NOVEL APPROACHES FOR THE CONTROL OF IRON CHLOROSIS IN FRUIT TREE CROPS

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Short summary

The works carried out in the project have confirmed the strong deleterious impact of lime-induced chlorosis on fruit yield and quality in several fruit tree species grown in Southern Europe. The results found in the project have established the principles to perform the early diagnosis (prognosis) of Fe chlorosis in trees from the mineral analysis of flowers. Two mineral analysis parameters in flowers, the concentration of Fe and the K/Zn ratio, have been shown to correlate well with tree chlorosis later in the year. Our work has demonstrated that other possibilities to control chlorosis, alternative to Fe-chelates, do exist and may be included among the routine fruit orchard managing practices. These agronomic practices include foliar sprays with inorganic Fe sources, growing graminaceous plants fertilised with Fe in the orchards and adding new Fe-containing products to the soil. Data obtained in this project have permitted to establish a new protocol for screening tests based on physiological characteristics associated to tolerance to Fe chlorosis in fruit trees. A standardised root tip test has been also developed to estimate root ferric chelate reductase activity *in vivo*. The biochemical characteristics of the root ferric chelate reductase enzyme in the roots (at the level of intact roots and purified plasma membrane) and leaves (at the level of mesophyll tissue, intact protoplasts and purified plasma membrane) have been characterised in Fe-deficient and sufficient plants. A very important finding is that the natural complexes of citrate and malate with ferric Fe are good substrates for the ferric chelate reductase enzyme, both in mesophyll tissue and in isolated leaf plasma membrane, opening new possibilities for applying these compounds to control Fe chlorosis in field conditions. Knowledge has been gained during the project on the molecular changes in proteins induced by Fe deficiency in leaf and root tissue, including the isolation and partial characterisation of the ferric chelate reductase from spinach leaves. Other approaches such as 2-D electrophoresis of root tip proteins have led to the identification of a series of polypeptides which are candidates to play a role in the Fe-efficiency responses of plants to Fe deficiency. The new knowledge on the photosynthetic changes induced by Fe deficiency has provided the background data to understand one of the characteristics less studied of Fe chlorosis, the light dependence of the deficiency. The changes in PS II efficiency in Fe-deficient leaves have been characterised in studies carried out in the project. Finally, we have demonstrated that a photosynthetic parameter, the chlorophyll content estimated from SPAD readings, is an excellent tool to be used as an indicator of Fe chlorosis.

Executive summary

The results obtained during the project have shown that chlorosis has major negative impacts in fruit yield and quality in different fruit species grown in the Mediterranean area. An increase in chlorosis symptoms led not only to severe reductions of total fruit yield per tree, but also affected adversely fruit size and quality. These data emphasise the importance of controlling chlorosis in fruit tree crops in Southern Europe.

The results found in the project have established the principles to perform the **early diagnosis (prognosis) of Fe chlorosis in trees** from the mineral analysis of tree flowers. Two mineral analysis parameters in flowers, the concentration of Fe and the K/Zn ratio, have been shown in the project to be well correlated with tree chlorosis later in the year. This suggests that the analysis of flowers of any previously non-analysed orchard or tree could be useful for estimating the future chlorosis status, as well as an index for assessing the Fe level of trees in the previous year. The proposed techniques for the prognosis of Fe chlorosis, however, would need validation from a larger set of orchards and in more diverse conditions.

The **Fe flower concentration** was proposed before the project as a tool for the prognosis of chlorosis in fruit trees. Our results have demonstrated that the correlation between Fe in fruit tree flowers and the leaf chlorophyll concentration later in the season is often significant. The use of the **K/Zn ratio in flowers** may offer advantages over the Fe flower concentration for the prognosis of tree chlorosis later in the year. We found that Fe deficiency causes only few changes in the nutrient concentrations in flowers, including increases in the K concentration, whereas the Zn concentration may decrease or not change significantly with chlorosis. The K/Zn ratio in flowers is well correlated to chlorosis every year. However, and conversely to what happens with Fe, the average concentration of K and Zn in flowers as well as the K/Zn ratio have quite consistent values from year to year.

The physiological basis for the changes in the concentrations of Fe, K and Zn with chlorosis has not been established yet. It is likely that most nutrients present in the flowers are already in the tree at the time of flower initiation, the vegetative season prior to flowering. The concentration of Fe, K and Zn in the flowers of fruit trees may reflect the size of the active or mobile pools of nutrients at the time floral buds were formed. If this hypothesis is correct, it will open other possibilities for very early nutritional diagnosis. For instance, one may envisage the possibility that the **nutrient concentration in floral buds** (or even in vegetative buds) during the dormancy period could also be a good early tool to estimate the chlorosis status of the next year. The first data on these very early tree materials have been obtained in the project and suggest that they would possibly offer new possibilities for the prognosis of chlorosis.

Agronomic means for controlling Fe chlorosis are still viewed with great interest by fruit growers. Since Fe chelates were introduced, there has been little effort in the research of alternative means for controlling the chlorosis. Our work has demonstrated that alternatives do exist and in the future may be included among the routine practices of managing fruit trees. The results found in the project have established that some agronomic practices, such as applying foliar sprays with inorganic Fe sources, growing graminaceous plants fertilised with Fe in the orchard and adding new Fe-containing products to the soil, could be as effective as synthetic Fe-chelates in controlling chlorosis in fruit trees. Alternatives to Fe chelates are of great importance in orchards that follow the guidelines of "Biological Production" or "Integrated Production".

Experiments carried out in the project have shown that foliar sprays of cheap Fe salts such as Fe sulphate could be effective in controlling chlorosis. Of course several applications need to be made during the growing season, and more work has to be done to optimise the

effects of foliar sprays in the regreening of leaves and minimising the possible deleterious effects in fruit quality in each crop. Also, some agronomic practices, such as growing graminaceous plants near the trees concomitantly to the addition of inorganic Fe salts to the soil or using new commercial compounds such as blood meal, are promising for controlling chlorosis. The proposed techniques, however, would need also validation under the integrated production management schemes used by commercial growers.

Research done in the project has also shown the importance of the Fe pools inactive in the chlorotic leaves of fruit trees, since when chlorosis was not too severe re-greening may occur by adopting strategies that remobilize Fe. The well-accepted phenomenon that chlorotic and green leaves have similar total Fe concentration, which has been termed "Fe paradox", indicates the existence of Fe pools in the chlorotic leaf which are somehow inactive. Data from our experiments demonstrate that sprays aiming to activate the Fe pools in the chlorotic leaf are effective, although rarely caused a full recovering from Fe chlorosis. This indicates that in a chlorotic leaf part of the Fe is inactivated outside the mesophyll cells. As both citric, sulphuric, ascorbic and indole-3-acetic acid caused re-greening, we could hypothesise that inactivation is due to several factors, including a high apoplast pH, high rates of oxidation of Fe(II) or low Fe reductase activity.

The results found in the project have established the principles for the design of screening tests for tolerance to Fe chlorosis based on physiological parameters. Data obtained in this project have permitted to establish a **new protocol for designing screening tests** based on physiological characteristics associated to tolerance to Fe chlorosis in fruit trees. We have produced consistently increases (20-fold) in the root ferric chelate reductase activity in tolerant fruit tree rootstocks, by re-supplying plants grown without Fe for several days with a small amount of Fe(III)-EDTA. Furthermore, we have found that this protocol may be used in

screening assays to select rootstock genotypes tolerant to Fe chlorosis, as shown from preliminary comparisons of several genotypes differing in chlorosis tolerance. The proposed new protocol could be used to assess in the future the germplasm available for chlorosis tolerance. The protocol has been shown to work well with micropropagated material, but its use with cuttings, more widely available than micropropagated plants for many rootstock genotypes, still needs further testing.

The possible use of **somaclonal variation methods** has given promising results with pear genotypes. These results are still preliminary but open a new way of finding tolerance to chlorosis in fruit trees.

The works developed within the project have provided light on several physiological changes induced by Fe deficiency in plants. The biochemical characteristics of the **root ferric chelate reductase enzyme** have been extensively studied on the level of intact roots *in vivo,* and on the level of purified plasma membranes *in vitro.* These characteristics include substrate dependence and optimal pH. Our finding that the enzymatic characteristics depend on the measuring pH has changed the view on the root ferric chelate reductase enzyme.

A standardised **root tip test** has been developed to estimate root ferric chelate reductase activity *in vivo*. After cultivation of the species or cultivar of interest in Fe-deficient and Fe-sufficient hydroponics solutions, information about Fe efficiency and patchiness of ferric chelate reductase activity was obtained from the performance of *in vivo* agar tests. Then, *in vivo* ferric chelate reductase activity was measured using either root tips or whole roots, depending on the agar tests.

The biochemical characteristics of the **leaf mesophyll ferric chelate reductase enzyme** have been studied at the level of mesophyll tissue *in vivo,* intact protoplasts and purified plasma membranes *in vitro*. The *in vivo* leaf ferric chelate reductase activity has been

confirmed to be light dependent. The ferric chelate reductase enzymatic characteristics of the leaf plasma membrane are remarkably similar to those of the root plasma membrane enzyme. However, the pH dependence of the ferric chelate reductase enzyme in isolated protoplasts is different from that of isolated plasma membranes, with its optimal pH being closer to that of the apoplastic pH. In any case, our data suggest that both the intrinsic decrease in ferric chelate reductase activity per protoplast surface and a possible shift in the pH of the apoplastic space could be responsible for the immobilization of physiologically inactive Fe pools in chlorotic leaves. Since no "Turbo" ferric chelate reductase activity could be found in leaves, screening leaf sources for ferric chelate reductase activity does not appear to be a successful approach to identify Fe-efficient fruit tree lines.

A very important finding is that the natural complexes of citrate and malate with ferric Fe are good substrates for the ferric chelate reductase enzyme, both in mesophyll tissue and in isolated plasma membranes. This opens new possibilities for applying these compounds to control Fe chlorosis in field conditions, as already shown in kiwifruit.

Knowledge has been gained during the project on the molecular changes in **proteins induced by Fe deficiency** in leaf and root tissue. The isolation and partial characterisation of the FC-R from spinach leaves has been carried out during the project. Other approaches such as 2-D electrophoresis of root tip proteins have led to the identification of a series of polypeptides which are candidates to play a role in the Fe-efficiency responses of plants to Fe deficiency. It is expected that these polypeptides will be characterised in the near future.

The new knowledge on the photosynthetic changes induced by Fe deficiency has provided the background data to understand one of the characteristics less studied of Fe chlorosis, the light dependence of the deficiency. The changes in PS II efficiency in Fe-deficient leaves have been characterised in studies carried out in the project. Chlorotic leaves

grown in both growth chambers and field conditions have increases in the molar ratios lutein/chlorophyll *a* and (V+A+Z)/chlorophyll *a* and show changes in leaf absorptance and reflectance. The low chlorophyll, Fe-deficient leaves showed no sustained decreases in PS II efficiency, measured after dark adaptation, except when the deficiency was very severe. However, the chlorotic leaves showed decreases in the actual PS II efficiency at steady-state photosynthesis, due to decreases in photochemical quenching and intrinsic PS II efficiency. Iron-chlorotic leaves were protected not only by the decrease in leaf absorptance, but also by down-regulation mechanisms enhancing non-photochemical quenching and thermal dissipation of the light absorbed by PS II within the antenna pigment bed.

Finally, we have demonstrated in the project that a photosynthetic parameter, the chlorophyll content estimated from **SPAD readings**, is an excellent tool to be used as an **indicator of Fe chlorosis**. This parameter, once properly calibrated for a given species, is far better than other indicators such as chlorophyll fluorescence, mineral content and visual ratings. We have used the SPAD apparatus in most experiments developed in the project, including chlorosis control experiments.

Chapter 1. Introduction

A significant part of the fruit tree industry in the Mediterranean area of the European Union is established in calcareous or alkaline soils, which favours the occurrence of Fe deficiency in trees thereafter referred to as Fe chlorosis. This deficiency occurs because of low Fe availability in high pH soils. Although differences among species and clones exist, several fruit tree crops, namely peach, pear, kiwifruit and citrus, are considered very susceptible to Fe chlorosis (Korcak, 1987). For example, in the Ebro valley in Northeastern Spain more that 90% of the peach orchards and almost 70% of the pear orchards suffer from Fe chlorosis (Sanz et al., 1992a). A similar occurrence of chlorosis is typical of pear and kiwifruit orchards in the Po valley of Italy, of peach orchards in the area of Imathia in Northern Greece and of peach and citrus orchards in Southern Portugal (Algarve).

Iron chlorosis in fruit trees has special characteristics not occurring in Fe-deficient annual field crops. First, one should consider the fact that trees are perennial, so that Fe deficiency and the development of chlorosis in one year would affect Fe nutrition and chlorosis development in the following year. Trees lose Fe when flowers, fruits and leaves fall naturally, and also when fruits are harvested and when wood is removed by agricultural practices such as pruning. It is generally accepted that trees may store large amounts of Fe (mainly adsorbed) in their root systems (Mengel, 1995; Tagliavini et al., 1993), which can be taken up or remobilised to the shoot later. It is also likely that the early growth stages and flowering in spring are sustained by Fe stored in the tree the previous season(s).

Another peculiar aspect of Fe nutrition in trees is related to their size and to the fact that, after absorption, Fe has to be transported for a long distance to reach the tree canopy. Problems in Fe transport through the xylem are therefore more likely in trees than in herbaceous crops and are possibly responsible for the fact that, within the same tree, leaf Fe

chlorosis is more severe on second or third order branches that on those directly inserted on the trunk. Also, fruit trees are usually composed of two individuals, one for the shoot (scion) and other for the root part (rootstock). Problems in the transport of Fe, as well as of other elements, may arise from the fact that in some species, such as in pear, a certain degree of scion/rootstock (usually quince) incompatibility is desirable as it allows a control of the tree size. Graft incompatibility, however, impairs both upward nutrient transportation through the xylem and carbohydrate replenishment to the roots through the phloem (Breen, 1975). Under these conditions, root responses to Fe deficiency, such as the increase of Fe-reduction activity and proton extrusion, may be impaired and might not last due to the lack of carbon skeletons and chemical energy coming from the shoot.

The occurrence of Fe chlorosis in orchards also varies during the lifetime of the trees. Chlorosis, once established, often becomes more intense with tree age. The cause of this phenomenon is not known. In some cases, when trees become older the root system may explore deeper soil layers whose characteristics, e.g. high calcium carbonate levels, high water content and low oxygen concentration, would be less favourable for Fe uptake.

The purpose of the Introduction section is to provide an overview of the knowledge existing at the starting date of the project, in January 1995. This has been done in separate sub-sections for the following issues related to Fe chlorosis: early diagnosis (task B of the project), alternative management techniques (task C), responses of rootstocks to chlorosis and screening techniques (Tasks D and E) and whole plant responses to iron deficiency (Task F). When appropriate reviews existed by 1995 they are quoted in the text. If no reviews on a specific subject existed, a succinct review on the information available at that time has been written and is included in the text.

1.1 Early diagnosis

The most straightforward approach to detect nutrient deficiencies in plants is to analyse the mineral content of leaves at the standard recommended time for analysis in each crop. In the case of fruit trees with Fe deficiency, however, this approach entails two major problems. First, Fe chlorotic leaves have often relatively high Fe concentrations, and present rather low correlation between leaf Fe and chlorophyll concentrations (Oserkowsky, 1933; Procopiou and Wallace, 1982; Abadía, 1992). This is the so-called "chlorosis paradox" (Römheld, 1997; Morales et al., 1998a; Römheld and Schmidt, 2000). A second problem is that at the recommended time of leaf sampling for analysis, 100-120 days after full bloom (DAFB), most of the peach tree varieties in the Mediterranean area are already harvested or very close to harvest. Therefore, the diagnosis of Fe deficiency at this point would be of no practical effect. An earlier standard sampling date (60 DAFB) has been proposed (Sanz et al., 1992b), although still at this date both problems are present.

The possibility of using the mineral content of flowers for the assessment of the mineral nutrition of fruit trees was first proposed in the pioneering work of Sanz and co-workers. The rationale for this approach is that flowers, that can be sampled in some species before leaves have emerged, may have mineral concentrations representative for the nutritional status of the tree later in the season. The use of flower analysis would permit to detect and correct any deficiencies at a very early stage, before fruits are set and several months before harvest, thus giving sufficient time for the nutrient amendments to improve yield and fruit quality.

Sanz and co-workers analysed in 1992 the concentrations of several elements in flowers at full bloom and in leaves at 60 and 120 DAFB in one hundred pear and one hundred peach orchards in Spain. Chlorophyll was not measured in these experiments. Orchards were selected as representative (from green to very Fe-chlorotic) of the Ebro valley area in

Northeastern Spain, in the summer of 1991. The results of that work were published in the proceedings of three Symposia held in 1993 (Sanz et al., 1994, Sanz and Montañés, 1995a; Sanz et al., 1995) and in two subsequent articles (Sanz et al., 1993; Sanz and Montañés, 1995b).

Three main observations were made in these preliminary trials. Firstly, Fe concentrations were markedly higher in flowers than in leaves. For instance, pear flowers and leaves had mean concentrations of 198 and 127 µg Fe g^{-1} DW, respectively (Sanz et al., 1993, 1994; Sanz and Montañés, 1995a). In peach, flowers and leaves had mean concentrations of 293 and 111 µg Fe g^{-1} DW, respectively (Sanz and Montañés, 1995a, 1995b). Secondly, the range of Fe concentrations was wider in flowers than in leaves. In pear, flowers had 70-471 µg Fe g^{-1} DW, whereas leaves had 43-210 µg g^{-1} DW. In peach, the ranges of Fe concentrations were 145-573 µg g^{-1} DW in flowers and 23-189 µg g^{-1} DW in leaves. This permitted to suggest that the Fe concentration in flowers, having a wider range of variation than the Fe concentration in leaves, could be potentially a better tool to predict chlorosis developed later in the year (Sanz et al., 1993). A third observation was that the correlation between the Fe concentrations in flowers and those in leaves later in the year was significant. In pear there were significant correlations between the Fe concentrations in flowers and leaves taken 60 ($r = 0.434^{**}$; $n = 100$) and 120 ($r = 0.349^{**}$; $n = 100$) DAFB (Sanz et al., 1993, 1994; Sanz and Montañés, 1995a). In peach there was also a significant correlation between Fe in flowers and Fe in leaves taken 60 DAFB ($r = 0.222^{*}$; $n = 100$) (Sanz and Montañés, 1995a, 1995b).

Therefore, at the beginning of the project it was thought that flower analysis was a promising tool for the early diagnosis of Fe chlorosis. However, no firm relationships between flower composition and the development of chlorosis had been demonstrated at that

time.

1.2 Alternative agricultural management techniques to control iron chlorosis

As Fe chlorosis has a tremendous economical impact on fruit industry, it makes unprofitable growing Fe chlorosis-susceptible species in calcareous soils in the absence of effective means of preventing or at least controlling chlorosis. Theoretically, the best way to solve the chlorosis problem in fruit trees would be the choice of tolerant rootstock genotypes (Socias et al., 1995, see section 1.3 below). However, this has been accomplished only in few circumstances, as in the case of the peach x almond hybrid rootstocks for peach. In many cases, Fe tolerant rootstocks are not very attractive from an agronomic point of view, because they may induce excessive growth in the scion (e.g. *Pyrus communis* seedlings for pear varieties and “Fercal” for grape). Several breeding programmes throughout the world are aimed to select Fe tolerant rootstocks, by exploring the variability within species or within genus and breeding properly chosen parents (Socias et al., 1995). This genetic approach could be successful in the long run, but until then tree growers have to rely on agronomic means for preventing or curing Fe chlorosis.

Agronomical approaches to control chlorosis were reviewed by Wallace (1991) and Morvedt (1991). As most fruit tree crops are high value crops, fruit growers often try to control chlorosis by applying routinely synthetic Fe chelates. Application rates are usually higher than 50 g of Fe-chelate per tree. Iron chelates are usually quite effective; however, they are very expensive. Estimates made in Italy and Spain indicate that in some orchards located in calcareous soils Fe chelate cost approaches 60% of the total fertiliser costs. Chelate cost is often as high as 250 Euro per hectare. Being soluble and very stable even at high pH, soil-applied Fe chelates are easily leached out of the root zone if excessive irrigation regimes are applied or during the autumn-winter period, when rains overcome evaporation of water from

soils. For these reason the Fe chelate applied in one year rarely prevent chlorosis from occurring the following year. When needed, Fe chelates have therefore to be applied every year. Moreover, the fate in soil of the chelating agents has received little attention. Chelates which are not taken up by the roots may leach out of the soil and then to aquifers, rivers and the sea. The total amount of Fe chelates used by European fruit tree growers is probably higher than 10,000 Tm per year.

At the beginning of the project there were preliminary informations by some growers that agronomic approaches other than Fe chelates could diminish Fe chlorosis in tree orchards. For instance, it was known that solid injections of ferrous Fe to the trees could cause tree regreening (Heras et al., 1976). Liquid injections could be also effective (Wallace, 1991). However, these procedures are expensive and labour-extensive. Other less expensive treatments that are applied in herbaceous crops to avoid Fe deficiency (Morvedt, 1991) could be applied also to trees. For instance, it was known by some growers that foliar sprays of Fe salts and acidic compounds could sometimes improve the green colour of fruit tree leaves in the Mediterranean area. Wallace (1991) and Morvedt (1991) reviewed early research on that topic. Also, soil management practices different from adding Fe-chelates were known to alleviate tree chlorosis in some instances. However, by 1995 these reports were little tested and documented in a scientific manner.

1.3 Responses of rootstocks to chlorosis and screening techniques

It is generally considered that the best way to solve the chlorosis problem in fruit trees would be the choice of tolerant rootstocks. The possibilities for finding new rootstocks for the

different fruit species through breeding was recently reviewed in the paper by Socias et al. (1995). Since classical breeding involves time-consuming testing in field conditions, the introduction of physiological markers in breeding programs is highly desirable. Possible physiological markers for Fe-efficiency include, among others, the ferric chelate reductase (FC-R) activity of roots, that is supposed to correlate with the capacity to acquire Fe from the medium when Fe is in short supply (Jolley et al., 1996).

Until 1995 few studies had been made on the responses of fruit tree species to Fe deficiency. Varanini and Maggioni (1982) first indicated the existence of a FC-R activity in *Vitis* spp. cuttings. In a study with *Malus domestica,* Ao et al. (1985) found that Fe deficiency induced chlorosis caused decreases in the pH of the nutrient solution and 10-fold increases in FC-R activity of whole plants, from 0.9 to 10 nmol Fe min^{-1} g^{-1} FW. Working with cuttings of *Vitis* spp. Bavaresco et al. (1991) found that conditions leading to chlorosis in sand culture promoted the formation of root hairs and an increase in root diameter. The excised root FC-R activities of stressed plants ranged from 0.6 to 3.6 nmol Fe min^{-1} g^{-1} FW, although no data were reported for non-stressed plants (Bavaresco et al. 1991).

A number of experiments involving FC-R activity measurements were carried out with *Prunus* spp. rootstocks until 1995 (Tagliavini and Rombolà, 1995). In some of them Fe deficiency led to the induction of the FC-R activity, whereas in other studies no such induction was found. In this work we will follow the nomenclature for *Prunus* rootstock species indicated in Casas et al. (1999). Studies including FC-R activities of *Prunus* spp. rootstocks were first carried out with rooted cuttings and micropropagated plants of a peach (Nemaguard), three plums (Brompton, San Julien A and Puebla de Soto 101) and two almond-peach hybrids (Adafuel and GF677) (Romera et al., 1991a, 1991b). These rootstocks developed chlorosis with bicarbonate in the medium in the order Nemaguard > Brompton,

San Julien A, Puebla de Soto, Adafuel and GF677. In a first series of experiments no FC-R induction was found with Fe deficiency (Romera et al., 1991a). However, in a second series of experiments with the same plant materials a 4 to 7-fold FC-R induction was found with Fe deficiency in the plums and almond x peach hybrids, but not in the peach rootstock (Romera et al., 1991b). A decrease in nutrient solution pH was also found with the plums and to a lesser extent with the peach-almond hybrids (Romera et al., 1991b).

Another study was carried out with two peach genotypes (Montclar and Nemaguard), and the peach x almond hybrid TNG (Titan x Nemaguard) (Egilla et al., 1994). The hybrid was found to be more resistant to chlorosis than the two peach genotypes. In these studies true FC-R was not measured, because $FeCl_3$ was used as Fe source. In this case Fe deficiency-stimulated FC-R activity was increased in some experiments (1 to 8-fold increase) in the hybrid (Egilla et al., 1994). These reductase values were apparently very high, although in other species the reduction rate with ferric salts is generally lower than with Fe-chelates (Schmidt, 1999). Both root hairs and root swellings were noted with low Fe. Iron reduction was found at the root hairs or in regions between the root tips. The first to detect FC-R activity in citrus seedlings were Treeby and Uren (1993) and Manthey et al. (1993). Root tip FC-R activities reported were in the ranges 4.1-6.3 and 2.0-56.1 nmol Fe min^{-1} g^{-1} FW for control and Fe-deficient seedlings; increases in FC-R activities for a given genotype ranged between less than 1 to 10-fold (Manthey et al., 1994).

In summary, at the beginning of the project root FC-R activity was not unequivocally demonstrated as being one of the Fe-efficiency responses of tree species. Although the FC-R activities of tree species were generally lower than those found in herbaceous plants (see Moog and Brüggemann, 1994), it was apparently difficult to find a repeatable induction of the FC-R activity under conditions of Fe deficiency. This fact made impossible to introduce this

physiological character in screening tests for chlorosis tolerance. On the other hand, hybrids obtained from classical breeding programs, for instance peach-almond hybrids, were known to be more tolerant than peach genotypes.

1.4 Whole plant responses to iron deficiency

At the beginning of the project the whole plant responses to Fe deficiency were incompletely known. Reviews summarising the knowledge on plant physiological responses to Fe deficiency at that time were those of Terry and Abadía (1986), Abadía (1992) and Zayed and Terry (1995) for chloroplasts and leaves and that of Welkie and Miller (1993) for roots.

Some areas where information was still lacking at the beginning of the project were the characteristics of the FC-R activities of intact roots and isolated plasma membranes (PM) and the nature of the ferric chelate reductase (FC-R) enzyme of the plasma membrane. The knowledge on the PM FC-R enzymes at the starting date of the project was summarised by Moog and Brüggemann (1994). Information on the characteristics of the mechanisms of Fe acquisition by the leaf cells was practically missing, with only one paper available on this issue (Brüggemann et al., 1993). Work in these areas has been carried out within the project. Finally, we have studied the role of photosynthetic parameters in the development of chlorosis and also its possible use as indicators of chlorosis.

2. Materials and methods

Describing in detail all the materials and methods used in the project by all participants will make this section extremely long. Therefore, we shall quote, whenever possible, scientific and technical reports written by project participants. In these widely available publications the materials and methods used are described in full detail.

Sampling of plant materials. Flower and leaf samples were taken by standard procedures, as indicated in Abadía et al. (1985), Belkhodja et al. (1998a) and Abadía et al. (2000). Flowers were taken at the full bloom stage. Fully developed leaves were taken from the distal third of the current year's growth.

Chlorophyll leaf concentration. Leaf chlorophyll was estimated non-destructively with portable instruments SPAD-502 (Minolta Corp.). In general, it was agreed to make two to four readings per leaf and to take into consideration fully expanded leaves belonging to expanding shoots. Usually 30 leaves per tree around the crown were measured. The SPAD meters were always calibrated for different plant species or genotypes by concomitant spectrophotometrical measurements of leaf extracts.

Chemical analysis. Samples were washed and analysed by standard procedures (Abadía et al., 1985; Belkhodja et al., 1998a). Procedures for measuring the mineral concentrations in plant materials followed the guidelines of Association of Official Analytical Chemists (AOAC). Active Fe determination was based on the procedures described by Pierson and Clark (1986). N was measured by Kjeldahl and P spectrophotometrically. Samples were dry-ashed by standard AOAC methods. To perform full dissolution of ashes samples were heated on a hot plate in the last step of the method (i.e. after adding HCl). K was measured by flame emission, and Ca (after La addition), Mg, Fe, Mn, Cu, Zn by AAS. Nitrate, sulphate, chloride and phosphate were separated by HPLC with a Dionex column and detected by conductivity. The mobile phase (10% Na carbonate) was set at a flow of 2 mL min^{-1}. Organic acids were separated by HPLC at room temperature and detected at 210 nm. The column used was a 7.8 x 300 mm Aminex HPX-87H (Bio-Rad). The mobile phase (8 mM sulphuric acid) was set at a flow of 0.6 mL min^{-1}. Total organic acids in leaves were extracted from frozen leaf discs. Discs were taken with a cork borer and ground in a mortar with 8 mM sulphuric acid. The extract was boiled for 3 min, filtered and kept at -30 °C until HPLC analysis.

Orchards for Early diagnosis. Many experiments have been conducted in this task during the project in Portugal, Italy, Greece and Spain in fruit tree orchards grown in calcareous soils.

P1 carried out experiments in Spain on flower and leaf analysis in the period 1995-1998 in peach and pear orchards located in 'El Temple' (El Temple, Huesca, Spain), 50 km North from Zaragoza. Full details on these orchards are given in Belkhodja et al., (1998a), Morales et al., (1998a), and Igartua et al. (2000). Other orchard used was in the 'San Bruno' site, in the Servicio de Investigación Agroalimentaria-Diputación General de Aragón farm, located in the Aula Dei Campus (Zaragoza, Spain) (Morales et al., 1998a).

The Italian group (P6) carried out work in 1995 and 1996 in peach orchards located in alkaline calcareous soils of the Province of Ravenna (Po Valley) in Italy. Two of the orchards were planted in November 1987 with

planting distances of 4.5 x 3.5 m. Trees (cvs. StarkRedGold, and SpringRed) were grafted on seedlings and trained as modified palmette. The main characteristics of the soil where SpringRed was planted were: pH (in water) 8.0, texture loamy, total carbonate 22.7%, active carbonate 6.0%, organic matter 1.31%, P (Olsen) 15 ppm, exchangeable K 252 ppm, exchangeable Mg 190 ppm, exchangeable Ca 2850 ppm, Fe-DTPA 16 ppm, Mn-DTPA 7 ppm, Zn-DTPA 3 ppm, Cu-DTPA 43 ppm. In addition to these two orchards, another 8-year old nectarine orchard of SpringRed was chosen in the same geographical area and with the same planting distances and used in 1996. No additions of Fe chelates or Fe salts were performed on the trees selected during the experimentation period. Fifteen trees per orchard (45 trees in total) were randomly chosen during winter and used for flower and leaf analysis. For the experiments in 1997, several trees of the cvs. StarkRedGold and SpringRed with different degrees of chlorosis were selected during 1996 in two orchards located in a calcareous area close to Imola (Bologna, Italy). In 1997, 10 leaves from chlorotic to green trees were collected 60, 90 and 120 days after full bloom (DAFB).

In Greece work was done in 1995-1998 in peach (cv. Flavour Crest on 5 rootstocks and 6 replications and 12 peach cvs. on 3 rootstocks, and Flavour Crest on 5 rootstocks).

In Portugal work was done in 1997 and 1998 with twenty trees of Valencia late grafted on *Citrange troyer* . Trees were randomly selected in a mature orchard, established in a calcareous soil (total calcium carbonate 22%, active calcium carbonate 10%, pH 7.7). In April, during full bloom, 30 flowers per tree were collected randomly. Leaf nutrient analysis and SPAD measurements were done. In February 1997 and March 1998 fruits were sampled in the selected trees. Three fruits were collected in each tree. Fresh weight, caliber, refraction index and citric acid percentage were determined in each fruit. In a second site, ‘Almancil’, twenty trees of 'Valencia late' grafted on *Citrange troyer* were randomly selected in a mature orchard, established in a calcareous soil (total calcium carbonate 47%, pH 7.8). This experiment was initiated in 1998, with leaf samplings on April, May and June and a flower sampling in April.

Alternative agricultural management techniques to control iron chlorosis. The techniques for treatment applications and recovery from Fe chlorosis in Spain (P1, P5), Greece (P3), Portugal (P2) and Italy (P4) were standardised during the first years of the project to allow for the use of similar experimental methods in field trials developed in the four Countries. We put emphasis on the characterisation of the soil conditions where treatments were applied, in particular soil pH, total and active (free) lime, soil texture, available soil Fe, etc. Beside untreated control plants, experimental plots treated with the same Fe chelate through the canopy (Fe-DTPA or EDTA) or the soil (Fe-EDDHA or Fe-EDDHMA) were present. Standardisation of treatment applications also included the use of surfactants, with the aim of improving the uniformity of distribution of the product on the leaf surface.

Experiments were performed (by P1 and P5 in Spain) in 1997 and 1998 to evaluate the losses of Fe and other nutrients due to leaf fall and pruning (Grasa et al., 1998). These experiments were done to quantify the yearly needs for Fe of peach trees.

An experiment was carried out in Italy to compare the autumn and the late winter application of Fe chelates in preventing Fe chlorosis development early in spring. The trial was carried out in a mature kiwifruit orchard, drip

irrigated and located in the hilly area close to Faenza (Ravenna, Italy). The soil had the following texture: sand 35%, silt 49%, clay 16% and a pH of 7.95; total $CaCO_3$ was 14% and active lime was 4.4%. Plants were assigned to 6 blocks. Treatments included an autumn soil supply of Fe or a late-winter soil supply of Fe. Autumn Fe supply was applied to the soil on October 30, 1997 in the form of Fe-EDDHMA (20 g/plant of a commercial Fe-chelate, equal to 1.4 g Fe/plant). The amount of Fe-chelate was split in two parts and localised under the drippers, which supplied a few litres of water to approach the chelate to the roots. In January 1998 the irrigation system was used again for leaching any residual Fe chelate from the root zone of all selected plants. Soil cores were taken at the root depth and analysed for DTPA extractable Fe and revealed similar Fe concentrations in the treated and untreated soils. The late-winter application of Fe was carried out on March 9, 1998, with the same amount of Fe as applied in autumn. At that stage the plants were at the phenological phase of bud swelling. On April 9, when shoots had an average length of 8-10 cm, two shoots per vine were collected and chlorophyll concentration was measured on the two largest leaves (not fully expanded). On April 27 1998, when average shoot length was 25-30 cm, leaf chlorophyll concentration was measured again.

The necessity of applying products against chlorosis every year was studied by P3 in two peach orchards where treatments to overcome Fe chlorosis were soil-applied in 1995. In 1996 the carry-over effect was assessed by measuring leaf chlorophyll concentration and fruit yield.

An experiment was carried out by P1 with the goal of lowering soil pH and improving Fe nutrition in two alkaline soils, both collected from agricultural land in the Po valley of Italy, but differing for calcium carbonate concentration (Tagliavini et al., 2000). As the distribution of fertilisers through the irrigation water (fertigation) has become largely adopted in intensive orchards and drip irrigation is often applied at daily intervals, the frequent addition into the soil of acidified water might bring about an improvement in Fe nutrition. To test this hypothesis P1 has undertaken a preliminary trial by lowering the pH of the irrigation water to 5.0. Soil solutions were collected at the depth of 20 and 30 cm and at 20 cm from the emitters by suction lysimeters (Neilsen et al., 1995), at regular intervals starting from the end of the irrigation period.

Responses of rootstocks to chlorosis and screening techniques. Participants in the different countries have performed many field rootstock evaluation trials.

In Italy two pear orchards, both with cv. Abbé Fetel, were used in 1997-1998. The first one was located close to Ferrara and planted in a soil with the following characteristics: pH (in water) 7.4-7.6, total carbonate 8.3-8.8%, active carbonate 1.7%. Trees were three-year-old and grafted on four quince rootstocks differing in vigour: quince C, Sydo, quince A and BA29. SPAD readings were taken at four different times on at least 15 randomly chosen trees. On each tree four leaves belonging to four shoots were analysed and four readings per leaf were taken. No Fe applications were done during the experimental period. A second orchard was located close to the town of Argenta (Ferrara) in a loam soil with the following characteristics: pH (in water) 7.8, total carbonate 31%, active carbonate 5%. The pear trees of the cv. Abbé Fetel, planted at 4 x 1 m during 1993 were grafted on the quince rootstocks BA29, Sydo and quince A and also on the pear rootstock Fox 11. The orchard received 10 kg/ha of a Fe chelate (6% Fe) by fertigation. In June ten trees were selected randomly and SPAD readings were taken, on five shoots each, in the most apical, fully expanded leaf. A rootstock comparison trial was also

established in March 1998 in a commercial farm located in the eastern Po Valley (Province of Ravenna). The loamy soil had the following characteristics: pH (in water) 7,97, total carbonate 18%, active lime 6.9%, DTPA Fe 23 ppm, organic matter 1.87%. Trees of the following cultivars were planted with distances of 3.6 x 1.4 m: Abbé Fetel, Conference, Bartlett, Comice and Bosc. Each cultivar was grafted on the quince rootstocks BA29 and C, and the pear rootstocks OHF 333, Fox 11 and 16. The experimental design was a randomised block design within each cultivar with 6 blocks and three plants per experimental unit.

The Greek participant carried out several field rootstock evaluation trials in 1995-1998. Generally, locally recommended cultural and management practices were followed. Five to six irrigations were provided yearly. The spraying programme included application of Bordeaux mixture at the leaf fall stage. At least 30 fully expanded leaves from the shoot apex were collected from each tree early in the morning and transferred to laboratory for SPAD and leaf mineral measurements. Flower and bark samples were also taken in some cases. Yield and fruit quality measurements and peach tree mortality were also recorded. Trees were generally pruned to a slender spindle bush system by hand pruning. A first experiment with a planting of 40 selected (among 1500) peach hybrids grafted with GF677 (with 3 replications of two trees each) established on a calcareous soil (active lime 20%) at a branch of Pomology Institute in Macrochori, Veria, Greece. Trees were planted at a spacing of 4 x 1.5 m. In a second experiment the peach cv. Flavour Crest grafted on five peach rootstocks (DSS, AN 1/6, Adafuel-J1, GF677 and ID 3) was established in 1984 on a calcareous soil, with a spacing of 5 x 5 m (randomised experimental design with 6 replications). Peach trees had not previously grown on the site. Trees were pruned to a vase shape by hand pruning and N was provided as $(NH_4)_2SO_4$ and $(NH_4)NO_3$ at 120 N units per ha. In a third experiment peach fruit samples of May Crest grafted on 13 peach rootstocks (GF677, AN 1/6, PR 204/84, KID1, DSS, KID2, wild seedling, IDS-37, Loadel, A/P Procopiou, MRS 2/5, Limnou, Damas GF1869) and May Crest self-rooted were taken weekly, freeze dried and analysed for their mineral content. Trees were planted in 1989, at a spacing 5 x 3 m (randomised experiment design with 5 replications of 2 trees each). In the same orchard pooled peach leaf (for four rootstocks, GF677, Pr 204/84, KID1, wild seedling) samples of the cv. May Crest were taken 90 and 150 DAFB and analysed for their chlorophyll (SPAD) and mineral concentrations. In a fourth experiment a planting of 476 peach trees established in January 1984 on a calcareous soil at a branch (Macrochori, Veria) of the Pomology Institute was used. One hundred and eight flower and leaf samples were taken (12 cvs. x 3 rootstocks x 3 replications of 2 trees each). Peach trees had not been grown on the site for at least 15 years. Ten trees (two as peripheral) each of 12 scion cultivars (Early Crest, May Crest, Flavour Crest, Sun Crest, Fayette, Katerina, Loadel, Andross, Everts, May Grand, Fire Bright and Fairlane) on four peach rootstocks (wild seedling, GF677, St Julien GF655/2 and Damas GF1869) were planted at spacing of 5 x 2.5 m (factorial experiment with four replications and two trees as experimental unit). No observations were taken from the rootstock Damas GF1869 because all trees grafted on this rootstock died at the first 6 years of cultivation. In a fifth experiment a planting of 24 selected peach hybrids of Andross and 13 peach cultivars (with 3 replications of two trees each on GF677) was established during 1994 on a calcareous soil at the Pomology Institute (Naoussa). Trees were planted at a spacing of 6 x 3 m.

Field peach rootstock evaluation trials were made in 1998 by P5 in Spain. Evaluation was based on visual symptoms of leaf chlorosis, tree vigour and fruit production. Two groups of rootstocks were studied, peach-

almond hybrids (GF-677 and GN-series, 9, 4, 15 and 22) and plum (PS-101). Trees were planted at 6 x 2 m and grafted with the same variety (Topcrest).

Plant cultivation in hydroponics. Different plant species were grown in growth chambers and greenhouses. Specific details could be found in the reports of the individual participants. The nutrient solutions were based in that of Hoagland with some variations. In the case of Citrus the Portuguese group adopted for some experiments the well-tested Carpena's solution. In some cases solid $CaCO_3$ or bicarbonate was added to simulate conditions usually found in the field that lead to Fe deficiency.

In 1997 in Germany seeds of *Phaseolus vulgaris* were sown on wet paper towels. After four days of germination at 20°C in the dark, plants were transferred to hydroponics. Iron-deficient beans were grown for eight days in half Hoagland nutrient solution in a greenhouse with supplemental light for 14 h/day and a day/night regime of 25/20 °C. Nutrient solutions were changed twice during that period. *Valerianella locusta*, *Spinacia oleracea* and *Actinidia deliciosa* were grown as described before (Individual report P4 -1996).

The Spanish laboratory (P1) carried out experiments in growth chambers with sugar beet and the rootstocks Ademir (*Prunus cerasifera*) and GF677 (*Prunus amygdalo-persica*), as fully described in Morales et al. (1998b), González-Vallejo (1999) and Gogorcena et al. (1998, 2000), respectively.

Experiments were done in 1995-1997 by the Greek group (P3) with peach growing in nutrient solutions. In a first type of experiment, peach plantlets of the cv. Sun Crest grafted on 5 rootstocks (wild seedling, GF677, Adafuel-J1, S.J. GF655/2, MRS 2/5) were grown in 10 L plastic containers (sand-perlite 1:1 mixture) in an air-conditioned greenhouse. Hoagland's nutrient solution with different pH values (from 4 to 9-10) was applied twice per week with a pumping device. The solution was renewed once per week. A second type of experiment was conducted with a similar set-up and five nutrient solutions containing different Fe concentrations (0, 2.5, 5, 10 and 20 mg/L).

Experiments were carried out by the Italian group (P6) in kiwifruit (*Actinidia deliciosa*) grown in liquid culture with and without Fe or $CaCO_3$. In a Bologna trial in 1996, micropropagated plants (cv. Hayward) were used and allowed to grow in nutrient solution without Fe, with 100 µM Fe or with 100 µM Fe and 10 mM bicarbonate. FC-R capacity was measured for three weeks after the imposition of the treatment, using root tips only and following the protocol prepared by the German group P4 and delivered to partners during the 1995 annual meeting. In a similar experiment in 1997, after three weeks from treatment imposition, xylem sap was extracted by applying a 3 bar pressure for 10 minutes to the root system of decapitated plants, and analysed for pH and organic acid composition.

In trials conducted jointly in 1996 and 1997 by P4 and P6 in Düsseldorf, two micropropagated clones of kiwifruit with different susceptibility to Fe chlorosis, Hayward and D1, were used. Plants were transplanted from pots filled with peat into 20 L plastic containers, filled with a continuously aerated half-strength Hoagland solution with 25 or 45 µM Fe-EDTA as source of Fe. After one week, treatments were imposed in a greenhouse with supplemental light and day/night air temperature of 25/20 °C. Two plants were used as controls, whereas 4 plants did not receive Fe. The initial pH was 6.5 and the solution was renewed twice a week. After 14 days from

imposing the treatments, the differences in FC-R at the root level were assessed as described below. To evaluate the degree of Fe chlorosis, at the end of the trial (14 days), foliar discs of a known area from apical leaves that were either fully or not fully expanded were collected and the chlorophyll concentration (on an area basis) was measured spectrophotometrically.

In 1997, one-year old micropropagated plants of two pear (*Pyrus communis*, L.) rootstocks recently released from Bologna University, A28 (FOX 11) and B21 (FOX 16) (Bassi et al., 1996), several pear clones under selection, D41, D69, A74, E82, E105 and E110 (Bassi et al., 1994) and several quince (*Cydonia oblonga* L.) rootstocks, BA29, quince A and quince C, were transferred to 500 mL containers filled with half-strength Hoagland solution continuously aerated containing either 90 _M Fe (as Fe-DTPA) or no Fe and 1 g/L of $CaCO_3$. Six plants per clone and Fe level were allowed to grow in a growth chamber with temperature control. Solutions were changed every three to four days.

In Portugal three *in vitro* propagated Citrus rootstocks were grown in nutrient solution: *Citrus taiwanica* Tan & Shim., *Citrange troyer* (*Citrus sinensis* (L.) Osb. *x Poncirus trifoliata* (L.) Raf.), which is a representative genotype in Algarve (Portugal), and *Citrumelo swingle*. Four-week old seedlings were transferred to a greenhouse with natural light and controlled environmental conditions. Air temperature was approximately 25°C and humidity 80%. These seedlings were transferred twenty days after germination, to a nutrient solution containing 5.0 mM $Ca(NO_3)_2$, 1.4 mM KNO_3, 0.6 mM K_2SO_4, 1.0 mM $MgSO_4$, 0.9 mM NaCl, 0.6 mM $(NH_4)_2HPO_4$, 3.0 mM $(NH_4)_2SO_4$, 0.2 mM $MgCl_2$, 41.8 µM H_3BO_3, 3.8 µM $ZnSO_4$, 3.9 µM $CuSO_4$, 6.9 µM $MnSO_4$ and 1.0 µM $(NH_4)_6Mo_7O_{24}$. Seedlings were grown in these conditions for 3 months, and the solutions were replaced once during that period. For each group of plants, five different Fe concentrations were used (0, 5, 10, 15 or 20 µM) as Fe(III)Na-EDTA. In order to simulate calcareous soil conditions, Fe chlorosis was artificially induced by the addition of 1 g of $CaCO_3$ L^{-1}. The pH of the solutions, considering all the treatments, was in the range 5.95 to 7.82.

In 1997 the Portuguese group carried out an experiment during 11 weeks with 100 plants of cv. Newall grafted on *Citrange troyer*. Three months after grafting, plants were transferred to the nutrient solution described above. Iron was added to the solutions as Fe(III)Na-EDTA at five different Fe concentrations (0, 5, 10, 15 or 20 mM). In some cases 1 g of $CaCO_3$ L^{-1} was added. Nutrient solution replacement was based on the electrical conductivity, which remained constant at 2 mmhos/cm during the first 6 weeks. The solution was replaced when ec dropped to 1.7 mmhos/cm. Initial pH was adjusted to 6.2 for all treatments. At the end of experiment pH readings were done in order to evaluate the proton excretion capacity of Citrange troyer. Leaf chlorophyll concentration was estimated by SPAD readings three days after placement in nutrient solution and then weekly in the second and third leaves, in five plants per treatment.

Preparation of leaf mesophyll tissue and mesophyll protoplasts. Mesophyll tissue of *Valerianella locusta* was prepared as described in the Individual Report P4-1996. Since stripping of the lower epidermis was not possible in *Actinidia deliciosa*, intact leaf discs (5-mm diameter) were used as described by de la Guardia et al. (1996). Mesophyll tissue of sugar beet and peach was used as described in Larbi (1999). Protoplasts from Fe-sufficient and Fe-deficient sugar beet leaves were obtained as described in González-Vallejo et al. (2000).

Root plasma membrane (PM) isolation. In Spain, vesicles enriched in root PM were purified from a sugar beet root microsomal fraction by differential centrifugation in an aqueous-polymer two-phase system (Susín et al., 1996). Leaf PM isolation was made as indicated in González-Vallejo et al. (1998a, 1999) using also aqueous two-phase partitioning. A PM-enriched fraction was obtained from kiwifruit as indicated in Rombolà et al. (2000). In Germany, purified PM from spinach leaves was obtained by the two-phase partitioning method as described in the Individual Report P4-1996.

In Germany, approximately 100 g of Fe-deficient bean root material was cut into pieces and homogenised in a Waring blender two times for 20 s in 330 mL homogenisation buffer, containing 50 mM Tris-HCl pH 8.8, 0.25 M sucrose, 5 mM EDTA, 0.6 g bovine serum albumin and 0.231 g dithiothreitol. PMSF and leupeptine were added to a final concentration of 0.5 and 0.02 mM, respectively. Crude microsomes were resuspended with 5 mM K phosphate pH 6.8 and 0.25 M sucrose. Root plasma membrane isolation was obtained from crude microsomes in an aqueous dextran-polyethylene glycol two-phase system. Maximum yields of bean root PM could be obtained with a polymer concentration of 6.6% (w/v) and without KCl. Typically, with this two-phase system a protein yield of 0.7 mg per 100 g root material could be obtained. The PM fraction was resuspended in 10 mM MOPS-BTP pH 6.8 containing 0.25 M sucrose.

Reductase activities. The FC-R activities of mesophyll tissues was performed in Germany as described in Rombolà et al. (2000) and in Spain as described in Larbi (1999). The FC-R activities of root and leaf plasma membrane preparations were measured according to Brüggemann et al. (1990), as explained in detail in Susín et al. (1996) and González-Vallejo et al. (1998a, 1999). In intact roots, the FC-R measurements were generally carried out in solution in the presence of 300 µM BPDS and 500 µM Fe-EDTA as described earlier (Moog et al., 1995). In Spain the root FC-R activity of intact, illuminated plants was measured as described in Susín et al. (1996).

Measurements were also performed with excised root tips. This methodology (see Individual Report P4-1996) includes (a) excising root tips and immersing them in micronutrient-free half-strength Hoagland nutrient solution; (b) transferring root tips to an Eppendorf cup containing micronutrient-free half-strength Hoagland solution pH 5.0, 400 _M BPDS and 300 _M Fe-EDTA; (c) incubation for 15 min at 22°C in the dark; (d) centrifuging the solution; (e) reading the absorbance of the solution at 535 nm against air and (f) weighing the root tips, after gentle drying on cellulose paper.

In the joint kiwi experiment between P4 and P6 differences in FC-R at the root level were assessed in an agar medium. Root systems were inserted in Petri dishes containing half-strength Hoagland solution with Fe-EDTA, BPDS and agar (7.5 g/L). FC-R activity was also measured *in vivo* by following the formation of the Fe(II)-BPDS complex from Fe(III)-EDTA (Bienfait et al., 1983). From each plant, part of the young root system (1-3 g) was removed and transferred to plastic tubes containing 25 mL of micronutrient-free 1/10 Hoagland's nutrient solution, at pH 6.0, with 300 µM BPDS and 400 µM Fe(III)-EDTA. In other experiments a pH of 6.3 and 300 µM BPDS and 500 µM Fe-EDTA were used. Solution was made up to a final volume of 26 mL. The plastic tubes were wrapped in aluminium foil to avoid light and the solution was continuously aerated. After 20 min of incubation, aliquots of the solution were removed, centrifuged in Eppendorf tubes and the absorbance at 535 nm

was measured. In order to estimate non-enzymatic FC-R (e.g. due to released compounds), parallel experiments were run with roots having no Fe chelate during the incubation period. In these cases Fe(III)-EDTA was added to the tubes only after removing the roots.

In an experiment carried out in 1997 in Portugal root FC-R activity was measured at the end of the experiment. Three plants from each treatment were selected and 5 root tips with a length of 2 cm were removed from the selected plants. The root tips were then placed in an Eppendorf tube wrapped with aluminium paper. Each tube contained 900 µL of micronutrient-free Hoagland solution, 300 µL buffer solution pH 6.5, 300 µM BPDS and 500 µM Fe(III)-EDTA. The reaction was allowed to take place for 3 h in the dark. Absorbances were measured in 1 mL of these solutions at 535 nm in a Shimadzu UV-160A spectrophotometer. Additional absorbance readings were made without root tips. Each treatment consisted of 10 plants distributed in a complete randomised design.

2-D Electrophoresis of root tips proteins, Electroblotting and Automated N-Terminal Sequencing. These procedures were carried out as described in González-Vallejo et al. (1998b) and González-Vallejo (1999).

Chlorophyll fluorescence. Continuous and modulated chlorophyll fluorescence measurements were described in detail in Morales et al. (1998b), Belkhodja et al. (1998b) and Morales et al. (2000b).

Table 10. Description of the field trials for low-input strategies to control iron chlorosis.

Country	year	**Crop**	**Cv.**	**Rootstock**	**Leaf sprays**	**Soil treatments**	**Soil characteristics**
Greece P3	1995-98	Peach	Katerina	GF677	YES	YES	pH=7.0-7.8 active $CaCO_3$>9.7%
	1995		Andross	Seedlings	YES	NO	pH=7.9-8.0 Calcareous active $CaCO_3$>8.8%
	1995		Everts	Seedlings	YES	NO	Calcareous active $CaCO_3$>8.8%
	1995-96		Firebright	Seedlings	NO	YES	Calcareous active $CaCO_3$>8.8%
Portugal P2	1995-96	Citrus	Valencia late	Citrange troyer	YES	No	Calcareous
			Valencia late	C. aurantium			
	1997-98		Encore				pH=7.8 Total $CaCO_3$=47.0% Active $CaCO_3$=12.0%
	1997-98						
Spain P1 P5	1995-98	Peach	SpringLady	Seedlings	YES	NO	Alkaline- Calcareous pH=8.0
	1996-97		Amarillo de Calanda	Seedlings	YES	NO p	Alkaline
	1995-96	Pear	Blanquilla	EMA quince	YES	NO	Calcareous total $CaCO_3$>25% Active $CaCO_3$>11.0%
			Starimson	EMA quince	YES	NO	
Italy P6	1995-96	Pear	Abbé Fetel	EMA quince	YES	YES	pH=7.9 Total $CaCO_3$=9.0% Active $CaCO_3$=4.4%
	1997-98	Pear	Abbé Fetel	EMA and EMC quinces	YES	YES	pH=7.5 Total $CaCO_3$=8.6% Active $CaCO_3$=1.7%
	1995-96	Kiwifruit	Hayward	ownrooted	YES	NO	pH=7.9-8.1 Total CaCO3=2.4% Active CaCO3=1.3%
	1995		Hayward	ownrooted	YES	NO	pH=8.3 Total CaCO3=14.5% Active CaCO3=7.0%
	1997		Hayward	ownrooted	YES	NO	Calcareous

3. Results

3.1 Early diagnosis

Many experiments have been conducted during the project in Greece, Italy, Portugal and Spain to investigate the possibility of using the mineral composition of flowers at the beginning of the season to predict chlorosis occurrence in summer. These experiments have been carried out in orchards where the chlorosis status (chlorophyll concentration) of each tree has been followed for several years. The mineral composition of flowers was also analysed in individual trees. Then, the mineral composition and chlorophyll concentrations in the leaves were measured at several dates after flowering in the same trees. Results obtained have been published in the Proceedings of a Symposium held in 1996 (Sanz et al., 1997a), and in recent papers (Belkhodja et al., 1998a; Abadía et al., 2000; Igartua et al., 2000). Some general conclusions found in these experiments are as follows.

Correlation between Fe in flowers and Fe in leaves.

The correlation between the Fe concentrations in flowers and those in leaves later in the year was confirmed to be generally quite good (Abadía et al., 2000). For instance, in an experiment carried out in Spain with peach the correlation between Fe in flowers and leaf Fe at 60 DAFB was significant in 1993, 1995 and 1996 (Table 1; in 1994 this experiment was not carried out). The correlations between Fe in flowers and leaf Fe were lower at 120 DAFB than at 60 DAFB in 1993 and 1996 (Table 1). This supports that in chlorotic leaves there is a process acting during the season that leads to the inactivation of Fe, since the leaf Fe concentration is generally less representative of the actual Fe status when the season is more advanced.

*Table 1. Coefficients of correlation between flower Fe concentration and leaf Fe concentration. Experiments were done in Spain with 50 individual peach trees cv. "Babygold 7" grafted on seedling. ***p<0.001, **p<0.01, *p<0.05 (data from Abadía et al., 2000).*

	1993	**1995**	**1996**
Flower Fe vs. leaf Fe 60 DAFB	0.306*	0.274*	0.488***
Flower Fe vs. leaf Fe 120 DAFB	0.239	0.340*	0.379**

Correlation between Fe in flowers and chlorophyll.

Chlorophyll during the growing season was better correlated with the flower Fe concentration than with the leaf Fe concentration (Abadía et al., 2000). It is well known that the correlation between total leaf Fe and chlorophyll concentrations could be poor in plants growing in the field (Procopiou and Wallace, 1982; Morales et al., 1998a; Römheld and Schmidt, 2000). In studies carried out in peach in Spain in 1996, the chlorophyll concentration at 120 DAFB was better correlated with the leaf Fe concentration at 60 DAFB (r = 0.605***; n = 50) than at 120 DAFB (r = 0.408**; n = 50). This indicates that the leaf Fe concentration early in the season is related to the development of chlorosis. The correlation between flower Fe and chlorophyll at 60 and 120 DAFB was highly significant in the three experimental years (in 1994 no experiment was carried out) (Table 1, Fig. 1A). This supports that the Fe concentration in flowers could be used to predict the occurrence of chlorosis later in the year.

*Table 2. Coefficients of correlation between flower Fe concentration and chlorophyll. Experiments were done in Spain with 50 individual peach trees cv. "Babygold 7" grafted on seedling. ***p<0.001 (data from Abadía et al., 2000).*

	1993	**1995**	**1996**
Flower Fe vs. SPAD 60 DAFB	0.483***	0.707***	0.624***
Flower Fe vs. SPAD 120 DAFB	0.470***	0.539***	0.705***

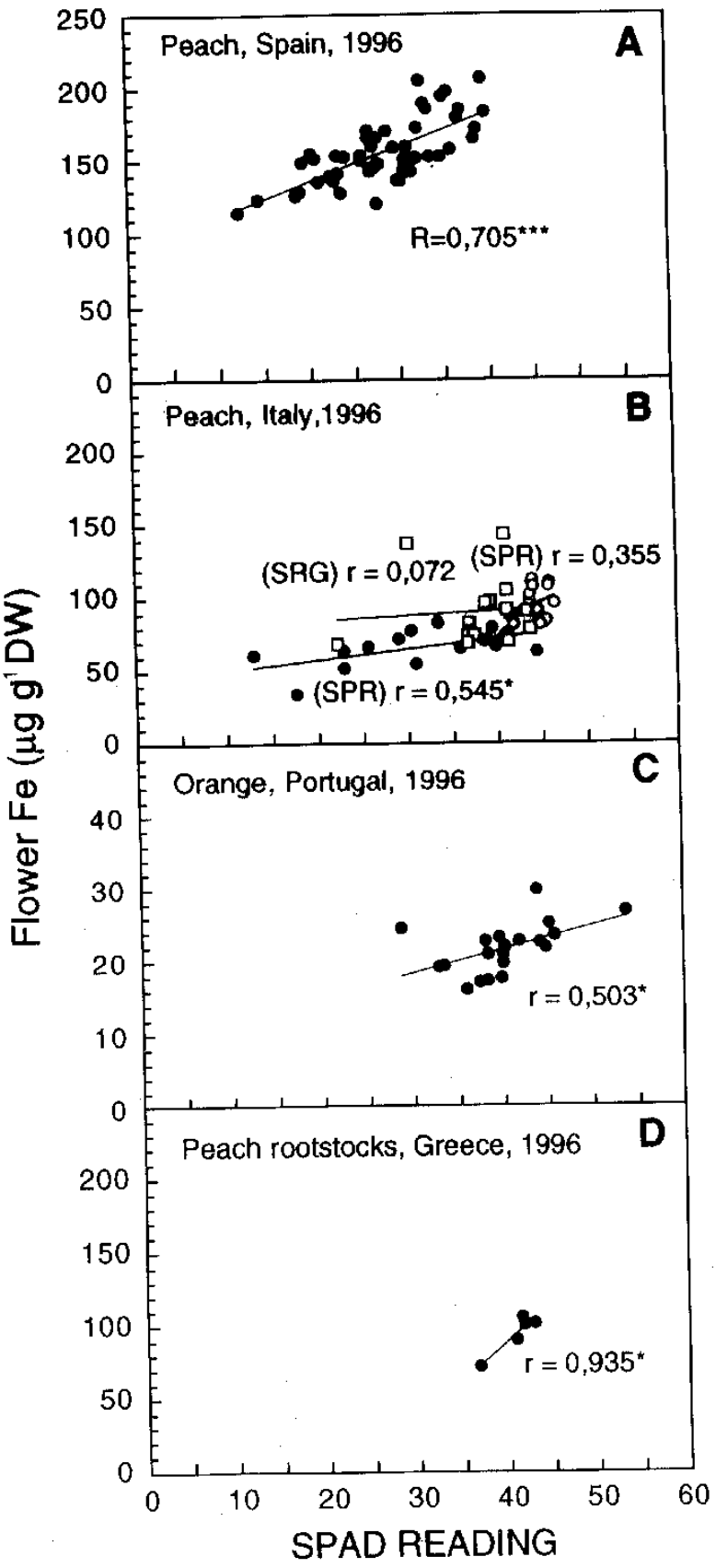

Figure 1: Correlation between flower Fe and chlorophyll estimated with the SPAD apparatus. (A) peach cv. 'Babygold 7' grafted on seedling in Spain in 1996 (SPAD measured at 120 DAFB); (B) peach cvs. 'SpringRed' (SPR) and 'StarkRedGold' (SRG) grafted on seedling in Italy (SPAD measured at 120 DAFB); (C) orange in Portugal cv. 'Valencia late' grafted on 'Citrange troyer' (flowers taken in April and SPAD measured at 60 DAFB); (D) peach cv. 'Flavour Crest' grafted on five peach rootstocks in Greece (SPAD measured at 120 DAFB).

A relationship between flower Fe and leaf chlorophyll has been found also in other Mediterranean areas (Abadía et al., 2000). For instance, in experiments carried out in 1996 in the Po valley area in Italy the correlation between flower Fe and SPAD at 120 DAFB was significant in a peach orchard having marked chlorosis (cv. Spring Red, r = 0.545*; n = 15), although it was not significant in two other orchards with less marked chlorosis (Fig. 1B). A similar correlation between flower Fe and chlorophyll (r = 0.503*; n = 20) was found for a perennial fruit tree, orange, in experiments carried out in southern Portugal (Fig. 1C).

The correlation may be also useful to assess the Fe-chlorosis tolerance of rootstocks (Abadía et al., 2000). In an experiment carried out in 1996 in the Imathia area in Greece a peach cv. (Flavour Crest) was grafted on five different peach rootstocks, DSS, AN 1/6, Adafuel, GF677 and ID 3. In this experiment a correlation was found (r = 0.935*; n = 5) between the flower Fe concentration and the SPAD at 120 DAFB (Fig. 1D).

Origin of the Fe in flowers.

An important issue that is related to the possible usefulness of this method is the origin of the nutrients present in flowers at bloom. Flower initiation occurs in peach and other fruit species in summer, once most of the vegetative growth has been completed (Westwood, 1993). The differentiation of the flowering structures occurs subsequently, and is usually completed before dormancy. It is therefore likely that there is a continuous requirement for nutrients during the whole period of flower bud differentiation in the year before their blossom. A second period when nutrient demand could be possibly high is in spring, coinciding with the opening of flowers and later on with the development of leaves.

In 1997 we carried out an experiment by treating some peach trees known to be chlorotic the past year with soil-applied Fe-EDDHA one month before flowering (Abadía et al., 2000). The flower Fe concentration in the treated trees was not different from that of Fe-

deficient trees not treated with Fe (Fig. 2), in spite of clear differences in the chlorophyll concentration of the leaves formed thereafter in both types of trees. This suggests that there was not a significant uptake of Fe at the time of flower opening, and that most, if not all, of the Fe in flowers was acquired from the Fe already present in the dormant tree.

This would contribute to explain why in the experiments carried out in trees whose chlorosis status has been followed for several years the flower Fe concentration was not only correlated with the Chl concentration later in the season, but also with the Chl concentration the previous year. This has been found in independent trials carried out separately in Italy and Spain (results not shown).

Relationship between flower Fe concentration and fruit yield.

Since flower Fe is correlated with chlorosis and chlorosis affects markedly fruit yield, one may expect that flower Fe may be related to yield. Indeed, the correlation between flower Fe concentration and fruit yield per tree was found to be significant ($r = 0.419^{**}$; $n = 50$) in an experiment carried out in a peach orchard in Spain in 1995 (Abadía et al., 2000; Fig. 3).

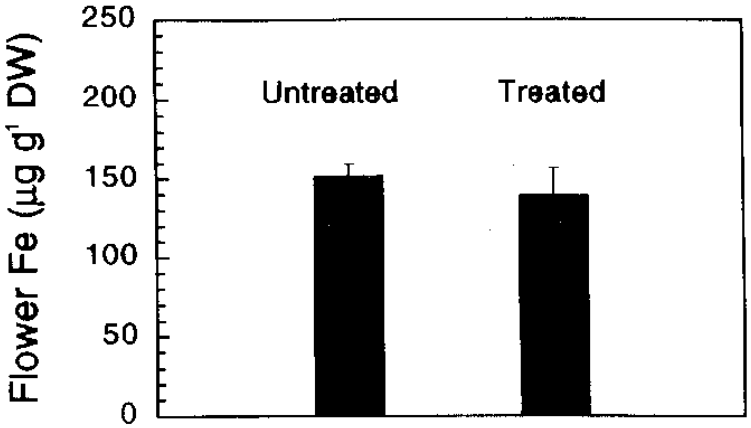

Figure 2: Flower Fe in peach trees treated and untreated with soil-applied Fe-EDDHA (60 g per tree of Sequestrene 138 –FeEDDHA- from Ciba-Geigy) one month before flower full bloom. All trees were known to be markedly Fe deficient the previous season. Untreated trees developed chorosis already in the first leaves formed, whereas the new leaves in treated trees were fully green.

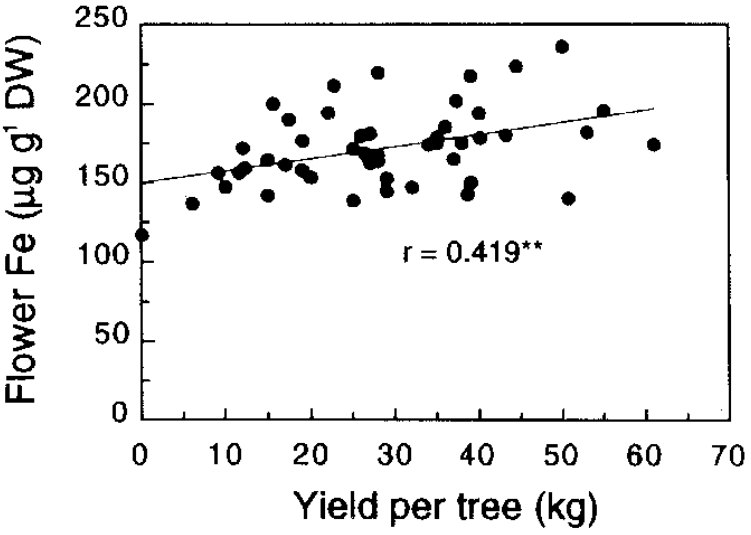

Figure 3: Correlation between flower Fe and yield per tree in peach cv. 'Babygold 7' grafted on seedling in Spain (1995).

Consistency of a nutrient balance over years in peach flowers.

Working with a five-year flower nutrient database obtained from a Spanish orchard we have found a consistent nutrient balance over years in peach flowers, with nutrient concentrations depending to some extent on the levels of other nutrients (Igartua et al., 2000). This was found with a principal component analysis, a practical technique for summarising a large table of correlation coefficients. Nutrient concentrations measured each year in flowers were included as variables, and the principal components are newly derived variables, which account for the main dimensions of variability existing in the original database. The analysis produced a dominant first principal component, which explained 21% of the total variance. The second component explained 13% of the variance, with further components explaining less than 9%. The first two principal components are represented in Fig. 4. This representation shows that some patterns were consistent across years. Iron, Ca, P, Cu and Zn had always positive loads on the first component (X axis in Fig. 4), whereas K, Mg and Mn had mostly negative loads. The position of N depended on years, and thus was not very influential overall on the appearance of this first component. Regarding the second component (Y-axis in Fig. 4), patterns were less clear, with most nutrients presenting positive loads (Mn, Mg, K, P, Zn and N), whereas Fe was the only one with consistently low or even negative loads.

Relationships between the principal components of the five-year flower nutrient database and chlorosis.

We have found a relationship between the nutrient balance existing in flowers at bloom, summarised mostly in the first principal component, and Chl concentration measured four months later in the season (at 120 DAFB) in the same trees (Igartua et al., 2000). This latter parameter describes well chlorosis in peach trees. The regression analysis of the principal components on Chl concentration was done for the average leaf Chl concentration across

years, and also for leaf Chl measured each year independently. Leaf Chl concentrations across years are highly correlated, with coefficients of correlation varying between 0.51 and 0.79 (not shown), for an average of 0.69; therefore, an average across years for each tree is a good summary of yearly Chl levels.

The results show that the first principal component explained 59% of average Chl concentration variance (between 40 and 52% in single year analysis), which is equivalent to a coefficient of correlation of 0.77. This relationship was always highly significant ($P<0.01$). Considering each year separately, the regression of Chl on the second principal component was significant ($P<0.05$) only for two years (but always with R^2 values much lower than for those for the first principal component). The second component explained 8% of average Chl concentration variance. This relationship has been graphically represented in Fig. 4 by drawing a vector proportional to the regression coefficients of Chl (averaged over years) on the two axes (components). A representation of a separate vector for each year actually produced a bundle of arrows, which were very close to the vector for average Chl over years (not shown).

Relationships between flower mineral composition and chlorosis.

The next step was to find out which element(s), or combination of elements, was mostly responsible for the relationship detected. Many combinations were tried, including some ratios previously pointed out as having a relationship with Fe chlorosis, such as K/Ca, K/P and P/Fe (Belkhodja et al., 1998a). The coefficients of determination for a number of models tried are shown in Table 3. The full model, i.e., including all nine nutrient concentrations determined in flowers (N, P, K, Ca, Mg, Fe, Mn, Cu and Zn) explained from 50 to 62% of the Chl variation among trees in the different seasons. Flower Fe was significantly correlated with Chl some years, whereas other years (1997) the correlation was very weak. The

combination of elements which better resumed the first principal component was the one combining the two elements showing consistently larger loads on that component, K and Zn. Multiple regressions considering these two elements explained a major part of the variation accounted for by the full model (Table 4). The significance of the regression coefficients for both K and Zn separately was tested each year. These coefficients were always significant ($P<0.05$), except for K in 1997. Also, the rest of the elements were included in the multiple regression models, after forcing the presence of K and Zn. This is a way of testing if there is any element that is significantly related to Chl concentration, after removing the relationship of Chl with K and Zn. Although in four years the inclusion of another element increased the R^2 value significantly, different elements were added to the K-Zn model each year, though Fe was included twice (Table 4). Thus, the best consistent model across years was the one including only K and Zn.

With a multiple regression approach we obtain a regression coefficient for each nutrient in the model, which is not convenient for predictive purposes. For the sake of simplicity, and potential usefulness for prognosis purposes, the ratio K/Zn (both concentrations in mg kg^{-1} of dry mass) was calculated, and the regression was repeated with it. The R^2 values obtained for this single regressor were almost as large as the ones obtained using K and Zn as separate variables (Table 5). Thus, a simple model including the flower K/Zn ratio explained a significant part of the variation of Chl concentrations 120 DAFB every year.

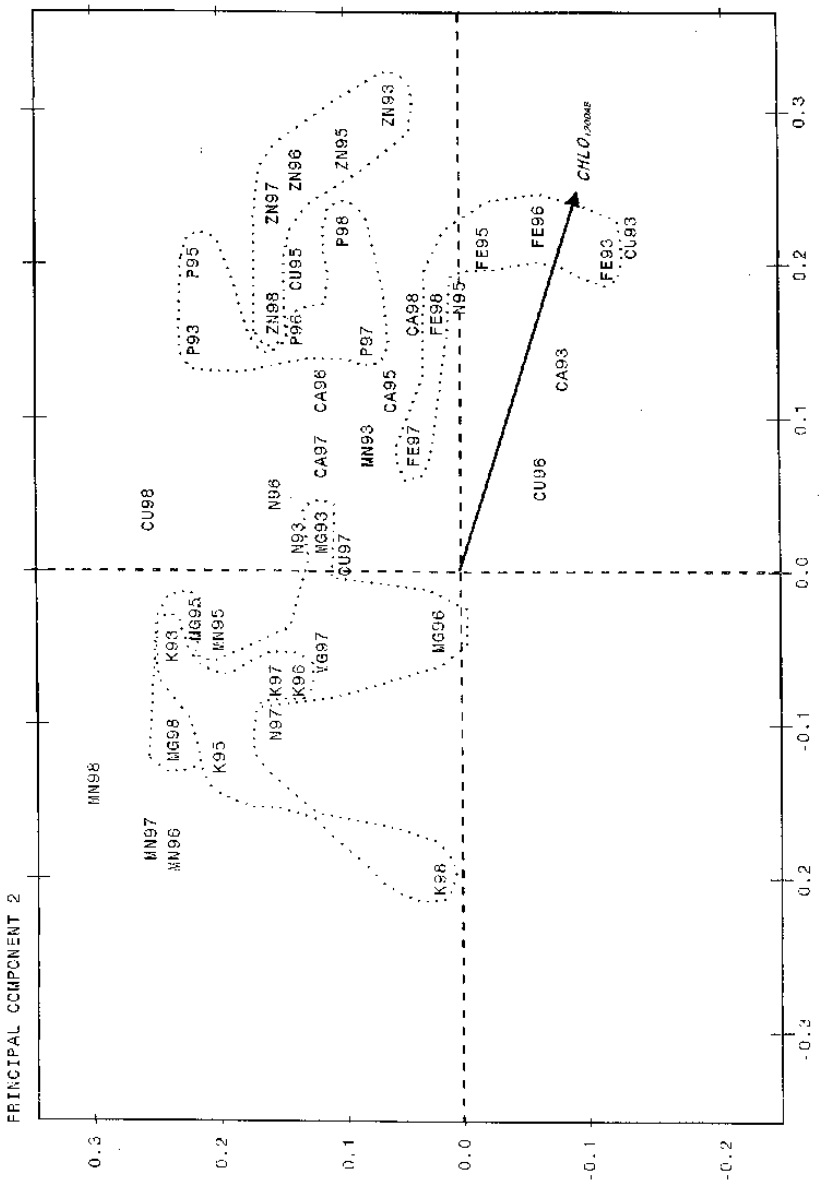

Figure 4: Principal component analysis of the five-year flower nutrient database. Nutrient concentrations measured each year in flowers were included as variables. The regression analysis of the principal components on Chl concentration is represented in the Figure by a vector (arrow) proportional to the regression coefficients of Chl (averaged over years) on the two axes (components). A representation of a separate vector for each year actually produced a bundle of arrows, which were very close to the vector for average Chl over years (not shown)

Table 3. Means and standard deviations for several inorganic nutrients in flowers, and ratios among them, determined at bloom in a peach orchard at El Temple, Zaragoza, (Spain). Chlorophyll concentrations 120 days after flower bloom were estimated from SPAD measurements (Igartua et al., 2000).

	1993 (50)		1995 (50)		1996 (50)		1997 (44)		1998 (43)	
Year	mean	st.dev.	mean	st.dev.	mean	st.dev.	mean	st.dev.	mean	st.dev.
					percentage of dry weight					
N	3.45	0.15	2.77	0.13	2.91	0.11	2.83	0.08	2.72	0.11
P	0.41	0.03	0.34	0.02	0.32	0.02	0.34	0.02	0.31	0.01
K	1.76	0.13	1.34	0.15	1.42	0.10	1.86	0.13	1.65	0.12
Ca	0.58	0.06	0.62	0.08	0.45	0.03	0.48	0.03	0.97	0.13
Mg	0.21	0.02	0.18	0.01	0.20	0.01	0.19	0.01	0.25	0.08
					mg kg^{-1} of dry weight					
Fe	121.3	22.5	172.7	24.7	156.4	21.9	108.5	18.4	302.9	32.8
Mn	24.9	3.3	30.1	5.0	25.6	4.7	18.8	3.9	39.4	8.3
Cu	199.8	42.3	288.3	33.7	138.8	23.7	253.0	33.6	287.8	14.3
Zn	41.9	5.7	36.1	5.0	37.0	4.2	41.6	4.5	46.9	2.6
					ratios					
K/Zn	427.3	60.8	374.5	56.1	388.2	50.6	450.9	58.0	353.8	33.4
P/Fe	34.7	6.1	20.0	3.0	20.9	2.8	32.5	5.5	10.2	1.1
K/P	4.3	0.4	3.9	0.4	4.4	0.3	5.4	0.4	5.4	0.5
K/Ca	3.1	0.4	2.2	0.4	3.2	0.3	3.9	0.3	1.7	0.3
					µmol m^{-2}					
$Chl_{120DAFB}$	233	93	233	72	220	68	217	66	244	64

Table 4. Coefficients of determination (R^2) of several regression models including flower nutrient concentrations, on leaf chlorophyll concentration determined 120 days after bloom. Elements whose inclusion in the K, Zn model supposed further significant increments of explained variation have been also included (Igartua et al., 2000).

		Single elements			Multiple regression	
Year	Total[a]	Fe	K	Zn	K, Zn	Next element[b]
1993	0.50**	0.26**	0.24**	0.15**	0.42**	Fe
1995	0.51**	0.28**	0.26**	0.14**	0.45**	-
1996	0.61**	0.50**	0.10*	0.25**	0.35**	Fe
1997	0.62**	0.03	0.05	0.23**	0.27**	N
1998	0.55**	0.14*	0.24**	0.11*	0.31**	Mg
Combined	0.36**	0.06**	0.05**	0.13**	0.29**	Mn

*,** regression model significant for P<0.05 and P<0.01, respectively

[a] full regression model, including all nine nutrients determined in flowers

[b] next element included in the multiple regression after K and Zn, which explained a significant part of remaining variation

Table 5. Parameters of the regression models for the leaf chlorophyll concentration at 120 days after bloom on the K/Zn ratio in flowers at bloom. Coefficients of determination (R^2) of regression models with other key ratios are included (Igartua et al., 2000).

	K/Zn ratio			Other ratios (R^2)		
Year	a[a]	b[b]	R^2	K/P	K/Ca	P/Fe
1993	602	-0.865	0.32**	0.16**	0.13*	0.24**
1995	545	-0.832	0.41**	0.41**	0.25**	0.23**
1996	526	-0.790	0.34**	0.19**	0.15**	0.42**
1997	471	-0.563	0.25**	0.20**	0.10*	0.00
1998	617	-1.055	0.30**	0.39**	0.16**	0.01
Combined	471	-0.607	0.27**	0.08**	0.07**	0.05**

*,** regression model significant for P<0.05 and P<0.01, respectively

[a] intercept of the K/Zn regression models

[b] slope of the K/Zn regression models

The regression lines of the K/Zn ratio on chlorophyll concentration are represented in Fig. 5. It is evident from the graph that the regression lines for each year are quite similar. Actually, a test of significance for the regression slopes failed to detect significant differences among them ($P<0.05$). This similarity of regression lines did not occur for any other nutrient or nutrient ratio tested (Fe, K/Ca, K/P and P/Fe, shown in Tables 4 and 5, among others). This consistency was remarked by the combined regression analysis across years of K/Zn ratio on Chl concentration (n=210), which explained 27% of leaf Chl concentration variance (Table 5). This coefficient of determination is equivalent to a correlation coefficient of -0.52. The relationship was quite constant across years, as shown by the slope, intercept and R^2 values (Table 5). The parameters of the equation derived from the combined regression analysis are also shown in Table 5.

The results suggest that a K/Zn ratio above 450 in flowers at bloom would indicate a true possibility of low Chl concentration later in the year (Fig. 5), with leaf Chl concentrations below 200 μmol m^{-2} at the 120 DAFB stage. Conversely, a K/Zn ratio below 375 would predict the absence of chlorosis at 120 DAFB.

The importance of having an early diagnosis of Fe chlorosis in peach is emphasised by the influence of chlorosis on yield. Incidentally, fruit yield per tree was recorded at this orchard in 1995. The correlation coefficient of yield with Chl concentration 120 DAFB was 0.80, which reveals a strong relationship. The correlation coefficient of yield with the K/Zn ratio at bloom was –0.66, suggesting a remarkable predictive power of this ratio for fruit yield much later in the season. This observation, however, should be confirmed by analysis for further years.

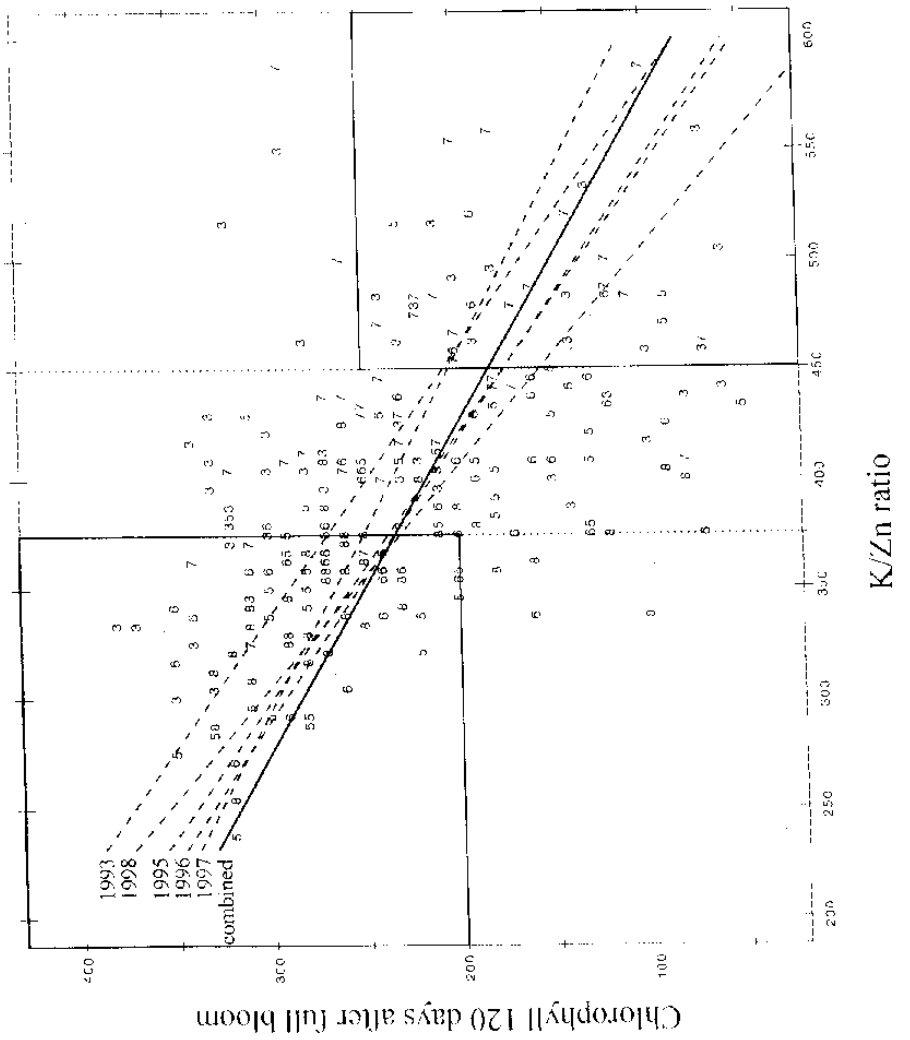

Figure 5: Regression lines of the K/Zn ratio (m/m) on chlorophyll concentration (µmol m^{-2}) for the five different years and for the whole database (continuous line marked "combined" in the Figure). Numbers represent data points for individual trees in the different years (i.e. 6 for data obtained in 1996)

Predictive power of the flower mineral composition in a different orchard.

The predictive power of the equation derived from the combined analysis across years was tested with a completely unrelated set of data (Igartua et al., 2000). Flower nutrient concentrations and Chl concentrations at 120 DAFB were available from a second peach orchard established in Sástago (Zaragoza, Spain). Samples were taken in this orchard for 12 pairs of trees (one sample per two trees) in 1996 and 24 individual trees in 1997. The characteristics of this second orchard could not be more different than those of the first one. The Sástago orchard is established on a lighter soil, drip irrigated and fertilised and has a much denser tree stand with smaller trees. Both flower nutrient and leaf Chl concentrations in Sástago were much lower, in general, than in the other orchard used. Also, the range of variation for Chl concentration was much narrower in Sástago; it varied from 100 to 210 μmol Chl m^{-2} in both years. Provided this narrow range of variation, it cannot be expected that the relationship between the K/Zn ratio at bloom and Chl will be as strong as in the first orchard.

Actually, the correlation coefficients between the flower K/Zn ratio and leaf Chl were not statistically significant at the $P<0.05$ level (-0.23 and -0.26 for 1996 and 1997, respectively), but presented the same overall trend than in the first orchard (higher Chl at lower K/Zn ratios). The combined regression equation obtained in the first orchard (Table 5) was applied to this new data set, to check the predictive value of the flower's K/Zn ratio on leaf Chl concentrations at 120 DAFB. The Chl concentrations predicted by the equation were, on average, very close to the actual ones (Table 6). Also, the predictions for the trees presenting the extreme Chl levels of the orchard were significantly higher (or lower) than the means (Table 6). It must be noted that the selection of the high and low Chl extreme samples

was made a priori, by looking at the shape of the Chl distributions, which clearly suggested the cutting points used.

Table 6. Predictive power of the K/Zn ratio for another peach orchard in Sástago (Zaragoza). Leaf chlorophyll concentrations are given in µmol m^{-2}. Predicted chlorophyll concentrations were calculated with the 'combined' equation for the K/Zn ratio presented in Table 5.

Year	Actual chlorophyll concentration			Predicted chlorophyll concentration		
	average	lowest[1]	highest[2]	average	lowest[1]	highest[2]
1996	160	146	192	175	167	193
1997	146	109	183	146	122	156

[1] Six samples with lowest chlorophyll concentrations in 1996, and four in 1997

[2] Three samples with highest chlorophyll concentrations in 1996, and four in 1997

3.2 Alternative agricultural management techniques to control iron chlorosis

Several approaches were followed in this task in experiments conducted during the project in Greece, Italy, Portugal and Spain. First, we have assessed the impact of Fe chlorosis on yields and the Fe requirements by deciduous fruit trees. We have tested a number of cheap and environmentally friendly Fe sources alternative to Fe chelates and explored the possibilities of causing re-greening of chlorotic leaves by activating the Fe inactive pools already present in the apoplast of chlorotic leaves. Also, we have conducted studies for optimising the timing of Fe applications. The results summarised below have been presented in several theses (Valli, 1997; García Laviña, 1998; Fidalgo, 1998; Folli, 1998; Rombolà, 1998) and research papers (Marangoni et al., 1997; Tagliavini et al., 1997; Rombolà et al., 1998a, 1998b, 2000; Pestana et al., 1999a, 1999b, 2000; Tagliavini et al., 2000).

Major concern was addressed in field trials to the control of the variability of soil conditions leading to the development of the chlorosis. Data from Partner 6 indicates a great

benefit of the use of the Covariance Analysis instead of Analysis of Variance alone. The chlorophyll content of the leaf before imposition of spray treatment should be used as covariate in the analysis, since this gives much precision to the comparisons. Whenever possible, as in the case of foliar applications alone, the experimental designs should consider the use of the tree as a block. Treatments should be randomly assigned to different shoots, within the same trees, showing similar symptoms of chlorosis.

Assessing the impact of Fe chlorosis on yields.

The results obtained during the project show conclusively that chlorosis has major negative impacts in fruit yield and quality (Tagliavini et al., 2000). Data relating leaf chlorophyll and fruit yield and quality have been obtained in Greece, Italy, Portugal and Spain and are shown in Figs 6-8 and in Table 7 (see also Sanz et al., 1997b). An increase in chlorosis symptoms led in all cases to severe reductions of total fruit yield per tree. Iron chlorosis also affected adversely fruit size in citrus, peach and kiwifruit, making part of the fruits to be unsuitable for marketing. In citrus, fruits from trees recovered from chlorosis had less acid concentration than those coming from chlorotic trees (Table 7).

Determining the amounts of Fe yearly removed by fruit trees.

We have obtained in the project estimations on the Fe balance in fruit orchards, by measuring in detail Fe losses due to leaf fall, pruning and fruit harvest. This has been done in peach.

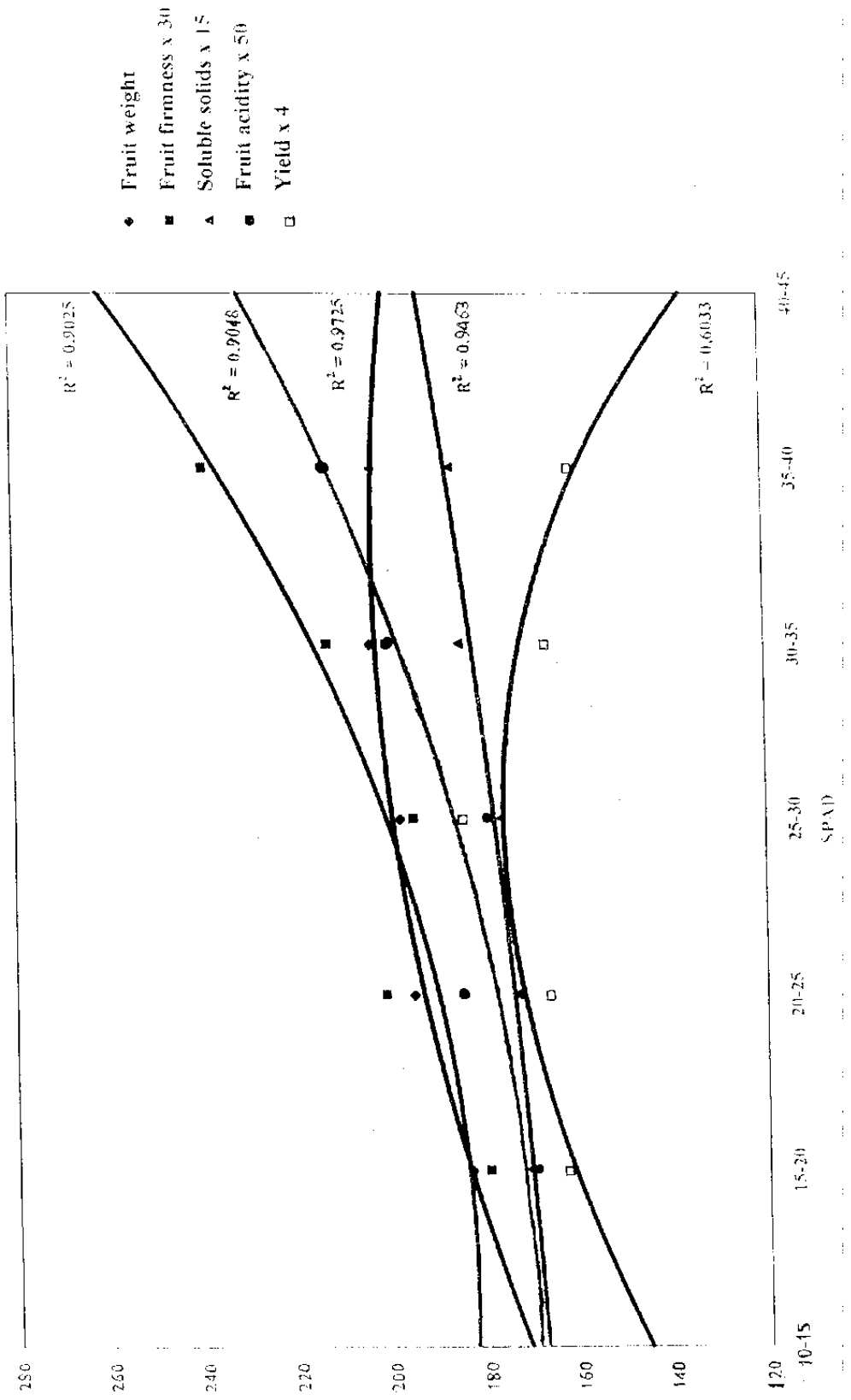

Figure 6: Effect of SPAD-502 level on yield and fruit quality characteristics (c.v. Katherina, 1997)

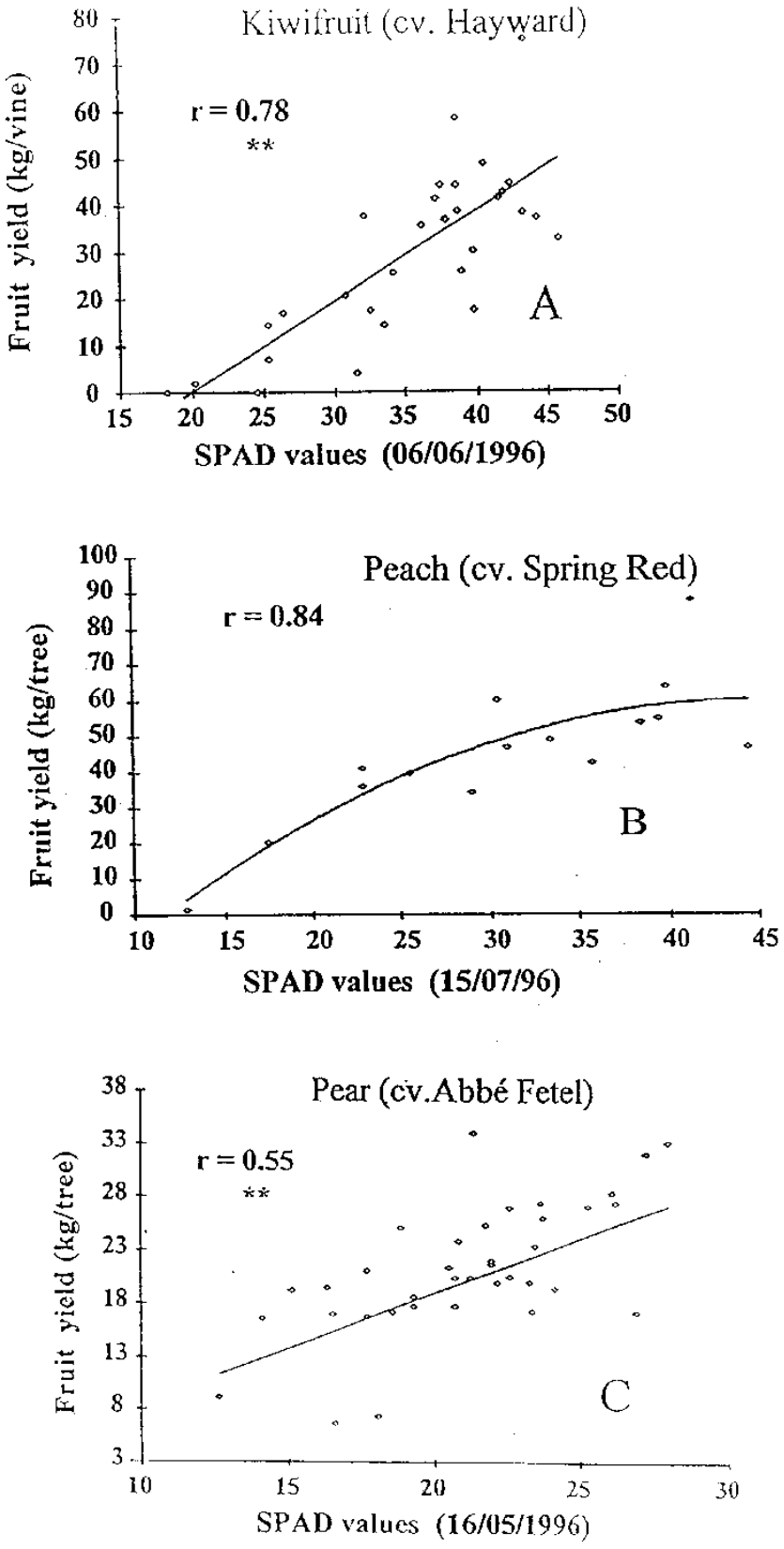

Figure 7: Relationships between SPAD values and yield as detected in Italy

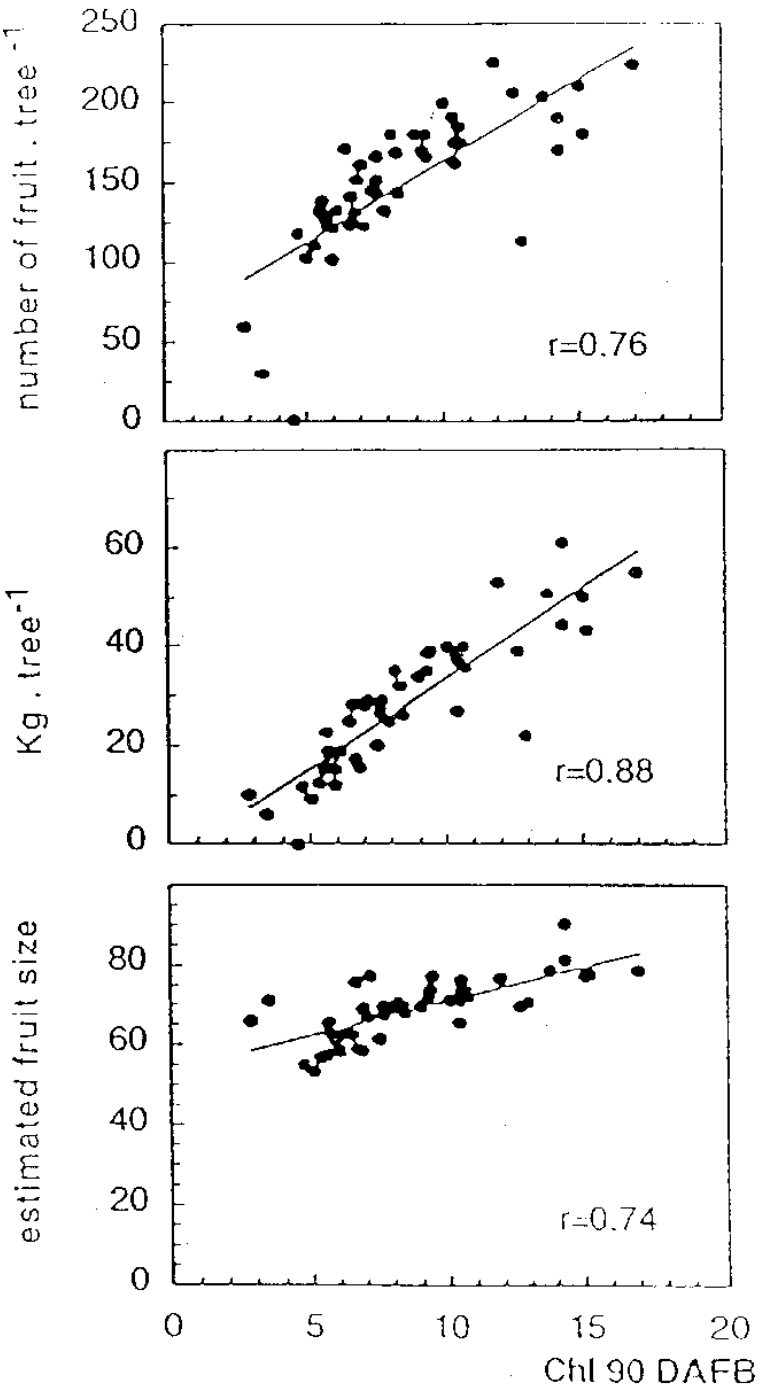

Figure 8: Relationships between SPAD values and yield as detected in Spain

A relatively large loss of Fe arises from leaf fall. Results obtained in Spain indicate that chlorotic peach trees had approximately the same number of leaves than control trees (Grasa et al., 1998). However, the number of leaves remaining attached to the trees when the season was advanced was significantly higher in control trees than in the ones affected by chlorosis. Chlorotic and control trees had total leaf DW of approximately 1.4 and 2.0 kg, respectively. Annual losses of Fe during leaf senescence were approximately 110 and 160 mg per tree in Fe-deficient and control trees, respectively (Grasa et al., 1998).

Table 7. The effect of foliar applications on the quality of oranges.

Treatment	FW (g fruit^{-1})	D (mm)	JC (ml)	**JC** (% FW)	TSC (º Brix)	CAC (% v/v J)
Chlorotic control	184 b	72 b	89 b	49 a	10.2 a	1.20 a
Sulphuric acid	179 b	72 b	90 b	50 a	9.9 a	1.07 ab
Iron sulphate	206 a	76 a	98 a	48 a	9.3 a	0.91 c
Iron chelate	193 ab	74 ab	94 ab	49 a	10.1 a	0.97 bc

FW – fresh weight; D – diameter; JC – juice content; TSC – total sugar content; CAC – citric acid concentration. Means in a column followed by the same letter are not significantly different at 5% (Duncan test).

Agricultural practices such as pruning remove a large amount of Fe from the tree. The analysis of pruning material in winter (mainly twigs that had previously supported fruit production) indicates that chlorotic and control trees may lose by pruning every year approximately 200 and 300 mg of Fe per tree, respectively (Grasa et al., 1998). No differences between chlorotic and control trees were found for the Fe content of the two year old material (cork or wood). However, chlorotic trees had much less shoot biomass than control trees, and consequently the Fe losses were smaller in chlorotic than in control trees. For an estimated

tree density of 800 tree/ha, the amounts of Fe lost from flower fall, fruit yield, leaf abscission and pruning in a non-chlorotic peach orchard would be close to 400 g Fe/ha.

Removal of Fe and other mineral nutrients in the fruit yield is reported in Table 8. Taking into account the yields of different crops it is possible to estimate the annual Fe removal in fruits, that may be lower than 50 g/ha in grape and peach, but could be significantly higher in kiwifruit and cherry (200-300 g/ha).

Optimising the timing for Fe application.

The timing of Fe chelate application significantly affected the development of Fe chlorosis after growth resumption of kiwifruit in spring (Table 9). Leaf chlorophyll was lower in shoots sampled from vines supplied with Fe in late winter than in those receiving Fe in autumn (Table 9). These results were confirmed by the analysis of leaf chlorophyll. We also recorded a lower number of chlorotic shoots in vines treated with Fe in autumn (25% of total shoot number) than in those treated in late winter (43%).

The carry over effect of soil applications of Fe in the previous year to prevent the occurrence of chlorosis the following year was studied by P3 in two peach orchards, where treatments to overcome Fe chlorosis were soil-applied in 1995. This trial established that the application of Fe sulphate or Fe-chelates to the soil in a given year had no long lasting effects during the following spring (data not shown).

Looking for low-expensive and environmental friendly alternatives to iron chelates.

Agronomic means for solving or alleviating the Fe chlorosis in fruit trees were evaluated during the 4-year period in calcareous or alkaline soils in Greece, Italy, Portugal and Spain. Trials were conducted with four crops very susceptible to Fe chlorosis (citrus, kiwifruit, peach and pear). The main features of these trials are shown in Table 10. Details of

treatment applications, with number and timings of Fe supply in the four years are reported in Tables 11-14. Both soil and foliar treatments were applied. The methodology for assessing the re-greening effect was always based on measurements by the portable chlorophyll reader SPAD 502.

Effectiveness of foliar sprays with Fe-containing compounds

Low input strategies to control Fe chlorosis by canopy sprays were mainly based on the use of Fe sulphate. This compound was applied to different crops, either alone or in combination with a variety of compounds aimed to increase its effectiveness by improving its penetration and its activity in the leaf. These compounds included inorganic and organic acids, aminoacids and other fertilisers. The potential phytotoxic effects on leaves and fruits of different rates of Fe sulphate, either alone or with citric acid, were also tested. Mn-chelates were also applied to test the possibility that Mn deficiency could contribute to the development of chlorosis.

Species	N	P	K	Ca	Mg	Fe	Mn	Zn	Source
	kg/t					g/t			
Apple	0.50-0.60	0.07-0.13	1.40	0.07	0.05-0.07	1.80	0.45	0.40	IFA, 1992; USDA, 1963
Chestnut	4.60	0.93	5.18	0.27	0.32	10.10	9.52	5.20	USDA, 1963
Apricot	0.87	0.19	2.96	0.14	0.08	5.40	0.79	2.60	USDA, 1963
Cherry (Sweet)	2.00-2.35	0.18-0.20	1.48-1.70	0.11-0.16	0.10-0.16	5.63-10.77	0.70-0.97	1.83-2.04	Huguet, 1980
Grape	1.30-1.90	0.30	2.20-2.80	0.14	0.20-0.30	2.90	7.18	0.40	Lohnertz, 1991 USDA, 1963
Kiwifruit	1.30-1.80	0.23	1.90-2.60	0.16-0.51	0.10	2.00-12.00	0.70-23.00	0.80-3.20	Smith *et al.*, 1988; Pailly, 1992
Peach	0.90-1.00	0.25	2.00	0.05	0.10	1.10	0.47	1.40	Marangoni and Rombolà, 1994; USDA, 1963
Pear	0.65-0.80	0.05-0.13	1.30-1.60	0.11	0.10-0.12	2.50	0.76	1.20	Word Fert. Use Manual, 1992; USDA, 1963
Pecan	14.6	1.28	3.92	0.36	1.28	0.02	0.045	0.055	USDA, 1963
Plum	0.49	0.10	1.72	0.04	0.07	1.00	0.49	1.00	USDA, 1963

Table 8: Amounts of the major nutrients removed in the fruit of selected tree species

Treatment	Leaf chlorophyll April 9 1998 µg/ cm^2	Leaf chlorophyll April 27 1998 µg/ cm^2
Autumn (1997) applied Fe	16 a	19 a
Late winter (1998) applied Fe	10 b	14 b

[x] Means followed by different letters are significantly different at P=0.05 level of probability (LSD test).

Table 9: Chlorophyll contents of kiwifruit leaves analysed on April, 1998, as affected by timing of Fe application

Table 11. Summary of the field trials for low-input strategies to control iron chlorosis in 1995.

Partner	Treatments	Rates applied	Fruit crop	Type of distribution	Number of applications
1,2,3,5,6	Control (No Iron)	-	pear, citrus, peach, kiwifruit		
1,3,5	Ascorbic acid	0.1-0.2%	peach	Foliar	4-5
1,2,3,5,6	$FeSO_4$	500 mg Fe/l	citrus, pear, peach, kiwifruit	Foliar	3-5
1,,2,3,5,6	$FeSO_4$ + Citric acid	500 mg Fe/l + 2 g/l citric acid	citrus, pear, peach, kiwifruit	Foliar	5
1, 5	$FeSO_4$ + sulphuric acid	500 mg Fe/l + 0.05-0.1%	peach, pear kiwifruit	Foliar	3
1, 3,5	$FeSO_4$ + Ascorbic acid	500 mg Fe/l + 0.1-0.2% ascorb. Acid	pear, peach	Foliar	4-5
3	$FeSO_4$ + KNO3	500 mg Fe/l + 0.1% KNO3	peach	Foliar	4-5
3	$FeSO_4$ + urea	500 mg Fe/l + 0.1% urea	peach	Foliar	4-5
1, 2,3,5,6	citric acid	2 g/l	peach, pear, citrus, kiwifruit	Foliar	3-4
1, 3, 5, 6	sulphuric acid	0.05-0.02%	peach, pear, kiwifruit	Foliar	3-4
1, 3, 2, 5,6	Fe-DTPA or Fe-EDTA or Fe-EDDHA	120 mg Fe/l	peach, citrus pear, kiwifruit	Foliar	3-4
3, 6	Mn-EDTA	120-360 mg Mn/l	pear, peach	Foliar	3
3, 6	Fe-DTPA or Fe-EDDHA and Mn-EDTA	same as above	pear, peach	Foliar	3
6	Fe-EDDHA	600 mg Fe/tree	pear	Soil	1
3	FeSO4	5.0 kg FeSO4/tree	peach	Soil	1
3	$FeSO_4$ plus organic matter or urea or KNO3 or gypsum	5.0 kg FeSO4 plus 50 kg/tree Organic matter, or 2.0 kg/tree urea or 5.0 kg/tree KNO3 or 5.0 kg/tree	peach	Soil	1
6	$FeSO_4$ and H_2SO_4	100 g FeSO4/tree	pear	Soil	1
6	$FeSO_4$ + Fe_2S or S	22 g Fe/tree	pear	Soil	1
6	$FeSO_4$ on grassed plots	22 g Fe/tree	pear	Soil	1

Table 12. Summary of the field trials for low-input strategies to control iron chlorosis in 1996.

Partner	Treatments	Rates applied	Fruit crop	Type of distribution	Number of applications
1,2,3,5,6	Control (No Iron)	-	pear, citrus peach, kiwifruit		
1,5	Ascorbic acid	0.1-0.2%	peach	Foliar	5
1,2,3,5,6	$FeSO_4$	250-500 mg Fe/l	citrus, pear, peach, kiwifruit	Foliar	3-5
1,,2,3,5,6	$FeSO_4$ + Citric acid	250-500 mg Fe/l + 2 g/l cit. A.	citrus, pear, peach, kiwifruit	Foliar	4-5
3	$FeSO_4$ + Citric acid + urea or KNO3	500 mg Fe/l + 2 g/l cit. A + 0.1%	peach	Foliar	4
1, 5	$FeSO_4$ + sulphuric acid	250-500 mg Fe/l, 0.3 ml/l sul. A.	peach, pear	Foliar	3
1, 5	$FeSO_4$ + Ascorbic acid	500 mg Fe/l + 0.1-0.2% asc. A.	pear	Foliar	5
3	$FeSO_4$ + KNO_3	500 mg Fe/l + 0.1% KNO3	peach	Foliar	4
3	$FeSO_4$ + urea	500 mg Fe/l + 0.1% urea	peach	Foliar	4
1, 2,3,5,6	citric acid	2 g/l	peach, pear, citrus, kiwifruit	Foliar	3-4
1, 5, 6	sulphuric acid	0.05-0.1%	peach, pear, kiwifruit	Foliar	3
1, 3, 2, 5,6	Fe-DTPA, Fe-EDTA or Fe-EDDHA	120 mg Fe/l	peach, citrus, pear kiwifruit	Foliar	3-4
2,6	Mn-EDTA	120-360 mg Mn/l	pear, citrus	Foliar	3
2, 6	Fe-DTPA or Fe-EDDHA and Mn-EDTA	same as above	pear, citrus	Foliar	3
3	Biomin-Fe (6% N, 5% Fe)	0.15 %	peach	Foliar	4
3, 6	Fe-EDDHA	600 mg Fe/tree	peach, pear	Soil	1
3	$FeSO_4$	3.0 kg/tree	peach,	Soil	1
3	$FeSO_4$ plus organic matter, urea, KNO3 or gypsum	3 kg FeSO4 plus 50 kg/tree manure, or 2.0 kg/tree urea or 5.0 kg/tree of KNO3 or gypsum	peach	Soil	1
6	$FeSO_4$ and H2SO4	100 g FeSO4/tree	pear	Soil	1
6	$FeSO_4$ + Fe2S or S	22 g Fe/tree	pear	Soil	1
3	Organic matter (manure)	50 kg/tree	peach	Soil	1
6	Grassed plots	22 g Fe/tree	pear	Soil	1

Table 13. Summary of the field trials for low-input strategies to control iron chlorosis in 1997.

Partner	Treatments	Rates applied	Amounts of Fe per hectare per year	Fruit crop	Type of distri-bution	Number of applications
6	Control (No Iron)	-		pear, citrus, peach, kiwifruit		
6	$FeSO_4$ + aminoacids and polypeptides	250 mg Fe/l	1250 g /Fe	pear, kiwifruit	Foliar	5
1,2,3,5	FeSO4	500 mg Fe/l	2500 g /Fe	citrus, pear, peach	Foliar	3-5
1, 5, 6	$FeSO_4$ + Citric acid	500 mg Fe/l + 2 g/l	2500 g /Fe	pear, peach	Foliar	5
1, 5,	$FeSO_4$ + sulphuric acid	500 mg Fe/l + 0.3 ml/l	2500 g /Fe	peach, pear,	Foliar	3
3	Biomin-Fe(N=6% - Fe=5%)	0.15 %	-	peach	Foliar	4
6	BLOOD MEAL	183 mg Fe/tree	500 g /Fe	pear	Soil	1
6	$FeSO_4$ after incubation with compost	2Kg compost /tree + 40g Fe/tree	108 kg Fe	pear	Soil	1
3	$FeSO_4$ +manure			peach	Soil	1
3	manure	50 kg/tree	-	peach	Soil	1
6	$FeSO_4$ on grassed plots	40 g Fe/tree	108 kg Fe	pear	Soil	1
6	$FeSO_4$ + aminoacids and polypeptides	300mgFe/tree	819 g Fe	pear	Soil	1
3	K_2SO_4	2 kg/tree	-	peach	Soil	1
3	KNO3	2 kg/tree	-	peach	Soil	1
3	$(NH_4)_2SO_4$	3.0 kg/tree	-	peach	Soil	1
3	Urea	1.4 kg/tree	-	peach	Soil	1
3, 5, 6	Fe - EDDHA	300-3000 mgFe/tree	819 g Fe	pear, peach	Soil	1
1, 3, 5	citric acid	2 g/l	-	peach, pear	Foliar	2-3
1, 2,5	sulphuric acid	0.3 ml/l	-	peach, pear	Foliar	3
1,5	ascorbic acid	0.2 %		pear	Foliar	
1,3,5,6	Fe-DTPA	120 mg Fe/l		pear, peach, kiwifruit	Foliar	2-3

Table 14. Summary of the field trials for low-input strategies to control iron chlorosis in 1998.

Partner	Treatments	Rates applied per tree or per liter	Amounts of Fe per hectare per year	Fruit crop	Type of distri-bution	Number of applications
1,2,3,5,6	Control (No Iron)	-		pear, citrus, peach		
6	$FeSO_4$ + aminoacids and polypeptides	125 mg Fe/l	337 g Fe	pear	Foliar	5
1,2,3,5	$FeSO_4$	500 mg Fe/l	-	citrus, peach	Foliar	2-5
1, 5, 6	$FeSO_4$ + Citric acid	250 mg Fe/l + 2 g/l citric acid	672 g Fe	pear	Foliar	5
1, 5	$FeSO_4$ + sulphuric acid	500 mg Fe/l + 0.3 ml/l	-	peach	Foliar	3
1, 5	citric acid	2 g/l	-	peach	Foliar	3
1, 2, 5	sulphuric acid	0.3 ml/l	-	peach, citrus	Foliar	3
1, 2, 3, 5	Fe-DTPA or Fe-EDDHA	120 mg Fe/l	-	peach, citrus	Foliar	2-3
3	Biomin-Fe(N=6% - Fe=5%)	0.15 %	-	peach	Foliar	2
6	Blood meal	183 mg Fe/tree	500 g Fe	pear	Soil	1
6	$FeSO_4$ after incubation with compost	2 Kg compost /tree + 40g Fe/tree	108 kg Fe	pear	Soil	1
6	$FeSO_4$ on grassed plots	40 g Fe/tree	108 kg Fe	pear	Soil	1
6	$FeSO_4$ + amino-acids and polypeptides	300mgFe/tree	810 g Fe	pear	Soil	1

The sprays with Fe sulphate caused re-greening of chlorotic leaves in all tested fruit crops. Iron sulphate had either similar or better leaf re-greening effect than Fe-DTPA. This was observed in pear (Figs. 9-10), peach (Fig. 11, Table 15), kiwifruit (Figs. 12-13) and citrus (Figs. 14-15). The addition of ascorbic acid to Fe sulphate hastened the re-greening effect of the Fe salt in pear (Fig. 9), whereas adding citric and sulphuric acids, KNO_3 and urea to Fe sulphate (Table 15) did not increase the effect of the Fe salt alone (Fig. 11). In pear and kiwifruit (P6) Fe sulphate applied with aminoacids and polypeptides derived from hydrolysis of proteins caused significant re-greening of chlorotic leaves (Figs. 16-17) and proved to be a good Fe source with high ability to penetrate the leaf (Table 16).

Iron sulphate did not show any phytotoxic effect in kiwifruit leaves up to concentrations of 4 g/L, but concentrations higher than 2 g/L caused some necrotic spots on fruits. The combined use of acid compounds and Fe sulphate did not increase the effectiveness of the Fe salt alone. No visible symptoms of phytotoxicity on pear and peach fruits were visible at rates used.

Re-greening following leaf treatments against Fe chlorosis was much more marked if the SPAD values of the treated leaves were approximately 10 than in the range 28-35 (García-Laviña, 1998). Data on kiwifruit also indicate that if Fe chlorosis becomes too severe, most kinds of canopy sprays may cause phytotoxicity on treated leaves and do not cause significant re-greening (Tagliavini et al., 1997). This also suggests the need for applying treatments against chlorosis well before chlorosis becomes severe.

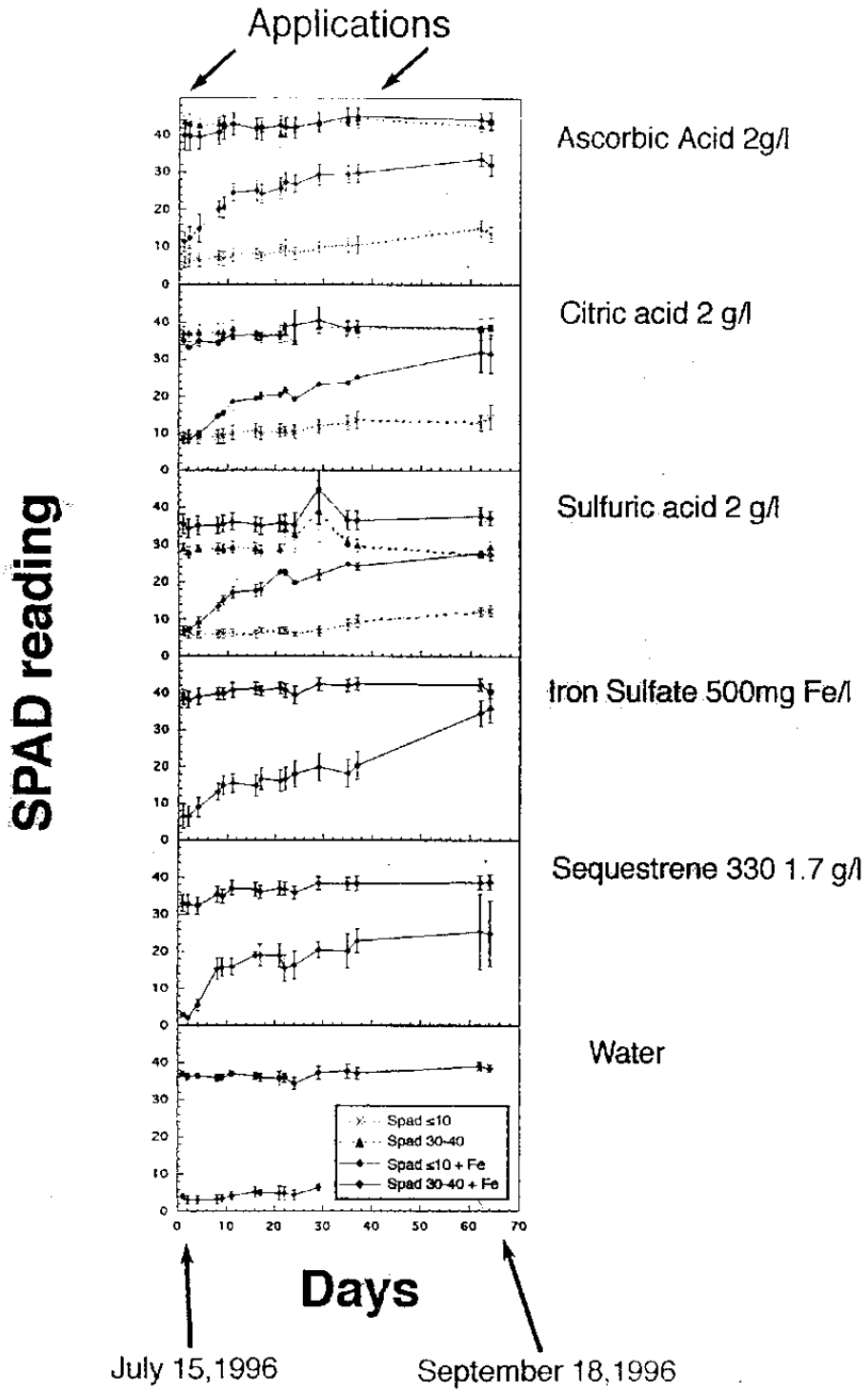

Figure 9: Changes of SPAD values on pear leaves as affected by foliar treatments

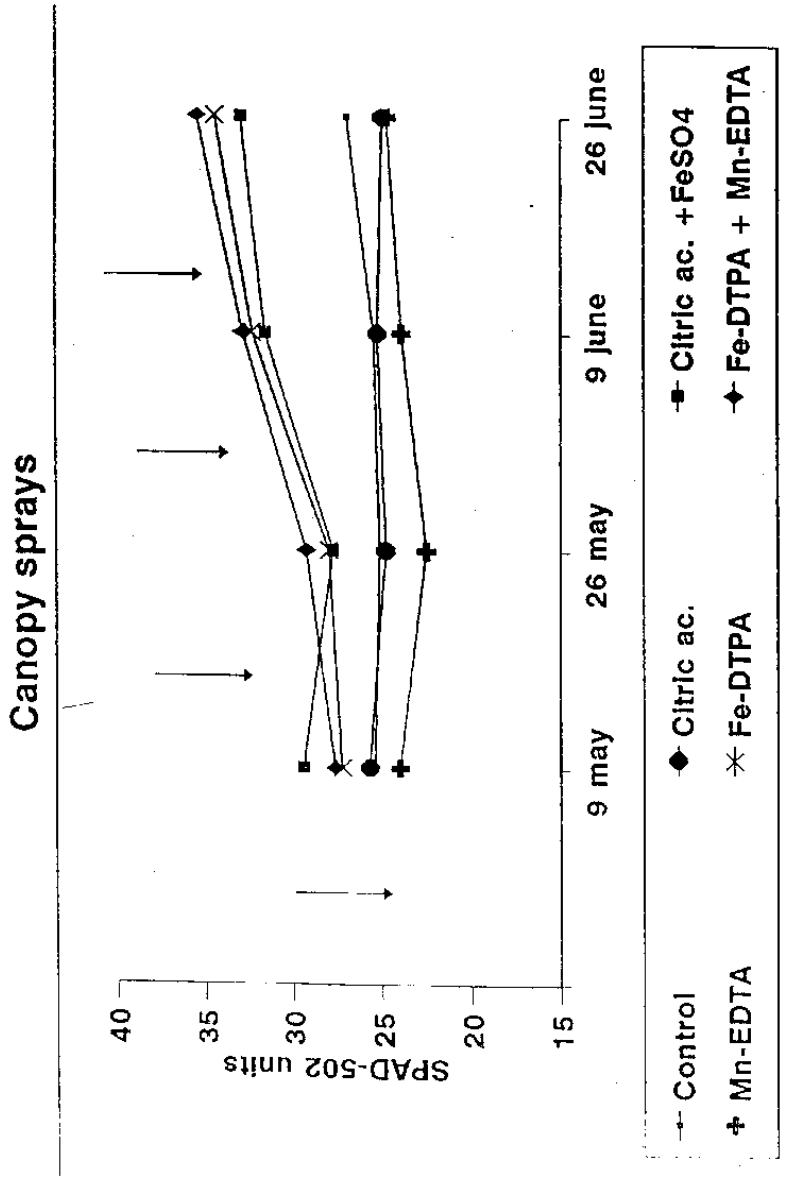

Figure 10: Changes of SPAD values on pear leaves as affected by foliar treatments

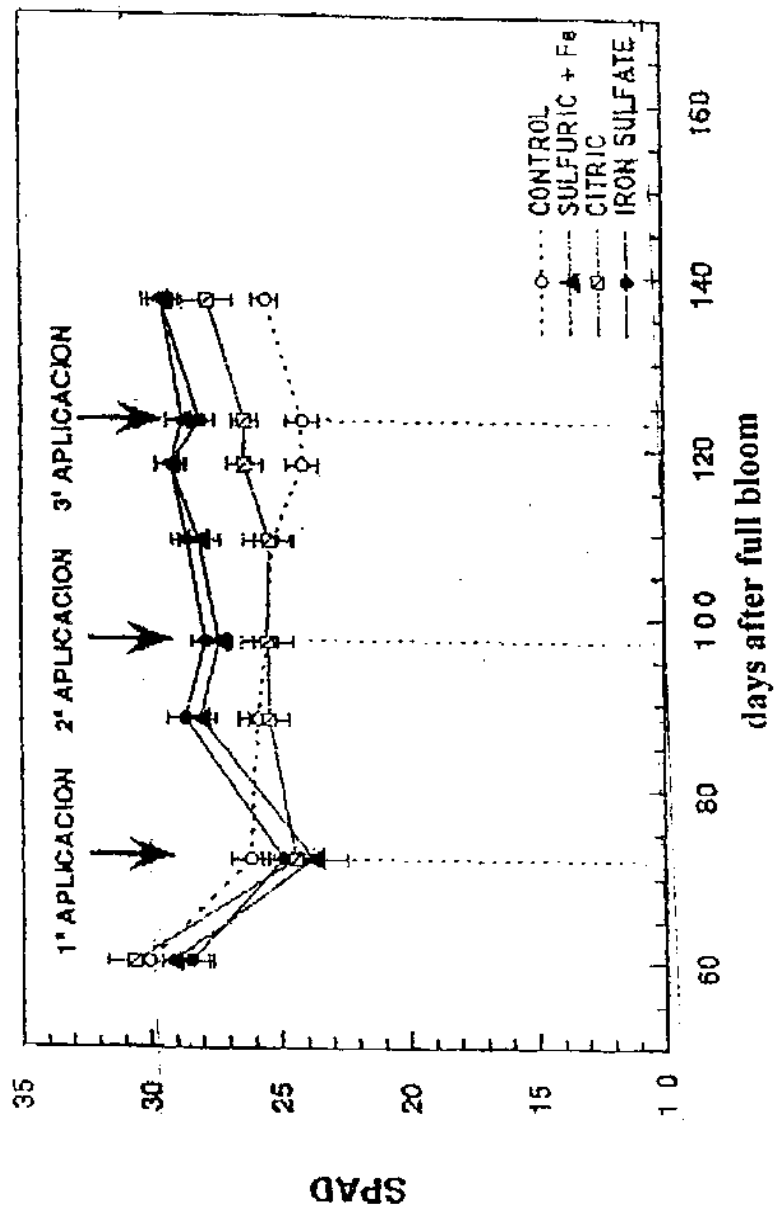

Figure 11: Values of SPAD recorded on peach leaves in Spain by P1-P5, as affected by sprays against chlorosis

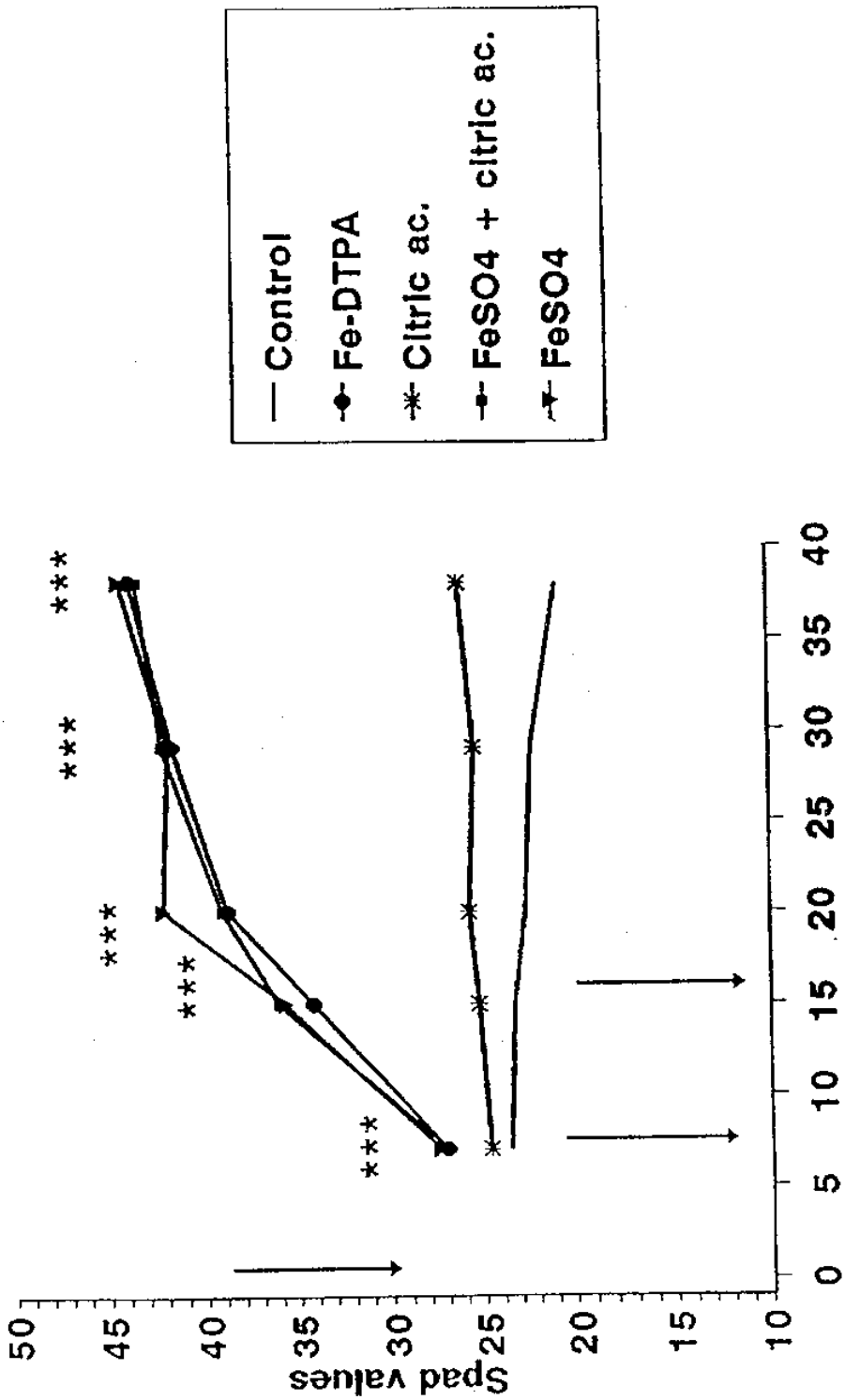

Figure 12: Changes of SPAD values on kiwifruit leaves as affected by foliar treatments

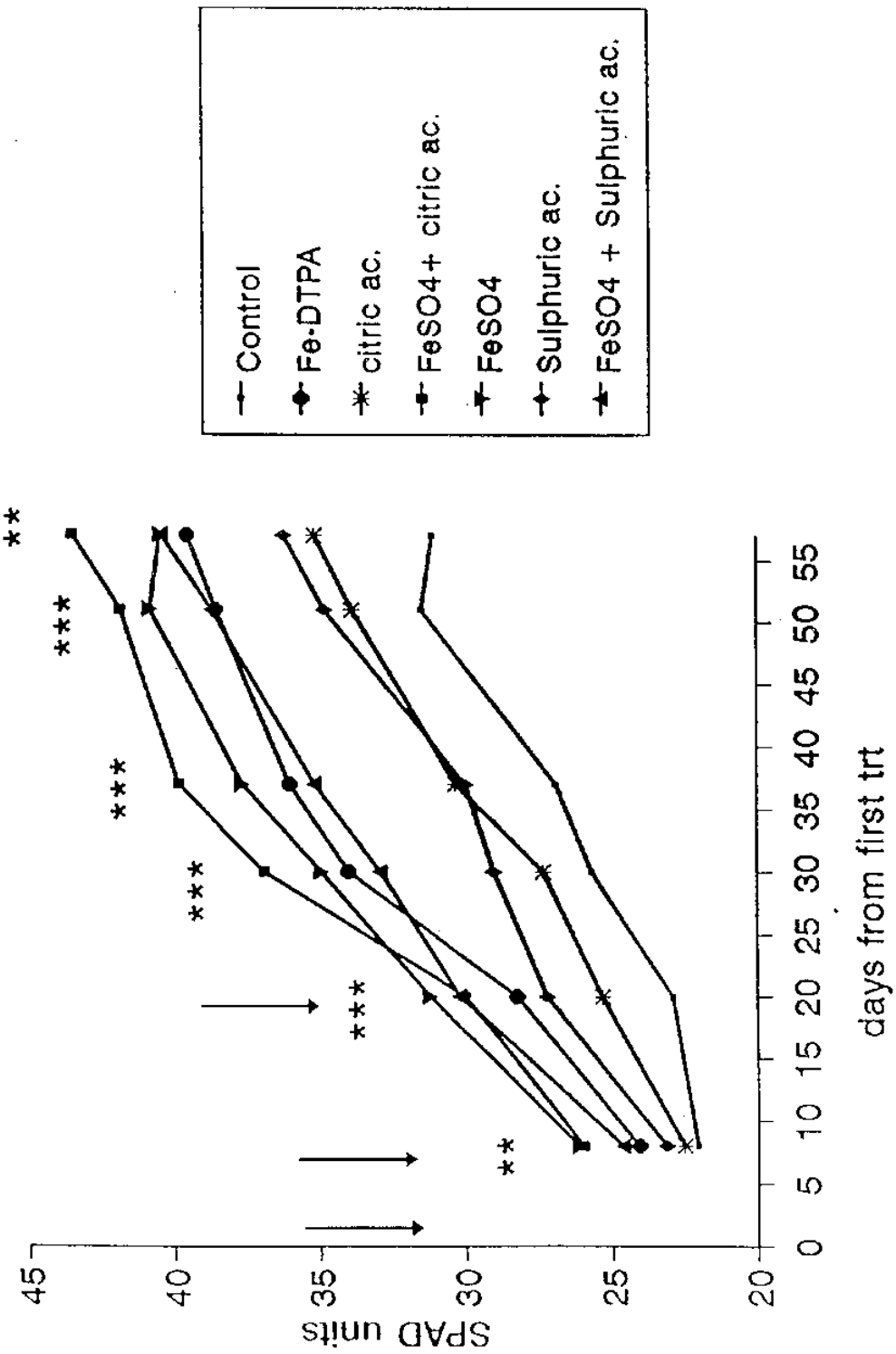

Figure 13: Changes of SPAD values on kiwifruit leaves as affected by foliar treatments

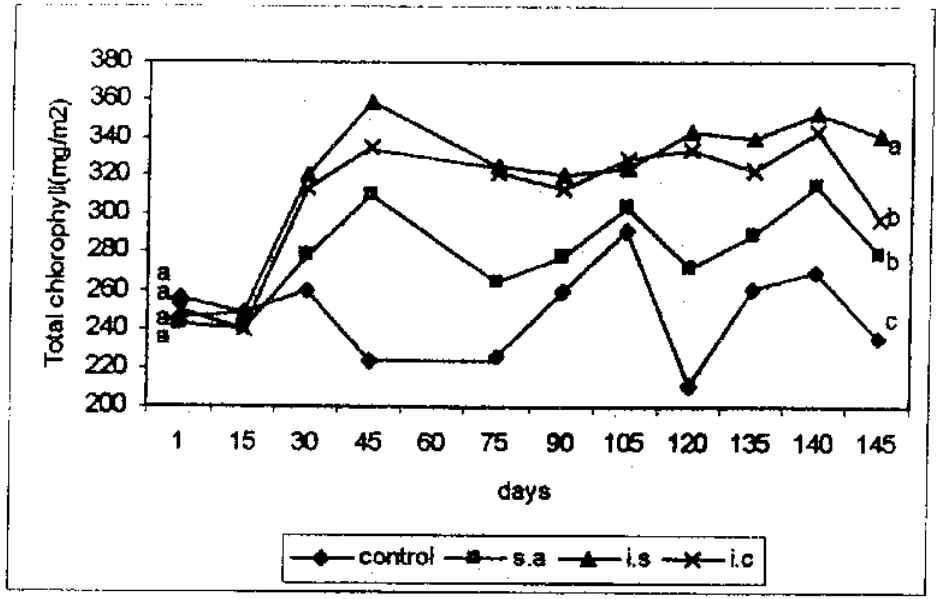

Figure 14: Variation of total chlorophyll concentration (mg m^{-2}) in Valencia late trees during the experiment. Treatments were : control (c), iron sulphate (is), sulphuric acid (sa) and iron chelate (ic). In each date, means followed by the same letter are not significantly different at 95% (Duncan test). Statistical analysis is shown for two dates only.

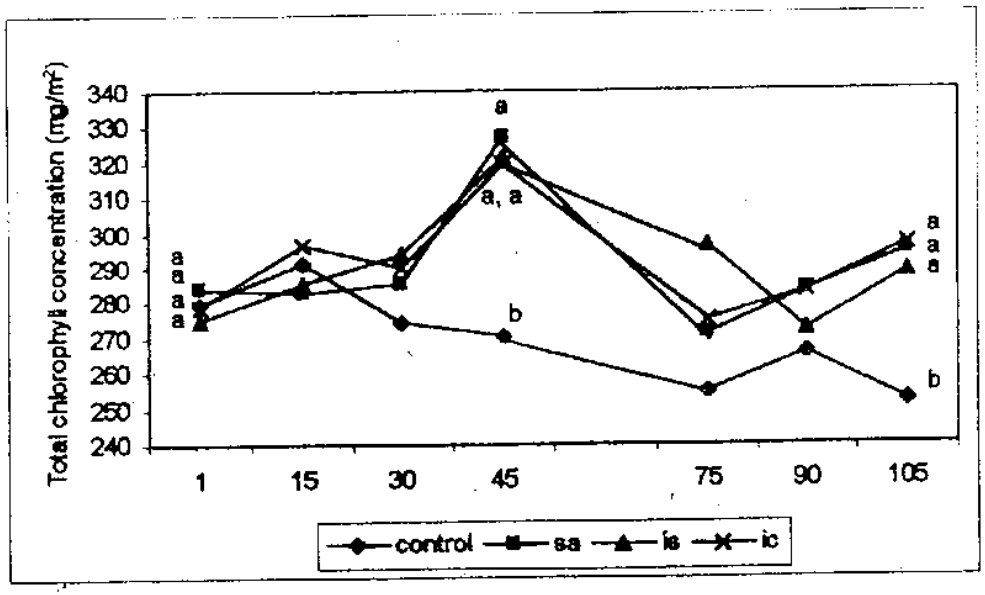

Figure 15: Variation of total chlorophyll concentration (mg m^{-2}) in Encore trees during the experiment. Treatments were : control (c), iron sulphate (is), sulphuric acid (sa) and iron chelate (ic). In each date, means followed by the same letter are not significantly different at 95% (Duncan test). Statistical analysis is shown for two dates only.

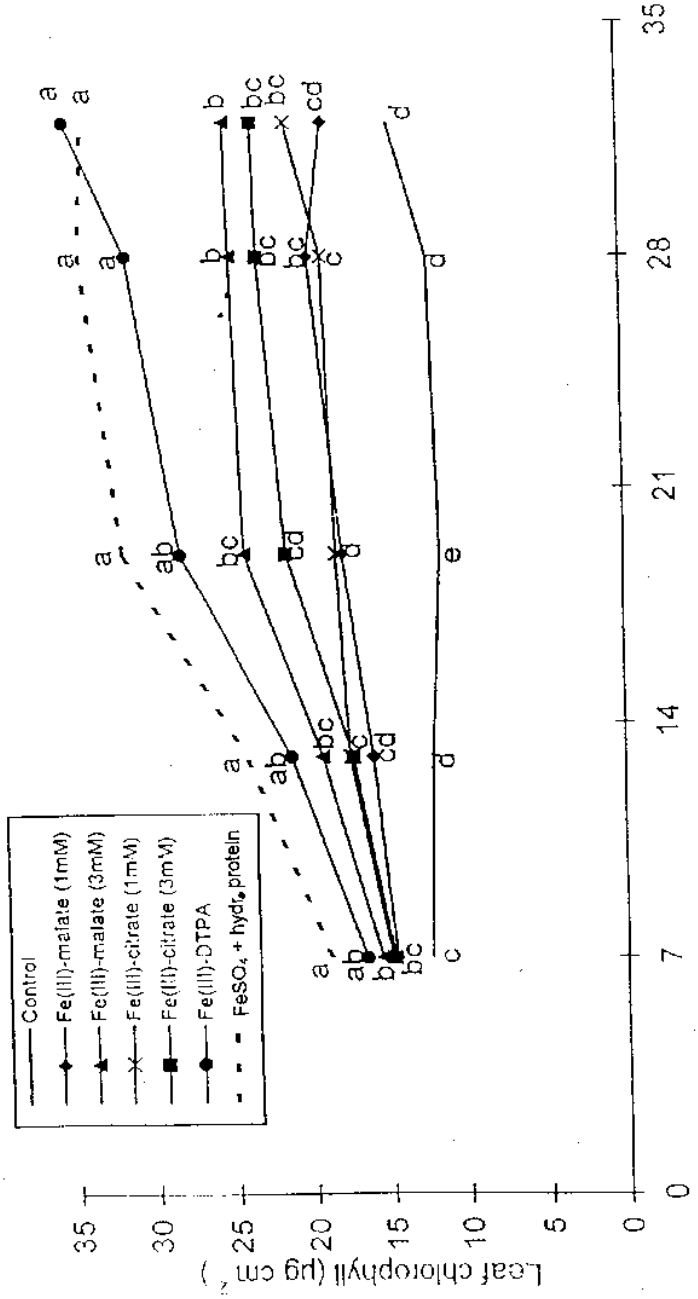

Figure 16: Changes of kiwifruit (cvHayward) leaf chlorophyll content as affected by foliar treatments. Values followed by the same letter are not statistically different at P=0.05

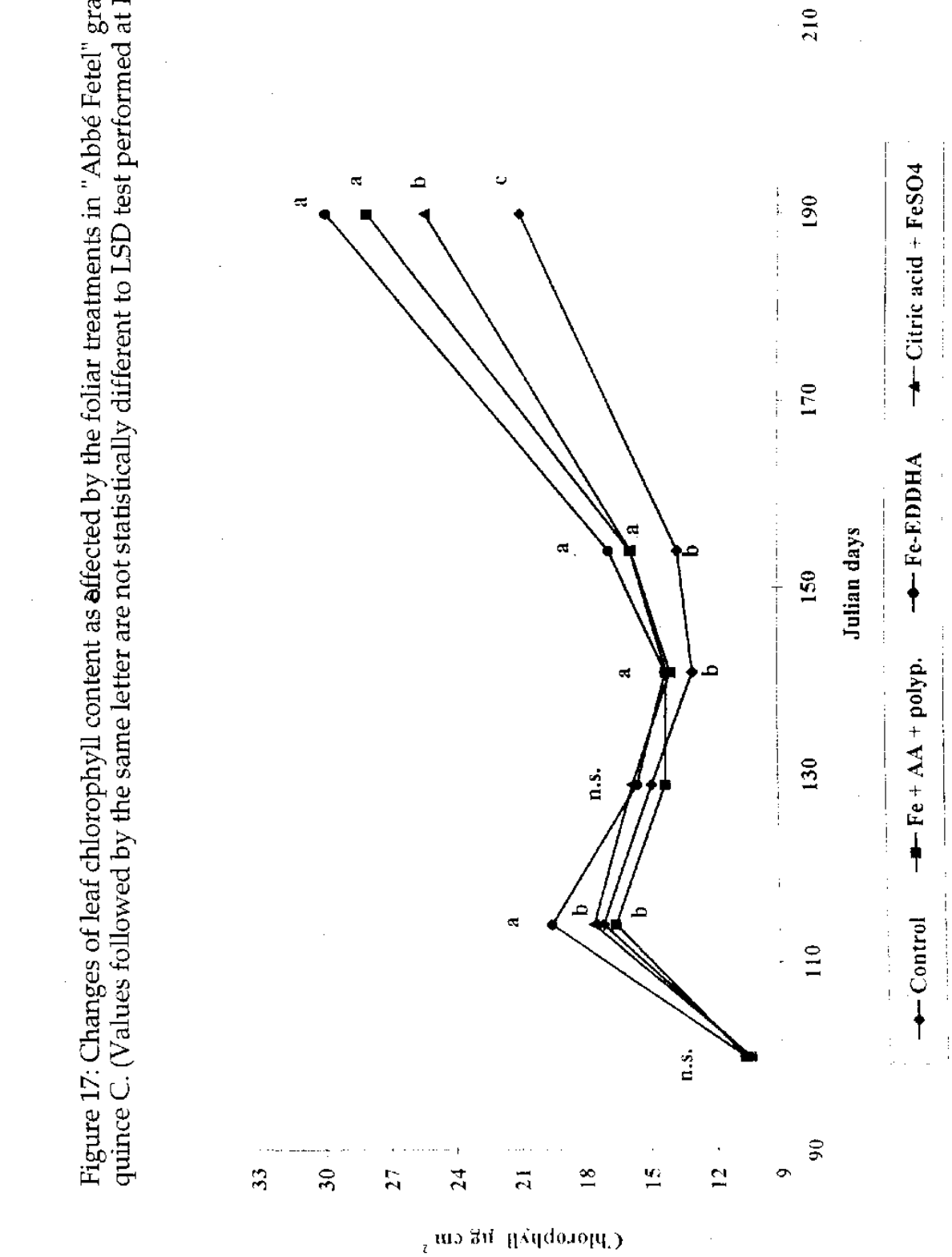

Figure 17: Changes of leaf chlorophyll content as affected by the foliar treatments in "Abbé Fetel" grafted on quince C. (Values followed by the same letter are not statistically different to LSD test performed at P=0.05

Table 15: Effect of foliar applied treatments on peach leaf chlorophyll content (cv Andross)

A/A	TREATMENS	S P A D			ACT.Fe ppm		
		60d	90d	120d	60d	90d	120d
1	Fe-EDTA	29,3	29,81	35,82		11,88	11,54
2	Citric Acid	32,7	30,28	35,27		12,65	10,49
3	FeSO4 .7H2O	30,9	30,58	36,75		16,16	19,13
4	FeSO4 .7H2O + Citric Acid	32,48	32,63	38,55		16,42	14,56
5	Control	32,6	31,8	32,28		12,42	14,43
6	Mn-EDTA	28,23	26,95	36,15		6,52	10,61
7	FeSO4 .7H2O + Mn-EDTA	29,38	28,82	34,28		10,09	14,2
8	FeSO4 .7H2O + KNO3	30,57	32,18	39,7		15,37	19,01

Table 16: Effects of foliar treatments on leaf Fe concentration and content on the amount of Fe taken up and on the proportion of applied iron retained by the leaves

Treatments	Fe concentration ($\mu g\ g^{-1}$)	Fe content ($\mu g\ dm^{-2}$)	Fe taken up ($\mu g\ dm^{-2}$)	Fe retained (% of applied)
Control	132 d [1]	94 d	-	-
Fe^{III}-malate (1mM)	190 c	145 c	51 c	24
Fe^{III} -malate (3mM)	297 b	234 b	140 b	22
Fe^{III} -citrate (1mM)	191 c	139 c	45 c	22
Fe^{III}-citrate (3mM)	344 ab	287 a	193 a	31
Fe^{III}-DTPA	339 ab	257 ab	163 ab	39
Fe^{II}-source	364 a	298 a	204 a	49

[1] Means followed by similar letters do not differ statistically by the Student Newman Keuls test (P=0.05)

The possible effects of surfactants alone, the possible synergetic effect of the addition of surfactants to leaf spray compounds and the effects of the way of application (spray or brush) were tested (Fidalgo, 1998). The surfactants used in the study (L-77 by OSI; and Mistol by Henkel) did not produce any effects on chlorosis when used alone (Fidalgo, 1998). The addition of the anionic tensoactive Mistol to Fe sulphate caused higher re-greening effect as compared to L-77 or Fe sulphate alone. The application of chemicals by spraying had generally similar effectiveness as the optimal wetting, performed using a brush, providing surfactants were applied. The efficiency of Fe salts applied to the upper (adaxial) or to the lower (abaxial) leaf surface was also compared (Fidalgo, 1998). Both Fe-sulphate and Fe-DTPA produced better re-greening in pear when Fe was applied to the lower leaf surface than to the upper one.

In Italy and Greece, the calcareous soils where pear and peach were grown did not depress Mn or other micronutrient level of trees. The application of Mn-EDTA alone was generally ineffective in overcoming the chlorotic symptoms (Table 15 and Fig. 10). The combination of Mn-EDTA with Fe-DTPA or Fe sulphate did not increase the effectiveness of the Fe applied alone (Fig. 10 and Table 15).

Effectiveness of soil treatments

Soil treatments were chosen as they represent a cheap and/or abundant source of natural, non-synthetic Fe. These alternative means to the Fe-EDDHA application included the use of Fe sulphate, alone or in combination with a variety of compounds known for their ability to lower the pH of the bulk soil, such as urea, pyrite and elemental sulphur. Fe sulphate was also distributed together with large amounts of organic matter in an attempt to prevent Fe from rapid transformation into hydroxide forms. The possibility of lowering the pH of the soil microsites involved in root development was also investigated under field conditions by

adding H_2SO_4. Iron sulphate was added in plots sowed with mixtures of graminaceous species, known to release phytosiderophores. Other strategies tested included the distribution of blood meal and of a vegetal compost (5.4 Tm/ha) previously enriched with $FeSO_4$.

Soil treatments against chlorosis were studied in pear and in peach orchards. A single application of Fe-chelate per year was generally very effective in controlling chlorosis. The applications of large amounts of Fe sulphate were generally ineffective if the salt was applied alone or in combination with acidic fertilisers such as urea and sulphur (Fig. 18). Analyses of pH and bicarbonate concentration in the soil solution from control plots and from those receiving the addition of the acid revealed that the attempt of acidification of the soil was ineffective in the medium term, since the soil buffering capacity was able to re-establish the original pH.

The buffer capacities of calcareous and non-calcareous soils are evident from data obtained by P6. In the calcareous soil, the addition of a low amount of protons decreased the soil pH after three hours (Fig. 19). With increasing amounts of protons the pH did not decrease any further, except following the addition of 1200 μeq H^+ g^{-1}, that caused a pH drop to approximately 3.0. After four days, the buffering power of the soil raised the pH of all the treated soils to about 7.3, excepting that receiving 1200 μeq g^{-1} (Fig. 19). After two months, however, the pH of all soils was buffered at 7.4-7.5. These results are not surprising, as a complete neutralisation of a soil with approximately 10% $CaCO_3$, would theoretically require the addition of about 2 meq H^+ g^{-1} of soil.

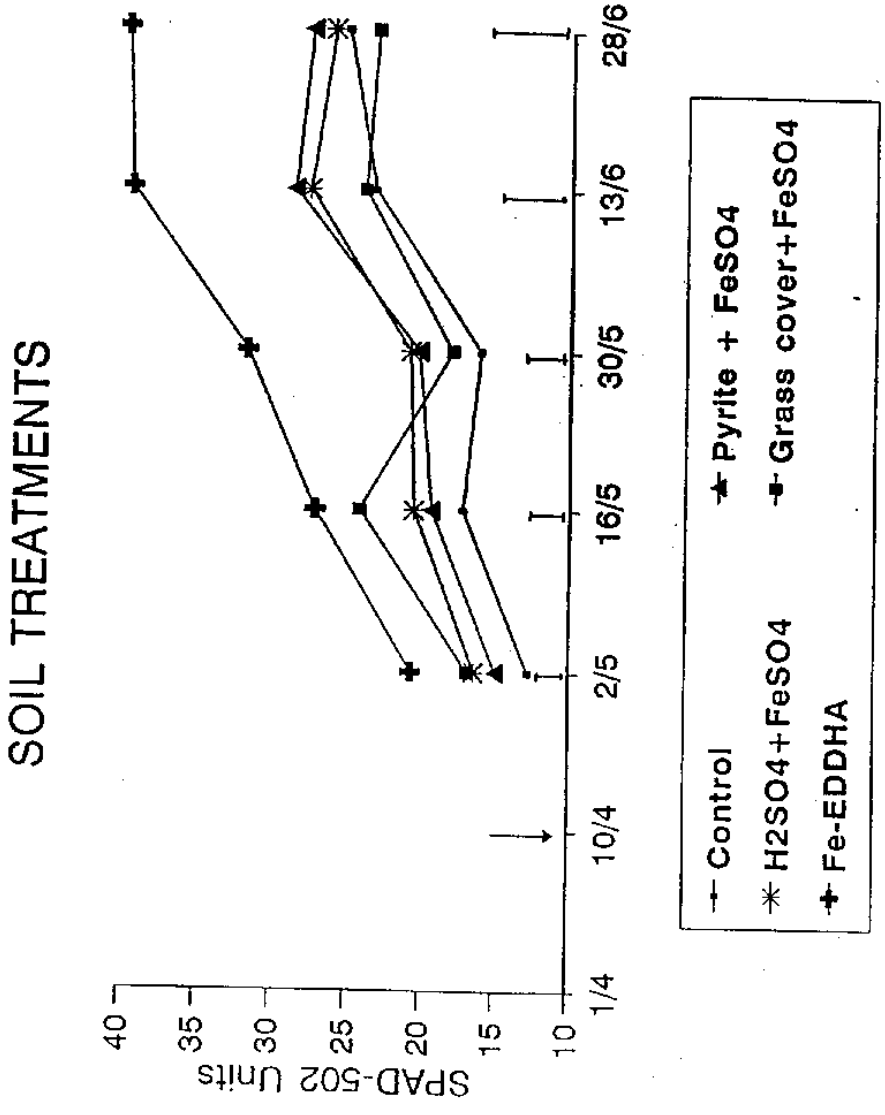

Figure 18: Seasonal pattern of leaf chlorophyll (SPAD values) as affected by soil tratments. Arrow indicates treatments application. Bars represent standard error within each date

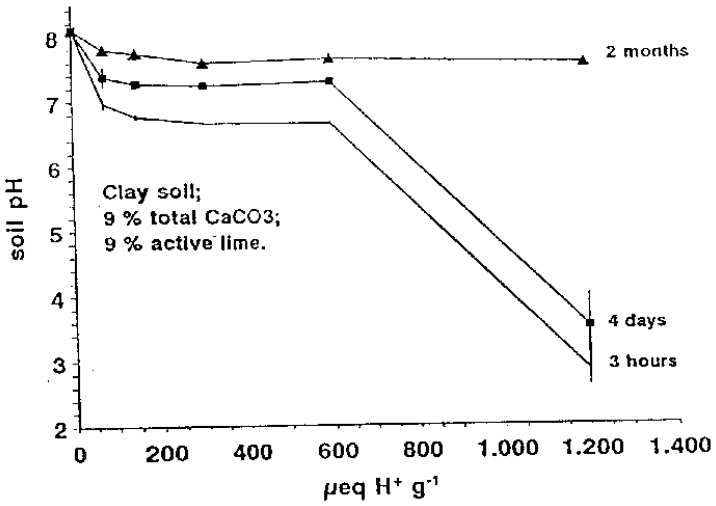

Figure 19: Changes of calcareous soil Ph as affected by the addition of protons

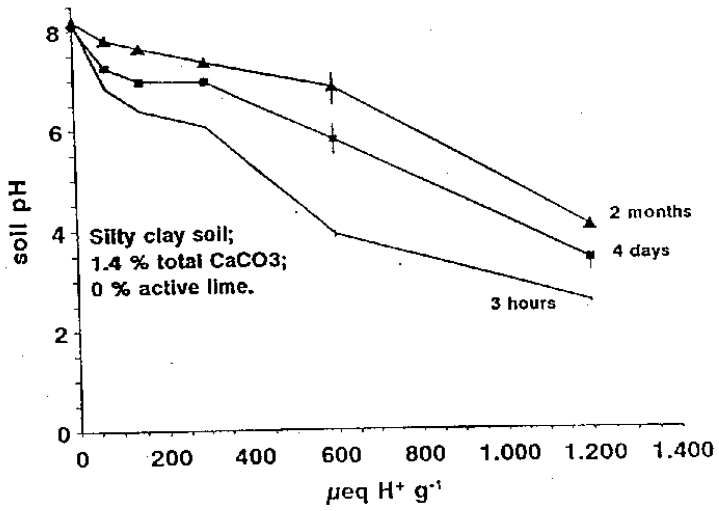

Figure 20: Changes of non-calcareous soil pH as affected by the addition of protons

In the non-calcareous soil (Fig. 20), the pH decreased progressively following the addition of increasing amounts of protons. The pH of soils also increased during the incubation period, as it occurred in the calcareous soil. All acid additions lowered the pH below 7.0 after 3 hours, but the two highest rates dropped the pH to values of 3-4. After 4 days, the 150 and 300 μeq g^{-1} proton doses had decreased the pH to about 7.0, with further pH decreases being linearly related to the proton doses applied (Fig. 20). After two months, the pH had decreased almost linearly with the proton doses added. Values slightly below 7.0 were recorded with the 600 μeq g^{-1} dose, with much lower values resulting from the highest proton dose. In both soils, the electrical conductivity of soil solutions increased with the acid addition from 0 to 300 μeq g^{-1} (Table 17). In the calcareous soil only, the electrical conductivity remained almost constant from 300 to 1200 μeq g^{-1} (Table 17), likely as a result of the formation of gypsum, which made Ca^{2+} and SO_4^{2-} precipitate.

Obtaining long-lasting decreases in the pH of calcareous soils is extremely difficult. For instance, in a soil with pH 7.6 and 8% total $CaCO_3$ fertigation was adopted, including the acidification of irrigation water to pH 5.0. The soil solution at 20-30 cm depth, 20 cm from the emitters, was collected with the aid of suction lisymeters at regular intervals, starting from the end of the irrigation period. Results indicate that once the acid solution enters to the soil it is quickly buffered, and at 20-30 cm depth the pH of the treated areas does not differ from that of the original soil solution. These evidences indicate that long lasting decreases of pH in calcareous soils are difficult to achieve. Also, the addition of strong acids to the soil in a single application may cause salinity problems to plants.

Table 17: Electrical conductivity (dS/m) of soil extracts (1:2 in water) after 2 months from the addition of increasing amounts of protons. Data are the average of three replicates + SE

Amount of Protons $\mu eq\ H^+\ g^{-1}$	Calcareous soil	Alkaline-not calcareous soil
0	0.28 ± 0.01	0.17 ± 0.01
75	0.94 ± 0.01	0.92 ± 0.07
150	1.37 ± 0.02	1.34 ± 0.13
300	2.48 ± 0.01	2.53 ± 0.02
600	2.54 ± 0.01	2.61 ± 0.02
1200	2.60 ± 0.03	2.92 ± 0.04

In the peach trials performed in Greece, the addition of Fe sulphate with organic matter was effective in causing leaf regreening. In Italy, the treatments applied to the soil of pear trees in the last two years caused significant differences at most measurement times (Fig. 21). In the trees grafted on quince C, significantly higher (relative to control) chlorophyll contents were recorded at the second measurement on leaves of trees treated with Fe-EDDHA, in those supplied with blood meal and in plots grassed and supplied with Fe sulphate (Fig. 21). Later on, untreated control leaves had the lowest chlorophyll content. The application of Fe chelate and the strategy that included grassed rows and Fe sulphate were the most effective in controlling chlorosis. The effectiveness of blood meal decreased with time. The applications of compost plus Fe sulphate and Fe-aminoacids-polypeptides were partially effective in causing leaf re-greening.

Leaf analyses performed in the different trials indicated that the highest total leaf Fe is often reached by foliar sprays of Fe-chelate or Fe sulphate, although these treatments have often less effectiveness than soil applied Fe-EDDHA. The evaluation of strategies for overcoming Fe chlorosis should therefore not be based on total leaf Fe concentration.

Causing re-greening of chlorotic leaves by activating the iron pool present in the apoplast of chlorotic leaves.

Experiments have been carried out to test the possibility of causing re-greening of chlorotic leaves by activating the Fe pool present in the apoplast of chlorotic leaves. Alternatives to Fe chelate (DTPA or EDTA) included treatments that should activate the Fe pools already present in leaves (Tagliavini et al., 1995b). For instance, sprays were done with acidic compounds as citric, indol-acetic and sulphuric acids, alone or in combination with Fe salts. Such acids have previously proven to cause some regreening as they may lower leaf apoplast

pH, increase leaf Fe reductase and prevent Fe inactivation in leaves. Ascorbic acid was also used alone or in combination with a Fe salt to prevent Fe-oxidation (see Tables 11-14).

Citric acid sprays caused a significant leaf regreening as compared to control in kiwifruit (Figs. 12-13) and peach (Fig. 11). The regreening caused by the sprays was generally of lower intensity than that caused by spraying Fe compounds, it started from the main veins and was firstly localised to the tissues close to them. In kiwifruit, the application of indole-3-acetic acid (50 μM) also promoted the synthesis of chlorophyll. In this case the regreening was very uniform on the whole leaf surface and lasted for one month (Fig. 22). In trials conducted in Spain on pear trees (Fig. 9), ascorbic acid sprays proved to be effective in causing re-greening, while in the pear trial carried out in Italy, citric acid alone was not effective (Fig. 10). Sulphuric acid was effective in causing re-greening in kiwifruit, pear and citrus (Figs. 9, 13-15).

3.3 Responses of rootstocks to chlorosis and screening techniques

Experiments have been conducted in this issue during the project in Germany, Greece, Italy, Portugal and Spain. Experiments were done with nutrient solution cultures and also in field conditions. Some of the results reported below have been reported in Symposia (Gogorcena et al., 1998) and Thesis (Rombolà, 1998; González-Vallejo, 1999) and have been published in research papers (Pestana et al., 1997; Gogorcena et al., 2000).

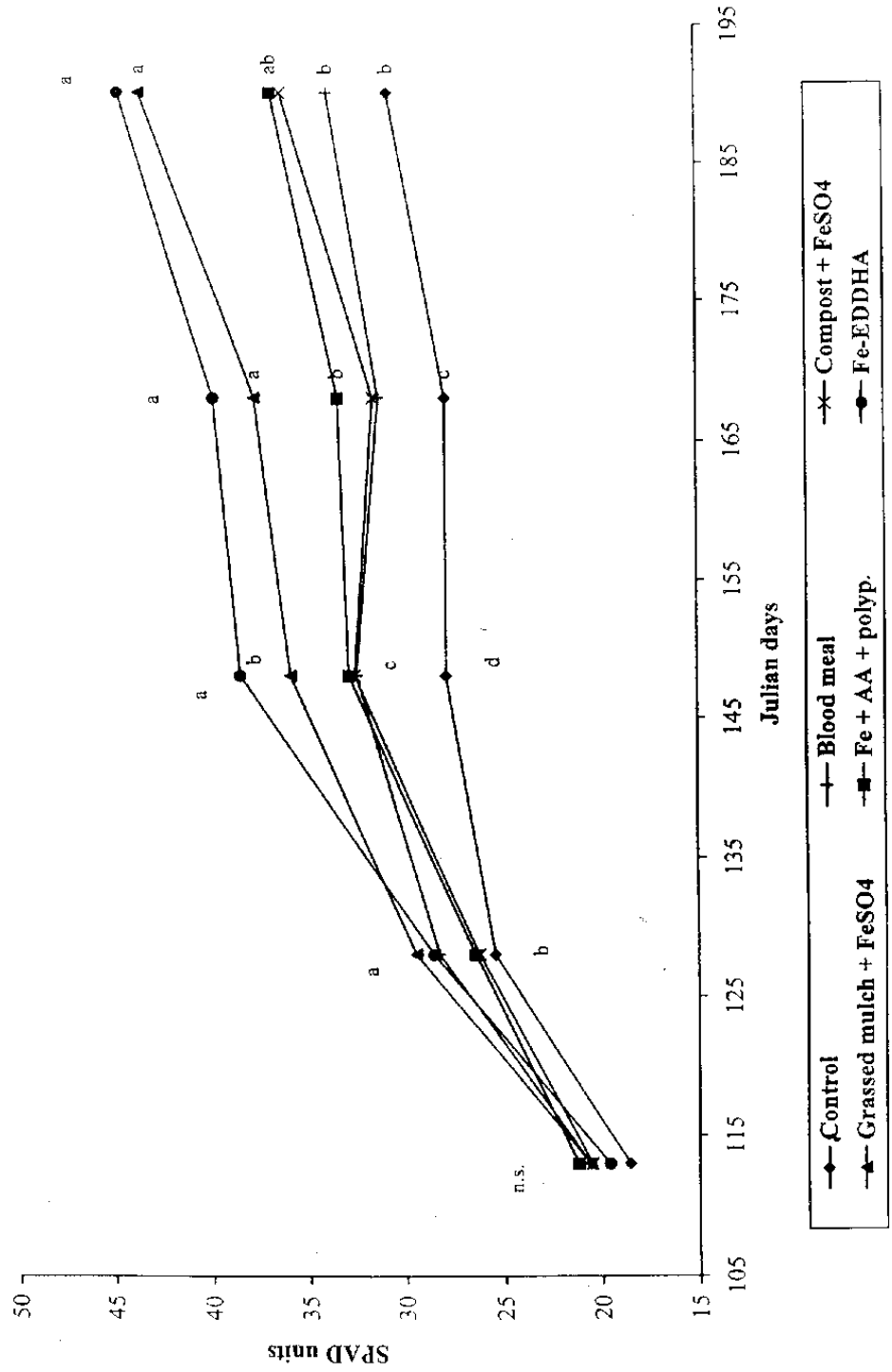

Figure 21: Changes of SPAD values on pear leaves as affected by the soil treatments in "Abbé Fetel" grafted on quince C. (Values followed by the same letter are not statistically different according to SNK test performed at P=0.05

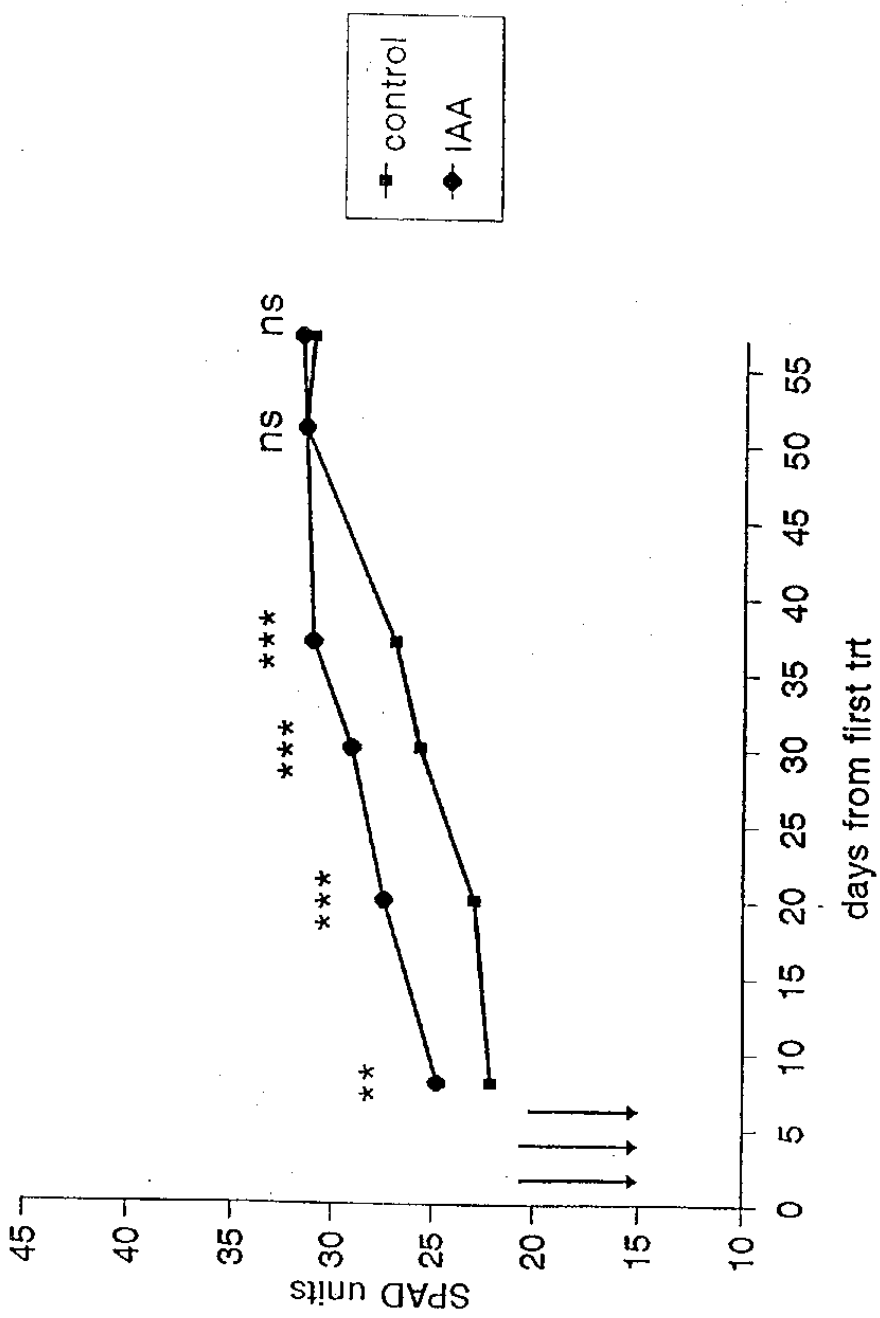

Figure 22: Changes of SPAD values on kiwifruit leaves as affected by indole-3-acetic sprays

Methods for screening based on physiological parameters

The first experiments carried out in the project trying to induce increases in root FC-R activity with Fe deficiency in nutrient solution were quite unsuccessful (González-Vallejo, 1999). Iron-deficient fruit tree rootstocks usually had FC-R activities similar or even lower than the Fe-sufficient controls, as previously reported by Tagliavini et al. (1995a). This confirmed that Fe deficiency not always induces increases in the root FC-R activity of fruit tree rootstocks.

A method has been developed to consistently induce increases in the root FC-R activity in the fruit tree rootstock GF 677 (*Prunus amygdalo-persica*) grown under Fe deficiency (Gogorcena et al., 1998, 2000). Clonal GF 677 plants were grown in a growth chamber in hydroponics with half-strength Hoagland nutrient solution with 90 µM Fe(III)-EDTA or without Fe. The root FC-R activity was measured *in vivo* using BPDS. Plants grown without Fe developed visible symptoms of chlorosis and had lower root FC-R activities than those grown with Fe (Fig. 23). Root FC-R activities were 0.1-1.9 and 0.6-5.3 nmol of Fe reduced per gram of fresh mass and minute, respectively, in Fe-deficient and sufficient plants.

However, when plants grown without Fe for several days were re-supplied with 180 µM of Fe(III)-EDTA, FC-R activities increased within 1 day (Fig. 24). The FC-R values after Fe ressupply were 20-fold higher than those found in Fe-deficient plants and 5-fold higher than those found in the Fe-sufficient controls. After three days of the Fe treatments the FC-R activities had decreased again to the control values. The reduction of Fe was localised at the subapical root zone. In the conditions used we have found no decreases of the nutrient solution pH values, indicating that this type of response is not strong enough to be detected in peach tree rootstocks.

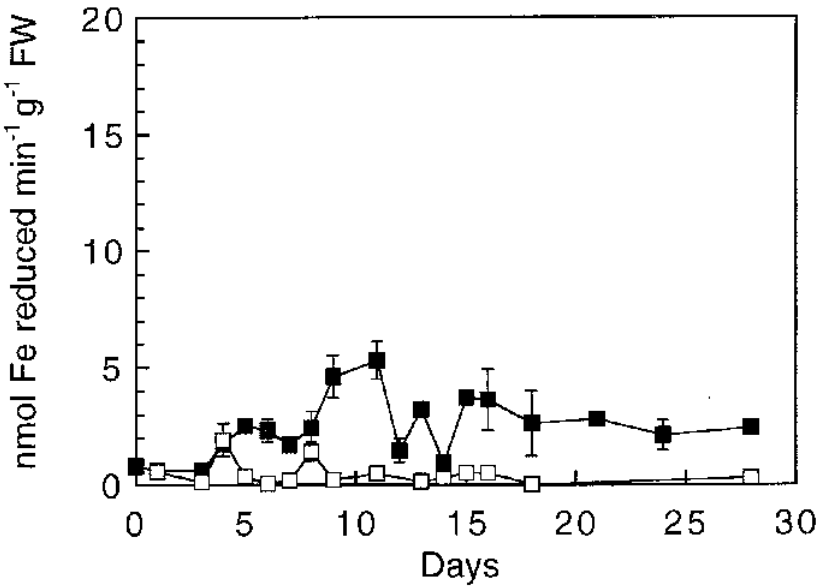

Figure 23: Root ferric chelate reductase activity of control (closed squares) and Fe-deficient whole GF 677 plants (open squares) at different times from the initiation of the treatment. Different plants were used for each assay. Data shown are from n=2-10.

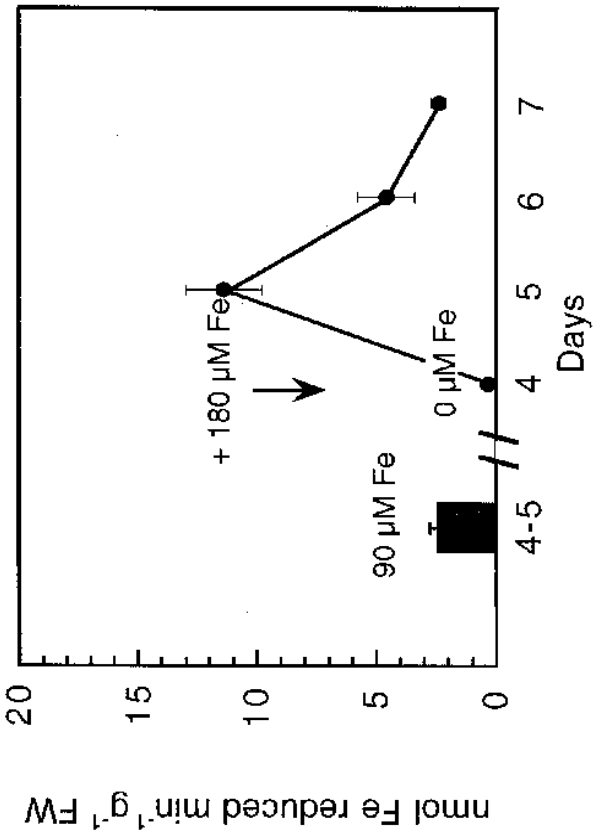

Figure 24: Root ferric chelate reductase activity of whole GF 677 plants submitted to different iron treatments. Data are the mean of n=5-15 plants for each treatment.

Also, no major changes in root morphology have been found in response to Fe deficiency.

The validation of this protocol for screening assays to select rootstock genotypes tolerant to Fe chlorosis has continued by using *in vitro* plants of four different rootstocks with different sensitivity to chlorosis, GF-677 (*Prunus amygdalo-persica*), Adesoto (*Prunus insititia*), Cadaman and Barrier 101 (both *Prunus persica x Prunus davidiana*). The rootstocks GF 677 and Adesoto behaved in the same way, showing a transient induction of the FC-R after the addition of 180 µM Fe to the nutrient solution (Table 18). The induction was 2-8 times over the values found in the controls. Conversely, the rootstocks Cadaman and Barrier did not show any induction of the FC-R activity under the same conditions. The rootstocks GF 677 and Adesoto are highly resistant to Fe chlorosis (Moreno et al., 1995) whereas Barrier and Cadaman are sensitive rootstocks. These results support that the method proposed could be a good tool for the selection of cultivars tolerant to Fe chlorosis.

Table 18. Reductase activity for the rootstocks GF 677, Adesoto, Cadaman and Barrier. Reductase values are expressed in nmol Fe g^{-1} fresh weight min^{-1}. Data are means at least of n=5±SE.

	90 µM Fe	**0 µM Fe**	**1 d after addition of 180 µM Fe**
GF 677	2.13±0.22	0.08±0.01	14.94±1.71
Adesoto	5.27±1.60	0.01±0.01	11.35±1.14
Cadaman	1.43±0.94	1.28±0.52	1.74±0.17
Barrier	0.79±0.35	2.35±1.31	1.92±0.57

Field evaluation and methods of propagation of fruit tree rootstocks.

Field evaluation of peach rootstocks has been done in Greece and Spain. Results have confirmed the good tolerance of the peach-almond hybrids (*Prunus amygdalo-persica*) Adafuel, GF 677 and the GxN series. Also, the good tolerance of the rootstock Adesoto 101 (PS-101) (*Prunus insititia*) in heavy soils has been demonstrated. Field evaluation of pear rootstocks, including several quince genotypes, has been done in Italy (Bassi et al., 1998).

Experiments have been done during the project to optimise propagation by hardwood cuttings and tissue culture. For instance, *in vitro* techniques allowed for a propagation success of 80-90% with the rootstock Adesoto 101 (PS 101) (*Prunus insititia*) that is very difficult to propagate by cuttings.

Somaclonal selection.

Work has been initiated by the Italian group with BA29 somaclones obtained through regeneration from leaves collected on shoots cultivated *in vitro* by standard micropropagation methods. The substrates used during the regeneration phase differed for their pH ranging from 5.7 (standard) to 8.0. In order to increase the selection pressure, each regenerated shoot was sub-cultivated at the same regeneration pH. The surviving shoots were transferred to a multiplication substrate so that from one single shoot one somaclone was obtained. Repeated subcultures were made on a standard substrate to eliminate possible carry-over effects due to regeneration and selection substrates.

The somaclones were compared to BA 29 mother plants grown *in vitro* as a control for their ability of growing, proliferating, rooting and decreasing the rhizosphere pH on substrates with a pH of 7.0, 7.5 and 8.0. Determinations of root FC-R activity were performed on root tips. The somaclones reacted differently to Fe withdrawal. After ten days from

treatment imposition, all the somaclones showed a stimulation of FC-R activity due to Fe deficiency, while Fe-deprived plants of control (BA29 mother plants) showed a 50% decrease in FC-R activity as compared to Fe-sufficient plants. These trials indicated the possibility of obtaining somaclones able to grow better than BA 29 mother plants at high pH *in vitro*, supporting that *in vitro* regeneration is able to create genotypical variation that may be useful for obtaining Fe-chlorosis tolerance.

3.4 Whole plant responses to Fe deficiency

Experiments have been made in this task in Greece, Germany, Italy, Portugal and Spain. The results reported below have been or will be published in research papers (Susín et al., 1996; Nedunchezhian et al., 1997; González-Vallejo et al., 1998a, 1999, 2000; Morales et al., 1998b, 2000; Abadía, 1998; Abadía et al., 2000), theses (Grünewald, 1996; Chatti, 1997; Belkhodja, 1998; Burg, 1998; López-Millán, 2000) as well as in several reports to Symposia (González-Vallejo et al., 1998b; Morales et al., 1998c).

Characterisation of iron acquisition mechanisms at the level of the root plasma membrane.

The physiological and biochemical characteristics of the root FC-R were extensively studied at the level of intact roots (*in vivo*) and at the level of purified PM (*in vitro*) by the Spanish, Portuguese, Italian and German groups.

In order to identify Fe-efficient species and cultivars without the need to perform field trials, a standardised method was developed by group P4 to carry out *in vivo* FC-R tests with root tips. The other groups tested this method with different fruit cultivars and genotypes, as summarised in Table 19. For some species the root tip method was suitable. Other species, for example kiwifruit, showed a remarkable patchiness in FC-R root activity, regardless of the age of the lateral roots (Rombolà, 1998). In such cases it was necessary to measure FC-R

activities with whole root systems. The standardised method was modified as described in the following protocol. After cultivation of the species or cultivar of interest in Fe-deficient and Fe-sufficient hydroponic solutions, information about Fe efficiency and patchiness of FC-R activity was obtained from the performance of *in vivo* agar tests. Then, *in vivo* FC-R activity was measured using either root tips or whole roots, depending on the agar tests. This method was submitted as technological offer to the EU International Relay Centre network.

Table 19: Data on root FC-R obtained from different species investigated during the project.

		FCR activity	pH-Optimum	k_m Fe-EDTA	Partner
kiwifruit					P4 + P6
Hayward + Fe		0.68			
Hayward - Fe		5.77			
D1 + Fe		1.77			
D1 – Fe	4.07				
almond-peach hybrid					P1
- Fe		0.57			
+Fe		2.20			
citrus					
+Fe		1.1			P2
-Fe		2.7			
bean					
+Fe		14	6.0	254	P4
-Fe		35	6.0	246	
sugar beet					
+Fe		11	no distinct	229	P1
-Fe		196	optimum	514	
Valerianella locusta					
+Fe		1.5			P4
-Fe		2.6			

The *in vitro* characterization of root FC-R was performed with PM isolated from Fe-deficient and Fe-sufficient roots of sugarbeet (Susín et al. 1996). In Fe-deficient plants the *in vivo* Fe(III)-EDTA reductase activity increased over the control values 10-20 times when

assayed at a pH of 6.0 or below ("turbo" reductase), but only 2-4 times when assayed at a pH of 6.5 or above. The *in vivo* ferricyanide reductase activity in Fe-deficient plants was 2-fold higher than that of controls, irrespective of the assay pH. The Fe(III)-EDTA and ferricyanide reductase activities of root PM preparations increased 2 and 3.5 times over the controls, irrespective of the assay pH. The K_m for Fe(III)-EDTA of the *in vivo* FC-R in Fe-deficient plants was approximately 510 and 240 µM in the pH ranges 4.5-6.0 and 6.5-8.0, respectively. The K_m for Fe(III)-EDTA of the FC-R in intact control plants and in PM preparations isolated from Fe-deficient and control plants was approximately 200-240 µM. Therefore, the "turbo" FC-R activity of Fe-deficient plants at low pH appears to be different from the "constitutive" FC-R. We also compared Fe-deficient plants grown in the nutrient solution with or without a solid phase of $CaCO_3$, and found that the development of the FC-R activities and their dependence on pH were similar in both cases, indicating that high concentrations of Ca^{2+} or CO_3^{2-} are not essential in triggering the "turbo" FC-R activity.

Characterisation of iron acquisition mechanisms at the level of the leaf plasma membrane.

The Fe acquisition mechanisms of the leaf PM have been studied at three different levels. These processes have been studied in mesophyll PM (kiwi, sugar beet and spinach, by P4/P6, P1 and P4, respectively), mesophyll cells (peach, sugar beet and *Valerianella*, by P1/P4, P1 and P4, respectively) and protoplasts (sugar beet and *Valerianella*, by P1 and P4). These sources were analysed with respect to biochemical characteristics (substrate dependence, pH-optimum, inhibitor and stimulation studies).

The FC-R activities of leaf PM isolated from the leaves of Fe-deficient and Fe-sufficient sugar beet have been characterised (González-Vallejo et al., 1999). Substrates used were the complexes of Fe(III) with EDTA, citric acid and malic acid. Iron deficiency was associated to 1.5- to 2-fold increases in leaf PM FC-R activity when rates were calculated on

a protein basis. The natural complexes of Fe with citrate and especially with malate were good substrates for the FC-R enzyme present in leaf PM preparations. The apparent affinities were higher for Fe(III)-malate. The optimal pH for the activity of the FC-R in sugar beet leaf PM was in the range 6.8-7.0. The FC-R activity decreased by approximately 30% when the assay pH was decreased to 5.8 or increased to 7.5. Therefore, these data provide evidence against the hypothesis that changes in apoplastic pH could decrease markedly the activity of the FC-R enzyme in PM preparations from the leaves of Fe-deficient plants.

Different assay conditions induce changes in the FC-R activities of leaf PM preparations of Fe-deficient and Fe-sufficient sugar beet (González-Vallejo et al., 1998a). Using an apoplast-type assay medium did not change significantly the FC-R activities when Fe(III)-EDTA was the substrate. However, when using ferric citrate as substrate, the effect depended on the citrate:Fe ratio. When the citrate:Fe ratio was 20:1, the effects were practically unappreciable. When using a lower citrate:Fe ratio of 5:1 the activities were, however, significantly lower with the apoplast-type medium than with the standard assay medium. Our data also indicate that anaerobiosis during the assay facilitates the reduction of Fe(III)-malate and Fe(III)-EDTA by PM preparations. Anaerobiosis increased by approximately 50% the PM FC-R activities when using Fe(III)-EDTA as substrate. When using Fe(III)-malate anaerobiosis increased activities by 70-90% over the values obtained in aerobic conditions. However, when using Fe(III)-citrate the increase in activity with anaerobiosis was not significant. The effects of riboflavin, FAD and FMN on the PM FC-R activities were also tested (González-Vallejo et al., 1998a). The presence of flavins generally increased activities in PM preparations from control and Fe-deficient plants. These increases were generally lower than 2-fold, and occurred with Fe(III)-EDTA and Fe(III)-citrate as substrates.

The FC-R activity of mesophyll was characterised with protoplasts isolated from Fe-sufficient and -deficient sugar beet leaves (González-Vallejo et al., 2000). Measurements were made in an ionic environment similar to that existing in the apoplastic space of the sugar beet mesophyll cells. The FC-R activity of Fe-sufficient and Fe-deficient protoplasts was dependent on light. Iron deficiency decreased markedly the FC-R activity per protoplast surface unit. The optimal pH for the activity of the ferric chelate reductase in mesophyll protoplasts was in the range 5.5 to 6.0, typical of the apoplastic space. Beyond pH 6.0 the activity of the ferric chelate reductase in mesophyll protoplasts decreased markedly in both Fe-sufficient and Fe-deficient protoplasts. These data suggest that both the intrinsic decrease in FC-R activity per protoplast surface and a possible shift in the pH of the apoplastic space could be responsible for the immobilisation of physiologically inactive Fe pools in chlorotic leaves.

The characteristics of the FC-R activity in leaf mesophyll disks have been investigated in control and Fe-deficient sugar beet and peach (Larbi, 1999). FC-R activity was higher when the leaf epidermis was removed in sugar beet, whereas in peach the epidermis could not be removed. The FC-R activity of mesophyll disks was light dependent. Iron(III)-citrate can be photo-reduced directly by light in the absence of plant materials. Total FC-R activity was the sum of enzymatic mesophyll reduction, reduction carried out by organelles at the disk cut edge and leakage of reducing substances both by the mesophyll and disk edge. Compounds excreted were shown by HPLC to include organic anions such as oxalate, citrate and malate. When expressed on a surface basis, Fe deficiency decreased the total mesophyll FC-R in sugar beet and peach. When expressed on a chlorophyll basis, however, control disks reduced less Fe than the Fe-deficient ones, both in sugar beet and peach. The optimal pH values for FC-R were in the range 6-6.7 and 5.5-6.9 for sugar beet and peach leaves, respectively. Fe(III)-malate was the substrate that led to the highest Fe reduction rates in both control and

Fe-deficient sugar beet leaves. In control sugar beet leaves Fe(III)-citrate led to higher Fe reduction rates than Fe(III)-EDTA, but the opposite was found in the case of the Fe-deficient leaves. The K_m of the control sugar beet leaves was similar using Fe(III)-EDTA and Fe(III)-malate, whereas it was lower with Fe(III)-citrate. In Fe-deficient sugar beet leaves the K_m for Fe(III)-EDTA was higher than for the other two substrates, which showed similar values. In peach leaves Fe(III)-malate and Fe(III)-EDTA led to the highest FC-R activity in control and Fe-deficient leaves, respectively.

The characteristics of the leaf FC-R was tested in Fe-sufficient and Fe-deficient kiwifruit leaves (Rombolà et al., 2000). For the first time the hypothesis that Fe(III)-malate is a suitable source of Fe for FC-R, in addition to Fe(III)-citrate, has been tested. The results demonstrated that, similarly to other species, mesophyll tissues of *A. deliciosa* leaves are able to perform an enzymatic Fe-reduction prior to Fe uptake. Plasma membrane enriched material extracted from Fe-sufficient leaves reduced Fe(III)-malate and Fe(III)-citrate. The pH optimum was 6.0-6.2 for Fe(III)-malate and 6.5 for Fe(III)-citrate. The substrate-dependence showed higher affinity for malate than for citrate. In contrast to the root level, the FC-R of kiwifruit leaves was not induced by Fe deficiency. On the contrary, after two weeks of Fe depletion, the reduction of Fe(III)-citrate was 4.5-fold lower in the Fe-deficient plants than in the Fe-sufficient ones, whereas the reduction of Fe(III)-malate was not significantly affected.

Under field conditions, we also tested the re-greening effects caused by the application of different Fe sources, including Fe(III)-malate, Fe(III)-citrate, Fe(III)-DTPA and an Fe(II)-source on chlorotic leaves (Rombolà et al., 2000). Under field conditions, Fe applied by spraying caused re-greening of chlorotic leaves, whose intensity and duration varied according to the source and concentration of Fe. The highest re-greening effect was caused by Fe(III)-DTPA and especially by Fe(II), whereas Fe(III)-citrate and Fe(III)-malate were less

effective in increasing leaf chlorophyll concentration. All treatments increased leaf Fe concentration. Although less Fe penetrated into the leaves from Fe(III)-malate than from Fe(III)-citrate, the re-greening effect from Fe(III)-malate was intermediate between that of Fe(III)-DTPA and the one caused by Fe(III)-citrate. The results suggest that if Fe(III)-malate can reach the PM it provides a good source of Fe for leaf Fe uptake.

Isolation and characterisation of the plasma membrane reductase.

A large part of the work done by the German group P4 in this project has been the isolation the FC-R from PM sources. First, large quantities of Fe-deficient, tomato root PM were isolated. In the progress of the isolation procedure, it became clear that the 6-week culture of tomato plants was too time-consuming. Consequently, the plant source was changed to spinach leaves and bean roots, which were harvested after only 12 days. PM from these sources were isolated, pooled, solubilized and further purified by procedures optimised for the separation of membrane proteins, such as preparative native gel electrophoresis, preparative IEF, FPLC with different columns (BIO Q 2, BIO Q 5, Sephadex P6, Econo Blue, Superose 12 and Mono P) (Wagner, 1999). After several drawbacks, FC-R was isolated starting from 120 mg protein of leaf spinach PM, by solubilization with CHAPS, gel filtration with Superose 12 and chromatofocusing via Mono P. All purification steps were monitored by SDS-PAGE. This procedure led to the identification of a 34.7 kD protein, which co-eluted with the FC-R activity. The protein was successfully transferred to a membrane and is currently being sequenced. The protein was not purified to homogeneity, which will only be possible by starting from an even larger amount of PM. The low amount of FC-R that exists in any purified PM fraction was the crucial difficulty in developing these purification techniques. This problem is still a major drawback for the isolation of FC-R from Fe-deficient

bean roots, since the yield of a PM preparation of bean roots is only one third of the yield obtained from spinach leaves.

A second approach to characterise the PM FC-R enzyme was taken by group P1. The Spanish group successfully carried out 2-D separation of the polypeptides present in whole root tips of Fe-deficient and sufficient sugar beet, and found a protein pattern which showed significant differences in several polypeptides (González-Vallejo et al., 1998b; González-Vallejo, 1999). These results clearly indicate that several different polypeptides are involved in Fe-efficiency responses. Some of these polypeptides have been identified (González-Vallejo, 1999) or will be sequenced in the near future.

Role of photosynthetic parameters in the development of chlorosis.

The changes in PS II efficiency in Fe-deficient leaves have been characterised in studies carried out in the project with pear trees (Morales et al., 2000a, 2000b), and also with the model plant sugar beet (Morales et al., 1998b). These data have been recently reviewed (Abadía et al., 2000). The Fe deficiency-induced leaf yellowing is due to decreases in the leaf concentrations of photosynthetic pigments, chlorophylls and carotenoids. However, carotenoids, and more specifically lutein and the xanthophylls of the V+A+Z (Violaxanthin + Antheraxanthin + Zeaxanthin) cycle are less affected than chlorophylls. Therefore, Fe-chlorotic leaves grown in both growth chambers and field conditions have increases in the molar ratios lutein/chlorophyll *a* and (V+A+Z)/chlorophyll *a*. These pigment changes are associated to changes in leaf absorptance and reflectance. In the chlorotic leaves the amount of light absorbed per unit chlorophyll increases. The low chlorophyll, Fe-deficient leaves showed no sustained decreases in PS II efficiency, measured after dark adaptation, except when the deficiency was very severe. This occurred when plants were grown in growth chambers or in field conditions. However, Fe-deficient leaves showed decreases in the actual

PS II efficiency at steady-state photosynthesis, due to decreases in photochemical quenching and intrinsic PS II efficiency. Iron-chlorotic leaves were protected not only by the decreases in leaf absorptance, but also by down-regulation mechanisms enhancing non-photochemical quenching and thermal dissipation of the light absorbed by PS II within the antenna pigment bed.

Photosynthetic parameters as indicators of the development of chlorosis.

We have quantified the relationship between SPAD readings and chlorophyll concentration in leaves in several tree crops (Figs. 25-28). In view of the excellent results obtained with the SPAD, it was considered not necessary to use other possible methods such as chlorophyll fluorescence. The relationship between SPAD readings and chlorophyll concentration is apparently linear when chlorophyll is expressed on a leaf weight basis, but is rather curvilinear when chlorophyll is given on an area basis. Differences in specific leaf weight between green and chlorotic leaves would possibly account for the different relationship found. In a peach study carried out by P6 with two peach cultivars it was found that similar SPAD values indicate lower leaf chlorophyll concentrations in leaves sampled on later stages than in those sampled earlier (Fig. 26). In peach, the relationships between chlorophyll and SPAD values were, within each sampling date, similar in the two cultivars. Of course, running calibration curves for each crop is mandatory, especially when working with leaves of very high and very low chlorophyll concentration.

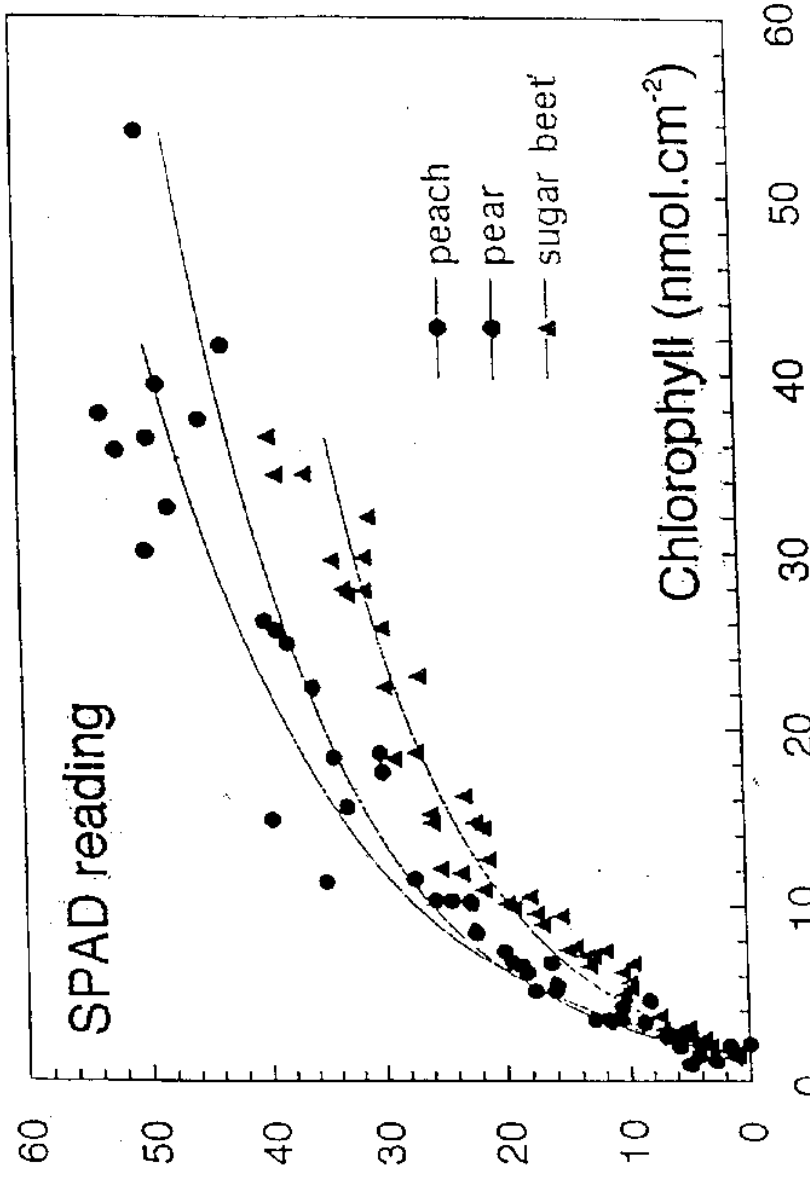

Figure 25: Relationships between leaf chlorophyll content and SPAD readings as detected on peach, pear and sugar beet

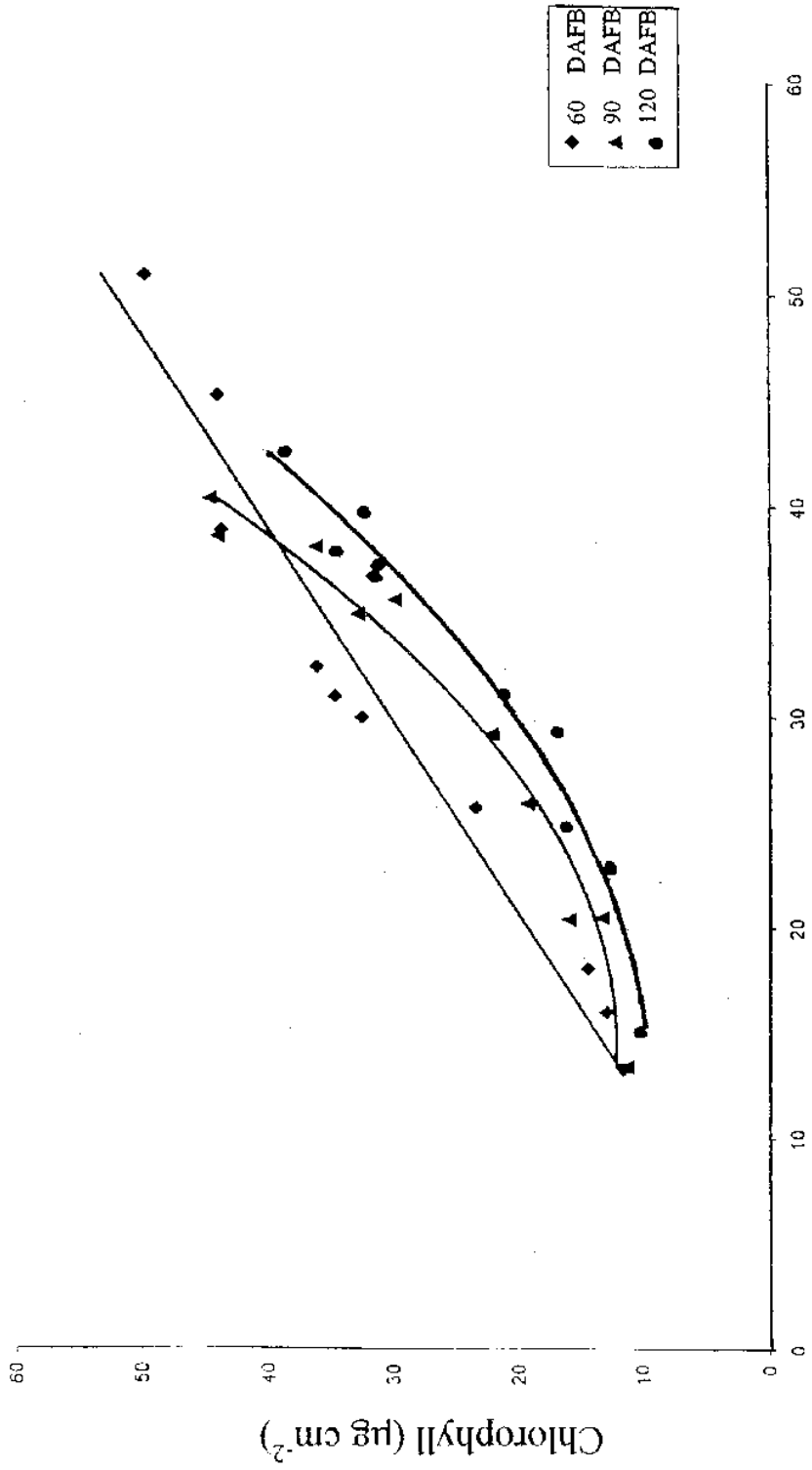

Figure 26: Relationships between leaf chlorophyll content and SPAD readings in peach leaves (cv Stark Redgold) as detected 60, 90 and 120 days after full bloom

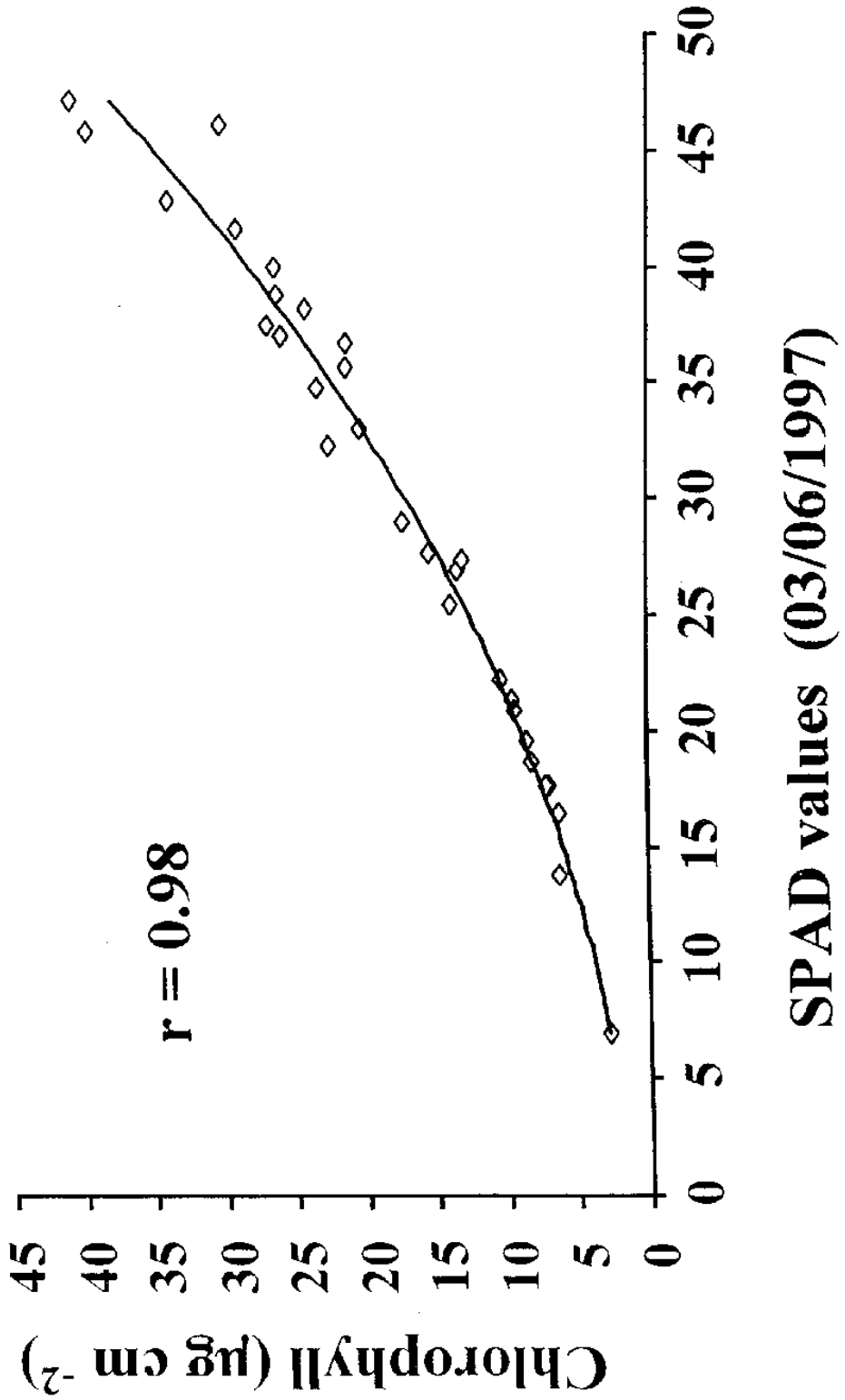

Figure 27: Quadratic correlation between chlorophyll content and SPAD values in pear leaves

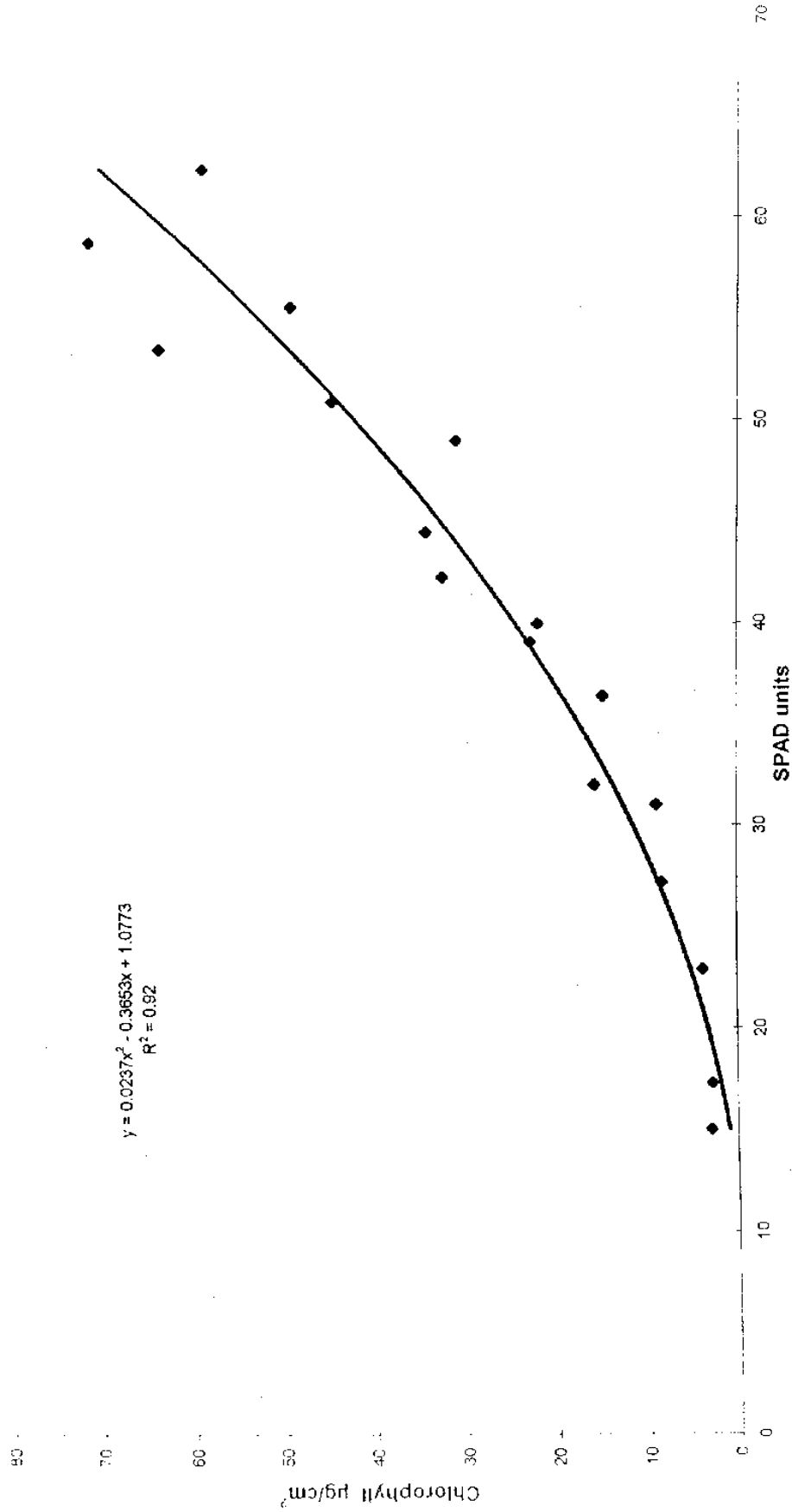

Figure 28: Quadratic correlation betwwen chlorophyll content and SPAD units in kiwifruit leaves

4. Discussion

The results obtained during the project have shown that chlorosis has major negative impacts in fruit yield and quality in different fruit species grown in the Mediterranean area (Tagliavini et al., 2000). An increase in chlorosis symptoms led not only to severe reductions of total fruit yield per tree, but also affected adversely fruit size and quality. These data emphasise the importance of controlling chlorosis in fruit tree crops in Southern Europe.

4.1 Early diagnosis

In this project we have found that the mineral composition of flowers in fruit trees at the begginning of the season could be used to predict tree chlorosis later in the year. Parameters found to have acceptable predictive values are the flower Fe concentration (Sanz et al., 1997a; Belkhodja et al., 1998a; Abadía et al., 2000) and the K/Zn ratio in flowers (Igartua et al., 2000). The Fe flower concentration was proposed before the project as a tool for the prognosis of chlorosis in fruit trees (Sanz et al., 1993; Sanz and Montañés, 1995b). Our results and others found recently (Sanz et al., 1998) have demonstrated that the correlation between Fe in fruit tree flowers and the leaf chlorophyll concentration later in the season is often significant. However, it was also found that the average Fe concentration in flowers, and thus the deficiency thresholds, could suffer large changes from year to year (Abadía et al., 2000; Igartua et al., 2000). This poses a major problem in using the concentration of Fe in flowers for the prognosis of the chlorosis status.

The use of the K/Zn ratio in flowers may offer advantages over the Fe flower concentration for the prognosis of tree chlorosis later in the year. We found that Fe deficiency causes only few changes in the nutrient concentrations in flowers, including increases in the K concentration, whereas the Zn concentration may decrease or not change significantly with

chlorosis (Belkhodja et al., 1998a). The K/Zn ratio in flowers is well correlated to chlorosis every year (Igartua et al., 2000). However, and conversely to what happens with Fe, the average concentration of K and Zn in flowers as well as the K/Zn ratio have quite consistent values from year to year (Igartua et al., 2000). Therefore, the K/Zn ratio in flowers seems a good candidate to predict chlorosis later in the year.

The physiological basis for the changes in the concentrations of Fe, K and Zn with chlorosis has not been established yet. It is likely that most nutrients present in the flowers are already in the tree at the time of flower initiation, the vegetative season prior to flowering. The concentration of Fe, K and Zn in the flowers of fruit trees may reflect the size of the active or mobile pools of nutrients at the time floral buds were formed. If this hypothesis is correct, it will open other possibilities for very early nutritional diagnosis. For instance, one may envisage the possibility that the Fe concentration in floral buds (or even in vegetative buds) during the dormancy period could also be a good early tool to estimate the chlorosis status of the next year. The first data on these very early materials have been obtained in the project and will be published soon.

It has been hypothesised that the increases in K concentration in flowers and leaves are the consequence of a physiological process that is active during the whole season in Fe-deficient trees (Belkhodja et al., 1998a). High K concentrations in the leaves and flowers of the deficient trees are possibly associated to two physiological processes that occur under Fe deficiency. These two processes are the increased activity of the plasma membrane ATPases, involved in root proton excretion (Marschner et al., 1986), and the accumulation of organic acids in all parts of the plant (Welkie and Miller, 1993). The pathways for Zn and Fe acquisition by roots and movement in the plant may share some common steps (Grusak, 1999). Indeed, Zn and Fe in flowers were generally correlated to some extent with Chl, and

these correlations were positive, lower Zn and Fe levels being associated to more chlorosis later in the year (data not shown; see also Belkhodja et al., 1998a). The relative decrease in flower Zn concentration with chlorosis may be also explained by a lower requirement for Zn when a large amount of K is available in the plant.

The results found in the project have established the principles to perform an early diagnosis (prognosis) of Fe chlorosis in trees from the mineral analysis of tree flowers. Two mineral analysis parameters in flowers, the concentration of Fe and the K/Zn ratio, are well correlated to tree chlorosis later in the year. This suggests that the analysis of flowers of any previously non-analysed orchard or tree could be useful for estimating the future chlorosis status, as well as an index for assessing the Fe level of trees in the previous year. The proposed techniques, however, would need validation from a larger set of orchards and in more diverse conditions. Furthermore, new techniques using winter tree materials, such as flower and vegetative buds, would possibly offer new possibilities for prognosis.

4.2 Alternative agricultural management techniques to control iron chlorosis

Agronomic means for controlling Fe chlorosis are still viewed with great interest by fruit growers. Since Fe chelates were introduced, there has been little effort in the research of alternative means for controlling the chlorosis. Our work has demonstrated that alternatives do exist and in the future may be included among the routine practices of managing fruit trees. Alternatives to Fe chelates are of great importance in orchards that follow the guidelines of "Biological Production" or "Integrated Production".

Experiments carried out in the project have proven that foliar sprays of cheap Fe salts such as Fe sulphate could be effective in controlling chlorosis. Of course several applications need to be made during the growing season, and more work has to be done to optimise the effects of foliar sprays in the regreening of leaves and minimising the possible deleterious

effects in fruit quality in each crop. Also, some agronomic practices, such as growing graminaceous plants near the trees concomitantly to the addition of inorganic Fe salts to the soil or using new commercial compounds such as blood meal, are promising for controlling chlorosis.

The whole study has also proved the importance of the Fe pools inactive in the chlorotic leaves of fruit trees, since when chlorosis was not too severe re-greening may occur by adopting strategies that remobilize Fe. The well-accepted phenomenon that chlorotic and green leaves have similar total Fe concentration has been termed “Fe paradox”. Data by Morales et al. (1998a) and Römheld and Schmidt (2000) suggest that this phenomenon depend, at least in part, on the fact that Fe chlorosis could impair leaf expansion. This would lead to a relative increase in the concentration (amount per dry weight), but not in the content (amount per leaf) of Fe in the chlorotic leaf. This would indicate that Fe chlorosis depends largely on an impaired Fe uptake and transportation. However, the existence of Fe pools in the chlorotic leaf which are somehow inactive has been suggested by several authors (Kosegarten and Englisch, 1994; Mengel, 1995; Tagliavini et al., 1995). Data from our experiments demonstrate that sprays aiming to activate the Fe pools in the chlorotic leaf are effective, although rarely caused a full recovery from Fe chlorosis. This indicates that in a chlorotic leaf part of the Fe is inactivated outside the mesophyll cells. Since citric, sulphuric, ascorbic and indole-3-acetic acids caused re-greening, we could hypothesise that inactivation is due to several factors, including a high apoplast pH, high rates of oxidation of Fe(II) or low Fe reductase activity.

The results found in the project have established that some agronomic practices, including foliar sprays and soil amendments, could be as effective as synthetic Fe-chelates in controlling chlorosis in fruit trees. The proposed techniques, however, would need also

validation under the integrated production management schemes used by commercial growers.

4.3 Responses of rootstocks to chlorosis and screening techniques

Data obtained in this project have permitted to establish a new protocol for designing screening tests based on physiological characteristics associated to tolerance to Fe chlorosis in fruit trees. We have produced consistently increases (20-fold) in the root FC-R activity in the fruit tree rootstock GF 677 (*Prunus amygdalo-persica*), by re-supplying plants grown without Fe for several days with 180 µM of Fe(III)-EDTA. Furthermore, we have found that this protocol may be used in screening assays to select rootstock genotypes tolerant to Fe chlorosis, as shown from preliminary comparisons of several genotypes differing in chlorosis tolerance.

Other authors have also found increases in FC-R activity when plants are supplied with a small amount of Fe. For instance, FC-R activity was higher when Fe concentrations in the growing medium were 0.32-2 µM than when Fe was in the range 0-0.1 µM. This was found in bean (Chaney et al., 1972), pea (Grusak, 1990) and sunflower (Romera et al., 1992). In cucumber plants grown in nutrient solutions without Fe (Romera et al., 1996b) the addition of a small amount of Fe (20 µM) produced a large and transient increase of the root Fe(III) reductase activity. In tomato, the addition of 2 µM Fe produced large increases in FC-R activity (Zouari, 1996). However, in sugar beet the FC-R activity is induced in the complete absence of Fe in the nutrient solution (Susín et al., 1996).

The addition of Fe may trigger increases in FC-R activity by at least two possible causes. First, a complete lack of Fe deficiency would cause a low activity of the Fe-dependent enzyme ACC synthase, an enzyme of the ethylene biosynthesis pathway; ethylene would be somehow required for the activity of the FC-R enzyme (Romera and Alcántara, 1994; Romera

et al., 1998, 1999b). Second, a Fe-containing component could be necessary for the functioning of the FC-R enzyme itself. For instance, a flavocytochrome has been recently demonstrated to be associated to FC-R activity in *Arabidopsis* (Robinson et al., 1999).

The finding that small amounts of Fe increase the FC-R activity of Fe-deficient plants suggests that the increases of FC-R activities found by other authors in tree rootstocks with Fe deficiency could be due to uncontrolled Fe sources. Small amounts of available Fe could come from the containers where plants are grown, or from the apoplast of the root upper part if not fully (and continuously) immersed in nutrient solution. Since the assay solution includes Fe(III)-EDTA, using long assay times (i.e. of several hours), as in Ao et al., (1985) or Cinelli et al., (1995), could also lead to Fe resupply to the plants being measured. Finally, using the same plants for sequential measurements with Fe(III)-EDTA, as in Romera et al., (1991b), could lead to inadvertently ressuply Fe to the plants being measured.

Since 1995, the physiological responses of fruit trees to Fe deficiency have been studied in several papers by other authors. The FC-R activity of quince (*Cydonia oblonga* L.) roots showed either small increases (Cinelli, 1995) or large decreases (Tagliavini et al., 1995a) with Fe deficiency. Excised roots of quince cuttings had FC-R activities of 0.6-2.1, 1.3-2.0 and 1.8-5.0 nmol Fe min^{-1} g^{-1} FW in the case of Fe-sufficient controls, plants grown without Fe and plants grown with Fe in the presence of $NaHCO_3$, respectively (Cinelli, 1995). Other authors (Tagliavini et al., 1995a) found FC-R activities of 0.23 and 0.02 nmol Fe min^{-1} g^{-1} FW in the case of whole *in vitro* control and Fe-deficient quince plants, respectively. The root FC-R activities of pear (*Pyrus communis* L.) whole plants obtained *in vitro* was decreased by Fe deficiency from 1.2 to 0.5 nmol Fe min^{-1} g^{-1} FW (Tagliavini et al., 1995a).

A study was carried out with cuttings of the peach cv. Nemaguard, and several almond x peach hybrid clones of the Garfi x Nemared series (de la Guardia et al., 1995). The peach

was more sensitive to chlorosis (and its growth was lower) than the hybrid genotypes. The FC-R activity was generally increased by Fe deficiency in the hybrids but not in Nemaguard. In this study values were not expressed on a root weight basis, but only on a plant basis, making comparison of results difficult. The FC-R activities of Nemaguard and the hybrid TNG (Titan x Nemared) were also compared by Cinelli et al. (1995). The hybrid was more resistant than the peach rootstock to chlorosis when treated for 30 d with $NaHCO_3$, although both rootstocks, when Fe-deficient, had similar FC-R values in a 90 min assay. Reduction was localised in root hairs and root tips. With longer reduction times the hybrid was claimed to have higher FC-R activities than the peach rootstock.

In *Vitis* spp. woody cuttings, Fe deficiency led to a decrease in the pH of the nutrient solution and an increase in organic acid content (Brancadoro et al., 1995). Stimulation of the FC-R activity of excised roots ranged from less than 1 to 4-fold, with values of 0.6-1.3 and 0.7-2.3 nmol Fe min^{-1} g^{-1} FW in the control and Fe-deficient plants, respectively. The root FC-R activity of *in vitro* kiwifruit (*Actinidia deliciosa*) whole plants was increased 5-fold by Fe deficiency, from 1.7 to 9.1 nmol Fe min^{-1} g^{-1} FW (Vizzotto et al., 1997). Iron deficiency induced chlorosis and acidification of the nutrient solution. The root tip FC-R activity of avocado (*Persea americana*) seedlings was increased 3.5-fold by Fe deficiency, from 2 to 6.9 nmol Fe min^{-1} g^{-1} FW (Manthey and Crowley, 1997).

The possible use of somaclonal variation methods has given promising results with pear genotypes. These results are still preliminary but open a new way of finding tolerance to chlorosis in fruit trees.

The results found in the project have established the principles for the design of screening tests for tolerance to Fe chlorosis based on physiological parameters. The proposed new protocol could be used to assess in the future the germplasm available for chlorosis

tolerance. The protocol has been shown to work well with micropropagated material, but its use with cuttings, more widely available than micropropagated plants for many rootstock genotypes, still needs testing.

4.4 Whole plant responses to iron deficiency

The works developed within the project have provided light on several physiological changes induced by Fe deficiency in plants. The biochemical characteristics of the root FC-R have been extensively studied on the level of intact roots *in vivo* (Susín et al., 1996; Zouari, 1996; Rombolà, 1998) and on the level of purified plasma membranes *in vitro* (Susín et al., 1996). These characteristics include substrate dependence and optimal pH. Our finding that the enzymatic characteristics depend on the measuring pH (Susín et al., 1996) has changed the view on the FC-R enzyme, as discussed in the recent review by Schmidt (1999).

A standardised method was developed to carry out *in vivo* FC-R root tip tests. After cultivation of the species or cultivar of interest in Fe-deficient and Fe-sufficient hydroponics solutions, information about Fe efficiency and patchiness of FC-R activity was obtained from the performance of *in vivo* agar tests. Then, *in vivo* FC-R activity was measured using either root tips or whole roots, depending on the agar tests. This method was submitted as technological offer to the EU International Relay Centre network.

The biochemical characteristics of the leaf mesophyll FC-R were little known before 1995. In the project these characteristics have been studied at the level of mesophyll tissue in vivo (Rombolà, 1998; Larbi, 1999), intact protoplasts (González-Vallejo et al., 2000) and purified plasma membranes (González-Vallejo et al., 1998a, 1999; Rombolà et al., 2000) *in vitro*. The *in vivo* leaf FC-R activity has been confirmed to be light dependent, as found recently by other authors (de la Guardia et al., 1996). The FC-R enzymatic characteristics of the leaf PM are remarkably similar to those of the root PM enzyme. However, the pH

dependence of the FC-R enzyme in isolated protoplasts is different from that of isolated PM, with its optimal pH being closer to that of the apoplastic pH. In any case, our data suggest that both the intrinsic decrease in FC-R activity per protoplast surface and a possible shift in the pH of the apoplastic space could be responsible for the immobilisation of physiologically inactive Fe pools in chlorotic leaves. Since no "Turbo" FC-R activity could be found in leaves, screening leaf sources for FC-R activity does not appear to be a successful approach to identify Fe-efficient fruit tree lines (Larbi, 1999; Rombolà et al., 2000).

A very important finding is that the natural complexes of citrate and malate with ferric Fe are good substrates for the FC-R enzyme, both in mesophyll tissue (Rombolà, 1998; Larbi, 1999), and in isolated plasma membranes (González-Vallejo et al., 1999; Rombolà et al., 2000). This opens new possibilities for applying these compounds to treat Fe chlorosis in field conditions, as already shown in kiwifruit by Rombolà et al. (2000).

Knowledge has been gained during the project on the molecular changes in proteins induced by Fe deficiency in leaf and root tissue. The isolation and partial characterisation of the FC-R from spinach leaves has been carried out during the project. Other approaches such as 2-D electrophoresis of root tip proteins have led to the identification of a series of polypeptides which are candidates to play a role in the Fe-efficiency responses of plants to Fe deficiency (González-Vallejo, 1999). It is expected that these polypeptides will be characterised in future projects.

The knowledge on the photosynthetic changes induced by Fe deficiency has provided the background data to understand one of the characteristics less studied of Fe chlorosis, the light dependence of the deficiency (Abadía, 1992; Terry and Zayed, 1995). The changes in PS II efficiency in Fe-deficient leaves have been characterised in studies carried out in the project with pear and peach trees and the model plant sugar beet (Abadía et al., 2000). Chlorotic

leaves grown in both growth chambers and field conditions have increases in the molar ratios lutein/chlorophyll *a* and (V+A+Z)/chlorophyll *a* and show changes in leaf absorptance and reflectance. The low chlorophyll, Fe-deficient leaves showed no sustained decreases in PS II efficiency, measured after dark adaptation, except when the deficiency was very severe. However, the chlorotic leaves showed decreases in the actual PS II efficiency at steady-state photosynthesis, due to decreases in photochemical quenching and intrinsic PS II efficiency. Fe-chlorotic leaves were protected not only by the decrease in leaf absorptance, but also by down-regulation mechanisms enhancing non-photochemical quenching and thermal dissipation of the light absorbed by PS II within the antenna pigment bed.

Finally, we have demonstrated in the project that a photosynthetic parameter, the chlorophyll content estimated from SPAD readings, is an excellent tool to be used as an indicator of Fe chlorosis. This parameter, once properly calibrated for a given species, is far better than other indicators such as chlorophyll fluorescence, mineral content and visual ratings. We have used the SPAD apparatus in most experiments developed in the project, including chlorosis control experiments.

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NOVEL APPROACHES FOR THE CONTROL OF IRON CHLOROSIS IN FRUIT TREE CROPS

Exploitation Report

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FINAL REPORTING

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FINAL REPORTING-EXPLOITATION REPORT

Project Title

NOVEL APPROACHES FOR THE CONTROL OF IRON CHLOROSIS IN FRUIT TREE CROPS

Organisations involved

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1 Description of the results and comparison of the innovative aspects to the state of the art

The results obtained during the project have shown that **chlorosis has major negative impacts in fruit yield and quality** in different fruit species grown in the Mediterranean area. An increase in chlorosis symptoms led not only to severe reductions of total fruit yield per tree, but also affected adversely fruit size and quality. These new data emphasise the importance of controlling chlorosis in fruit tree crops in Southern Europe. No such clear data on the effect of Fe chlorosis on fruit yield and quality were available before the project.

The results found in the project have established the principles to perform the **early diagnosis (prognosis) of Fe chlorosis in trees** from the mineral analysis of tree flowers. Two mineral analysis parameters in flowers, the concentration of Fe and the K/Zn ratio, have been shown in the project to be well correlated with tree chlorosis later in the year. This suggests

that the analysis of flowers of any previously non-analysed orchard or tree could be useful for estimating the future chlorosis status, as well as an index for assessing the Fe level of trees in the previous year. The **Fe flower concentration** was proposed before the project as a tool for the prognosis of chlorosis in fruit trees. Our results have demonstrated that the correlation between Fe in fruit tree flowers and the leaf chlorophyll concentration later in the season is often significant. We have found that a new parameter, the **K/Zn ratio in flowers** may offer advantages over the Fe flower concentration for the prognosis of tree chlorosis later in the year. The K/Zn ratio in flowers is well correlated to chlorosis every year. However, and conversely to what happens with Fe, the average concentration of K and Zn in flowers as well as the K/Zn ratio have quite consistent values from year to year.

Agronomic means for controlling Fe chlorosis are still viewed with great interest by fruit growers. Since Fe chelates were introduced, there has been little effort in the research of alternative means for controlling the chlorosis. Our work has demonstrated that alternatives do exist and in the future may be included among the routine practices of managing fruit trees. The results found in the project have established that some agronomic practices, such as applying foliar sprays with inorganic Fe sources, growing graminaceous plants fertilised with Fe in the orchard and adding new Fe-containing products to the soil, could be as effective as synthetic Fe-chelates in controlling chlorosis in fruit trees. Alternatives to Fe chelates are of great importance in orchards that follow the guidelines of "Biological Production" or "Integrated Production".

Experiments carried out in the project have shown that **foliar sprays of cheap Fe salts** such as Fe sulphate could be effective in controlling chlorosis. Of course several applications need to be made during the growing season, and more work has to be done to optimise the effects of foliar sprays in the regreening of leaves and minimising the possible

deleterious effects in fruit quality in each crop. Also, some **agronomic practices**, such as growing graminaceous plants near the trees concomitantly to the addition of inorganic Fe salts to the soil or using new commercial compounds such as blood meal, are promising for controlling chlorosis. The proposed techniques, however, would need also validation under the integrated production management schemes used by commercial growers.

Research done in the project has also shown the importance of the Fe pools inactive in the chlorotic leaves of fruit trees, since when chlorosis was not too severe re-greening may occur by adopting strategies that remobilise Fe. The well-accepted phenomenon that chlorotic and green leaves have similar total Fe concentration, which has been termed "Fe paradox", indicates the existence of Fe pools in the chlorotic leaf which are somehow inactive. Data from our experiments demonstrate that sprays aiming to activate the Fe pools in the chlorotic leaf are effective, although rarely caused a full recovery from Fe chlorosis. This indicates that in a chlorotic leaf part of the Fe is inactivated outside the mesophyll cells.

Data obtained in this project have permitted to establish a **new protocol for screening tests** based on physiological characteristics associated to tolerance to Fe chlorosis in fruit trees. We have produced consistently increases (20-fold) in the root ferric chelate reductase activity in tolerant fruit tree rootstocks, by re-supplying plants grown without Fe for several days with a small amount of Fe(III)-EDTA. Furthermore, we have found that this protocol may be used in screening assays to select rootstock genotypes tolerant to Fe chlorosis, as shown from preliminary comparisons of several genotypes differing in chlorosis tolerance. The proposed new protocol could be used to assess in the future the germplasm available for chlorosis tolerance. The works made with fruit tree rootstocks before the project showed that the root ferric chelate reductase activity was not always induced by Fe deficiency, and the reason for that was unknown.

The possible use of **somaclonal variation methods** has given promising results with pear genotypes. These results are still preliminary but open a new way of finding tolerance to chlorosis in fruit trees.

The works developed within the project have provided light on several physiological changes induced by Fe deficiency in plants. The biochemical characteristics of the **root ferric chelate reductase enzyme** have been extensively studied on the level of intact roots *in vivo*, and on the level of purified plasma membranes *in vitro*. Our finding that the enzymatic characteristics depend on the measuring pH has changed the view on the root ferric chelate reductase enzyme.

A standardised **root tip test** has been developed to estimate root ferric chelate reductase activity *in vivo*. This test could be also useful in screening programs.

The biochemical characteristics of the **leaf mesophyll ferric chelate reductase enzyme** have been studied at the level of mesophyll tissue *in vivo*, intact protoplasts and purified plasma membranes *in vitro*. The *in vivo* leaf ferric chelate reductase activity has been confirmed to be light dependent. The pH dependence of the ferric chelate reductase enzyme in isolated protoplasts is different from that of isolated plasma membranes, with its optimal pH being closer to that of the apoplastic pH. Both the intrinsic decrease in ferric chelate reductase activity per protoplast surface and a possible shift in the pH of the apoplastic space could be responsible for the immobilisation of physiologically inactive Fe pools in chlorotic leaves. The knowledge on this enzyme before the project was very limited, with only one paper published on that issue.

A very important finding is that the natural complexes of citrate and malate with ferric Fe are good substrates for the ferric chelate reductase enzyme, both in mesophyll tissue and in

isolated plasma membranes. This opens new possibilities for applying these compounds to control Fe chlorosis in field conditions, as already shown in kiwifruit.

Knowledge has been gained during the project on the molecular changes in **proteins induced by Fe deficiency** in leaf and root tissue. The isolation and partial characterisation of the FC-R from spinach leaves has been carried out during the project. Other approaches such as 2-D electrophoresis of root tip proteins have led to the identification of a series of polypeptides which are candidates to play a role in the Fe-efficiency responses of plants to Fe deficiency. It is expected that these polypeptides will be characterised in the near future.

The new knowledge on the photosynthetic changes induced by Fe deficiency has provided the background data to understand one of the characteristics less studied of Fe chlorosis, the light dependence of the deficiency. The changes in PS II efficiency in Fe-deficient leaves have been characterised in studies carried out in the project.

Finally, we have demonstrated in the project that a photosynthetic parameter, the chlorophyll content estimated from **SPAD readings**, is an excellent tool to be used as an **indicator of Fe chlorosis**. This parameter, once properly calibrated for a given species, is far better than other indicators such as chlorophyll fluorescence, mineral content and visual ratings. We have used the SPAD apparatus in most experiments developed in the project, including chlorosis control experiments.

2 Description of the practical applications

1. - Chlorosis has major negative impacts in fruit yield and quality in different fruit species grown in the Mediterranean area. This emphasises the importance of controlling chlorosis in fruit tree crops in Southern Europe.

2. - Early diagnosis (prognosis) of Fe chlorosis in trees could be made at least from two mineral analysis parameters in flowers, the concentration of Fe and the K/Zn ratio.

3. - Alternatives to Fe-chelates do exist and in the future may be included among the routine practices of managing fruit trees. Agronomic practices such as applying foliar sprays with inorganic Fe sources, growing graminaceous plants fertilised with Fe in the orchard and adding new Fe-containing products to the soil could be as effective as synthetic Fe-chelates in controlling chlorosis in fruit trees.

4. - A new protocol for screening tests based on physiological characteristics associated to tolerance to Fe chlorosis in fruit trees has been developed. The proposed new protocol, which induces root ferric chelate reductase activity in Fe deficiency-tolerant fruit tree rootstocks, could be used to assess germplasm for chlorosis tolerance.

5. - The possible use of somaclonal variation methods has given promising results with pear genotypes.

6. - A standardised root tip test has been developed to estimate root ferric chelate reductase activity *in vivo*.

7. - The natural complexes of citrate and malate with ferric Fe are good substrates for the leaf ferric chelate reductase enzyme. This opens new possibilities for applying these compounds to control Fe chlorosis in field conditions.

8. - The new knowledge on the photosynthetic changes induced by Fe deficiency has provided the background data to understand one of the characteristics less studied of Fe chlorosis, the light dependence of the deficiency.

9. - A photosynthetic parameter, the chlorophyll content estimated from SPAD readings, is an excellent tool to be used as an indicator of Fe chlorosis.

3 Exploitation plan

3.1 Patents or registered designs

None.

3.2 Means of exploitation

Several activities designed to disseminate the results of the project have been or are going to be developed in the near future. This includes the following:

3.2.1. - **Project brochure**. A diptych-type brochure has been produced by the Coordinator, indicating some of the practical results found in the project. This diptych would be sent to the extension services of the different countries for dissemination.

3.2.2. - **Web Project page**. A new web page has been created (www.spicom.es/chloropage). This page is expected to contain the Project information summarised in the brochure, papers already published by Project participants and the text of the final Project Report.

3.2.3. - **Dissemination papers**. An effort will be made to publish some summary papers in farmer-oriented Journals, such as the two recently published in the "Rivista di Frutticoltura e di Ortofloricoltura" and in "Phytoma" (see numbers 24 and 31 in list **4.1** below).

3.2.4. - **Joint presentations in Symposia**. Joint presentations summarising the results of the project will be made in the next Plant Nutrition Symposia of Cairo (2000) and Hannover (2001) and in the Houston Iron Meeting (2000).

4 Information of publications

From the works developed in the project we have published research and technological papers (see list **4.1** below), and several Theses (see list **4.3** below). Recently we have submitted for publication a number of new papers (see list **4.2** below).

Since a large part of the work is still unpublished, it is expected that at least during two years there will be a continuous flow of papers being sent for publication. In this context, we shall do an effort to publish results in publications oriented to growers.

4.1 Published Research and Technical Papers

1 **Tagliavini M, Rombolà AD 1995** Nuove prospettivi puor superare la clorosi ferrica negli alberi da frutto. **Rivista di Frutticoltura** 9, 11-21.

2 **Susín S, Abadía A, González-Reyes JA, Lucena JJ, Abadía J 1996** The pH requirement for *in vivo* expression of the Fe-deficiency-induced "turbo" ferric chelate reductase. A comparison of the Fe deficiency-induced Fe reductase activities of intact plants and isolated plasma membrane fractions in sugar beet (*Beta vulgaris* L.). **Plant Physiology** 110, 111-123.

3 **Tagliavini M, Scudellari S, Marangoni B, Pelliconi F, Valli S 1997** Valutazione di metodi alternativi ai chelati per il controllo della clorosi ferrica nell'actinidia. In: **Proceedings Convegno Nazionale sulla Coltura dell'Actinidia,** Faenza, Italia, 197-202.

4 **Marangoni B, Tagliavini M, Toselli M 1997** La chlorosi ferrica del pesco: conoscenza, prevenzione e terapia. In: **Proceedings of the XXII Convegno Peschicolo,** Cesena, Italia, 108-113.

5 **Pestana M, Ferreira P, Correia PJ, David M, de Varennes A, Faria** EA. 1997 Efeito da clorose férrica em porta-enxertos de citrinos: estudo de alguns parâmetros fisiológicos. **Actas Horticultura** 18, 44-51.

6 **Nedunchezhian N, Morales F, Abadía A, Abadía J 1997** Decline in photosynthetic electron transport activity and changes in thylakoid protein pattern in field grown iron deficient peach (*Prunus persica* L.). **Plant Science** 129, 29-38.

7 **Sanz M, Belkhodja R, Toselli M, Montañés L, Abadía A, Tagliavini M, Marangoni B, Abadía J 1997** Floral analysis as a possible tool for the prognosis of iron deficiency in peach. In: **Mineral nutrition and fertilizer use for deciduous fruit crops**. **Acta Horticulturae** 448, 241-245. Val J, Montañés L, Monge E eds. ISBN 90-6605-759-9.

8 **Morales F, Grasa R, Abadía A, Abadía J 1998** The iron "chlorosis paradox" in fruit trees. **Journal of Plant Nutrition** 21, 815-825.

9 **Morales F, Abadía A, Abadía J 1998** Photosynthesis, quenching of chlorophyll fluorescence and thermal energy dissipation in iron-deficient sugar beet leaves. **Australian Journal of Plant Physiology** 25, 403-412.

10 **Rombolà AD, Tagliavini M, Scudellari D, Quartieri M, Malaguti D, Marangoni B 1998** La clorosi ferrica delle piante arboree da frutto: aspetti generali e strategie di cura. **Proceedings Technical Meeting "Novel approaches to Mineral Nutrition of Fruit Trees", Notiziario Tecnico CRPV** 54, 35-50.

11 **Bassi D, Tagliavini M, Rombolà AD, Marangoni B 1998** Il progetto di selezione di portinnesti clonali per il pero, serie Fox. **Rivista di Frutticoltura e di Ortofloricoltura** 4, 17-19.

12 **Rombolà AD, Brüggemann W, Tagliavini M, Moog PR 1998** Meccanismi biochimici di tolleranza alla clorosi ferrica in Actinidia (*A. deliciosa*). In: **Proceedings of 4th "Giornate Scientifiche della Società Orticola Italiana ".** pp. 395-396.

13 **Rombolà AD 1998** Report sul "9th International Symposium on Fe Nutrition and Interaction in Plants". **Notiziario SOI di Ortoflorofrutticoltura** 6, 201-203.

14 **Belkhodja R, Morales F, Quílez R, Abadía A, Abadía J 1998** Iron deficiency causes changes in chlorophyll fluorescence due to the reduction in the dark of the photosystem II acceptor side. **Photosynthesis Research** 56, 265-276.

15 **Belkhodja R, Morales F, Sanz M, Abadía A, Abadía J 1998** Iron deficiency in peach trees: effects on leaf chlorophyll and nutrient concentrations in flowers and leaves. **Plant and Soil** 203, 257-268.

16 **González-Vallejo EB, Susín S, Abadía A, Abadía J 1998** Changes in sugar beet leaf plasma membrane Fe(III)-chelate reductase activities mediated by Fe-deficiency, assay buffer composition, anaerobiosis and the presence of flavins. **Protoplasma** 205, 163-168.

17 **Abadía J 1998** Absorción y transporte de hierro en plantas. In: Actas del VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas. pp. XIII-XXIV. Ediciones de la Universidad Autónoma de Madrid, ISBN 84-8497-927-X.

18 **Gogorcena Y, Abadía J, Abadía A 1998** Inducción in vivo de la reductasa de patrones frutales de *Prunus persica* L. In: Actas del VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas. pp. 27-32. Ediciones de la Universidad Autónoma de Madrid, ISBN 84-8497-927-X.

19 **Morales F, Abadía A, Abadía J 1998** Mecanismos de protección frente al exceso de luz en hojas deficientes en hierro. In: Actas del VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas. pp. 101-106. Ediciones de la Universidad Autónoma de Madrid, ISBN 84-8497-927-X.

20 **López-Millán AF, Morales F, Abadía A, Abadía J 1998** Implicaciones metabólicas en la respuesta bioquímica a la deficiencia de hierro en remolacha (*Beta vulgaris* L.). In: Actas del VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas. pp. 143-148. Ediciones de la Universidad Autónoma de Madrid, ISBN 84-8497-927-X.

21 **González-Vallejo EB, Abadía A, Herbik A, Stephan U, Rémy R, Abadía J 1998** Determinación de patrones polipeptídicos de raíz de remolacha (*Beta vulgaris* L.) en condiciones de deficiencia de Fe. In: Actas del VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas. pp. 119-124. Ediciones de la Universidad Autónoma de Madrid, ISBN 84-8497-927-X.

22 **Grasa R, Morales F, Abadía A, Abadía J 1998** Contenido foliar de nutrientes en árboles de melocotonero y pérdida de los mismos por abcisión y poda. In: Actas del VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas. pp. 131-136. Ediciones de la Universidad Autónoma de Madrid, ISBN 84-8497-927-X.

23 **Pestana M, Gonçalves DA, de Varennes A, Faria, EA 1999** The recovery of citrus from iron chlorosis using different foliar applications. Effects on fruit quality. In: **Improved Crop Quality by nutrient management.** D. Anaç, D. Martin-Prével eds. pp 201-204. Kluwer Academic Publishers, Dordrecht, The Netherlands.

24 **Rombolà AD, Quartieri M, Scudellari D, Marangoni B, Abadía J, Tagliavini M 1999** Strategie di cura della clorosi ferrica in frutticoltura integrata. **Rivista di Frutticoltura e di Ortofloricoltura** 5, 59-64.

25 **González-Vallejo EB, González-Reyes JA, Abadía A, López-Millán AF, Yunta F, Lucena JJ, Abadía J 1999** Reduction of ferric chelates by leaf plasma membrane preparations from Fe-deficient and Fe-sufficient sugar beet. **Australian Journal of Plant Physiology** 26, 601-611.

36 **Abadía J, Morales F, Abadía A 1999** Photosystem II efficiency in low chlorophyll, iron-deficient leaves. **Plant and Soil** 215, 183-192.

27 **Pestana M, Correia PJ, de Varennes A, Faria EA 1999** Relações entre a composição mineral de flores de laranjeira e qualidade dos frutos. **Revista das Ciências Agrárias** 22,55-61.

28 **Igartua E, Grasa R, Sanz M, Abadía A, Abadía J 2000** Prognosis of iron chlorosis from the mineral composition of flowers in peach. **Journal of Horticultural Science and Biotechnology** 75, 111-118.

29 **Gogorcena Y, Abadía J, Abadía A 2000** Induction of in vivo root ferric chelate-reductase activity in the fruit tree rootstock *Prunus amygdalo-persica*. **Journal of Plant Nutrition** 23, 9-21.

30 **González-Vallejo EB, Morales F, Abadía A, Abadía J 2000** Iron deficiency decreases the Fe(III)-chelate reducing activity of leaf protoplasts. **Plant Physiology** 122, 337-344.

31 **Sanz M 2000** El diagnóstico nutricional en agricultura y viabilidad de programas de fertilización. **Phytoma** 114, 44-50.

32 **Morales F, Belkhodja R, Abadía A, Abadía J 2000** Photosystem II efficiency and mechanisms of energy dissipation in iron-deficient, field-grown pear trees (*Pyrus communis* L.) **Photosynthesis Research** 63, 9-21.

33 **M Tagliavini, Abadía J, Rombolà AD, Abadía A, Tsipouridis C, Marangoni B 2000** Agronomic means for overcoming Fe chlorosis in deciduous fruit plants. **Journal of Plant Nutrition** 23, in press.

34 **Rombolà AD, Brüggemann W, Tagliavini M, Marangoni B, Moog PR 2000** Iron source affects Fe reduction and re-greening of kiwifruit (*Actinidia deliciosa*) leaves. **Journal of Plant Nutrition** 23, in press.

35 **Abadía J, Tagliavini M, Grasa R, Belkhodja R, Abadía A, Sanz M, Faria EA, Tsipouridis C, Marangoni B 2000** Using the flower Fe concentration for estimating crop chlorosis status in fruit tree orchards. A summary report. **Journal of Plant Nutrition** 23, in press.

37 **Morales F, Belkhodja R, Abadía A, Abadía J 2000** Energy dissipation in iron-chlorotic, field-grown pear. **Journal of Plant Nutrition** 23, in press.

38 **Toselli M, Marangoni B and Tagliavini M 2000** Iron content in vegetative and reproductive organs of nectarine trees in calcareous soils during the development of chlorosis**. European Journal of Agronomy**, in press.

4.2 Papers Submitted

38 **Pestana M, Correia PJ, de Varennes A, Abadía J, Faria EA 2000** Effectiveness of different foliar applications to correct for iron chlorosis in citrus grown on a calcareous soil. Submitted to **Journal of Plant Nutrition**.

39 **Pestana M, Correia PJ, de Varennes A, Abadía J, Faria EA 2000** The use of floral analysis to diagnose the nutritional status of orange trees**.** Submitted to **Journal of Plant Nutrition**.

40 **Pestana M, David M, de Varennes A, Abadía J, Faria EA 2000** Responses of Newhall Orange Trees to Iron Deficiency in Hydroponics: Effects on Leaf Chlorophyll, Photosynthetic Efficiency and Root Ferric Chelate Reductase Activity. Submitted to **Journal of Plant Nutrition**.

41 **Larbi A, Morales F, López-Millán A.F., Gogorcena Y, Abadía A, Moog PR, Abadía J 2000** Reduction of Fe(III)-chelates by mesophyll leaf disks of sugar beet. Multi-component origin and effects of Fe deficiency. Submitted to **Planta**.

42 **Zouari M, Abadía A and Abadía J 2000** Iron is Required for the Induction of Root Ferric Chelate Reductase Activity in Fe-deficient Tomato. Submitted to **Journal of Plant Nutrition**.

43 **López-Millán A.F.,, Morales F, Abadía A and Abadía J 2000** Changes induced by iron deficiency in the composition of the leaf apoplastic fluid from field-grown pear (Pyrus communis L.) trees. Submitted to **Journal of Experimental Botany**.

44 **López-Millán AF, Morales F, Andaluz A, Gogorcena Y, Abadía A, de las Rivas J, Abadía J 2000** Protective mechanisms in roots of iron deficient sugar beet: changes in carbon assimilation and oxygen use. Submitted to **Plant Physiology**.

45 **López-Millán AF, Morales F, Abadía A, Abadía J 2000** Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport. Submitted to **Plant Physiology**.

46 **López-Millán AF, Morales F, Abadía A, Abadía J 2000** Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (Beta vulgaris L.) leaves. Submitted to **Physiologia Plantarum**.

4.3 Theses

1 **Grünewald, Sussanne** (1996) **Diploma Thesis** (Diplomarbeit). Eisenchelatreduktion durch Mesophyllzellen. Heinrich-Heine-Universität Düsseldorf, Germany.

2 **Zouari, Mohamed** (December 1996) **Master Thesis**. Reponses radiculaires face à la deficience en fer chez differents genotypes de tomate et de betterave. International Center for Advanced Mediterranean Studies-Instituto Agronómico Mediterráneo de Zaragoza (CIHEAM-IAMZ), Spain.

3 **Chatti, Jameleddine** (January 1997) **Master Thesis**. Quelques changements de la composition chimique de la sève du xylème sous deficience en Fe chez la tomate, le pêcher et l'amandier. International Center for Advanced Mediterranean Studies-Instituto Agronómico Mediterráneo de Zaragoza (CIHEAM-IAMZ), Spain.

4 **Valli, Stefano** (1998) **Tesi di Laurea in Scienze Agrarie (Master).** Sviluppo di metodi alternativi ai chelati per superare la clorosis ferrica nell'actinidia. Dipartimento di Coltture Arboree- Facoltà di Agraria , Università di Bologna, Italia.

5 **Fidalgo, Carolina** (February 1998) **Trabajo Fin de Carrera** (Diploma). Efecto de distintos factores en la corrección de clorosis férrica en peral. Escuela Universitaria Politécnica de Huesca. Universidad de Zaragoza, Spain.

6 **García-Laviña**, **Pilar** (February 1998) **Trabajo Fin de Carrera** (Diploma). Tratamientos foliares para la corrección de la clorosis férrica. Escuela Universitaria Politécnica de Huesca. Universidad de Zaragoza, Spain.

7 **Belkhodja, Ramzi** (July 1998) **PhD Thesis** (Tesis Doctoral). Evaluación de la tolerancia a estreses ambientales en plantas cultivadas mediante técnicas de fluorescencia de clorofila, análisis de pigmentos y contenido mineral. Universidad de Lleida, Spain.

8 **Burg, Sascha** (1998) **Diploma Thesis** (Diplomarbeit). Characterization of the reducing capacity of iron-deficient and non-deficient bean roots. Heinrich-Heine-University Düsseldorf, Germany.

9 **Folli, Federico** (1998) **Tesi di Laurea in Scienze Agrarie (Master) -** Diagnosi e prevenzione della clorosi ferrica nelle colture arboree da frutto. Dipartimento di Coltture Arboree- Facoltà di Agraria , Università di Bologna, Italia.

10 **Rombolà, Adamo Domenico** (1998) **PhD Thesis** (Tesi di Dottorato di Ricerca). Aspetti fisiologici e biochimici della clorosi ferrica in Actinidia (*A. deliciosa*). Università di Bologna, Italia.

11 **González-Vallejo, Elena B** (May 1999) **PhD Thesis** (Tesis Doctoral). Caracterización de mecanismos de adquisición de Fe en plantas superiores. Universidad de Zaragoza, Spain.

12 **Larbi, Ajmi** (June 1999) **Master Thesis**. Effet de la chlorose ferrique sur la réduction de fer par le mésophyle de feuilles de la betterave a sucre (*Beta vulgaris* L.) et du pêcher (*Prunus persica* L.). International Center for Advanced Mediterranean Studies-Instituto Agronómico Mediterráneo de Zaragoza (CIHEAM-IAMZ), Spain.

13 **López-Millán, Ana Flor** (2000) **PhD Thesis** (Tesis Doctoral). Adquisición y transporte a larga distancia de hierro en las plantas. Universidad de Zaragoza, Spain (to be presented in April 2000).

14 **Wagner, Volker** (2000) **PhD Thesis.** Isolation of plasma membrane-bound ferric chelate reductase from spinach leaves. Heinrich-Heine-University Düsseldorf, Germany (to be presented).

15 **Weyrauch, Katharina** (2000) **PhD Thesis**. Isolation of plasma membrane-bound ferric chelate reductase from iron deficient bean roots. Johann Wolfgang Goethe-University Frankfurt, Germany (to be presented).

5 Supplementary investments

Some supplementary funds were obtained from the Spanish National Research Plan at the beginning of the project to acquire related durable equipment.

6 Dissemination activities other than publications

Results of the project have been disseminated so far by means of communications to technical and scientific meetings (see list **6.1** below), formal presentations to the agroindustrial sector (see list **6.2** below) and technological offers already sent to the IRC centres (see section 7 below).

Other way of disseminating the results has been through the continuous flow of visits of farmers, agricultural companies and agricultural schools to the partner Institutes in Faro (Portugal), Zaragoza (Spain), Bologna (Italy) and Naoussa (Greece). In these visits results have been disseminated by providing reprints and preprints of the work carried out, as well as by discussing details of the work.

6.1 Communications to Technical and Scientific Meetings

1995

1 ***International Conference on Bioiron.*** *Asheville, North Carolina, USA, April 1995.*

Belkhodja R, Morales F, Quílez R, Abadía A, Abadía J The redox state of the photosystem II acceptor side in iron-deficient sugar beet (*Beta vulgaris* L.) leaves: Evidence for an incomplete plastoquinone reoxidation in the dark (Communication /Poster).

2 ***8th Congress of Algarve.*** *Faro, Algarve, Portugal, April 1995.*

Varennes de A, Faria EA, Pestana M, Quelhas dos Santos J The project “Novel approaches for the control of iron chlorosis in fruit tree crops" (Communication/Poster).

3 ***17th Congress of the Greek Horticultural Society****.*

Tsipouridis K, Ferios I, Stilianidis D Evaluation of 59 peach varieties for iron chlorosis tolerance (Communication /Poster).

4 ***XXII Convegno Peschicolo.*** *Cesena, Italia, September 1995.*

Marangoni B, Tagliavini M, Toselli M Iron chlorosis of peach trees: causes, prevention and methods of control (Communication).

1996

5 ***Third International Symposium on Mineral Nutrition of Deciduous Fruit Trees.*** *Zaragoza, Spain, July 1996.*

Sanz M, Belkhodja R, Toselli M, Montañés L, Abadía A, Tagliavini M, Marangoni B, Abadía J Floral analysis as a possible tool for the prognosis of iron deficiency in peach (Communication /Poster).

6, 7 ***Symposium of the German Botanical Society.*** *Düsseldorf, Germany, August 1996.*

Grünewald S, Brüggemann W, Moog PR Vergleichende Charakterisierung der in-vivo Eisenchelat-Reduktion durch Mesophyll unterschiedlich eisenversorgter Dicotyledonen. Poster P-4.012. page 78 (book of abstracts of the symposium).

Wagner V, Brüggemann W, Moog PR

Charakterisierung der Plasmalemma-gebundenen Eisenchelat-Reduktaseaktivitaet von Spinat (Spinacea oleracea). Poster P-13.033. page 312 (book of abstracts of the symposium).

8 ***Convegno Nazionale sulla Coltura dell'Actinidia.*** *Faenza, Italia, September 1996.*

Tagliavini M, Scudellari S, Marangoni B, Pelliconi F, Valli S. Alternatives to iron chelates for the control of Fe chlorosis in kiwifruit (Communication).

1997

9-16 ***9th International Symposium on Iron Nutrition and Interactions in Plants.*** *Hohenheim, Germany, July 1997.*

Abadía J, Tagliavini M, Abadía A, Sanz M, Tsipouridis C, Araujo-Faria E, Marangoni B Using the flower Fe concentration for estimating crop chlorosis status in fruit tree orchards. A summary report (Communication/Keynote Lecture).

Tagliavini M, Abadía J, Abadía A, Tsipouridis C, Marangoni B. Alternatives to Fe-chelates for overcoming fruit tree iron chlorosis in Mediterranean countries (Communication/Keynote Lecture).

López-Millán AF, Abadía A, Abadía J Organic acid concentrations in the apoplast of iron-sufficient and iron-deficient sugar beet (*Beta vulgaris* L.) (Communication /Poster).

Morales F, Belkhodja R, Abadía A, Abadía J Photosystem II photochemical efficiency and mechanisms of energy dissipation in the leaf of iron-deficient, field-grown pear (*Pyrus communis* L.) (Communication /Poster).

González-Vallejo EB, Abadía A, González-Reyes JA, Abadía J Characterization of the Fe(III)-chelate reductase activities of plasma membrane preparations isolated from leaves of iron-sufficient and iron-deficient sugar beet (*Beta vulgaris* L.) (Communication /Poster).

Moog PR, Grünewald S Ferric reduction by intact mesophyll cells (Communication /Poster).

Wagner V, Moog PR Biochemical characterization of plasma membrane-bound Ferric chelate reductase activity isolated from spinach leaves (Communication /Poster).

Rombolà AD, Brüggemann W, Tagliavini M, Moog PR Iron deficiency in kiwifruit (*Actinidia deliciosa*): Responses of roots and leaves (Communication /Poster).

17 ***II Congresso Iberoamericano, III Congresso Ibérico de Ciências Hortícolas.*** *Vilamoura, Portugal, March 1997.*

Pestana M, Ferreira P, Correia PJ, David M, de Varennes A, Faria EA. Efeito da clorose férrica em porta-enxertos de citrinos: estudo de alguns parâmetros fisiológicos (Communication /Poster).

1998

18 ***International Symposium on Plant Nutrition.*** *Hohenheim, Germany, February 1998.*

Abadía J, Morales F, Abadía A Photochemical efficiency in low-chlorophyll, Fe-deficient leaves (Communication/Keynote Lecture).

19 ***4th Giornate Scientifiche della Società Orticola Italiana.*** *Sanremo, 1-3 April 1998.*

Rombolà AD Meccanismi biochimici di tolleranza alla clorosi ferrica in Actinidia (*A. deliciosa*) (Oral Communication).

20 ***International Conference "Plasma membrane redox systems and their role in biological stress and disease".*** *Antwerp, Belgium, April 1998.*

González Vallejo EB, Abadía A, González-Reyes JA, Lucena JJ, Abadía J Fe(III)-chelate reductase activities of plasma membrane preparations affected by iron deficiency in sugar beet (*Beta vulgaris* L.) leaves. (Communication/Poster).

21-22 ***XIth International Congress on Photosynthesis.*** *Budapest, Hungary, August 1998.*

Morales F, Abadía A, Abadía J Photosynthetic induction in iron-deficient sugar beet leaves: a time-resolved, laser-induced chlorophyll fluorescence study (Communication/Poster).

Dauborn BE, Moog PR Iron deficiency under low light causes changes in the capacity of the antioxidative system in *Phaseolus vulgaris* L (Communication/Poster).

23 ***International Workshop on Photosynthesis under Biotic and Abiotic Stress. Stress Sinergisms in Plants.*** *Tata, Hungary, August 1998.*

Morales F, Abadía A, Abadía J Photosynthetic induction in iron-deficient sugar beet leaves: a time-resolved, laser-induced chlorophyll fluorescence study (Communication/Poster).

24-29 ***VII Simposio Nacional-III Ibérico sobre Nutrición Mineral de las Plantas.*** *Madrid, España, September 1998.*

Abadía J Absorción y transporte de hierro en plantas (Keynote speech).

Gogorcena Y, Abadía J, Abadía A Inducción in vivo de la reductasa de patrones frutales de *Prunus persica* L. (Oral Communication).

Morales F, Abadía A, Abadía J Mecanismos de protección frente al exceso de luz en hojas deficientes en hierro (Oral Communication).

López-Millán AF, Morales F, Abadía A, Abadía J Implicaciones metabólicas en la respuesta bioquímica a la deficiencia de hierro en remolacha (*Beta vulgaris* L.) (Communication/Poster).

González-Vallejo EB, Abadía A, Herbik A, Stephan U, Rémy R, Abadía J Determinación de patrones polipeptídicos de raíz de remolacha (*Beta vulgaris* L.) en condiciones de deficiencia de Fe (Communication/Poster).

Grasa R, Morales F, Abadía A, Abadía J Contenido foliar de nutrientes en árboles de melocotonero y pérdida de los mismos por abcisión y poda (Communication/Poster).

1999

30 ***10º Symposium Internacional. La sanidad de los frutales en condiciones Mediterráneas.*** *Valencia, España, November-December 1999.*

Sanz M El diagnóstico nutricional en agricultura y viabilidad de programas de fertilización (Keynote speech).

2000

31-33 ***International Symposium on the Optimization of Plant Nutrition.*** *Cairo, Egypt, April 2000.*

Abadía J, Abadía A, Faria EA, Pestana M, Tsipouridis C, Moog PR, Brüggemann W, Negueroles J, Marangoni B, Tagliavini M Results of the European Project "Novel Approaches for the Control of Iron Chlorosis in Fruit Trees (Poster).

Gogorcena Y, Abadía J, Abadía A A new protocol that can be used as a tool in screening fruit tree rootstocks for tolerance to iron chlorosis (Poster).

Pestana M, Correia PJ, de Varennes A, Abadía J, Faria EA Iron chlorosis in two *Citrus* rootstocks: effect on some physiological parameters (Poster).

34 ***10th International Symposium on Iron Nutrition and Interactions in Plants.*** *Houston, U.S.A., May 2000.*

Abadía J, López-Millán AF, Abadía A Apoplastic organic acids and their role in the availability of iron for leaf cells (Keynote speech).

35-37 ***"5th International Conference on Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease".*** *Hamburg, Germany, March 26th-29th, 2000*

Wagner V, Moog PR Isolation of Ferric Chelate Reductase from Spinach Leaves

Weyrauch K, Moog PR Isolation of Ferric Chelate Reductase from roots of Phaseolus vulgaris

Moog PR, Weyrauch K , Wagner V Characteristics of Plasma Membrane-bound Ferric Chelate Reductases isolated from Roots and Leaves. - What are the Differences ?

6.2 Other formal presentations to the agro-industrial sector

1995

1 ***Meeting with the Public field advisors of Regione Emilia Romagna.*** *Ferrara, Sala Convegni del Centro Operativo Ortofrutticolo.*

Tagliavini M "Iron chlorosis in fruit tree crops".

2 ***Joint Project Meeting in Naoussa. Dissemination Report.*** *Naoussa, Makedonia, Greece.*

Tsipouridis C "Results with practical economical interest".

1997

3 ***Fruttiflor exhibition.*** *Faenza, Italia, October 10, 1997.*

B Marangoni, Tagliavini M "Strategies for overcoming the Fe chlorosis in deciduous fruit trees".

4 ***Consorzio Provinciale Agrario di Ravenna.*** *Ravenna, Italia, November 14, 1997.*

Tagliavini M "Summary results of the iron chlorosis project".

5 ***Consorzio Provinciale Agrario di Forli Cesena-Rimini.*** *Forli Cesena-Rimini, Italia, November 28, 1997.*

Tagliavini M "Summary results of the iron chlorosis project".

6 ***Center of Educational Training (K.E.K.).*** *Kopanos, Naoussa, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

7 ***Center of Educational Training (K.E.K.).*** *Lamia, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

8 ***Center of Agricultural Education (K.E.T.E.).*** *Veria, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

9 ***Center of Educational Training (K.E.T.E.).*** *Rhodes, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

1998

10 ***Demonstration activities of C.R.P.V, Cesena.*** *Ferrara, June 1998.*

Scudellari D "Visit by field technicians of the Emilia-Romagna Region to a pear trial managed with strategies alternative to Fe chelates ".

11 ***Cooperative P.A.F. of Faenza and UNICOOP.*** *Faenza, October 6 1998*

Rombolà AD, Tagliavini M "Results of trials carried out under the chlorosis project".

12 ***Center of Educational Training (K.E.K.).*** *Kopanos, Naoussa, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

13 ***Center of Educational Training (K.E.K.).*** *Lamia, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

14 ***Center of Agricultural Education (K.E.T.E.).*** *Veria, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

15 ***Center of Educational Training (K.E.T.E).*** *Rhodes, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

39 ***Escuela Universitaria Politécnica de Huesca,*** *Huesca, Spain.*

García-Laviña, P and Fidalgo, C Lecture including results of the iron chlorosis project.

40 ***Escuela Superior de Ingenieros Agrónomos,*** *Universidad de Lleida, Spain.*

Belkhodja, R Lecture including results of the iron chlorosis project.

1999

18 ***Center of Educational Training (K.E.K.).*** *Kopanos, Naoussa, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

19 ***Center of Educational Training (K.E.K.).*** *Lamia, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

20 ***Center of Agricultural Education (K.E.T.E.).*** *Veria, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

21 ***Center of Educational Training (K.E.T.E).*** *Rhodes, Greece (2 days).*

Tsipouridis C Lecture including results of the iron chlorosis project.

22 ***I Jornadas Hydro de Fertirrigación,*** *Zaragoza, Spain.*

Sanz M Fertirrigación (Lecture including results of the iron chlorosis project).

23 ***Day of Science,*** *Faro, Portugal (24 November).*

Faria, EA Poster including results of the iron chlorosis project.

24 ***Citriculture Meeting,*** *Feira de Silves, Portugal (1-5 December).*

Faria, EA Lecture including results of the iron chlorosis project.

2000

25 ***Jornadas Técnicas sobre Fruticultura. Actualización de Técnicas de Cultivo en Frutales. Escuela Universitaria Politécnica de la Almunia de Doña Godina****. 24 February. Zaragoza, Spain.*

Sanz M Diagnóstico nutricional (Lecture including results of the iron chlorosis project).

7 Further support through technology transfer organisations

Part of the most practically oriented results of the project have been sent to the corresponding IRC centres for dissemination among member states. The technological offers disseminated so far are as follows:

7.1 Technological offers sent to IRC

Flower analysis for early diagnosis of nutritional status of fruit trees.
From Innovation Relay Centre-Zaragoza, Spain.
(Reference 250199)
Posted January, 1999

Low input treatments to control iron chlorosis.
From Innovation Relay Center- ICR IRENE, c/o ENEA Bologna, Italy.
Posted January, 1999

Effectiveness of Different Foliar Applications to Correct for Iron Chlorosis in Citrus Grown on a Calcareous Soil.
From Innovation Relay Center Faro, Portugal.
Posted January, 1999

Screening for iron chlorosis tolerance.
From Innovation Relay Centre Hessen /Rheinland –Pfalz, Germany
Posted January, 1999

8 Dissemination by the Commission services

A short information on the project could be accessed through the Cordis web page.