DOCTORAL THESIS

Specific growth and micro elements absorption of Orange Jasmine (*Murraya* sp.) under the iron deficient environment

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GRADUATE SCHOOL OF BIOSPHERE SCIENCE
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We hereby recommend that the dissertation by Mrs. Cahyo Wulandari entitled “Specific growth and micro elements absorption of Orange Jasmine (Murraya sp.) under the iron deficient environment” be accepted in partial fulfillment of the requirement for the degree of Doctor of Agriculture.

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CHAPTER 1

General Introduction

1.1 Fe deficiency in citrus plants

Citrus fruit is familiar all over the world and is performed in the world fruit trade by the first of value although production shows geographical concentration in certain areas. Some important citrus farms have calcareous soils that are not suitable for citrus trees, for example in Florida, USA, and Mediterranean area such as Portugal, Spain, Italy, also Indonesia. Trees planted in these soils often suffer from Fe deficiency. The most prevalent cause of Fe deficiency is the presence of high levels of bicarbonate (HCO_3^-), leading to high pH and low availability of Fe, the condition known as lime-induced chlorosis (Pestana, 2005). Elevated HCO_3^- concentration makes Fe insoluble, thereby inhibits Fe uptake of roots and translocation into shoots (Nikolic et al., 2000). Besides Fe, P and other transition metals, which are essential micronutrients (including Cu, Mn, and Zn) can also be limited (Gries et al., 1998).

Fe deficiency affects the biochemistry, morphology, and physiology of the whole plant because Fe is an important cofactor for many enzymes, including those involved in the biosynthesis pathway of chlorophylls (Molassiotis et al., 2006).

In fruit trees, Fe deficiency in calcareous soils causes considerable loss of yield (Pestana et al., 2003), delayed fruit ravening, and impaired fruit quality as reported in peach and orange (Pestana et al., 2001). Based on Álvarez-Fernández`s et al. definition (2006), fruit quality is determined by fruit size, fruit color, firmness and amount of juice, and also the concentrations of chemical compounds such as organic acids, vitamins, phenolic compounds, etc., which may affect organoleptic characteristics. In such cases, commercial yield and marketable quality depend on the application of Fe fertilizers such as synthetic chelate, which do not represent a sustainable management approach due to high cost and potential pollution of the soil and water

environment (Rombola and Tagliavini, 2006). One of the best alternatives to prevent Fe chlorosis problems is introducing of Fe-deficiency tolerant plant species or genotypes.

1.2 Strategies of higher plants in Fe acquisition

Although Fe is one of the most abundant elements in the earth's crust, its availability to plants is very low under upland condition. Fe availability is dictated by soil redox potential and is reduced by soil pH increasing. Under the aerobic or higher soil pH, Fe is readily oxidized, and is predominately in the form of insoluble ferric oxides. At lower pH, the Fe^{3+} solubility is increased and become more available for the plant utilization (Morrissey and Guerinot, 2009).

Not only plants suffer from Fe deficiency, but also about two thirds of the world`s population is at risk of Fe deficiency induced anemia, the most prevalent nutrient-related human disease. Because plants are the primary source of Fe for humans, understanding the mechanisms that underlie Fe homeostasis is of interest for addressing agricultural problems and Fe malnutrition of humans. Besides these important consequences, the complex regulation of Fe nutrition in plants represents a fascinating series of adaptations to a limited resource (Schmidt, 2003).

Because of the fundamental Fe role in many vital processes, living organisms (including plants) have evolved mechanisms to acquired Fe. General agreement exists as to the existence of at least two distinct root responses mechanism (strategies) to Fe deficiency in higher plants. These mechanisms have been referred to as Strategy I and Strategy II, respectively (Marschner and Romheld, 1994). Strategy I exists in dicotyledonous and monocotyledonous species, with the exception of the graminaceous species (grasses) (Strategy II). Citrus trees are classified as Strategy I type plants. In Strategy I species, the response to Fe deficiency has various

components. The Fe uptake is enhanced by secretion of chelators, proton extrusion, ferric reduction and enhanced activity of a ferrous transporter in the root plasma membrane. Morphological and cytological changes, e.g. formation of root hairs and transfer cell in the root epidermis, have proved to be induced concomitantly with or prior to the expression of physiological responses; this has led to the assumption that structural and ultra-structural alterations are a prerequisite for the functioning of the Fe efficiency apparatus (White, 2012; Lansdberg, 1986).

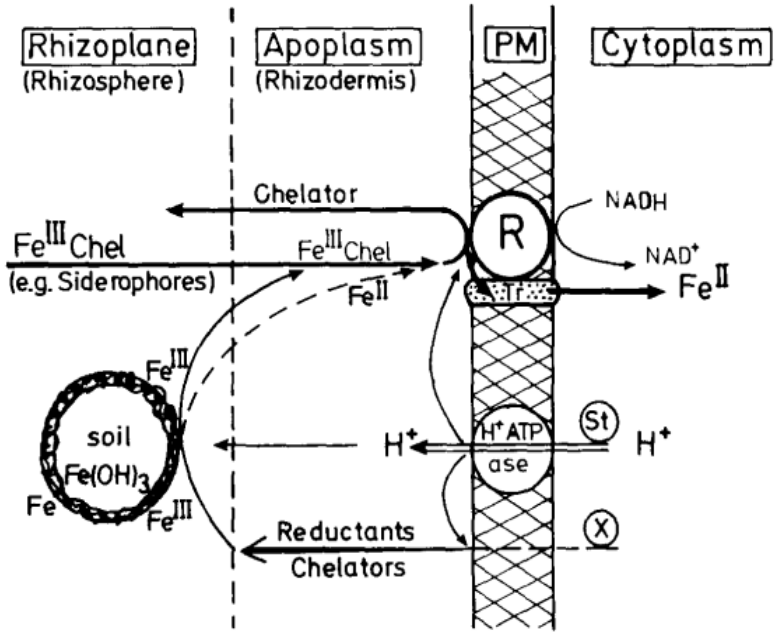

Figure 1.1 Model from root responses to Fe deficiency in dicots and non-graminaceous monocots: Strategy I. R=inducible reductase; Tr=transporter for Fe^{II}; St=stimulated H^+ pumping ATPase; X=increased production/release of chelateor/reductants (Modified from Marschner et al., 1986; Romheld, 1987 in Marchner and Romheld, 1994).

One of the best alternatives to prevent Fe chlorosis problems is introducing of tolerant plant species or genotypes. Differences in tolerance have been found in many herbaceous and woody species. In fruit trees, it is common to use the commercial varieties grafted on tolerant rootstocks, which the plant tolerance is mainly determined by the rootstock genotype. The search for new citrus rootstocks with better performance than currently used (including Fe

tolerant ones) is the major aim of the citrus industry in many countries. The different methods have been used for selecting tolerant plant material. The most general approach is field experiments, growth in pots with calcareous soil and hydroponic culture with high bicarbonate and low Fe concentration. In hydroponic culture the plants are grown under controlled and uniform conditions, and so avoid the variability of soil and environmental conditions characteristic of field experiments (Alcantara et al., 2003). Those methods require many months or years because the citrus plants grow slowly. A much short experimental period is clearly required.

1.3 Orange Jasmine

Orange Jasmine (*Murraya* sp.) is also known as mock orange, satin wood, honey bush, China-box, café de la India. It is an evergreen shrub or occasionally a small tree, usually 2 to 3 m in height but reaching 7.5 m and 13 cm in stem diameter. Older Orange Jasmine normally has multiple stems from the ground level. The stems are supported by taproots with lateral roots and abundant fine roots. The native range of Orange Jasmine includes India to Malaya. In addition, the shrub has been planted throughout the tropics and has naturalized in many locations (Pacific Island Ecosystems at Risk, 2002).

Orange Jasmine is adapted to a wide range of areas that receiving from about 750 mm to 1,900 mm of annual precipitation. These species also grows from nearly sea level to elevations of 1,300 m. It grows on most well-drained soils derived from both sedimentary and igneous rocks, although said to favor limestone areas. Plants survive temperatures to about – 4 º C (Pacific Island Ecosystems at Risk, 2002). Orange Jasmine is widely cultivated as an ornamental

tree or hedge because of its hardiness, wide range of soil tolerance (alkaline, clay, sand, acidic, loam, drought), and suitable for larger hedges (Gilman, 1999).

The resistance of fruit trees to Fe deficiency is determined by the rootstocks which influence the factors such as tree vigor, mineral nutrition, water balance, and finally fruit yield and quality. Management of orchards using resistant rootstocks would represent a better alternative than common remediation strategies. Unfortunately, there are a few studies about Fe deficiency tolerance of citrus rootstocks with favorable agronomical characteristic compared with the study about diseases resistance of citrus rootstock, such as the citrus tristeza virus (CTV). For example, sour orange (*Citrus aurantium* L.) is a commonly used commercial rootstock because it leads to production of high quality fruit and increased resistance to several diseases and adverse conditions, including calcareous soils. However, the use of this citrus rootstock is limited to areas that are still free from CTV (Pestana et al., 2011).

One of the alternative ways to search for potential citrus rootstocks is using related citrus species for selecting tolerant plant material. Orange Jasmine is one of the related citrus species having a potential as a citrus rootstock. However, there is a little information on Fe tolerant of Orange Jasmine except for ferric chelate reductase (FCR) activity (Castle et al., 2009).

1.4 Nutritional Fe and Mn Interaction

Citrus trees are widely cultivated in tropical and subtropical climates. In tropical regions, citrus mainly cultivated in acidic soils that usually show soil Mn excess. On the other hand, in subtropical area the main citrus industries are located in calcareous soils that show Fe and also Mn deficiency problem.

In plant nutrition, Fe and Mn are closely related because of similar properties. It is well documented that two elements are antagonistic in microelements absorption by plants and one may inhibit the uptake of the other and vice versa. Normally, when Fe availability is lower in soils, Mn absorption increases (Sanchez-Raya et al., 1974) in the roots of both Fe-sensitive (Heenan and Campbell, 1983; Leidi et al., 1987) and Fe-tolerant (Alam et al., 2001b) plants. Increasing Mn absorption under Fe deficient condition can introduce Mn toxicity. Manganese is an essential element for plants, mainly for photosynthesis and as an enzyme antioxidant-cofactor. Nevertheless, an excess amount of Mn reduces biomass and photosynthesis, and causes biochemical disorder such as oxidative stress (Millaleo et al., 2010). In any case, the use of different genotypes of rootstocks for citrus tree may permit the plant to overcome antagonistic conditions associated with active lime (calcareous soil) and/or Mn excess (acidic soil) resulting in no impact on growth and production of cultivars.

1.5 Objectives

The present study was performed to clarify the Fe-deficient tolerance responses of Orange Jasmine and compare them with the Fe-sensitive plant Trifoliate Oranges (*Poncirus trifoliata* spp.) that is known as susceptible citrus rootstocks to Fe deficiency (Castle et al., 2009; Benyahia, 2011).

1. To clarify Fe-deficient tolerance responses of Orange Jasmine within a shorter experimental period in uniform medium conditions with easiness to control (water culture).

2. To examine the influences of Fe treatments in the water culture on the Mn absorption of Orange Jasmine under four different Mn concentrations.
3. To confirm what kind`s functions do Orange Jasmine exists when grown under high pH soil condition.

CHAPTER 2

Responses of Orange Jasmine

Under Fe Deficiency

2.1 Introduction

The Fe is an essential nutrient for plant growth and development. In spite of abundance in soils, Fe often forms highly insoluble ferric hydroxide precipitates that limit its availability to plants (Guerinot and Yi, 1994). Plants like soybean show different susceptibilities to Fe deficiency and these differences were shown to be related to root reduction by Brown (1961). Two absorption strategies have been proposed in plants that are tolerant to Fe deficiency (Marschner et al., 1986) while plant metabolic responses to Fe deficiency are well documented (Brown and Jolly, 1989). Nongraminaceous monocots and dicots (Strategy I plants) induce the activity of FCR on the root cell membrane, an enzyme involved in the conversion of Fe^{3+} to Fe^{2+}. Strategy II function is observed in monocots, which exude mugineic acid from the root to dissolve Fe^{3+} compounds. Compared to Strategy II plants, Strategy I plants have many complex functions that involve not only FCR but also proton extrusion (Brown and Ambler, 1974; Brown and Jolley, 1989). Fe^{3+} reduction has been used to predict the resistance of Fe-deficiency in soybean (Jolley et al., 1992), whereas the release of H^{+}, reductants and reduction of Fe^{3+} to Fe^{2+} in tomato and soybean roots have been documented (Camp et al., 1987). In addition, Fe deficiency stress causes root branching (Hagström et al., 2001; Jin et al., 2008) and ferric chelate compound exudation (Noguchi et al., 1994) which also chelate Fe^{3+}. Cucumber grows thin lateral roots (Dell'Orto et al., 2002), while red clover has a different root branching pattern (Jin et al., 2008). Many kinds of phenols are released from Fe deficient alfalfa (Koshino et al., 1993) and red clover (Jin et al., 2007) roots.

Citrus trees are classified as Strategy I type plants. However, there is little information about their Fe dissolving capability, which presents a problem because of their sensitivity to Fe deficiency, especially plants in commercial production. Screening has identified some Fe tolerant

species suitable as rootstock by focusing on FCR activity (Brown and Jolley, 1988; Castle et al., 2009), leaf chlorosis (Benyahia et al., 2011), leaf chlorophyll fluorescence (Pestana et al., 2005), and extrusion of phenolic compounds and protons (Treeby and Uren, 1993). These studies required many days because citrus plants grow slowly. Fe-deficiency treatments require between 6 and 10 weeks (Castle et al., 2009, Benyahia et al., 2011, Pestana et al., 2005). A shorten experimental period is clearly needed by comparing responses of Fe-deficient tolerant rootstock, such as Orange Jasmine (OJ) (Castle et al., 2009), and a Fe-deficient sensitive rootstock, Flying Dragon (FD, *Poncirus trifoliata* var. *monstrosa* (T. Ito) Swingle) (Benyahia et al., 2011). Orange Jasmine is a promising rootstock, as it favors limestone soils (Stone, 1970). However, there is no information on its response to Fe deficiency except for its FCR activity (Castle et al., 2009).

Therefore, this study was conducted to investigate the time course of FCR activity, proton exudation, root development, chlorophyll content, SPAD value, biomass production and Fe, Cu, Mn and Zn concentration.

2.2 Materials and Methods

2.2.1 Analysis of FCR activity

Orange Jasmine (OJ) seeds, imported from the United States of America, and Flying Dragon (FD) seeds, obtained from the Agricultural Technology Center of Hiroshima Prefecture, Japan, were soaked in distilled water overnight at 30°C.

After rinsing three times, the seeds were transferred to multiple pots containing a mix of commercial soil and peat moss (Ikubyou baido, Takii Seed Co, Kyoto, Japan) for germination at 30°C. About three months later, when the plants had grown to approximately 10 cm high, each root was washed with tap water and soil was gently removed from the root surface. Containers

holding 24 L were prepared for hydroponic culture, filled with a complete nutrient solution and aerated continuously. The solution had the following composition (in μM): $Ca(NO_3)_2$, 3,000; $MgSO_4$, 500; NaH_2PO_4, 300; K_2SO_4, 200; H_3BO_3, 3; $ZnSO_4$, 0.4; $CuSO_4$, 0.2; $MnCl_2$, 0.5; $(NH_4)_6Mo_7O_{24}$, 0.01 and Fe EDTA, 100. The container top was covered by a plastic plate, which had 24 of 2 cm diameter holes each. Plants having nearly the same height and size were selected. Three plants were transfered into each hole of the container's top plate as one treatment unit. Replication was in triplicate. In initial experiments, when the Fe concentration in hydroponic culture was 20 μM, the symptoms of Fe deficiency appeared in young leaves of FD, but not in OJ. Therefore, we used 100 μM Fe to avoid this problem. The pH of nutrient solution was adjusted to 5.5 twice a day, in the morning and evening, using 1 N HCl. The nutrient solution was renewed every 3 days. The experiment was carried out in a growth chamber with a 16 h light-period at 30°C and an 8 h dark period at 28°C. After two weeks of pre-culturing in the complete nutrient solution, Fe-deficiency treatment started and continued for 21 days to compare the Fe deficiency influences between OJ and FD. Roots were sampled every 3 days after the initiation of treatment, on days 3, 6, 9, 12, 15, 18, and 21. Roots near the tip were sampled and used to measure FCR activity according to Zheng et al. (2003). About 0.5 to 1 g of excised roots (< 5 cm from the root tips) was placed in 10 mL of assay solution in a plastic test tube. The assay solution contained 50 mM Tris (2-amino-2-hydroxymethyl-1, 3-propanediol), 0.5 mM $CaSO_4$, 0.3 mM 4,7-diphenyl-1,10-bathophenanthrolinedisulfonicacid (BPDS) and 50 mM Fe EDTA. The pH was adjusted to 7.5 with H_2SO_4. The tubes were placed in an air-conditioned laboratory with the temperature of 26 ± 2°C for 90 min with periodic swirling once every 10-15 min. The absorbance of the Fe (II)$[BPDS]_3$ complex at 535 nm was recorded by a spectrophotometer

(Hitachi U-3310). The concentration of Fe (II) in the reaction medium was calculated based on an Fe (II) calibration curve.

2.2.2 Proton extrusion, root development, leaf greenness and chlorophyll analysis

The OJ seeds, imported from China, and FD seeds, obtained from the Agricultural Technology Center of Hiroshima Prefecture, Japan were used. The methods for germination and growth in hydroponic solution were almost similar to the experiment 1. The differences were the treatment unit and long period of experiment. In this experiment, one plant was transfered into each hole of the container top plate as one treatment unit, and Fe-deficiency treatment started and continued for 28 days.

To visually confirm proton extrusion, roots treated for 28 days of Fe deficiency were placed in a polystyrene 9 cm diameter petri dish on 0.75% (w/v) type II agarose (Sigma Chemical Co.) containing a pH indicator (bromocresol purple, 0.006%) dissolved in 0.5 mM $CaCl_2$ at pH 6.5 according to the method of Römheld et al. (1984). The color pattern was recorded after 30 min of incubation.

The leaf greenness of the youngest new expanded leaves was analyzed with a SPAD-502 Chlorophyll meter (Minolta Camera Co. Ltd., Japan) at the end of the experiment. After leaf greenness recording, the chlorophyll concentration was determined at the same leaf according to the Lichtenthaler (1987) method. The sub-apical leaves (1 to 2 leave(s) per plant) were harvested, immediately weighed, crossed in disc, and then used for the extraction of chlorophyll assessment. Two milliliters of 80 % acetone were added to fresh leaf samples (approximately 200 mg). The extraction took place after 72 h in darkness, at room temperature. The extract's absorbance was measured at 645 and 663 nm.

2.2.3 Plant growth and micro mineral analysis (Fe, Cu, Mn and Zn)

The influence of Fe deficiency on biomass production and the balance of Fe, Cu, Mn and Zn in the roots and shoots were compared between the two citrus rootstocks. Seeds of OJ and FD were soaked and cultured following the method used in the experiment 1. After 21 days of Fe-deficiency treatment in aqueous culture, the roots and shoots were rinsed with distilled water three times; the excess root moisture was removed by blotting on a paper towel. The roots were separated from the shoots. Each OJ shoot had branches with complex leaves; the upper one-third of branches was segregated as upper leaves and the other leaves were grouped as lower leaves. In this time, FD did not have branches, so upper leaves were taken from the top one-third of the stem. After removing all leaves, the tree trunks and stems were separated from the leaves and mixed, with both trunks and stems considered as stem tissue. The fresh and dried (at 70°C for 48 h) weights of each plant part were determined. Dried samples were ground (1 mm screen) by hand and digested in concentrated HNO_3 and $HClO_4$. Fe, Cu, Mn and Zn were analyzed by Inductively Coupled Plasma (ICP) (iCAP 6000, Thermo Fisher Scientific Inc., UK).

2.2.4 Statistics

All the statistical analyses were performed using the SAS software (SAS Institute). Means were compared using Student's t-test at $P < 0.05$ and $P < 0.01$ in all cases.

2.3 Results

2.3.1 FCR activity

For each of the three experiments, it observed that new leaves yellowed under Fe deficiency at one week after the initiation of stress and the yellowing became more evident each day. The FCR activity of the Fe-deficient OJ roots started to increase after 12 days of treatment (Figure 2.1). The activity increased to about two times higher than the initial activity after 15 days of treatment but decreased by 21 days. During Fe-deficiency treatment, FCR of OJ showed numerical values that were consistently higher than those of FD. The differences in FCR activity changes in FD were almost negligible for the entire 3 weeks treatment.

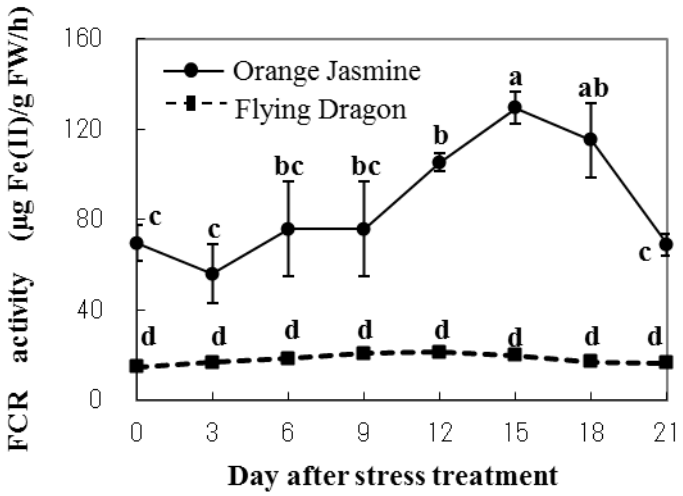

Figure 2.1 Effect of Fe deficiency on ferric chelate reductase (FCR) activity of roots after 21 days treatment. Data are the means ± SD (n=3). Values with the same letter are not significantly different at $P < 0.05$ in each plant.

2.3.2 Proton extrusion and root development

Figure 2.2 and 2.3 show the results of the experiment on proton extrusion based on a colored pH indicator. Turning yellow from purple on the root surface indicates proton extrusion. This reaction was noted 30 min after the start of the experiment. Fe-deficient OJ roots showed an intense yellow, which means proton extrusion was enhanced in the Fe-deficient roots. Fe-sufficient OJ roots showed a little yellow, which means proton extrusion was also detected although activity was not very high. Proton extrusion was observed slightly in FD roots. Figure 2.4 showed the increased formation of lateral roots and root hairs of Fe-deficient OJ at 28 days treatment. The OJ roots began to increase formation of lateral roots and/or root hairs from 10 days or 2 weeks treatment and concomitant with chlorosis development.

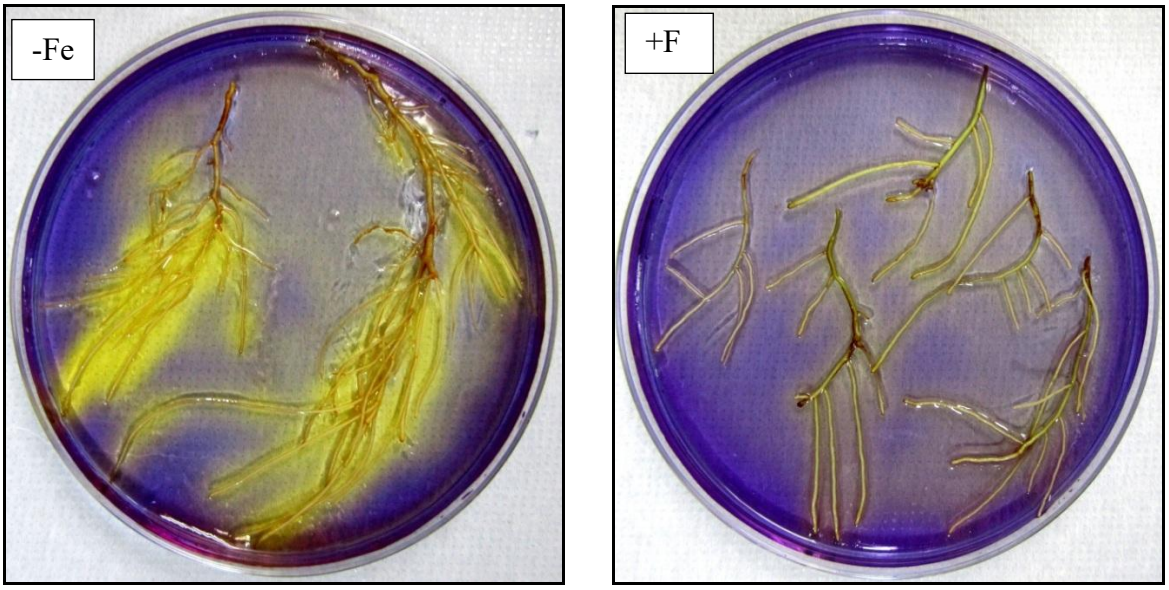

Figure 2.2 Effects of the Fe treatments on proton extrusion in the Orange Jasmine (OJ) roots after 28 days treatments. Yellow color indicates proton pattern recorded after 30 min of incubation.

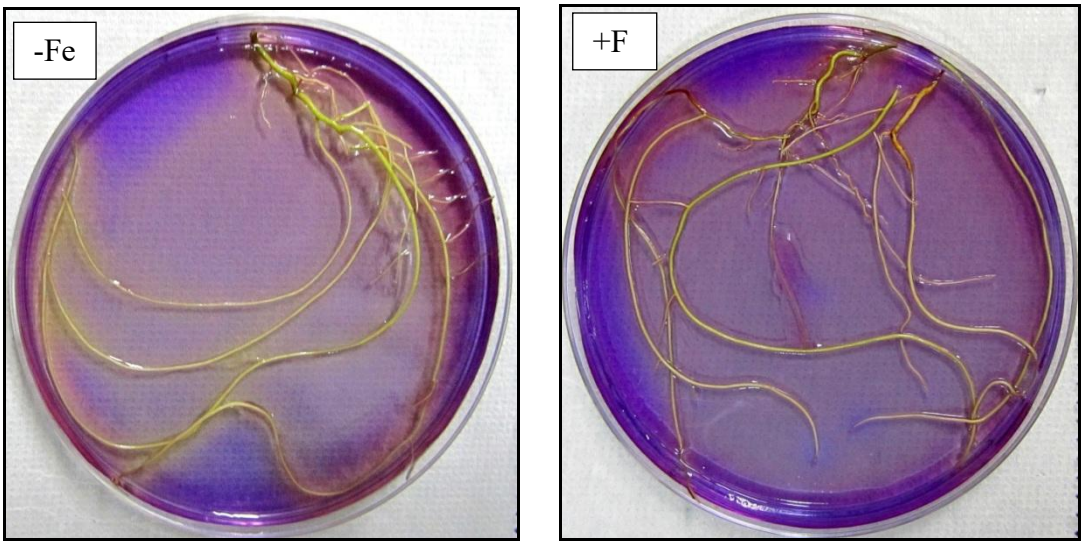

Figure 2.3 Effects of the Fe treatments on proton extrusion in the Flying Dragon (FD) roots after 28 days treatments. Yellow color indicates proton pattern recorded after 30 min of incubation.

Figure 2.4 Effects of the Fe treatments on the formation of lateral root and root hairs of Orange Jasmine (OJ) after 28 days treatments.

2.3.3 Leaf greenness and chlorophyll content

In Fe-sufficient treatment, no significant difference was recorded in the leaf greenness (SPAD value) and chlorophyll content of both plants. However, differences appeared under Fe-deficiency conditions. Fe deficiency led to decrease of leaf greenness and chlorophyll content in both plants, but the reduction rate was lower in OJ than FD (Figure 2.5).

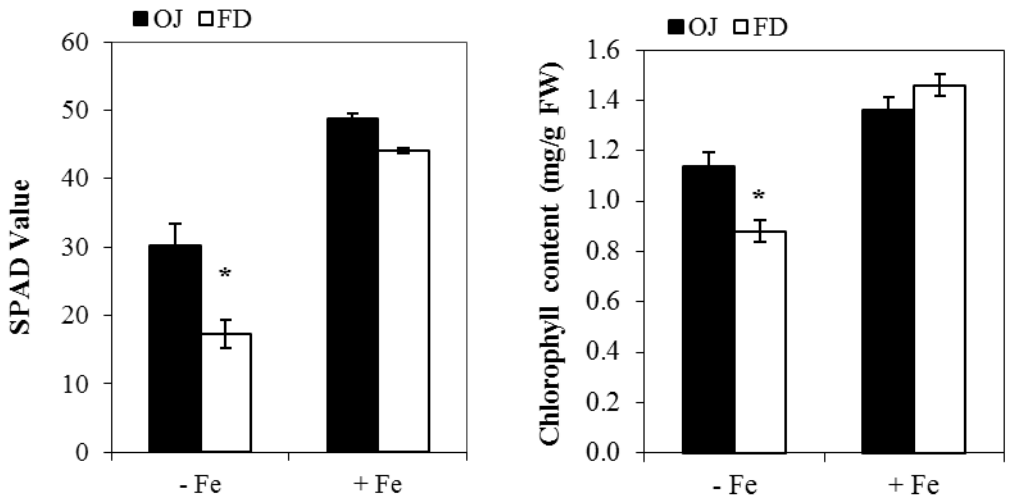

Figure 2.5 Effects of the Fe treatments on SPAD value and chlorophyll content in Orange Jasmine (OJ) and Flying Dragon (FD) after 28 days treatments. Data are the means ± SE (n = 3). * and **, significant differences ($P < 0.05$ and $P < 0.01$) between the two treatments.

2.3.4 Biomass production

Figure 2.6 shows the biomass production after 21 days of Fe-deficiency treatment. Though the biomass of each plant was small because of the young plant age, the mean biomass increased after 21 days treatment compared to the initiation stage. The increase in root dry weight of OJ was two times greater under Fe deficiency than Fe sufficiency but was 16% lower that of Fe-deficient than Fe-sufficient FD plants. No significant difference in OJ shoot dry

weight was observed between Fe treatments, but FD shoot biomass decreased 46% under Fe-deficiency treatment.

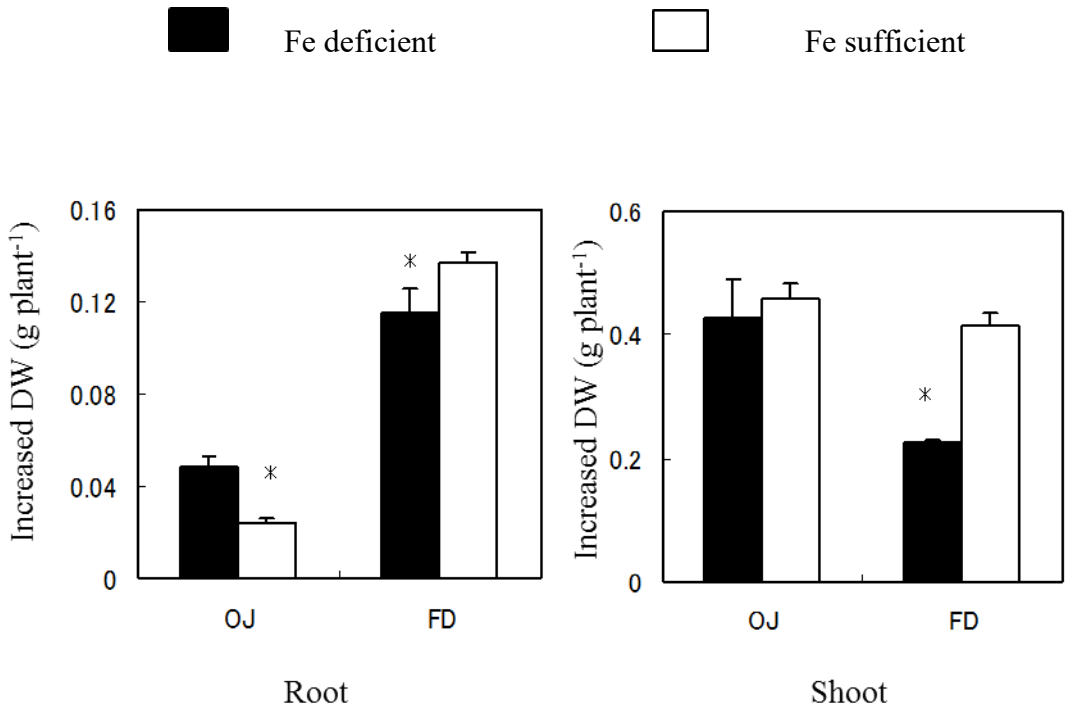

Figure 2.6 Effects of Fe treatment on increased root and shoot dry weight of Orange Jasmine (OJ) and Flying Dragon (FD) after 21 days treatment. Each value shows the dry weight of one plant. Data are the means ± SE (n = 3). * and **, significant differences ($P < 0.05$ and $P < 0.01$) between the two treatments in each species.

2.3.5 Fe, Cu, Mn and Zn concentration in roots, leaves and stems

Figure 2.7 and 2.8 show Fe, Cu, Mn and Zn concentration in the roots and shoots of OJ and FD. The Fe concentration in the Fe-sufficient roots of OJ was two times higher than in FD. The higher Fe concentration in the Fe-sufficient OJ roots was noteworthy among these four microelements, although the Fe concentration in the OJ lower leaves was one-third lower than in FD. The concentrations of the four microelements were lower in OJ leaves than in FD leaves.

The Cu, Mn and Zn concentrations of OJ were about one half to one quarter lower than in FD in leaves and stems.

Fe deficiency increased the concentration of Cu, Mn and Zn in FD roots. This phenomenon was also observed in the leaves and stems (Figure 2.8). The Fe concentration in OJ upper leaves was significantly different between Fe-deficiency (20 μg g^{-1} DW) and Fe-sufficiency (44 μg g^{-1} DW) treatments, although the Fe concentration in lower leaves was not significantly different between treatments. In FD, the Fe concentration in all plant parts of Fe-deficient plants including the roots, both upper and lower leaves, and stems, was significantly lower than in Fe-sufficient plants. In the upper leaves, the Fe concentration was 43 μg g^{-1} in Fe-deficient and 83 μg g^{-1} in Fe-sufficient plants, and in the lower leaves, was 108 and 135 μg g^{-1}, respectively, on a dry weight basis.

An Fe-deficient OJ roots showed a remarkable increase in concentration of Cu, but the stems and leaves no change. The Cu concentration in OJ roots increased from 5 μg g^{-1} DW under Fe-sufficiency to 64 μg g^{-1} DW under Fe-deficiency treatment. The Cu content of FD roots increased from 7 μg g^{-1} DW under Fe-sufficiency to 49 μg g^{-1} DW under Fe-deficiency treatment. There was 12 times more Cu accumulation than the controls in OJ and 7.1 times more in FD.

Notably, the Mn concentration of OJ decreased in Fe-deficient roots to 220 μg g^{-1} DW compared to the value under Fe-sufficient of 251 μg g^{-1} DW, although the Mn content of Fe-deficient FD roots was two times more than the control (228 μg g^{-1} compared to 110 μg g^{-1}). The Zn concentration of the FD roots under Fe deficiency increased almost four fold over the control (193 μg g^{-1} compared to 52 μg g^{-1} DW), although the Zn concentration of OJ roots was only two times higher under Fe deficiency (60 μg g^{-1} compared to 26 μg g^{-1}). These responses of

a decrease in Mn concentration and an increase in Zn concentration being regulated by Fe deficiency in OJ were also apparent in the leaves and stems.

The higher Fe shoot/root ratio at the end of the treatments was shown (Figure 2.9). It ratio was 1.6 times higher in OJ and 0.7 times lower in FD compared to the initial stage. Each ratio was lower at 21 days in Fe-sufficient plants of both OJ and FD. A larger decline of 3.1-fold at 21 days was seen in Fe sufficient OJ compared to the initiation of Fe deficient because of the extensive Fe accumulation in the roots; in FD, it was only a 2.1-fold difference.

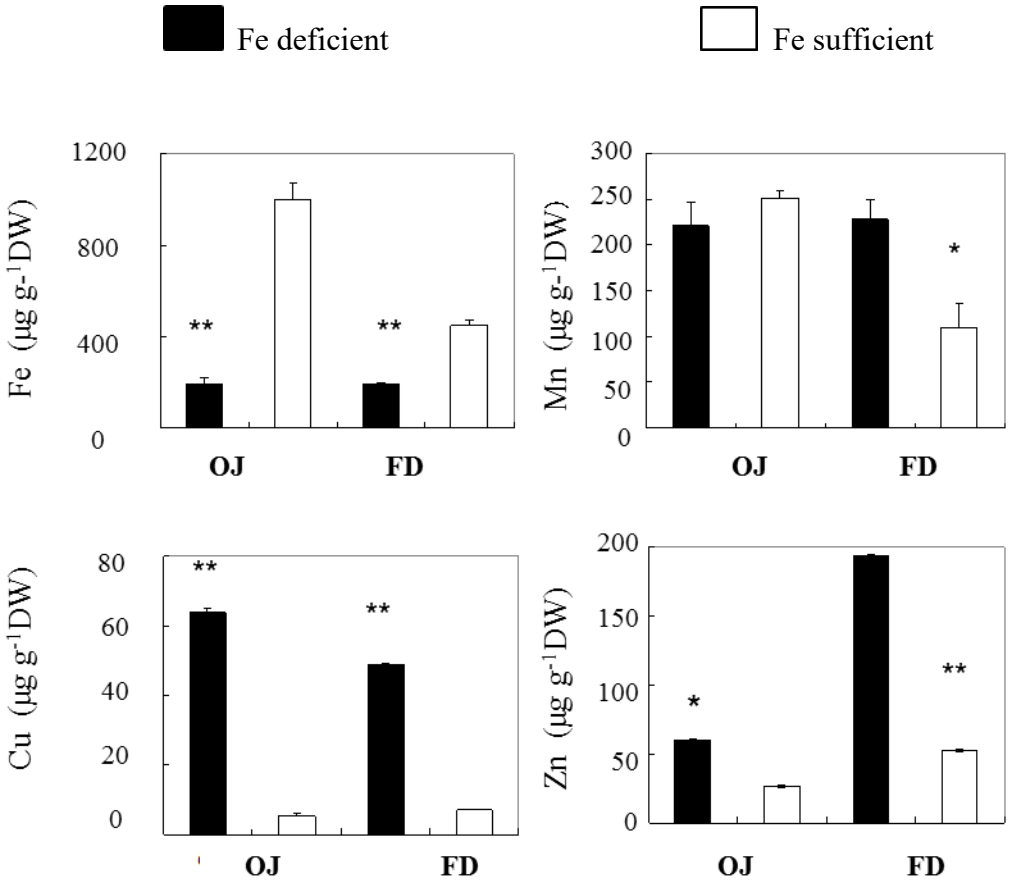

Figure 2.7 Effect of Fe treatments on Fe, Cu, Mn and Zn concentration in Orange Jasmine (OJ) and Flying Dragon (FD) roots. Data are the means, ± SE (n = 3). * and **, significant differences ($P < 0.05$ and $P < 0.01$) between the two treatments in each species.

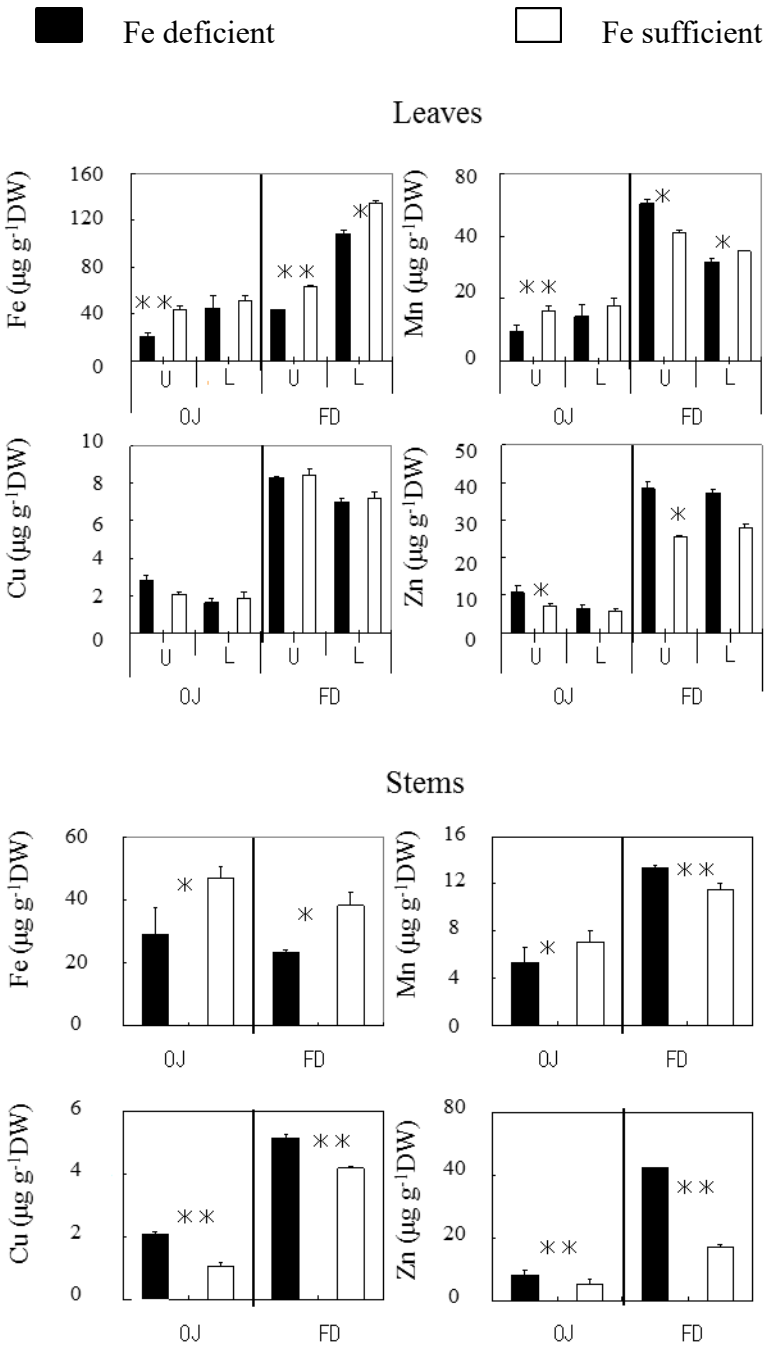

Figure 2.8 Effect of Fe treatments on Fe, Cu, Mn and Zn concentration in Orange Jasmine (OJ) and Flying Dragon (FD) leaves and stems. U = upper leaves and L = lower leaves. Data are the means, ± SE (n = 3). * and ** are significant differences ($P < 0.05$ and $P < 0.01$) between Fe treatments in the same organ of each plant.

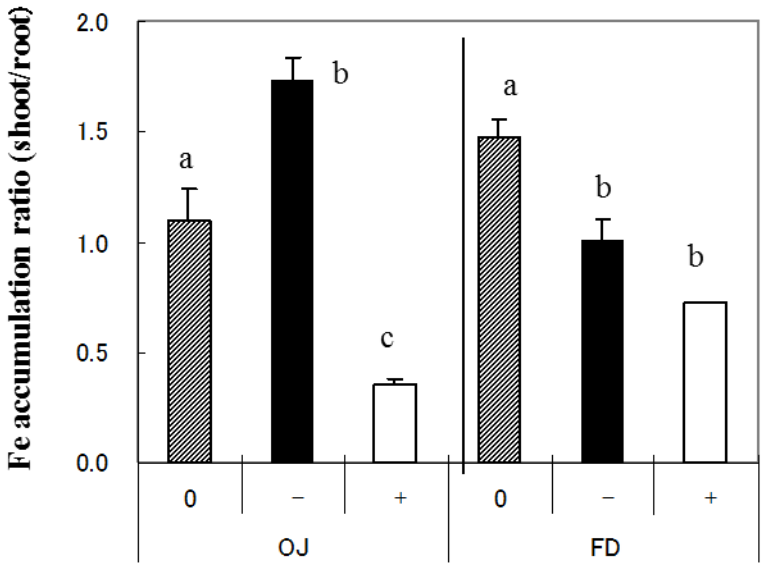

Figure 2.9 Effect of Fe treatments on Fe accumulation ratio (shoot/root) in Orange Jasmine (OJ) and Flying Dragon (FD) after 21 days of treatment. 0 = plants at the initiation of Fe-treatments. - and + mean 21 days treated plants under Fe treatments . Data are the means ± SE (n = 3). Values with the same letter were not significantly different ($P < 0.05$).

2.4 Discussion

2.4.1 FCR activity

The time course of FCR activity in OJ was found to be similar to that of red clover, an annual plant (Zheng et al., 2003), though OJ showed one enhanced peak. Reduction activity of Fe^{3+} to Fe^{2+} increased and peaked at 15 days after treatment (Figure 2.1). Fe reduction has been suggested as a major contributor to a suite of activities resulting in Fe uptake and utilization (Camp et al., 1987). FCR activity depends on the Fe concentration in the root apoplast and whether Fe can be utilized (Bienfait et al., 1985). The Fe accumulation in the root apoplast and translocation of large quantities of Fe to the shoot are considered important characteristics of Fe-deficiency tolerant in soybeans (Longnecker and Welch, 1990). The shoot/root Fe ratio increased

in Fe stressed OJ, but not in FD. Because OJ more accumulated Fe in the root, much Fe might be stored in the root apoplast and would be reused due to enhanced FCR activity. The observation of a decrease in FCR activity in red clover is indicative that Fe from the root apoplast pool could be consumed and that permanent damage could occur after a further decrease in FCR (Zheng et al., 2003). The OJ roots still looked healthy, with no apparent damage after 21 days of treatment. However, it can be confirmed the classification of OJ as a Fe-tolerant plant based on its responses to 21 days of Fe-deficiency treatment. It regards OJ as showing the typical Fe acquisition strategy, in which proton release is synchronous with an increase in FCR activity.

2.4.2 Proton extrusion

The OJ grown in the absence of Fe within 28 days exhibited stronger proton extrusion than in FD (Figure 2.2 and 2.3). This meant that OJ had a greater tolerance to Fe deficiency than FD, because active proton extrusion induced by Fe-deficiency stress is a sophisticated and important mechanism that involves other biochemical and physiological processes in plants and chemical processes in soil. The pH decrease in the rhizosphere favors an increase in the mobilization of sparingly available inorganic Fe^{3+}, because the solubility of Fe^{3+} depends on pH. For example, pH decrease from 8.0 to 4.0 will increase the concentration of Fe^{3+} from 10^{-20} to 10^{-8} M (Romheld and Marschner, 1986). Proton extrusion has also been proposed to play role in the stimulation of Fe (III) chelate reductase activity (Toulon et al., 1992) since these two responses occur simultaneously and the inducible reductase has a distinct optimal pH around 5.5 (Moog and Bruggemann, 1994). Many authors have also found high proton extrusion by Fe-deficient roots in linked to an increase in the synthesis and in the accumulation of organic acids, in particular citric and malic acids (Landsberg, 1981; de Vos et al., 1986). The link between higher

proton extrusion and enhanced synthesis of organic acids could be explained by the fact that the proton efflux causes cytoplasm alkalinization, which in turn activates the dark fixation of CO_2, a reaction that is catalyzed by the PEP carboxylase (pH-stat theory) (Bienfait et al., 1989).

From those facts, the determination of medium acidification capacity (decrease nutrient solution or the rhizosphere pH and proton extrusion) has been used as one of the screening technique for selecting Fe chlorosis tolerant plants (Gao and Shi, 2007; Gogorcena, 2001; Jejali et al., 2010; M'Sehli et al., 2008).

2.4.3 Leaf greenness and chlorophyll content

As shown in the results, leaf greenness (SPAD value) and chlorophyll content of young leaves was significantly affected by Fe deficiency in both plants (Figure 2.5), resulting in the appearance of Fe chlorosis. Fe-deficient plants of OJ were more able to maintain greater leaf greenness and chlorophyll content compared to FD, suggesting that OJ is more tolerant to Fe chlorosis. This was also observed in several other Fe-tolerant species (Mahmoudi et al., 2007; Marschner and Romheld, 1994; Ksouri et al., 2006; Pestana et al., 2005).

2.4.4 Biomass production

The OJ root biomass increased under Fe-deficiency treatment. This response was similar to that observed in *Spathiphyllum* (Yeh et al., 2000) and *Medicago ciliaris* (M'Sehli et al., 2008). Although Pestana et al. (2005) reported that Troyer citrange (*Citrus sinensis* (L.) Osb. X *Poncirus trifoliate* (L.) Raf.) and Taiwanica orange (*Citrus taiwanica* Tan. And Shim.) plants are tolerant to Fe deficiency, they had less root biomass under Fe-deficient than under Fe-sufficient conditions. Red clover maintains shoot and root biomass (Zheng et al., 2003) by developing

lateral root hairs and increasing their numbers (Jin et al., 2008). There was almost no difference in dry weight between Fe-deficiency and Fe-sufficiency treatments of the shoots of OJ. New leaves of OJ emerged and grew under Fe deficiency. OJ might have better carbon and Fe transfer from the roots into the shoots that allowed a biomass increase compared to FD.

The increase of root biomass of OJ in Fe-deficiency treatment may be explained by several reasons. A general characteristic of Fe-deficient plants is an increase in the concentration of organic acids in cells, in particular malate and citrate (Landsberg, 1981). A hypothesis is that the increased amounts of citrate are a consequence of cytoplasmic alkalinization upon proton pumping and subsequent induction of the biochemical pH-stat mechanism. The increase in the activity of PEP carboxylase in conditions of low Fe (Rabotti et al., 1995) also produces more organic acids through the involvement of anaplerotic CO_2 fixation that at the end would increase the fresh weight of the root. Another reason is a relative higher growth of the roots in soil with HCO_3^- and low available Fe, as in calcareous soils, can be a mechanism for increasing the soil volume exploration by the roots, which can help with acquisition of elements with low nutrient availability like P, K, Fe, Zn, Mn and Cu (Rengel, 2001).

2.4.5 Fe, Cu, Mn and Zn concentration in roots, leaves and stems

The Fe concentration of OJ roots was almost three times higher than FD. The Fe ratio of shoot/root increased in Fe-deficient OJ, but not in FD. Because OJ accumulated Fe in the roots, much Fe might be stored in the root apoplast and would be reused due to enhanced FCR activity (Jin et al., 2007).

In Fe-deficiency treatment, Cu concentration in OJ roots was over 60 $\mu g\ g^{-1}$ DW and was 12 times higher than in Fe-sufficient roots. Kuhns and Sydnor (1976) reported that this amount

was not toxic and was lower than the levels in plants like Delaware Valley azalea (*Rhododendron obtusum* Planch), common boxwood (*Buxus sempervirens* L.) and spreading cotoneaster (Cotoneaster divaricata Rehd. & Wils.)**.** An annual plant, Rhodes grass (*Chloris gayana* Knuth.), showed no changes in the growth of roots or shoots under the same conditions (Sheldon and Menzies, 2005). The Cu concentration of OJ in Fe-deficiency treatment is considered to be not a limiting factor for its growth.

The Mn concentration in OJ roots decreased under Fe-deficiency conditions, in contrast to FD. Normally, Fe and the other metal elements, like Cu, Mn and Zn, have antagonistic effects on root absorption. When Fe concentration is lower, Mn absorption increases (Sanchez-Raya et al., 1974) in the roots of both Fe-sensitive plants (Heenan and Campbell, 1983; Leidi et al., 1987) and Fe-tolerant plants (Alam et al., 2001b). Mn is an essential element for plants, mainly for photosynthesis and as an enzyme antioxidant cofactor. Nevertheless, an excess of Mn reduces biomass and photosynthesis, and causes biochemical disorders such as oxidative stress (Millaleo et al., 2010). Mn excess decreases CO_2 assimilation and also oxidizes root tissue (Li et al., 2010). Nevertheless, the levels of some antioxidant enzymes and stress inducible proteins increase to protect against metal stress in Fe-efficient plants (Masaoka et al., 1998). Pittman (2005) reviewed molecular mechanisms of Mn transport and suggested that the oxidized forms of Mn (III) and Mn (IV) are not bioavailable to plants and neither can be absorbed. Mn (II) is the reduced form, which is taken up by cells as the divalent cation Mn^{2+}. Under Fe deficiency, FCR can reduce Mn (III) and Mn (IV) to Mn (II), causing Mn accumulation. Fe-efficient muskmelon (*Cucumis melo* L.) cultivars which have higher FCR activity can reduce Mn absorption in the root and shoot once cellular Mn concentration reaches toxicity level in the absence of Fe (Jolley et al., 1991).

Citrus trees are cultivated in humid and tropical or subtropical regions of the world, mainly in acidic soil. These regions usually show soil Mn excess. There are almost no reports that plant can decrease Mn concentration under Fe deficiency, as observed in OJ. This phenomenon shows that Mn absorption is regulated by Fe nutritional conditions. Thomine et al. (2003) proposed that *Arabidopsis thaliana* AtNRAMP3 protein, an Fe transporter, influences metal accumulation and that its overexpression downregulates Mn accumulation under Fe starvation. Cailliatte et al. (2010) reported that NRAMP1 restores the capacity of the Fe-regulated-transporter1 mutant to take up Fe and cobalt (Co), indicating that it has broad substrate selectivity *in vivo* and is a Mn transporter in *Arabidopsis*. *MbNRAMP1* from apple tree stock encodes a functional metal transporter capable of mediating the distribution of ions as well as transport of the micronutrients Fe and Mn and the toxic metal Cd (Xiao et al., 2008). These observations suggest that OJ which has strong FCR activity may control Mn absorption like Fe-tolerant muskmelon (Jolley et al., 1991).

Under Fe-deficient conditions, the Zn concentration of the FD roots increased almost four times above the unstressed control (from 52 to 193 $\mu g\ g^{-1}$ DW), but Zn concentration in OJ roots increased by about two times (from 26 to 60 $\mu g\ g^{-1}$ DW). This value is still lower than the critical toxicity levels in leaves of crop plants, which range from as low as 100 to more than 300 $\mu g\ g^{-1}$ DW (Marschner, 1995). This same response also was apparent in the leaves and stems. Some studies have also reported that Fe deficiency accelerates accumulation of Zn and leads to excess Zn accumulation (Kobayashi et al., 2003). The lower Zn content of OJ roots than that of FD showed that OJ has a stronger capacity to regulate metal homeostasis than FD.

In conclusion, OJ, tolerant to Fe deficiency, showed an increase in root FCR activity and proton extrusion within a short period of Fe deficiency (3 until 4 weeks). Nevertheless, OJ

roots contained a much higher level of Cu than FD, which does not influence root or shoot production but can regulate Mn absorption and Zn accumulation. These results showed that OJ plants have some mechanisms to avoid Mn and Zn toxicity, thus maintaining the balance of cellular heavy metals concentration within a Fe-deficient root environment. Simultaneously, the root biomass increased, resulting in a larger surface area to allow intake of Fe from the soil. This leads to greater tolerance of Fe deficiency and the maintenance of metal homeostasis.

CHAPTER 3

Nutritional (Fe – Mn) Interactions

in Orange Jasmine

3.1 Introduction

In plant nutrition, Fe and Mn are closely related because of similarities properties. It is well documented that two elements are antagonistic and one may inhibit the uptake of the other. Normally when Fe concentration is lower, Mn absorption increases (Sanchez-Raya et al., 1974) in the root of Fe-sensitive plant and also in Fe-tolerant plant. Increasing Mn absorption under Fe-deficient condition can introduce Mn toxicity. Mn is an essential element for plants, mainly for photosynthesis and as an enzyme antioxidant-cofactor. Nevertheless, an excess amount of Mn reduces biomass and photosynthesis, and causes biochemical disorder such as oxidative stress (Millaleo et al., 2010). Decreased plant growth due to Mn toxicity is more severe than Mn deficiency in many parts of the world.

It has been reported that Fe treatments alleviate and/or correct Mn toxicity in many crops such as flax (Moraghan, 1978) and soybean (Heenan et al., 1983). Most studies on Mn and Fe interaction have found a negative correlation between Fe and Mn accumulation in the shoots of susceptible Mn cultivars. In cotton, the Fe content was higher and the Mn/Fe ratio was lower in the Mn-tolerant cultivar than in the Mn-sensitive cultivar (Foy et al., 1995).

In the chapter 2, it was found that OJ maintained Mn concentration at low levels in Fe-deficiency condition under standard Mn concentration (0.5 μM). This result was different from the common knowledge on Mn accumulation under Fe-deficient condition. This means that OJ roots may have the ability to regulate Mn absorption to avoid toxicity. Influences of Fe medium concentration on OJ growth and Mn absorption were conducted under four different Mn concentrations.

3.2 Materials and Methods

3.2.1 Plant culture

Experiments were carried out on OJ. The OJ seeds were imported from China. The methods for germination and growth in hydroponic solution were similar to the chapter 2. One L pots were prepared for culture medium, filled up with the complete nutrient solution and aerated continuously. The Fe was supplied as FeEDTA at 0 and 100 μM and Mn was provided at 0, 0.5, 5 and 50 μM as $MnCl_2$. The container top was covered by a plastic plate which had one holes of 2 cm diameter. Almost the same height and volume of the plants were selected. Three plants were transfered in each hole of the container top plate as 1 treatment plant unit. The experiment was set up with 3 replication. The pH of nutrient solution was buffered by 1 mM Mes (2-(N-morpholino) ethanesulfonic acid) adjusted to pH 5.5 with 1 N NaOH. The nutrient solution was replaced every other day. The experiment took place from July to September 2011 in a greenhouse with daylight condition. The average temperature was about 28 ºC/ 20 ºC (day/night). After 2 weeks pre-culture in the complete nutrient solution, Fe and Mn treatment started and continued for 28 days.

3.2.2 Plant growth and mineral nutrition analysis

The methods for analysis plant growth and mineral nutrition were similar to the chapter 2.

3.2.3 Statistics

Data were analyzed using ANOVA and presented as the mean ± S.E. for each treatment species (n=3). The means were compared by LSD test at a 5 % level (performed with SPSS Version 16.0 for Windows).

3.3 Results

3.3.1 Plant growth

The plant biomass volume of Fe-deficient OJ was larger than that of Fe-sufficient in the every Mn concentration in the medium (Figure 3.1 and 3.2). The growths of OJ also tended to be accelerated under no Fe medium compare to sufficient Fe both in the shoots and roots part except at 0 µM Mn. The OJ was alive under wide range of Mn medium concentration, from 0 to 50 µM.

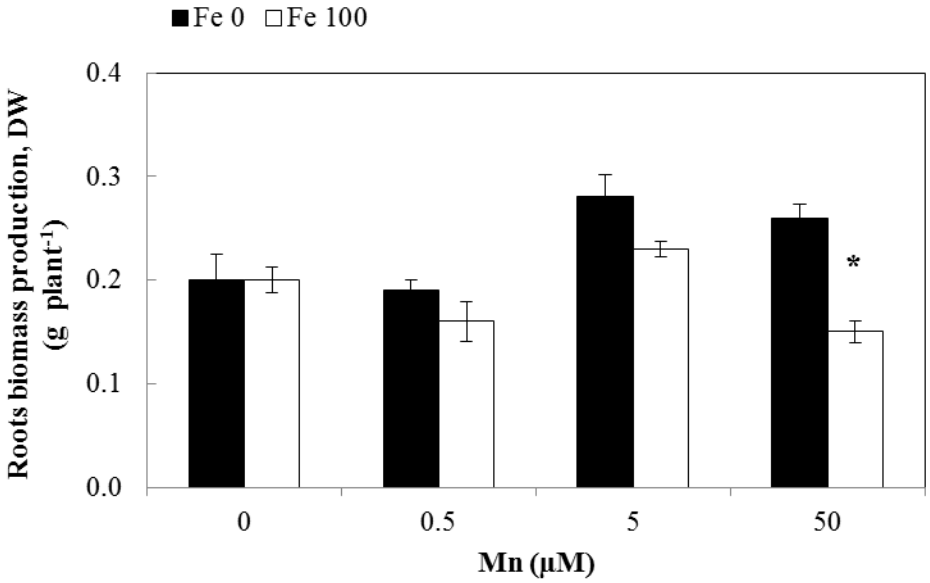

Figure 3.1 Effect of Fe and Mn treatments on roots biomass production of Orange Jasmine (OJ) grown in nutrient solution culture. Data are the means ± SE (n = 3). *, significant differences at $P < 0.05$ between the two treatments.

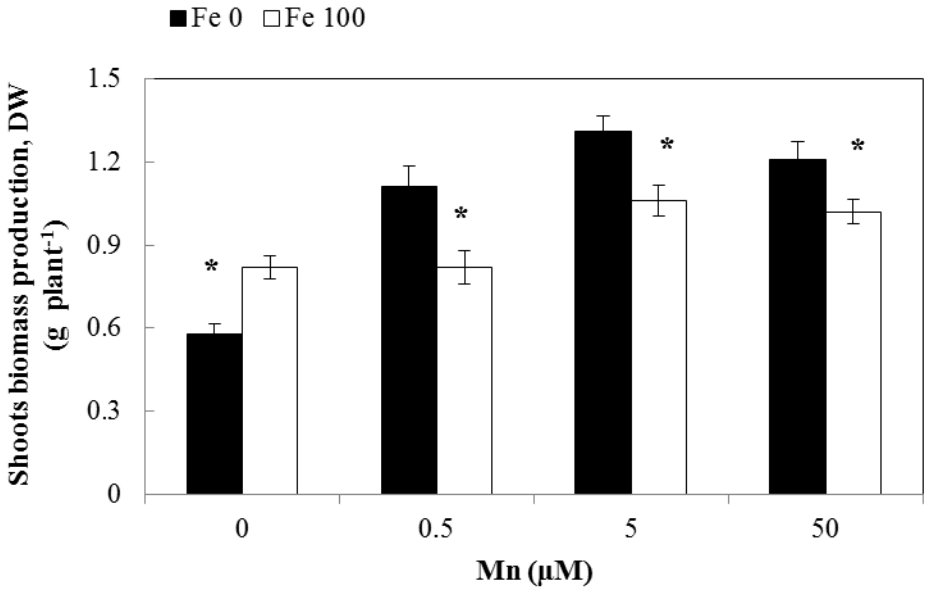

Figure 3.2 Effect of Fe and Mn treatments on shoots biomass production of Orange Jasmine (OJ) grown in nutrient solution culture. Data are the means ± SE (n = 3). *, significant differences at $P < 0.05$ between the two treatments.

3.3.2 Fe and Mn concentration and interaction

The Fe concentration roots and leaves of OJ was not so influenced by Mn concentration in both Fe treatments. Even there was a little different Fe concentration in Fe deficiency condition under Mn treatment, the differences might be because of the initial condition (Table 3.1). Under Fe sufficient, almost no influence in Fe concentration from Mn treatments.

The Mn concentration in roots and leaves also increased as Mn concentration in the medium increased (Figure 3.3, 3.4 and 3.5). There was no different between Fe-deficiency and Fe-sufficiency treatment on Mn concentration of roots and leaves OJ until 10 times Mn concentration in medium (5 µM). Finally, Mn concentration in the Fe-deficient roots of OJ increased when Mn concentration in solution was 100 times

higher than the standard medium (50 μM). The interaction between Fe and Mn was clearer when Mn concentration in medium was 100 times higher compared to the standard (50 μM).

Table 3.1 The effect of Mn medium concentration on Fe concentration in roots, lower leaves and upper leaves of Orange Jasmine (OJ). Data are the means ± SE (n = 3).

Treatment		Fe concentration (μg g^{-1} DW)		
Fe (μM)	Mn (μM)	Root	Lower leaves	Upper leaves
0	0	306.7 ± 24.2 b	82.3 ± 6.0 ab	42.0 ± 1.3 a
	0.5	366.6 ± 23.2 ab	72.8 ± 5.1 b	29.1 ± 2.9 b
	5	452.4 ± 61.4 a	90.8 ± 2.5 a	31.2 ± 3.8 b
	50	383.3 ± 16.6 ab	93.4 ± 4.2 a	26.5 ± 3.8 b
100	0	1334.3 ± 94.8 a	94.3 ± 2.4 a	55.2 ± 9.7 a
	0.5	1258.3 ± 68.2 a	104.9 ± 4.8 a	57.0 ± 1.9 a
	5	1356.9 ± 98.5 a	104.4 ± 6.1 a	49.6 ± 5.2 a
	50	1067.4 ± 130.6 a	102.3 ± 3.4 a	47.0 ± 2.4 a

Values with the same letter were not significantly different at $P < 0.05$ between Mn treatments in the same Fe concentration.

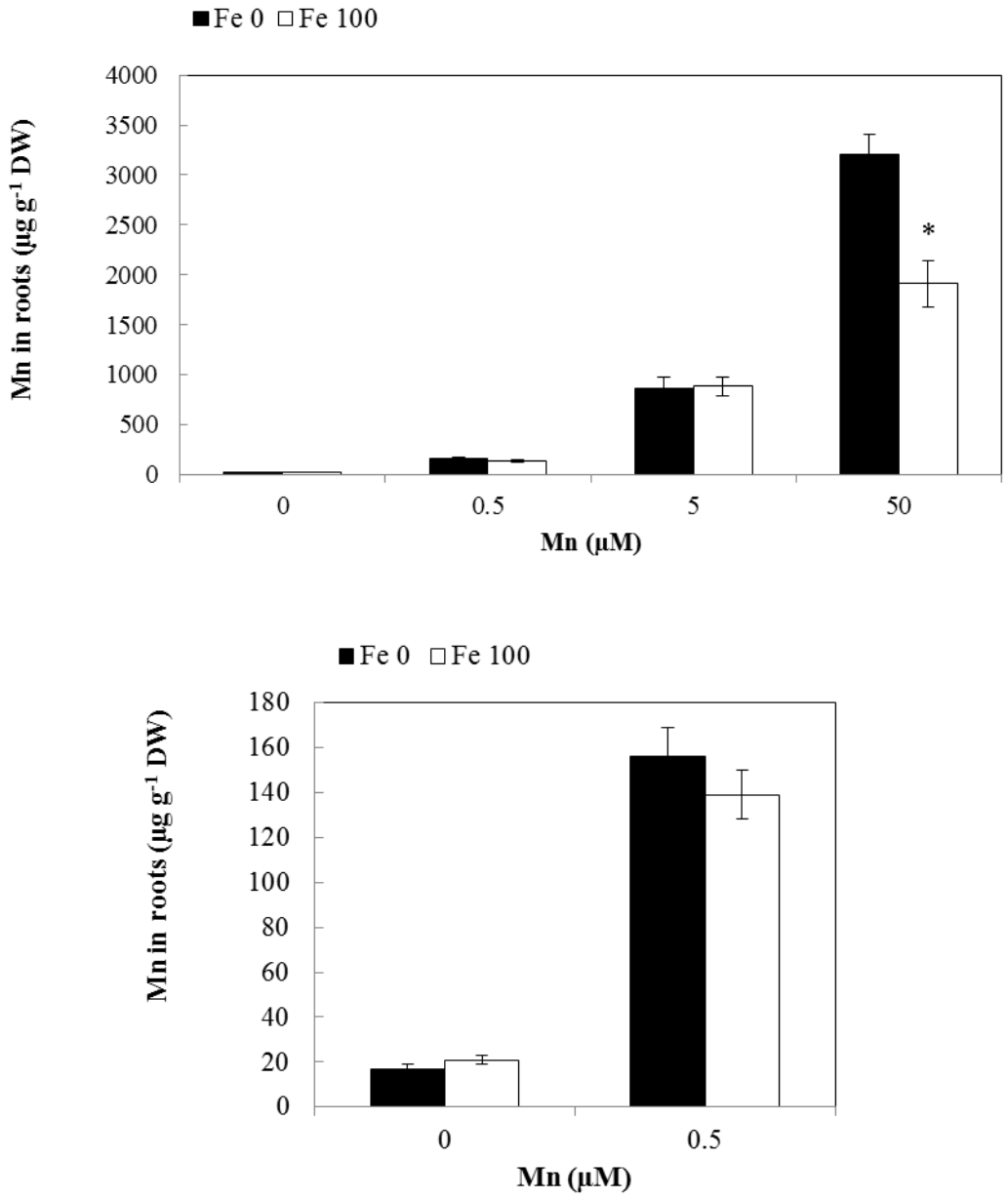

Figure 3.3 Effect of Fe and Mn treatments on Mn concentration in roots of Orange Jasmine (OJ). Data are the means ± SE (n = 3). * , significant differences at $P < 0.05$ between the two treatments.

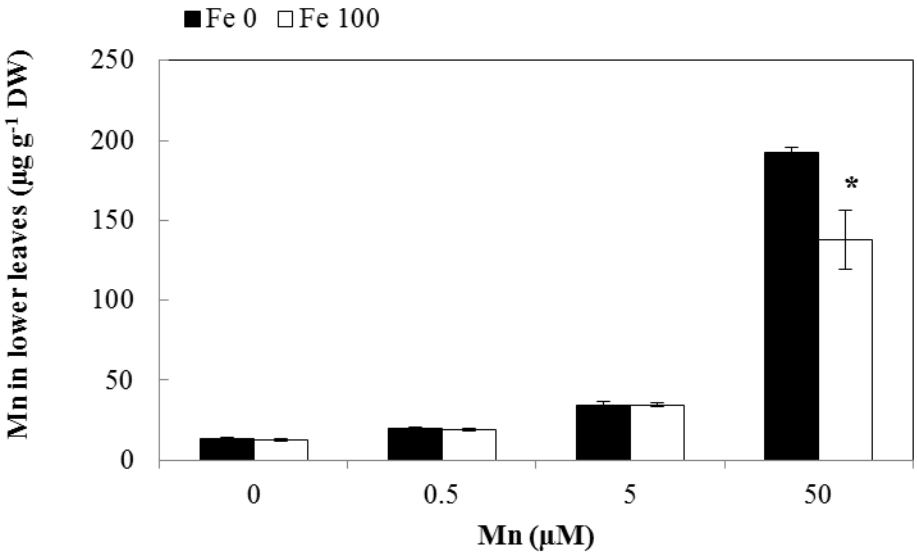

Figure 3.4 Effect of Fe and Mn treatments on Mn concentration in lower leaves of Orange Jasmine (OJ). Data are the means ± SE (n = 3). *, significant differences at $P < 0.05$ between the two treatments.

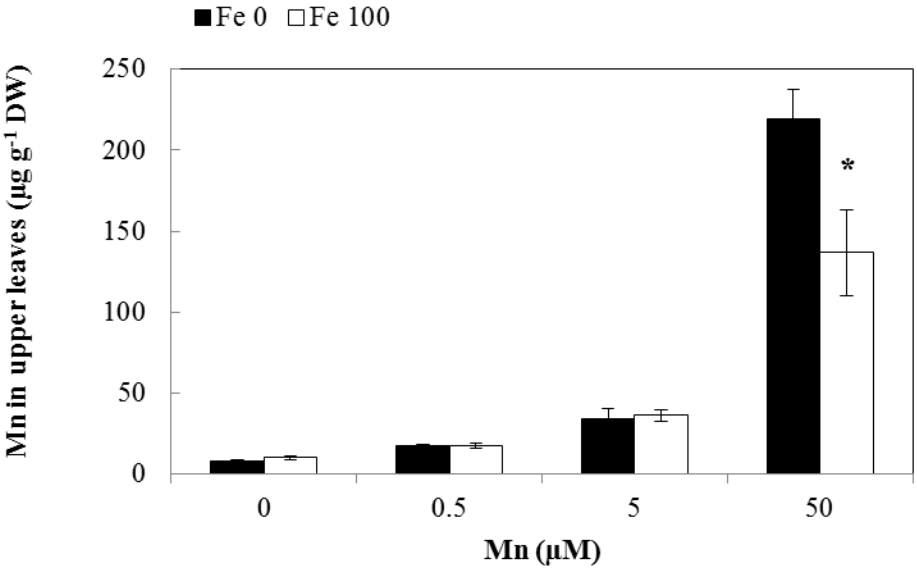

Figure 3.5 Effect of Fe and Mn treatments on Mn concentration in upper leaves of Orange Jasmine (OJ). Data are the means ± SE (n = 3). * , significant differences at $P < 0.05$ between the two treatments.

3.4 Discussion

3.4.1 Plant growth

Fe-deficient OJ produced new roots and leaves much more than Fe-sufficient. This symptom was seen in every Mn medium treatment (Figure 3.1 and 3.2). OJ was also observed to be adapted to wide concentration ranges of Mn medium (from 0 to 50 μM). Reducing plant growth under excess Mn were reported in soybean (Izaguirre-Mayoral, 2005), barley (Alam, 2001a) and rapeseed genotypes (Moroni, 2003). However, OJ could grow in the excess Mn concentration (50 μM). It is considered, OJ can adapt wide range of Mn medium concentration.

3.4.2 Fe and Mn concentration and interaction

In OJ, the Mn concentration in the Fe-deficient roots and leaves could be regulated and maintained at the similar level with Fe sufficient until 5 μM Mn treatment (10 times higher compared to the standard). Finally, Mn concentration increased in the Fe-deficient roots when Mn concentration in solution was 100 times higher than the standard (50 μM). This increasing was not so high in the upper leaves of OJ, and they still produced new roots and leaves. There are almost no reports that Mn concentration maintain/decreases in other plants under Fe deficiency as observed OJ. This phenomenon shows that Mn absorption is regulated by Fe nutritional conditions. OJ might like an Fe-efficient muskmelon (*Cucumis melo* L.) cultivars which have higher FCR activity that can reduce Mn absorption in the root and shoot once cellular Mn concentration reaches toxicity level in the absence of Fe (Jolley et al., 1991). Or OJ might has NRAMP metal transporter like proposed in *Arabidopsis thaliana* AtNRAMP3. A protein an Fe transporter, that influences metal accumulation and overexpression down regulates Mn accumulation under Fe starvation (Thomine et al., 2003).

Under high Mn concentration, Fe-deficient OJ showed can control Mn absorption in the upper leaves. Upper site leaves are important part for plant to keep healthy from metal stress, related to photosynthesis activity. Increasing Fe treatment also could alleviate high Mn absorption in OJ. Generally, plant tolerant to excess Mn by accumulated high concentrations of Mn in roots and proportionally low Mn concentration in shoots, while the sensitive ones exhibited large translocation Mn from roots to shoots, such as in perennial ryegrass (Mora et. al., 2009) and grape (Mou et al., 2011).

In conclusion, OJ can regulate Mn absorption under Fe-deficient condition even in the higher Mn concentration in the medium.

CHAPTER 4

Responses of Orange Jasmine

Grown Under Calcareous Soil

4.1 Introduction

Calcareous soils are characterized by the low bioavailability of plant nutrients and by a high base status and pH between 7.5 and 8.5 depending on the quality and quantity of carbonate mineral present. Typically, Fe and P are the two main nutrients that limit plant growth on calcareous soil (Marschner, 1995). Despite of Fe and P, other transition metals, which are essential micronutrients (including Cu, Mn, and Zn) can also be limiting due to poor solubility at high pH (Gries et al., 1998). Consequently, many vascular plant species are unable to colonize calcareous sites and the floristic composition of calcareous soil and acid silicate soils differs markedly (Conti et al., 1999).

Tyler (1994) have shown calcifuge plants (those which cannot establish well on calcareous soils) to be primarily excluded from growth in calcareous soil due to poor P use efficiency, and in a small proportion of species due to their Fe use efficiency. It has been proposed that such mechanism may exist in calcicole plants (i.e. those that can establish on calcareous soils) to overcome nutrient deficiency allowing them grow in calcareous soil (Störm et al., 1994). Root responses purported to be responsible for overcoming these nutrient deficient environments. Recent studies have suggested that root exudation may be a significant nutrient acquisition mechanism operating in calcareous soil (Jones, 1998). OJ is adapted to a wide range of soil pH (Gilman, 1999), although it is said to favor limestone areas (calcicole plants).

In chapter 2 and 3 OJ has tolerance responses under Fe-deficient condition in water culture medium. Under calcareous soil that provides Fe deficiency and the other influences, what kinds of responses do OJ exist to overcome not only Fe deficiency but also the other micro nutrients deficiency? In this chapter, Trifoliate Orange (TO) (*Poncirus trifoliata* spp.), as known

the susceptible citrus rootstocks to Fe deficiency (Benyahia, 2011, Castle et al., 2009) were also used for comparative analysis.

4.2 Materials and Methods

4.2.1 Plant cultivation

The two related citrus and two citrus rootstocks were studied: Orange Jasmines (*Murraya* sp. Indonesia and China) (M Indo and M China) and Trifoliate Oranges, *Poncirus trifoliata* (L.) (P) Raf and Flying Dragon trifoliate orange (*Poncirus trifoliata* var. montrosa) (Pm). Orange Jasmine seeds were imported from Indonesia and China and Trifoliate Oranges seeds were obtained from the Fruit Research Center of the Hiroshima Prefectural Research Institute. These seeds were soaked in distilled water overnight at 30 C. After rinsing three times, the seeds were transferred to a pot containing mix of commercial soil and peat moss (Ikubyou baido, Takii Seeds Co, yoto, Japan) for germination at 30 C. About 3 months later, when the plants grew up to around 15 cm height, each plant roots were washed by tap water and soil was removed from the surface gently. After that, the uniform plants of OJ and TO were transplanted to plastic pots (300 ml), filled with soil that amended with 0% (original soil pH, 5.8, Non-calcareous soil) and 10 % $CaCO_3$ (pH 7.3, Calcareous soil) by weight . The same soil that used for germination was used. One pot contains one plant. The plants were harvested at 8 weeks after treatments. The experiment was set up with 3 replicates of 8 treatments.

4.2.2 Growth parameters

The plant height and whole plant biomass (fresh weight) were measured before treatments and the end of the experiment. After that, each plant was separated into shoots (stems and leaves) and roots. The fresh weight and dry weight were determined. The dry weight was determined after drying at 70 °C for at least 48 h until constant weight.

4.2.3 Leaf greenness

The leaf greenness of the first new expanded leaves showing after treatment were recorded by taking SPAD reading with a SPAD-502 Chlorophyll meter (Minolta Camera Co. Ltd., Japan) at the end of the experiment.

4.2.4 Chlorophyll fluorescence

The maximum quantum yield of PS II photochemistry (Fv/Fm) was measured by a chlorophyll fluorometer (Imaging-PAM micro-ver, Effeltrich, Germany) after dark adaptation of samples for 1 hour.

4.2.5 Soil pH

The soil pH was determined after plant sampling. The dried soil samples that were screened through a 2 mm sieve were diluted in distilled water with 1:25 (w/v) ratio respectively.

4.2.6 Available metal in soil

At the beginning and the end of the experiment, available of micronutrients in original soil and soil with bicarbonate treatment were determined by Diethylenetriaminepentaacetic acid

(DTPA) extractable. The dried soil samples that screened through a 2 mm sieved mixed with DTPA extractable with ratio 1:10 (w/v), shacked for 2 h using a shaker table at 200 rpm. Samples decanted into appropriate filters and transferred filtrate to referenced test tubes for ICP analyses (Thermo Fisher Scientific Co. Ltd.; iCAP 6000).

4.2.7 Mineral nutrition analysis

Plant materials were rinsed with taps water and finally with distilled water, dried and weight as described above. Dried sample was powdered by hand and digested in concentrated HNO_3 and $HClO_4$. Fe, Mn, Cu and Zn were analyzed by ICP (Thermo Fisher Scientific Co. Ltd; iCAP 6000, UK) method. Calcium (Ca) and Magnesium (Mg) were determined by an atomic absorption spectrophotometer (U-3310 Hitachi Co. Ltd. Tokyo, Japan). Potasium (K) was determined by a flame photometer (ANA 135, Eiko Instruments Inc., Tokyo, Japan).

4.2.8 Statistics

Data were analyzed using ANOVA and presented as the mean of 3 replicates ± S.E. for each treatment species (n=3). The means were compared by LSD test at a 5 % and 1 % level (performed with SPSS Version 16.0 for Windows).

4.3 Results

4.3.1 Plant growth

The dry weight of the shoot (stems and leaves) and the whole plant dry weight decreased significantly in the all TO species under calcareous soil. On the other hand, OJ could maintain the dry weight and the total plant dry weight tended to increase by increasing of root biomass (Figure 4.2 and 4.3). The plant height of TO decreased more than OJ under calcareous soil (Figure 4.1). The root biomass of all species did not decreased under bicarbonate treatment and there was seen a tendency to increase in OJ (Figure 4.4).

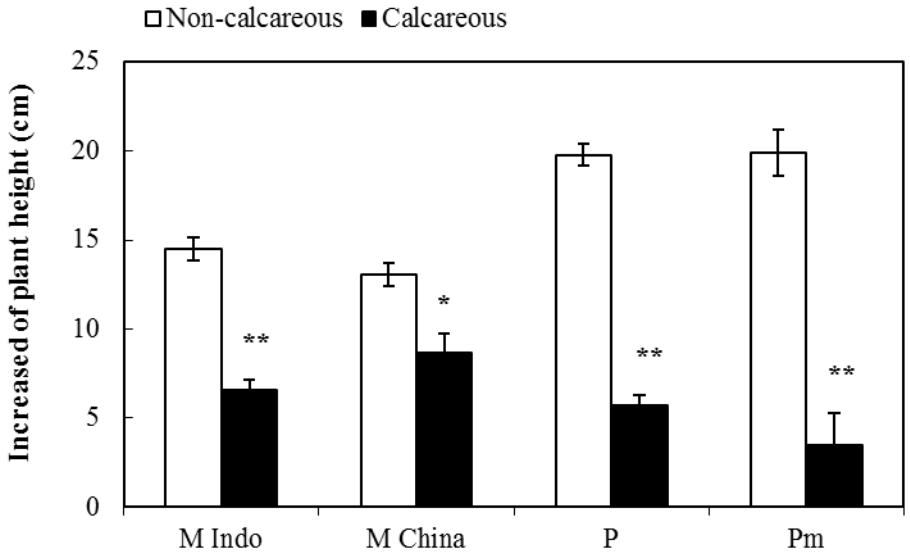

Figure 4.1 Effects of bicarbonate treatments on increased of plant height of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. Data are the means ± SE (n=3), * and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species.

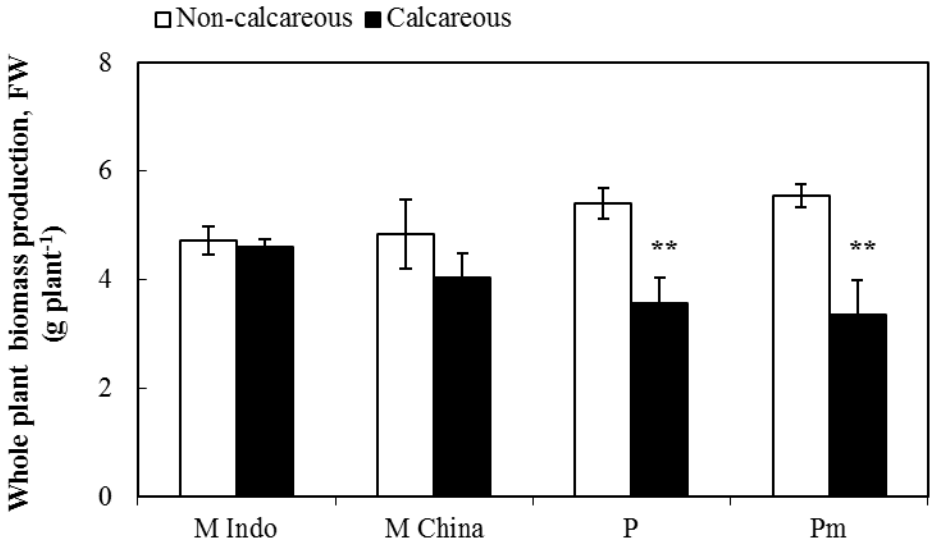

Figure 4.2 Effects of bicarbonate treatments on whole plant biomass production of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. Data are the means ± SE (n=3), * and **, significant differences at 5 % and 1% by LSD test between two treatments in the same species.

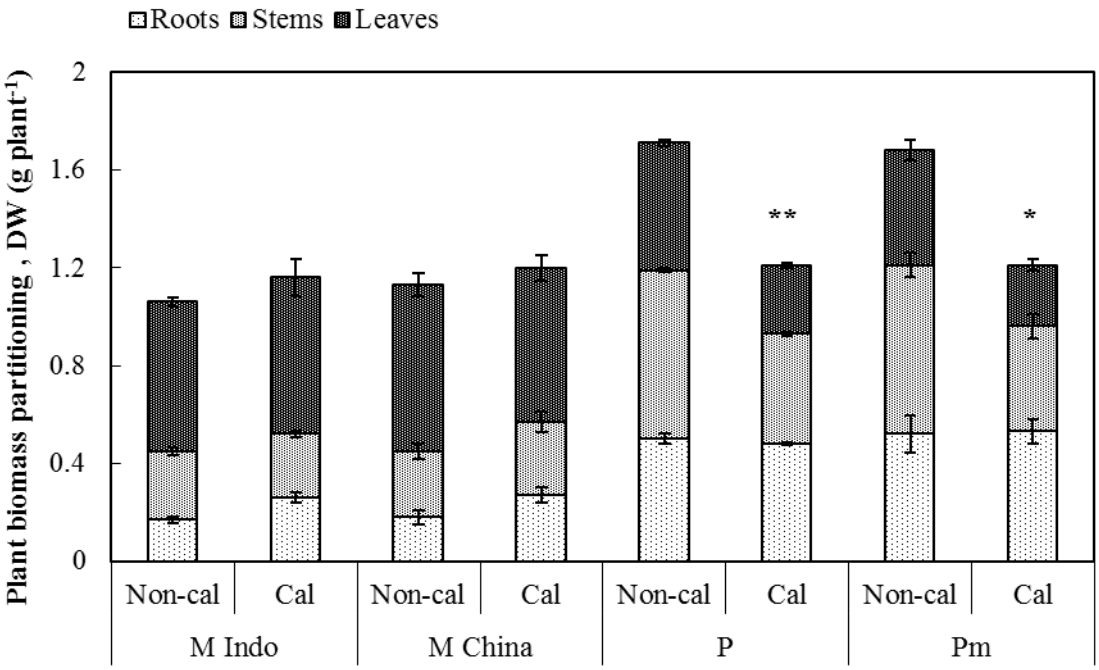

Figure 4.3 Effects of bicarbonate treatments on plant biomass and dry matter partitioning of Orange Jasmine (M Indo and M China) and Trifoliate Orange (M Indo and M China) after 8 weeks treatments. Data are the means ± SE (n=3), * and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species. Non-cal : non-calcareous soil; Cal : calcareous soil.

Figure 4.4 Effects of bicarbonate treatments on root biomass of Orange Jasmine after 8 weeks treatments.

4.3.2 Leaf greenness and chlorophyll fluorescence

The leaf greenness and chlorophyll fluorescence values were analyzed on the first new expanded leaves that showing after treatment, not on the youngest leaves, because the youngest leaves of TO be very small to take analysis. Leaf greenness and maximum quantum yield of PS II photochemistry (Fv/Fm) in both plants were low under calcareous soil. The higher SPAD ratio values between treatments to control were found in OJ (52.2% and 58.1%) compared to TO (36.3% and 36.1%) (Figure 4.5). The ratios of the maximum quantum yield of PS II photochemistry (Fv/Fm) were also higher in OJ (47.6% and 49.1%) than TO (31.2% and 33.1%).

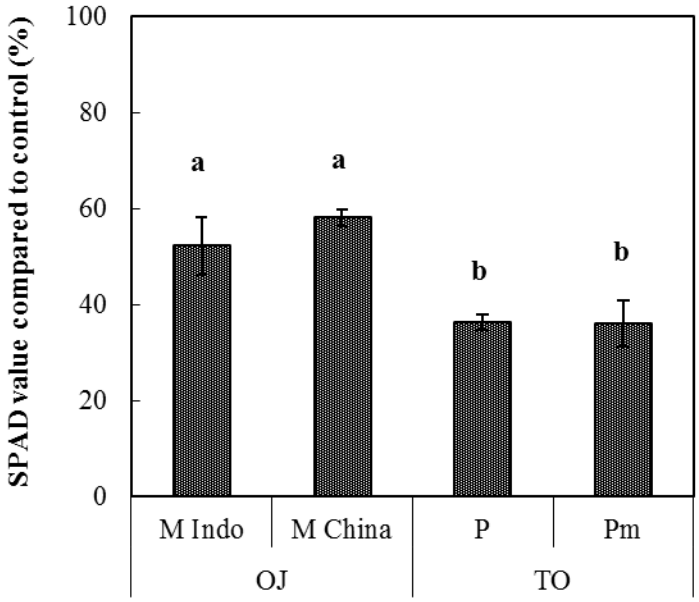

Figure 4.5 Effects of bicarbonate treatments on leaf greenness ratio between control and treatment of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. The values are the means ± SE (n=3). Values followed by the same latter are not significantly different at 5 % level, by LSD test, between the species.

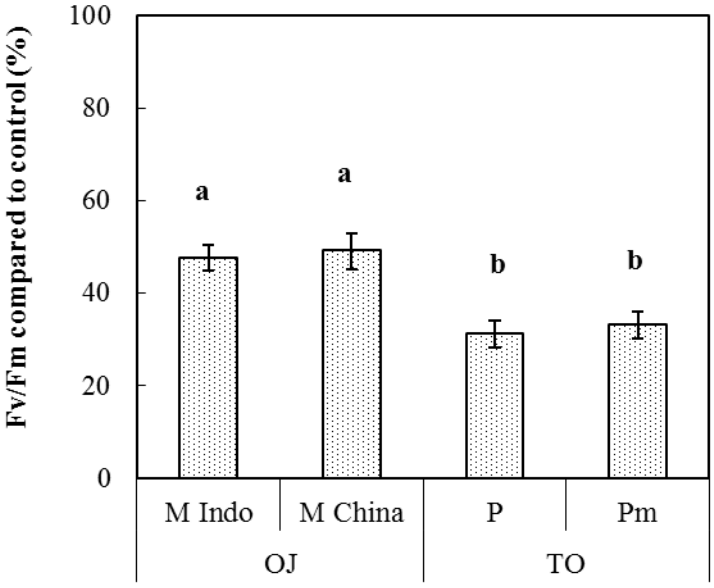

Figure 4.6 Effects of bicarbonate treatments on the ratio of maximum quantum yield of Photosystem II (ratio of variable (Fv) to maximum (Fm) chlorophyll fluorescence) between control and treatment of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. The values are the means ± SE (n=3). Values followed by the same latter are not significantly different at 5 % level, by LSD test, between the species.

4.3.3 Soil pH

There was a significant difference of soil pH between OJ and TO in both conditions (Figure 4.7). Even in small differences, OJ had a tendency to lowering soil pH compared to TO. In non-calcareous soil, the pH was 6.2 (M Indo) and 6.2 (M China) for OJ soils and 6.4 (P) and 6.5 (Pm) for TO soil. Under calcareous soil, Orange Jasmine`s pH were 7.5 (M Indo) and 7.4 (M China), and Trifoliate Orange were 7.6 (P) and 7.7 (Pm).

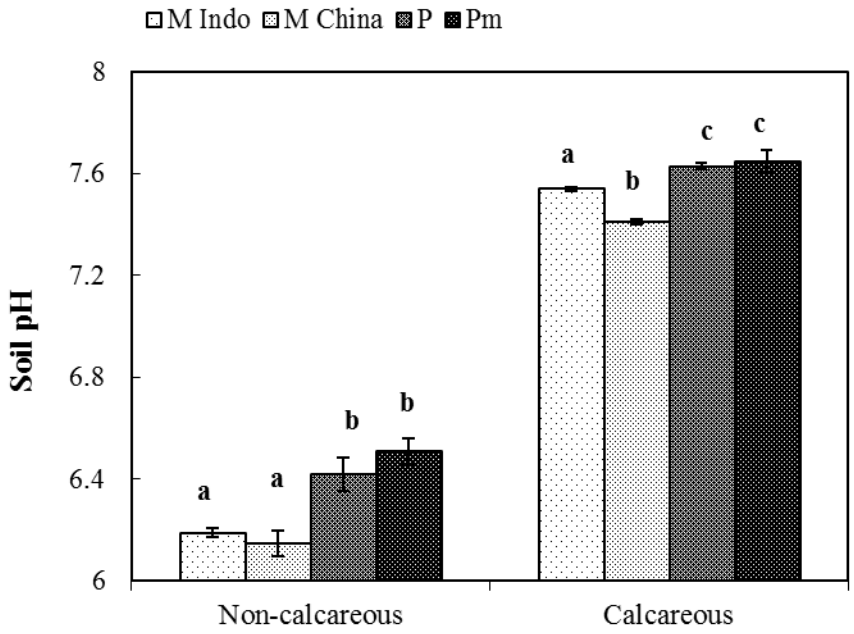

Figure 4.7 Effects of bicarbonate treatments on soil pH of Oranges Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. The values are the means ± SE (n=3). Values followed by the same latter are not significantly different at 5 % level, by LSD test, between the species in the same treatment.

4.3.4 Soil micro nutrients availability

Availability of micronutrients in soils significantly decreased by bicarbonate treatment (under calcareous soil) (Figure 4.8) at the beginning of the experiment. In the end of the experiment, there was no difference between OJ and TO in non-calcareous soil (Figure 4.9). On the other hand, under calcareous soil, OJ showed greater soil micro nutrient availability compared to TO (Figure 4.10), especially Fe that had the highest value than the other metals.

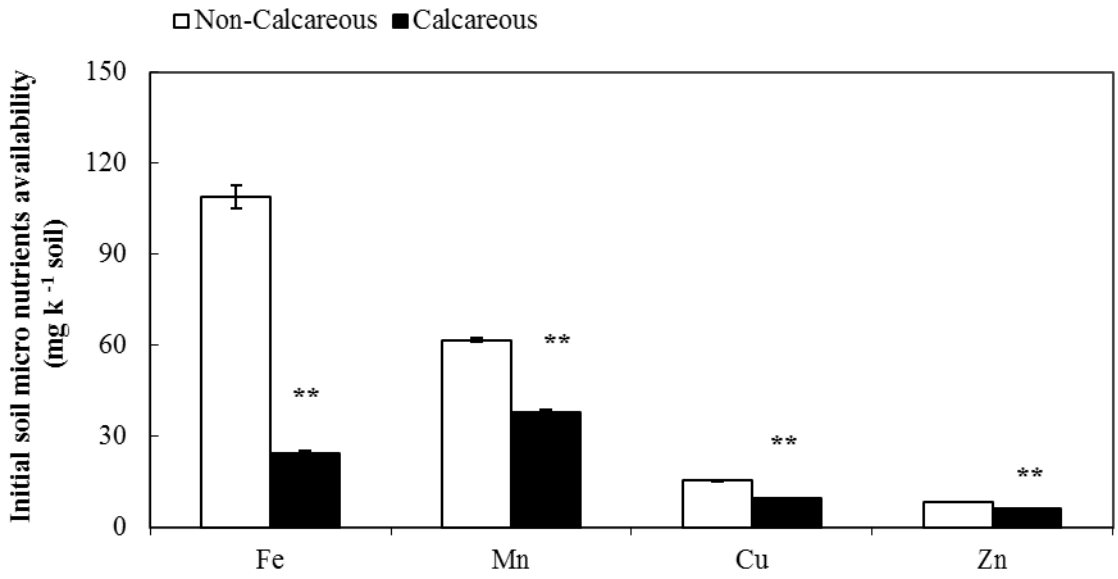

Figure 4.8 The initial soil micronutrients availability. Data are the means ± SE (n=3), * and **, significant differences at 5 % and 1 % by LSD test between two treatments.

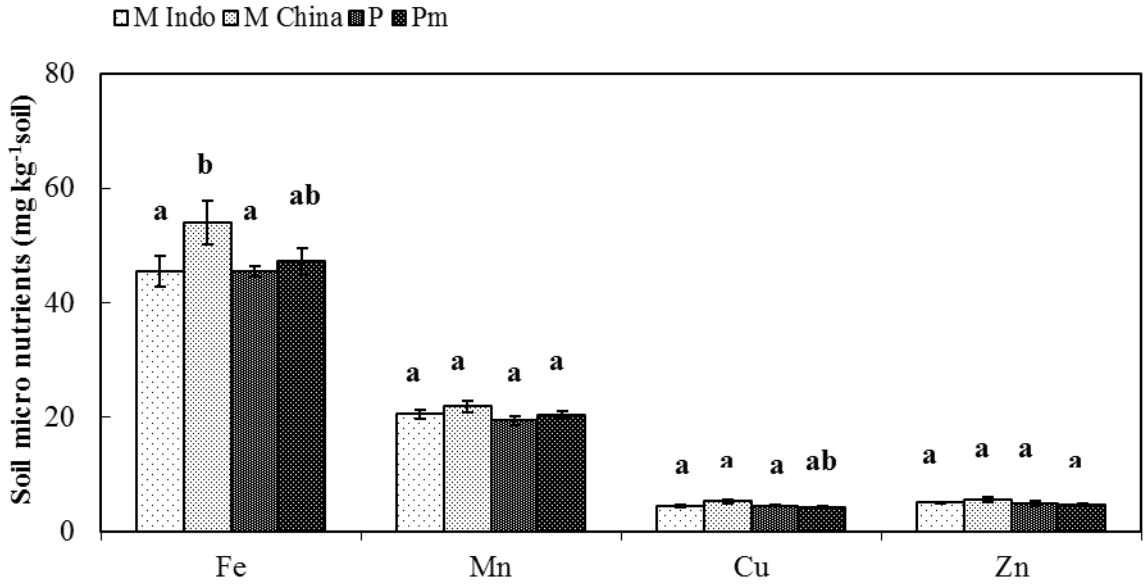

Figure 4.9 The availability of soil micronutrients of Oranges Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments on non-calcareous soil. The values are the means ± SE (n=3). Values followed by the same latter are not significantly different at 5 % level, by LSD test.

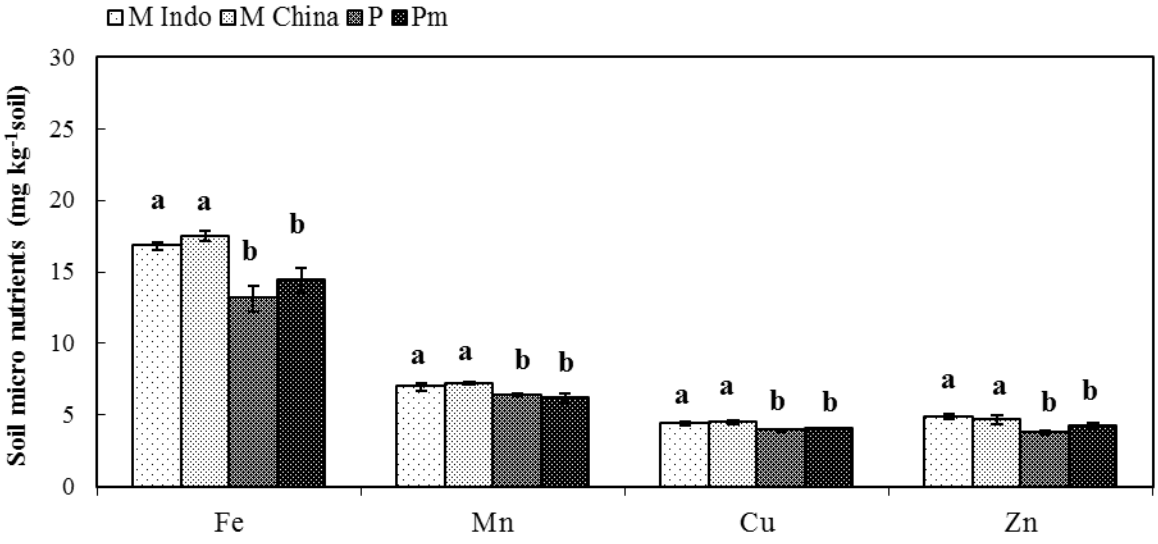

Figure 4.10 The availability of soil micronutrients of Oranges Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments on calcareous soil. The values are the means ± SE (n=3). Values followed by the same latter are not significantly different at 5 % level, by LSD test.

4.3.5 Micro nutrient concentration and accumulation in plants

The micro nutrient concentration in all plant parts of the genotypes tested reduced under calcareous soil (Table 4.1). In case of TO, they seemed to maintain micro nutrient concentration in leave but it was not reflect in accumulation.

The Fe absorption in all plants was significantly decreased except in M Indo (Figure 4.11 A). OJ showed ability to maintain Mn absorption under calcareous soil compare to TO (Figure 4.11 B). In case of Cu, all plants also showed decreased absorption significantly (Figure 4.12 A). Zn absorption showed different pattern under calcareous soil (Figure 4.12 B), M China and Pm could maintain Zn absorption, but M Indo and P showed decreased.

Tabel 4.1 Effects of bicarbonate treatments on Fe, Mn, Cu, and Zn concentration in the Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm). The values are the means ± SE (n=3).

Genotypes	Treatment	Micro nutrients concentration (µg g^{-1} DW)			
		Fe	Mn	Cu	Zn
Leaves					
M Indo	Non-cal	77.0 ± 4.9	17.0 ± 2.3	4.3 ± 0.3	18.1 ± 2.1
	Cal	47.5 ± 3.5**	15.4 ± 1.5	2.2 ± 0.1**	10.2 ± 0.6*
M China	Non-cal	84.5 ± 3.4	20.0 ± 3.2	5.0 ± 0.8	16.0 ± 1.2
	Cal	36.8 ± 1.6**	27.2 ± 1.0	1.0 ± 0.1*	18.2 ± 1.9
P	Non-cal	111.6 ± 4.9	28.0 ± 1.2	7.5 ± 0.3	17.9 ± 0.3
	Cal	109.9 ± 4.9	29.2 ± 6.1	3.8 ± 0.1**	13.8 ± 0.1
Pm	Non-cal	146.6 ± 13.6	32.4 ± 3.6	5.6 ± 0.1	18.2 ± 2.1
	Cal	123.7 ± 9.3	43.9 ± 4.8	5.3 ± 0.4	20.7 ± 0.1
Stems					
M Indo	Non-cal	38.2 ± 1.9	7.1 ± 0.8	3.5 ± 0.2	14.4 ± 0.6
	Cal	30.9 ± 5.0	6.7 ± 0.9	2.3 ± 0.2*	11.1 ± 2.5
M China	Non-cal	49.6 ± 4.6	9.7 ± 0.5	4.0 ± 0.6	20.2 ± 1.2
	Cal	38.9 ± 2.2	6.6 ± 0.3**	1.7 ± 0.1*	24.8 ± 2.1
P	Non-cal	37.4 ± 7.0	8.9 ± 0.2	3.9 ± 0.1	10.7 ± 0.9
	Cal	26.3 ± 2.9	8.5 ± 1.0	2.0 ± 0.1**	11.1 ± 2.5
Pm	Non-cal	30.0 ± 2.8	10.0 ± 0.2	3.6 ± 0.2	13.1 ± 1.6
	Cal	25.8 ± 2.8	10.8 ± 0.4	2.4 ± 0.1**	9.2 ± 1.0
Roots					
M Indo	Non-cal	59.9 ± 3.6	33.3 ± 4.7	7.6 ± 0.5	39.4 ± 6.9
	Cal	27.9 ± 1.4**	27.7 ± 2.3	4.3 ± 0.2**	20.2 ± 1.1
M China	Non-cal	56.9 ± 5.7	64.1 ± 4.3	8.6 ± 1.6	34.3 ± 5.5
	Cal	36.9 ± 0.4*	40.5 ± 1.2**	4.1 ± 0.5	24.7 + 4.9
P	Non-cal	87.7 ± 4.7	56.3 ± 5.4	8.5 ± 0.4	38.7 ± 2.7
	Cal	34.6 ± 3.1**	29.6 ± 3.5*	3.5 ± 0.4**	26.3 ± 2.4*
Pm	Non-cal	73.2 ± 3.7	71.0 ± 3.6	6.2 ± 0.2	33.3 ± 5.3
	Cal	37.9 ± 5.4**	43.7 ± 6.2*	4.4 ± 0.5*	44.9 ± 9.7

* and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species. Non-cal : non-calcareous soil; Cal : calcareous soil.

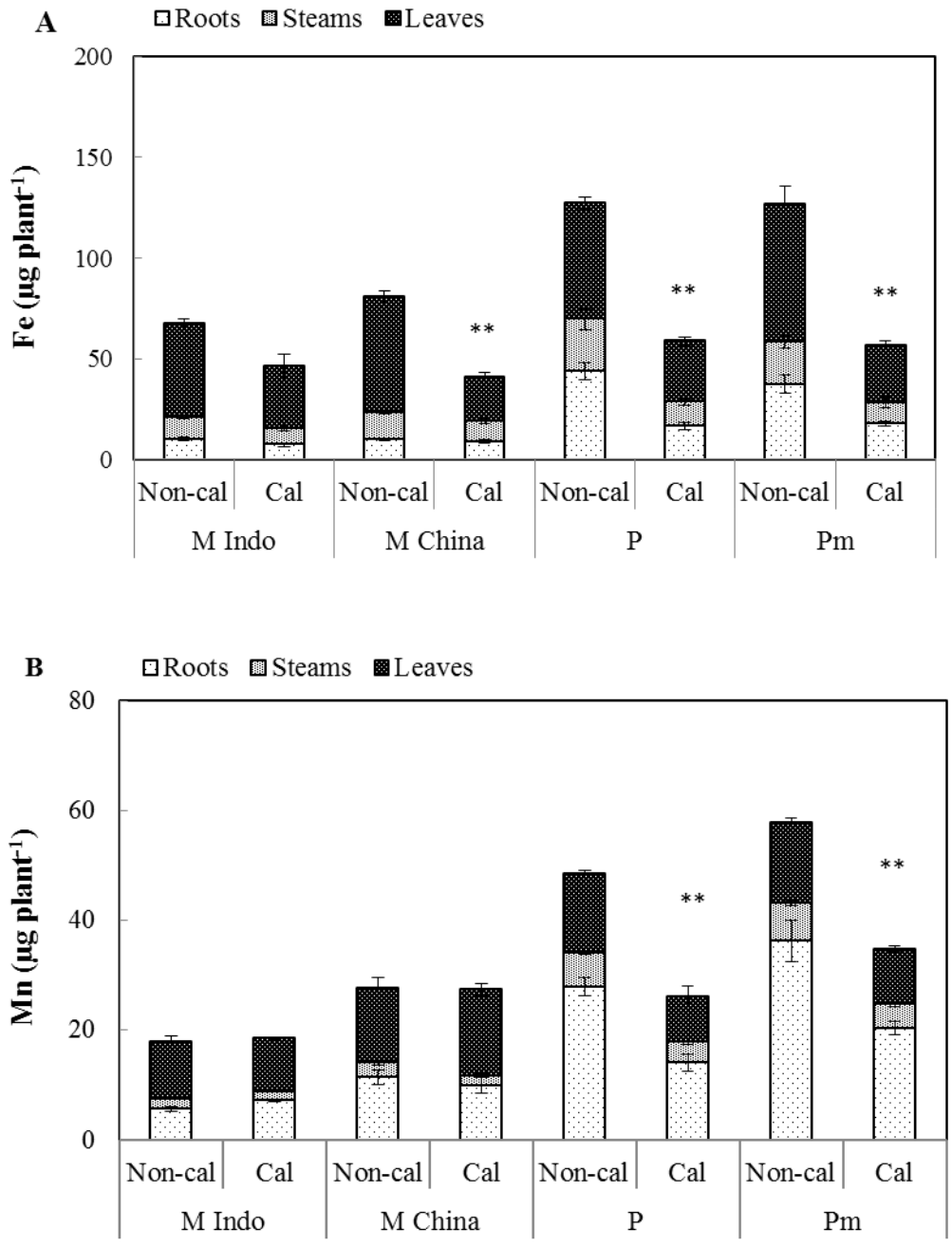

Figure 4.11 Effects of bicarbonate treatments on Fe (A) and Mn (B) accumulation in leaves, stems and roots of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. Data are the means ± SE (n=3). * and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species. Non-cal : non-calcareous soil; Cal : calcareous soil.

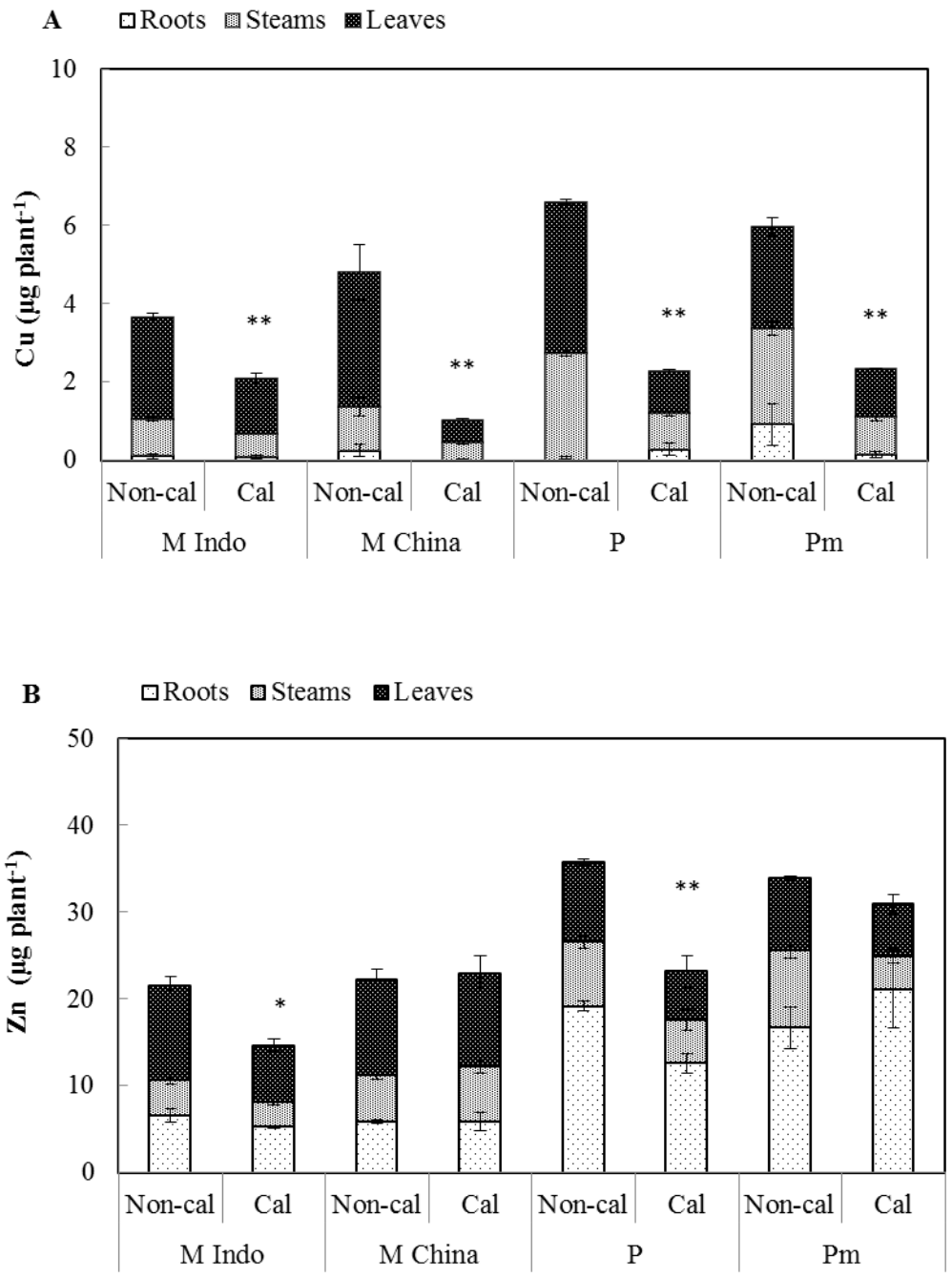

Figure 4.12 Effects of bicarbonate treatments on Cu (A) and Zn (B) accumulation in leaves, stems and roots of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. Data are the means ± SE (n=3). * and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species. Non-cal : non-calcareous soil; Cal : calcareous soil.

4.3.8 Macro nutrient (Ca, K, Mg) concentration and accumulation in plants

Under calcareous soil, OJ and TO plants acquired significantly higher Ca in all plant segments as compared to those in non-calcareous soil. In this case, TO acquired less Ca than Orange Jasmine (Table 4.2). The K concentration in the leaves of all genotypes also increased. Especially in OJ, K concentration also increased in roots, but decreased in TO. Mg concentrations tended to decrease in all part of the plants.

OJ showed larger increase in Ca accumulation when it was grown on calcareous soil. On the other hand, TO maintained or little decreased Ca absorption (Figure 4.13). Increasing absorption of K and Mg also showed by OJ, except M China that show little decreased in Mg. On the contrary, K and Mg absorption of TO was significantly decreased (Figure. 4.14 A and 4.14 B).

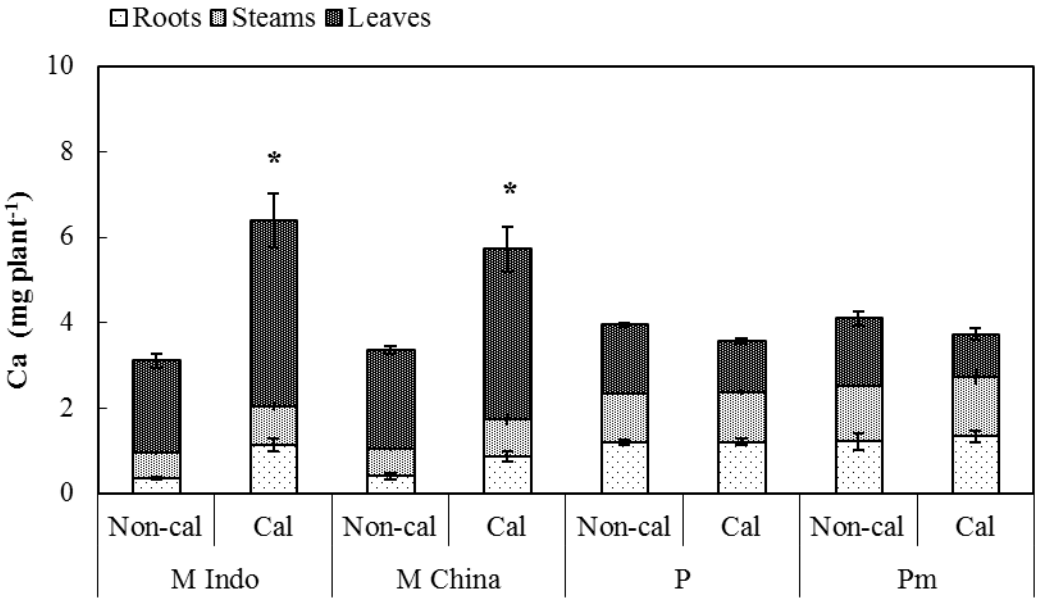

Figure 4.13 Effects of bicarbonate treatments on Ca accumulation in leaves, stems and roots of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. Data are the means ± SE (n=3). * and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species. Non-cal : non-calcareous soil; Cal : calcareous soil.

Table 4.2 Effects of bicarbonate treatments on macro nutrients (Ca, K and Mg) concentration in the Orange Jasmines (M Indo and M China) and Trifoliate Oranges (P and Pm). The values are the means ± SE (n=3)

Genotypes	Treatment	Macro nutrients (mg g^{-1} DW)		
		Ca	K	Mg
Leaves				
M Indo	Non-Cal	3.6 ± 0.2	39.0 ± 1.8	2.2 ± 0.1
	Cal	6.8 ± 0.2**	48.2 ± 2.3*	1.9 ± 0.0*
M China	Non-Cal	3.5 ± 0.4	41.6 ± 0.3	2.0 ± 0.2
	Cal	6.8 ± 0.4**	46.5 ± 1.3*	1.8 ± 0.1
P	Non-Cal	3.1 ± 0.0	27.6 ± 0.8	1.6 ± 0.0
	Cal	4.3 ± 0.1**	31.5 ± 0.7*	1.2 ± 0.0**
Pm	Non-Cal	3.4 ± 0.1	29.3 ± 0.3	1.3 ± 0.0
	Cal	4.3 ± 0.2*	32.4 ± 1.0*	1.2 ± 0.1
Stems				
M Indo	Non-Cal	2.1 ± 0.0	28.1 ± 0.8	0.9 ± 0.0
	Cal	3.5 ± 0.4*	27.7 ± 0.3	1.0 ± 0.1
M China	Non-Cal	2.3 ± 0.1	27.6 ± 1.0	1.0 ± 0.1
	Cal	3.3 ± 0.1**	27.5 ± 0.7	0.7 ± 0.0*
P	Non-Cal	1.6 ± 0.0	18.7 ± 0.2	0.9 ± 0.0
	Cal	2.6 ± 0.1**	18.0 ± 1.6	0.9 ± 0.0
Pm	Non-Cal	1.9 ± 0.1	18.8 ± 0.4	0.8 ± 0.0
	Cal	3.5 ± 0.1**	19.6 ± 0.8	1.0 ± 0.1*
Roots				
M Indo	Non-Cal	2.0 ± 0.1	22.9 ± 2.6	1.3 ± 0.0
	Cal	4.3 ± 0.3**	29.8 ± 0.8	1.8 ± 0.3
M China	Non-Cal	2.2 ± 0.0	25.0 ± 3.0	1.3 ± 0.0
	Cal	3.5 ± 0.1**	31.5 ± 0.9	1.4 ± 0.1
P	Non-Cal	2.4 ± 0.1	31.6 ±1.2	0.9 ± 0.1
	Cal	2.5 ± 0.2	28.9 ± 1.9	0.7 ± 0.0
Pm	Non-Cal	2.3 ± 0.1	34.5 ± 0.3	0.9 ± 0.0
	Cal	2.8 ± 0.0**	31.2 ± 1.8	0.9 ± 0.0

* and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species. Non-cal : non calcareous soil; Cal : calcareous soil.

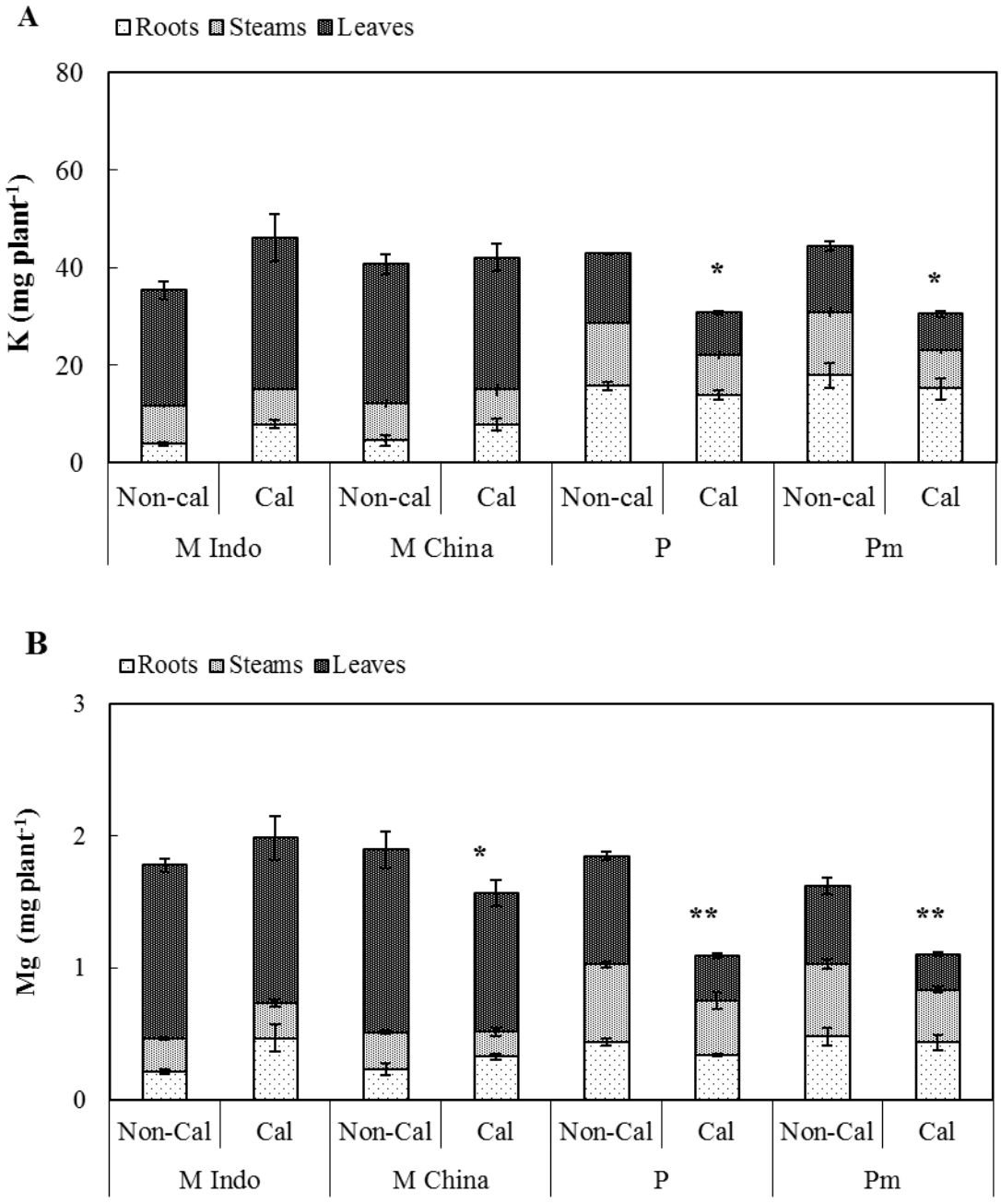

Figure 4.14 Effects of bicarbonate treatments on K (A) and Mg (B) accumulation in leaves, stems and roots of Orange Jasmine (M Indo and M China) and Trifoliate Orange (P and Pm) after 8 weeks treatments. Data are the means ± SE (n=3). * and **, significant differences at 5 % and 1 % by LSD test between two treatments in the same species. Non-cal : non-calcareous soil; Cal : calcareous soil.

4.4 Discussion

4.4.1 Plant growth

The development of Fe-deficient symptoms as growth depression and yellowing of the young (new) leaves are common responses when plants grow in calcareous soil (Alcantara et al., 2003; De la Guardia and Alcantara, 2002; Gruber and Kosergaten, 2001; Ma et al., 2006). The TO genotypes, which are known to be susceptible to Fe deficiency (Benyahia, 2011, Castle et al., 2009), showed significant growth depressions prior to yellowing of youngest leaves. The impairment of growth expressed clear in shoots part by restricting of plant height and new leaves production. If new leaves came out, they could not change to green in leaf color and sometimes dropped. It is suggested that these growth depressions were directly caused by insufficient supply of physiologically available Fe in plant. Under such conditions, poor leaf growth may be followed by leaf chlorosis as was the case with sunflowers (Masalha et al., 2000) and also with vine cultivars Silvaner and Riparia IG which the youngest leaves became yellow at the end of the vegetation period (Gruber and Kosegarten, 2001). It is also suggested that insufficient Fe availability in the plant cell restricts the synthesis of ribonucleotide reductase which is a Fe containing enzyme producing deoxyribonucleotide diphosphate. Deoxyribonucleotide diphosphate is a precursor of DNA and a lack of DNA will severely affect cell division and thus merismatic growth (Gruber and Kosegarten, 2001). On the contrary, OJ could maintain the shoot growth under calcareous soil and still produced new leaves even in chlorotic condition and changed to green in leaf color day by day (regreening, data not shown). This condition supposed if OJ could show better performance under low Fe content compared to TO. These responses also happen in Fe tolerant peach rootstock, *P. xerophila* that shows maintenance of plant height and shoot dry weight in high soil pH (Ma et al., 2005). The ability of OJ to maintain shoot

growth even under calcareous soil may be explained by the accumulation of organic acids in Fe-deficient plant parts. The first effects of the organic acid were, by mechanisms directed to facilitate Fe acquisition by excretion of organic acids and proton from roots, and also to supply the FCR enzymes with enough reducing power. The second, carbon export to the leaves via the xylem could facilitate the survival of the shoot parts while roots try to acquire enough Fe from the soil. A possible function of the export of organic acids to the leaves is the use of these carbon compounds for basic maintenance processes such as respiration (Abadia et al., 2002). The occurrence of a significant anaplerotic carbon fixation in the roots of Fe-deficient plants could provide explanation for the relatively small effect of Fe deficiency on sugar beet leaf growth under controlled conditions in spite of the markedly reduced photosynthesis in the same leaves (Terry, 1980). Fe deficiency causes a marked reduction in photosynthetic rates, but has a relatively small effect on leaf growth (Terry, 1980).

Shoot growth of TO was depressed by approximately 50% without affecting root dry weight when grown in the calcareous soil (Figure 4.3), thus inhibition of shoot growth might occur independently from root growth. This result is in agreement with the results of Gruber and Kosegarten (2001), who reported that insufficient Fe supply impaired shoot growth more strongly than root growth. In this case, OJ showed a tendency increasing of root biomass (Figure 4.3 and 4.4) under calcareous soil.

The increasing root biomass of OJ under calcareous soil is in agreement with the other works (De la Guardia and Alcantara, 2002) and may be explained by several reasons. First, a general characteristic of Fe-deficient plants is an increase in the concentration of organic acids, in particular malate and citrate (Landsberg, 1981). A hypothesis is that the increased amount of citrate is a consequence of cytoplasmic alkalization upon proton pumping and subsequent

induction of the biochemical pH-stat mechanism. The increase in the activity of PEP carboxylase in conditions of low Fe (Rabotti et al., 1995) produces more organic acids through the involvement of anaplerotic CO_2 fixation that at the end would increase the fresh weigh of the root. Second, a relative higher growth of the roots in soil with HCO_3^- and low available Fe, as in calcareous soils, can be a mechanism for increasing the soil volume exploration of roots, which can help acquisition of elements with low availability like P, K, Fe, Zn, Mn and Cu (Rengel, 2001).

4.4.2 Leaf greenness and chlorophyll fluorescence

Increasing soil pH under calcareous soil made decrease Fe supply to the leaves, and that affected chlorophyll synthesis, chloroplast development and the maximum quantum yield of PS II photochemistry in both plants. In this case, OJ showed greater availability with lower Fe concentration. This condition was also made OJ had better shoot growth even under calcareous soil compared to TO. It should be noted that OJ could grow well under lower Fe concentration compared TO.

4.4.3 Soil pH

There was significant difference of soil pH between OJ and TO in both treatments (Figure 4.7). Even in small differences, OJ had a tendency to lowering soil pH compared to TO.

The rhizosphere pH may differ from the bulk soil pH by up to two units, depending on plant and soil factors, with important consequences for the pH-dependent solubility of nutrients and toxic elements in the soil solution. The most important factor for root-induced changes in rhizosphere pH is the uptake of nutrients, which is coupled with proton (H^+) transport in higher

plants. The decreasing soil pH of OJ may be explained by several reasons. First, cation-anion exchange balance. The increasing absorption Ca and K by OJ supposed balanced by a net release of protons which leads to rhizosphere acidification (Romera et al., 1991). Second, acidification medium by proton (H^+) extrusion, and third organic anion release. Proton extrusion and organic anion release are included the responses to Fe deficiency of Strategy I plant. Romheld and Marschner (1986) stated the availability of Fe^{3+} depends on the pH, and its decrease from 8.0 to 4.0 will increase the concentration of Fe^{3+} from 10^{-20} to 10^{-8} M (for every one unit drop in pH, Fe^{3+} becomes a 1000-fold more soluble).

Rhizosphere pH may be also influenced by released and uptake of HCO_3^-, respiratory CO_2 production by roots and rhizosphere microorganisms, and release of low-molecular-weight organic compounds which may also be coupled with proton transport.

The pH buffering capacity of the soil and the initial soil pH are the main factors determining the extent to which plant roots can change the rhizosphere pH. However, a lack of significant pH change in a soil with high pH buffering capacity (like happen in OJ soil) does not necessarily mean the absence of proton flux in the rhizosphere. Indeed, the protons may replace other cations from the cation exchange sites of the soil and thereby affect the mobilization/immobilization of nutrients (Hinsinger et al., 2009).

4.4.4 Soil micro nutrient availability

The availability of soil micro nutrient was significantly decreased by bicarbonate treatment (calcareous soil) (Figure 4.8). Decreasing avalaibililty of essential micronutrients (Fe, Cu, Mn and Zn) was due to their poor solubility in high pH (Gries et al., 1998). In the end of the experiment, there was no difference of soil micro nutrient availability between OJ and TO in

non-calcareous soil (Fig. 4.9). On the other hand, under calcareous soil, even in small differences, Orange Jasmine showed greater soil micro nutrient availability compared to Trifoliate Orange (Figure 4.10).

The availability of soil micro nutrient has positive correlation with soil pH. In this case OJ showed a tendency to lowering soil pH compared to TO. Acidification of the rhizosphere is presumably important in solubilization of soil Fe particularly in soils high pH and/or with a high buffering capacity. On the other hand, the excretion of protons in response to Fe deficiency is often accompanied by increased synthesis and consequent accumulation of organic acids (mainly malic and citric acids) near the root tips (Landsberg, 1981). Dinkelar et al. (1989) found an increase in the amount of available micro nutrients, such as Fe, Mn and Zn, in the rhizosphere soil sampled near proteoid roots of white lupin, which were also shown to be the root zones responsible for intense excretion of citrate. Carboxylic acid excretion into the rhizosphere under Fe-limiting conditions might enhance the solubility of Fe oxides in soil through chelation, or by a transport process that acidifies the substrate in a similar way as described in lupins under P deficiency (López-Bucio, 2000). Another role of Fe deficiency induced H^+ excretion is seen in stimulation of Fe(III) reductase activity (Toulon et al., 1992). Those mechanisms might exist in OJ under calcareous soil, because all micro nutrients availability was significantly higher in Orange Jasmine's soil compared to Trifoliate Orange even in a small difference. Especially, Fe availability was the highest compared to other metals.

4.4.5. Micro nutrients concentration and accumulation in the plants

Under calcareous soil, the micro nutrient concentration decreased in all plant parts of all the genotype tested (Table 4.1). The severe inhibition shoots growth of TO under calcareous soil made the concentration of metals in leaves constant same or higher than the control (no dilution effect). These responses are contrary with TO metals accumulation value that showed decreased under calcareous soil.

OJ leaves showed micro nutrients deficiency symptoms as a result of Fe deficiency as well as other micro nutrients such as Mn, Zn and Cu simultaneously. Based on Broadley et al. (2012), the critical deficient concentration of Fe and Mn in leaves is the range of 50 – 150 mg kg^{-1}DW for Fe and 10 – 20 mg kg^{-1}DW for Mn. The critical concentration of Cu is 1 – 5 mg kg^{-1}DW for Cu (Robson and Reuter, 1981) and Zn is 15 – 20 mg kg^{-1}DW for Zn (Cakmak et al., 1997). However, OJ still produced new leaves even in chlorotic condition and changed to green day by day (regreening). On the contrary, TO hardly produced new leaves, and if new leaves came out, they could not change to green in leaf color and sometimes dropped. Therefore, it is considered that OJ could grow well under lower micro nutrient content compare to TO.

4.4.6 Macro nutrients concentration and accumulation in plants

The K concentration in the leaves of all genotypes also increased under bicarbonate stress. In case of OJ, K concentration also increased in roots, but decreased in TO. On the other hand, Mg concentration tended to decrease in all part of all plants. Similar in micro nutrients concentration, the severe inhibited shoots growth of TO under calcareous soil made the concentration of macro nutrients in leaves perceived same or higher than control (no dilution

effect). These responses were contrary with value macro nutrient accumulation that showed decreased under calcareous soil.

OJ showed larger increasing Ca absorption when grown on calcareous soil. On the other hand, TO maintained or little decreased Ca absorption (Figure 4.13). The higher absorption of Ca recorded by plants grown under increasing bicarbonate might be related to larger amounts of available Ca in soil solution. Increasing absorption of Potassium (K) and Magnesium (Mg) also showed by OJ, except M China that showed little decreased in Mg. On the contrary, K and Mg absorption of TO were significantly decreased (Figure 4.14 A and 4.14 B).

Regardless of the mechanisms of cation uptake, it implies in charge balance, that uptake of cation must be accompanied by either the uptake of an anion(s) of equal but opposite charge, or by the extrusion of cation (e.g. H^+) (Haynes, 1990). Besides proton extrusion that made rhizosphere acidification, one of the primary factors determining organic acids levels in roots is their degree of cation-anion imbalance. In situations where roots take up an excess of cations (particularly K), the negative charge required to balance this often provided by organic acids, such us malate, malonate, citrate and aconitate (Chang and Roberts, 1991). This symptom was similar in Fe-tolerant plant, sugar beet that showed increasing concentration of cations (K, Ca and Mg) in Fe-deficient plant respect to control thus tending to balance the organic acid increases (López-Millan, et al., 2000).

In conclusion, OJ has similar response when grown under calcareous soil and Fe-deficiency solution. The response is increasing root biomass. Based on this response, it could be considered that the first mechanism of OJ to survive under calcareous soil is overcome Fe deficiency by increasing root biomass that made larger soil volume absorption. OJ also can grow not only under low level Fe but also the other micro elements. OJ also tends to decrease soil pH.

CHAPTER 5

General Discussion

The search for new citrus rootstock with better performance than those currently used (including Fe tolerant ones) is the major aim of the citrus industry in many countries. The ordinary screening methods require many months or years because the citrus plants grow slowly. A much shorter experimental period is clearly required. One of the alternative ways to search for potential citrus rootstock is using related citrus species for selecting tolerant plant material such as Orange Jasmine (*Murraya* sp.). This plant is one of the most Fe-efficient rootstock plants because it favors limestone soil and easily naturalized in many locations with wide range of conditions. However, there is little information on OJ constant with Fe-deficiency tolerant except for ferric chelate reductase (FCR) activity (Castle et al., 2009). Examined the specific Fe-tolerant responses of OJ and compared them with the Fe-sensitive plant Trifoliate Orange (*Poncirus* sp.) that is known as an Fe-susceptible citrus rootstock (Benyahia, 2011) within a shorter experimental period by using water and soil culture methods were done.

Under Fe-deficient condition, OJ showed the increasing FCR activity (Chapter 2) that is similar to an Fe-tolerant plant, red clover (Zheng et al., 2003). Reduction activity of Fe^{3+} to Fe^{2+} has been suggested as a major contributor to a suite of activities resulting in Fe uptake and utilization (Camp et al., 1987). The FCR activity depends on the volume of Fe in the root apoplast and whether the Fe can be utilized (Bienfait et al., 1985). The shoot/root Fe ratio increased in Fe stressed OJ, but not in TO. Because OJ accumulated a high content of Fe in the root, much more Fe might be stored in the root apoplast and would be reused due to enhanced FCR activity (Chapter 2 and Chapter 3).

OJ grown in the absence of Fe within 28 days exhibited stronger proton extrusion than TO (Chapter 2). This response also reflected under calcareous soils that favor natural condition constant with Fe deficiency. OJ showed higher activity of lowering soil pH compared to TO

(Chapter 4). The availability of soil micro nutrients has positive correlation with soil pH. Acidification of the rhizosphere is presumably important in solubilization of soil Fe particularly in soils high pH and/or with a high buffering capacity. On the other hand, the excretion of protons in response to Fe deficiency (Chapter 2) is often accompanied by increased synthesis and consequent accumulation of organic acids (mainly malic and citric acids) near the root tips (Landsberg, 1981). Dinkelar et al. (1989) found an increase in the amount of availability of micro nutrients, such as Fe, Mn and Zn, in the rhizosphere soil sampled near proteoid roots of white lupin, which were also shown to be the root zones responsible for intense excretion of citrate. Carboxylic acid excretion into the rhizosphere under Fe-limiting conditions might enhance the solubility of Fe oxides in soil through chelation, or by a transport process that acidifies the substrate in a similar way as described in lupins under P deficiency (López-Bucio, 2000). Those mechanisms might be existed in OJ when grown under calcareous soil, by evidence that all micro nutrients availability was significantly higher in OJ's soil compared to TO even in a small differences. Especially, Fe availability was the highest compared to the other metals (Chapter 4). The decreasing soil pH of OJ may be explained by several reasons. First, cation-anion exchange balance. The increasing absorption Ca and K by OJ supposed balanced by a net release of protons which leads to rhizosphere acidification (Romera et al., 1991). Second, acidification medium by proton (H^+) extrusion, and third organic anion release. Besides H^+ extrusion that made rhizosphere acidification, one of the primary factors determining organic acids levels in roots is their degree of cation-anion imbalance. In situations where roots take up an excess of cations (particularly K^+), the negative charge required to balance this often provided by organic acids, such as malate, malonate, citrate and aconitate (Chang and Roberts, 1991) (Chapter 4). This symptom found in Fe-tolerant plant, sugar beet that showed increasing cations

(K, Ca and Mg) concentration in Fe-deficient plant respect to control thus tending to balance the organic acid increases (Lopez-Millan, et al., 2000).

The remarkable result using OJ under Fe deficiency included, OJ could regulate Mn absorption in Fe deficient roots (Chapter 2 and Chapter 3). Normally Fe and the other metal elements such as Mn have antagonistic effects on root absorption. The Mn absorption increases under Fe deficient condition which can introduce Mn toxicity. In this case, OJ regulated Mn concentration and maintained Fe and Mn balance in the Fe deficient root. The concentration of Mn in the Fe-deficient roots could be regulated and maintained under similar level with Fe-sufficient root until 5 μM Mn treatment (10 times higher compared to standard). When Mn in solution was 100 times higher than the standard (50 μM), Mn concentration finally increased in the Fe deficient root of OJ but did not increase so high in the leave and still produced new roots and leaves (Chapter 3). In this study (Chapter 3), OJ was observed to adapt to wide ranges of Mn concentration in medium and still survived under high Mn concentration (50 μM) and low Fe concentration in the medium. There are almost no reports that Mn content decreases/maintain under Fe deficiency as observed in OJ. This phenomenon shows that Mn absorption is regulated by Fe nutritional conditions. Thomine et al. (2003) proposed that Arabidopsis thaliana AtNRAMP3 protein, an Fe transporter, influences metal accumulation and that its overexpression downregulates Mn accumulation under Fe starvation.

Despite of Fe and P, other transition metals, which are essential micronutrients (including Cu, Mn, and Zn) can also be limiting due to poor solubility at high pH (Gries et al., 1998). In the early stage of treatment (Chapter 4), OJ leaves showed micro nutrients deficiency symptoms as a results of Fe deficiency as well as other micro nutrients such as Mn, Zn and Cu simultaneously. However, OJ still produced new leaves even in chlorotic condition and changed to green day by

day (regreening). On the contrary, TO hardly produced new leaves, and if new leaves came out, they could not change to green and sometimes dropped. It is considered that OJ could grow under lower micro nutrient content compare to TO. OJ also showed maintains Mn absorption under calcareous soil compare to TO that significant decreased. OJ could grow day by day and become stronger to adapt calcareous soil condition that has many constraints in mineral nutrients.

The other remarkable results using OJ under Fe-deficiency treatment increase of root biomass compared to those under Fe-sufficient condition (almost 2 times), in contrast to TO which tended to decrease (Chapter 2 and Chapter 3). There are a few reports wherein Fe-efficient plants increase their root biomass under Fe deficiency, such as in *Spathiphyllum* (Yeah et al., 2000) and *Medicago ciliaris* (M`Sehli et al., 2008). In OJ, increasing root biomass depended on the increased formation of lateral roots and root hairs that was well correlated with increasing root FCR activity and proton extrusion. Generally, under Fe-deficient condition, root biomass decreases or remains unchanged even in Fe tolerant plants. Increasing root biomass was also observed when OJ grown under calcareous soil that provide Fe-deficient condition (Chapter 4). Based on this response, it could be considered that the first mechanism of OJ to survive under calcareous soil is overcome Fe deficiency by increasing root biomass that made larger soil volume absorption.

OJ also showed maintains shoot growth under Fe-deficiency condition. There was almost no difference in dry weight between Fe-deficiency and Fe-sufficiency treatments of the shoots of OJ . New leaves of OJ emerged and grew under Fe deficiency (Chapter 2 and Chapter 3). Result of these studies (Chapter 2 and Chapter 3) is consistent with result under calcareous soil (Chapter 4). OJ still produced new leaves even in chlorotic condition and changed to green day by day (regreening). On the contrary, TO hardly produced new leaves, and if new leaves came out, they

could not change to green and sometimes dropped. Eight weeks in calcareous soil provided almost no decrease of shoot biomass compared to neutral pH soil. This condition supposed if OJ could grow under low Fe content compared to TO. The Fe deficient plants of OJ also were more able to maintain greater leaf greenness and chlorophyll content compared to TO (Chapter 2, Chapter 3 and Chapter 4), suggesting that the former is more tolerant to Fe chlorosis. OJ might have better carbon and Fe transfer from the roots into the shoots that allowed a biomass increase compared to TO.

In conclusion, OJ, a plant tolerant to Fe deficiency, showed increasing FCR activity in root and decreasing pH rhizosphere. OJ also can regulate Mn absorption under Fe-deficient condition. This result showed that OJ have some mechanism to avoid Mn toxicity. Simultaneously, the root biomass increased, resulting in a larger surface area to allow intake of Fe from the soil. This leads to greater tolerance of Fe deficiency and the maintenance of metal homeostasis.

Summary

Citrus fruits are familiar all over the world and rank first in international fruit trade in terms of value, although production shows geographical concentration in certain areas. Some important citrus farms have calcareous soils that are not suitable for citrus trees. Trees planted in these soils often suffer from Fe deficiency. The most prevalent cause of Fe deficiency is the presence of high levels of carbonate in soils, leading to a high pH and low availability of Fe and the condition known as lime induced chlorosis. Fe deficiency affects the biochemistry, morphology, and physiology of the whole plant because Fe is an important cofactor of many enzymes, including those involved in the biosynthetic pathways of chlorophylls. In calcareous soils, citrus productivity depends on the availability of a suitable rootstock that is tolerant to low Fe. The search for new citrus rootstocks with better performance than those currently used (including Fe tolerant ones) is the major aim of the citrus industry in many countries. The ordinary screening methods require many months or years because the citrus plants grow slowly. A much short experimental period is clearly required. One of the alternative ways to search for potential citrus rootstocks is using related citrus species for selecting tolerant plant material such as Orange Jasmine (*Murraya* sp.). This plant is Fe-efficient rootstock plants because it favors limestone soils and easily naturalized in many locations with a wide range of conditions. However, there is little information on Orange Jasmine except for Ferric Chelate Reductase (FCR) activity. I investigated the specific Fe-tolerant responses of Orange Jasmine and compared them with the Fe-sensitive plant Trifoliate Orange that is known as an Fe susceptible citrus rootstock within a shorter experimental period by using water and soil culture methods.

1. Responses of Orange Jasmine under Fe deficiency (Experiment 1)

Within a short experiment period of three or four weeks, selecting Fe-tolerant citrus rootstocks became possible, comparable to an annual plant experiment. An increase in root ferric chelate reductase activity and proton extrusion was confirmed under Fe deficiency as similar to Fe-deficient tolerance dicotyledonous plants.

Remarkable results using Orange Jasmine under Fe deficiency included increase of root biomass compared to those under sufficient Fe treatment (almost 2 times), in contrast to Flying Dragon which tended to decrease. There are a few reports wherein Fe-efficient plants increase their root biomass under iron deficiency. In Orange Jasmine, increasing root biomass depended on the increased formation of lateral roots and root hairs that was well correlated with increasing root FCR activity and proton extrusion. Generally, under Fe-deficient conditions, root biomass decreases or remains unchanged even in Fe-deficient tolerant plants.

The second remarkable result showed that Mn concentration in Fe-deficient roots of Orange Jasmine did not increase. Normally Fe and the other metal elements such as Mn have antagonistic effects on root absorption. Mn absorption increases under Fe-deficient condition which may induce Mn toxicity. In this case, Orange Jasmine regulated Mn concentration and maintained Fe and Mn balance in the Fe-deficient root. These results showed that roots of Orange Jasmine have specific functions under Fe-deficient conditions.

2. Nutritional (Fe-Mn) interactions in Orange Jasmine (Experiment 2)

In the experiment 1, using standard Mn concentration (0.5 μM) in the medium, the Fe-deficient roots of Orange Jasmine maintained Mn concentration at low levels. This means Orange Jasmine root has the ability to regulate Mn absorption to avoid toxicity. Influences of Fe concentration in the water culture on the Mn absorption were studied under four different Mn concentrations in Orange Jasmine.

The concentration of Mn in the Fe-deficient roots could be maintained at similar level in Fe-sufficient roots until 5 μM Mn treatment (10 times higher compared to the standard). When Mn in solution was 100 times higher than the standard (50 μM), Mn concentration finally increased in the Fe-deficient root of Orange Jasmine but did not increase so high in the leave and still produced new roots and leaves. It was also found that Orange Jasmine preferred lower Fe medium and still grew well under poor Fe medium. In this study, Orange Jasmine was observed to be adapted to wide concentration ranges of Mn and still survived under high Mn concentration (50 μM) and low Fe concentration in the medium.

3. Responses of Orange Jasmine grown under calcareous soil (Experiment 3)

The specific Fe-deficient tolerance responses of Orange Jasmine were proven by increasing root biomass and regulating manganese accumulation. In calcareous soil, Orange Jasmine was tested what kinds of functions do when soil pH was adjusted to 7.3 by amending with 10 % $CaCO_3$ by weight and cultivated for 8 weeks.

In the early stage of treatment, Orange Jasmine leaves showed of Fe-deficient symptom as well as the other micro nutrients such as Mn, Zn and Cu, simultaneously. However, Orange Jasmine still produced new leaves even in chlorotic condition and changed to green day by day

(regreening). On the contrary, Trifoliate Orange hardly produced new leaves, and if new leaves came out, they could not change to green and sometimes dropped. Eight weeks in calcareous soil provided almost no decrease of shoot biomass compared to neutral pH soil. Remarkable root biomass increase was also observed in Orange Jasmine under calcareous soil. These responses meant that Orange Jasmine developed strategy to increase Fe absorption by lateral roots formation. Under calcareous soil, the DTPA soluble micro elements were higher and the pH was slightly lower in soil with Orange Jasmine compared to that with Trifoliate Orange. These results suggested that lateral root development could influence the soil environment. Orange Jasmine can grow not only under low Fe but also other micro elements.

In conclusion, under Fe-deficient condition Orange Jasmine increase root biomass, maintain Mn absorption and accelerate root FCR activity and proton extrusion. Orange Jasmine does need high Fe in the medium. Orange Jasmine increase root biomass in all Mn concentration and also maintain Mn absorption until 10 time`s standard Mn concentration under Fe-deficient condition. Orange Jasmine also increase root biomass under calcareous soil, can grow not only under low Fe but also other micro elements and can decrease soil pH.

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