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Appropriate micropropagation regimes were developed for four *Pyrus* species (*P. amygdaliformis* Vill., *P. betulaefolia* Bunge, *P. calleryana* Dcne., and *P. communis* L.) and *Cydonia oblonga* L.. Shoot multiplication was optimal at 10 or 20µM N^6-benzyladenine (BA) and high light intensity (135$\mu Em^{-2}s^{-1}$). Root formation of the *Pyrus* species was stimulated by exposure of shoots to high levels (10 or 32µM) of ß-indolebutyric acid (IBA) for 7 days or a dip in 10mM IBA for 15s, followed by a passage on auxin-free medium. α-Naphthaleneacetic acid (NAA) was more effective than IBA in stimulating rooting of *C. oblonga*.

Adventitious shoots of *Cydonia oblonga* Quince A were induced from leaves cultured on MS-N6 medium containing thidiazuron (TDZ) and NAA. Optimal regeneration (78% of the cultured leaves with 3.2 shoots per leaf) occurred with 32µM TDZ plus 0.3µM NAA on young leaves obtained from micropropagated shoots. Adventitious shoots of *Pyrus amygdaliformis* and *P. communis* were obtained, but at much lower frequency.

Effects of Fe-limiting conditions *in vitro* were determined by comparing the

responses of shoots and rooted plantlets to media containing FeEDTA or $FeSO_4$, with or without bicarbonate. Symptoms of Fe deficiency were genotype-dependent and most severe in the presence of $FeSO_4$ and bicarbonate. Chlorosis was pronounced in *Cydonia*, absent in *P. amygdaliformis* and *P. communis*, and intermediate in *P. betulaefolia* and *P. calleryana*, indicating parallel responses between *in vitro* and field conditions. Similar responses were obtained with rooted and unrooted shoots.

Tolerance to Fe-deficiency chlorosis was correlated with maintenance, under Fe-stress, of a high Fe^{2+}/total Fe ratio, high Fe^{3+}-reducing activity, and medium acidification. This adaptive response was diminished by bicarbonate. Roots of plantlets, shoot bases, root cultures and cell suspension cultures all manifested Fe-stress inducible Fe^{3+}-reducing activity.

The adventitious shoot regeneration protocol was successfuly used to select somaclonal variants of *C. oblonga* with increased tolerance to Fe-deficiency chlorosis. Two variants, IE1 and IE2, were recovered which displayed higher Fe^{3+}-reducing ability and acidification of the medium than the original *C. oblonga* clone. These variants may be useful as pear rootstocks in regions with calcareous soils.

Somatic Manipulation of Pyrus and Cydonia: Characterization and Selection for Iron Efficiency

by

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This thesis is dedicated to

my wife Rosa and daughter Rosa Maria

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SOMATIC MANIPULATION OF *PYRUS* AND *CYDONIA*:
CHARACTERIZATION AND SELECTION FOR IRON EFFICIENCY

INTRODUCTION

Fe-deficiency chlorosis in pears is a wide-spread problem, particularly in regions with calcareous soils. Although high levels of Fe may be present, the occurrence of $CaCO_3$ limits the uptake, transport or use of Fe, resulting in reduced chlorophyll formation and leaf chlorosis (Marschner, 1986). There are large variations among *Pyrus* species in tolerance to Fe-deficiency chlorosis (Lombard and Westwood, 1987) and the response of the scion is generally determined by the genotype of the rootstock (Chaplin and Westwood, 1980; Römheld, 1987). *Cydonia oblonga* L. (quince) is used extensively in Europe as a dwarfing rootstock for intensive orchards of pear (Sansavini, 1990; Stebbins, 1990; Kappel, 1990). Quince is highly sensitive to low Fe availability, resulting in leaf chlorosis, a prevalent problem in pear-growing regions with calcareous soils.

The only approach thus far to prevent Fe-deficiency chlorosis in fruit trees is soil application of Fe chelates (FeEDTA, FeEDDHA) (Hamze et al., 1987; Wallace, 1989). However, this practice is expensive, contributes to contamination of underground water, and provides only temporary control (Raese and Parish, 1984; Swietlik and Faust, 1984; Hamze et al., 1985; Raese and Staiff, 1988). The development of tolerant rootstocks, a primary goal of pear breeding in regions with calcareous soils (Lombard and Westwood, 1987), would overcome the problem. Sexual hybridization between *Pyrus* and *Cydonia* has been proposed to obtain tolerant rootstocks, however, hybrid

rootstocks have not been developed (Trabut, 1916; Shimura et al., 1983).

Tissue culture techniques are used extensively for genetic modification of crop plants. Such manipulations are especially important for the improvement of tree fruit species due to the long juvenility and high heterozygozity which often complicate conventional breeding (James, 1987). Somatic hybridization between *Cydonia* and *Pyrus* species (Ochatt et al., 1989) and selection of somaclonal variants (Hammerschlang, 1990; Viseur, 1987a; Brisset et al., 1990) could provide rootstocks with improved Fe efficiency. Alternatively, *Agrobacterium*-mediated and other transformation vectors (James et al., 1989) may be used to incorporate genes promoting Fe^{3+} reduction or chelation, cloned from bacteria or plants (Wallace, 1990).

The uptake and utilization of nutrients can be studied using cultured plantlets or tissues if field conditions can be simulated *in vitro*. In this study, cultured plantlets and tissues were used to characterize Fe-deficiency responses in *Pyrus* and *Cydonia*. Tissue cultures have been useful in selection of soybean and sugarcane cell lines tolerant to Fe-deficiency (Sain and Johnson, 1984 and 1986; Stephens et al., 1990; Naik et al., 1989). However, plant regeneration from cell suspensions is difficult in these species and also in fruit tree species (James, 1987). To overcome this problem, screening for tolerance to Fe-deficiency chlorosis was performed with adventitious shoots of *C. oblonga*.

As a prerequisite for *in vitro* studies of Fe-deficiency chlorosis and selection of tolerance to low Fe availability, appropiate tissue culture protocols, including shoot multiplication, rooting of shoots, and adventitious shoot regeneration from leaves, were established for *Pyrus* and *Cydonia*. Subsequently, an *in vitro* screening system was developed to select for tolerance to Fe-deficiency chlorosis. The rootstocks which showed

large differences in tolerance to low Fe availability were characterized with regard to Fe levels, Fe^{3+} reduction, and acidification of the medium. The final objective was the selection and characterization of somaclonal variants with increased tolerance to low Fe availability.

LITERATURE REVIEW

Pear production

Pear production is centered in areas with dry and warm summers, which facilitate fruit set, improve fruit quality and reduce problems with the bacterial disease fire blight. Leading countries in pear production are China, Italy, USA, USSR and Spain. The most commonly cultivated pear varieties in Europe and North America belong to the species *Pyrus communis* L. World-wide, only a few cultivars are grown. In the United States 'Bartlett', 'Anjou' and 'Bosc' account for 95% of the production.

Many of the 22 *Pyrus* species are used as rootstocks. Seedling rootstocks are most widely used (Kappel, 1990), while clonal rootstocks have been limited mainly to selections of quince, *Cydonia oblonga* L., for cultivation in milder regions of Europe, the United States, South Africa, and Israel. More recently, clonal stocks of *P. communis* have been introduced in the United States and South Africa, and of *P. calleryana* in Australia. Pear rootstocks are clonally propagated by hardwood stem cuttings and quince rootstocks by trench or mound layerage. The desired variety is grafted on the rootstock. All *Pyrus* species are graft-compatible. Quince and pear are often graft-incompatible (Moore, 1986), but a interstem is used to overcome this problem (Westwood, 1988).

Pyrus species are self-sterile diploids (2n=2x=34). Variability among *Pyrus* species has been used to select rootstocks resistant to diseases and pests and tolerant to soil and climate conditions (Lombard and Westwood, 1987). The species *P. communis, P. betulaefolia*, and *P. calleryana* offer many useful traits as rootstocks. They are more difficult to propagate than quince, but are resistant to many pests and diseases, and are

more tolerant to cold climates and alkaline soils. Difficulties in clonal stock propagation can be overcome by application of tissue culture techniques.

C. oblonga has the same somatic chromosome number as pear. Quince is used in intensive European orchards because of its precocity, yield efficiency, fruit quality, and dwarfing capacity, resulting in reduced labor input (Sansavini, 1990; Stebbins, 1990; Kappel, 1990). 'E.M. Quince A' is a widely used clonally propagated rootstock (Tehrani, 1987; Kappel and Quamme, 1988). Most European quince breeding programs focus on tolerance to lime-induced chlorosis, winter hardiness, and graft-compatibility.

Pear tissue culture

The increase in planting density of tree fruit orchards has promoted the development of micropropagation techniques. *In vitro* propagation offers the advantage of rapid propagation of the desired cultivars and rootstocks under controlled conditions in a pest-free environment. However, except for apple and peach, the commercial use of tissue culture to propagate temperate fruit and nut crops has been limited to a few cultivars due to the high cost of production (Hammerschlag, 1986b).

Micropropagation techniques developed for apple by Quoirin (1974), Jones (1976) and Abbot and Whiteley (1976), have been used for clonal propagation of pear rootstocks and self-rooted pear cultivars. Micropropagation of a few rootstocks, including *P. communis* OHxF51 and *P. betulaefolia*, has been reported (Cheng, 1978 and 1979; Banno et al., 1988). The potential of self-rooted cultivars, which would eliminate the time and expense involved in grafting, has been supported by initial field evaluation results (Zimmerman, 1986). Micropropagation of pear varieties, *P.*

communis cvs. Conference, Doyenne du Comice, Bartlett, Seckel, Kaiser, and Beurre Bosc among others, has been reported by a number of authors (Lane, 1979; Singha, 1980; Shen and Mullins, 1984; Marino, 1984; Singha, 1982; Singha, 1984; Maarri et al., 1986b; Viseur, 1987b; Rodriguez et al., 1991). A few reports describe *in vitro* propagation of cultivars of other species, such as *P. pyrifolia* 'Nijusseiki' and 'Hosui' (Bhojwani et al., 1984; Hirabayashi et al., 1987; Banno et al., 1989) and *P. calleryana* 'Bradford' (Stimart and Harbage, 1989). Little has been published on clonal micropropagation of *Cydonia oblonga* L. rootstocks (Duron et al., 1989). Nevertheless, the technique used for *Pyrus* has been successfully applied to quince rootstocks Provence (Maarri et al., 1986a), BA29, A and C (Duron et al., 1989). Micropropagation has been used for long term storage of *Pyrus* species (Wanas et al., 1986; Reed, 1990), and mineral nutrition studies (Singha et al., 1985 and Singha et al., 1987).

The medium devised by Murashige and Skoog (1962), with small modifications, has been used most often for micropropagation of *Pyrus* species and *C. oblonga.* The medium is supplemented with N^6-benzyladenine (BA) to initiate axillary branching, and ß-indolebutyric acid (IBA) or α-naphthaleneacetic acid (NAA) to induce rooting. Shoot tips have been more amenable than meristems for establishment of cultures. Multiplication (3 to 10-fold increase every 4 weeks) and rooting rates (30 to 90%) are often genotype dependent. In cases such as *P. calleryana* 'Bradford' rooting has failed (Stimart and Harbage, 1989), while 100% of quince shoots may be rooted (Duron et al., 1989).

Other tissue culture techniques which have great potential in breeding of fruit trees have been reviewed by Singha (1986) and James (1987). These methods include adventitious regeneration for the induction of somaclonal variation, mutagenesis

followed by regeneration, ploidy manipulations, somatic hybridization, and *Agrobacterium*-mediated transformation. Since these fruit tree species are highly heterozygous, have long reproductive cycles, and are asexually propagated, the use of somatic methods is very appropriate for genetic improvement.

Leaves from micropropagated shoots are an abundant source of uniform material, and have been used for regeneration in *Malus* (James, 1987). Regeneration in *Pyrus* species was first reported by Yehia et al. (1985) and Viseur (1986) using roots and shoot internodes as explants, but the regeneration frequencies were very low. Adventitious regeneration has been improved by Chevreau et al. (1989), using leaves of *P. communis* and *P. bretschneideri* cultivars. Regeneration from leaf discs may facilitate applications of biotechnology to pear breeding, e.g. selection of somaclonal variants and genetic engineering through *Agrobacterium*-mediated transformation, as shown for apple by James et al. (1989).

Regeneration from protoplasts has been achieved for selected *Pyrus* materials (Ochatt and Caso, 1986; Ochatt and Power, 1988), and somatic hybrids between *P. communis* and *Prunus avium* x *pseudocerasus* have been obtained (Ochatt et al., 1989).

Importance of Fe in plants and consequences of Fe deficiency

Fe is a micronutrient involved in many metabolic processes, among others photosynthesis and respiration. A shortage of Fe results in decreased synthesis of Fe-containing proteins, decreased production of chlorophyll, and subsequently lower photosynthetic capacity. Chlorosis in the youngest leaves is the first visible symptom of Fe deficiency. In general, Fe deficiency occurs at Fe levels below 50µg/g dry weight

(Clark, 1983). In green plants, up to 80% of Fe is located in the chloroplasts, stored in the stroma as Fe^{3+} in ferritin (Seckbach, 1982; Smith, 1984). Fe^{3+} is covered with a protein coat (apoferritin) which allows storing Fe at concentrations otherwise toxic. The resulting ferritin also mobilizes Fe^{3+}, and prevents Fe from being used by pathogens (Seckbach, 1982). Under Fe deficiency the stromal Fe content decreases and lamellar Fe increases (Terry and Low, 1982). Apparently, Fe^{3+} stored in ferritin is rapidly used for lamellar components involved in photosynthesis.

Fe is also stored in roots (Bienfait et al., 1985; Chaney, 1989). Root Fe pools are mobilized when Fe deficiency occurs (Chaney, 1989). In general, total Fe content is a poor indicator of the Fe-nutritional status of the plant and is not well correlated with the chlorophyll content of leaves (Tong et al., 1986; Mengel and Geurtzen, 1988; Rashid et al., 1990; Lang et al., 1990). Precipitation of Fe^{3+} in the leaf apoplast due to alkalinization, caused by nitrate nutrition or alkaline soils, decreases Fe uptake into mesophyll cells and subsequently induces chlorosis, while at the same time total Fe in the leaf could be relatively high (Mengel and Geurtzen, 1988). Fe^{2+}, the physiologically available form of Fe in the cell, is better correlated with the chlorophyll content, and seems to be a suitable indicator for critical Fe deficiency levels (Katyal and Sharma, 1980; Hamze et al., 1985; Häussling et al., 1985; Marschner, 1986). Other factors involved in the degree of Fe-deficiency chlorosis are: Fe uptake, translocation, distribution, and dilution by leaf growth (Häussling et al., 1985; Hamze et al., 1987; White and Robson, 1990).

Green plants deprived of Fe become deficient in the new leaves, while older leaves remain green, because Fe in plants is not retranslocated and a continuous supply of Fe is necessary. Fe is usually available in the soil as Fe^{3+}. Fe absorption into the root

vascular tissues requires prior reduction to Fe^{2+} at the root surface of all plants except grasses (Chaney et al., 1972; Römheld and Marschner, 1983). In the xylem the predominant forms of Fe are Fe^{3+}-citrate, -malate and -phytosiderophores (Brown and Ambler, 1974; Marschner, 1986; Chaney, 1989). Transfer cells in the shoot may regulate the selective transport of Fe^{3+} to the Fe-stressed leaves where its reduction may occur prior to physiological utilization (Landsberg, 1984).

Numerous enzymes and redox proteins, vital to plant metabolic processes including photosynthesis, respiration and nitrogen fixation, contain Fe. The major metabolic function of Fe is based on its reversible oxidation-reduction capability. These redox systems include the hemoproteins, and the Fe-S or nonheme Fe proteins (Miller et al., 1984).

The most important hemoproteins are the cytochromes, which are one-electron carriers with Fe-porphyrin as prosthetic group. These are present in the photosystem II of chloroplasts, and in the mitochondrial respiratory chain. Cytochromes are also part of cytochrome oxidase which reduces oxygen to water in the last step of the mitochondrial electron transport chain.

Nitrogen fixation in the nodules of plants infected with *Rhizobium* is possible because of anaerobic conditions maintained by leghemoglobin (Tang et al., 1990). This hemoprotein, synthesized by the plant, is abundant in the nodules and binds O_2.

Other hemoproteins are catalase and peroxidase. The first facilitates H_2O_2 dismutation to H_2O and O_2, and the second leads to H_2O formation and oxidation of organic hydrogen donor. Both enzymes, together with superoxide dismutase, are essential in protection of cells against reactive oxygen species. Active oxygen species such as superoxide can affect the photosynthetic apparatus. Fe-deficiency chlorosis is associated

with higher superoxide formation and superoxide dismutase activity (Ostrovskaya et al., 1990). The catalase and peroxidase activities decrease with Fe deficiency and are suitable indicators of the Fe-nutritional status (Bar-Akiva et al., 1978; Bar-Akiva, 1984). Reduced peroxidase activity in Fe-deficient plants is associated with the deteriorating suberization of the rhizodermal cell wall in the roots and accumulation of phenolics in the rhizosphere (Sijmons et al., 1985). Certain phenolics, such as caffeic acid, chelate and reduce inorganic Fe^{3+}. With a decrease in peroxidase, which oxidizes NADH in the process of polymerization of aromatic domains of suberin, NADH accumulates at the plasma membrane. As a consequence, the Fe^{3+} reducing capacity of the membrane increases.

The most important Fe-S protein is ferredoxin (Smith, 1984; Marschner, 1986). This serves as a redox protein in Photosystem I, nitrate reduction and N_2 reduction. Ferredoxin is the final recipient of electrons from the photosynthetic light reactions. Most of the reduced ferredoxin is used to reduce $NADP^+$ in the chloroplasts. Nitrate reduction to ammonia is facilitated by nitrate and nitrite reductases. The nitrate reductase enzyme complex contains a cytochrome, and nitrite reductase contains an Fe-S protein. Ferredoxin provides electrons to both steps. The nitrogenase system, mediating N_2 reduction to ammonia in selected organisms, contains Fe-S proteins which receive electrons from ferredoxin. Other Fe-S proteins, electron carriers with ferredoxin as final recipient, are found in both photosystem I and II. Finally, nonheme Fe-S proteins are found in the three multiprotein reductase complexes of the mitochondrial respiratory chain.

The tricarboxilic acid cycle enzyme aconitase which catalyzes the isomerization of citrate to isocitrate in the mitochondria, contains an Fe-S center. Inhibition of this

enzyme by Fe deficiency causes accumulation of organic acids such as malic and citric acids in the roots (Bienfait, 1988a). Elevated levels of organic acids are most likely the source of enhanced H^+ efflux in Fe-deficient dicotyledoneous plants. The inhibition of aconitase in Fe-deficient apple seedlings is responsible for succinic, malic and citric acid accumulation in the whole plant (Sun et al., 1987). Another Fe containing enzyme is xanthine oxidase, an enzyme present in the cytoplasm and required In adenine and guanine degradation. In Fe-deficient plants purine metabolism is impaired and as a consequence riboflavin accumulates in the roots (Marshner, 1986).

Since most of the Fe is located in the chloroplasts and Fe is involved in chlorophyll biosynthesis, the most apparent symptom of Fe deficiency is interveinal chlorosis and necrosis in young leaves. Fe is first required in the formation of ∂-aminolevulinic acid (ALA), precursor of heme and chlorophylls (Miller et al., 1984). Fe is also present in the enzyme coproporphyrinogen oxidase which mediates the biosynthesis of chlorophyllides and chlorophylls from Mg-protoporphyrin IX. Under Fe deficiency, biosynthesis of structural proteins of the grana, ferredoxin and other photosystem I components decrease. This results in reduced chloroplast volume, amount of protein per chloroplast, chlorophyll content and photosynthesis per unit leaf area. In chlorotic apple seedlings, chloroplasts are small, the grana does not develop, and photosynthesis of affected leaves decreases (Zhou et al., 1984b; Sun et al., 1987). Light and electron microscope examinations have established that Fe deficiency leads to disorganization of the chloroplast structure. It inhibits grana stack formation, induces parallel aggregations of lamellae, and increases number of lipid globuli. The bundle sheath chloroplasts of C_4 plants, which do not contain grana, have swollen thylacoids under Fe deficiency (Hecht-buchholtz, 1983; Marschner, 1986).

Lime-induced chlorosis

Nutrition is one of the most effective ways to improve productivity in fruit trees. Fruit tree nutrition affects not only the vegetative parts but also fruit production and quality. As a consequence of breeding for big fruit, nutritional deficiencies affecting fruit quality and storability have developed. The functions, requirements, and effects of macronutients and micronutrients in growth of fruit trees, flower bud initiation and development, and fruit quality and storability are discussed in Marschner (1986) and Faust (1989).

Lime-induced or bicarbonate-induced chlorosis are terms often used for plant chlorosis associated with impaired Fe availability in calcareous soils. More than 25% of the world land surface, predominantly in semi-arid and arid climates, is calcareous (Vose, 1982). These soils may contain 20% to 95% free CaCO3 in the upper horizon, with pH buffered to 7.5-8.5. Plant species vary greatly in their susceptibility to Fe deficiency, and within species there are varietal differences (Olsen and Brown, 1980; Hamze et al., 1987; White and Robson, 1990). Crops greatly affected include soybean, sorghum, dryland rice, peanut, grapevine, citrus, peach, plums, cherry, pear and apple (Vose, 1982; Vose 1983; Chen and Barak, 1982). Fruit trees are affected when grown in these soils with high moisture conditions. The most sensitive deciduous fruit trees are peaches and pears (Faust, 1989). Variability in field tolerance to lime-induced chlorosis is found among *Pyrus* species which may be ranked from sensitive to tolerant (1 to 5) as follows: *Pyrus betulaefolia* (2), *P. calleryana* (2), *P. communis* (3), *P. amygdaliformis* (5). Quince is more susceptible to lime-induced chlorosis than any

Pyrus species (Lombard and Westwood, 1987).

Since mineral nutrient uptake depends on soil factors and the rootstock, chlorosis symptoms are generally determined by the rootstock. In a study performed with 'Williams' as the scion cultivar, in a slightly acid soil in western Oregon, *P. amygdaliformis* and *P. elaeagrifolia* had higher Fe uptake than *C. oblonga* and *P. calleryana* (Chaplin and Westwood, 1980). In alkaline soils the differences might have been even more pronounced. Fe chlorosis also varies among *P. communis* varieties grafted on the same rootstock (Faust, 1989). However, studies involving reciprocal grafts of Fe-efficient and inefficient tomato (Brown et al., 1971) and chickpea cultivars (Römheld, 1987) show that, independent of the scion, the rootstock controls Fe acquisition, amount of chlorophyll, and growth.

Discrepancy between Fe solubility and plant demand for Fe is the primary cause of Fe-deficiency chlorosis in calcareous soils. The estimated concentration of chelated Fe (FeEDTA) required in the soil solution for optimal plant growth is 10^{-6} to 10^{-5}M (Lindsay and Schwab, 1982). Although high levels of Fe may be present in soils (Vose, 1982), Fe concentrations in the soil solutions of well-aerated soils with pH between 7 and 9 may be less than 10^{-10}M (Lindsay, 1974). Soluble Fe in calcareous soils occurs mainly in the chelated form. Major chelating compounds such as humic acids, organic acids and phenolics are derived from organic matter, microorganisms, and root exudates (Lindsay, 1989). Hydroxamate siderophores, produced by microorganisms, are major Fe^{3+} chelating compounds at high pH (Powell et al., 1982). Plants can utilize Fe from these siderophores, in some cases with more efficiency than from synthetic chelates (Cline et al., 1984; Reid et al., 1984; Jurkevitch et al., 1988). In alkaline soils with high organic matter the concentration of chelated Fe in the soil solution can be 10^{-4} to

10^{-3}M. Chelates increase uptake of Fe by increasing the concentration gradient of diffusible Fe (O'Connor et al., 1971; Mashhady and Rowell, 1978). Unfortunately calcareous soils are frequently low in organic matter.

Although elevated phosphorus levels and/or poor aeration may be involved, the bicarbonate ion (HCO_3^-) which forms in calcareous soils is the most important soil factor associated with lime-induced chlorosis in plants (Mengel et al., 1984, Marschner, 1986; White and Robson, 1990), including pears grafted on quince (Boxma, 1972). In liquid cultures, apple seedlings had aggravated chlorosis when the pH of the nutrient solution or the bicarbonate concentration was increased (Zhou et al., 1984a, 1984b, and 1985). Only in wet calcareous soils are bicarbonate concentrations high enough to cause major problems in Fe uptake. Bicarbonate formation by $CaCO_3$ hydrolisis depends on the CO_2 pressure and the presence of H_2O (Korcak, 1987).

The mechanism by which high bicarbonate induces chlorosis is poorly understood. Marshner (1986) lists several possible means by which bicarbonate may affect Fe utilization. Fe uptake may be impaired by a buffered pH of the soil solution resulting in inhibition of root H^+ efflux, inhibition of Fe^{3+} reduction at the plasma membrane (Römheld et al., 1982; Römheld and Marschner, 1983), and inhibition of phenolics release (Dofing et al., 1989). Transport of Fe to the shoot and expanding leaves may be reduced by sequestration of Fe in vacuoles by organic acids, which accumulate with Fe stress (Lee and Woolhouse, 1969). Unavailability of Fe for chlorophyll formation in the leaves and uneven distribution of Fe within the leaf tissue can be the result of alkalinization of the leaf apoplast and cytoplasm (Kolesch et al., 1984; Mengel and Geurtzen, 1988; Mengel, 1989). Bicarbonate inhibition of root growth may result in decreased cytokinin transport to the leaves and chlorophyll formation (Marschner,

1986). Few data are avaliable to determine which of these would be the most probable cause. Fleming et al. (1984) indicate that bicarbonate exerts a greater effect on Fe-efficiency responses than Fe translocation to the shoot. The fact that chlorosis is better correlated with Fe^{2+} levels than total Fe in leaves may indicate the importance of bicarbonate inhibition of Fe reduction (Abadia et al., 1984; Kathyal et al., 1980; DeKock et al., 1979).

Correction of Fe deficiency in trees has been attempted in several ways: application of manure, which icreases organic matter, Fe solubility and uptake (Mathers et al., 1980; Lindsay, 1989), trunk injection or direct soil application of Fe salts (Raese and Parish, 1984; Horesh et al., 1986). The most efficient approach has been soil application of Fe chelates (FeEDTA, FeEDDHA) (Hamze et al., 1987). A high chelator-to-Fe ratio in apple seedlings reduces chlorosis (Tong et al., 1986). A useful way to control lime-induced chlorosis in fruit trees is to correct soil drainage and continuously provide Fe-EDDHA by drip irrigation (Wallace, 1989). Foliar and soil Fe chelate applications are temporary measures (Raese and Parish, 1984; Swietlik and Faust, 1984; Hamze et al., 1985; Raese and Staiff, 1988). In annual crops such as soybean, seed treatment with FeEDDHA has been economically useful (Karkosh et al., 1988). Bacterial siderophores applied to calcareous soils may also serve as a remedy for lime-induced chlorosis (Jurkevitch et al., 1988; Szaniszlo et al., 1989; Bar-Ness et al., 1989). The most efficient alternative to greatly alleviate this problem is the development of tolerant rootstocks. These would reduce costs and underground water contamination. But among all fruit tree species, only peach-almond hybrids have been developed as tolerant peach rootstocks.

Plant adaptive mechanisms for Fe acquisition

When the Fe concentration within the plant decreases to below a critical level, Fe acquisition is enhanced by the induction of adaptive mechanisms, which are depressed when the Fe requirement is met (Römheld, 1987). Adaptive mechanisms enhance Fe availability mainly by improving Fe solubitity in the rhizosphere. Based on studies of more than a hundred plant species, plants have been grouped in two strategies depending on the adaptive mechanisms used for Fe acquisition. All dicotyledons and most monocotyledons belong to Strategy I, while monocotyledons in the order of Poales posses the characteristics of Strategy II (Römheld and Marschner, 1986b; Marschner et al., 1986; Römheld, 1987; Bienfait, 1988a; Wallace, 1990).

Strategy I: dicotyledons and most monocotyledons

Strategy I plants respond to Fe stress with biochemical and morphological root changes. All plants within this group improve Fe acquisition primarily by inducing an Fe^{3+}-reducing activity at the root surface, H^+ release by an ATPase-dependent H^+-pump, and release of phenolics with reducing and chelating properties. These physiological processes can be further promoted by an increase in the number and length of root hairs.

In Strategy I plants, Fe absorption across the plasma membrane requires prior reduction to Fe^{2+} (Chaney et al., 1972; Romheld and Marschner, 1983). Thus, enhanced Fe^{3+} reduction results in an increased Fe^{2+} uptake. Reduction of Fe^{3+} chelates, by electron transfer from the cytosol, was first hypothesized by Chaney et al. (1972). Bienfait (1985 and 1988a) proposed the presence of two plasma membrane-bound Fe^{3+}

reducing systems: a basic reductase, also called "constitutive" or "standard", and an inducible reductase, called "adaptive" or "turbo". However, it can not be excluded that the inducible reductase is a result of a change in the constitutive reductase, e.g., the disappearance of an Fe-containing electron carrier, leading to an increased availability of electrons to Fe^{3+} chelates (Bienfait, 1988a). Because no clear evidence in support of this has been reported, this review will distinguish between the two reductases.

The basic Fe^{3+}-reductase, seems to be constitutively expressed in all plant cells, regardless of the Fe status of the plant, and reduces ferricyanide but not Fe^{3+} chelates. This reductase is also present in Strategy II plant species. The transfer of electrons from the cytosol to ferricyanide was first found in carrot cells (Craig and Crane, 1981), and subsequently in tobacco callus (Barr et al., 1984) and roots of maize, bean, oats and tomato (Bienfait, 1987).

The adaptive Fe^{3+} reductase, responsible for high Fe^{3+}-reduction rates in Fe-deficient Strategy I plant species occurs in the epidermis of young roots. Evidence for this plasma membrane-bound Fe^{3+} reductase was found by Bienfait et al. (1982), Barrett-Lennard et al. (1983) and Römheld and Marschner (1983). This "turbo" reductase has low specificity to substrate, reducing ferricyanide, a wide variety of ferric chelates, such as EDTA and EDDHA, and ferrated phytosiderophores (Römheld and Marschner, 1990). Ferric hydroxide or ferrated microbial siderophores are poorly accessible (Römheld, 1987; Bar-Ness et al., 1989). Both Fe and Mn are mobilized by the adaptive reductase in response to Fe deficiency. This can lead to Mn toxicity in plants growing in calcareous soils with low Fe levels (Moraghan, 1979).

NADPH was found to be the electron donor to the adaptive Fe^{3+} reductase (Sijmons et al., 1984a), although Schmidt et al. (1990) indicated that NADPH may not be a

cofactor of the reductase but a NADPH/NADH electron shuttle may provide electrons for the reaction. Studies using plasma membrane vesicles from soybean (Sandelius et al., 1986), tomato (Buckhout et al., 1989), and *Plantago lanceolata* (Schmidt et al., 1990) have given direct evidence that the adaptive Fe^{3+} reductase is a membrane-bound NADH dependent reductase, enhanced in Fe-deficient Strategy I plant species (Buckhout et al., 1989). The NADH oxidizing part of the Fe^{3+} reductase may be inside the plasma membrane and an electron transport chain transfers the electrons to external Fe^{3+}-EDTA (Schmidt et al., 1990).

Another Fe-reducing system, operating in the cell wall, has recently been proposed (Tipton and Thowsen, 1985). In cell-wall preparations from soybean roots Fe^{3+} reduction occurred with L-malate secreted from the Fe-deficient roots as the source of electrons. However, this reducing activity appears to be less dependent on the Fe-nutritional status of the plant and may be of little significance to the overall Fe^{3+} reduction activity of intact roots (Bienfait, 1987; Römheld, 1987).

Associated with the enhanced Fe^{3+}-reducing activity in Strategy I plant species under Fe deficiency is ATPase-driven H^+-pumping, which lowers the pH in the apoplast and rhizosphere (Römheld and Marschner, 1981b; Römheld et al., 1984). Acidification results in increased solubilization of inorganic Fe^{3+} compounds in the apoplast and rhizosphere, resulting in enhanced Fe uptake (Bienfait et al., 1985). Organic acids, e.g. citrate and malate, in roots and shoots of Fe-deficient plants constitute one source of H^+ (Vos et al., 1986). As part of the Fe-efficiency response of Strategy I plant species, citrate and malate accumulation is stimulated during H^+ extrusion. The acids couple to

K^+ or NH_4^+ ions taken up in exchange for the excreted H^+ (Landsberg, 1981). Although Strategy II plant species accumulate malate and citrate and excrete H^+, they do not acidify the rhizosphere because of their tendency to excrete OH^- during ion uptake (Landsberg, 1981; Bienfait, 1987; Mengel and Geurtzen, 1988).

Another adaptive response by Strategy I plant species is the excretion of reducing compounds by roots. Chaney et al. (1972) showed that compounds capable of reducing Fe^{3+} chelates were excreted from the roots. High levels of phenolics and organic acids, such as citric and malic acids, were found in roots of Fe-deficient plants (Brown and Ambler, 1973; Olsen and Brown, 1980; Olsen et al., 1981; Landsberg, 1981; Romheld and Marschner, 1981a, 1981b and 1983). Excreted phenolics and organic acids can help mobilize inorganic Fe^{3+} compounds by acidification, chelation, and reduction. Chelation of Fe^{3+} facilitates diffusion and reduction at the plasma membrane (Bienfait, 1988a). Reduction of sparingly soluble Fe^{3+} complexes facilitates Fe diffusion as Fe^{2+} (Kojima and Bates, 1981). However, excretion of organic compounds can not explain the high Fe^{3+} reducing capacity of Fe-deficient roots (Römheld and Marschner, 1983; Barrett-Lennard et al., 1983; Lang et al., 1990). Organic compounds may have other functions, for instance, they may lead to proliferation of microorganisms which decrease the concentration of oxygen and increased Fe^{2+} concentration when Fe^{3+} is used as electron acceptor (Smith, 1984).

Under Fe deficiency Strategy I plant species show morphological changes including root shortening, swelling of apical root zones, increased root hair formation, and appearance of transfer cells in the rhizodermis of apical root zones. A region of a few mm just behind the root tip is responsible for Fe^{3+}-reduction, but under Fe stress this

region is extended to several cm long. In many plant species, the formation of rhizodermal transfer cells coincides in location and time with the activation of Fe^{3+}-reduction and H^+-pumping around the apical root zones. Transfer cells have protuberances in the cell wall and plasmalemma, providing vigorous transport of solutes. These cells have high metabolic activity and their cytoplasm contains numerous large mitochondria. These cells are important in solute phloem loading and are also present in salt glands and nitrogen fixing root nodules (Brown and Ambler, 1974; Kramer et al., 1980; Romheld and Marschner, 1981a; Landsberg, 1982; Hecht-Buchholz, 1983; Römheld and Kramer, 1983; Landsberg, 1984; Bienfait, 1985).

Considering the adaptive mechanisms of Strategy I plant species individually, i.e. Fe^{3+} reductase, H^+-pumping and the release of phenolics and organic acids, they have limited significance for Fe acquisition in high pH buffered calcareous soils (Römheld, 1987). However, when considered together, these mechanisms may explain Fe-efficiency in Strategy I plant species. The cooperative action of the Fe^{3+} reductase and the H^+-pump has been explained by their simultaneous activation and spatial coincidence in the rhizodermal cell layer and root hairs of the apical root zones, where Fe uptake is also higher (Römheld and Marschner, 1981b; Marschner et al., 1982; Römheld and Marschner, 1984; Hagemeyer and Waisel, 1989). Fe^{3+} reduction is increased 10 fold by a decrease of pH from 7.5 to 5.5 (Römheld and Marschner, 1983). Release of phenolics and organic acids, and reduction by the cell wall-localized reductase, which has a higher optimal pH range (7.5-8.0) (Tipton and Thowsen, 1985), may play a major role in Fe mobilization from calcareous soils (Römheld, 1987).

The Fe-nutritional status of the plant regulates the activity of the adaptive Fe^{3+}

reductase and H^+-pumping, and consequently Fe uptake, maintaining growth and chlorophyll formation. When the plant recovers from Fe deficiency, the adaptive mechanisms drop to normal levels within 1 day (Chaney et al., 1972; Römheld and Marschner, 1981a and 1981b; Bienfait, 1985). Interestingly, although the Fe^{3+}-reductase activity is regulated by the Fe-nutritional status of the plant, the Fe^{2+}-transport protein transports any Fe^{2+} generated by the plasmalemma Fe^{3+}-reductase regardless of the Fe-nutritional status of the plant (Grusak et al., 1990). The Fe^{2+}-transport protein saturates in the range of 10^{-5} to 10^{-4}M Fe^{2+}, which is higher than the concentration resulting from reduction of the total Fe in the soil solution of well aerated soils (10^{-8} to 10^{-6}M, Römheld and Marschner, 1986b).

The Fe^{2+}-level of the leaf may be translated in a signal which induces the Fe-efficiency reactions and Fe translocation in the roots. Several mechanisms have been postulated: phloem Fe changes the Fe-status of the root (Bienfait, 1988a); phloem sugars may change H^+-pumping and Fe^{3+}-reductase activity (Marschner, 1986; Bienfait, 1988a); and auxin transport to roots could stimulate H^+-pumping and cause morphological changes (Olsen and Brown, 1980; Römheld and Marschner, 1981a; Landsberg, 1981, 1982, and 1984). Application of indoleacetic acid (IAA) to leaves of chlorotic maize stimulated the H^+-pumping in the plasmalemma of mesophyll cells, resulting in Fe dissolution in the apoplast and Fe uptake by cells (Mengel and Geurtzen, 1988). Reduced growth and chlorophyll content of callus cultures were attributed to low auxin concentrations and subsequent low H^+-pumping (Stephens et al., 1990). However, activation of auxin transport to roots of Fe-deficient plants has not been reported.

The Fe-nutritional status of the plant may also regulate Fe translocation to xylem. When Fe^{2+} is taken up by roots of Fe-sufficient plants, it could be stored as Fe^{3+}-citrate in the roots. When Fe deficiency occurs, cytoplasmic Fe^{3+}-citrate is reduced and pumped into the xylem. Fe^{3+}-complexes are then transported to the leaf apoplast where Fe^{3+}-reduction occurs, and subsequently Fe^{2+} crosses the plasma-membrane (Mengel, 1989; Chaney 1989).

Alternatively, roots may control the development of all visible and measurable Fe-efficiency reactions on their own, without the need for a signal from the shoot (Bienfait et al., 1987 and 1988a). It is possible that the enhanced activity of the adaptive Fe^{3+}-reductase in Fe-deficient roots is not only regulated by the amount of enzyme in the membranes but also by an increased $NAD(P)H/NAD(P)^+$ internal ratio (Sijmons et al, 1984a; Bienfait, 1985; Sandelius et al., 1986; Schmidt et al., 1990). NADPH may accumulate due to inhibition of Fe-containing enzymes, which need large amounts of NADPH (Schmidt et al., 1990). Since the levels of aconitase (an Fe-S protein required for the isomerization of citrate) are not affected by Fe deficiency, Bienfait (1987 and 1988a) proposed that accumulated citrate and malate drive the reduction of $NADP^+$ by isocitrate dehydrogenase. Consequently, roots of Fe-deficient plants increase their capacity to reduce Fe^{3+}-chelates by accumulation of malate and citrate (Vos et al., 1986; Bienfait, 1988a). The accumulation of citrate occurs also in roots and leaves of apple seedlings, indicating whole plant involvement in the increase of reducing power (Sun et al., 1987). Furthermore, the increased activity of the adaptive Fe^{3+}-reduction is the same for cultured cells as for Fe-deficient roots, indicating that the mechanism may be controlled at the cellular level (Cornett and Johnson, 1989).

Sijmons et al. (1984b) and Marre et al. (1988) proposed that the increased H^+-ATPase activity under Fe stress could be regulated indirectly by the activitation of the adaptive Fe^{3+}-reductase in the plasma membrane. Fe^{3+}-reductase transfers $2e^-$ across the plasma membrane to reduce Fe^{3+} and $1H^+$ in the cytoplasm. As a consequence of H^+ accumulation in the cytoplasm, H^+-ATPase activity increases. Recently, the involvement of the cytoplasmic Fe^{2+} concentration in the regulation of the adaptive H^+-ATPase and Fe^{3+}-reductase has been proposed (Zocchi and Cocucci, 1990). A low Fe^{2+} cytoplasmic concentration in cucumber roots and plasma membrane vesicles increases the activity of the H^+-ATPase and Fe^{3+}-reductase, resulting in an enhanced Fe uptake capacity. When the cells are Fe^{2+}-sufficient both the H^+-ATPase and Fe^{3+}-reductase activities are repressed. Activation of H^+-ATPase acidifies the external medium and creates a highly negative transmembrane electrical potential. This polarized membrane potential inhibits H^+-ATPase activity when the cell is Fe^{2+}-sufficient, while in Fe^{2+}-stressed cells the H^+-ATPase activity is released from such a repression. In support of this regulatory mechanism, the synthesis of a polypeptide, a component or activator of the H^+ ATPase activity, is induced by Fe^{2+} and other divalent metal ions in Fe-deficient roots (Bienfait et al., 1989).

Differences in genetic control are found between species, and cultivars used to evaluate resistance to Fe-deficiency chlorosis. Fe inefficiency in tomato mutant T3228fer (Brown et al., 1971) is inherited as a Mendelian recessive allele (*fer*) (Wann and Hills, 1973). A single dominant gene controls tolerance to lime-induced chlorosis in chickpea (Hamze et al., 1987; Saxena et al., 1990) and in soybean (Weiss,

1943). More recently, research on soybean (Prohaska and Fehr, 1981; Cianzio and Fehr, 1982) has shown that resistance to lime-induced chlorosis is not controlled by a single gene but by genes with additive effects. In bean Coyne et al. (1982) found complete dominance for resistance, and they suggest that 2 genes are involved. Analyses of only one of the responses to Fe stress, acidification capacity of roots, indicate that this trait was determined by 2 genes (Alcantara et al., 1990).

Research performed on an Fe-inefficient tomato mutant T3228fer (Brown et al., 1971) indicates that all Fe-efficiency reactions are controlled by one mechanism. This mutant does not develop any of the Fe-efficiency reactions (Brown and Ambler, 1974). The gene apparently controls all Fe-efficiency responses such as Fe^{3+}-reduction and formation of rhizodermal transfer cells, although it does not affect the formation of other transfer cells or other transmembrane electron transport chains in the plant (Bienfait, 1987). The gene (FER) probably codes for the primary branching control point responsible for the translation of the cell Fe^{2+}-status into Fe-efficiency responses (Bienfait, 1988a). The postulated Fe^{2+}-sensor may work as the fur protein in the aerobactin Fe assimilation system of *E. coli* (Bagg and Neilands, 1987; Crosa, 1989) with the difference that FER protein is most likely a transcriptional activating factor of the operon responsible for Fe-efficiency responses. When the concentration of Fe^{2+} in the cell is sufficient, Fe^{2+} may bind FER protein and block transcription of the operon. Two plasma membrane proteins, synthesized under the control of the FER gene, have been identified in Fe-deficient roots (Bienfait, 1988b).

A pea mutant, E107, controlled by a monogenic recessive gene, has been described (Welch and LaRue, 1990). Under normal Fe conditions this mutant releases H^+ and Fe^{3+} reductants, and takes up Fe in larger amounts than the normal parent, resulting in high

Fe concentrations in leaves (Welch and LaRue, 1990).

Although few studies have been performed to characterize fruit trees, some reports indicate they belong to the Strategy I group of plants. Fe-deficient apple and peach seedlings responded with higher root Fe^{3+} reduction rates than Fe-sufficient plants (Tong et al., 1985; Romera et al., 1989). Although Tong et al. (1985) found that enhanced Fe^{3+}-reduction by Fe-stressed roots is not due to exudates, others indicate that organic acids, e.g. succinic, malic, and citric decrease the pH and increase reduction of Fe^{3+}EDTA (Sun et al., 1987). Released organic acids may enhance Fe^{3+} reduction by chelating Fe and transporting it to the rhizodermal cells (Tong et al., 1985; Faust, 1989). As with other Strategy I plant species, $CaCO_3$ or high pH decrease the ability of fruit trees to lower the pH (Sun et al., 1987) and reduce Fe^{3+} (Tong et al., 1985).

Strategy II: monocotyledons of the order Poales

The system used by the Poaceae (Gramineae) to alleviate Fe stress has only been known for a few years. Previously, they were considered "Fe inefficient", because increased Fe reduction of the roots and acidification of the rhizosphere were not observed in these plants (Olsen and Brown, 1980; Kramer, 1983; Longnecker and Welch, 1986). In contrast, graminaceous species are generally less susceptible to lime-induced chlorosis than dicotyledoneous. Takagi (1976) found that root washings of oat and rice contain Fe-solubilizing substances. Roots of barley were found to secrete far more Fe-chelating substances than other gramineous plants. It is known now that grasses have a constitutive plasma membrane-bound reductase, similar to Strategy I plant species, and two inducible mechanisms: an inducible release of Fe-mobilizing nonproteinogenic

amino acids, phytosiderophores (PS), and an Fe^{3+}PS-membrane transport system. Both activities are located in apical root zones (Römheld, 1987; Nomoto et al., 1987).

Excretion of PS and uptake of Fe^{3+}PS are regulated by the Fe-nutritional status of the plant. The release of PS is enhanced under Fe deficiency by a factor of up to 20. Although PS are degraded rapidly by microbes, concentrations of up to 1µM have been found in the rhizosphere of barley plants grown in calcareous soils (Römheld and Marschner, 1990). The pH of the nutrient solution has little effect on the release of PS. Within 1 day after Fe resupply to Fe-deficient plants, the release of PS is depressed.

PS are strong chelators of Fe^{3+} (e.g. ferric hydroxide), even at high pH, but not of Fe^{2+}. PS of plant species and cultivars differ in their chemical nature and Fe solubilizing activities (Sugiura et al., 1985). Fe reduction is not required in the solubilization of Fe by PS. Various PS are as effective as the microbial siderophore ferrioxamine B and better than some synthetic chelates (e.g. EDTA, DTPA) in mobilizing Fe^{3+} from a calcareous soils. However, PS are not selective and also mobilize Zn^{2+}, Mn^{2+} and Cu^{2+}. Some of the PS have been identified, such as mugineic acid, avenic acid, and distichonic acid A. These PS are closely related to nicotianamine, which is an Fe^{2+} carrier between and within plant cells. In response to Fe deficiency grasses accumulate nicotianamine in their roots transform nicotianamine into PS, which are released in the rhizosphere and chelate Fe^{3+}. The biosynthesis of nicotianamine and PS, and its regulation by Fe is unknown (Sugiura et al., 1981; Powell et al., 1982; Fushiya et al., 1982; Mino et al., 1983; Benes et al., 1983; Takagi et al., 1984; Nomoto et al., 1987; Stephan and Scholz, 1990).

The Fe^{3+}PS-transport system is selective and may be Fe-regulated, as in

microorganisms (Bagg and Neilands, 1987; Crosa, 1989). $Fe^{3+}PS$ are transported and taken up into the root cells, without previous reduction, in the same way as hydroxamate siderophores in microorganisms. Different genetic control mechanisms may exist for excretion of PS and uptake of $Fe^{3+}PS$. The uptake rates of various $Fe^{3+}PS$ are similar in a given plant species, and different between species. Under Fe stress up to 5 times more $Fe^{3+}PS$ are transported into the roots, but synthetic chelates (e.g. FeEDDHA) or microbial siderophores (e.g. ferrioxamin B) are used sparsely. Concentrations as low as 10^{-8} to 10^{-9}M $Fe^{3+}PS$ are sufficient for optimal growth of barley and sorghum in nutrient solutions. These values are much lower than the reported values of 10^{-6} to 10^{-5}M FeEDTA required for optimal plant growth (Lindsay and Schwab, 1982; Marschner et al., 1986; Römheld and Marschner, 1986a; Nomoto et al., 1987; Römheld, 1987; Nomoto et al., 1987; Awad et al., 1988; Römheld, 1989; Takagi, 1989; Römheld and Marschner, 1990).

It has been postulated (Nomoto et al., 1987; Takagi 1989) that the process of Fe acquisition by grasses involves the synthesis and storage of PS in the apical root zones. Regulated by the Fe-nutritional status of the plant, stored PS is secreted through a transport system controlled by a circadian clock, and the ferrated PS is taken up into the root cells by an active transport system (Römheld, 1987). Inside the root cell the complex is reduced to Fe^{2+}-PS, followed by release of Fe^{2+}. Subsequently the free PS is re-used for repeated Fe chelation outside the root. Support for this mechanism has been found by supplying ^{59}Fe-[^{14}C]PS to barley, which results in uptake of the ferrated PS across the membrane and release of Fe^{2+}. Subsequently, free PS become available for repeated Fe chelation outside the root (Römheld, 1987). The reduction potential of the

mugineic acid-Fe^{3+} complex indicates that it is reducible by physiologically available reductants such as NADPH (Nomoto et al., 1987). Microbial ferrated siderophores are cleaved by a plasmalemma transport system in oat root cells, but only the Fe^{2+} is transported into the cell (Szaniszlo et al., 1989), which indicates the specificity of the membrane transport system.

There is initial evidence that the mechanism responsible for secretion of PS in barley is operative in suspension cultures and thus also controlled at the cellular level (Kishi-Nishizawa et al., 1989). Furthermore, plasma membrane vesicles from the roots of Fe-stressed barley possess the specific PS transport activity, and at the same time Fe-stress induced polypeptides are found (Mihashi et al., 1989).

The constitutive NADH-dependent Fe^{3+}-EDTA reductase has been biochemically characterized in plasma membranes of barley roots (Brüggemann and Moog, 1989). This trans-membrane electron transport is due to one single enzyme, which may or may not be identical to the NADH-dependent ferricyanide or nitrate reductase (Omholt and Boyer, 1988; Brüggemann and Moog, 1989).

Selection for tolerance to Fe stress

Because Fe deficiency is a problem for many plants grown on calcareous soils, attempts have been made to screen for higher efficiency (Clark, 1983; Hecht-Buchholtz, 1983).

Within each strategy there are quantitative differences among species and cultivars for Fe-efficiency and resistance to lime-induced chlorosis, related to the expression of the adaptive mechanisms (Olsen and Brown, 1980; Hamze et al., 1987; White and

Robson, 1990). Characterization of differences in the responses to Fe deficiency is important for development of screening methods for adaptation to calcareous soils. Strategy I plants should be selected for the capacity of their roots to reduce Fe^{3+} chelates and to take up Fe. Recent research, using soybean, and peach and grapevine rootstocks in nutrient solutions, has shown a correlation between tolerance to Fe-deficiency chlorosis and capacity to reduce Fe^{3+} and lower the medium pH (Römheld and Marschner, 1984; Römheld, 1987; Romera et al., 1989; Bavaresco et al., 1989; Lang et al., 1990). No correlation was found between tolerance of soybean to Fe-deficiency chlorosis and Fe reduction by reducing compounds released by the roots (Dofing et al., 1989). Strategy II plants should be selected for their rate of Fe^{3+} mobilization by PS released by the roots. Tolerance of strategy II plants to Fe deficiency was found to be correlated with secretion rate of PS (Nomoto et al., 1987) or solubilization of Fe^{3+} by PS (Römheld, 1987).

Differences between genotypes in their capacity to reduce Fe^{3+} or mobilize Fe^{3+} by PS indicate the genetic potential for selection and breeding of Fe-efficient cultivars. Among annual crop species, cultivars with improved tolerance to lime-induced chlorosis have been released for soybean (Fehr and Bahrenfus, 1980; Froehlich and Fehr, 1981; Prohaska and Fehr, 1981; Fehr et al., 1984; Jessen et al., 1988), sorghum, dry bean, and oat (Cianzio, 1989). Screening available germplasm for tolerance to Fe-deficiency chlorosis has been performed for chickpea (Saxena and Sheldrake, 1980; Hamze et al., 1987; Saxena et al., 1990), peanut (Hartzook et al., 1974), pepper, and clover (Cianzio, 1989).

Conventional screening for resistance to Fe-deficiency chlorosis involves field evaluation in calcareous soils. The development of a nutrient solution system (Coulombe

et al., 1984) has simplified the evaluation of soybean genotypes for Fe-deficiency chlorosis (Jessen et al., 1986) by reducing the number of genotypes to be tested in the field. Nutrient solutions are often used, containing different Fe, phosphate, and bicarbonate levels, nitrogen sources and pH. Responses in nutrient solutions are usually, but not always, comparable to those in calcareous soils. Addition of excess $CaCO_3$ to nutrient solutions improves the correlation with results in calcareous soils (Hamze et al., 1987). Tests of peach rootstocks in nutrient solutions give a good correlation with field chlorosis ratings (Romera et al., 1989; Rashid et al., 1990). However, nutrient solution evaluation is expensive and the number of plants that can be evaluated is limited.

Evaluation of Fe efficiency has also been attempted by measuring cell suspension growth (Sain and Johnson, 1986) and callus growth in soybean (Stephens et al., 1990). Screening *in vitro* can help to eliminate Fe-inefficient genotypes before field evaluation, and select single plants with high Fe-efficiency. Soybean and sugarcane cell lines with continuous growth under Fe-limiting conditions have been selected with the objective of regenerating plantlets with high Fe-efficiency in the field (Sain and Johnson, 1986; Naik et al., 1989). However, regeneration from cell suspensions is difficult for many species, including fruit trees.

In fruit trees, selection of rootstocks with high tolerance to lime-induced chlorosis has increased the adaptability of peach (Syrgiannidis, 1985) and citrus (Wutscher et al., 1970; Hamze and Nimah, 1982) to calcareous soils. Several approaches may improve tolerance to lime-induced chlorosis of pear rootstocks. The use of *P. communis* clones as rootstocks could improve tolerance to Fe stress considerably. Intensive orchards require the use of dwarfing rootstocks, such as *C. oblonga* clones. Selection of

dwarf *P. communis* rootstocks, such as Brossier's selections at Angers or particular OH x F clones, is being pursued (Lombard and Westwood, 1987). The use of selfrooted *P. communis* L. varieties may reduce lime-induced chlorosis, althought the plants obtained may be too vigorous and irregular.

Interspecific hybrids have been made between peach and almond for use as peach rootstocks (Syrgiannidis, 1985; Rashid et al., 1990). These hybrids exhibit consistently higher Fe efficiency than peach seedlings, both grafted with peach. Sexual hybrids between *Pyrus* and *Cydonia* have been obtained (Trabut, 1916; Shimura et al., 1983), but horticultural evaluation of the intergeneric hybrids as rootstocks has not been reported. *P. amygdaliformis* or *P. elaeagrifolia* would be good sources of tolerance to lime-induced chlorosis to be used for interspecific crosses (Lombard and Westwood, 1987).

Somatic hybridization between *Cydonia* and *Pyrus* species could provide an alternative source of rootstocks, possibly dwarf with better graft-compatibility, and with tolerance to lime-induced chlorosis (James, 1987).

MATERIALS AND METHODS

Plant materials

The genotypes used in this study were *Pyrus betulaefolia* Bunge OPR (Oregon Pear Rootstock)-266, *P. communis* L. cvs. Seckel and Anjou, *P. communis* L. OPR-1 and Angers Selection P-2462, *P. calleryana* Dcne. OPR-191, *P. amygdaliformis* Vill. NCGR (National Clonal Germplasm Repositiory) Pear Acc. No. 633 and *Cydonia oblonga* L. East Malling Quince A. They were selected based on their importance as rootstocks or cultivars, and their differential responses to Fe-limiting conditions (Lombard and Westwood, 1987). Vernalized shoots (1400h below 7°C) of 'Seckel', OPR-266 and Quince A were obtained in February from 10-year-old trees and kept in water at room temperature until new shoots of about 5cm were formed. Shoots of the other genotypes were obtained in April from screenhouse-grown trees.

Establishment and multiplication of shoot cultures

The micropropagation sequence for *Pyrus* and *Cydonia* is schematically presented in Figure 1. Shoot explants with leaves removed were surface-sterilized in ethanol (70%) for 1min, followed by sodium hypochlorite (0.5%) + Tween-20 (1 drop/100ml) for 10min, and rinsed three times with sterile distilled water. The explants were cut to 2-3cm and cultured in tubes (15 x 2.5cm) containing 20ml MS-M medium supplemented with 5µM N^6-benzyladenine (BA), added before autoclaving. MS-M medium consisted of mineral nutrients devised by Murashige and Skoog (1962),

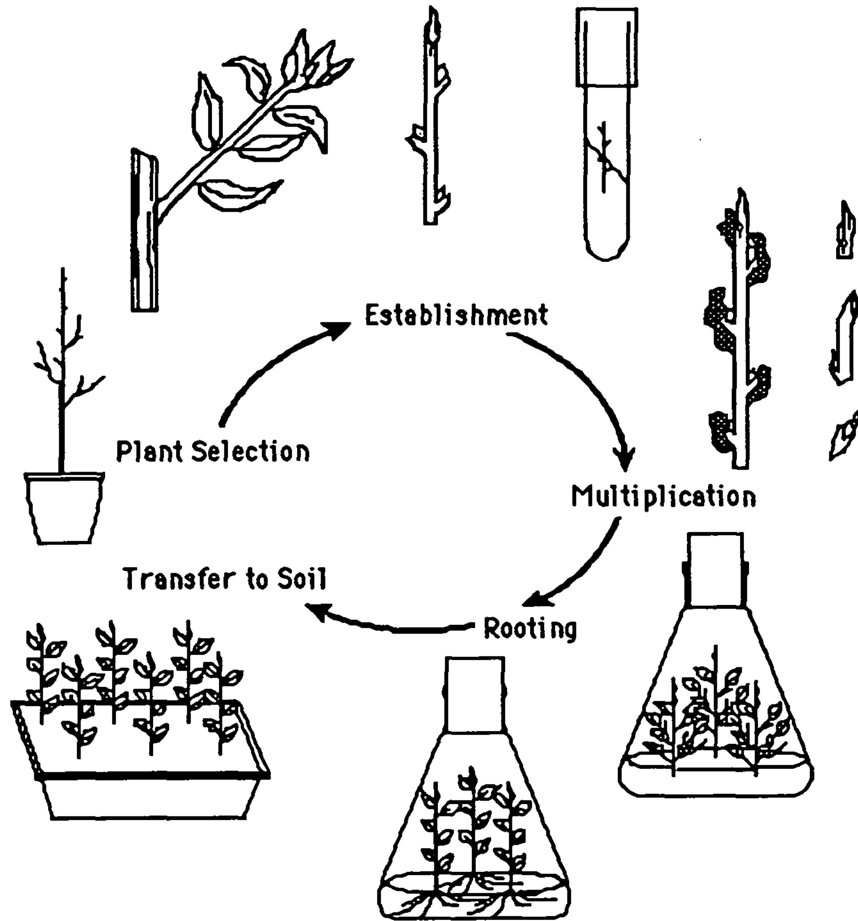


Figure 1. Micropropagation scheme for *Pyrus* and *Cydonia*

sucrose (30g/l), *myo*-inositol (100mg/l), thiamine-HCl (1mg/l), nicotinic acid (1mg/l), pyridoxine-HCl (1mg/l) and Difco Bacto agar (6g/l). The pH was adjusted to 5.7 before autoclaving. Cultures were kept at a temperature of 25°C under a photoperiod of 16h of cool-white fluorescent light (40µmol $m^{-2}s^{-1}$).

After 14 days, shoots were subdivided into sections bearing one or two growing buds. Three sections were planted per Erlenmeyer flask with 50ml of MS-M medium supplemented with 5µM BA. For shoot multiplication, segments of 2cm were transferred monthly using medium of the same composition.

To test whether further modifications of this procedure could increase shoot proliferation in *P. communis*, *P. calleryana* and *P. amygdaliformis*, the effects of shoot position (horizontal vs. vertical), BA concentration (0, 2.5, 5, 10, 20µM), and light intensity (13, 40 and 135µmol $m^{-2}s^{-1}$) were determined in two experiments each with nine shoots per treatment.

Rooting of micropropagated shoots

Appropriate rooting regimes were first established for *P. betulaefolia*. The choice of this species was based on our earlier observations that published rooting conditions for other *Pyrus* species (Cheng, 1978; Lane,1979; Maarri et al., 1986; Shen and Mullins, 1984; Singha, 1980; Singha, 1986) were not effective for *P. betulaefolia*. The experiments were conducted using micropropagated shoots in the third through fifth subculture. The medium used for rooting (MS-R) was similar to MS-M medium but contained one-half of the NH_4NO_3 and KNO_3 concentrations. Growth regulators used for rooting experiments were dissolved in dimethylsulfoxide (DMSO) (Schmitz and Skoog,

1970) and added to the medium after autoclaving (25µl DMSO per 50ml medium).

In the first experiment (third subculture) the effects of various concentrations (0, 0.3, 1, 3.2, 10 and 32µM) of α-naphthaleneacetic acid (NAA) and ß-indolebutyric acid (IBA) were tested. Shoots were grown on auxin-containing medium for 28 days and then on auxin-free medium for 28 days. In the second experiment (fourth subculture) the effects of the duration (7, 14 and 28 days) of IBA treatment (0, 0.3, 1, 3.2, 10 and 32µM) were examined. In the third experiment (fifth subculture) shoots were exposed to 10µM IBA for 7 days and then to auxin-free medium for 28 days with the following variations:

- pre-treatment of shoots in the dark at 9°C for 7 days before transfer to IBA-containing medium (Monette, 1987);
- keeping shoot cultures in the dark during exposure to IBA (Hirabayashi et al., 1987; Marino, 1984 ; Zimmerman, 1984);
- addition of 0.01mM riboflavin to medium in both passages (with and without IBA) (Duron et al.,1989);
- etiolation for 7 weeks before root induction (Zimmerman, 1984).

The optimal conditions for rooting of *P. betulaefolia* (as established above) and a number of published methods (Table 1) were applied to the four *Pyrus* species and *Cydonia* in the sixth and seventh passage. In addition, the effects of a 15sec dip in a 10mM IBA (in 50% methanol) were tested.

Each experiment consisted of 12 replicate flasks with three shoots each per treatment. The percentage of rooted shoots, number of roots per rooted shoot, average root length per rooted shoot, and callus growth (quantified by the largest area in mm^2) were recorded. The treatment effects were analysed by one or two way analyses of

variance, Fisher's Protected LSD multiple comparison of means and regression analyses to fit a polynomial response model to the increasing levels of IBA (Petersen, 1985). IBA concentrations were converted to the Log_{10} scale to linearize the sigmoid response.

Table 1. Rooting regimes used for *Pyrus* species and *C. oblonga*.

Treatment	Passage 1 + Auxin			Passage 2 - Auxin		
Number	Type	Conc (µM)	Time (wks)	Time (wks)	Reference	Species
1	NAA	5	1	4	Maarri et al., 1986a	*C. oblonga*
2	NAA	10	4	0	Lane, 1979	*P. communis*
					Singha, 1980	
					Shen and Mullins, 1984	
3	IBA	1	1 *	4	Hirabayashi et al., 1987	*P. communis*
					Zimmerman, 1984	*M. domestica*
4	IBA	10	1	4	-	
5	IBA	32	1	4	-	
6	IBA	10mM	15sec	4	-	
7	-	-	-	4	-	

* In the dark. The rest of the treatments at a 16h photoperiod (40 µmol $m^{-2}s^{-1}$)

Adventitious shoot regeneration from leaves

Leaves were obtained from three-week-old micropropagated shoots of *C. oblonga* and *P. amygdaliformis*. In the initial experiment, leaves were dissected into four transverse sections. For the remaining experiments, transverse incisions, 2mm apart, were made on the leaves, leaving the sections together at one margin. Three leaves were placed, with the abaxial side down, in petri dishes containing 25ml MS-N6 medium. MS-N6 medium consisted of a combination of MS (Murashige and Skoog, 1962) and N6 (Chu, 1978) minerals (with N, P, K and S from N6), sucrose (30g/l), *myo*-inositol

(100mg/l), thiamine-HCl (1mg/l), nicotinic acid (1mg/l), pyridoxine-HCl (1mg/l) and Difco Bacto agar (6g/l). The pH was adjusted to 5.7 before autoclaving. The growth regulators BA, N-phenyl-N[1]-1,2,3-thiadiazol-5-ylurea (thidiazuron or TDZ), and NAA were dissolved in DMSO and added to MS-N6 medium after autoclaving.

In the initial experiment to determine optimal growth regulator concentrations, leaves of *C. oblonga* and *P. amygdaliformis* were cultured on medium with BA (1 to 100μM) and TDZ (0.001 to 10μM) in combination with NAA (0.1 to 3μM), with 24 leaves per treatment. Based on the results of that experiment, TDZ concentrations of 1 to 100μM and NAA concentrations of 0.3, 1 and 3μM were chosen for *C. oblonga*. The same TDZ concentrations but NAA concentrations of 0.01 to 3μM were chosen for *P. amygdaliformis*. Four replicate experiments were performed, each with 12 leaves per treatment. In subsequent experiments, casein hydrolysate (100mg/l), yeast extract (100mg/l), malt extract (100mg/l), coconut water (100ml/l), glycine (2mg/l), folic acid (1mg/l), biotin (1mg/l), pantothenic acid (1mg/l), putrescine (161mg/l), and higher sucrose concentration (60g/l) were tested for *C. oblonga*. Culturing leaves with the abaxial part up or in continuous dark conditions were also tested. Finally, the optimal medium was used to determine if higher regeneration frequencies could be attained by careful selection of *C. oblonga* leaves (as explants). Two replicate tests were conducted, each with 50 uniform young leaves.

All cultures were kept at 25°C in the dark for the first three weeks, and at a 16h photoperiod (40μmol $m^{-2}s^{-1}$) for the following three weeks. Leaf sections with adventitious shoots were transferred to multiplication medium (MS-M) containing 5μM BA (Lane, 1979; Dolcet-Sanjuan, 1990).

To enhance genetic variation in *C. oblonga*, 200 leaves were dissected, placed on

regeneration medium, and irradiated using a 93 Curie 60-Cobalt Irradiator. They were exposed to 2 Krads and subsequently moved to fresh regeneration medium. Regeneration was compared with a control sample of 50 leaves.

To determine the anatomy of organogenic tissue, leaf samples were taken after the three-week dark period and fixed in FAA (2% formalin, 5% acetic acid and 60% ethanol). Tissue sections (10μm) were stained with 0.25% toluidine blue.

Rapid screening for tolerance to Fe-deficiency chlorosis

Micropropagated shoots (2cm in length) and rooted plantlets were cultured on MS-M medium with 5μM BA and MS-R medium respectively with the following modifications:

1. + FeEDTA, pH 5.7
2. + FeEDTA + $KHCO_3$ (1mM), pH 6.3
3. + $FeSO_4 \cdot 7H_2O$, pH 5.7
4. + $FeSO_4 \cdot 7H_2O$ + $KHCO_3$ (1mM), pH 6.3

The Fe concentrations were 0.1mM, the same as in MS-M medium. Ten flasks with three shoots each were used per treatment. The experiment was repeated twice. After 28 days the chlorophyll content of the leaves was determined.

Systems used for characterization of Fe-deficiency responses

To characterize Fe efficiency, micropropagated shoots, plantlets, roots, and cell suspensions were incubated in liquid medium with the following variations:

1. + Fe, pH 5.7

2. + Fe + $KHCO_3$ (1mM), pH 6.3

3. - Fe, pH 5.7

4. - Fe + $KHCO_3$ (1mM), pH 6.3

Fe was supplied as Fe^{3+}EDDHA (0.1mM). FeEDDHA was used instead of FeEDTA, because at pH higher than 6.0 EDDHA forms a more stable chelate with Fe^{3+} (Halvorson and Linsay, 1972; Lindsay, 1974; Orphanos and Hadjiloucas, 1984). Liquid conditioning medium was used to prevent any interference from agar. Conditions differed slightly with the type of culture as described below.

Shoots

Shoots (2cm) were obtained from four-week-old cultures and placed in culture tubes (15 x 2.5cm) containing 5ml MS-M medium amended as described above and supplemented with 5µM BA. Shoots were kept at 25°C under a photoperiod of 16h of cool white fluorescent light (40µmol $m^{-2}s^{-1}$).

Plantlets

Roots of micropropagated plantlets were rinsed in sterile distilled water. Plantlets were cultured in flasks (8 x 4cm) containing 15ml liquid MS-R medium amended as described above. Plantlets were kept at 25°C under a photoperiod of 16h of cool white fluorescent light (40µmol $m^{-2}s^{-1}$).

Root cultures

Roots were removed from micropropagated plantlets, rinsed in sterile distilled

water, and placed in 125ml Erlenmeyer flasks with 40ml MS-R medium amended as described above. Root cultures were placed on an orbital shaker (60rpm) at 27°C in the dark.

Cell suspensions

Leaves were obtained from micropropagated shoots, cut into transverse strips, and placed in petri dishes (3 leaves per petri dish) containing 25ml MS-N6 medium with 5μM kinetin and 10μM 2,4-dichlorophenoxyacetic acid (2,4-D). After a culture period of four weeks in the dark at 27°C, callus which had formed at the cut edges was transferred to fresh medium. Cell suspensions were established by transfer of three-week-old callus to 125ml Erlenmeyer flasks with 40ml liquid MS-M medium containing 1μM kinetin and 5μM 2,4-D. Cultures were incubated on an orbital shaker (120rpm) at 27°C in the dark.

Cells were collected from pooled two-week-old cell suspensions by centrifugation (1400g for 10min) and washed by suspension in conditioning medium followed by centrifugation (1400g for 10min). The conditioning medium contained the ingredients of the cell suspension medium, with the amendments described above. Equal volumes (5ml packed volume) were suspended in 125ml Erlenmeyer flasks with 40ml conditioning medium. Cultures were placed on an orbital shaker (120rpm) at 27°C in the dark.

A completely randomized experiment, with five repetitions per conditioning treatment and assay, was repeated twice for *P. amygdaliformis* and *C. oblonga* cultures. Shoots, plantlets and roots were conditioned for 10 and 20 days, and cell suspensions for

4, 12 and 20 days. All assays were performed for shoots and plantlets. For root cultures and cell suspensions, Fe reduction in the tissue, location of Fe reduction sites and medium acidification were determined.

Assays measuring responses to Fe stress

Chlorophyll content

The chlorophyll content of leaves was determined using the procedures described by Moran (1982). Briefly, each of the upper three leaves of the main shoot was cut into six pieces, weighed and extracted by incubation for 24h in 15ml N,N-dimethylformamide (DMF) at 4°C in the dark. The absorbance of the extracts was determined using 1cm quartz cuvettes and a Beckman model 34 spectrophotometer at 647 and 664.5 nm. The amount of chlorophyll (µg/ml) was determined by the equation 17.90 A_{647} + 8.08 $A_{664.5}$ (Inskeep and Bloom, 1985). The chlorophyll content of the leaves was expressed as µg per mg fresh weight of leaf tissue.

Growth rate and total Fe content

Before conditioning, shoots and plantlets were cut, rinsed with double distilled water, blotted dry, and weighed. After conditioning, they were blotted dry and weighed. For Fe content determination, the part of the shoot or plantlet which had been in contact with conditioning medium was removed, and the rest of the tissue was weighed, dried for 2 days at 70°C, ashed at 500°C for 6 h, and dissolved in 5% HNO_3 overnight. Total Fe was determined by atomic absorption spectrophotometry and expressed as µg of total Fe per gram of fresh weight.

Reduced Fe content in the tissue

The Fe^{2+} content in tissues was determined with 2,2'-bipyridyl (Bipy) as described by Abadia et al. (1984). The part of the shoot or plantlet which had been in contact with conditioning medium was removed, and the rest of the tissue rinsed with double distilled water, blotted dry, and cut into 1 to 3mm pieces. Tissues were weighed and 50-100mg tissue was incubated per glass tube (10 x 1.5cm) with 10ml Bipy (83mM), pH 3, on an orbital shaker (100rpm) in the dark for 24h. After filtration through Whatman No. 1 filter paper, the absorption of extracts at 522nm was determined. $Fe^{2+}SO_4 \cdot 7H_2O$, reduced by treatment with hydroxylamine-HCl, was used to prepare a standard curve. Fe^{2+} content was expressed as µg per gram fresh weight.

Fe reduction in tissues

The Fe^{3+} reduction capacity of shoots, plantlets, roots and cell suspensions was determined spectrophotometrically with the ferrous color reagent BPDS (bathophenanthrolinedisulfonic acid, sodium salt). This is a chemical chelator which forms a red colored complex with Fe^{2+} ($[Fe^{2+}(BPDS)_3]^{4-}$) but not with Fe^{3+}. The molar absorption of the red $FeBPDS_3$ is 22140 M^{-1} cm^{-1} at 535nm (Kojima and Bates, 1981).

Because Co^{2+}, Mn^{2+}, Zn^{2+} or Cu^{2+} interfere with Fe^{3+} reduction, they were omitted from the liquid assay medium (Römheld and Marschner, 1983). For all types of cultures the assay medium contained 0.1mM $Fe^{3+}EDTA$ and 0.3mM BPDS (Marschner et al., 1982), and the pH was adjusted to 6.0 before filtersterilization. Fe reduction was

always measured after 48h, calculated from the absorption at 535nm after subtraction of the absorption of the blank, which had no tissue, and expressed as nmoles of Fe^{2+} per gram fresh weight over 24h or per ml of packed cells over 24h. Conditions differed slightly with the type of culture, as described below.

Shoots were rinsed in sterile distilled water and placed with their bases in vials (5.5 x 1.5cm) containing 7ml MS-M medium amended as described above. The vials were wrapped with aluminium foil to prevent photoreduction of Fe, and placed inside tubes (15 x 2.5cm) to maintain humidity (Figure 2). Shoots were kept at 25°C under a photoperiod of 16h of cool white fluorescent light (40µmol $m^{-2}s^{-1}$). The part of the shoot in contact with the medium was weighed and Fe^{3+} reduction was determined.

Plantlets were rinsed in sterile distilled water and placed with their roots in vials (8 x 4cm) with 60ml MS-R medium amended as described above. The vials were wrapped with aluminium foil and placed inside a flask (12 x 6cm) (Figure 2). Plantlets were kept at 25°C under a photoperiod of 16h of cool white fluorescent light (40µmol m $^{2}s^{-1}$). Roots were weighed and Fe^{3+} reduction was determined.

Roots were rinsed in sterile distilled water and placed in a Erlenmeyer flask with 40ml MS-R medium amended as described above. Cultures were placed on an orbital shaker (60rpm) at 27°C in the dark. Roots were weighed and Fe^{3+} reduction was calculated.

Cells were collected from 5ml cell suspension by centrifugation (1400rpm for 5min), washed, and resuspended in 10ml of assay medium. The assay medium contained the ingredients of the cell suspension culture medium, with the amendments described above. Cultures were kept in tubes (10 x 1.5cm) placed on an orbital shaker (120rpm) at 27°C in the dark. Packed volume of cells was measured by centrifugation (1400rpm

for 5min) and Fe^{3+} reduction calculated.

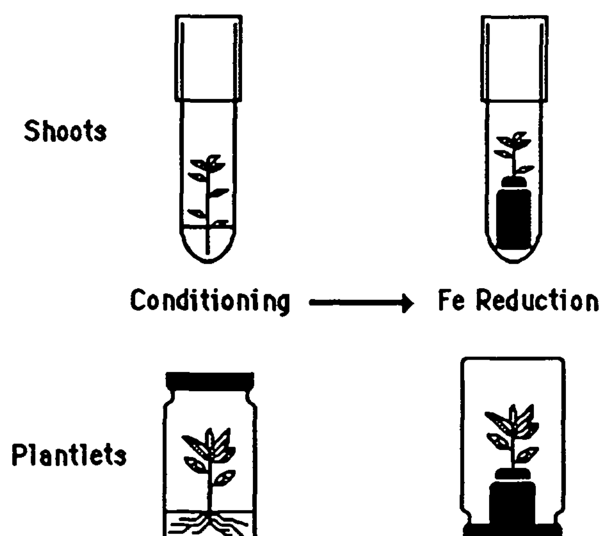


Figure 2. Shoot and plantlet manipulation for the assay measuring Fe reduction in tissues

Fe reduction by exudates

Fe^{3+} reducing activity of exudates from shoots or plantlets was determined using the ferrous chemical chelator PDTS (ferrozine or [3-[-pyridyl]-5,6-bis[4-phenyl-sulfonic acid]-1,2,4, triazine]) (Mozafar, 1989).

Shoots or plantlets were rinsed in sterile distilled water and cultured as in the previous assay. MS-M medium was used for shoots and MS-R medium for plantlets, but Fe, Co, Mn, Zn or Cu were omitted and the pH adjusted to 6.0. After 48h, the parts of the shoots in contact with the medium were weighed and 5ml (shoots) or 20ml (plantlets) medium were taken. After addition of 1ml of 5mg/l $Fe_2(SO_4)_3 \cdot 5H_2O$ and 1ml of 2g/l PDTS per 20ml solution, the pH was adjusted to 5 with Na-acetate buffer (pH 13), and the final volume adjusted to 25ml (Mozafar, 1989). The solution was kept in the dark at

25°C for 90min. Fe^{3+} reduction was calculated from the absorption at 562nm after subtracting the absorption of a blank, without shoot or plantlet, and expressed as nmoles Fe^{2+} released per gram of fresh weight over 24h.

Location of Fe reduction sites

A Prussian blue staining procedure was used to locate the sites of rapid Fe^{3+} reduction (Bell et al., 1988). The liquid assay medium contained 100µM ferricyanide ($K_3Fe[CN]_6$) and 100µM $FeNH_4(SO_4)_2$ as sources of Fe^{3+}. When reduction of either of the Fe^{3+} sources occurs, the insoluble Prussian blue stain forms and adheres to the tissue.

Shoots, plantlets, roots and cell suspensions were incubated under the conditions for the assay measuring Fe reduction in tissues. The medium was also the same except for omission of Fe and BPDS, and the pH was 3.0. After 5 to 10h, sites of Fe^{3+} reduction were observed under the microscope.

Proton extrusion

The medium pH was determined after conditioning. In addition, the agar embedding technique with bromocresol purple described by Marschner et al. (1982) and Römheld et al. (1984) was used to locate proton extrusion. Shoots and plantlets were rinsed with distilled water (pH 7.0), and placed on petri dishes with 50ml MS-M medium (pH 7.0) containing 0.3% agar. Bromocresol purple indicator (0.006% w/v) was added. This indicator is purple at pH 7.0, red at pH 6.0, and yellow at pH 4.5. Shoots and plantlets were kept illuminated and humid during the assay. Changes in the pH were observed after

1h.

Fe chelation by phytosiderophores

The indicator used to measure excretion of phytosiderophores, by shoots or plantlets, is a blue complex, composed of chrome azurol S (CAS), Fe^{3+}, and hexadecyltrimethyl ammonium bromide (HDTMA), which has an extinction coefficient of 10^5 M^{-1} cm^{-1} at 630nm and pH 5.6 (Schwyn and Neilands, 1987). When siderophores are present, the Fe^{3+} from CAS indicator is bound to the ligand, resulting in a color change to orange. To avoid interference with EDDHA, only shoots or plantlets conditioned in medium without FeEDDHA and $KHCO_3$ were used in this assay.

Shoots and plantlets were rinsed in distilled water and moved to liquid MS-M (shoots) or MS-R (plantlets) medium without Fe^{3+}EDTA. Conditions were the same as for the assay measuring Fe reduction by exudates. After five days, 5ml nutrient solution was mixed with 5ml CAS assay solution. The presence of phytosiderophores in the exudates was determined from the absorption at 630nm after subtracting the absorption of a blank, without shoot or plantlet.

Preparation of Fe chelates

To prepare a stock solution of 10mM Fe^{3+}EDDHA, 4.0044g EDDHA (ethylenediamine di[o-hydroxyphenylacetic acid], 90%) was dissolved in 50ml 1N NaOH, and 2.7030g $FeCl_3 \cdot 6H_2O$ was dissolved in 50ml 1N HCl. After the solutions cleared, the chelate was added slowly to the Fe solution under stirring, and the volume was adjusted to 1 liter.

A 10mM Fe^{3+}EDTA stock solution was prepared following the same procedure with some exceptions: 2.7030g $FeCl_3 \cdot 6H_2O$ was dissolved in 50ml 0.1M HCl, and mixed with 3.7224g $EDTANa_2 \cdot 2H_2O$ ((ethylenedinitrilo)tetraacetic acid) dissolved in 50ml 0.1N NaOH.

Selection for Fe efficiency

Adventitious *C. oblonga* shoots from 549 regenerating leaf sections were transferred to MS-M medium with 5µM BA. Three weeks later, 1921 individual shoots were subcultured on the same medium. Cultures were kept at a temperature of 25°C under a photoperiod of 16h of cool-white fluorescent light (40µmol $m^{-2}s^{-1}$).

Three shoots originating from each of the 1921 regenerants were cultured on MS-M medium with FeEDTA replaced by equimolar amounts of $FeSO_4 \cdot 7H_2O$ plus 1mM $KHCO_3$ (pH 6.3) and supplemented with 5µM BA. Four weeks later, nine lines were selected which showed less chlorosis than the others and Quince A control. The same multiplication and selection procedure was repeated with these 9 cultures.

The selected adventitious shoots were multiplied and rooted using standard protocols for *C. oblonga*. These putative somaclonal variants (Numbers 1-4) were characterized and compared to *C. oblonga* controls (micropropagated Quince A and adventitious regenerant No. 5) and *P. amygdaliformis*. Chlorophyll content, Fe reduction of tissues, and medium acidification were determined after Fe-deficiency conditioning.

RESULTS

Multiplication of cultured shoots

Shoot multiplication of *Pyrus* species and *C. oblonga* was promoted by BA (Table 2). The regime of 5µM BA and 40µmol $m^{-2}s^{-1}$ light was most conducive for *C. oblonga* and *P. betulaefolia*, but less stimulatory for the rest of the species. Shoot proliferation was better when the explants were placed flat on the medium. Proliferating shoot cultures of *P. amygdaliformis* and *P. betulaefolia*, two species for which micropropagation procedures had not been established before, and *C. oblonga*, are shown in Figure 3.

Table 2. Multiplication rates (No. shoots longer than 1cm/explant) under the initial culture conditions.

	Position on the medium	
Genotype	Upright	Flat
P. amygdaliformis Vill., 633	1.7±0.4	3.6±0.9
P. communis L., Seckel	3.3±0.9	6.6±0.8
P. communis L., Anjou	3.9±0.9	7.2±1.1
P. calleryana Dcne., OPR-191	2.8±0.6	3.1±0.7
P. betulaefolia Bunge, OPR-266	2.5±0.7	14.6±2.9
C. oblonga L., E.M. Quince A	9.5±2.7	10.2±2.3

Measurements were taken after four weeks of culture. Means pooled from the first ten passages.

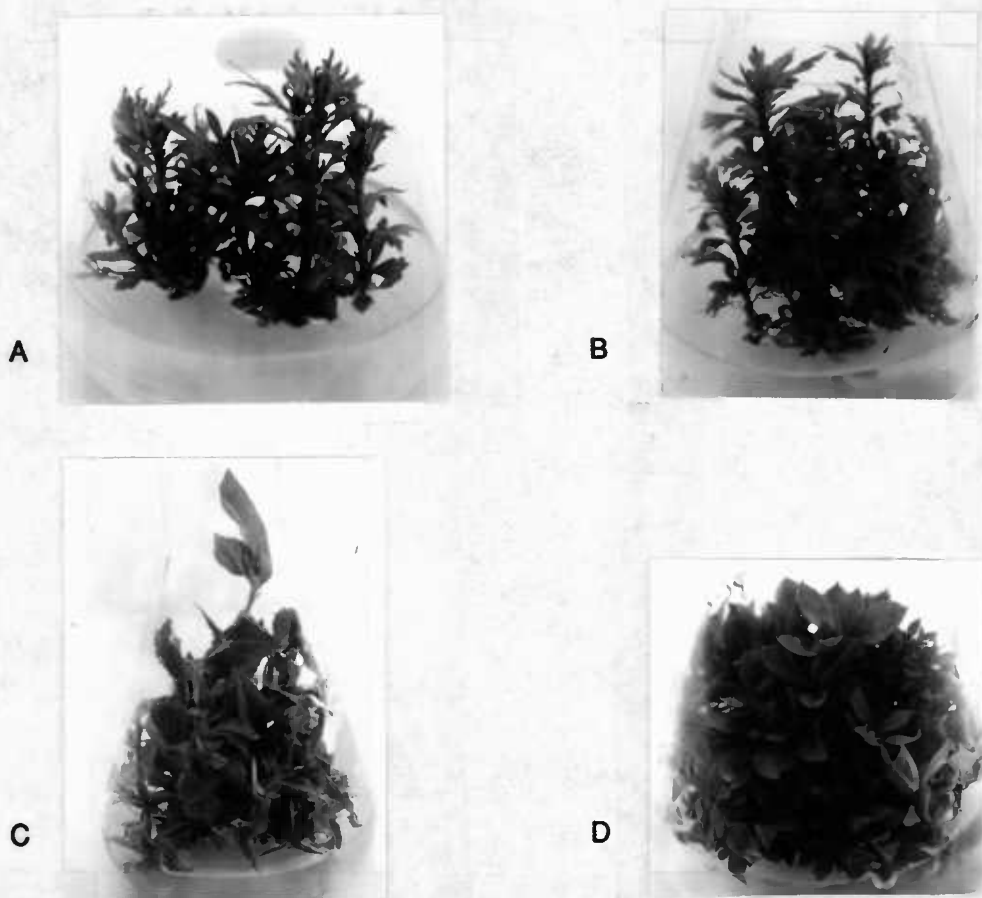


Figure 3. Proliferating shoot cultures of (A) *P. amygdaliformis*, (B) *P. betulaefolia*, (C) *C. oblonga*, and (D) *P. communis* P-2462.

To determine whether further modifications could lead to higher rates of multiplication in *P. communis*, *P. calleryana* and *P. amygdaliformis*, the effects of shoot orientation (vertical vs. horizontal), a range of cytokinin concentrations (2.5 to 20μM BA) and light intensity (13, 40 or 135μmol $m^{-2}s^{-1}$) were tested (Tables 3, 4, and 5). The analysis of variance indicated a highly significant interaction between the three factors for all genotypes. Placing the shoots horizontally was generally better. Increasing the cytokinin concentration to 10 or 20μM BA and the light intensity to 135μmol $m^{-2}s^{-1}$ also markedly increased shoot proliferation. These effects were particularly pronounced in *P. amygdaliformis* (Table 5), resulting in 26.6 shoots per explant at 20μM BA under the higher light intensity. However, only slight improvement was obtained for *P. calleryana*.

Two additional genotypes, *P. communis* L. OPR-1 and *P. communis* L. Angers Selection P-2462, were cultured flat on medium with 5μM BA under high light intensity (135μmol $m^{-2}s^{-1}$), resulting in multiplication rates of 12.4±3.2 and 24.1±5.4 respectively (Figure 3D).

One interesting observation concerned the morphology of the leaves of micropropagated *Pyrus* shoots. The leaf size was dependent on the orientation of the shoots, the light intensity, and BA concentration. Shoots formed on explants in the horizontal position had generally larger leaves than those in the vertical position. Also, leaves were larger at higher light intensities and lower BA concentrations. Differences in the leaf size were most pronounced in *P. communis*, *P. amygdaliformis*, and *P. calleryana* cultures (Figure 4).

Table 3. Effects of BA concentration, light intensity and explant position on shoot multiplication of *P. communis* 'Seckel'.

Upright position on the medium

Light intensity (μmol $m^{-2}s^{-1}$)		BA concentration (μM) 0	2.5	5	10	20
13	m.r. [a]	1.0	1.4	2.7	2.1	2.0
	mm [b]	-	16.5	12.3	13.1	11.4
40	m.r.	1.0	1.3	3.6	3.0	2.2
	mm	-	19.5	12.7	11.5	7.6
135	m.r	1.0	3.3	5.1	10.4	15.4
	mm	-	13.3	18.7	14.2	9.6

Flat position on the medium

Light intensity (μmol $m^{-2}s^{-1}$)		BA concentration (μM) 0	2.5	5	10	20
13	m.r.	1.0	2.4	3.2	3.2	3.1
	mm	-	14.7	15.8	11.0	5.5
40	m.r.	1.0	1.3	7.6	5.2	2.9
	mm	-	13.5	14.3	9.1	8.6
135	m.r.	1.1	3.2	7.4	14.7	16.8
	mm	11.0	13.2	11.1	14.4	11.0

Means were recorded after four weeks of culture, from two experiments with nine repetitions each.

[a] Multiplication rate = No. shoots longer than 1cm/explant.

[b] Mean shoot length (mm) of the new shoots.

Table 4. Effects of BA concentration, light intensity and explant position on shoot multiplication of *P. calleryana.*

Upright position on the medium

Light intensity ($\mu mol\ m^{-2}s^{-1}$)		BA concentration (μM) 0	2.5	5	10	20
13	m.r. [a]	1.3	2.7	2.3	2.0	2.6
	mm [b]	5.0	12.0	11.6	12.7	11.6
40	m.r.	1.0	2.3	3.0	2.6	2.6
	mm	-	19.7	18.7	13.4	8.9
135	m.r	1.1	2.2	2.3	3.3	2.4
	mm	5.0	10.7	14.0	12.3	13.7

Flat position on the medium

Light intensity ($\mu mol\ m^{-2}s^{-1}$)		BA concentration (μM) 0	2.5	5	10	20
13	m.r.	1.3	3.7	3.9	3.2	3.4
	mm	6.7	23.9	14.3	13.5	10.8
40	m.r.	1.3	2.3	2.8	3.3	3.6
	mm	5.0	13.4	18.7	16.9	13.4
135	m.r.	1.3	2.2	3.4	4.6	4.4
	mm	5.0	7.9	12.4	13.9	7.9

Means were recorded after four weeks of culture, from two experiments with nine repetitions each.

[a] Multiplication rate = No. shoots longer than 1cm/explant.

[b] Mean shoot length (mm) of the new shoots.

Table 5. Effects of BA concentration, light intensity and explant position on shoot multiplication of *P. amygdaliformis*.

Upright position on the medium

Light intensity (μmol $m^{-2}s^{-1}$)		BA concentration (μM) 0	2.5	5	10	20
13	m.r. [a]	1.1	1.7	1.8	2.8	5.6
	mm [b]	10.0	12.4	10.4	15.2	10.7
40	m.r.	1.0	1.2	2.0	1.9	6.7
	mm	-	5.5	10.6	7.3	6.7
135	m.r	1.3	3.2	3.9	10.4	16.7
	mm	7.5	6.6	6.0	8.3	9.8

Flat position on the medium

Light intensity (μmol $m^{-2}s^{-1}$)		BA concentration (μM) 0	2.5	5	10	20
13	m.r.	1.2	1.6	3.3	5.0	5.7
	mm	7.0	9.1	8.9	8.8	13.0
40	m.r.	1.8	3.2	4.3	4.6	7.0
	mm	6.3	8.0	7.2	6.3	7.9
135	m.r.	1.2	5.2	7.3	23.3	26.6
	mm	9.0	9.5	8.9	10.9	11.5

Means were recorded after four weeks of culture, from two experiments with nine repetitions each.

[a] Multiplication rate = No. shoots longer than 1cm/explant.

[b] Mean shoot length (mm) of the new shoots.

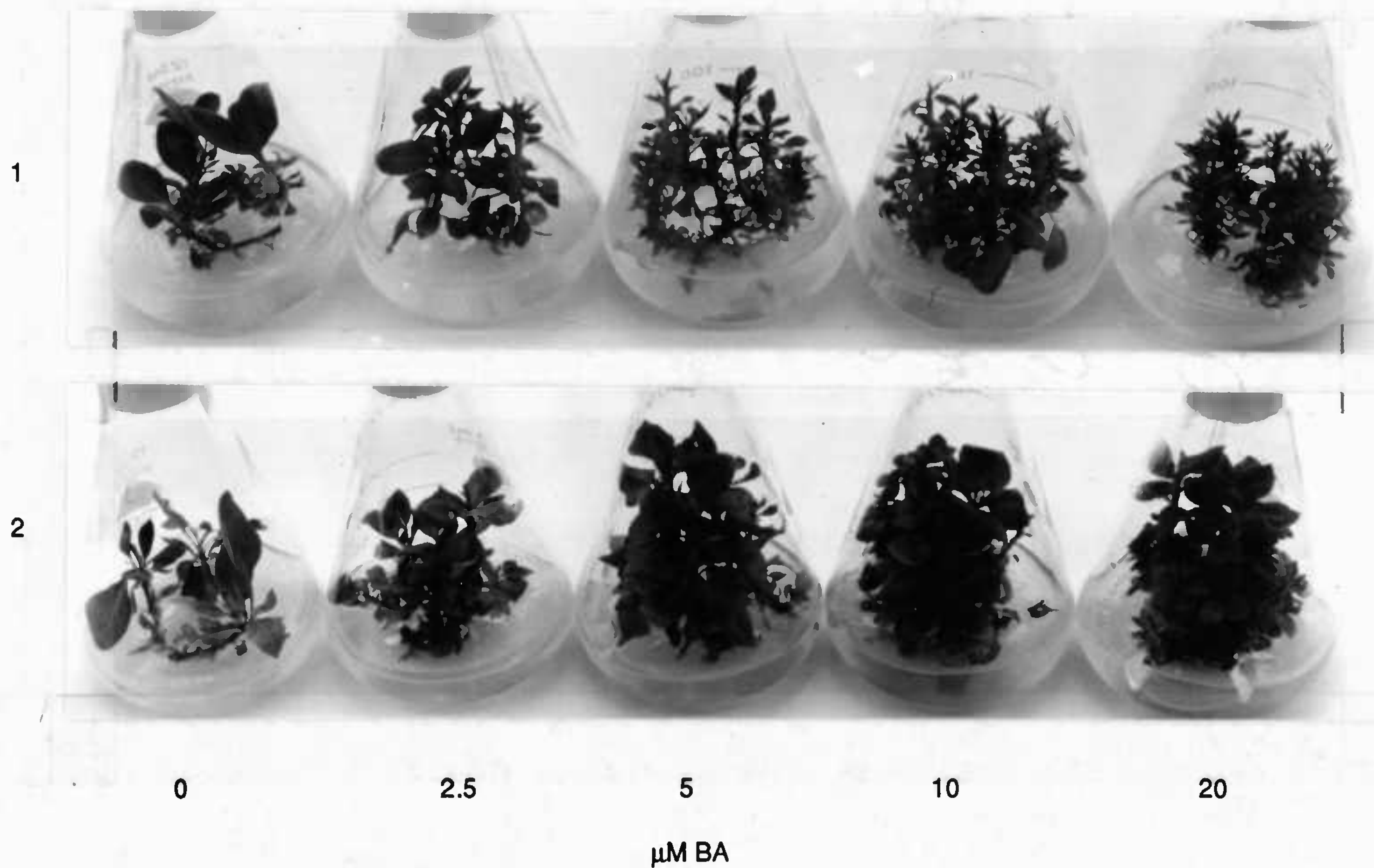


Figure 4. Effects of BA and light intensity on shoot cultures.
P. calleryana after four weeks on MS-M medium with 0, 2.5, 5, 10 and 20µM BA under a 16h photoperiod of 40 µmol $m^{-2}s^{-1}$ (1) or 135µmol $m^{-2}s^{-1}$ (2).

Rooting of micropropagated shoots

In the first rooting experiment, the effects of several concentrations of IBA and NAA were tested. Regression analyses indicated no differences between IBA and NAA after 28 days on auxin-containing medium (Figure 5A). Rooting decreased with increasing auxin concentration. However, when the shoots were transferred to auxin-free medium for 28 days, the positive effects of prior exposure to IBA became apparent (Figure 5B). Linear regression curves for IBA and NAA were significantly different ($p<0.01$). Higher concentrations of IBA (10 and 32μM) were most effective and resulted in rooting of a large proportion of the shoots. However, callus formation was also profuse. None of the NAA treatments followed by a passage of auxin-free medium stimulated root formation.

In a second experiment, the optimal concentration and duration of IBA treatment were determined by culturing shoots for 7, 14 and 28 days on IBA-containing medium followed by a period of 28 days on auxin-free medium. The results of this experiment are presented in Figure 6. ANOVA and planned contrasts, for the number of roots, root length and callus formation, indicated that the control treatment, without IBA, was significantly different ($p < 0.01$) from the rest of treatments. Regression analyses indicated that when the auxin concentration was presented on a logarithmic scale, the responses in rooting percentage (Figure 6A), number of roots per shoot (Figure 6B) and callus growth (Figure 6C) displayed linear increases, with two exceptions which showed quadratic responses. The linear regression curves for rooting percentage (7 and 14 days) were not significantly different and neither were those for root number (7, 14 and 28 days). The average root length increased with the auxin concentration and exposure time. Average root lengths were 7 and 9mm respectively for 7 and 14 days

treatment with 10μM IBA, and 10 and 12mm for the same treatment times at 32μM IBA. Callus growth also increased significantly with both duration and concentration of IBA treatment (Figure 6C). Considering all parameters it appeared that 7-day treatment with high concentrations (10 and 32μM) of IBA was most effective for induction of root formation in *P. betulaefolia.*

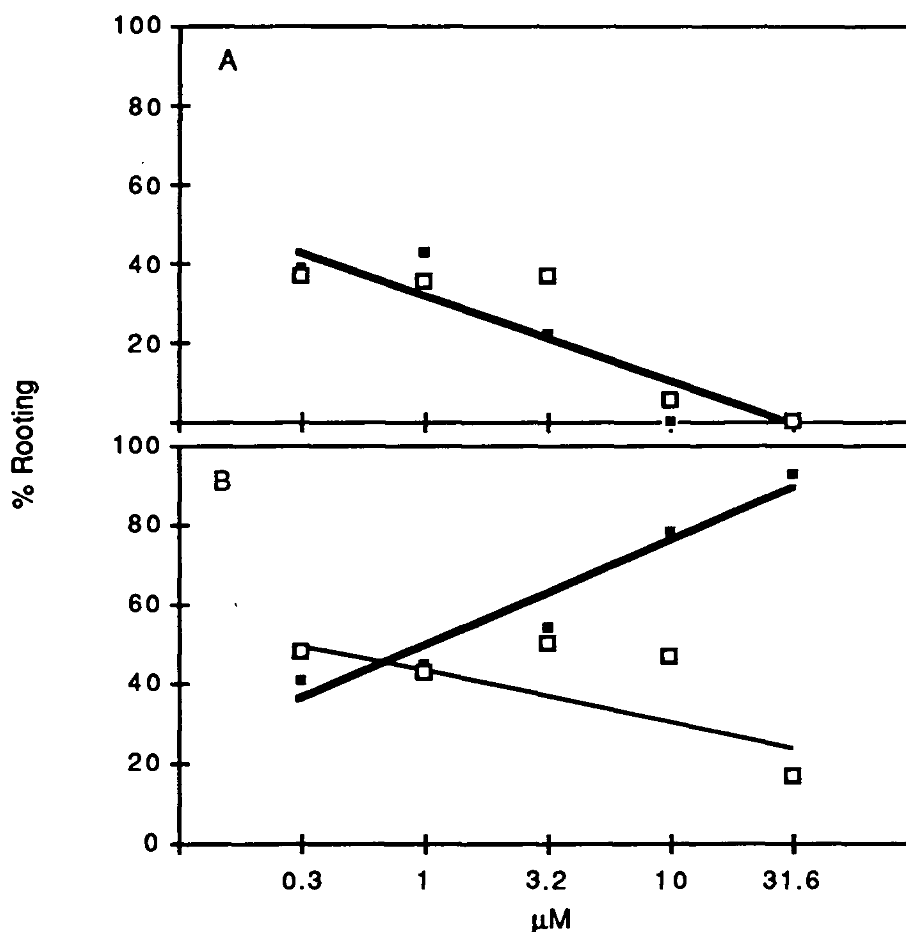


Figure 5. Effects of NAA and IBA on rooting of *P. betulaefolia.*
Rooting percentages were determined after a passage of 28 days on medium containing NAA (□) or IBA (■) (A) and an additional passage of 28 days on auxin-free medium (B). One common regression line represents the effects of both auxins in A (——). Two separate regression lines represent responses to NAA (——) or IBA (——) in B.

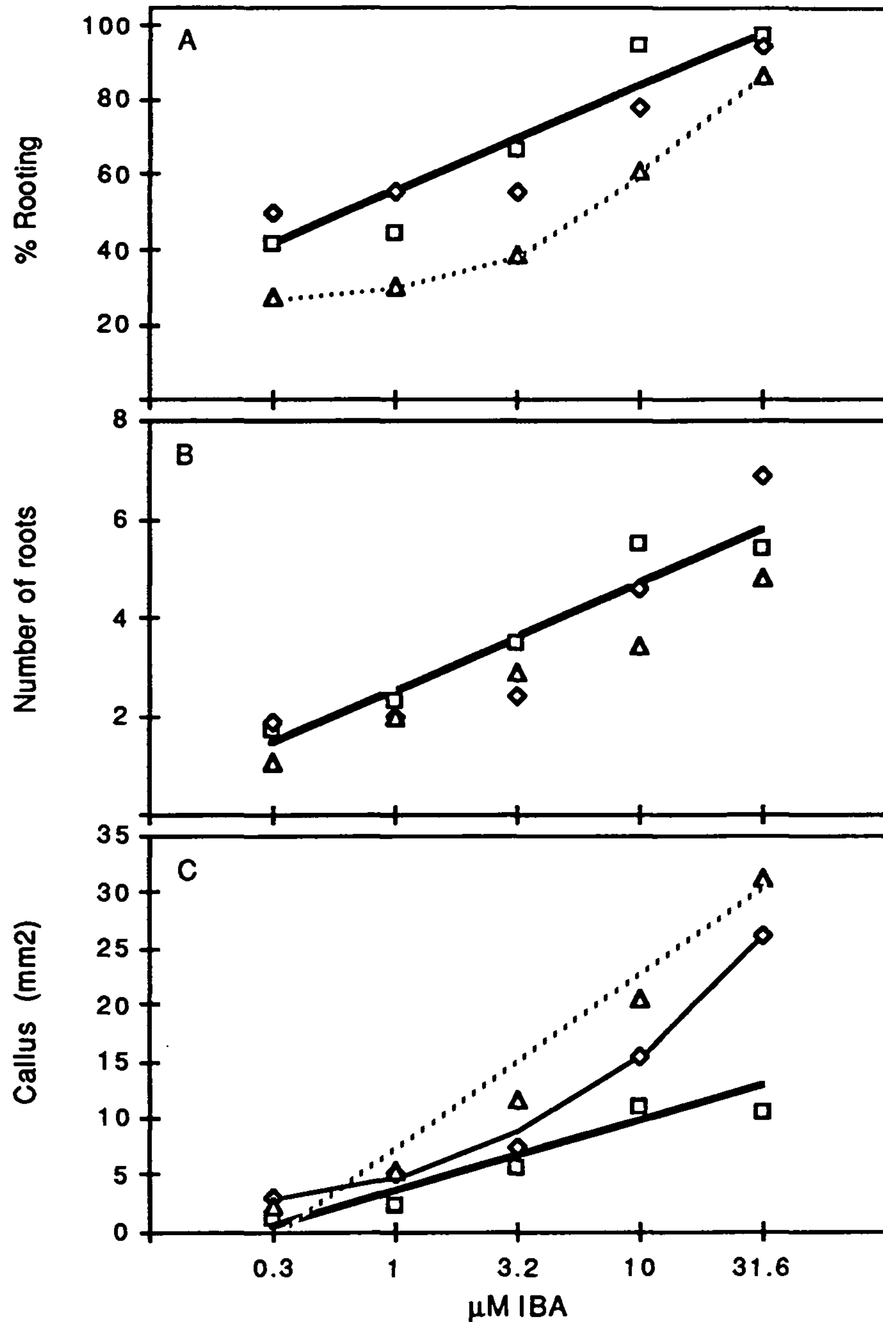


Figure 6. Effects of concentration of and exposure time to IBA on *P. betulaefolia* rooting. Rooting percentage (A), number of roots (B) and callus growth (C) measurements were taken after a passage of 28 days on auxin-free medium following a passage of 7 (□), 14 (◇), or 28 (△) days on IBA-containing medium. One common regression line represents responses to the 7 and 14 day treatments in A (——) and responses to 7, 14 and 28 day treatments in B (——). The curves for the 28 day treatment in A (·······) and 14 day treatment in C (——) reflect a quadratic response. For the 7 day treatment (——) and 28 day treatment (·······) in C a linear response applies.

Other variations tested in conjunction with exposure to IBA, pre-treatment at 9°C (Monette, 1987), keeping shoots in the dark (Hirabayashi et al., 1987; Marino, 1984; Zimmerman, 1984), the addition of riboflavin (Duron et al., 1989) and etiolation before root induction (Zimmerman, 1984), were not beneficial to rooting (ANOVA and Fisher's Protected LSD analyses). Specifically, riboflavin and etiolation caused a reduction in rooting percentage and root number over the control ($p < 0.05$).

The effects of the two optimal treatments for *P. betulaefolia* (treatments 4 and 5 in Table 1) as well as a number of additional rooting regimes (Table 1) were determined using other *Pyrus* species and *C. oblonga* (Table 6). All *Pyrus* species responded well to the rooting regimes optimal for *P. betulaefolia*, and even a dip in IBA solution (treatment 4) seemed sufficient for rooting of most of the *Pyrus* material. However, *C. oblonga* did not root well after these short treatments with IBA but required exposure to NAA (treatment 1) which was inadequate for the *Pyrus* species. The results suggest that one or several of the treatments tested are appropriate to induce a high percentage of rooting for each of the species examined. Even rootstocks difficult to root with traditional methods, such as *P. communis* OPR-1 and Angers selection P-2462 (Figure 7), could be rooted easily *in vitro* (one week on medium containing 10µM IBA followed by 3 weeks on auxin-free medium). The method of dipping shoots in high concentrations of IBA (treatment 6) should be of particular interest for commercial micropropagation since it eliminates a passage in culture.

Table 6. Responses of *Pyrus* species and *C. oblonga* to different rooting regimes.

Genotype	Treatment*	Rooting %	Number of roots	Root length (mm)	Callus (mm^2)
P. amygdaliformis	1	53	7.1 a**	11 b	12 b
	2	27	4.9 a	3 c	39 a
	3	37	4.7 a	6 c	4 b
	4	80	5.7 a	9 b	9 b
	5	90	5.9 a	9 b	32 a
	6	73	6.7 a	6 c	8 b
	7	7	5.5 a	24 a	0 b
P. communis, 'Seckel'	1	67	3.2 b	43 a	16 c
	2	90	10.1 a	16 c	63 c
	3	77	2.5 b	36 ab	13 c
	4	90	3.2 b	30 b	50 c
	5	87	2.7 b	10 c	194 a
	6	70	2.5 b	15 c	150 b
	7	20	1.5 b	49 a	0 c
P. communis, 'Anjou'	1	47	4.1 b	37 a	10 cd
	2	87	11.2 a	17 c	17 b
	3	63	3.6 bc	37 a	4 de
	4	97	3.5 bc	28 b	13 bc
	5	97	2.9 cd	16 c	46 a
	6	97	2.3 d	20 c	17 b
	7	23	1.6 d	39 a	0 e
P. calleryana	1	57	4.0 b	37 b	24 d
	2	87	11.4 a	15 cd	73 c
	3	73	3.5 b	29 b	21 d
	4	93	4.1 b	32 b	60 c
	5	97	3.4 b	12 d	237 a
	6	80	3.0 b	20 c	189 b
	7	13	2.0 b	54 a	0 d
P. betulaefolia	1	60	6.1 ab	10 b	10 c
	2	17	3.4 c	3 c	59 a
	3	40	3.7 c	5 c	3 de
	4	97	5.4 bc	8 b	8 cd
	5	90	6.3 ab	8 b	29 b
	6	87	7.2 a	6 c	8 cd
	7	23	6.9 ab	27 a	0 e
C. oblonga	1	97	4.2 b	48 a	33 d
	2	77	9.2 a	15 b	204 a
	3	23	1.7 b	28 ab	26 d
	4	50	2.7 b	38 a	57 c
	5	17	2.6 b	39 a	125 b
	6	17	2.8 b	32 ab	44 cd
	7	3	1.0 b	61 a	0 d

* Treatments are described in Table 1. ** Treatment means for each genotype with the same letter are not significantly different in the Fisher's Protected LSD ($p = 0.05$).

Figure 7. Rooted shoots of *P. communis* Angers Selection P-2462.

Adventitious shoot regeneration from leaves

In preliminary experiments, a combination of MS and N6 medium was more effective than MS in preventing browning of cultured leaves of *P. amygdaliformis* and *C. oblonga*. Exposing leaves to a dark-light sequence improved regeneration as compared to culturing leaves under continuous light (16h). Therefore, subsequent experiments were conducted using a combination of MS and N6 medium, with 3 weeks of dark followed by 3 weeks of light (16h) regimes.

After 3 weeks of culture on medium containing NAA (0.3 to 3μM), most *C. oblonga* leaves had white nodular callus and adventitious roots. Half of the *P. amygdaliformis* leaves also formed adventitious roots. After exposure to light, adventitious shoots were observed in *C. oblonga* and *P. amygdalifomis* on medium with NAA and TDZ. TDZ was more effective in inducing adventitious shoot formation and preventing senescence than BA. In *C. oblonga*, shoot regeneration occurred at 0.1μM to 10μM TDZ, with the highest rate at 10μM, in contrast, shoot formation on medium with BA was sporadic. The favorable effects of TDZ was also observed in *P. amygdaliformis*. At high auxin concentrations, callus formation was greater in *P. amygdaliformis* than *C. oblonga*.

Based on the results of the experiment described above, concentrations of 1 to 100μM TDZ were tested in combination with 0.3 to 3μM NAA for *C. oblonga* (Table 7). A narrower range of auxin concentrations (0.01 to 3.2μM) were used for *P. amygdaliformis* (Table 8). Most treatments resulted in adventitious shoot formation, but the highest proportion of regeneration occurred using 32μM TDZ and 0.3μM NAA for *C. oblonga*, and 3μM TDZ and 1μM NAA for *P. amygdaliformis*.

Table 7. Effects of TDZ and NAA on adventitious shoot regeneration from *C. oblonga* leaves.

TDZ conc. (μM)	Percentage NAA conc. (μM)			No. shoots/leaf NAA conc. (μM)		
	0.3	1	3	0.3	1	3
1	6	17	6	1.0	1.6	2.0
3	19	38	27	1.2	1.9	2.2
10	33	21	33	2.4	2.3	2.4
32	48	25	31	3.6	3.1	2.5
100	0	2	0	-	1.0	-

Treatment percentages and means were pooled from four experiments with 12 repetitions each.

Table 8. Effects of TDZ and NAA on adventitious shoot regeneration from *P. amygdaliformis* leaves.

TDZ conc. (μM)	Percentage NAA conc. (μM)					No. shoots/leaf NAA conc. (μM)				
	0	0.01	0.1	1	3	0	0.01	0.1	1	3
1	0	6	2	2	4	-	1.3	1.0	1.0	1.0
3	2	2	4	21	2	1.0	1.0	1.0	3.4	2.0
10	0	0	2	8	19	-	-	1.0	1.3	1.9
32	0	0	0	0	0	-	-	-	-	-
100	0	0	0	0	0	-	-	-	-	-

Treatment percentages and means were pooled from four experiments with 12 repetitions each.

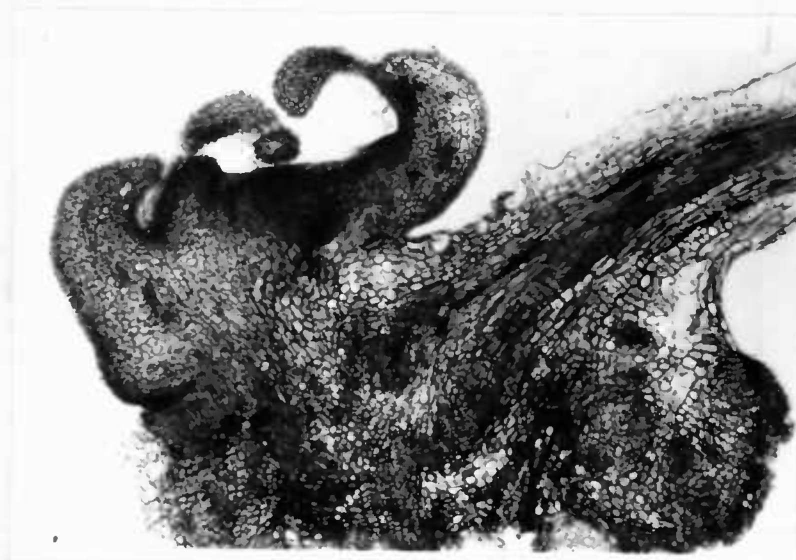
Addition of vitamins (folic acid, biotin, and pantothenic acid) or complex organic substances (casein hydrolysate, yeast extract, malt extract, and coconut water), glycine or putrescine did not enhance regeneration. Increased sucrose concentration (60 g/l) slightly repressed the regeneration rate, and placing leaves with the abaxial part up or continous culture in the dark did not enhance regeneration.

By careful selection of leaves as explants, regeneration was improved further. Using young and uniformly developed leaves from micropropagated shoots with vigorous growth, the frequency of regenerating leaves of *C. oblonga* reached 78%, with 3.2 shoots per leaf.

Irradiation of *C. oblonga* leaves with 2 Krads reduced regeneration to 22% with an average of 1.7 shoots per leaf, while the control sample showed 68% regeneration with an average 3.4 shoots per leaf.

Anatomical studies revealed that small callus tissues initiated at the cut edges of *C. oblonga* leaves, and buds subsequently formed in the calli after 3 weeks of culture (Figure 8). Usually several shoots could be observed in close proximity during the 3 weeks of light culture (Figure 9).

Shoot elongation was promoted by culturing regenerating leaf sections in multiplication medium. After three weeks, regenerated shoots could be easily dissected and multiplied (Figure 10).



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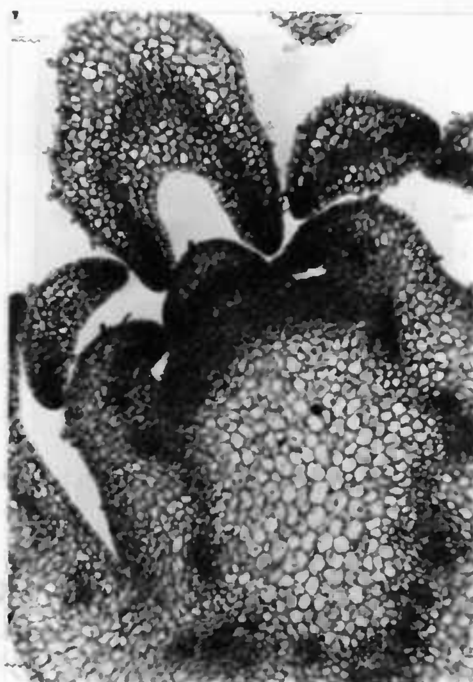


B

Figure 8. Adventitious shoot regeneration from *C. oblonga* leaves.
Cross section of a regenerating leaf (A) and adventitious shoot meristem (B) after three weeks in the dark.

Figure 9. Adventitious shoots regenerated from leaves of *C. oblonga* (A) and *P. amygdaliformis* (B) cultured for six weeks.

Figure 10. Adventitious shoots of *C. oblonga* after six weeks on regeneration medium and three weeks on multiplication medium.

Screening for tolerance to Fe-deficiency chlorosis

Unrooted and rooted shoots of the six genotypes were exposed to four different media, containing $FeSO_4$ or FeEDTA with or without potassium bicarbonate. The development of Fe-deficiency symptoms was extremely uniform between replicates, which facilitated the detection of genotypic responses and the effects of individual treatments. *C. oblonga*, which is highly sensitive to alkaline soils, was also very sensitive to simulated conditions in vitro (Figures 11 and 12). The replacement of FeEDTA in the control medium by $FeSO_4$ was sufficient to induce chlorosis, and inclusion of bicarbonate with $FeSO_4$ caused more severe symptoms. In contrast, *P. amygdaliformis* and *P. communis*, tolerant species in field trials, did not exhibit chlorosis under any of the conditions tested. Various treatments resulted in significant differences in chlorophyll content (Table 9) for *C. oblonga*, *P. betulaefolia* and *P. calleryana*, but no significant variation was detected in *P. communis* and *P. amygdaliformis*. In addition, the effects of the treatments were similar for rooted or unrooted shoots of each genotype.

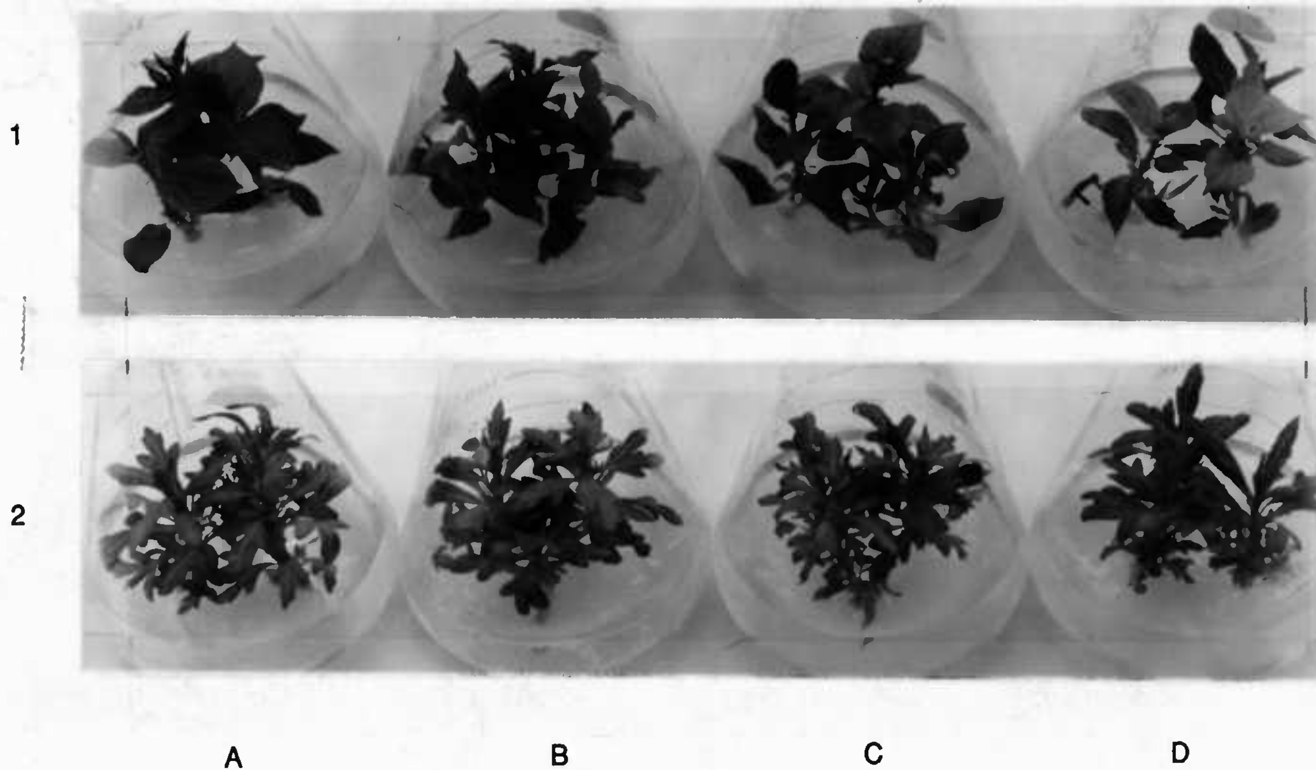


Figure 11. Responses of *C. oblonga* and *P. amygdaliformis* shoots to Fe stress. *C. oblonga* (1) and *P. amygdaliformis* (2) were grown for 28 days on medium containing FeEDTA (A), FeEDTA + $KHCO_3$ (B), $FeSO_4$ (C), and $FeSO_4$ + $KHCO_3$ (D).

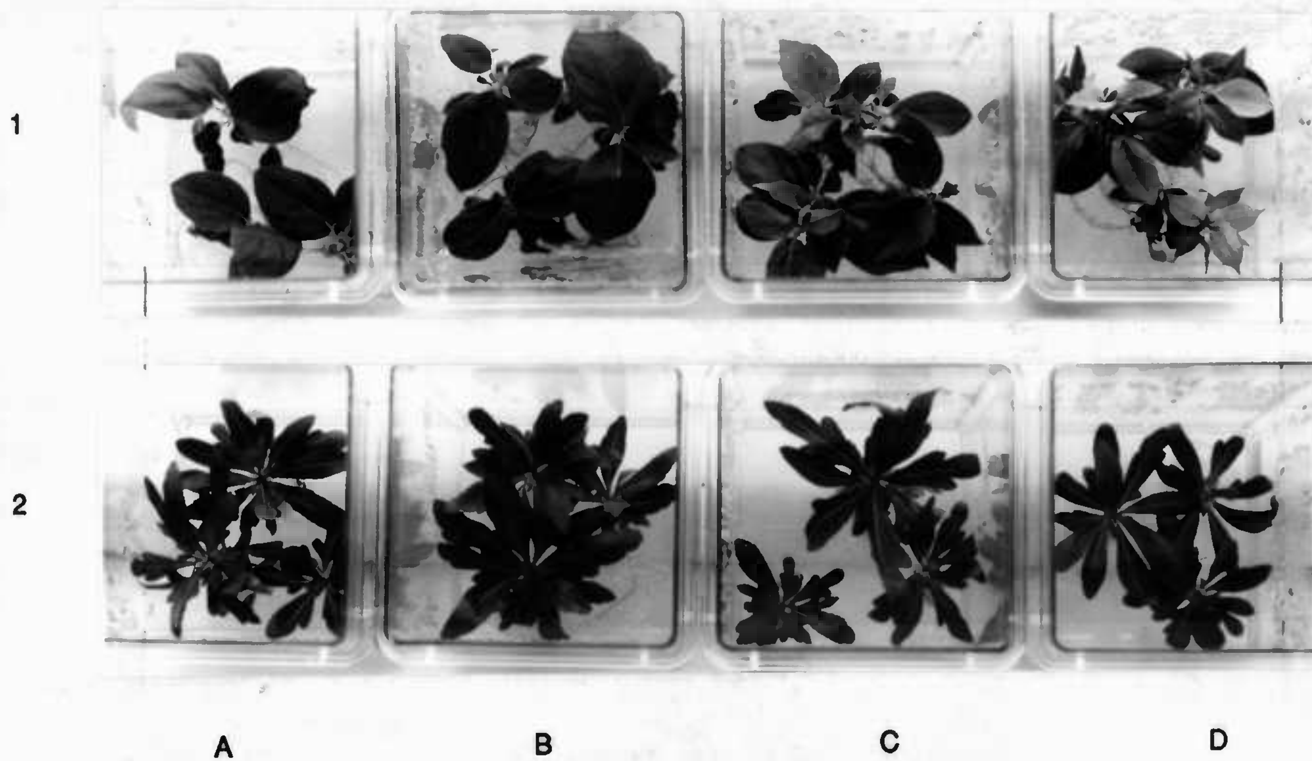


Figure 12. Responses of *C. oblonga* and *P. amygdaliformis* plantlets to Fe stress. *C. oblonga* (1) and *P. amygdaliformis* (2) were grown for 28 days on medium containing FeEDTA (A), FeEDTA + $KHCO_3$ (B), $FeSO_4$ (C), and $FeSO_4$ + $KHCO_3$ (D).

Table 9. Chlorophyll concentration (µg/mg fresh weight) of shoots and plantlets of *Pyrus* species and *C. oblonga*.

	Treatment				
Genotype	FeEDTA	FeEDTA + HCO_3^-	FeSO4	FeSO4 + HCO_3^-	p *
Unrooted					
P. amygdaliformis	2.8	2.9	2.7	2.7	0.873
P. communis, 'Seckel'	2.9	2.6	2.7	2.5	0.315
P. communis, 'Anjou'	2.4	2.4	2.6	2.4	0.823
P. calleryana	2.8 ab**	3.0 a	2.4 bc	2.2 c	0.014
P. betulaefolia	3.1 a	2.8 a	2.2 b	2.0 b	<0.001
C. oblonga	3.7 a	3.1 b	2.4 c	0.9 d	<0.001
Rooted					
P. amygdaliformis	5.2	5.0	5.3	5.1	0.599
P. communis, 'Seckel'	4.4	4.3	4.3	4.2	0.674
P. communis, 'Anjou'	3.7	3.8	3.2	3.5	0.152
P. calleryana	4.3 a	4.0 ab	3.8 ab	3.6 b	0.048
P. betulaefolia	4.3 a	4.1 a	3.6 ab	3.3 b	0.020
C. oblonga	4.2 a	4.7 a	2.5 b	1.3 c	<0.001

* Significance level of the difference between treatments within each genotype.
** Treatment means with the same letter for each genotype are not significantly different in the Fisher's Protected LSD (p = 0.05).

Characterization of differential responses to Fe stress

Conditioning in liquid medium without Fe led to a decrease in chlorophyll content of the leaves of *C. oblonga* shoots and plantlets, but did not affect that of *P. amygdaliformis* (Figure 13). The presence of bicarbonate further aggravated chlorosis in *C. oblonga* when conditioning was extended to 20 days. These results confirm

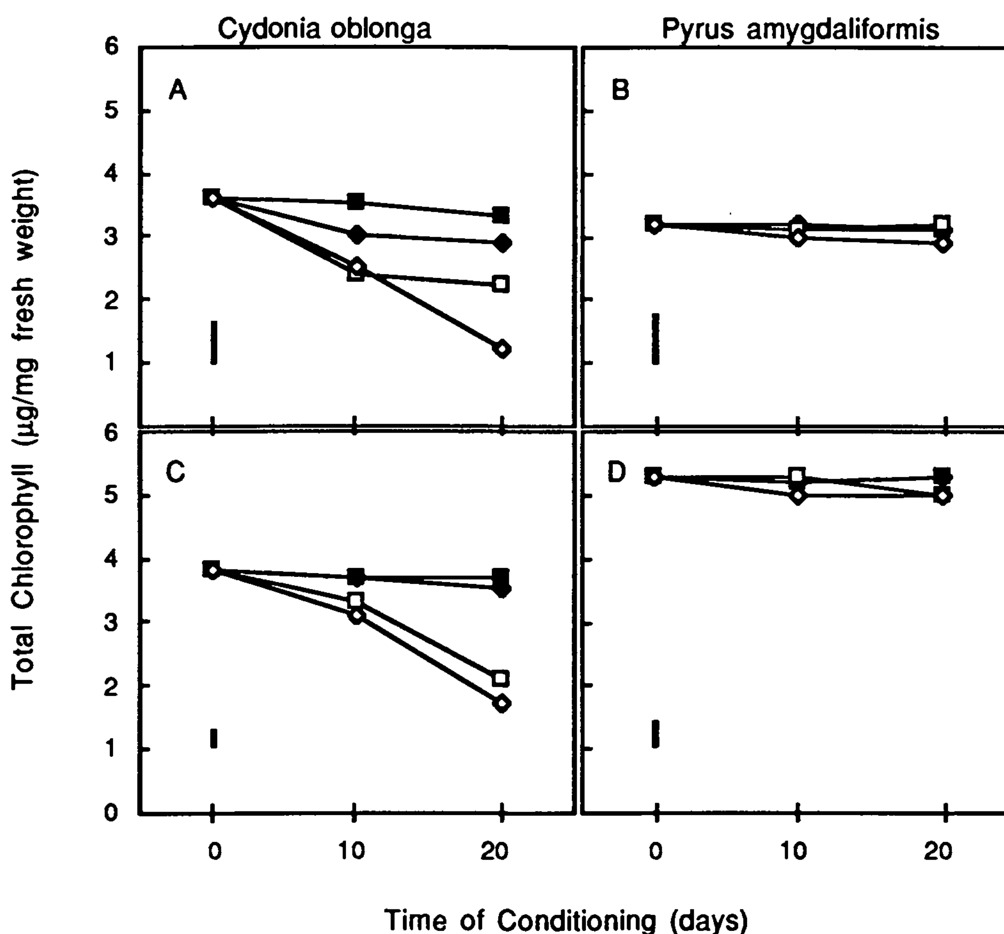


Figure 13. Chlorophyll content of unrooted (A, B) and rooted (C, D) shoots of *C. oblonga* and *P. amygdaliformis*.
Measurements taken before and after conditioning for 10 and 20 days in medium + Fe - $KHCO_3$ (■), + Fe + $KHCO_3$ (◆), - Fe - $KHCO_3$ (□), - Fe + $KHCO_3$ (◇). Means were pooled from two experiments with five repetitions each. The bar in each graph represents the Fisher's Protected LSD (p=0.05) between treatment means.

the findings of the screening tests (Table 9) and demonstrate the usefulness of the conditioning system. The 10-day conditioning period was selected for further characterization of traits possibly related to Fe deficiency since chlorosis was evident after this length of treatment. Tests of Fe reduction in tissues, and medium acidification were performed after 10 and 20 days.

During conditioning, fresh weight of shoots and plantlets continued to increase for both *C. oblonga* and *P. amygdaliformis*, but were significantly larger for *P. amygdaliformis* than *C. oblonga* (Table 10). Therefore, higher chlorophyll content in *P. amygdaliformis* was not related to growth differentials. The fresh weight increases for both genotypes did not differ between treatments, which simplifies interpretation of results from further tests.

Table 10. Increase in fresh weight (mg) of shoots and plantlets of *C. oblonga* and *P. amygdaliformis* over 10 days of conditioning.

	Conditioning treatment						
	+ Fe		- Fe		p-value		
Genotype	$-KHCO_3$	$+KHCO_3$	$-KHCO_3$	$+KHCO_3$	G	T	G x T
			Unrooted				
C. oblonga	51	67	63	63	<0.001	0.245	0.634
P. amygdal.	119	146	153	122			
			Rooted				
C. oblonga	40	37	38	33	<0.001	0.600	0.936
P. amygdal.	64	52	56	51			

Means were pooled from two experiments with five repetitions each. The p-value is the significance level of the differences between genotypes (G), treatments (T), and interaction (G x T). Treatment means with the same letter are not significantly different in the Fisher's Protected LSD ($p = 0.05$).

The total Fe content in shoots was higher in *C. oblonga* than *P. amygdaliformis* under high Fe conditions and decreased to similar levels in both under Fe stress (Table 11). Therefore, difference in total Fe content could not account for the stable chlorophyll levels of *P. amygdaliformis* under Fe stress. In contrast, the Fe^{2+} level and the proportion of the total, was higher in *P. amygdaliformis* than *C. oblonga* under all conditions. The differential appearance of Fe chlorosis between the two species correlating with Fe^{2+} content is in agreement with current theories concerning Fe-chlorosis in dicots (Marschner, 1986; Mengel and Geurtzen, 1988).

As differences in Fe^{2+} content and proportion of Fe should be reflected in conversion of Fe^{3+} to Fe^{2+}, reducing ability was measured in exudates, and in intact roots, shoots and cultured cells. Fe reduction was detected in root but not shoot exudates. The reduction rate was significantly higher in *P. amygdaliformis* than *C. oblonga* (Table 12). The most striking difference in Fe reduction was observed between plantlets (Figure 14B) and isolated roots (Figure 14C) of the two species. Fe reduction was generally highest when both Fe and bicarbonate were absent. Bicarbonate alone inhibited Fe reduction in the two species, mostly in shoot cultures. A longer (20-day) conditioning period resulted in higher Fe-reducing ability (in shoots and plantlets). However, in root cultures, Fe reduction was high after a 10-day conditioning period and was little increased by an additional 10 days.

Table 11. Total Fe and Fe^{2+} concentration (µg/g fresh weight) of the shoots of non-rooted and rooted *C. oblonga* and *P. amygdaliformis* before and after conditioning for 10 days.

Iron Form	Genotype	Before Conditioning	After conditioning: + Fe, - $KHCO_3$	After conditioning: + Fe, + $KHCO_3$	After conditioning: - Fe, - $KHCO_3$	After conditioning: - Fe, + $KHCO_3$	p-value: G	p-value: T	p-value: G x T
					Unrooted				
Total	*C. oblonga*	44.4	38.5	38.7	17.7	17.7	<0.001	<0.001	<0.001
	P. amygdal.	29.3	31.4	27.4	16.3	16.8			
Fe^{2+}	*C. oblonga*	19.1	16.3	19.6	14.2	14.8	<0.001	<0.001	0.191
	P. amygdal.	24.3	24.7	22.4	18.1	18.4			
		a	a	a	b	b			
					Rooted				
Total	*C. oblonga*	45.2	45.7	43.6	24.6	24.5	<0.001	<0.001	<0.001
	P. amygdal.	34.2	30.5	29.1	20.1	21.1			
Fe^{2+}	*C. oblonga*	18.1	18.1	17.9	15.1	13.8	<0.001	<0.001	0.335
	P. amygdal.	26.7	25.0	28.3	21.8	20.5			
		a	a	a	b	b			

Means were pooled from two experiments with five repetitions each. The p-values are the significance levels of the differences between genotypes (G), treatments (T), and the interaction (G x T). Treatment means with the same letter are not significantly different in the Fisher's Protected LSD (p = 0.05).

Table 12. Fe reduction (nmoles Fe(II)/g fresh weight x 24h) by exudates from bases of shoots, and roots of plantlets of *C. oblonga* and *P. amygdalifomis* after 10 days of conditioning.

	Conditioning treatment						
	+ Fe		- Fe		P-value		
Genotype	- KHCO3	+ KHCO3	- KHCO3	+ KHCO3	G	T	G x T
	Unrooted						
C. oblonga	0.0	0.0	0.0	0.0	N.S.	N.S.	N.S.
P. amygdal.	0.0	0.0	0.0	0.0			
	Rooted						
C. oblonga	0.3	0.2	0.2	0.2	<0.001	0.014	0.077
P. amygdal.	0.9	0.6	0.5	0.9			
	a	b	b	a			

Means were pooled from two experiments with five repetitions each. The p-value is the significance level of the differences between genotypes (G), treatments (T), and interaction (G x T). Treatment means with the same letter are not significantly different in the Fisher's Protected LSD (p = 0.05).

Since the differences in Fe reduction between the two species occurred in root tissues as well as shoot bases, cultured cells were examined to determine if such differences extend to undifferentiated cells. Interestingly, Fe reduction in cultured cells of *P. amygdaliformis* was also higher than those of *C. oblonga* (Figue 15). The increases in reducing capacity due to Fe stress were very similar to those of roots, indicating the genotypic differences occur at the cellular level. The only difference resided in the earlier peak, after a 12-day conditioning period, in cultured cells. This may be related to depletion of other nutrients and the need to subculture after two weeks.

Changes in the medium pH of shoot, plantlet, root and cell suspension cultures were measured after a 20-day conditioning period. The pH was lower for *P. amygdaliformis* than *C. oblonga* in all types of cultures (Table 13). Thus acidification of the medium was

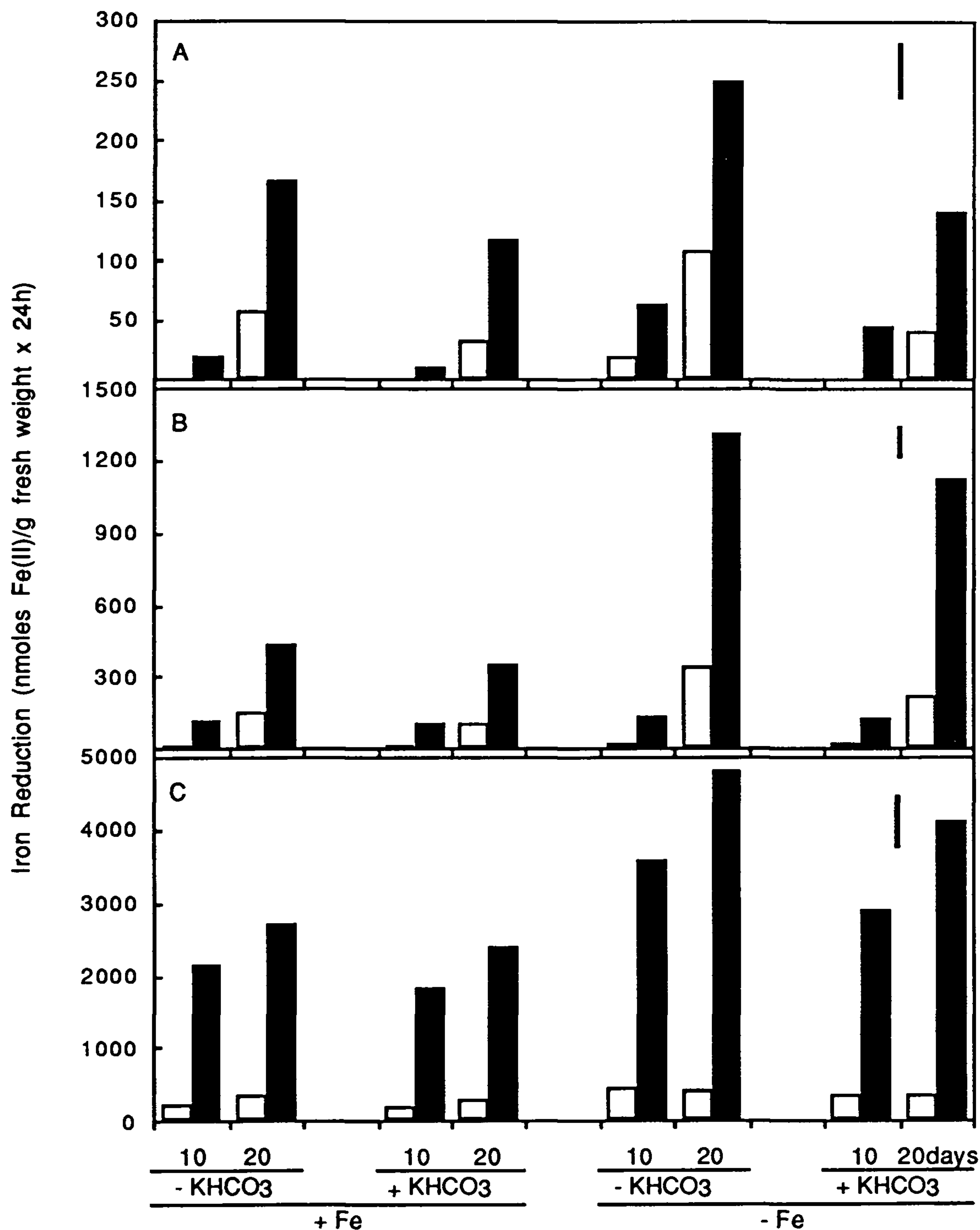


Figure 14. Fe reduction in bases of shoots (A), roots of plantlets (B), and root cultures (C) of *C. oblonga* (□) and *P. amygdaliformis* (■) after 10 and 20 days of conditioning. Means were pooled from two experiments with five repetitions each. The bar in each graph represents the Fisher's Protected LSD (p=0.05) between treatment means.

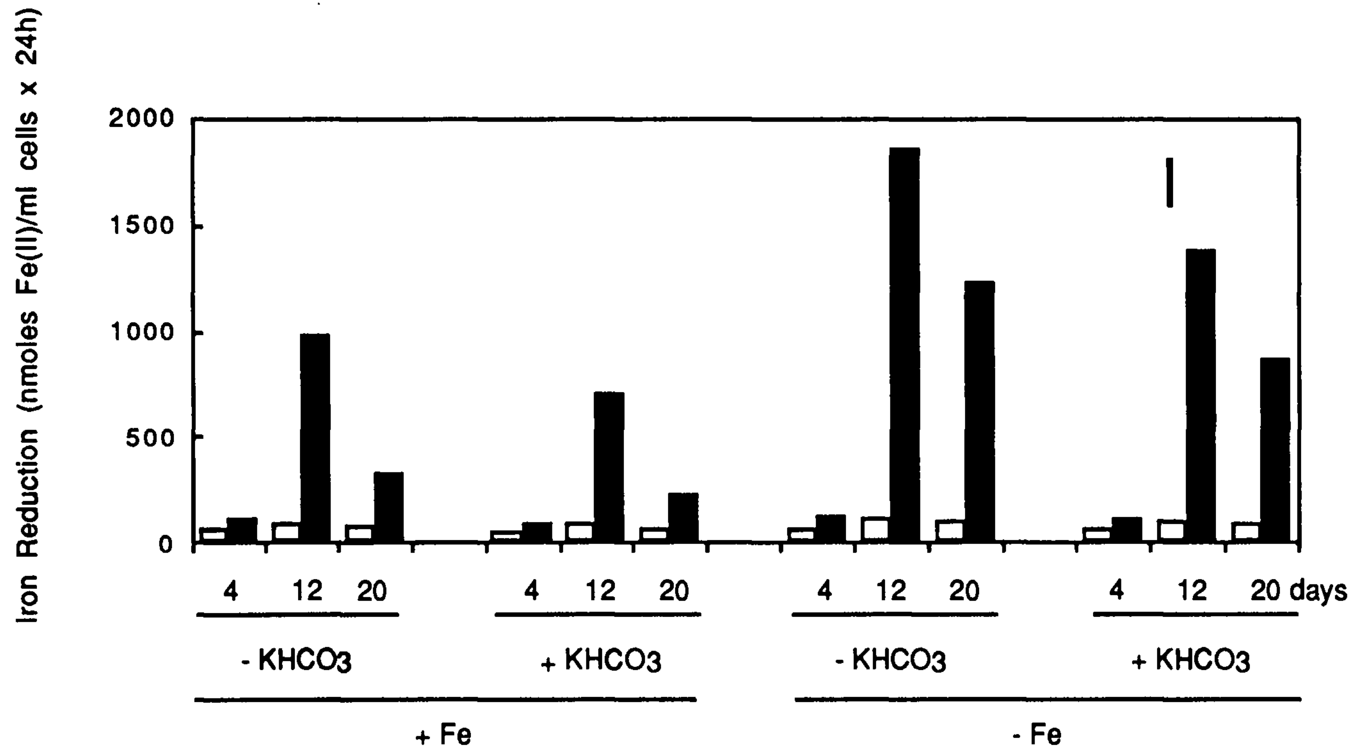


Figure 15. Fe reduction in cell suspensions of *C. oblonga* (□) and *P. amygdaliformis* (■) after 4, 12 and 20 days of conditioning. Means were pooled from two experiments with five repetitions each. The bar represents the Fisher's Protected LSD (p=0.05) between treatment means.

accompanied by a higher reducing ability in *P. amygdaliformis*.

Developmental changes were observed between the two species after Fe stress conditioning. Swollen root tips were more frequent in *P. amygdaliformis* than *C. oblonga* plantlets and root cultures. Fe reduction sites were located 5 to 10mm from the tips of cultured roots (Figure 16) while in cell suspension cultures, they were observed outside the cells (Figure 17). Such sites were more abundant in *P. amygdaliformis*, which is in agreement with the results of Fe reduction assays.

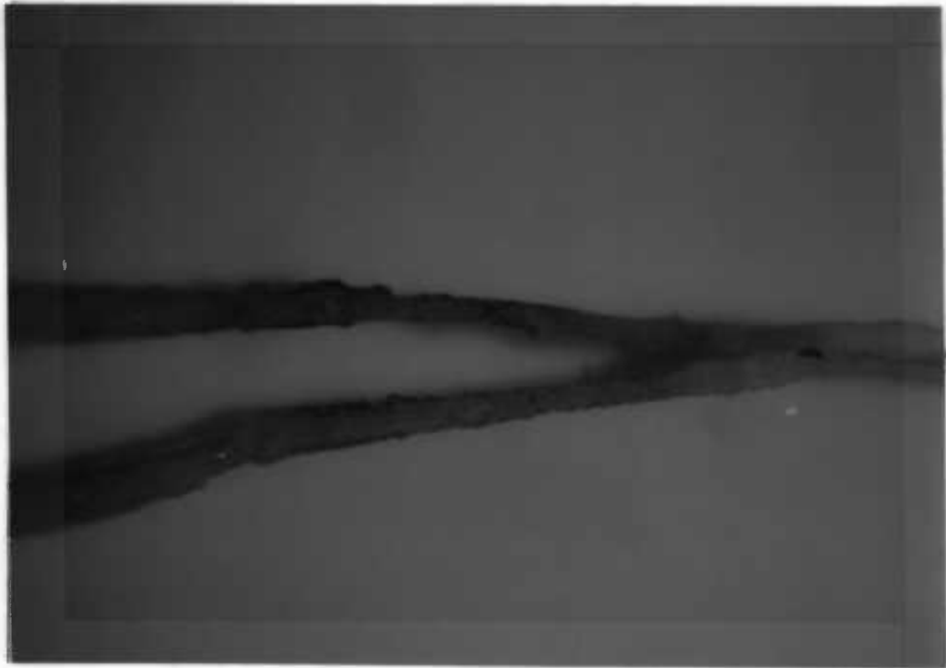
Tests for the occurrence of phytosiderophores excreted by shoots and roots of plantlets in both genotypes gave negative results. Thus, responses of *P. amygdaliformis* and *C. oblonga* to low Fe availability follow the path reported for Strategy I species. Quantitative differences between the two species in Fe reduction and medium acidification, were in agreement with the occurrence of Fe-deficiency chlorosis.

Table 13. Medium pH of shoot, plantlet, root and cell suspension cultures of *C. oblonga* and *P. amygdalifomis* after 20 days of conditioning.

	Conditioning treatment						
	+ Fe		- Fe		p-value		
Genotype	- KHCO3	+ KHCO3	- KHCO3	+ KHCO3	G	T	G x T
	Shoot cultures						
C. oblonga	5.4	5.1	4.5	4.9	0.060	<0.001	0.318
P. amygdal.	5.1	5.2	4.2	4.7			
	a	a	c	b			
	Plantlet cultures						
C. oblonga	4.4	4.4	4.1	4.2	<0.001	0.008	0.966
P. amygdal.	3.7	3.8	3.5	3.5			
	a	a	b	b			
	Root cultures						
C. oblonga	4.3	4.3	4.1	4.1	<0.001	0.002	0.726
P. amygdal.	4.1	4.0	3.8	3.7			
	a	a	b	b			
	Cell suspensions						
C. oblonga	5.8	5.9	5.5	5.6	<0.001	<0.001	<0.001
P. amygdal.	5.6	5.7	5.1	5.2			

Means were pooled from two experiments with five repetitions each. The p-value is the significance level of the differences between genotypes (G), treatments (T), and interaction (G x T). Treatment means with the same letter are not significantly different in the Fisher's Protected LSD ($p = 0.05$).

A



B

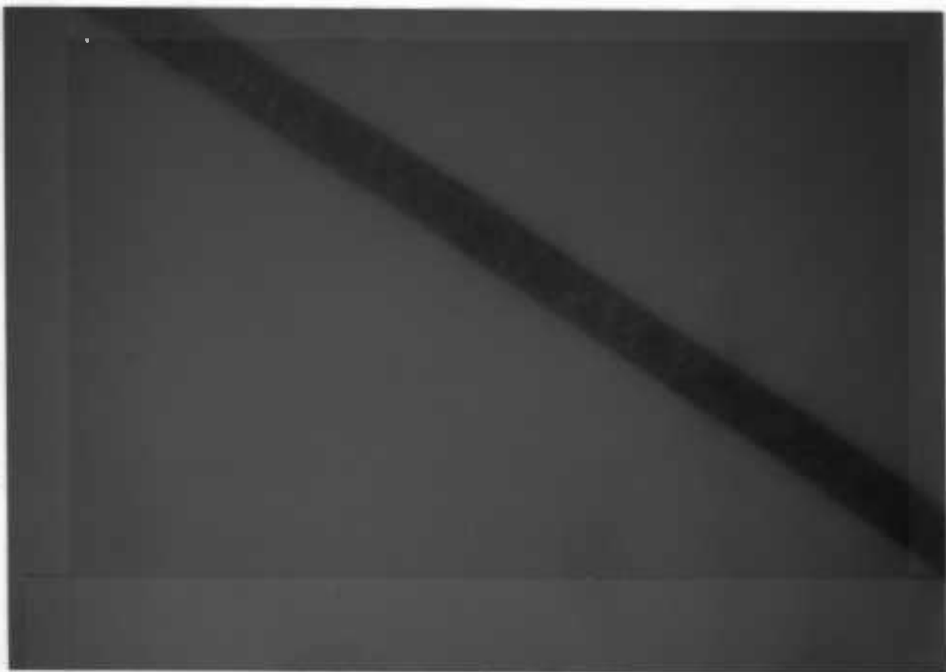


Figure 16. Fe reduction sites in roots of *P. amygdaliformis* (A) and *C. oblonga* (B) stained with Prussian blue.

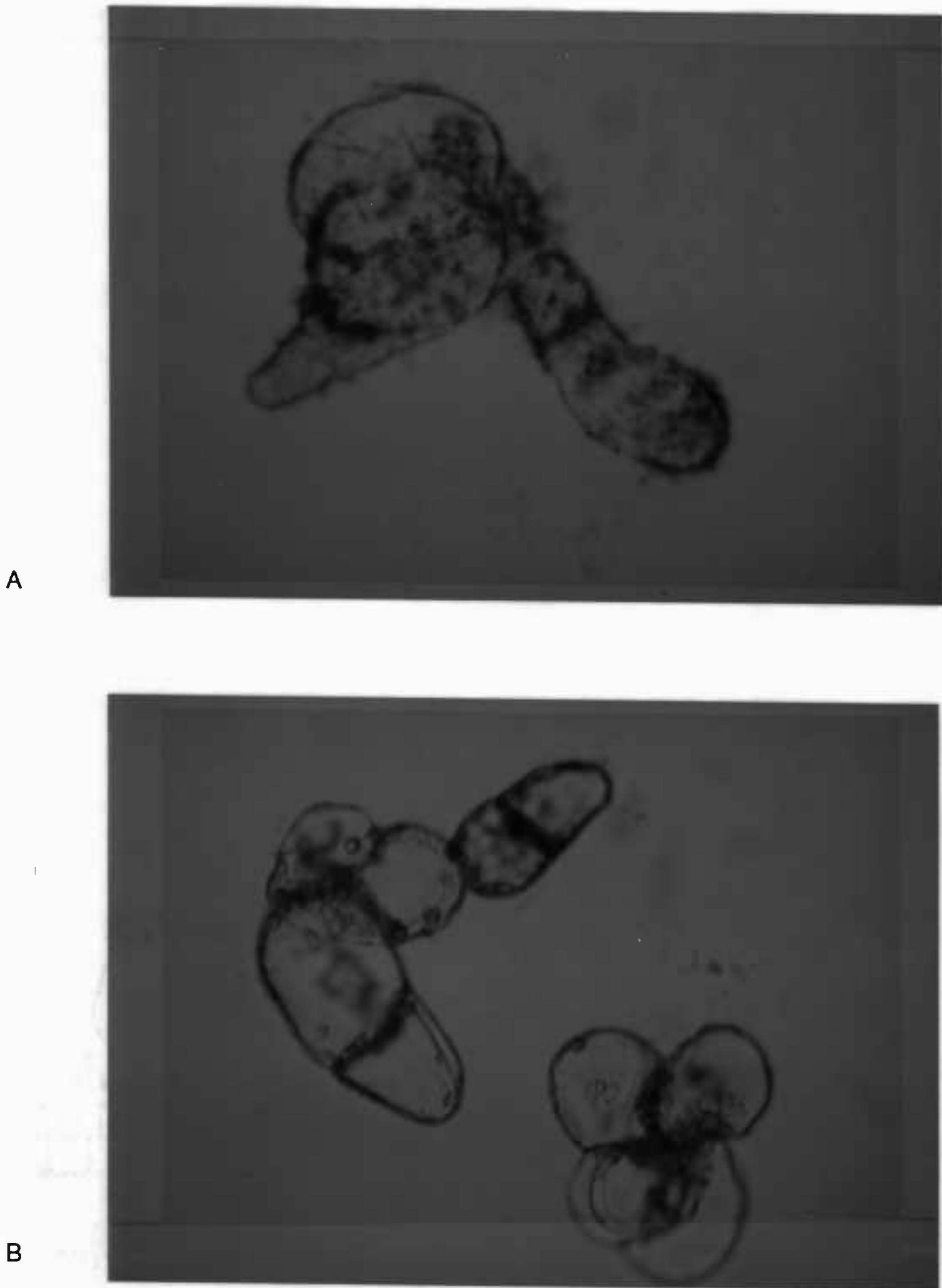
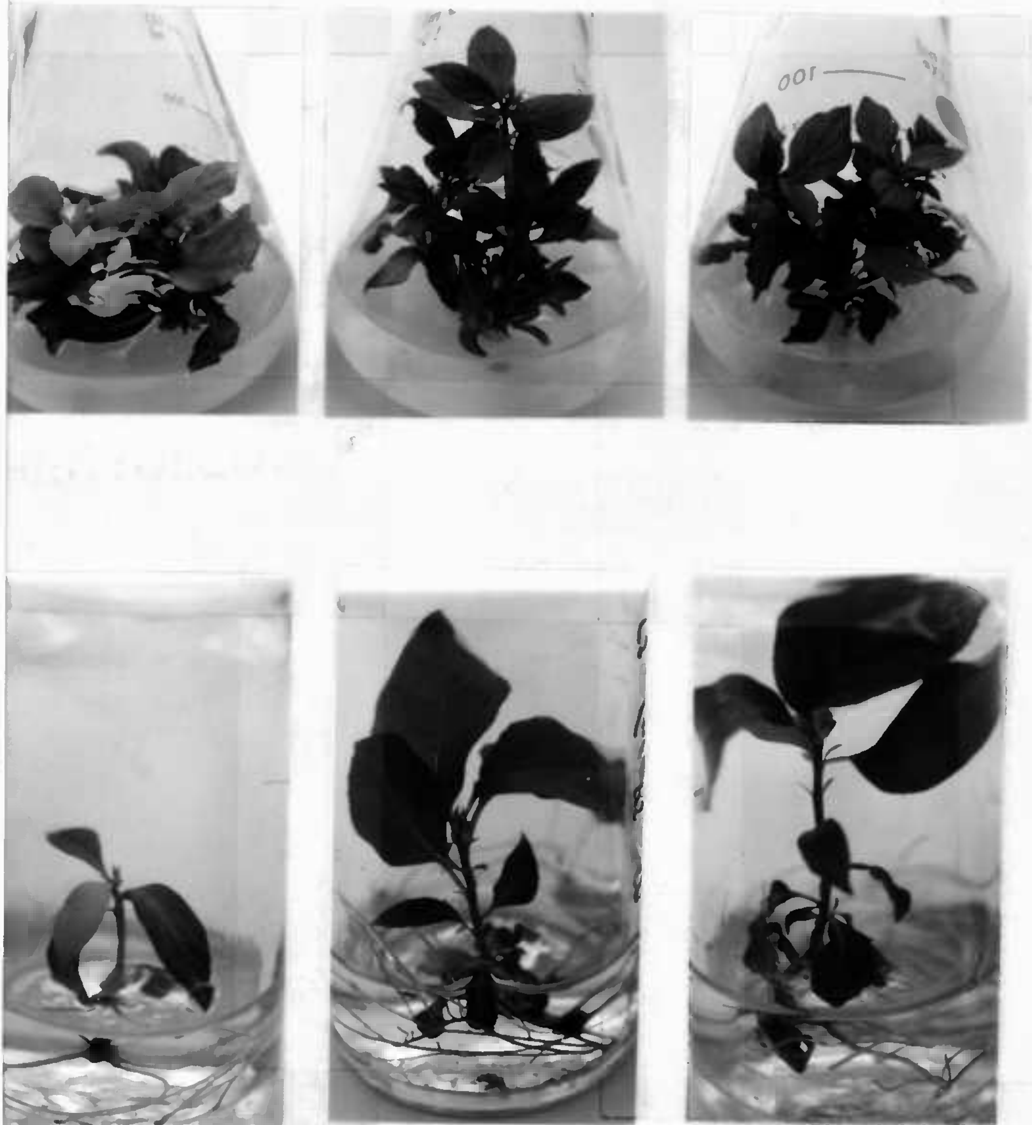


Figure 17. Fe reduction sites in cell suspensions of *P. amygdaliformis* (A) and *C. oblonga* (B) stained with Prussian blue.

Selection for Fe efficiency

Variability in tolerance to Fe deficiency chlorosis was observed among adventitiously regenerated quince clones. Four variants (No. 1 to 4) with higher tolerance than the parental line, *C. oblonga* Quince A, were selected from 1921 clones were more tolerant than the parental line. Two out of these (No. 1 and 2) grew vigorously in subsequent cultures under selective conditions (Figure 18A).

Differential Fe-deficiency chlorosis was also observed with plantlets after 20 days of Fe stress conditioning (Figure 18B). Selected adventitious *C. oblonga* clones number 1 and 2 (IE1 and IE2) had higher leaf chlorophyll content, higher Fe-reducing activity in their roots and a higher capacity to lower the medium pH than the original *C. oblonga* Quince A (Table 14). Although the Fe efficiency of these clones was not as high as that of the *P. amygdaliformis* controls, their chlorophyll content was comparable to Quince A conditioned in the presence of Fe (Figure13).



18. Shoots (A) and plantlets (B) of unselected *C. oblonga* Quince A (Left), clones
Center) and No.2 (Right) grown in the absence of Fe.

Table 14. Total chlorophyll concentration, Fe reduction in the roots, and medium pH after 20 days of conditioning of plantlets of unselected *C. oblonga* Quince A, clones numbers 1, 2, 3, 4 and 5, and *P. amygdaliformis* in the absence of Fe.

Genotype	Total chlorophyll (µg/mg fresh weight)		Fe reduction (nmoles Fe^{2+}/ g fresh weight x 24h)		Medium pH	
C. oblonga Quince A	2.0	c	396	c	4.2	c
C. oblonga No. 5	2.1	c	545	c	4.1	c
C. oblonga No. 4	2.3	c	666	c	4.1	c
C. oblonga No. 3	2.3	c	664	c	4.0	c
C. oblonga No. 2	3.1	b	1010	b	3.8	b
C. oblonga No. 1	3.2	b	1011	b	3.8	b
P. amygdaliformis	4.9	a	1625	a	3.5	a

Means were pooled from ten repetitions. Treatment means with the same letter are not significantly different as determined by the Fisher's Protected LSD ($p = 0.05$).

DISCUSSION

Adventitious shoot regeneration from leaves of *C. oblonga* and screening of shoots under Fe-stress *in vitro* conditions were used to recover two somaclonal variants, IE1 (Iron Efficient) and IE2, with higher tolerance to Fe-deficiency chlorosis. The Fe^{3+}-reducing activity and acidification of the medium by roots of plantlets were higher for IE1 and IE2 than the original *C. oblonga* clone. The selected variants could be useful pear rootstocks in calcareous soils.

As a prerequisite to studies of Fe-chlorosis *in vitro*, micropropagation and regeneration methodologies for *Pyrus* and *Cydonia* were optimized. Multiplication of all genotypes was adequate when 2cm long shoots were cultured on MS medium with 5μM BA at 40μmol $m^{-2}s^{-1}$, although it could be further promoted by increasing the BA concentration to 10 or 20μM and the light intensity to 135μmol $m^{-2}s^{-1}$. The orientation of the shoot (horizontal vs. vertical) also affected the multiplication rate, although not as much as observed with apple (Yae et al., 1987). Since BA as the only growth regulator gave excellent results, other growth regulators were not included in our experiments. Howerver, reports of beneficial effects of auxins (Bhojwani et al., 1984; Cheng, 1978; Marino, 1984; Stimart and Harbage, 1989; Wanas et al., 1986), gibberellins (Duron et al., 1989; Singha, 1986) and combinations of different cytokinins (Shen and Mullins, 1984) have been noted.

Leaf size was dependent on the genotype and culture conditions. The orientation of the shoots, light intensity, and BA concentration were found to affect leaf size of micropropagated shoots. Shoots formed on explants in horizontal position, higher light intensities, and lower BA concentrations generally had larger leaves. These leaves were

appropiate for organogenesis experiments.

Rooting experiments demonstrated that optimal conditions for root induction varied widely among *Pyrus* species. All *Pyrus* species responded well to the rooting regimes optimal for *P. betulaefolia*, and even the dip in IBA seemed sufficient for rooting of most of the *Pyrus* materials. However, *C. oblonga* did not root well after treatment with IBA but required exposure to NAA, which was inadequate for the *Pyrus* species. One or several of the treatments tested were appropriate to obtain a high percentage of rooting for each of the species examined. Suitable regimes could be established for each of the diverse materials including species such us *P. calleryana*, for which root formation has not been successful (Stimart and Harbage, 1989), and *P. amygdaliformis*, *P. communis* OPR-1 and *P. communis* Angers selection P-2462 for which it has not been reported before. The method of inducing root formation by dipping shoots in high concentrations of IBA should be of particular interest for commercial micropropagation since it eliminates a culture passage.

Although adventitious shoot regeneration from leaves has been reported for *P. communis* and *P. bretschneideri* (Chevreau et al., 1989), success had not been reported for *P. amygdaliformis* and *C. oblonga* species. This study shows that adventitious shoots can be regenerated from both species starting with leaves from micropropagated shoots. As reported before for apple (Welander, 1988) the use of nitrate, ammonium, and phosphate concentrations of N6 medium, instead of the higher concentration in MS medium, prevented browning of leaf discs. In addition, exposing leaves to a sequence of dark and light treatments improved regeneration compared to a continuous light regime of 16h.

TDZ was found to be more effective than BA in preventing leaf senescence and

inducing adventitious shoot formation. The two cytokinins, BAP (Browning et al., 1987; Fasolo et al., 1989; James et al., 1988; Welander, 1989; Predieri et al., 1989) and TDZ (Chevreau et al., 1989; Fasolo et al., 1989; Swartz et al., 1990) have been effective in stimulating regeneration from leaves of apple and pear. TDZ is generally more active and stable than adenine-type cytokinins (Mok et al., 1982; Mok and Mok, 1985). Thus the efficacy of TDZ in regeneration of quince may be due to the particularly high cytokinin requirement of this species. Addition of NAA was required to prevent leaf senescence and promote regeneration.

In contrast to results for apple or pear (James et al., 1988; Chevreau et al., 1989), additional vitamins, complex organic substances, glycine, putrescine, or sucrose did not enhance but slightly repressed regeneration in leaves of quince. Also, placing the leaves with abaxial part up or keeping the leaf cultures in the dark increased regeneration rates in apple (James et al., 1988; Welander, 1988; Fasolo et al., 1989; Pedrieri, 1989), but not in quince. Leaf irradiation reduced regeneration, which is in agreement with results obtained for apple (James et al., 1988; Pedrieri et al., 1989). Regeneration of quince was greatly improved by careful selection of leaves.

Anatomical studies showed that several shoots could be obtained at one site of regeneration, suggesting possible proliferation of axillary buds from the first shoot or the formation of multiple adventitious buds. Structural analyses of a number of tissues indicated the latter course of development. The occurrence of multiple adventitious shoots may indicate the presence of a number of competent cells close to each other and possibly of common origin. Alternatively, the first bud may stimulate formation of additional buds.

In vitro shoot cultures are very useful for studies of Fe-deficiency chlorosis. One

of the advantages is that large numbers of uniform shoots can be evaluated easily. In addition, experiments using a defined medium are much simpler than field evaluation in calcareous soils, since changes in medium ingredients and pH can be precisely administered. Although nutrient solution evaluations have been used (Coulombe et al., 1984; Tong et al., 1986; Zhou et al., 1984; Romera et al., 1989; Rashid et al., 1990) and offer some of the advantages of *in vitro* cultures, the physical arrangements are usually more complex and expensive, and the number of plants that can be evaluated is limited. Moreover, it is difficult to keep the solutions free of contaminating microorganisms.

The results show clearly that Fe-chlorosis can be induced *in vitro* by manipulating the culture medium. Reducing the chelator to Fe ratio increased Fe-deficiency chlorosis, as observed with apple seedlings grown in nutrient solutions (Tong et al., 1986). More importantly, the responses of *Pyrus* species and *C. oblonga* corresponded to those observed in the field. Quince A, a rootstock with extreme sensitivity to the presence of calcium carbonate in the soil (Lombard and Westwood, 1987), also displayed severe symptoms *in vitro*. *P. amygdaliformis* and *P. communis*, a tolerant and moderately tolerant species under field conditions, did not show any chlorosis, even when Fe was supplied in non-chelated form and bicarbonate was present. *P. betulaefolia* and *P. calleryana*, two intermediate species, were chlorotic only under the most Fe-limiting conditions *in vitro*. Interestingly, unrooted shoots showed very similar Fe-deficiency chlorosis to rooted shoots, indicating that the same mechanisms may be operative in rooted and unrooted shoots.

Two species, *C. oblonga* and *P. amygdaliformis*, were used to characterize the differences in tolerance to Fe-deficiency chlorosis. The total chlorophyll content in

unrooted or rooted shoots clearly reflected the higher tolerance of *P. amygdaliformis*. The genotypic differences in Fe-deficiency chlorosis could not be explained by differences in growth rates, or in total Fe content in the tissue. In both genotypes total Fe decreased with Fe-stress, although plantlets were less affected than unrooted shoots, possibly because roots could provide stored Fe to the shoots. However, the Fe^{2+} concentration and proportion of total Fe as Fe^{2+} was higher in *P. amygdaliformis*. The ability of *P. amygdaliformis* to maintain a higher level of Fe^{2+} in the tissue than *C. oblonga* was correlated with its higher Fe^{3+}-reducing activity.

Fe-stress conditioning or Fe depletion during culture increased reduction of Fe^{3+}EDTA in shoots, plantlets, roots and cell suspensions of the two genotypes. The increase in Fe^{3+} reduction, decrease of medium pH, and absence of secreted siderophores that chelate Fe^{3+} conform to the classification of these species as Strategy I plants (Römheld, 1987). Also the morphological changes occurring in Strategy I plants under Fe-stress such as swolled root tips and increased root hair formation close to the root tips, were more intense in *P. amygdaliformis* than *C. oblonga*.

Bicarbonate inhibited Fe reduction primarily in shoots and cell suspensions, but not as much in plantlets and root cultures. This may have been related to the higher ability of plantlets and root cultures to decrease the medium pH. In general, bicarbonate had only a slight effect, probably due to its low level in the medium. The concentration of HCO_3^- and CO_3^{2-} may be 1 to 2meq/l in agricultural soils, although it could be even as high as 5meq/l. The medium used in this study had HCO_3^- concentrations equivalent to the low range of what is found in the soil. Higher concentrations of $KHCO_3$ (5mM) resulted in explant death, probably because of the high pH (above 7).

In agreement with Bienfait et al. (1987), isolated roots had the same adaptive responses to Fe-stress as roots of plantlets indicating that roots are able to control the development of Fe-efficiency reactions on their own without a signal from the shoot. Although adaptive mechanisms to Fe-deficiency can be observed also in isolated roots, base of shoots, and at the cellular level, regulation in the whole plantlet may involve a signal from the Fe-deficient leaves to the roots as currently proposed (Marschner, 1986; Bienfait, 1988a).

The fact that roots, shoots, and cell suspensions had similar adaptive responses to Fe-stress indicates that genotypic differential tolerance to Fe-deficiency chlorosis may be the result of differences in Fe^{3+} reduction and H^{+}-pumping throughout the whole plant and not only in the roots. This is in agreement with reports on isolated roots (Bienfait et al., 1987; Zocchi and Cocucci, 1990), cell suspensions (Cornett and Johnson, 1989), and plasma membrane vesicles (Buckhout et al., 1989; Schmidt et al., 1990). Furthermore, the large differences in Fe^{3+} reduction between cell cultures of the two species suggest that the genetic potential can be realized even in unorganized tissues, which is in agreement with previous results in soybean (Sain and Johnson, 1984 and 1986; Naik et al., 1989).

Regeneration followed by *in vitro* screening has been used to recover somaclonal variants of *C. oblonga* with tolerance to low Fe. Screening of adventitious shoots has overcome the regeneration problems encountered by selection of cell lines (Sain and Johnson, 1986; Naik et al., 1989). A total of 1921 independent adventitious regenerants have been exposed to Fe-deficient medium. New leaves formed on most shoots were typically chlorotic, although shoots from four isolates were green. Further characterization of the adventitiously regenerated plantlets showed altered responses to

Fe-limiting conditions. Two variants of *C. oblonga* with higher tolerance to Fe-limiting conditions had a significantly higher ability to reduce Fe^{3+} and acidify the medium than the original *C. oblonga* clone. These variants, IE1 and IE2, could be a potential source of horticulturally superior rootstocks in calcareous soils.

The leaf regeneration system described here may be used to select somaclonal variants with other improved characteristics, such as salt-tolerance, and disease resistance (Viseur, 1987a; Hammerschlang, 1986a and 1990; Bajaj, 1990; Brisset et al., 1990). In addition, it may be adapted for *Agrobacterium*-mediated transformation of *C. oblonga*, as demonstrated for apple (James et al., 1989), which would be a first step towards genetic engineering of this pear rootstock. Eventually, Fe-efficiency of quince and other species may be enhanced by incorporation of genes promoting Fe^{3+} reduction.

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