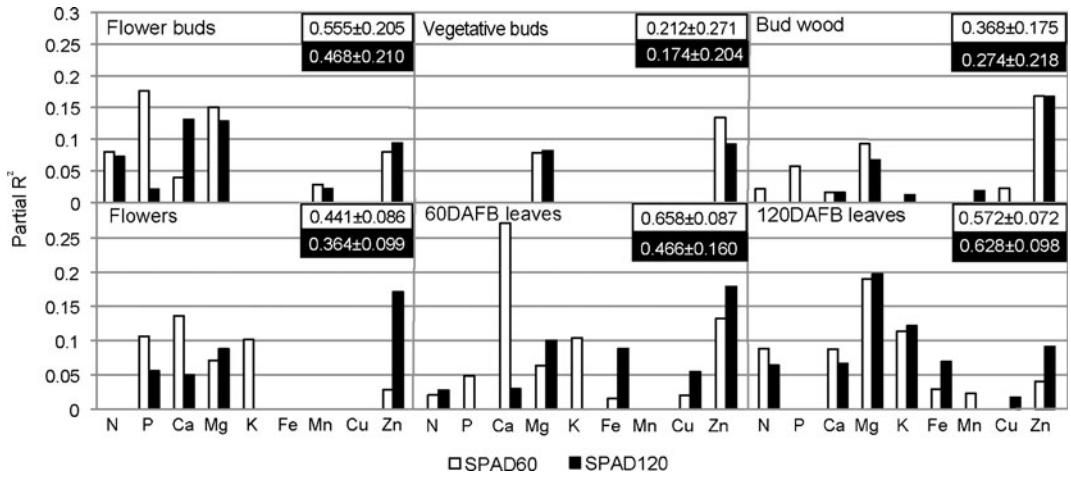

Fig. 5 Determination coefficients (partial R^2) between peach tree nutrients in different tissues and SPAD values at 60 and 120 DAFB. Coefficients of determination for the stepwise multiple analyses (global R^2) are also shown in each graph in insets (white and black background for SPAD at 60 and 120 DAFB, respectively). In all cases, values shown are multi-year averages of the individual values found in the different years of study

indexes were N and Zn. Similarly to what occurred in peach trees, Zn was the only common element in the final regression model for all pear tree materials excepting buds (data were available only for a single year in that case; Fig. 6). Iron was included in the regression in the case of flowers and 60 DAFB leaves, whereas Mn, conversely to what occurs in peach trees, participated in the case of flowers and 60 DAFB leaves.

The global R^2 values for pear trees were higher when using SPAD60 than when using SPAD120 in all materials, excepting in bud wood (insets in Fig. 6). Values were (SPAD60/SPAD120): 0.682/0.248, 0.094/0.108, 0.435/0.381, 0.609/0.393 and 0.695/0.608 for buds, bud wood, flowers, leaves at 60 DAFB and leaves at 120 DAFB, respectively. Therefore, R^2 values were (in decreasing order): for SPAD60, leaves at 120 DAFB>buds>leaves at 60 DAFB>flowers>bud wood, and for SPAD120, leaves at 120 DAFB> leaves at 60 DAFB>flowers>buds>bud wood.

Evaluation of the regression equations to predict chlorosis in different years

The reliability of the best-fit regression equations obtained for the prediction of SPAD60 from the nutrient concentrations was assessed, taking as a proof of concept the case of peach (flower buds and flowers). The best-fit equations obtained, using data from all years of study combined (3 years for flower buds and 5 years for flowers), were:

- Flower buds: SPAD60=18.84+61.55 P+0.02 Fe+ 9.18 K-39.25 Mg (R^2=0.357, P<0.0001).
- Flowers: SPAD60=29.27+51.9 P+20.14 Ca-0.21 Mn-97.26 Mg-0.02 Fe (R^2=0.490, P<0.0001).

Both R^2 values are rather high, especially considering the heterogeneity of the peach tree population used. To assess the reliability of these equations across years, the SPAD value observed experimentally in each tree every year was plotted *vs.* the SPAD values predicted by the equations above (Fig. 7). Results show that there were no major differences in the slopes of the lines corresponding to each year of study, indicating that the multi-year equations obtained were sufficiently reliable. In the case of pear trees the reliability of the models was not as good as that in peach trees; the best-fit equations obtained and the regression lines obtained using pear tree flowers and bud wood data are shown as an example in Online Resource 4.

We have also assessed the percentages of correct chlorosis assignment when using the best-fit regression curves (Fig. 8). The trees were distributed in the same three chlorosis categories indicated above (markedly chlorotic, moderately chlorotic and green). In a first approach, we grouped moderately chlorotic and green trees, considering that

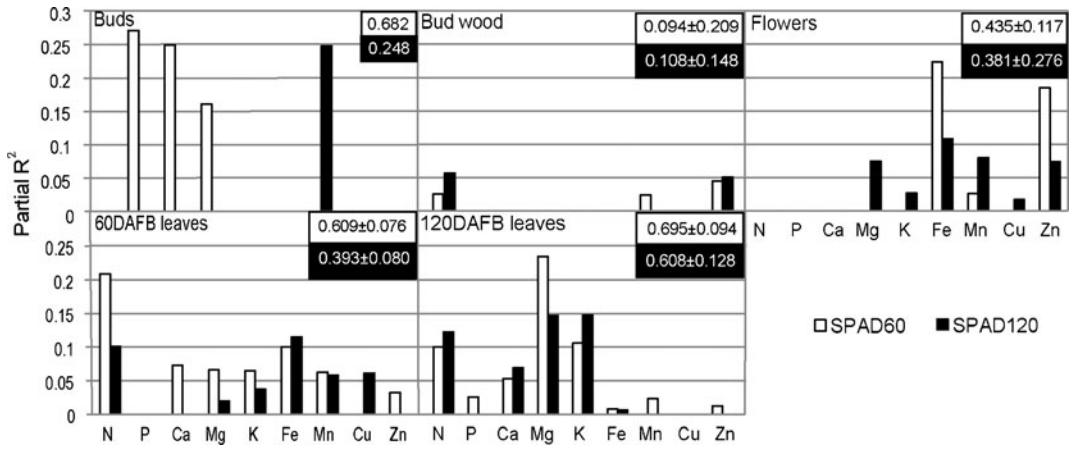

Fig. 6 Determination coefficients (partial R^2) between pear tree nutrients in different tissues and SPAD values at 60 and 120 DAFB. Coefficients of determination for the stepwise multiple analyses (global R^2) are shown in each graph in insets (white and black background for SPAD at 60 and 120 DAFB, respectively). In all cases, values shown are multi-year averages of the individual values found in the different years of study

moderately chlorotic ones would not need any Fe fertilization (Fig. 8, left). In that case, the equation correctly assigned as markedly chlorotic 12–18% of the total number of trees (in the case of flowers and flower buds, respectively), whereas in 10–11% of the trees the prediction was incorrect. Therefore, the chlorosis prediction was correct in approximately 54 (12 out of 22%) and 63% (18 out of 29%) of the cases using flowers and flower buds, respectively. In a second approach, we grouped moderately and markedly chlorotic trees, considering that moderately chlorotic ones would need Fe fertilization (Fig. 8, right). In that case, the equation assigned correctly as chlorotic 44 and 57% of the total number of trees (in the case of flowers and flower buds, respectively), whereas in 7–8% of the trees the prediction was incorrect. Therefore, the chlorosis prediction was correct in approximately 86 (44 out of 51%) and 88% (57 out of 64%) of the cases (using flowers and flower buds, respectively). These are the percentage of times that a producer in our region will be right in the decision of applying or not a corrective treatment for Fe chlorosis using the equations above.

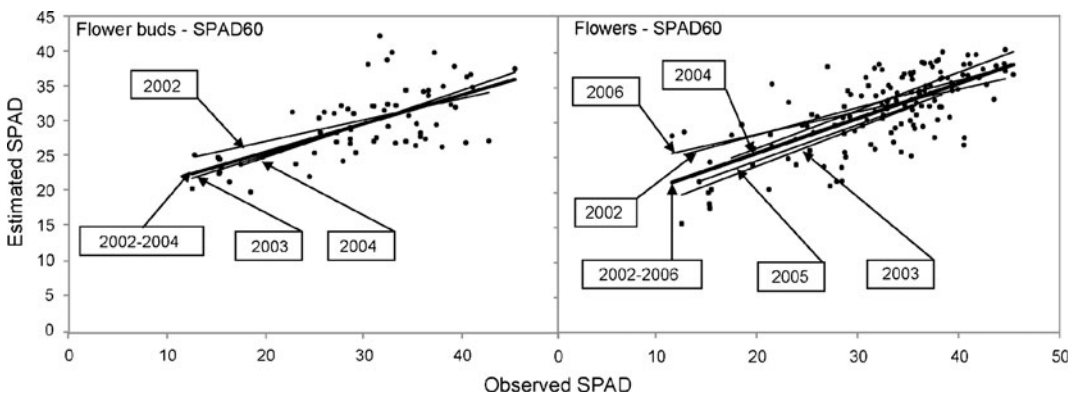

Fig. 7 Relationships between the SPAD60 peach tree values predicted by the best-fit equation obtained taking into account all data (including all years of study) *vs.* the SPAD60 values observed experimentally every year. Each point corresponds to a single tree of the database, using flower bud (**a**) and flower (**b**) mineral nutrient data. The thin lines correspond to regressions for individual years, and the thick line to the multi-year regression

Furthermore, as an additional test of reliability of the regression method, we developed equations from peach tree flower datasets considering data from only 4 years, and tested the model with the data from the remaining year, i.e., we used a jackknife procedure to draw conclusions about the validity of the method. Five different equations were obtained, the first using data from 2002, 2003, 2004 and 2005 to obtain an equation and validating it for 2006, the second using data from 2003, 2004, 2005 and 2006 to obtain an equation and validating it for 2002, and so on. Then, the percentages of correct chlorosis assignment were estimated as indicated above for the five different best-fit regression curves, and an average was calculated. The results, presented in Online Resource 5 (means±SE), suggest that the percentage of correct chlorosis assignment was consistent across years, and not very different from the results for the best possible equation (the one calculated with all years available).

Discussion

This study provides a database of mineral concentrations from early materials, including buds and flowers, in peach and pear trees affected to different extents by Fe-deficiency chlorosis. The SPAD values reported represent a wide range, spanning from markedly chlorotic individuals to trees that were fully green. These SPAD values are representative of the ranges found previously in pear and peach fruit trees growing in the same area (Belkhodja et al. 1998; Morales et al. 1994, 1998). Therefore, the trees analyzed were an adequate sample to accomplish one of the main goals of this study, to explore the relationship of mineral nutrient concentrations with iron chlorosis in a situation as close to reality as possible. In the case of peach, the mineral concentrations of flowers and leaves were within the nutrient ranges observed in previous studies in the area (Abadía et al. 1985; Belkhodja et al. 1998; Igartua et al. 2000; Sanz et al. 1993), whereas those of buds (flower and vegetative buds and bud wood) are reported here for the first time. In the case of pear, the mineral concentrations of flowers and leaves were within the nutrient ranges common in the area (Morales et al. 1998; Sanz et al. 1993), and those of buds are reported here for the first time. Studies reporting the mineral concentrations of fruit tree buds are not common. The concentration of B in buds was used to assess the B nutritional status of apple trees (Wójcik 2002), and the flower bud B concentrations was also reported in olive trees by Rodrigues and Arrobas (2008). Also, the nutrient concentrations in pistachio flower buds have been reported (Mehdi et al. 2006; Vemmos 1999). Data

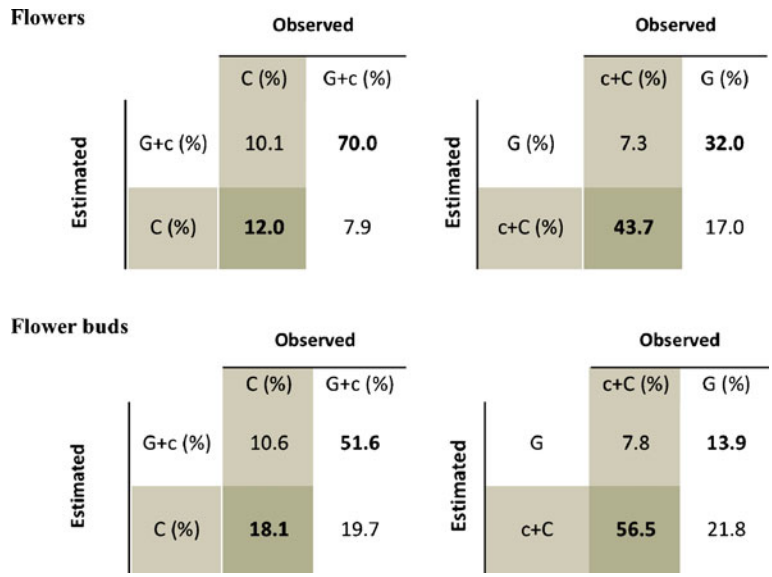

Fig. 8 Assessment of the chlorosis (SPAD60) prediction power of the best-fit regression curves using peach tree flower (**a**) and flower bud (**b**) nutrient concentrations. G, c and C refer to percentages of green, moderately chlorotic and markedly chlorotic trees. Trees were grouped considering that moderately chlorotic trees either do not need (left part of the Figure) or need Fe fertilization (right part of the Figure)

presented in this work will constitute a framework for future studies on the underlying mechanisms of the fluxes of mineral nutrients from the bud to the flower and leaf stages.

Results obtained support that it is possible to carry out the prognosis of Fe chlorosis using early materials such as buds and flowers. In general, the elemental composition of flower buds and flowers allowed to predict chlorosis (at 60 DAFB) almost as well as the leaf elemental composition at 60 DAFB, as indicated by the R^2 values of the multiple regressions (Figs. 7 and 8). This indicates that the tree nutrition status at flowering, or even prior to it, is almost as good for the prediction of future foliar Fe-chlorosis as that of the leaves themselves. However, the relationships found here, where a heterogeneous tree population (from many different orchards) was sampled, were markedly different from those obtained in previous studies, where a single orchard was used (Belkhodja et al. 1998, Igartua et al. 2000). This study has been carried out sampling trees from a set of commercial orchards, with no control over other factors that may affect chlorosis, such as soil characteristics, cultivars or orchard management practices. Therefore, we can expect a much larger variability of responses than in single orchard experiments, due to this variety of uncontrolled factors. The trends found in such unfavorable conditions may have a better translational potential, as the experimental set up matches the conditions encountered by commercial producers. On the other hand, the results presented here indicate that in early materials (buds and flowers) of peach trees that will show Fe chlorosis later in the year the concentrations of P would tend to be relatively low, and those of Mg would tend to be relatively high. However, the reason for these associations between P, Mg and Fe status are not yet known, and unraveling the mechanisms behind these relationships will require further experiments.

Leaf chlorophyll measured at 60 DAFB (SPAD60) tended to present very large loadings in all principal component analyses, across all tissues and in both tree species. In most cases, SPAD60 had a large loading on the first component and, where this did not occur (as for buds and bud wood in pear), the loading on the second component was large. This means that this trait showed relatively more relationships with the rest of the variables, and thus can be explained by them to some extent. These mathematical relationships do not indicate causality, but they can be useful to derive predictive equations (see below). On the other hand, SPAD120 tended to present slightly lower loadings, meaning that its relation with mineral elements was weaker than for SPAD60. This was confirmed by the regression analysis, where, in all cases excepting one, the model chosen explained better (i.e., have larger R^2 values) SPAD60 than SPAD120. This situation was expected, since SPAD120 represents a physiological stage more distant in time from the other samplings than SPAD 60. Another possibility is that SPAD120 may be affected by the application of corrective treatments between 60 and 120 DAFB. In some cases, trees at 120 DAFB may present a better Fe-nutrition status due to application of Fe corrective treatments, which are usually done along the season.

Consistently significant associations between nutrient concentrations and SPAD found by the different methods in each of the peach and pear tree materials are summarized in Table 6. These results indicate that different statistical analysis methods can provide complementary data, since in some cases only one or two methods indicated significant associations, whereas in other cases three or four of the methodologies used detected such associations (shaded cells in Table 6). The most marked associations were detected by any of the four methodologies, whereas more subtle associations were only detected with the principal component and multiple stepwise regression analysis.

In our experimental conditions, the general best-fit regression equations obtained for the prediction of SPAD60 from nutrient concentrations of peach flower buds and flowers were quite reliable over the different years. Also, such equations could predict, in more than 86% of the cases, whether a tree in our region will show chlorosis later in the year, using only flower bud or flower mineral data. The formal validation of the relationships found must be tested in further studies, using mineral nutrient datasets different to those employed to develop the equations. Furthermore, the possibility that the relationships could be even stronger when using a single cultivar should be also explored. The development of this type of predictive tools will offer the producer the possibility of taking a very early decision, having potentially a large impact on final fruit yield, although

Table 6 Summary of the associations found between nutrient concentrations in the different plant materials and SPAD values in peach (A) and pear (B) trees, using four different approaches: mean comparison depending on chlorosis level (1), correlation analysis (2), principal component analysis (3), and stepwise multiple regression (4)

A	SPAD	N	P	Ca	Mg	K	Fe	Mn	Cu	Zn
Flower bud	**SPAD60**	4	**2,3,4**	4	**2,3,4**			4		2,4
	SPAD120	4	**2,3,4**	4	**1,2,3,4**			4		2,4
Vegetative buds	**SPAD60**				**2,3,4**					3,4
	SPAD120				**1,2,3,4**					3,4
Bud wood	**SPAD60**	4	2,4	2,4	**2,3,4**				4	**2,3,4**
	SPAD120		2	2,4	**1,2,3,4**	4		4	1	**1,2,3,4**
Flowers	**SPAD60**	3	**2,3,4**	4	**2,3,4**	3,4				2,4
	SPAD120	3	**1,2,3,4**	4	**1,2,3,4**	3			1	**1,2,4**
60 DAFB	**SPAD60**	4	4	**2,3,4**	4	**2,3,4**	**2,3,4**		2,4	**2,3,4**
	SPAD120	4	1	**2,3,4**	4	2,3	**2,3,4**		2,4	**1,2,3,4**
120 DAFB	**SPAD60**	4		3,4	**2,3,4**	**2,3,4**	**2,3,4**	4		**2,3,4**
	SPAD120	4		3,4	**2,3,4**	**2,3,4**	**2,3,4**		4	**2,3,4**
B	**SPAD**	**N**	**P**	**Ca**	**Mg**	**K**	**Fe**	**Mn**	**Cu**	**Zn**
Buds	**SPAD60**		4	3,4	3,4	3		2,3	3	
	SPAD120		2	3	3	3		**1,2,3,4**	3	
Bud wood	**SPAD60**	4								2,4
	SPAD120	4								2,4
Flowers	**SPAD60**		3	3	3	3	2,4	**2,3,4**		**2,3,4**
	SPAD120		3	3	**1,3,4**	3,4	**1,2,4**	**1,2,3,4**	4	**1,2,3,4**
60 DAFB	**SPAD60**	2,4		4	4	**2,3,4**	2,4	**2,3,4**	2,3	4
	SPAD120	**1,2,4**	3		4	**2,3,4**	**1,2,4**	**1,2,3,4**	**2,3,4**	
120 DAFB	**SPAD60**	2,4	**2,3,4**	4	**2,3,4**	**2,3,4**	3,4	**2,3,4**	3	4
	SPAD120	**1,2,4**	**1,2,3**	4	**1,2,3,4**	**2,3,4**	3,4	**1,2,3**	3	

these benefits can only be confirmed after further experiments.

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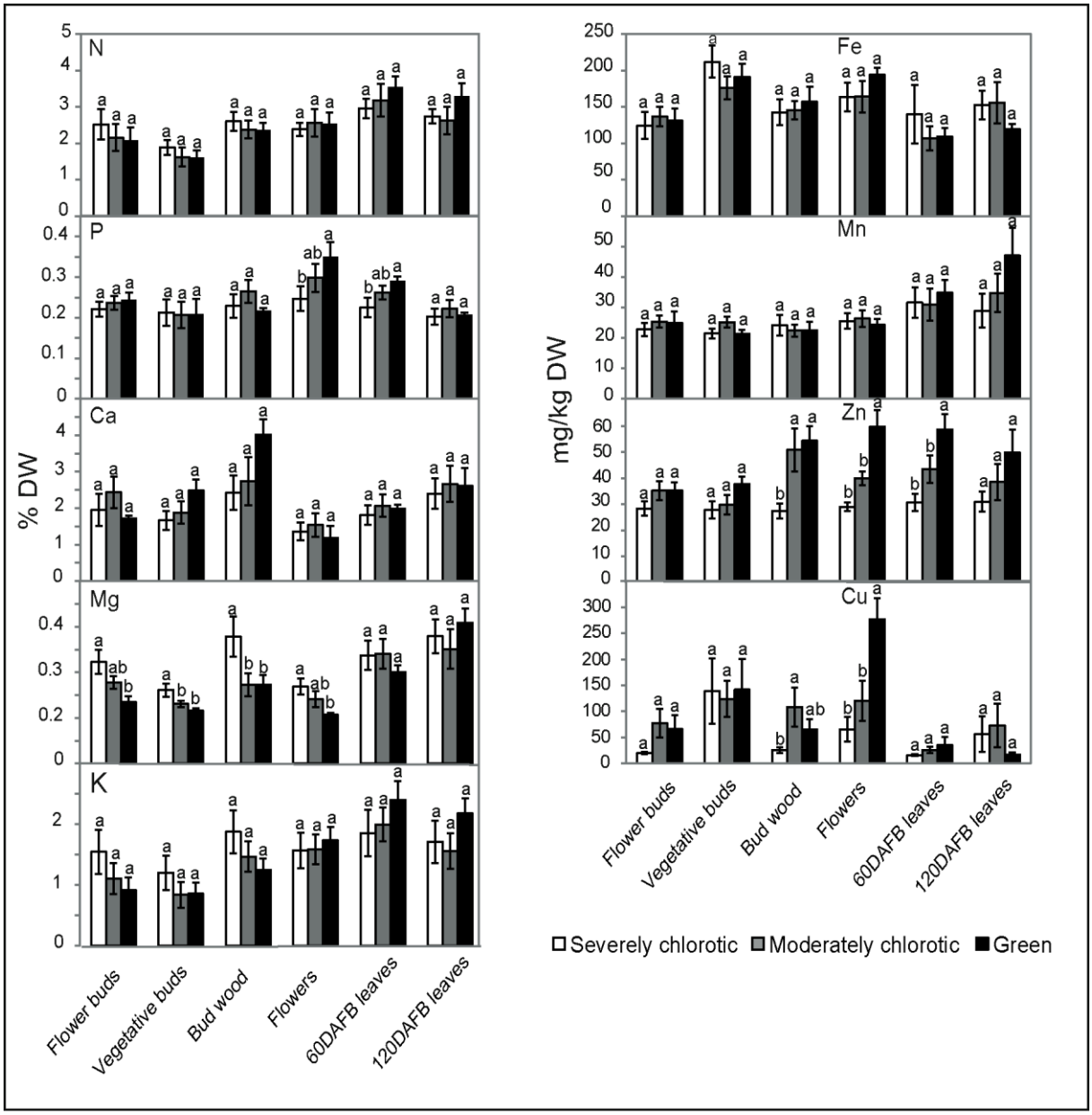
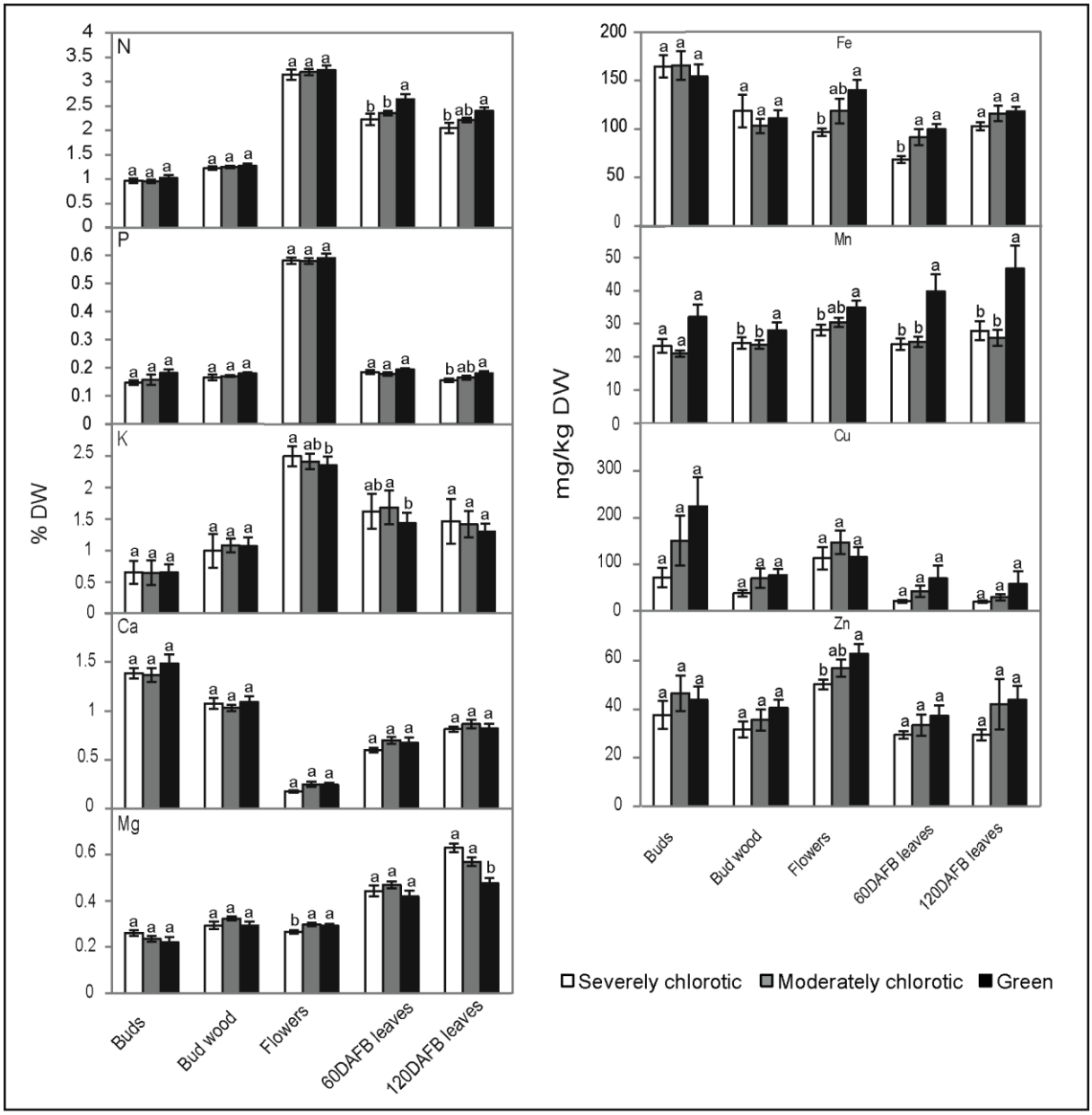
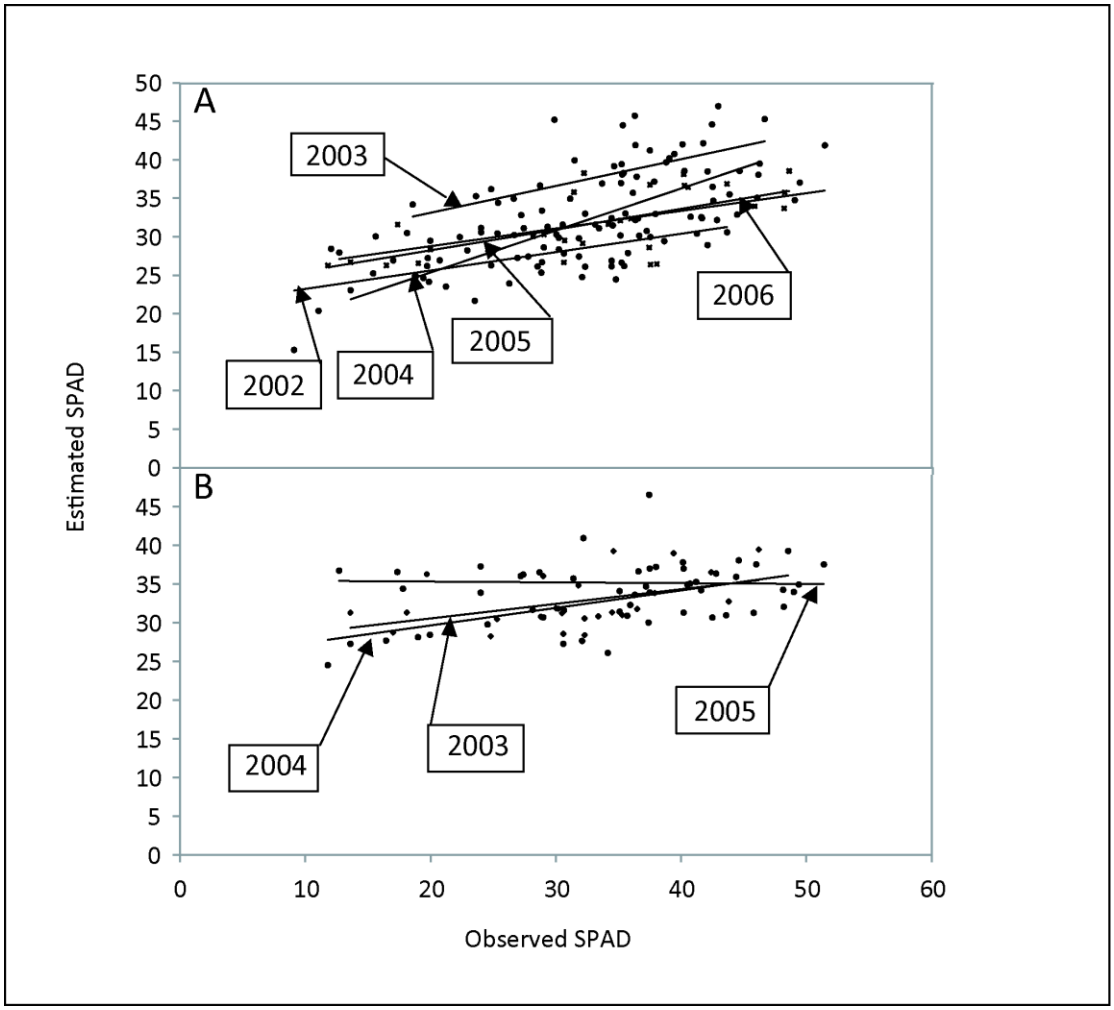


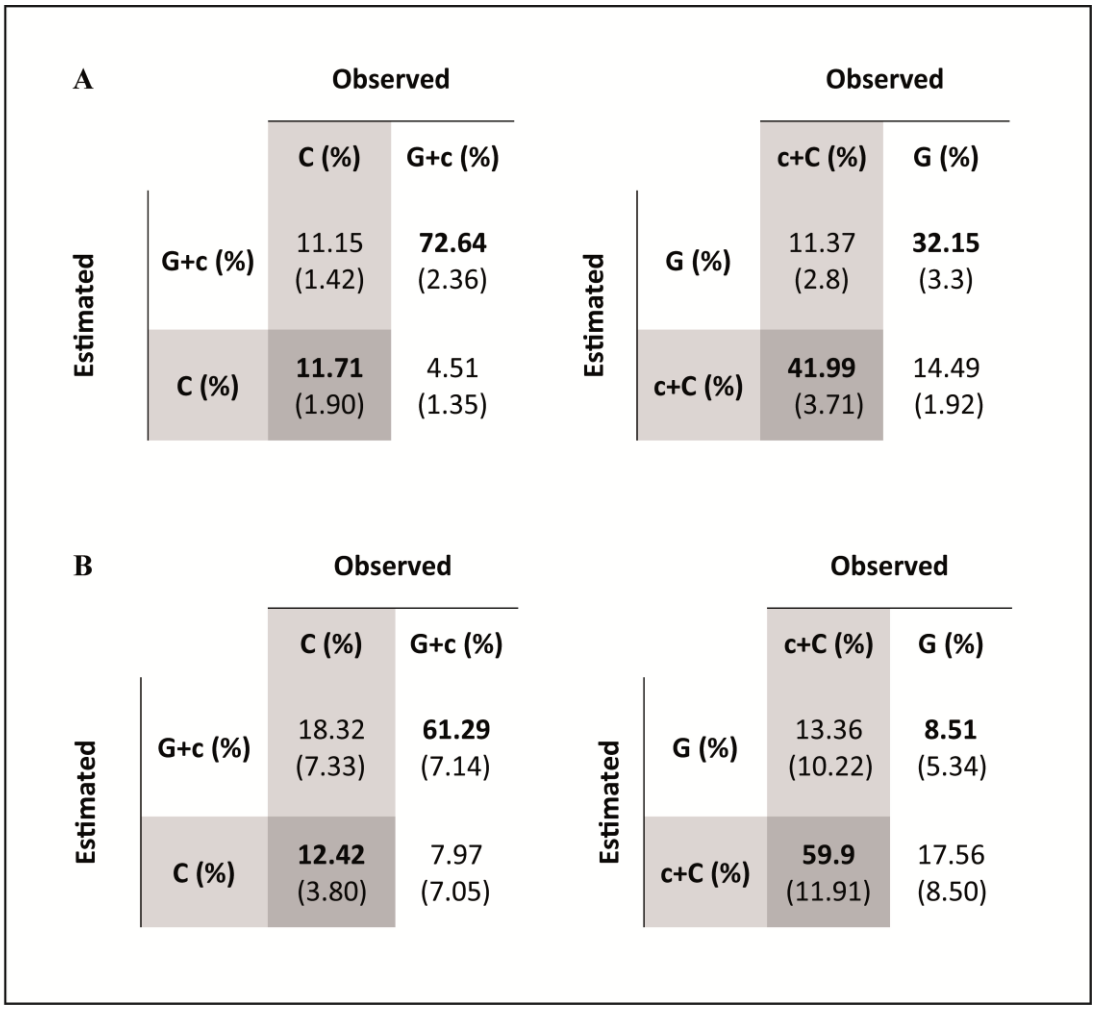
Online resource 2. Changes in nutrient concentrations in peach buds, flowers and leaves with different degrees of iron chlorosis. Data are means ± SE of 32 trees. Bars marked with the same letter were not significantly different (Duncan's test) at the $P \leq 0.05$ probability level.



Online resource 3. Changes in nutrient concentrations in pear buds, flowers and leaves with different degrees of iron chlorosis. Data are means ± SE of 30 trees. Bars marked with the same letter were not significantly different (Duncan's test) at the $P \leq 0.05$ probability level.



Online resource 4. Relationships between the SPAD60 pear tree values predicted by the best-fit equation obtained taking into account all data (including all years of study) *vs.* the SPAD60 values observed experimentally every year. Each point corresponds to a single tree of the database, using flowers (A) and bud wood (B) mineral nutrient data.



Online resource 5. Further assessment of the iron chlorosis (SPAD60) prediction power of the best-fit regression curves using peach tree flower (A) and pear tree flower (B) nutrient concentrations. Data presented in this Figure are similar to those shown in Figure 8 in the paper. However, in this case, we developed equations from peach tree flower datasets considering data from only four years, and tested the model with the data from the remaining year, i.e., we used a jackknife procedure to draw conclusions about the validity of the method. Five different equations were obtained, the first using data from 2002, 2003, 2004 and 2005 to obtain an equation and validating it for 2006, the second using data from 2003, 2004, 2005 and 2006 to obtain an equation and validating it for 2002, and so on. Then, the percentages of correct chlorosis assignment were estimated as indicated in the text for the five different best-fit regression curves, and an average was calculated (the corresponding SE are in parenthesis). G, c and C refer to percentages of green, moderately chlorotic and markedly chlorotic trees. Trees were grouped considering that moderately chlorotic trees either do not need (left part of the Figure) or do need Fe fertilization (right part of the Figure).

Effects of foliar Fe application on nutrient and photosynthetic pigment composition and Chl fluorescence parameters in peach trees grown in the field and sugar beet grown in hydroponics

Abstract

Background and Aims

The aim of this study was to assess the effects of foliar $FeSO_4$ applications on two plant species grown in different environments: peach trees grown in the field and sugar beet grown in hydroponics.

Methods

The distal half of peach and sugar beet leaves was treated by leaf dipping and using a paint brush, respectively. The re-greening of the distal (Fe-treated) and proximal (untreated) leaf areas was assessed with a SPAD apparatus on a weekly basis, during 8 weeks in the case of peach leaves and on a daily basis for 7 days in sugar beet leaves. At the end of the experimental period, leaves were excised, and tissue Fe, N, P, K, Ca, Mg, Mn, Zn and Cu concentrations were determined in treated and untreated leaf areas. Also, the changes in leaf photosynthetic pigment composition were characterized in both peach tree and sugar beet leaves. The Chl fluorescence imaging was measured in peach tree leaves one week after the treatment. Low temperature-scanning electron microscopy microanalysis (LT SEM-EDX) and Perls Fe staining was carried out in peach tree leaves at the end of the experiment.

Results

The treated distal leaf parts of both species showed a significant uptake of Fe, as well as marked re-greening, with significant increases in the concentrations of all photosynthetic pigments, decreases in the (Z+A)/(V+A+Z) ratio and increases in the F_V/F_M ratios. In the untreated basal leaf parts, Fe concentrations increased slightly, but little re-greening occurred. No changes in the concentrations of other nutrients were found.

Conclusions

Results obtained indicate that $FeSO_4$ applications are effective at the site of application both in peach trees grown in the field and sugar beet grown in hydroponics. The effects of the foliar fertilizer were very minor outside the leaf surface treated, with Fe lateral movement in the leaf suffering major restrictions.

Introduction

Iron (Fe) deficiency (Fe chlorosis) is a common disorder affecting plants in many areas of the world, and is mainly associated with calcareous, high pH soils (Abadía et al. 2011, El-Jendoubi et al. 2011). Plant Fe deficiency has great economical significance, because crop quality and yield can be severely compromised (Álvarez-Fernández et al. 2011, El-Jendoubi et al. 2011). Therefore, the use of expensive fertilization procedures is often required (Álvarez-Fernández et al. 2004).

The correction of Fe chlorosis in crops grown on calcareous soils is an old problem with no easy solution. Until rootstocks tolerant to Fe chlorosis having favorable agronomical characteristics become available, the prevention or correction of Fe chlorosis is of paramount importance to fruit growers (Pestana et al. 2003). Foliar sprays can be a cheaper, environmental-friendly alternative to soil treatments for the control of Fe chlorosis. Foliar fertilization is most effective when soil nutrient availability is low, topsoil dry, and root activity is decreased during the reproductive stage (Wójcik 2004). The success of treatments with Fe-containing formulations depends on their capacity to penetrate the cuticle and/or stomata, undergo transport through the apoplast and cross the plasma membrane of leaf cells to reach the cytoplasm and then the chloroplast (Abadía et al. 2011, Rombolà et al. 2000, Fernández et al. 2009).

Iron(II)-sulphate has been tested as a foliar fertilizer in several studies. It was reported to increase leaf chlorophyll concentrations in kiwi (Rombolà et al. 2000), citrus (Pestana et al. 2001, Pestana et al. 2003), pear (Álvarez-Fernández et al. 2004) and peach (Fernández et al. 2006, Fernández et al. 2008). This type of treatment could improve fruit size and quality, as observed in *Citrus* species (El-Kassa 1984, Pestana et al. 2001, Pestana et al. 1999). The effectiveness of foliar application of $FeSO_4$ with and without acids and Fe-DTPA to re-green chlorotic pear trees was studied by Álvarez-Fernández et al. (2004), and it was concluded that foliar fertilization cannot offer a good alternative for the full control of Fe chlorosis and proposed that it could be a technique complementary to soil Fe-chelate application. Nevertheless, Fe fertilization is also an usual practice in crops where the use of chelates is too expensive. However, there are still few indexed references dealing with the foliar treatments for the correction of iron chlorosis, and therefore the scientific background for the foliar fertilization practice is still scarce (Abadia et al. 1992, Abadía et al. 2011).

In this study, we assessed the effect of an Fe-containing formulation (2 mM $FeSO_4$ supplemented with a surfactant), estimated to have a good re-greening effect in previous studies (Fernández et al. 2006, Fernández et al. 2008), on Fe-deficient peach and sugar beet leaves. The distal half of peach leaves was treated with the solution by leaf dipping, first at the beginning of the trial and then 4 weeks later, and those of sugar beet was treated with a paint brush, first at the beginning of the trial and then two days later. Afterwards, the re-greening of treated (distal) and untreated (proximal) leaf areas was estimated with a SPAD apparatus, on a weekly basis during 8 weeks for peach leaves and on a daily basis during 7 days for sugar beet leaves. At the end of the experimental period, leaves were excised and tissue Fe, N, P, K, Ca, Mg, Mn, Zn and Cu concentrations were determined in Fe-treated and untreated leaf areas. In treated and untreated peach leaves, Chl fluorescence imaging was performed one week after treatment and low temperature-scanning electron microscopy and microanalysis (LT SEM-EDX) and Perls Fe-specific staining were carried out at the end of the experiment.

Material and Methods

Peach tree orchard

A peach tree orchard was selected, near the village of Plasencia de Jalón (Zaragoza province), in the Ebro river valley in North-Eastern Spain (41°40'27.72"N, 1°13'33.46"O). Trees were of the variety 'Miraflores' grafted on GF677 rootstock, 16-year old and with a frame 5 x 4 m. Trees were flood-irrigated every approximately 2-3 weeks. Normal fertilization practices were used, with the exception of Fe fertilization, which was totally excluded from the grower treatments in the selected trees. This orchard is known to be affected by Fe chlorosis as many others in the area.

Sugar beet growth in hydroponics

Sugar beet (*Beta vulgaris* L. 'Orbis') plants were grown in a controlled environment chamber with a photosynthetic photon flux density at leaf height of 350 µmol m^{-2} s^{-1} photosynthetic active radiation and a 16 h-22 ºC/8 h-19 ºC, day/night regime. Seeds were germinated and grown in vermiculite for two weeks. Seedlings were grown for three more weeks in half-strength Hoagland nutrient solution with 45 µM Fe(III)-EDTA [Fe(III)-ethylenediaminetetraacetate]. Then, seedlings were transferred to 20 L plastic buckets containing half-strength Hoagland nutrient solution with either 0 (-Fe) or 45 µM Fe(III)-EDTA (+Fe, Fe-sufficient control plants). The pH of the Fe-free nutrient

solutions was buffered at approximately 7.7 by adding 1 mM NaOH and 1 g L^{-1} of $CaCO_3$, a treatment that simulates conditions usually found in the soils associated with Fe deficiency (Susin et al. 1996). After growing for 14 days under these conditions, plants grown in the zero Fe treatment showed clear Fe-deficiency symptoms, including leaf chlorosis (Fig. 3.1).

Figure 3.1 *Sugar beet plants grown in hydroponic conditions. In the two left buckets plants were grown in Fe-deficient conditions and in the right bucket plants were grown in Fe-sufficient conditions.*

Iron treatments in peach trees

Sixteen peach trees with a similar leaf chlorosis level were chosen in June. Some of them were used as negative controls (untreated Fe-deficient chlorotic trees), others were fertilized with soil-applied Fe(III)-EDDHA and used as positive controls, and leaves in other trees were treated with a foliar-applied $FeSO_4$ solution (treated trees). Also, some trees without any Fe-deficiency symptoms (green controls) were selected in the same orchard at the beginning of the trial and used in the experiments. All Fe-deficient trees were not treated with Fe at the beginning of the season. Before treatment Fe-deficient trees had SPAD values of approximately 15-20, indicative of Fe chlorosis, whereas Fe-sufficient trees had SPAD values of approximately 31-35.

To carry out the soil application in June, five wells (approximately 20 cm-deep, 20 x 20 cm-wide) were excavated in the soil around each tree, approximately 100 cm from the trunk, and ten g of Fe(III)-EDDHA was placed in the uncovered soil surface of each well. The wells were topped again with soil and four L of water per well were added.

Leaf sampling for foliar analysis, (30 leaves/tree) was made on July 29. In each treatment, one leaf was used for the elemental micro-localization study and the remaining for the determination of Fe concentration.

Figure 3.2 *Treatment of the distal half part of (A) peach leaves by dipping and (B) sugar beet leaves using a paint brush with a solution containing 2 mM $FeSO_4$ and 0.1% surfactant.*

For the foliar application, 40 similar shoots per tree were selected, and 20 of them were kept as Fe-deficient controls and the other 20 were treated. In each shoot, leaves at the positions 4^{th}-5^{th} from the top (young and fully developed) were labeled with color tape. In mid June, the distal half part of the labeled leaves was immersed briefly (2 s) in a solution containing 2 mM $FeSO_4$ and 0.1% BreakThrough S-233 (a surfactant, organo-silicon compound, polyether- modified polysiloxane, from Goldschmidt GmbH, Essen, Germany) (Fig. 3.2A) (Abadía et al. 2011). The solution was kept at pH 4.0 and was applied immediately after preparation to minimize atmospheric oxidation (Fernández et al. 2006). A second application with the same formulation was made four weeks later.

Experiments were made in the summers of 2009, 2010 and 2011. In 2009, only the assessment of re-greening effects and the analysis of mineral elements were carried out. In 2010 and 2011, all parameters were measured.

Iron treatments in sugar beet leaves

A solution containing 2 mM $FeSO_4$ and 0.1% BreakThrough S-233 was applied to the distal half part of the leaf, on both the adaxial and abaxial leaf sides, using a paint brush (Fig. 3.2B). The application was made twice, the first one at the beginning of the experiment and then two days later.

Re-greening effect assessment

In peach tree leaves, the assessment of the leaf re-greening was carried out weekly by measuring the leaf chlorophyll concentration in the 40 labeled shoots in each of the 4 trees. Leaf chlorophyll was estimated in every leaf using a SPAD 502 meter (Minolta Co., Osaka, Japan), carrying out one measurement in the midst of the distal treated area, and one more in the midst of the basal untreated area. In the unfertilized, control leaves measurements were also made in the distal and basal leaf parts. Values shown are means ± SE (n = 4 trees, with 20 leaves/tree). Total Chl (in μmol m^{-2}) was calculated from the SPAD indexes (Álvarez-Fernández et al. 2004, Fernández et al. 2006).

In sugar beet, the re-greening effect was assessed estimating daily leaf chlorophyll concentration. Four measurements were made in the distal treated area and four more in the basal untreated area. In the unfertilized control leaves, measurements were also made in the distal and basal leaf parts. Values shown are means ± SE (n = 4 plants, with 4 leaves per plant).

Leaf mineral analysis

At the end of the experimental period (8 weeks after the first application in peach trees and 7 days after the first application in sugar beet), leaves were excised and the mineral element concentration of the above described different leaf parts (distal and basal areas from fertilized and unfertilized leaves), was analyzed according to standard laboratory procedures (Igartua et al. 2000). Treated leaves were divided in two parts, discarding a 5-mm strip in the intersection zone. Prior to processing, both leaf sides were carefully washed with 0.1% detergent (Mistol, Henkel) solution to remove surface contamination. Thereafter, leaves were washed thoroughly in tap water and then in ultrapure water. Results were expressed as % of dry weight (DW) for macronutrients (N, P, K, Ca and Mg) and as μg g^{-1} DW for micronutrients (Fe, Cu, Mn and Zn).

Photosynthetic pigment measurements

At the end of the experimental period, 4 disks per leaf part and treatment were taken with a calibrated cork borer, wrapped in aluminum foil, frozen in liquid N_2 and taken to the laboratory to be stored (still wrapped in foil) at -20 ºC. Leaf pigments were later extracted with acetone in the presence of Na ascorbate and stored as described previously (Abadía and Abadía 1993). Pigment extracts were thawed on ice, filtered

through a 0.45 μm filter and analyzed by HPLC (Larbi et al. 2004). All chemicals used were HPLC quality. The analysis time for each sample was 15 min.

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

Sections of fresh peach leaf tissue (2.5 x 2.5 mm leaf pieces) were mounted on aluminum stubs with adhesive (Gurr®, optimum cutting temperature control; BDH, Poole, UK), cryo-fixed in slush nitrogen (-196 ºC), cryo-transferred to a vacuum chamber at -180 ºC, and then 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 were overlaid with gold for observation and microanalysis. Fractured samples were observed at low temperature with a digital scanning electron microscope (Zeiss DSM 960, Oberkochen, Germany) using secondary and back-scattered electrons. Secondary electron images (1024 × 960 pixels) were obtained at 133 eV operating at 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.

Microprobe analysis was carried out with an Energy Dispersive X-ray microanalysis (EDXA) Pentaflet microanalytical system (Pentaflet, Oxford, UK). Measurements were made on the SEM-BSE samples simultaneously during SEM observation at a resolution of 133 eV, 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. Only smooth surfaces were taken for microanalysis (Hess et al. 1975). Semi-quantitative element analysis was carried out using standard ZAF (atomic number, absorption and fluorescence) correction procedures with Link Isis v.3.2 software (Link Isis, Oxford, UK). One-way ANOVA was used to compare the results obtained in the different leaf tissues (adaxial epidermis, palisade parenchyma, xylem vessels, spongy parenchyma and abaxial epidermis), followed by a post hoc multiple comparison of means with Duncan's test ($P < 0.05$; $n = 8$). Eight points of analysis per leaf tissue and three leaves per treatment were analyzed. All calculations were made using SPSS v.17.0 software.

Iron staining (Perls-DAB)

Representative areas (25 mm^2) from the midst of peach leaflet blades adjacent to main veins were embedded in 5% agar and sectioned transversally at 70 μm thickness using a vibrating blade microtome (VT1000 S, Leica Microsystems GmbH, Wetzlar, Germany). Perls-DAB staining was performed according to (Roschzttardtz et al. 2009). Fresh

sections were incubated with a 4% $K_4[Fe(CN)_6]$, 4% HCl solution for 30 min at RT and 100% relative humidity. Negative control of the staining was performed incubating fresh sections with 4% HCl. After three washes with deionized water, a second incubation with methanol containing 0.01 M NaN_3 and 0.3% H_2O_2 was carried out for one h at RT. Sections were washed for three times with 0.1 M phosphate buffer pH 7.4 and then incubated with the same buffer containing 0.025% DAB, 0.005% H_2O_2 and 0.005% $CoCl_2$ for 30 min at RT. Finally, sections were washed with deionized water and bright light images (2592 x 1994 pixels) were taken using an inverted microscope (DM IL LED, Leica) with a CCD camera (Leica DFC 240C).

Chlorophyll fluorescence imaging

Chlorophyll fluorescence imaging of peach leaves was used to investigate the spatial heterogeneity of Chl fluorescence parameters after the foliar treatment using an imaging-PAM fluorometer (Walz, Effeltrich, Germany). All light sources were placed in a ring arrangement and directed at a fixed angle and distance onto the leaf area. Two outer LED-rings (a total of 96 LEDs) provided the measuring and actinic light and the saturating pulses, with a peak wavelength at 470 nm. A good homogeneity of the actinic light intensity was obtained in the whole illuminated leaf area. The inner LED-ring (a total of 16 LEDs) provided the pulse-modulated light for assessment of PAR-absorptivity at 650 and 780 nm. The charge-coupled device (CCD) camera has a resolution of 640 x 480 pixels. Pixel value images of the fluorescence parameters were displayed with help of a false color code, ranging from black through red, yellow, green, blue to pink (from 0.000 to 1.000) (Berger et al. 2004). All measurements were carried out with a maximal distance between camera and leaf (measuring area of 26 x 34 mm). Plants were kept in the dark for 30 min prior to measurement, and leaves were kept in the dark between measurements for 5 min. The minimum (dark) fluorescence F_O was obtained by applying measuring light pulses at low frequency (1 Hz). The maximum fluorescence F_M was determined by applying a saturating blue light pulse (10 Hz). The Chl fluorescence parameters were named according to (Larbi et al. 2006). Dark-adapted, maximum PSII efficiency was calculated as F_V/F_M, where F_V is F_M - F_O. Then, actinic illumination (204 µmol photon m^{-2} s^{-1}) was switched on and saturating pulses were applied at 20 s intervals for 5 min in order to determine the maximum fluorescence yield during saturating pulses (F_M'), and the chlorophyll fluorescence yield during actinic illumination (F_S). For each interval, saturation pulse images and values of

various Chl fluorescence parameters were captured. Actual (Φ_{PSII}) PSII efficiency was calculated as ($F_M´-F_S$)/$F_M´$ (Genty et al. 1989). Photochemical quenching (qP) was calculated as ($F_M´-F_S$)/$F_V´$ (Larbi et al. 2006), and non-photochemical quenching (NPQ) was calculated as ($F_M/F_M´$)-1 (Bilger and Björkman 1991).

Results

Re-greening effect of foliar Fe fertilization in peach tree and sugar beet leaves

Re-greening of the Fe-treated distal part of Fe-deficient peach leaves was already observed one week after the first treatment. The increase was approximately 20 µmol Chl m^{-2} (Fig. 3.3A). The re-greening continued in the following weeks and also after the second treatment. Eight weeks after the first Fe treatment, the treated area of peach leaves showed a marked re-greening when compared to the untreated part (Fig. 3.4). At the end of the experiment, the treated leaf areas had approximately 236 µmol Chl m^{-2}, and therefore the Chl concentration increase was 68% with respect to the initial leaf Chl concentration. The same formulation (combination of Fe compound and surfactant) had been reported to cause a relative Chl increase of approximately 120% (Fernández et al. 2008). However, and conversely to what was indicated in peach trees by (Fernández et al. 2008) re-greening did not extend into the untreated area (Fig. 3.4A). The untreated basal part of the Fe-treated leaves and both parts of leaves dipped in Fe-free solutions only showed a slight re-greening at some sampling times (increases were always ≤14% when compared to the initial Chl concentration). In all chlorotic untreated leaves, the Chl concentrations of the distal part were always slightly higher (6-19%) than those of the basal part.

In sugar beet leaves, leaf re-greening was already observed after one day. The increase was approximately 18 µmol Chl m^{-2} (Fig. 3.3B). At the end of the experiment, the treated distal areas of the sugar beet leaves had a Chl concentration of 127 µmol Chl m^{-2}, an increase of 171% with respect to the initial leaf Chl concentration. However, the re-greening of the leaf surface was not totally homogenous (Fig. 3.4B). On the other hand, the untreated basal part of treated leaves and both parts of the untreated chlorotic controls had only minor Chl concentration changes. In all chlorotic and green untreated leaves, the Chl concentrations of the distal part were always higher (22-41%) than that of the basal part. Also, some but not all of the leaves showed necrosis symptoms near the border of the untreated basal part (Fig. 3.4C). Iron sufficient control green leaves

also had a Chl concentration increase during the experimental period (from 200 to 290 and from 230 to 300 µmol Chl m^{-2} in the basal and distal leaf parts, respectively).

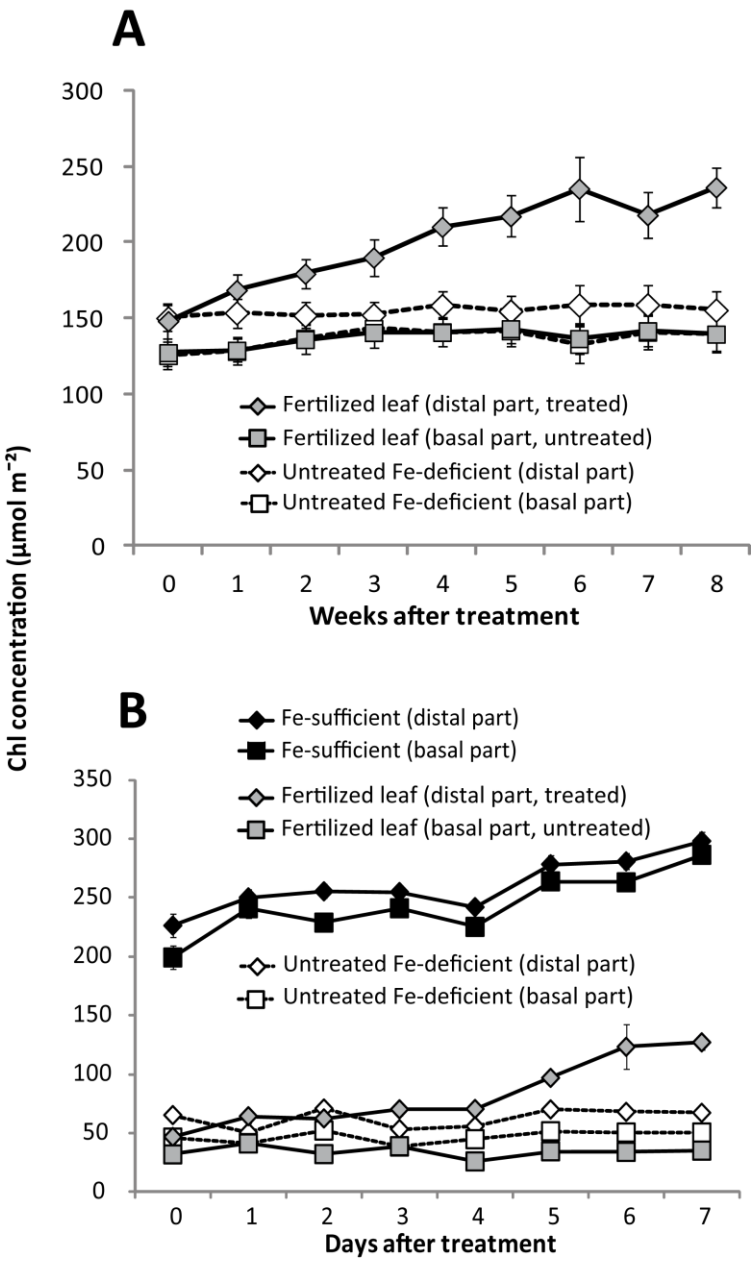


Figure 3.3 *Time course of the changes in leaf Chl concentration in peach tree (A) and sugar beet (B). The treatment was carried out with a solution containing 2 mM $FeSO_4$ and 0.1% surfactant. In peach leaves, foliar treatment was made at weeks 0 and 4 and SPAD index was measured each week. In sugar beet leaves, treatment was made at days 0 and 2 and SPAD was measured daily. Data are means ± SE (n = 12, 4 plants per bucket).*

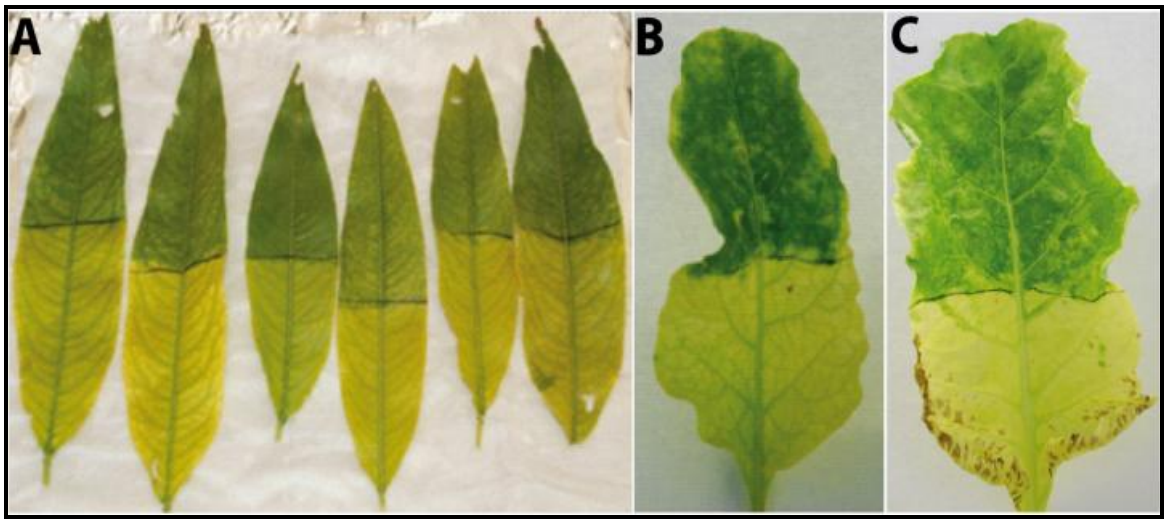


Figure 3.4 *Images of peach tree leaves 8 weeks after the first foliar Fe treatment (A) and two different sugar beet leaves 8 days after the first treatment (B and C). The green areas are the result of treatments with a solution containing 2 mM $FeSO_4$ and 0.1% surfactant.*

Leaf mineral concentration in peach tree and sugar beet leaves

The effects of foliar Fe-fertilization on the concentration of macro- and micro-elements are shown in Table 3.1. Nitrogen concentrations were lower in the basal than in the distal parts of both Fe-treated and control-untreated leaves, but the differences were not statistically significant. The concentrations of P were similar in all samples. Concerning Ca, concentrations were lower in the distal than in the basal parts of both Fe-treated and control-untreated leaves, but the differences were not statistically significant. The Mg and K concentrations were quite similar in all samples.

Concerning the microelement concentrations, foliar fertilization induced significant changes only in the case of Fe, which increased significantly in the distal treated part (Table 3.1). Also, the basal untreated part of fertilized leaves had slight Fe increases when compared to the basal part of untreated leaves, although the differences were not significant. In the case of Mn, there was a significant difference between the distal and basal areas, both in Fe-treated and control leaves, and Fe-fertilization did not have any significant effect. Copper and Zn concentrations were similar in all samples.

In the sugar beet experiment, results were quite different from those obtained in the peach tree experiment (Table 3.2). Nitrogen concentrations were similar in all samples, with the exception of the distal parts of Fe-sufficient plants, which were higher than the

rest. Phosphorus concentrations were higher in the distal and basal leaf parts of the Fe-sufficient plants than in the rest of samples. No significant differences in K concentrations were found. In the case of Ca, the concentration was higher in the Fe-deficient leaves (distal and basal parts) than in the Fe-sufficient controls, and the concentration increased (although not significantly at $p \leq s\ 0.05$) upon Fe fertilization. The highest Ca and Mg concentrations were found in the distal part of treated leaves.

Table 3.1 *Concentrations of macro (N, P, Ca, Mg and K; in % DW) and microelements (Fe, Mn, Cu and Zn; in µg g^{-1} DW) in distal and basal parts of Fe-fertilized and Fe-deficient, untreated peach tree leaves, 8 weeks after the first treatment with 2 mM $FeSO_4$ and 0.1% surfactant.*

	Fe-deficient		Fe-fertilized	
	Distal part	**Basal part**	**Distal part**	**Basal part**
N	3.78±0.20a	3.46±0.18a	3.88±0.23a	3.29±0.23a
P	0.24±0.01a	0.23±0.01a	0.22±0.02a	0.22±0.01a
Ca	2.97±0.22a	3.54±0.33a	3.11±0.22a	3.64±0.33a
Mg	0.97±0.04a	0.91±0.03a	0.93±0.03a	0.88±0.33a
K	2.87±0.08a	2.91±0.10a	2.79±0.09a	2.89±0.07a
Fe	126.0±15.3b	103.1±7.3b	176.7±16.4a	126.7±16.9b
Mn	67.5±3.8b	89.4±6.1a	70.8±6.4b	92.8±5.4a
Cu	15.6±2.0a	15.0±2.4a	15.3±1.7a	14.9±2.3a
Zn	28.8±1.5a	26.4±1.5a	28.8±1.8a	27.9±1.6a

Data are means ± SE (n =11 trees, 3 in 2009, 4 in 2010 and 4 in 2011). Values followed by the same letter within the same row were not significantly different (Duncan test) at the $p \leq 0.05$ level.

Iron concentrations in sugar beet leaves increased upon fertilization, although differences were significant only in the case of the distal treated part (at $p \leq 0.10$) (Table 3.2). Iron concentrations after fertilization were still lower than those found in leaves of green, sufficient plants. On the other hand, Fe concentrations in the distal leaf part were generally higher than those in the basal part. In the case of Mn, values found were higher in fertilized leaves, although not as high as in green Fe-sufficient plants. On the other hand, Mn concentrations in the distal part were generally higher than those in the basal part. In the case of Cu, concentrations decreased with Fe fertilization, especially in the distal part. Finally, Zn concentrations were little affected by Fe fertilization, and the concentrations in Fe-sufficient plants were always much higher than those in Fe-deficient materials (especially in the distal leaf part).

Table 3.2 *Concentrations of macro (N, P, Ca, Mg and K; in %DW) and microelements (Fe, Mn, Cu and Zn; in µg g^{-1} DW) in distal and basal parts of Fe-fertilized, untreated Fe-deficient and green Fe-sufficient sugar beet leaves, 7 d after the first treatment with solution containing 2 mM $FeSO_4$ and 0.1% surfactant.*

	Fe-deficient		Fe-fertilized		Fe-sufficient	
	Distal part	**Basal part**	**Distal part**	**Basal part**	**Distal part**	**Basal part**
N	3.69±0.12b	3.54±0.32b	3.24±0.16b	3.33±0.16b	5.34±0.13a	4.00±0.42b
P	0.34±0.04c	0.28±0.03c	0.19±0.02c	0.25±0.03c	1.04±0.28a	0.74±0.07b
Ca	6.69±0.54ab	5.77±0.16b	7.43±0.55a	6.73±0.36ab	2.09±0.08c	2.08±0.08c
Mg	2.13±0.25ab	2.02±0.21b	2.75±0.29a	2.42±0.19ab	2.16±0.08ab	1.81±0.06b
K	4.78±0.41a	4.30±0.17a	4.97±0.43a	4.89±0.28a	4.93±0.31a	4.40±0.06a
Fe	145.8±11.7bc	104.3±16.0c	207.0±15.0ab	135.3±14.8bc	265.±48.4a	151.1±22.7bc
Mn	135.9±23.0b	73.5±13.4c	161.5±8.0b	111.4±18.2bc	226.2±13.4a	126.1±20.4bc
Cu	19.0±3.6b	13.4±1.9bc	9.6±1.5c	10.6±1.5c	34.4±5.8a	17.7±2.8bc
Zn	23.6±1.5c	27.6±1.9c	20.8±1.5c	18.5±1.5c	110.4±12.3a	61.5±11.2b

Data are means ± SE (n=8 plants). Values followed by the same letter within the same row were not significantly different (Duncan test) at the p<0.05 level.

Pigment concentrations in peach tree and sugar beet leaves

In peach trees, the concentrations per area of all pigments, with the exception of zeaxanthin (Z), showed increases in the treated distal area of fertilized leaves 8 weeks after the first foliar application (Table 3.3). The increase was largest in the case of Chl *b*, Chl *a* and total Chl (2.6-, 2.4- and 2.4-fold, respectively), and less important in the case of the carotenoids neoxanthin, lutein and β-carotene (84-87%). The pool of violaxanthin (V) cycle pigments (V+A+Z) increased by 54%, mostly due to a 74% increase in V. The concentration of photosynthetic pigments in the basal leaf part did not change after Fe fertilization. On the other hand, the pigment concentrations in the distal part of untreated peach leaves were slightly higher (12-21%) than those in the corresponding basal leaf parts.

The Chl *a* /Chl *b* ratio was 3.7 and 3.9 in distal and basal parts of Fe-deficient leaves, respectively, and decreased to 3.2 and 3.5 in distal and basal parts of the Fe-fertilized leaves, respectively (Table 3.3). Changes upon Fe fertilization were also found in the V+A+Z cycle. The proportion of the epoxidized form V in the pool (V+A+Z) increased

from 0.56-0.59 in the untreated controls to 0.76 in the Fe treated area, whereas the proportion of A+Z decreased from 0.43-0.44 to 0.24.

Table 3.3 *Concentrations of photosynthetic pigments (in μmol m^{-2}; neoxanthin, lutein, V+A+Z, β-carotene, Chl a and Chl b) in distal and basal parts of Fe-fertilized and Fe-deficient untreated peach tree leaves, 8 weeks after the first treatment with a solution containing 2 mM $FeSO_4$ and 0.1% surfactant.*

	Fe-deficient		**Fe-fertilized**	
	Distal part	**Basal part**	**Distal part**	**Basal part**
Chl *a*	82.4±3.4 b	73.6±4.0 b	197.7±7.7 a	77.8±6.5 b
Chl *b*	24.5±1.8 b	20.1±1.4 b	63.3±2.9 a	25.0±3.2 b
Chl total	106.9±4.9 b	93.7±5.2 b	261.0±10.5 a	102.8±8.5 b
Neoxanthin	7.3±0.3 b	6.3±0.3 b	13.7±0.7 a	6.5±0.5 b
Lutein	17.2±0.7 b	14.7±0.7 b	31.6±1.8 a	15.5±1.0 b
β-carotene	17.1±0.7 b	14.7±0.6 b	31.3±1.3 a	15.0±1.1 b
(V+A+Z)	21.3±1.2 b	20.2±1.1 b	32.7±2.3 a	18.7±1.6 b
Chl *a*/Chl *b*	3.7±0.1 a	3.9±0.1 a	3.2±0.0 b	3.5±0.2 a
(A+Z)/(V+A+Z)	0.40±0.04 a	0.44±0.04 a	0.24±0.04 b	0.43±0.05 a
V/(V+A+Z)	0.59±0.04 b	0.56±0.04 b	0.76±0.04 a	0.57±0.05 b

Data are means ± SE (n=8 trees, 4 disks per tree, 4 each in 2010 and 2011). Values followed by the same letter within the same row were not significantly different (Duncan test) at the p<0.05 level.

In sugar beet, the foliar Fe treatment also led to an increase in the concentration of photosynthetic pigments in the distal treated leaf area (Table 3.4). The increase was largest in the case of β-carotene, Chl *b* and Chl *a*, (8.8-, 6.4- and 6.0-fold, respectively), and less important in the case of neoxanthin and lutein (4.8- and 4.6-fold, respectively). All pigment values found after fertilization was still lower (ca. 44-76%) than those found in leaves of Fe-sufficient plants. Increases in pigments were also found in the basal treated leaf parts (especially in the case of Chl *b*), although the differences were not statistically significant at $p \leq 0.05$. On the other hand, the pigment concentrations in the distal part of untreated sugar beet leaves were quite similar to those in the corresponding basal leaf parts.

On the other hand, the Chl *a*/Chl *b* ratio did not decrease after the Fe treatment in the distal treated parts, but showed decreases in the basal part (from 5.1 to 3.2) (Table 3.4). The V/(V+A+Z) ratio increased after the Fe treatment both in the basal and distal part,

and in the latter case values became close to the ratio found in the green leaves. The highest value of the (Z+A)/(V+A+Z) ratio was found in the chlorotic leaves and the lowest in the green leaves. This ratio decreased markedly in the distal treated leaf part after the Fe treatment, and also showed decreases, although to a lower extent, in the basal untreated one.

Table 3.4 *Concentrations of photosynthetic pigments (in μmol m^2; neoxanthin, lutein, V+A+Z, β-carotene, Chl a and Chl b) in distal and basal parts of Fe-fertilized, Fe-deficient untreated and Fe-sufficient green sugar beet leaves.*

	Fe-deficient		**Fe-fertilized**		**Fe-sufficient**	
	Distal part	**Basal part**	**Distal part**	**Basal part**	**Distal part**	**Basal part**
Chl *a*	33.0±1.4b	33.8±2.0b	199.4±28.1a	57.5±12.0b	263.8±27.8a	272.0±46.0a
Chl *b*	8.5±0.4b	6.6±0.2b	54.4±11.6a	20.4±6.2b	83.5±10.8a	82.52±12.9a
Chl total	41.5±1.0b	40.4±2.2b	253.9±39.3a	77.9±16.7b	347.3±37.4a	354.6±58.8a
Neoxanthin	1.4±0.3c	1.8±0.2c	6.7±1.4b	2.1±0.3c	15.3±3.7a	14.1±1.8a
Lutein	5.5±0.8c	7.1±1.0c	25.5±0.6b	9.5±6.6c	54.0±8.8a	44.6±8.4a
β-carotene	2.4±1.2c	2.4±0.8c	21.1±3.8b	5.3±0.6c	41.1±9.4a	30.6±5.6b
(V+A+Z)	8.2±1.3b	10.4±1.3b	14.5±1.7b	10.2±2.0b	27.9±5.9a	22.5±4.1a
Chl *a*/Chl *b*	3.9±0.4b	5.1±0.2a	3.8±0.3b	3.2±0.6b	3.2±0.2b	3.3±0.8b
(Z+A)/(V+A+Z)	0.78±0.5a	0.77±0.04a	0.16±0.08c	0.57±0.14b	0.04±0.01c	0.02±0.01c
V/(V+A+Z)	0.23±0.03c	0.23±0.05c	0.84±0.03a	0.43±0.09b	0.96±0.02a	0.98±0.01a

Data are means ± SE (n = 4 plants). Values followed by the same letter within the same row were not significantly different (Duncan test) at the p ≤ 0.05 level.

Localization of iron by Perls-DAB stain in peach tree leaves

The Perls-DAB staining method indicates the localization of Fe with a dark color. In control, foliar Fe-fertilized and soil Fe-fertilized samples, Fe was located in most leaf parts, with a lower intensity in the upper epidermal layer (Fig. 3.5A, C and E). In Fe-deficient and the basal untreated part of Fe-fertilized leaves, Fe was mainly accumulated in vascular tissues and to a minor extent in the parenchymal areas (Fig. 3.5B and D).

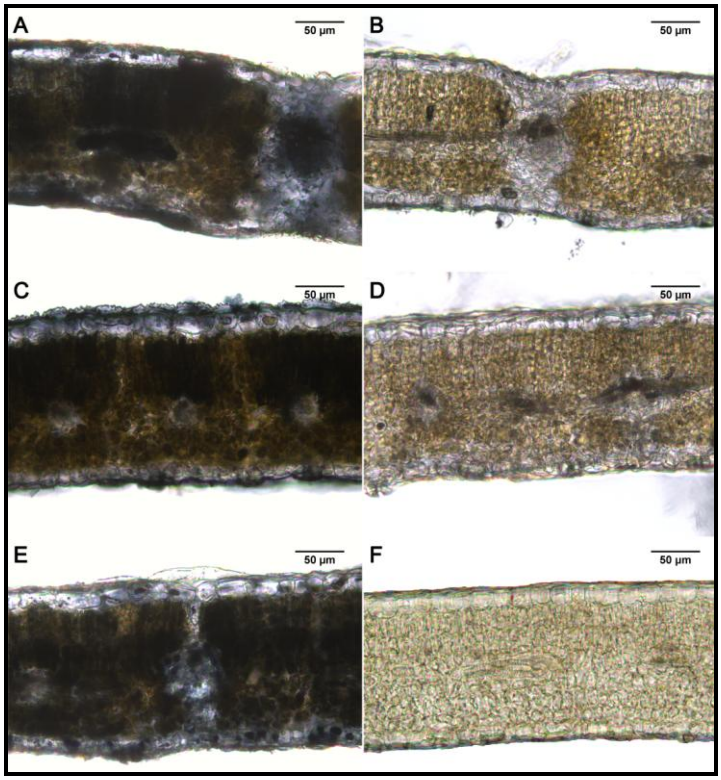


Figure 3.5 *Iron staining (Perls-DAB) in leaf peach tree transversal sections: (A) Fe-sufficient control; (B) Fe-deficient chlorotic; (C) distal treated leaf part (2 mM $FeSO_4$ with 0.1% surfactant); (D) basal untreated leaf part in the same leaves used for C; (E) leaves of a soil fertilized tree (Sequestrene, 50 g/tree); and (F) negative control.*

Leaf structure and localization of Fe (LT-SEM-EDX) in peach tree leaves

Leaf tissue structural information of the different layers, including adaxial epidermis, palisade parenchyma, xylem vessels, spongy parenchyma and abaxial epidermis, was obtained using LT-SEM of cryo-fractured peach leaves (Fig. 3.6). Generally speaking, chlorotic leaves had a lower total thickness with a more compact mesophyll tissue (Fig. 3.6B) when compared to the green ones (Fig. 3.6A, C).

The distribution of the relative Fe signal in the leaf-cross sections by EDX analysis is also shown in Fig. 3.6 (right panels). Iron signals were more intense in leaf sections of control and Fe-fertilized samples (Fig. 3.6A, C and D) than in those of Fe-deficient and foliar untreated ones (Fig. 3.6B and E). Also, the Fe signal in the untreated area of the half treated leaves was slightly more intense than in the Fe-deficient leaves. All these data are in general agreement with the leaf Fe concentrations shown in Table 1.

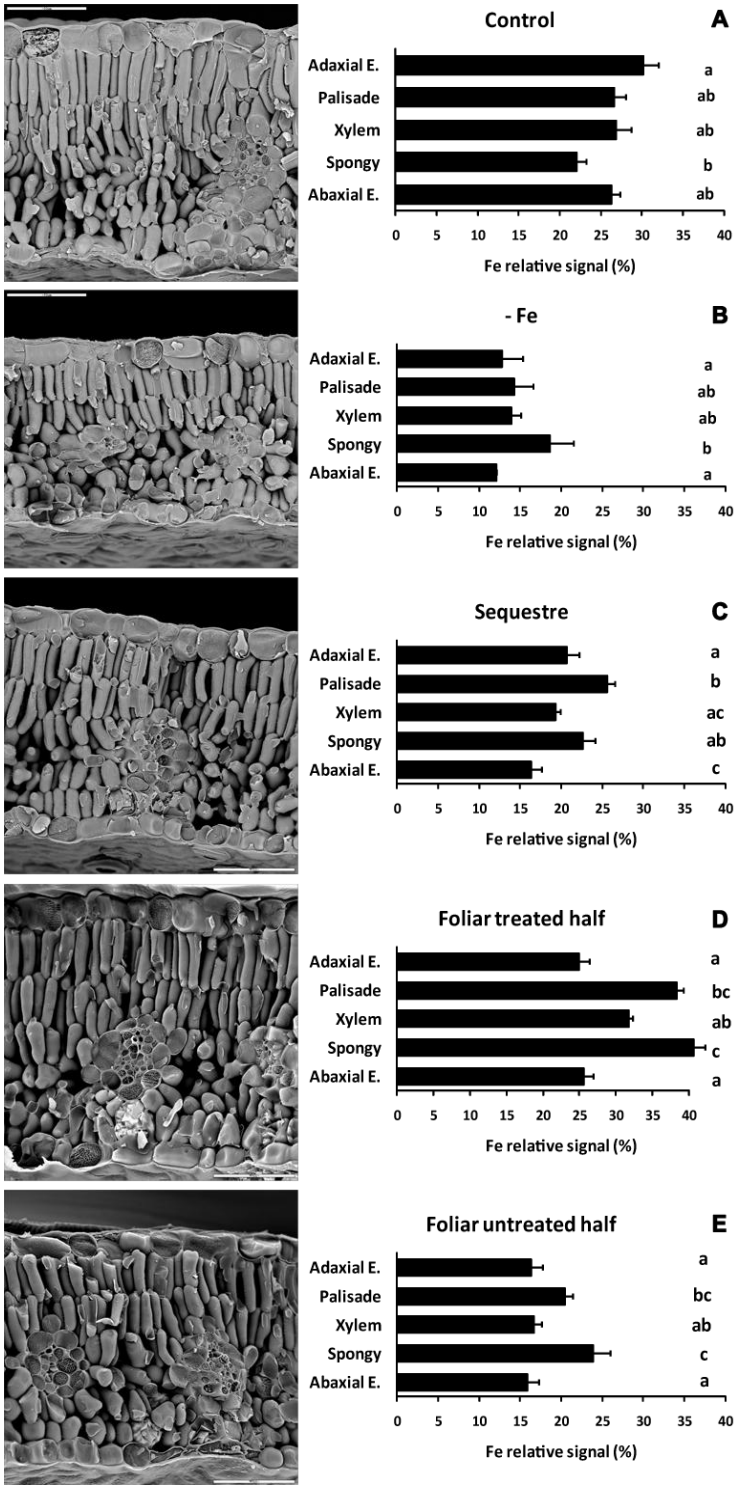


Figure 3.6 *LT-SEM micrographs (left panels) and EDX analysis (spot mode, right panels) of transversal sections obtained by cryo-fracture from peach tree leaves: (A) Fe-sufficient control; (B) Fe-deficient chlorotic; (C) soil fertilized (Sequestrene, 50 g per tree); (D) distal Fe-treated leaf part (2 mM $FeSO_4$ with 0.1% surfactant); and (E) basal untreated leaf part in the same leaves used for D. Relative Fe signals are means (± SE). Significant differences among plant tissues are indicated by different letters ($p \leq 0.05$; $n = 8$). Bars in the images are 50 µm.*

In Fe-deficient leaves, Fe was more abundant in the spongy parenchyma in comparison with the other leaf tissues (Fig. 3.6B), whereas in control leaves Fe was more abundant in both epidermal layers and somewhat lower in spongy parenchyma (Fig. 3.6A). In the distal sections of Fe-fertilized leaves, more intense Fe signals were present in palisade and spongy parenchyma and to a lower extent in the xylem area, and this occurred both after soil (Fig. 3.6C) and foliar Fe-fertilization (Fig. 3.6D). Also, some increases in the intensity of the Fe signal occurred in the palisade and spongy parenchyma in the basal untreated leaf part (Fig. 3.6E).

Chl fluorescence in peach tree leaves

Chl fluorescence was measured in peach leaves one week after the first foliar Fe application. Measurements were done (in triplicate) in different leaf areas (marked in red in Fig. 3.8) of the severely chlorotic untreated (A), Fe-deficient untreated (B), Fe-sufficient (C) and Fe-fertilized (D-F) leaves (Fig. 3.7). Parameters measured included F_V/F_M, Φ_{PSII}, qP and NPQ, and numerical values shown in Table 5 are means ± SE of the values obtained in the different measurement areas.

Images in Fig. 3.8 are typical of those obtained in the different treatments for F_V/F_M. The image in Fig. 3.8A is from a severely deficient leaf, which had very low Chl concentrations and also a low F_V/F_M ratio (Table 5). Images from Fe-deficient and Fe-sufficient controls indicate some differences in F_V/F_M values visible in the picture (Fig. 3.8B and C, respectively) but not statistically different at $p \leq 0.05$ (Table 5). In all Fe-deficient leaves, areas close to the veins had a higher F_V/F_M ratio than interveinal areas (Fig. 3.8A and B). One week after the treatment, the more distal areas show F_V/F_M ratios similar to those of the Fe-sufficient controls, whereas the distal area near the treatment line border had slightly lower ratios. In the basal untreated part, F_V/F_M ratios decreased progressively from the treatment line border.

Concerning Φ_{PSII}, it was lower in the severely Fe-deficient leaves than in moderately Fe-deficient and Fe-sufficient ones (Table 5). Upon Fe resupply, the distal treated parts showed an increase of Φ_{PSII} values. A small increase in this parameter was also observed in the basal part close to the treatment border. In the case of qP, values were higher in the Fe-deficient leaves than in the Fe-sufficient one. Upon Fe resupply, values decreased slightly in all areas. In the case of NPQ, Fe-deficient leaves had lower values

than the Fe-sufficient one. Upon Fe resupply, all leaves maintained low NPQ values, that were especially low in the more basal area.

Figure 3.7 *Peach tree leaves used for the Chl fluorescence measurements. (A) Severely chlorotic leaf, with a very advanced chlorosis status, taken from the distal part of the shoot; (B) Fe-deficient leaf taken at the 4-5th position in the shoot, one week after treatment by dipping the upper half of the leaf in a solution containing 2 mM $FeSO_4$ and 0.1% surfactant; (C) Positive control: Fe-sufficient leaves taken in the same position in the shoot but from a Fe-sufficient tree; (D) distal part of an Fe-treated leaf; (E) middle part of an Fe-treated leaf, showing the black line delimiting the treatment area; and (F) basal part of an Fe-treated leaf.*

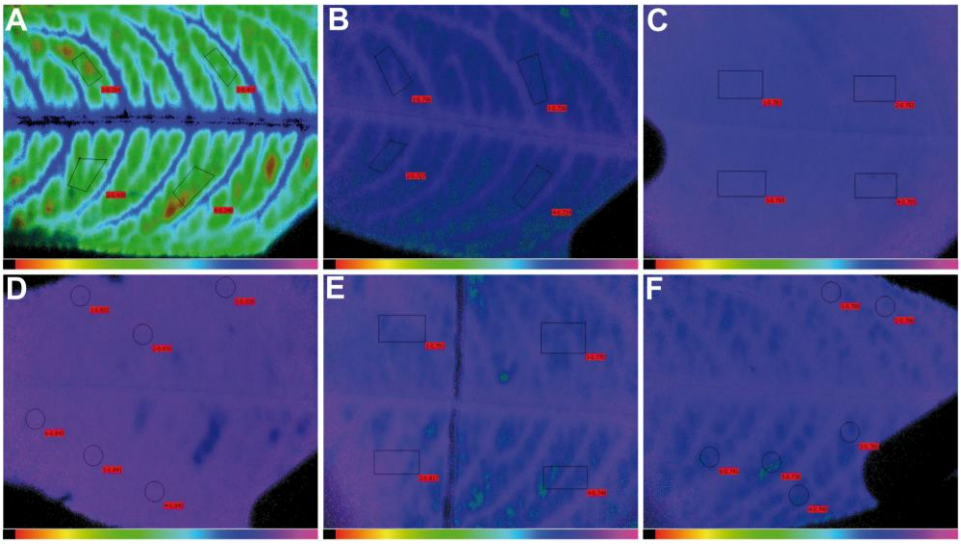


Figure 3.8 *Images showing the difference in maximum quantum yield in dark adapted samples (F_V/F_M): (A) a severely Fe-deficient leaf, having 61 µmol Chl m^{-2}; (B) an Fe-deficient leaf, having 95 µmol Chl m^{-2}; (C) an Fe-sufficient leaf having 370 µmol Chl m^{-2}; (D) distal part of a Fe-treated leaf; (E), middle part of an Fe-treated leaf, showing the black line delimiting the treatment area; and (F) basal part of an Fe-treated leaf. Areas measured are marked in red.*

Table 3.5 *Chl fluorescence parameters (F_V/F_M, Φ_{PSII}, qP and NPQ) in severely Fe-deficient, Fe-deficient, distal treated and basal untreated areas of fertilized leaves, and Fe-sufficient leaves.*

	Severely chlorotic	Fe-deficient	Fe-fertilized				Fe-sufficient
			More distal part	Distal part	Basal part	More basal part	
Fv/Fm	0.61±0.05d	0.74±0.01bc	0.82±0.01a	0.80±0.01ab	0.77±0.01abc	0.71±0.01c	0.80±0.01ab
Φ_{PSII}	0.38±0.04c	0.51±0.01ab	0.55±0.01a	0.55±0.00a	0.54±0.01a	0.48±0.02b	0.49±0.02ab
qP	0.80±0.01a	0.78±0.02ab	0.73±0.01bc	0.76±0.01abc	0.79±0.01b	0.74±0.02bc	0.71±0.02c
NPQ	0.16±0.01b	0.13±0.01b	0.14±0.01b	0.15±0.01b	0.14±0.01b	0.09±0.01c	0.20±0.02a

Data are means ± SE (n =12, 4 areas of interest in each of the 3 leaves). Data followed by the same letter within the same row are not significantly different (Duncan test) at the $p \leq 0.05$ level.

Discussion

Foliar Fe treatments were effective at the site of application both in peach trees grown in the field and in sugar beet grown in hydroponics. Application of 2 mM $FeSO_4$ to the distal parts of peach tree and sugar beet leaves caused similar increases in the Fe concentrations in the treated parts (41-42%). Iron entered most of the leaf tissues, as shown by Perls stain, with the increases being large in palisade and spongy parenchima and vascular tissues, as indicated by LT-SEM-EDX. The leaf entrance of Fe resulted in significant leaf re-greening, confirming data found in previous studies with peach trees (Fernández et al. 2006, Fernández et al. 2008). Increases in Chl were already significant at the first sampling dates after the treatment, 1 d in sugar beet and 1 week in peach trees. This kinetics in the time course re-greening is also in good agreement with previous data for sugar beet (Larbi et al. 2004) and peach trees (El-Jendoubi et al. 2011). At the end of the experiment, Chl had increased, when compared to the initial leaf Chl concentrations, by approximately 70% in peach and 1.7-fold in sugar beet. In previous studies with peach and pear trees the Chl increases after foliar Fe fertilization were 95 (Fernández et al. 2006) and 275% (Álvarez-Fernández et al. 2004), respectively. Regarding the relative increases in photosynthetic pigments, the increases were in the order Chl *b* > neoxanthin > Chl *a* > β-carotene > lutein in peach tree and Chl

a > Chl *b*> lutein > β-carotene > VAZ > neoxanthin in sugar beet. These changes were accompanied by decreases in the (Z+A)/(V+A+Z) ratio in both species, as well as in small increases in F_V/F_M. Iron deficiency has been shown to induce decreases in F_V/F_M and ΦPSII in sugar beet, peach and pear (Nedunchezhian et al. 1997, Abadía et al. 1999, Morales et al. 2000), and similar changes in photosynthetic pigments and Chl fluorescence after Fe-resupply to the nutrient solution were reported to occur in sugar beet by Larbi et al. (2004).

Foliar Fe treatments also had some effects in the basal, untreated leaf parts. Application of $FeSO_4$ to the distal parts of peach tree and sugar beet leaves caused similar increases in the Fe concentrations in the untreated parts (23-30%, respectively; significant only at $p \leq 0.10$). The use of LT-SEM-EDX suggested that some Fe entered the palisade parenchyma, although the Perls stain also suggested an increase of Fe in some vascular areas. This small Fe increase is unlikely to result from surface mass flow movement of Fe compounds at the moment of application, because all treated leaf surfaces dried within a few minutes. The leaf entrance of Fe, however, resulted in only minor leaf re-greening. Regarding the relative increases in photosynthetic pigments, the only changes noticed in the basal untreated part was a decrease in the (Z+A)/(V+A+Z) ratio and a decrease in the Chl *a*/Chl *b* ratio when compared to the untreated controls.

Previous results indicating that Fe foliar fertilization could lead to a switch in nutrient composition in peach tree leaves, from a high (K–N–P)/low (Ca–Mg) to a high (Ca–Mg)/low (K–N–P) state (Fernández et al. 2008), were not confirmed in the present study. The origin of this discrepancy is unclear, although in the Fernandez et al. (2008) study, nutrient concentrations used were the average of those found with several foliar Fe-treatments, using $FeSO_4$ and other Fe-containing formulations.

The changes in Chl fluorescence parameters found with Fe deficiency and Fe-resupply in this study were less marked than those found in previous studies (Morales et al. 1994, Nedunchezhian et al. 1997, Abadía et al. 1999, Morales et al. 2000). The differences found in many parameters between previous studies and this one could be assigned to the differences in the Chl fluorescence devices used. For instance, comparisons made in a wide range of Chl concentrations in Fe-deficient sugar beet showed that lower F_V/F_M values were found with the PAM-2000 device (used in earlier works) than with the imaging-PAM (this study) (not shown). There are examples in the

literature reporting significant differences in Chl fluorescence parameters, depending on the device used (Peguero-Pina et al. 2009). Iron-deficient peach leaves in the present study had F_V/F_M values of approximately 0.6-0.7, similar to those obtained in previous works using the PAM-2000 device (Larbi et al. 2006). However, this similarity is only apparent, because with the PAM-2000 it is possible to use a protocol that includes a far-red (FR) pre-illumination after the dark-adaptation of leaves, and this causes increases in the F_V/F_M values of Fe-deficient leaves (Belkhodja et al. 1998). Unfortunately, with the imaging-PAM it is not possible to use FR pre-illumination. In any case, changes found in most parameters with Fe-deficiency and resupply had a similar trend, with parameters approaching values found in the controls in distal treated areas and also basal areas close to the application, although in the latter case to a lesser extent. The case of qP merits a brief commentary, since although there was no significant difference between the qP values in both parts of the treated leaves, values were always high. The highest qP value (0.80) was found in severely chlorotic peach leaves (0.80), and the lowest one in the green leaves (0.71). A similar result was obtained in an earlier work with sugar beet (Morales et al. 1998).

In summary, the application of a foliar fertilizer containing $FeSO_4$ was effective enough at the leaf treated surface, both in peach trees grown in the field and sugar beet grown in hydroponics. Iron was incorporated in the leaves and the re-greening was very marked. The effects of the foliar fertilizer, however, were very minor outside the leaf surface treated, with lateral Fe movement in the leaf suffering major restrictions. New formulations should be aimed to extend the reach of the Fe fertilizers beyond the treated surface, although new knowledge on the Fe mobilization pathways in the leaf will be necessary to reach this goal.

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Using the xylem sap composition as a tool to study iron deficiency in field grown peach trees

Abstract

Background and Aims

Xylem, the main conduit for water and minerals from roots to the aerial plant parts, also transports organic solutes, including carbohydrates, amino acids, organic acids, proteins, hormones and other signal molecules. The aim of this study was to set up a reliable protocol for obtaining sufficient amounts of peach tree xylem sap from field grown trees to characterize the metabolite and protein composition changes with Fe chlorosis.

Methods

Xylem sap was obtained using a Schölander chamber from peach tree shoots. The concentration of Fe and the pH were measured in Fe-sufficient, Fe-deficient, and Fe-fertilized trees. Changes in the metabolite and protein profiles were studied using gas chromatography-mass spectrometry (GC-MS) and a gel based 2D approach, respectively.

Results

Xylem sap Fe concentrations in Fe-deficient plants were in the 2-10 µM range, whereas pH values were in the 5.6-6.7 range. Iron concentrations and pH were found to change with sampling time. Soil Fe-fertilization led to increases in xylem sap Fe in the short term but did not cause pH changes. Metabolomics techniques were successfully applied, and a number of metabolites changing in relative amount with Fe deficiency were identified. Multivariate analysis was able to separate adequately xylem sap samples from Fe-deficient and Fe-sufficient trees. Reproducible xylem sap protein profiles were also successfully obtained with a 2D gel-based proteomics approach.

Conclusions

The present study provides the basis for further studies on the characterization of the fruit tree xylem sap composition and the changes in such composition with nutrient deficiencies.

Introduction

The movement of solutes from roots to the aerial parts of the plant takes place in the tracheary elements of the xylem. Xylem is traditionally considered as the main conduit for water and minerals (Evert 2006). However, the xylem sap also contains organic solutes, including carbohydrates, amino acids, organic acids, hormones and other metabolites, as well as proteins (Satoh 2006).

The interaction between different plant organs is essential to coordinate growth, development and defense reactions, because plants are immobile and need to cope with changes occurring in their environment (Oda et al. 2003). The communication between roots and shoots is mediated by signal molecules, which are supplied from the root system *via* xylem (Dodd 2005) and whose concentrations change in case of biotic or abiotic stresses (Buhtz et al. 2010, Cánovas et al. 2004, Kehr et al. 2005).

One of the most prevalent abiotic stresses in crops in the Mediterranean region is Fe-deficiency (El-Jendoubi et al. 2011, Abadía et al. 2011). Plant adaptation to Fe shortage not only includes an increase in the mechanisms involved in root Fe uptake from the soil, but also involves different metabolic changes occurring at the root, xylem, leaf and fruit levels. In roots, there are increases in the activities of phospho*enol*pyruvate carboxylase (PEPC) (Andaluz et al. 2002) and several enzymes of the glycolytic pathway and the tricarboxylic acid (TCA) cycle (Brumbarova et al. 2008, Herbik et al. 1996, Li et al. 2008, Rellán-Álvarez et al. 2010a, Rodriguez-Celma et al. 2011). The increased anaplerotic C fixation mediated by PEPC leads to an accumulation of organic acids (Abadía et al. 2002), that may play important roles in the transport of Fe and C to the leaf *via* xylem (López-Millán et al. 2000, Rellán-Álvarez et al. 2010a).

Xylem sap and leaf apoplastic fluid organic acid concentrations are markedly increased with Fe deficiency in several plant species (Jiménez et al. 2007, Larbi et al. 2003b, López-Millán et al. 2001b, López-Millán et al. 2009, López-Millán et al. 2000). At the leaf level, the most characteristic Fe-deficiency symptom is the yellow color of young leaves, caused by a relative enrichment in carotenoids (Abadía, 1992), associated to changes in the light-harvesting pigment-protein complex composition (Abadía 1992, Laganowsky et al. 2009, Larbi et al. 2004, Timperio et al. 2007). Iron deficiency-induced leaf chlorosis leads to reduced photosynthetic efficiency and electron transport, with less C being fixed *via* photosynthesis (Abadía 1992, Larbi et al. 2006). In fruits, Fe

deficiency leads to changes in maturity and chemical composition, depending on the availability of C (Álvarez-Fernández et al. 2011).

Changes in plant metabolism occurring shortly after Fe resupply have been only partially characterized. Whereas Fe resupply leads to rapid (within 3-6 h) increases in the concentration of Fe in the xylem sap (Orera et al. 2010, Rellán-Álvarez et al. 2010), significant increases in leaf chlorophyll concentrations and photosynthetic rates has only been observed after one or two days in controlled environments or one week in the field (Larbi et al. 2004, Larbi et al. 2003, Timperio et al. 2007). Also, Fe-resupply, either to leaves or to roots, leads to a rapid (within 24 h) de-activation of transcripts associated to root Fe acquisition mechanisms, including *FRO* and *IRT* (Abadía et al. 2011, Enomoto et al. 2007, López-Millán et al. 2001c), whereas the activities of FRO and PEPC decrease more slowly (Abadía et al. 2011, Enomoto et al. 2007, López-Millán et al. 2001c). Xylem sap and leaf apoplastic carboxylate concentrations decrease progressively after Fe resupply in Fe-deficient sugar beet plants (Larbi et al. 2010). In roots, organic acid concentrations and metabolite profiles reach control levels only within a few days after Fe-resupply (Abadía et al. 2011, Rellán-Álvarez et al. 2010). Also, Fe resupply leads to progressive decreases in the concentration of organic acids in the whole plant (López-Millán et al. 2001a, López-Millán et al. 2001c).

Relatively little information is available about the effects of Fe deficiency on xylem sap composition of fruit trees. In peach tree xylem sap, small increases in malate concentration with Fe deficiency were reported in a preliminary study (Chatti 1997). The changes in apoplastic fluid composition with Fe deficiency have been studied in pear trees (López-Millán et al. 2001b). Also, increases in xylem sap Fe concentrations and decreases in organic acid concentrations after placing solid implants containing Fe sulfate in branches of Fe-deficient pear and peach trees were reported (Larbi et al. 2004). The proteomic profiles of xylem sap have only been studied in herbaceous plants such as tomato (Rep et al. 2003), cucumber (Masuda et al. 2001) and maize (Alvarez et al. 2006, 2008).

The aim of this study was to test the hypothesis that Fe deficiency may cause consistent changes in the xylem sap metabolite and protein profiles in peach trees. "Omics-" technologies have been recently applied in Fe-deficiency studies, focusing mainly on whole plants, whole roots and isolated thylakoid membranes: these include transcriptomic (Buhtz et al. 2010, Thimm et al. 2001, Yang et al. 2010) and proteomic

studies (Andaluz et al. 2006, Brumbarova et al. 2008, Donnini et al. 2010, Gollhofer et al. 2011, Herbik et al. 1996, Laganowsky et al. 2009, Lan et al. 2010, Li et al. 2008, Li and Schmidt 2010, Rellán-Álvarez et al. 2010b, Rodriguez-Celma et al. 2011, Timperio et al. 2007). Recently, a study combining metabolomics and proteomics has been published using sugar beet roots (Rellán-Álvarez et al. 2010b). In the present Thesis chapter, xylem was obtained from peach trees grown in Fe-sufficient and Fe-deficient conditions in the field, and the Fe concentrations, pH and metabolite and protein profiles were characterized. Metabolomics data obtained with this approach have been included in a recent paper (Rellán-Álvarez et al. 2011), whereas proteomics data are still being prepared for publication.

Material and Methods

Field plant material

Two peach tree orchards were used. In 2008 and 2009, an orchard located in Peñaflor (Zaragoza province, Spain; 41º46'42.65''N and 0º47'38.70''O) was used. The orchard had 14 year-old peach trees (*Prunus persica* (L.) Batsch, cv. 'Catherina' grafted on 'GF677' rootstock, with a frame of 2.5 × 6 m and grown on a flood-irrigated calcareous soil. In summer 2010, a different peach tree orchard, selected near the village of Plasencia de Jalón (Zaragoza province, Spain; 41°40'27.72"N, 1°13'33.46"O) was used. Trees were 16 year-old, cv. 'Miraflores' on GF677 rootstock, with a frame 5 x 4 and grown on a flood-irrigated calcareous soil. Normal fertilization practices were used, with the exception of Fe fertilization, which was totally excluded from the grower treatments in the selected trees. The orchards were appropriately maintained in terms of nutrition, pruning and pest and disease control. Trees did not receive any Fe fertilization for two years prior to the beginning of the trial. Tree Fe status was monitored by estimating leaf Chl concentration with a hand-held Chl meter (SPAD-502, Minolta Corp., Ramsey, NJ) using leaves 3rd and 4th from the shoot tip. All SPAD data were measured at xylem sampling time.

In July 2008, three trees with no chlorosis symptoms in the springtime season but with a slight chlorosis at the sampling time (control trees, +Fe, with SPAD 18-32; Fig. 4.1A) and two trees with severe Fe-deficiency symptoms (-Fe, with SPAD 9-17; Fig. 4.1B) were selected. Seven current year shoots (25-30 cm in length) were taken from each tree in July 2008 between 7:00 and 8:00 AM solar time. Then, shoots were

protected with a wet paper towel and immediately brought to the laboratory for xylem extraction. Xylem samples were used for metabolomics analysis (see below).

In the summer of 2009, xylem sap extraction was carried out from shoots sampled in the same orchard. Two trees considered as Fe-deficient received a soil Fe(III)-EDDHA (50 g per tree) treatment, whereas another Fe-deficient tree did not receive any Fe fertilization. These xylem samples were used to set up the proteomics analysis.

Figure 4.1. *Peach trees appearance at sampling time in Peñaflor, Zaragoza, Spain. (A): tree grown in Fe-sufficient conditions; (B): tree grown in Fe-deficient conditions.*

Figure 4.2. *Appearance of the second peach tree orchard in Plasencia de Jalón, Zaragoza, Spain. (A): summer 2009; (B): summer 2010; (C) summer 2011.*

In 2010 two experiments were carried out in the Plasencia de Jalón orchard (Fig. 4.2). The aim of the first experiment was to characterize the changes in xylem sap Fe concentrations, pH and protein profiles during the season in Fe-sufficient and Fe-deficient trees. A second experiment was aimed to study changes in xylem sap Fe concentrations, pH and protein profiles after soil Fe fertilization with Fe(III)-EDDHA. In these two experiments, eighteen trees were chosen at the beginning of the trial, six of them without Fe-deficiency symptoms (Fe-sufficient) and 12 with deficiency symptoms (Fe-deficient) and similar SPAD values (11-14 SPAD units). The Fe-deficient trees

were divided in two groups, with six being used as Fe-deficient controls and the remaining six trees being treated with soil-applied Fe (III)-EDDHA (50 g per tree).

In the case of the experiment on xylem changes during the season, two shoots (current-year shoots without fruits) were taken for xylem sap extraction from six Fe-deficient and six Fe-sufficient trees. Shoots were cut at 7:00-8:00 AM solar time, and had similar SPAD values, position in the tree (1.5-2.0 m high), length (25-30 cm) and external diameter (3-4 mm). These parameters were the optimal found in previous experiments in 2008 and 2009. Xylem sap sampling was done in June, July and August. Sampling was carried out in three consecutive sampling days; June 1, 2 and 3, July 14, 15 and 16 and August 31, September 1 and 2. In each of these days, two shoots were taken for xylem sap extraction from each of the twelve trees. Therefore, six shoots in total were taken from each of the twelve trees in the three-day sampling period. A sample of 30 leaves per tree was collected for mineral analysis at the three sampling datea (young, fully developed leaves, located in the position 4^{th}-5^{th} from the top).

In the case of the experiment on Fe fertilization, xylem sap extraction was also made on three dates: June 9, before soil Fe(III)-EDDHA application (week 0), June 16 (one week after Fe application; week 1) and June 23 (2 weeks after Fe application; week 2). At each sampling date, six shoots per tree (with six trees per treatment) were taken for xylem sap extraction and subsamples for Fe and metabolomic analyses were taken as described above. To carry out the soil application in June, five wells (approximately 20 cm deep, 20 x 20 cm wide) were excavated in the soil around each tree (Fig. 4.3), approximately 100 cm from the trunk, and 10 g of fertilizer was placed on the uncovered soil surface of each well. Wells were topped again with soil and 4 L of water per well were added.

Xylem sampling

Extraction of peach xylem sap from peach tree shoots was carried out as described elsewhere (Chatti 1997, Larbi et al. 2003) with some modifications. The final protocol was as follows: the shoot cutting was devoid of the basal bark (3-4 cm), washed with distilled water and placed in a Schölander chamber (Solfranc Tecnologies, Tarragona, Spain) with the distal end, including leaves, inside the pressure chamber. Then, pressure was increased progressively from 5 to 22 bars, with higher-pressure values resulting in cytosolic contamination (data not shown). The first few drops of sap were discarded to

avoid contamination, the cut surface was wiped out with paper tissue, cleaned with Type I water and blotted dry. Then, the xylem sap was collected for 4 min in Eppendorf tubes fitted with a 0.45 µm filter (Ultrafree centrifugal filters, Durapore-PVDF 0.45 µm from Millipore). After rapid centrifugation, 20 and 10 µl aliquots were taken for Fe concentration and metabolomic analysis, respectively. The remaining sample was used for proteomic analysis. Samples were kept on ice during manipulation. Cytosolic malate dehydrogenase c-mdh (EC 1.1.1.37) was used in all cases as a cytosolic contamination marker (López-Millán et al., 2000b).

Figure 4.3. *Iron(III)-EDDHA application to the soil. Five wells 20 cm deep and 20 x 20 cm wide excavated in the soil around a peach tree, at approximately 100 cm from the trunk.*

Iron determination in xylem sap

Iron in the xylem sap was determined by graphite furnace atomic absorption spectrometry (Varian SpectrAA with Zeeman correction). Samples were analyzed with six biological and three technical replications each.

Metabolomic analysis

Metabolite extraction was carried out as previously described for xylem sap (Fiehn 2003). Dried extracts were derivatized as described elsewhere (Fiehn et al. 2008). Derivatized samples (1 µL) were injected randomly in split-less mode with a cold injection system (Gerstel, Mülheim an der Ruhr, Germany) and analyzed by a GC device (Agilent 6890, San Jose, CA, USA) using an integrated guard column (Restek, Bellefonte, PA, USA) and a Rtx 5Sil MS column (30 m x 0.25 mm, 0.25 µm film thickness). The GC device was connected to a Leco Pegasus IV time-of-flight mass spectrometer (TOFMS) controlled with Leco ChromaTOF software v.2.32 (Leco, St. Joseph, MI, USA). Peak detection and mass spectra deconvolution were performed with Leco Chroma-TOF software v.2.25, and GC-MS chromatograms were processed as

described previously (Fiehn et al. 2008). Metabolite data were normalized using the sum of all metabolite peak heights in a single run, to account for small GC injection variations. The resulting data were multiplied by a constant factor in order to obtain values without decimal figures. Data were analyzed to check for possible correlations between peak height values and peak variance, and since a positive correlation was found a log10 transformation of the data was carried out to avoid variance-mean dependence.

Proteomic analysis

Proteins in two mL of xylem sap were precipitated by adding five volumes of 10% tricloroacetic acid in water. The solution was incubated overnight at -20 ºC and then centrifuged during 15 min at 10000 g at 4 ºC. Protein pellets were washed twice with metanol and dried with N_2. Proteins were solubilized in 250 µL of a buffer containing 8 M urea, 2% (w/v) CHAPS, 50 mM DTT, 2 mM PMSF and 0.2% (v/v) IPG buffer pH 3-10 (GE Healthcare, Uppsala, Sweden) in agitation during 2 h at 29 º C. Proteins were quantified using the Bradford method using BSA as standard. Each sample was measured three times using three dilutions.

Proteins in the extracts were separated by 2D-electrophoresis (IEF-SDS-PAGE) using the methods described by Andaluz et al. (2006). The first dimension IEF separation was carried out on 7 cm ReadyStrip IPG Strips (BioRad, Hercules, CA, USA) with a linear pH gradient pH 3-10 in a Protean IEF Cell (BioRad). Strips were rehydrated for 16 h at 20 ºC in 125 µL of rehydration buffer containing 70 µg of xylem sap proteins and a trace of bromophenol blue, and then transferred onto a strip electrophoresis tray. IEF was run at 20ºC, for a total of 14000 V h (20 min with 0-250 V linear gradient; two h with 250-4000 V linear gradient and 4000 V until 10000 V h; Rodriguez-Celma et al. 2011). After IEF, strips were equilibrated (reduction and alquilation process) for 10 min in equilibration solution I [6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT] and for another 10 min in equilibration solution II [6M urea, 0.375 M Tris-HCl pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide]. For the second dimension, polyacrylamide gel electrophoresis (SDS PAGE), equilibrated IPG strips were placed on top of vertical 12% SDS-polyacrylamide gels (8 x 10 x 0.1 cm) and sealed with melted 0.5% agarose in 50 mM Tris-HCl (pH 6.8) containing 0.1% SDS. SDS PAGE was carried out at 20 mA per gel for approximately 1.5 h, until the bromophenol blue reached the plate

bottom, in a buffer containing 25 mM Tris Base, 1.92 M glycine, and 0.1% SDS, at 4 ºC. Gels were subsequently stained with Coomassie Colloidal R-250 (Sigma, Barcelona, Spain)

Data analysis

For metabolomics analysis, statistical analysis of the normalized log10 transformed data was carried out with Statistica software (v.9.0. StatSoft, Inc., Tulsa, OK, USA). Only those metabolites present in at least 80% of either the Fe-deficient or the control samples were considered. Significant changes in metabolite levels with Fe deficiency were detected for each plant species and tissues using one-factor analysis of variance (ANOVA; $p \leq 0.05$). Metabolite response ratios were defined as the level in the Fe deficiency treatment divided by the level in the Fe-sufficient controls; when ratios were lower than one the inverse was taken and the sign changed. Only metabolites with mean response ratios above 2.0 or below -2.0 were considered relevant and are discussed in this study. Multivariate analysis (supervised Partial Least Square, PLS) was used to study the clustering of the Fe-deficient and control samples, as well as to find the set of metabolites responsible of the separation between samples. Correlations between selected metabolites were also analyzed, to reveal processes that may be consistently present in Fe-deficient materials.

Results

Changes in SPAD, xylem Fe concentrations and pH during the season

The SPAD index increased gradually during the summer in control trees whereas in the Fe-deficient trees no changes were found (Table 4.1). Considering the changes in Fe xylem concentrations during the season, the Fe concentration in control trees ranged between 6 and 4 µM in June and July and decreased to approximately 2 µM in August (Table 4.1). This trend was similar in the Fe-deficient trees, which had a xylem sap Fe concentration of approximately 4-5 µM in June and July and approximately 2 µM in August. The xylem sap pH of control trees decreased progressively from June (pH 6.6) to August (pH 5.7) and in Fe-deficient trees from 6.7 in June to 5.6 in August (Table 4.1).

Table 4.1. *Changes in SPAD values Fe and xylem Fe concentration and pH during the season in Fe-deficient and Fe-sufficient trees (2010 experiment in Plasencia de Jalón).*

Data are means ± SE (n = 6 trees, 6 shoots per tree). Data followed by the same letter within the same column are not significantly different (Duncan test) at the p<0.05 level.

Treatment	Date	Fe (µM)	SPAD	pH
Fe-sufficient	June	5.5±0.6a	30.4±0.6c	6.6±0.1a
	July	4.1±0.9a	34.0±0.4b	6.2±0.1b
	August	2.1±0.3b	39.4±0.5a	5.7±0.1c
Fe-deficient	June	4.5±0.7a	13.9±0.6a	6.7±0.1a
	July	4.3±1.2a	12.0±0.9a	6.1±0.1b
	August	2.4±0.6b	12.1±0.8a	5.6±0.1c

Changes in SPAD, xylem Fe concentrations and pH with Fe fertilization

The SPAD index did not change in Fe-deficient trees during the experiment, whereas Fe fertilization led to major increases in SPAD (67%), from 14 at the beginning to 23 at the end of the experiment (two weeks after Fe supply) (Table 4.2). The Fe concentration in xylem sap of Fe-deficient trees ranged from 5 to 10 µM (Table 4.2). In the trees undergoing Fe fertilization, Fe concentration in xylem sap increased from the initial value of 5 to 9 µM after one week of the treatment and decreased to 3 µM two weeks after Fe supply (Table 4.2). The xylem sap pH was quite similar during the two weeks of the experiment, in both Fe-deficient and Fe-EDDHA fertilized trees. Values ranged between 6.1 and 6.5 for Fe-deficient trees and between 6.6 and 6.7 for Fe-EDDHA fertilized trees.

Table 4.2. *Changes in SPAD values Fe and xylem Fe concentration and pH with Fe fertilization (June 2010 experiment in Plasencia de Jalón). Data are means ± SE (n = 6 trees, 6 shoots per tree). Data followed by the same letter within the same column are not significantly different (Duncan test) at the p<0.05 level.*

Treatment	Date	Fe (µM)	SPAD	pH
Fe-deficient	Week 0	7.6±2.1a	12.6±0.6a	6.1±0.1a
	Week 1	4.9±2.1a	11.0±0.7ab	6.5±0.1a
	Week 2	10.3±1.9a	12.4±0.84a	6.3±0.1a
Fe(III)-EDDHA	Week 0	4.9±1.1b	13.5±0.5c	6.6±0.1a
	Week 1	9.1±2.1a	18.5±1.0b	6.7±0.1a
	Week 2	2.8±0.8b	22.6±1.0a	6.7±0.1a

Xylem sap metabolite profiles change with iron deficiency

The xylem sap from Fe-deficient and Fe-sufficient peach tree plants was analyzed, taking into account only metabolites present in at least 80% of either Fe-deficient or Fe-

sufficient samples. A total of 251 of such consistently present metabolites were detected in peach tree xylem sap. Using the Fiehn Lib databases, 77 of them were identified. Iron deficiency caused significant (at $p \leq 0.05$) and consistent changes (present in at least 80% of either Fe-deficient or Fe-sufficient plants and with mean response ratios above 2.0 or below -2.0) in the levels of six of the identified metabolites (Table 4.3), and also in height of the unknowns (Table 4.4). The corresponding response ratios, defined as the level in the -Fe treatment divided by the level in the +Fe treatment, are also shown in Tables 4.3 and 4.4. In peach tree xylem sap, the only metabolite that increased more than 2-fold was the non-proteinogenic aminoacid nicotianamine (NA). The largest decrease was found for the carbohydrate 3-phosphoglyceraldehyde, whereas four other metabolites: benzoic acid, butane-2,3-diol, gluconic acid and 2-deoxyerythritol decreased approximately by 50%. On the other hand, the height unknown metabolites accounted for 60% of the total metabolites changing significantly in response to Fe deficiency in peach tree. Two unknowns increased 8- and 5-fold with Fe-deficiency, whereas another unknown also showed large decreases (Table 4.4).

Xylem sap metabolite levels cluster separately in Fe-deficient and sufficient samples

The clustering of metabolites was studied using PLS analysis, including both the identified and unknown metabolites (Fig. 4.4). Iron-deficient and Fe-sufficient samples were well separated in clusters. The first vector (*v1*) explained 13% of the variability in peach tree xylem sap, with the second vector (*v2*) explaining 9.5% of the variability.

The separation between clusters was associated with those metabolites with a large contribution (X-weight) to *v1* (Table 4.5). Identified metabolites with large positive X-weights were carbohydrates (glucose, arabitol and sucrose) and glycolysis related compounds (ribose and gluconic acid), whereas those with large negative X-weights were aminoacids and other N related metabolites (2-hydroxyglutaric acid, proline), carbohydrates (galactinol and threonine) and some organic acids such as fumaric. Approximately 45% of the metabolites with a high contribution to cluster separation were unknowns (Table 4.5).

Table 4.3. *Main effects of iron deficiency on identified xylem sap metabolite levels (2008 experiment in Peñaflor). The relative metabolite ratio is defined as the level of the metabolite in the -Fe treatment divided by its level in the +Fe treatment. When the response ratio was lower than 1, the inverse was taken and the sign changed. See full details in Rellán-Álvarez et al. (2011).*

		Relative metabolite ratio
Aminoacid and Nitrogen Metabolism		
	nicotianamine	2.4
	benzoic acid	-2.3
	butane-2,3-diol	-2.4
Carbohydrate Metabolism		
	gluconic acid	-2.1
Glycolysis and Pentose Phosphate Metabolism		
	PGA	-3.9
Others		
	2-deoxyerythritol	-2.1

Table 4.4. *Main effects of iron deficiency on unknown xylem sap metabolite levels (2008 experiment in Peñaflor). The relative metabolite ratio is defined as the level of the metabolite in the -Fe treatment divided by its level in the +Fe treatment. See full details in Rellán-Álvarez et al. (2011).*

Metabolite Id	Relative metabolite ratio
231075	-2.19
231213	7.66
231216	3.05
231227	3.33
231255	4.54
231280	3.93
231380	-3.56
231353	-4.41

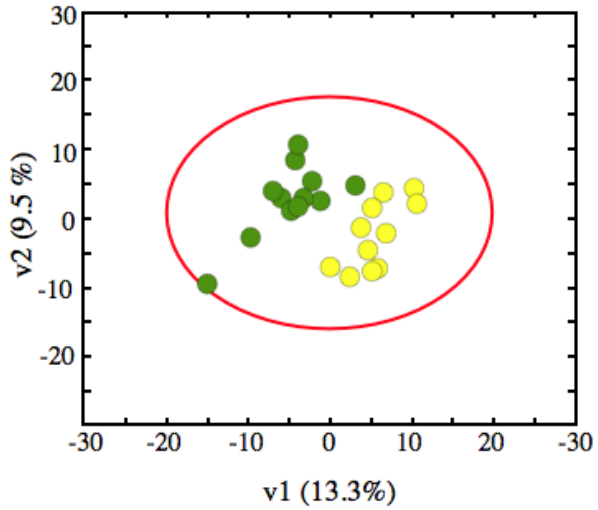

Figure 4.4. *Partial least square (PLS) analysis of xylem sap and leaf extract metabolites as affected by Fe deficiency. Score scatter plot of PLS vector1 (v1) vs. PLS vector2 (v2) of all detected metabolites (identified ones plus unknowns) in control (green circles) and Fe-deficient (yellow circles) sample. The percentage of variability explained by each vector is indicated in parenthesis in the corresponding axes.*

Table 4.5. *Metabolite X-weights in the Partial Least Square (PLS) analysis of xylem sap shown in Figure 4.4. The X-weight indicates the contribution of each metabolite in the explanation of the horizontal distribution of spots in the PLS output.*

Positive X-weight value				Negative X-weight value			
231300	0.10	231075	0.10	231213	-0.20	231280	-0.19
2-deoxyerythritol	0.09	231353	0.09	231255	-0.19	233425	-0.18
231217	0.09	glucose	0.09	231227	-0.17	233408	-0.17
ribose	0.09	232807	0.08	pipecolic acid	-0.17	2-hydroxyglutaric a.	-0.16
231151	0.08	ribonic acid	0.08	231328	-0.15	2-ketoisocaproic acid	-0.14
231106	0.07	arabitol	0.07	231216	-0.13	**galactinol**	-0.13
sucrose	0.07	chlorogenic acid	0.06	citramalate	-0.13	proline	-0.13
231385	0.07	glycerol	0.06	fumaric acid	-0.12	231333	-0.12
gluconic acid	0.06	N-ac-D-hexosamine	0.05	**galactonic acid**	-0.12	phenylalanine	-0.11
threonic a.	0.05	butane-2,3-diol	0.05	threonine	-0.11	233412	-0.11
233428	0.05	231106	0.07	lysine	-0.11	231216	-0.13

Proteomic analysis optimization

The study of the differences in the xylem sap protein profile between Fe-sufficient and Fe-deficient trees was carried out by a 2D (IEF-SDS-PAGE) gel-based technique. In an initial attempt to optimize the protein purification protocol, proteins present in 1.8 mL of xylem sap were precipitated with 4 volumes of 80% acetone, 0.07% of β-mercaptoetanol was added and the mixture was kept at -20 C overnight. After re-solubilization with the buffer indicated in the proteomic analysis section of Materials and Methods, total protein was quantified in the extract using the Bradford method. The protein profiles were analyzed by 2D-electrophoresis (2DE). In the first dimension, proteins were separated (IEF) by their isoelectric point, using a linear pH gradient from pH 3-10. Then, the 1D strip was loaded onto the second dimension SDS-PAGE gel. The 2DE gel obtained was subsequently stained with colloidal Coomassie Blue (Fig. 4.5).

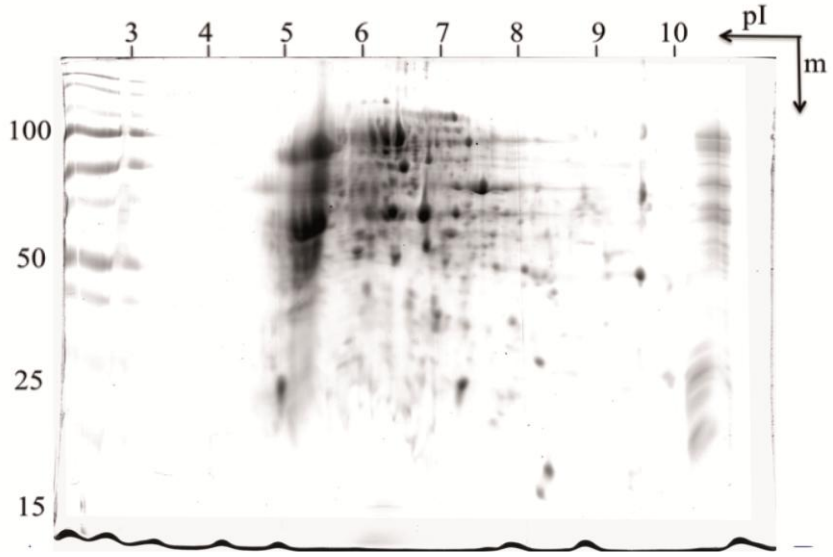

Figure 4.5. *2D electrophoresis gel of a peach xylem sap sample. Xylem sap proteins were precipitated with 80% acetone, and 50 µg of solubilized xylem sap proteins were separated in a first dimension by IEF (3-10 strip) and then in a second dimension by SDS-PAGE (12%) buffer.*

Results shown in Fig. 4.5 indicate that the protein extraction method was not the optimal for the xylem sap sample used. Two major problems can be inferred from the observation of the gel: first, proteins were not well focused, especially those having a pI below 6.0; second, a high background was observed throughout the central part of the gel, probably causing a lack of focalization and suggesting the presence in the sample of contaminants that interfere with the IEF.

Therefore, we used a different precipitation method using 10% TCA in water. After re-solubilization and 2DE, gels showed a good protein focalization in all the pI-range and a clear background free of interferences (Fig. 4.6). This method was subsequently chosen for further experiments. The total protein amount applied in each gel also required optimization, since an excessive protein load usually has a negative effect on resolution. We found that 70 µg of protein was an adequate quantity to obtain a satisfactory resolution. 2DE gels obtained from samples from a green control peach tree and a Fe-deficient peach tree are shown in Fig. 6A and B, respectively. These two gels were obtained using the conditions described above and with a linear pH gradient 3-10.

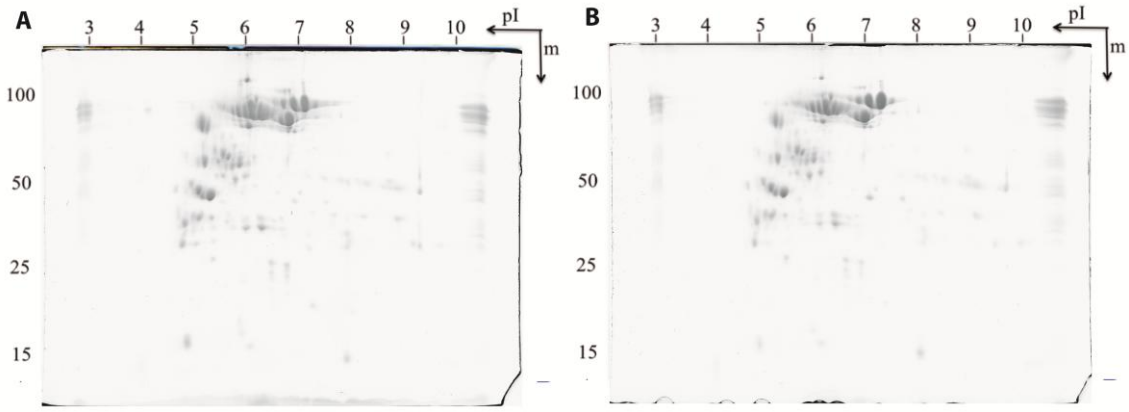

Figure 4.6. *2D electrophoresis gels obtained from Fe-sufficient (A) and Fe-deficient (B) peach xylem sap samples. Xylem sap proteins were precipitated with 10% TCA, and 70 µg of solubilized xylem sap proteins were separated in a first dimension by IEF (3-10 strip) and then in a second dimension by SDS-PAGE (12%) buffer.*

For the differential proteomics experiment, xylem sap samples obtained from the 6 shoots sampled in each tree (six trees per Fe condition) were pooled, and the resulting single sample for each tree was used to obtain a 2DE gel. The 12 different 2DE gels obtained for the Fe-deficient (6 individuals) and Fe-sufficient (6 individuals) trees are shown in Fig. 4.7. Results show that protein profiles have a high reproducibility. Although the possible differences are still under study, no major differences appear to occur between the Fe-deficient and Fe-sufficient xylem sap protein profiles. In the next step, a detailed image analysis using PDQuest 8.0 software (BioRad) will be carried out, including statistical analysis of the possible differences in the protein profiles between treatments. Identification of the spots that show significant changes between samples may be carried out by digesting the samples with trypsine and subsequent identification by MS/MS using MASCOT as a search engine.

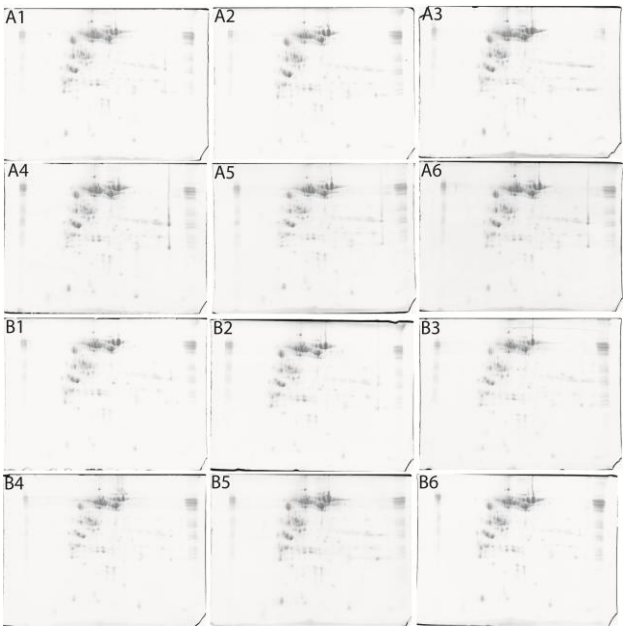

Figure 4.7. *2D electrophoresis gels from peach tree xylem sap. Gels A1-A6 were obtained from six green trees and Gels B1-B6 from six Fe-deficient trees. Each sample pooled the xylem sap from 6 different shoots of the same tree.*

Discussion

In this study we describe how to obtain xylem sap from peach tree shoots in enough quantity to proceed to the characterization of the sap in terms of the metabolite and protein relative abundance. In previous studies in our laboratory the isolation of xylem sap from peach and pear trees was achieved, but the protocol was not fully standardized (Chatti 1997, Larbi et al. 2003).

The xylem Fe concentration of Fe-deficient trees was in the range 2-10 µM, depending on the specific tree and the sampling date (the highest value was found in August). Our data indicate that different Fe-deficient trees with a similar level of chlorosis may have different xylem sap Fe concentrations. Upon fertilization in June with soil-applied Fe(III)-EDDHA, the Fe concentration in the xylem sap increased in the first week from 5 to 9 µM to decrease after another week to 3 µM (all these values are for the same trees). These values are somewhat different to those found in previous studies with pear and peach, where the Fe-concentrations in Fe-deficient trees were close to 2-3 µM, and those of trees fertilized with solid Fe implants ranged from 3 to 7 µM (Larbi et al. 2003). Xylem sap Fe concentrations in the µM range have been previously reported in a number of plant species (see Supplementary Table I in Rellán-Álvarez et al. 2010). For instance, xylem sap Fe concentrations were approximately 2 and 6 µM in Fe-deficient and Fe-sufficient in sugar beet (López-Millán et al. 2000),

whereas values of approximately 5-10 µM were observed in Fe-deficient tomato (Orera et al. 2010, Rellán-Álvaréz et al. 2009).

Regarding pH, values found are in the ranges 5.6-6.7 and 5.7-6.7 in the Fe-deficient and Fe-sufficient (including fertilized) trees, respectively, with values decreasing progressively from June to August. Values found previously were in the ranges 6.4-7.2 and 6.4-7.4 in peach and pear trees, respectively (Larbi et al. 2003). Xylem sap pH values found were somewhat higher than those found in sugar beet and tomato, which are close to 5.5 (López-Millán et al. 2000, Rellan-Alvarez et al. 2009). On the other hand, no major changes in xylem sap pH occurred as a consequence of Fe fertilization. The application of solid Fe implants to the branches, caused increases in xylem sap Fe concentrations accompanied by small pH decreases, both in pear and peach trees (Larbi et al. 2003). Iron deficiency has been reported previously to cause only small increases in peach xylem sap pH (Chatti 1997), whereas in other plants xylem sap pH may either increase (white lupin, Pissaloux et al. 1995), decrease (tomato, Bialczyk and Lechowski 1992, sugar beet, López-Millán et al. 2000) or not change (faba bean, Nikolic and Römheld 1999) with Fe deficiency.

This has been the first time that a GC-MS metabolomics approach has been used to obtain peach tree xylem sap metabolic profiles (Rellan-Alvarez et al. 2011). It should be remarked that when using this approach not all metabolites in a given sample can be measured, since for each component this depends on several matrix-dependent factors (including ionization efficiency, derivatization efficiency, etc.). The PLS analysis was able to separate Fe-deficient from Fe-sufficient trees. However, some of the metabolites with a large X-weights in the PLS analysis are unknowns. One of the major changes observed in the metabolomic profile between control and deficient trees was an increase in NA, a non-proteinogenic aminoacid related with intracellular Fe trafficking (von Wirén et al., 1999), which increased 2-fold in peach xylem sap when the Fe deficiency was severe. This may suggest that NA could play a role in long-distance Fe transport in peach trees, especially in severely deficient trees where the xylem organic acid concentrations could be very high (Larbi et al. 2010). At the pH found in some peach tree xylem sap samples (6.5-7.5), NA can chelate efficiently Fe, as it has been shown by *in vitro* experiments (Rellán-Alvarez et al. 2008). On the other hand, no changes in carboxylates were found, although malate was found to increase with Fe deficiency in a

previous study (Chatti 1997), and after an Fe-implant treatment organic acid concentrations were found to decrease (Larbi et al. 2004).

Some xylem sap carbohydrates such as gluconic acid decreased significantly with Fe deficiency, whereas others such as galactinol and galactonic acid did not increase significantly, but had a large X-weight in the PLS analysis. This is interesting, because galactinol and other RFO sugars have been found to increase with Fe-deficiency in roots (Rellán-Álvarez et al. 2010). Other changes occurring in sugar beet (López-Millán et al. 2000) could not be confirmed to occur in peach xylem sap. Xylem carbohydrate levels may be affected by xylem/phloem transfer processes, which are largely species dependent and are very different in woody species. Also, the fact that the trees were mature individuals, as opposed to the young plants grown in controlled environments, can have an effect on the number of metabolites affected by Fe deficiency.

The preliminary proteomic study carried out in this work reveals that it is feasible to obtain, purify and separate proteins from the xylem sap of field-grown trees, and obtain good protein profiles with very reproducible 2D gels. Once the statistic analysis and protein identification is carried out, this experiment may reveal important players in the xylem sap of peach trees. The experiments are also likely to shed light on the mechanisms that fruit trees use to cope with abiotic stresses and to respond once fertilization has been implemented. This is of special relevance, due to the low number of high-throughput studies in woody species and field grown crops.

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Setting good practices to assess the efficiency of iron fertilizers

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Review

Setting good practices to assess the efficiency of iron fertilizers

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ABSTRACT

The most prevalent nutritional disorder in fruit tree crops growing in calcareous soils is Fe deficiency chlorosis. Iron-deficient, chlorotic tree orchards require Fe-fertilization, since chlorosis causes decreases in tree vegetative growth as well as fruit yield and quality losses. When assessing the effectiveness of Fe-fertilizers, it is necessary to use sound practices based in the state-of-the art knowledge on the physiology and biochemistry of Fe deficiency. This review provides an overview on how to carry out the assessment of the efficiency of Fe-fertilizers, discussing common errors found in the literature, outlining adequate procedures and giving real examples of practical studies carried out in our laboratory in the past decade. The review focuses on: i) the design of Fe-fertilization experiments, discussing several issues such as the convenience of using controlled conditions or field experiments, whether fertilizer assessment experiments should mimic usual fertilization practices, as well as aspects regarding product formulations, dosages, control references and number of replicates; ii) the assessment of chlorosis recovery upon Fe-fertilization by monitoring leaf chlorophyll, and iii) the analysis of the plant responses upon Fe-fertilization, discussing the phases of leaf chlorosis recovery and the control of other leaf nutritional parameters.

1. Introduction

The most prevalent nutritional disorder in fruit tree crops growing in calcareous soils is Fe deficiency (see reviews in [1,2]). The main symptom of Fe deficiency in plants is leaf yellowing, which is usually called leaf chlorosis; this occurs both in growth chamber and field-grown plants (*e.g.*, in sugar beet and peach trees, respectively; Fig. 1). In field conditions, chlorosis in the orchards is often heterogeneous, with individual trees affected to different extents. Images of fruit tree field orchards affected by Fe-chlorosis are shown in Fig. 2 (A: peach tree orchard; B: pear tree orchard). Iron-deficient, chlorotic tree orchards are usually fertilized with Fe every year, because chlorosis causes decreases in tree vegetative growth, a shortening of the orchard lifespan as well as losses in fruit yield [2] and changes in fruit quality [3,4]. The diagnosis of Fe deficiency, conversely to what happens with other nutrient disorders, cannot be adequately assessed using leaf elemental composition, because Fe-deficient field-grown leaves often have Fe concentrations as high as that of Fe-sufficient ones (the "chlorosis paradox"; [5]). This is possibly associated to an accumulation of Fe in or near the vascular system [6,7]. Therefore, leaf chlorophyll (Chl) concentrations (generally monitored using a hand-held device) are used most of the times to assess the Fe nutritional status.

Iron fertilization in trees can be carried out in several ways, including the addition to the soil or irrigation water of Fe-containing compounds [8], as well as providing Fe directly to the plant by spraying tree canopies or injecting trunks or branches with Fe-compounds in solid or liquid forms [1]. There is a very large number (several hundred) of Fe-containing fertilizers, many of them containing the same active principles and others consisting of a mixture of Fe-compounds [8,9]. These Fe-fertilizers often have different degrees of effectiveness due to many different factors [1,8,10]. Therefore, it is necessary to compare the efficiencies of Fe-fertilizers, and many studies are published every year assessing and comparing Fe-containing products (*e.g.*, see [11,12]). In particular, any new Fe-fertilizer must be assessed using this type of studies. The recovery after Fe-fertilization is generally monitored using the leaf Chl concentration, for the reasons explained above, although leaf Fe concentrations are still sometimes used. However, divergences in specific methodological details could be found in the literature, and

Abbreviations: Chl, chlorophyll; EDDHA, ethylenediamine-N-N′bis(*o*-hydroxyphenylacetic) acid; SPAD, Soil and Plant Analyzer Development.

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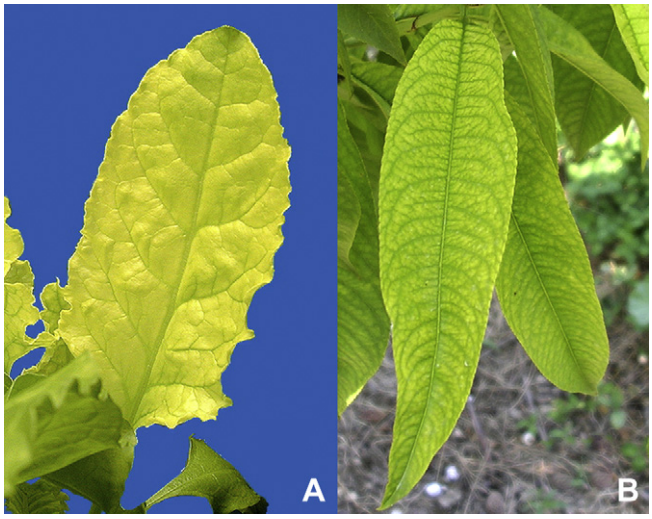

Fig. 1. Iron-deficient leaves of *Beta vulgaris* grown in a controlled-environment growth chamber in nutrient solution (A) and *Prunus persica* grown in the field (B).

this could make difficult the comparison of results obtained in different experiments.

This paper proposes good practices to assess the efficiency of Fe-fertilizers, by examining a number of factors that are crucial in this type of assessment studies. The rationale for most of them is provided by the current knowledge on plant physiology and biochemistry [13], which is not always taken into account in Fe-fertilizer efficiency agronomical studies.

Fig. 2. Pictures of field orchards affected by iron chlorosis, showing the heterogeneity among trees in *Prunus persica* (A) and the heterogeneity among branches in the same tree in *Pyrus communis* (B).

2. Design of iron fertilization experiments

Iron fertilizers should be first tested for stability of the Fe-containing active agent in the conditions prevailing in the media to be used (*e.g.*, spray solutions, nutrient solutions, soils, etc.) [14]. Many factors (light, pH, etc.) can affect its stability and availability for plants. Once the possible usefulness of a Fe-fertilizer is predicted, controlled conditions or field experiments must be designed for assessing efficiency.

2.1. Controlled conditions vs. field experiments

Many studies in the scientific literature assess the efficiency of Fe-fertilizers using controlled conditions environments such as in growth chambers and greenhouses [12]. The plant material used consists in plant tissues, seedlings, plantlets or adult plants, grown in different media. The most commonly used media are synthetic ones such as nutrient solutions and agar (the latter for plant tissues and seedlings), and solid media such as perlite, vermiculite and other inert substrates, and mixtures of inert substrates and soils. Sometimes these experiments use soil as substrate [15]. Water supply is usually unrestricted, and environmental parameters such as light intensity (usually around 500 μmol quanta m^{-2} s^{-1}), temperature and humidity are (more or less) tightly controlled.

A second type of experiments uses instead adult plants (trees, for instance) grown in farm fields. These involve plants grown on soils in the field under natural light, temperature and humidity conditions (for instance, see [16]). Water supply in these experiments is usually not as well controlled as in growth chambers and greenhouses, since it depends on the irrigation practices prevalent in the area.

Using the same Fe-fertilizer in these two types of experiments could give very different results. An example of the differences found when the same commercial fertilizer was applied to Fe-deficient peach plants in a growth chamber and in a field experiment is shown in Fig. 3. The commercial product was very effective in correcting Fe deficiency in peach grown in nutrient solution in

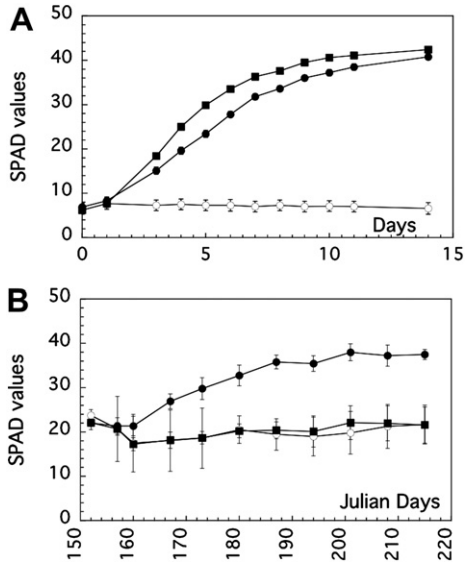

Fig. 3. Time-course of leaf SPAD values after Fe-fertilization in *Prunus* plants. Plants were grown in nutrient solution in a controlled-environment growth chamber (A) and in the field (B). The same Fe-fertilizer commercial product (black squares) was compared to Fe(III)–EDDHA (black circles). Untreated controls are represented as white circles (in all cases $n = 4$ trees). Concentrations in the nutrient solution were 90 and 45 μM Fe for the commercial product and Fe(III)–EDDHA, respectively. In the field experiment, 500 and 50 g of the commercial product and Fe(III)–EDDHA, respectively, were added to the soil near each tree.

a growth chamber (Fig. 3A), but it was totally ineffective when applied in large amounts to Fe-deficient peach trees growing on a calcareous soil in the field (Fig. 3B). The most likely explanation for these differences is that the growth chamber experiment usually disregards factors that are very important in field conditions, including the fertilizer–soil interactions and the plant–soil interactions. Also, light intensity (up to 2000 μmol quanta m^{-2} s^{-1} in field conditions *vs.* less than 500 μmol quanta m^{-2} s^{-1} in controlled conditions) and quality (full sun spectrum in field conditions *vs.* lamp spectra in controlled conditions) can be markedly different.

In summary, experiments using controlled environments and field conditions may address different issues. Controlled conditions experiments assess the plant Fe uptake, transport and utilization, in a growth media where a limited number of factors can affect Fe availability. On the contrary, field experiments assess the full depth of the issue. Therefore, any specific product should be tested in environments as close as possible to the final destination. We can say that the efficiency of any Fe source proven in controlled conditions does not assure in principle its efficiency in field conditions, and field tests are always mandatory for field-destined products, after carrying out preliminary tests in controlled conditions.

2.2. Should fertilizer assessment experiments mimic normal fertilization practices?

Sometimes, studies aimed to assess differences in Fe-fertilizer efficiency are designed trying to imitate the timeframe and doses used in common farmer's preventive fertilization practices. In deciduous tree crops, preventive Fe-fertilization is often carried out in early spring, before leaf appearance [1,2]. However, carrying out fertilizer assessment experiments using early fertilization could lead to inconclusive results for several different reasons. First, Fe-chlorosis in orchards is known to be quite heterogeneous, and within a single orchard it is usual to find trees affected strongly by Fe-chlorosis, whereas other trees are only moderately affected and others are green and healthy (Fig. 2A; [17]). In some species heterogeneity may exist also within a given tree, with some branches chlorotic and other green in the same individual (*e.g.*, in the pear tree in Fig. 2B; see also [4]). The chlorosis degree may also vary in given tree from year to year, probably because during its lifespan each individual tree may gain access randomly to some soil Fe resources. Therefore, when tree leaves are not present (*e.g.*, in experiments that include early spring application of Fe-fertilizers) it is not possible to assure the Fe-status homogeneity of individual trees. This usually leads to a wide data variability, both in the control untreated trees and in the fertilized ones, and frequently impedes obtaining any valid conclusion.

In our experience, the best way to compare the efficiency of Fe-treatments is to carry out a corrective fertilization in homogeneously Fe-deficient trees, *i.e.*, having a similar degree of leaf chlorosis. This homogeneity is better assessed when leaves are present and chlorosis is already well established (*e.g.*, at the end of May or beginning of June in the Northern hemisphere). Of course, it must be first confirmed that chlorosis is due to Fe deficiency and not to other nutrient deficiencies (see below). Within each treatment, several trees with very similar leaf Chl levels (*e.g.*, mean ± 2 SPAD units) should be chosen as replicates. The level of chlorosis should be sufficient to observe clear responses to Fe-fertilizers in the crop in question; for instance, initial SPAD levels of 15–22 are adequate in peach trees grown in the field (corresponding to approximately 116–153 μmol Chl m^{-2}). These values are approximately 40–55% of the maximum SPAD reading in this species [16]. The possible interactions between Fe-fertilization and fruit harvest date have not been studied so far, and some fruit cultivars may have commercial harvest dates (*i.e.*, early-June in the case of peach) close to the experimental dates proposed here. Since fruits are one of the largest sinks for Fe (not shown), further experiments should address these interactions.

Although this late spring, corrective fertilization timing is not so common in farmer's practice, the main question we must address is whether a given Fe-fertilizer is effective or not in field conditions. Only if the answer is yes, further tests can be designed using the agronomical standard, early spring preventive application.

2.3. Product formulations, dosages, control references and number of replicates

Product formulation (*i.e.*, the specific details of the product preparation) is a very important, often disregarded factor, which can affect Fe-fertilizer efficiency. For instance, the same active principle could be applied to the soil either in solution or as a powder, granules, etc., and this would largely influence Fe availability. When applying fertilizers to the growth media, Fe-fertilizers in solution or in powder could be rapidly inactivated (*e.g.*, inorganic Fe-sources; [18]) or leached (*e.g.*, Fe-chelates; [8]), whereas other immobilized formulations could slowly release Fe for the plant [19]. Another example is foliar Fe-fertilization, where formulation (both co-adjuvants and surfactants) is essential in determining the efficiency of Fe-fertilizers [10,16].

Whereas the effect of Fe-fertilizers on Fe-chlorosis is dose-dependent, different fertilizers could have different optimal doses [20]. Therefore, when comparing different fertilizers, the real effects could be masked by a dose effect; for instance, if an efficient fertilizer is applied in an amount that is too low, no Fe-chlorosis correction will be observed. It is always advisable, when assessing a new Fe-fertilizer, to apply it first in generous amounts, because the main question we have to answer is whether they can provide or not Fe to the tree. Thereafter, the efficiency of a given Fe-fertilizer compared to others can be addressed separately at a later stage, to find the adequate amounts for standard agronomical management practices.

Another important issue is the Fe-compound to be used as a reference in Fe-fertilized assessment studies: it is advisable to use as a positive control the most effective Fe-fertilizer in each type of fertilization. Therefore, Fe–EDDHA should be used as a reference in calcareous soils, but not in foliar applications [10]. Iron sulphate can be used as a positive control in foliar applications, but not when comparing soil fertilizer applications in calcareous soils. On the other hand, untreated (zero Fe-treated) plants should be always used as a negative control.

The number of chlorotic trees needed is a major issue, given that they must be as homogeneous as possible concerning leaf Chl (SPAD value ± 2, see above). In fact, the availability of such homogeneous individuals in the field could be limited in many cases. In our experience, having at least four initially homogeneous tree replications per treatment is fully adequate in this type of experiments. When this is not possible, trees with different degrees of chlorosis can be used, and covariance analyses should be carried out.

3. Assessment of chlorosis recovery upon iron fertilization

The most appropriate way to assess the efficiency of Fe-fertilizers is to follow the evolution of leaf Chl after Fe-fertilization. This has been carried out traditionally by using visual scale ratings [21–23]. In the last two decades, however, hand-held apparatus, such as the SPAD (Soil and Plant Analyzer Development) from Minolta and others, have become popular for the diagnosis of

plant nutrient status (of Fe and other elements; [24–28]). These devices measure the leaf transmittance at two wavelengths, one corresponding to the Chl absorption maximum and the other to the near infrared as a reference value. In fact, most studies nowadays use this kind of devices to assess leaf Chl concentration changes upon Fe-fertilization [11,12]. A number of factors must be considered when assessing leaf Chl concentration using SPAD-type devices.

Since the objective of the assessment is to have a representative measurement of the Chl in the tree canopy, it is not sufficient to measure a small number of leaves. Leaf pigment concentrations depend on the leaf orientation and incident light intensity, being therefore affected by depth into the canopy, shadowing effects, etc. [29]. A common practice is to measure at least 30–60 leaves at mid tree-height around the canopy; this is in line with common leaf sampling practices, which advise 25–100 leaves for mineral nutrient status assessment [30]. This sample size provides a quite robust assessment, resulting in small deviations from the mean when making independent measurements on the same day in a given tree (data not shown). When several persons make the measurements it is advisable to standardize sampling as much as possible, and results are often more repeatable when the same operator makes all measurements. Also, care must be taken when working with leaves showing internerval-chlorosis, which may occur in some species (*e.g.*, in peach, see Fig. 1B). In any case, the number and type of leaves used must be explicitly written in the report. In some plant species such as pear trees, chlorosis could be very heterogeneous (Fig. 2B) and it may be better to follow chlorosis recovery in specific branches, instead of considering the whole tree, in order to limit leaf chlorosis heterogeneity.

Another important point concerns the selection of leaves to be used during the Fe-chlorosis correction assessment. Generally, young, fully expanded leaves in the distal third of the current year's growth (4th and 5th position from the branch tip) are used; those are the same leaves generally used for mineral analysis [31]. Leaves can be marked with colour tape at the beginning of the experiment, and chlorosis recovery can be followed in the same marked leaves with the SPAD at each time point [16]. However, since the experiment will last for several weeks, shoots will grow and the position of the marked leaves in the canopy will change. Leaves that were in the 4th and 5th position from the branch tip will be located after a few weeks deeper in the canopy, in position 7th and 8th, and these leaves in the inner part of the canopy will adapt by having more pigments than those in the canopy surface [29,32]. Therefore, the change in Chl concentration caused by the correction of chlorosis may be masked by changes in leaf age and position in the canopy. When using the same – marked – leaves for measurements, the Chl concentration of the Fe-deficient (non Fe-treated) controls would tend to increase (Fig. 4). This would complicate the interpretation of results, since moderately efficient treatments also give a Chl increase difficult to distinguish from that of the untreated control. Therefore, it is advisable to measure instead leaves in the same position (*e.g.*, 4th and 5th) during the full duration of the experiment. Using this method, untreated controls generally remain chlorotic (Fig. 4) or undergo a further decrease in leaf Chl concentration, whereas moderately efficient treatments will often result in slightly increased SPAD values.

A very common incorrect assumption is that the SPAD-type devices do measure Chl; this is not correct, since all they do is to make an approximate estimation of the Chl concentration from the differences in transmittance at two different wavelengths, and changes in the SPAD values can be caused by other reasons [27,33]. Therefore, it is always advisable to run a calibration curve, by quantitatively measuring Chl extracted from leaves using organic solvents, and plotting Chl concentrations *vs.* SPAD values (Fig. 5; [34,35]). As already indicated in early works, in some cases leaves can be very thick and/or opaque (*e.g.*, leaves of olive trees, *Quercus* spp., etc.), and the SPAD device will saturate at readings of 60 or more, with further increases in Chl not having any effect on the SPAD value [36]. The relationship between Chl and SPAD reading can also change with the leaf developmental stage (young leaves are thinner and less opaque than adult ones), and also between plant species and cultivars [25]. Examples of this are given in Fig. 5, which shows different SPAD/Chl concentration curves for two fruit tree species at two different sampling dates. In summary, it is generally acceptable to use SPAD value changes to estimate the leaf Chl concentration when a single species and cultivar is used and leaves are not too thick or opaque. In any case, it is always advisable to present a SPAD *vs.* Chl concentration calibration curve.

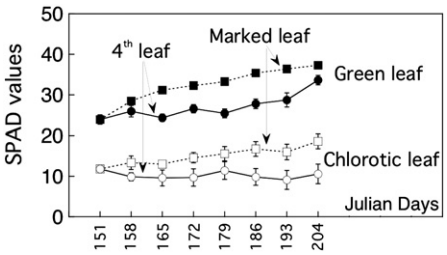

Fig. 4. Changes in leaf SPAD values in Fe-sufficient leaves (green; black symbols) and Fe-deficient leaves (chlorotic; white symbols) using two different leaf samples. Developed leaves in the distal branch tip were marked at the beginning of the experiment with colour tape and SPAD was measured in the same leaves throughout the experiment (marked leaves; squares) or in leaves at the 4th position in the branch (circles) (in all cases $n = 6$ trees).

4. Analysis of the plant responses upon iron fertilization

Besides assessing the effectiveness of Fe-fertilizers in chlorosis recovery, the evaluation of their effects on crop yield and quality will be also desirable. However, the normalization of the tree Fe-status *via* corrective Fe-fertilization is likely to have major fruit quality effects only in the following growth season, and not in the season when corrective fertilization is carried out. The most likely explanation for this fact is that the recovery of the tree physiological processes after fertilization takes quite a long time.

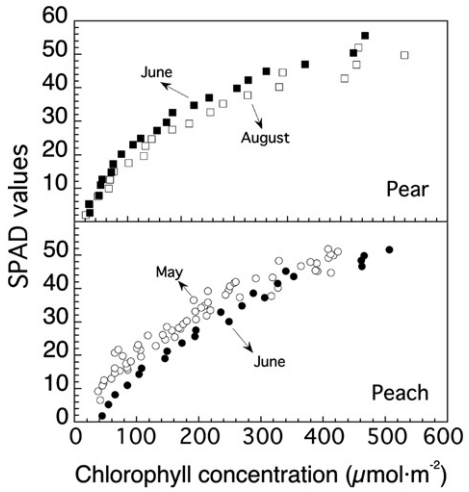

Fig. 5. Correlations of SPAD values *vs.* leaf chlorophyll concentrations at different dates in *Prunus persica* (A) and *Pyrus communis* (B) grown in the field.

4.1. The three phases of leaf chlorosis recovery

When considering the corrective effects of Fe-fertilizers, three different response parameters can be assessed: rapidity, maximal intensity and persistence [37]. When using soil applications to fruit trees, once the Fe-fertilizer is applied, there is usually an approximately one-week lag phase where no changes in leaf Chl are observed. Then, leaf Chl concentrations begin to increase, and the rapidity of this phase of the response can vary among different fertilizers during the first month after Fe-application (Fig. 6A). These differences likely reflect different Fe uptake and transport rates, which may depend on the specific product used. After approximately 1–1.5 months the maximal intensity of the response is usually observed, and this may also differ among Fe-fertilizers (Fig. 6B). Finally, the persistence of the response can be observed in the following months, with some Fe-fertilizers leading to a sustained leaf Chl concentration, whereas in others the effects are weakened with time, and trees progressively develop Fe-chlorosis symptoms (Fig. 6C). This is likely the result of the growth effects caused by the correction of Fe deficiency, which inevitably lead to a further increase in tree Fe demand that cannot be met adequately by some fertilizers or dosages. In fact, some fertilization practices such as branch solid injections may be efficient one or several years after Fe-application [38]. In the case of foliar sprays, several applications per year may be needed (for a review, see [10]).

New Fe-fertilizers for fruit trees should preferentially be aimed to improve overall intensity and persistence rather than rapidness of recovery, which would be less important considering that major effects on fruit quality and yield would be expected to occur only in the following growth season.

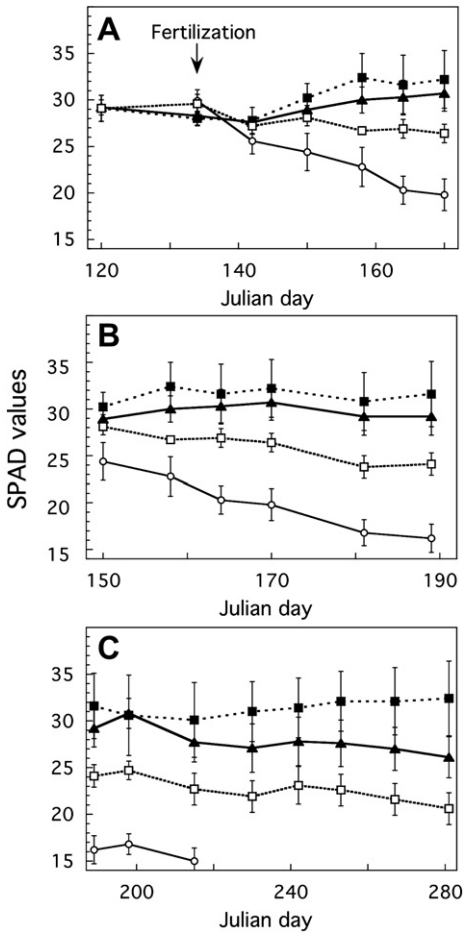

Fig. 6. Time-course of leaf SPAD values after soil Fe-fertilization with different Fe-products in field-grown *Prunus persica* trees showing the three phases of response to Fe-fertilizers. Untreated trees are represented as white circles, the reference product (Fe–EDDHA) as black triangles and two different commercial products as black and white squares (in all cases $n = 4$ trees). The rapidity of the response can be estimated in the first weeks after the Fe-fertilizer application (A). The intensity of the response can be estimated in the approximately 1–1.5 month after the Fe-fertilizer application (B). The persistence of the response can be estimated during the following months (C).

4.2. Control of other leaf nutritional parameters

In field experiments there is always a possibility that other biotic or environmental factors could result in decreases in leaf Chl contents. These include pathogens [39], as well as other nutrient deficiencies such as those of N [40], Zn [41,42] and Mn [43,44]. In plant species other than fruit trees, several metal toxicities [45,46], have been reported to decrease Chl concentrations. Therefore, it is mandatory to have a previous knowledge of the orchard where the experiment will be carried out (*e.g.*, for at least two years), to reduce the possibilities that such interfering factors could be present. The best way to assure that leaf chlorosis is due to Fe deficiency is to use the so-called "biological diagnosis", using local applications of Fe salts (*via* leaf sprays, petiole treatment or leaf injection) to check that re-greening occurs [27,47]. It is always advisable to analyse mineral concentrations in leaf samples at the beginning and at the end of the treatment, as well as on the standard mineral analysis dates, 60 or 120 days after flower full bloom. These analyses constitute an additional and very useful monitoring tool, since they permit monitoring other parameters (Fe and K concentrations, K/Ca and P/Fe ratios, etc.) that also change with the tree Fe nutrition status [48–51].

5. Concluding remarks

When assessing the effectiveness of Fe-fertilizers, it is necessary to use sound practices based in the state-of-the art knowledge on the physiology and biochemistry of Fe deficiency [13]. This includes using appropriate choosing of experimental orchards and individuals (taking special care in assuring the presence of Fe deficiency and the homogeneity of chlorosis) as well as an adequate methodology to measure leaf Chl concentrations. It should be always taken into account that the effectiveness of a given Fe-fertilizer will depend on the specific conditions imposed in the particular study, and in many cases a positive result will not grant efficiency in other scenarios.

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General Discussion

In this Thesis I try to make a contribution to answer several major questions related to nutritional disorders in plants: (i) how much a plant normally needs from a given element?; (ii) is it possible to prognose -not only diagnose- the nutritional disorder en question?; (iii) is it possible to take into account the state of-the-art of all related scientific knowledge, integrating physiological, biochemical and agronomical data to improve practical correction methods?; (iv) are there additional effects of the nutritional disorder which are not yet studied, and how to improve sampling methods in this respect?; and (v) which kind of methodological and practical advices can be delivered to guide people working in the field of correction of these nutritional disorders? We took iron chlorosis as a typical nutritional disorder in the Mediterranean region (Abadía et al. 2011), and peach tree as the main plant species of study because it is the most affected crop by Fe chlorosis in the area (Sanz et al. 1992). Therefore, most parts of the study were focused on peach tree, even though some parts were carried out with pear trees and sugar beet grown in hydroponics.

In order to answer to the first question mentioned, the amounts of nutrients removed by peach trees, and in particular Fe, were characterized, taking into account all the events at which trees lose nutrients: flower abscission, fruit thinning, fruit harvest, summer and winter pruning and leaf fall, as well as immobilization in permanent structures of the tree measured after tree excavation. The approach adopted in this part of the Thesis was based on the following rationale: (i) considering different orchard conditions; (ii) comparing of all nutrient concentration and contents found in three different peach tree cultivars; and (iii) analyzing all observations and reaching conclusions.

Concerning the nutrients removal, we characterized it quantitatively, in terms of total amounts per tree and year and also in terms of amounts per fruit yield. Furthermore, we analyzed the data qualitatively by making a breakdown of the relative contribution of each event to the global removal of each element. This approach could be called a "complete fruit tree nutritional scenario". We concluded that, for example, in case of Fe the tree needs were larger than those suggested previously (Abadía et al. 2004), and this could be attributed to the differences in the evaluation of the amounts stored in the permanent structure of the tree. A similar but less complete study, using the means of nutrient concentrations in three cultivars, was published previously (Grasa et al. 2006). Moreover, the breakdown of the nutrient requirements was quite similar in the three

peach tree cultivars used, in spite of the large differences in orchard yield and management, with each nutrient exhibiting a characteristic "fingerprint" breakdown allocation pattern. This study supports that values obtained can be used for estimation of the nutritional needs of other cultivars, provided they have a similar age, since this factor can cause nutrient concentration changes (Stassen et al. 2010). This kind of results could be considered as a new insight in the fruit tree nutrition field. On the other hand, from the time-course evolution of the nutrient concentration in leaves during the season we could confirm results obtained by other authors, who indicated a partial remobilization and storage of N and P before leaf fall and a major loss of Ca and Mg.

Concerning possibility to carry out the prognosis of Fe chlorosis, the elemental composition of early plant materials such as flower buds and flowers allowed the prediction of chlorosis (at 60 days after full bloom; DAFB) almost as well as the own leaf elemental composition at 60 DAFB. Such a relationship between mineral nutrient concentrations and Fe chlorosis was explored in a situation close to reality, with several peach and pear trees being sampled in different commercial orchards. Sampling included flower buds, vegetative buds, bud wood, flowers and leaves at 60 and 120 DAFB and was repeated for 3–5 years. The large database generated was exploited using different statistical approaches to: (i) evaluate the consistency of the results; (ii) show an adequate strategy for the database analysis; and (iii) extract all kind of conclusions that can be taken, since no single statistical method can provide all conclusions desired.

We used the following statistical approaches: (i) a comparison of means depending on the chlorosis level, to see which elements increase and decrease with Fe chlorosis; (ii) correlation and principal component analyses to assess the possible relationships that can exist between nutrient concentrations and SPAD indexes; and (iii) a stepwise multiple regression, to distinguish which nutrients contribute more than others to the explanation of the SPAD variance. Results revealed a consistent relationship, as indicated by all of the statistical methods used, between Mg (in all materials excepting 60 DAFB leaves), Zn (en particular bud wood and leaves), P (flower buds and flowers) and Fe (in the case of 60 and 120 DAFB leaves) and Fe chlorosis in the case of peach trees. Relationships between Mn (buds, flowers and leaves), Fe (flowers and 60 DAFB leaves), K (60 and 120 DAFB leaves), Mg (120 DAFB leaves), N (60 and 120 DAFB leaves and Zn (flowers) and Fe chlorosis were found in the case of pear trees.

The next step was to obtain equations for the prediction of chlorosis. The best-fit regression equations for the prediction of SPAD60 from nutrient concentrations of peach flower buds and flowers were quite reliable over the different years. Such equations could predict, in more than 86% of the cases, whether a tree in our region will show chlorosis later in the year, using only flower bud or flower mineral analysis data. Results were consistent despite the different peach and pear cultivars sampled, wich included different orchard conditions (such as soil characteristics and management practices), and the different statistical methods used. The development of this type of predictive tools will offer the farmer the possibility of taking a very early decision, having potentially a large impact on final fruit yield. Apart from the practical consequences of this study, the results also point out that some other elements may also interact consistently with the Fe chlorosis development.

Regarding the foliar iron chlorosis correction, several objectives were sought to improve the knowledge about foliar treatments and to evaluate the treatment effects. These include the assessment of the leaf mineral composition changes, re-greening and photosynthetic pigment concentration changes, Fe penetration capacity, and Chl fluorescence parameters. By using the different methods of evaluation, a consistent scenario was found, with a significant Fe uptake and a re-greening effect in the treated leaf part. However, in the untreated one small Fe concentration increases were found with no appreciable re-greening. Although the re-greening level obtained in the present work was not fully complete, we consider that in the case of orchards where the soil proprieties lead to a rapid precipitation of Fe and where soil treatment is laborious, a foliar fertilization alternative would be a good one. Moreover, we would expect that two foliar iron applications to the whole tree, wetting it very well with 5 l solution and giving to the Fe-compound more time for penetration, may be an effective solution for Fe chlorosis control. In summary, we consider that these advances in the understanding of the Fe penetration and allocation have shed some light on the complex scenario ruling the performance of Fe spray formulations.

Furthermore, as the performance level of a given fertilizer is not only affected by the fertilizer proprieties themselves but also by the evaluation methodology applied, we find it useful to present all advices and comments arising from the practical experience (problems, doubts, etc.) gathered during the different experiments related to fertilizer effect evaluation. We conclude that when assessing the effectiveness of Fe-fertilizers, it

is necessary to use sound practices based in the state-of-the art knowledge on the physiology and biochemistry of Fe deficiency. This includes using appropriate choosing of experimental orchards and individuals (taking special care in assuring the presence of Fe deficiency and the homogeneity of chlorosis) as well as an adequate methodology to measure leaf Chl concentrations. It should be always taken into account that the effectiveness of a given Fe-fertilizer will depend on the specific conditions imposed in the particular study, and in many cases a positive result will not grant efficiency in other scenarios.

Another chapter gives new perspectives for the understanding of nutrient transport in the xylem sap of woody plants, and in particular fruit trees, using metabolomic and proteomic approaches. In particular, the second approach has been very little used in woody plants, and no studies reporting protein profiles have been published in fruit tree species so far.

Conclusions

Conclusions

1- The allocation of all nutrients analyzed in the different plant parts was similar in different types of peach trees. Each element has a typical allocation pattern. All this indicates that the nutrient allocations found could be used as a guide for the estimation of nutrient requirements in other cultivars.

2- Peach tree materials removed in tree pruning and leaf fall include substantial amounts of nutrients that could be recycled to improve soil fertility and tree nutrition. Poorly known tree materials such as flowers and fruit stones contain measurable amounts of nutrients.

3- Significant associations between nutrient concentrations in the different plant materials and leaf SPAD were found using four different statistical approaches: i) comparison of means depending on the chlorosis level, ii) correlation analysis, iii) principal component analysis, and iv) stepwise multiple regression.

4- It is possible to carry out a prognosis of Fe-chlorosis using early materials such as buds and flowers. For instance, the flower bud composition could be used to predict accurately whether a tree will show chlorosis later in the season

5- A foliar $FeSO_4$ treatment can be effective in promoting Fe-chlorosis correction in the leaf treated areas, and this is associated to increases in leaf Fe and changes in pigment photosynthetic concentrations and Chl fluorescence parameters. In the untreated areas adjacent to treated one, small increases in Fe concentration were found, but re-greening was not observed.

6- The optimization of peach xylem sap sampling makes possible to obtain sufficient amount of xylem sap to carry out proteomic and metabolomic analysis in parallel.

7- A preliminary metabolomics analysis of peach tree xylem shows changes with Fe chlorosis in the concentration of some compounds, including the non-proteinogenic amino-acid nicotianamine. This suggests that it could play a role in long-distance Fe transport in peach trees.

8- The good resolution and reproducibility of the 2D gels obtained with peach xylem sap indicate that this technique could be a powerful tool for the study of changes in xylem composition with Fe deficiency.

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