Research in Plant Biology, 2(6): 23-29, 2012 ISSN : 2231-5101
www.resplantbiol.com

Regular Article

In vitro evaluation of iron-deficiency tolerance in an endemic putative apple rootstock

Hassan Mahdavikia and Nasser Mahna*

Departmen of Horticultural Sciences, Faculty of Agriculture, University of Tabriz, 51666-16471, Tabriz, Iran
*Corresponding author: mahna@tabrizu.ac.ir & n.mahna@gmail.com

In most species of fruit trees, iron deficiency-induced chlorosis causes several economic damages. Recently, in vitro culture techniques have been used to assess rootstocks for susceptibility to iron deficiency-induced chlorosis. In the present study, the in vitro shoots of three apple genotypes including *Malus baccata*, MM.106 and Gami-Almasi, a native putative apple rootstock, were evaluated on MS medium supplemented with four concentrations of Fe (0, 9, 18, 36 mg/L) from Fe-NaEDTA and the concentration of Fe in MS medium as the control. Visual chlorosis index, leaf chlorophyll index, leaf area, fresh weight increment and dry weight of shoots were determined 26 days after culture. Iron concentrations affected the visual chlorosis index of *M. baccata* and MM.106. However, there were no chlorosis symptoms in Gami-Almasi. Leaf chlorophyll index were affected significantly by genotypes and Fe concentration. Leaf area and fresh weight increment differed significantly in various genotypes; nevertheless, the effect of Fe concentration was not significant. Leaf area and fresh weight were recorded the highest in *M. baccata* compared to the other two genotypes. Genotypes and Fe concentration treatments were not affected dry weight of the shoots.

Keywords: apple rootstocks, *in vitro* evaluation, iron deficiency.

Chlorotic reaction of plants to iron deficiency is a common nutritional disorder and a major contributing factor in reducing production level and horticultural products quality (Tagliavini et al, 2000). This nutritional disorder occurs frequently in calcareous soils and often results in appearance of chlorosis (Marschner & Romheld, 1994). Most of leaf iron content has been found in chloroplasts (about 80%), about 60 percent of which is stored in thylakoid membrane (Terry and Abadia 1986). Therefore, iron deficiency affects chloroplast structure and activity at first, because of the iron-dependent enzymes being mostly inactivated or damaged (Soldatini et al. 2000). For example, antioxidant enzymes such as Ctalase (CAT), Peroxidase (POX) and one of Superoxide dismutase (SOD) isoforms require iron in their active sites. These enzymes inactivate the reactive oxygen species (ROS) which could bind to DNA, protein and membrane lipid and lead to mutation, reduction of photosynthesis, senescence and finally death (Scandalios 1990).

Perennials, especially fruit trees, when planted in alkaline and calcareous soils, normally demonstrate iron deficiency symptoms (Romera et al. 1991). Such conditions could result in diminished production level, fruit quality, and

Received: 5.10.2012; Revised: 28.11.2012; Accepted: 30.11.2012

reproductive output and even failure to develop flower buds in the next year. To ease this problem and obtain an acceptable yield, Fe-chelating fertilizers have been employed. However, such strategies are highly demanding and may exert bioenvironmental hazards (Wallace 1984). Therefore, utilization of iron deficiency tolerant rootstocks is served as the first choice to manage production costs and alleviate the relevant biological concerns may arise when using fertilizers (Tagliavini et al. 2000).

Breeding programs for *in situ* selection of chlorosis tolerant rootstocks are time consuming and mostly the achieved results are uncertain (Fairbanks 2000). *In vitro* techniques can be successfully used in a shorter time for identification of genotypes with a higher tolerance to iron shortage chlorosis (Bavaresco et al. 1993). These methods have been deployed for selection of quince (Marino et al, 2000) and pear tolerant cultivars (Dolcet-San Juan et al. 1992). Furthermore, in grape cultivars, the effect of three concentrations of Fe-NaEDTA has been evaluated on five growth variables and the results have proved *in vitro* techniques as a fast method to screen tolerant and susceptible cultivars for planting on alkaline soils (Serpil et al. 2008). In another study, physiological and biochemical responses to iron deficiency of a peach rootstock (Mr. S 2/5) have been investigated under *in vitro* conditions (Lombardi et al. 2003). There is no apple rootstock, genetically improved or commercially available, which is tolerant to iron deficiency and suitable for planting in Iran's calcareous soils. Therefore, this research has been conducted to evaluate *in vitro* the effect of iron shortage stress on some genotypes including a member of a native population of a putative apple rootstock which has long been growing in our region with a calcareous soil.

Materials and Methods:

In vitro shoots of three apple genotypes including: MM.106, *Malus baccata* and Gami-Almasi were cultured in iron-free MS medium containing four different concentrations of Fe as Fe-NaEDTA (0, 9, 18 and 36 mg/l afterwards T1, T2, T3 and T4, respectively) as well as in intact MS medium (as the control). Then, plantlets were maintained in a growth chamber at 24±1 °C, with 40 $\mu molm^{-2}s^{-1}$ light intensity and 16/8 (light/dark) photoperiod. All treatments were replicated 5 times with 3 samples in each replication. The effect of different treatments was determined at the end of 26 days. Plantlets were evaluated for the following parameters: fresh weight increment (FWI: final fresh weight - primary fresh weight), leaf area (LA) of the plantlets measured using a leaf area meter (LA Meter System Corp., Model Li-1300, USA), shoots initial dry weight (DW_0) by oven drying at 70°C and chlorophyll index of the first and second young leaves (LCI) measured with a Minolta SPAD-502 meter (Osaka, Japan). A visual chlorosis rating of 1 to 4 was performed following Krouma et al (2003) after 7, 14 and 21 days and determined based on observations on the first, second and third leaves of plantlets. Quantitative characters were evaluated in a factorial design based on randomized complete block design and VCI data analyzed through the non-parametric method Kruskal-Wallis using SPSS software (SPSS Inc., Chicago, Illinois).

Results and Discussion:

The iron-deficiency stress has been mostly studied by field trials and hydroponics culture experiments. A serious impediment to evaluate iron chlorosis using calcareous soils under field and greenhouse conditions is the lack of reliable replication of chlorosis symptoms among experiments which can be due to the complexity of the chemical and physical criteria in both plant and soil needed to be met in order for chlorosis to occur. Actually, nutrient solution systems that mimic soil–plant interactions responsible for chlorosis have been exploited effectively for inducing chlorosis symptoms (Fairbanks 2000). The *in vitro* system, compared to the traditional methods, requires less space, time and plant

material for the experiments and permits a better control of growth conditions. Tissue culture techniques have been used as a rapid method to screen tolerant genotypes to iron-deficiency stress in some of fruit trees such as quince (Muleo et al. 1995), grapevine (Tangolar et al. 2008), pear (Dolcet-Sanjuan et al. 1992), peach (Lombardi et al. 2003) and so forth.

Evaluation of visual chlorosis index showed variation among genotypes after treating with different concentrations of iron. Analysis of VCI data through non-parametric Kruskal-Wallis method showed that there was no significant difference between genotypes at 7 days after treatment, but at 14 days and 21 days after treatment, there was significant differences at $p<0.01$. The same statistical procedure was carried out to evaluate the difference between iron treatments and the results showed that treatments after 7 days had no effect on the genotypes. However, 14 and 21 days after treatment, there was a significant difference between treatments at $p<0.05$ and $p<0.01$, respectively (Table 1).

Table 1: Kruskal-Wallis analysis separately carried out in genotypes and Fe treatments for visual chlorosis index measured at 7, 14 and 21 days after treatment.

Source of variation	Df	VCI_7§	VCI_{14}	VCI_{21}
Genotype	2	4.645 ns	10.257**	10.405**
Fe treatment	4	2.992 ns	10.885*	17.194**

ns: Non-significant; *, **: Significant at $p<0.05$ and $p<0.01$, respectively; df: degree of freedom; §: Visual chlorosis index at 7 days after treatment.

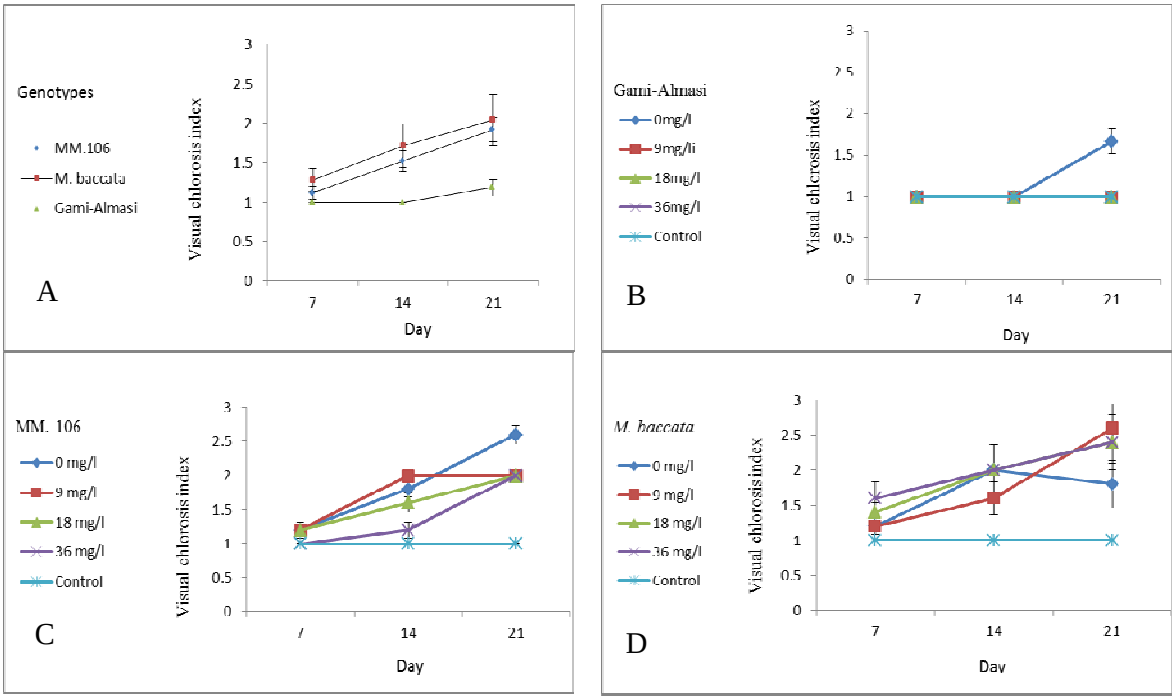

Figure 1: Reaction norm diagrams for visual chlorosis index (VCI) measured at 7, 14 and 21 days after treatment in different genotypes and iron treatments. A) The trend of chlorosis in three genotypes; B, C and D: VCI observed in different Fe treatments in Gami-Almasi, MM. 106 and M. baccata, respectively. Error bars represent Mean ± SE.

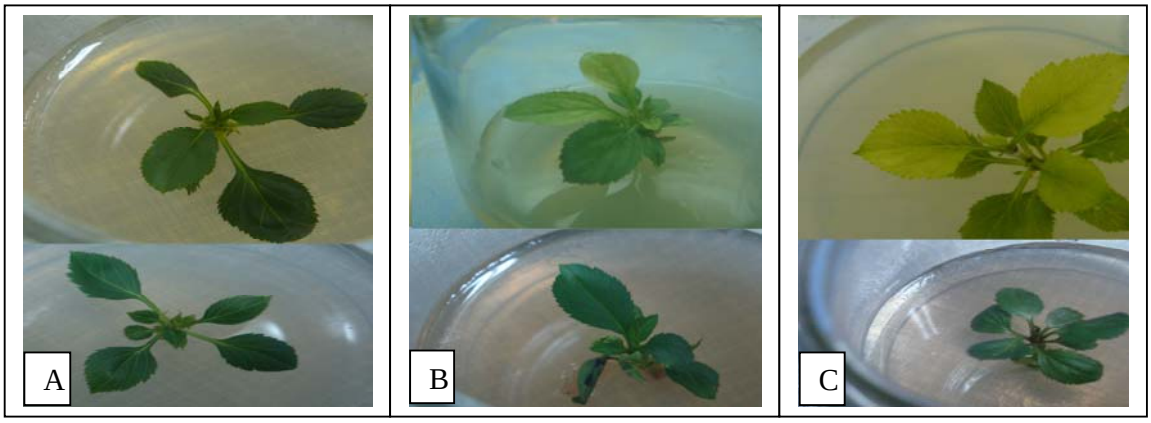

Figure 2: Responses of A) Gami-Almasi; B) MM.106; C) *Malus baccata* to iron treatments based visual chlorosis index.

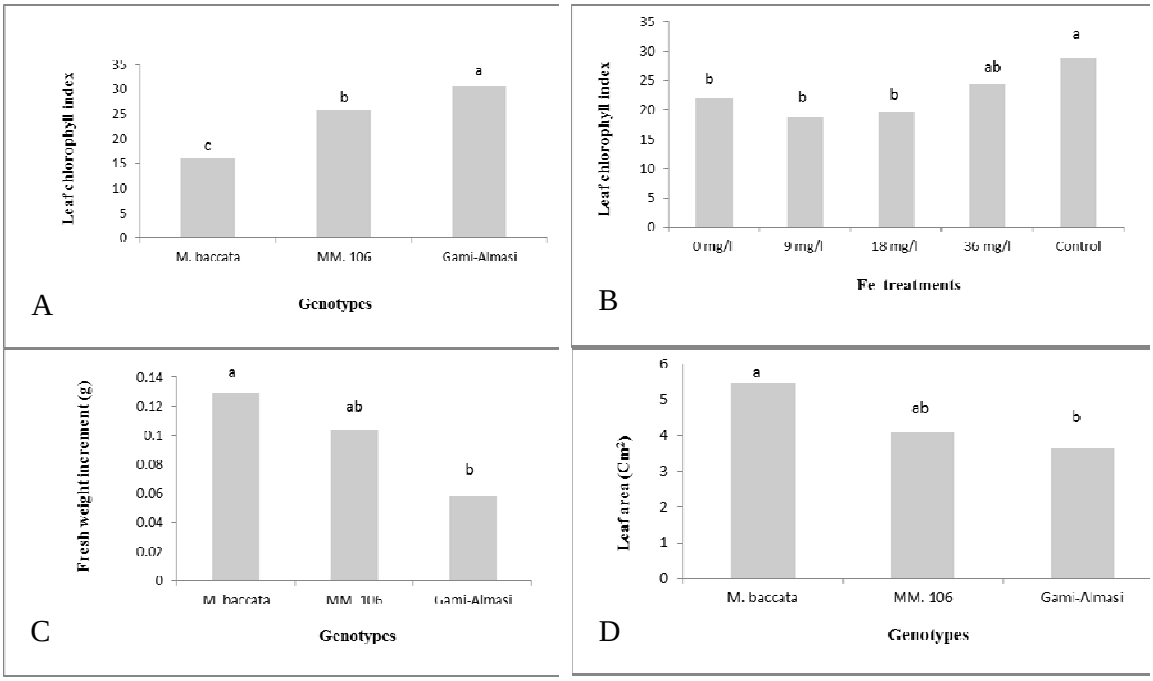

Figure 3: A, B and C: Responses of *Malus baccata*, Gami-Almasi and MM.106 to iron treatments based on leaf chlorophyll index, leaf area and fresh weight increment; D: The effect of different Fe treatments on leaf chlorophyll index averaged over all genotypes. Different letters represent significance at p<0.05.

Table 2: Analysis of variance of leaf chlorophyll index, leaf area and fresh weight increment.

Source of variation	df	LCI	LA	$FW_{inc.}$
Genotype	2	978.165**	37.546*	0.030*
Fe treatment	4	141.818*	4.971 ns	0.011 ns
Fe treatment × Genotype	8	38.617 ns	2.467 ns	0.010 ns

ns: Non-significant; *, **: Significant at p<0.05 and p<0.01, respectively; df: degree of freedom.

Gami-Almasi exhibited remarkably better tolerance to iron deficiency, so that even after 14 days, no symptom was observed. Afterwards, the symptoms were observed gradually as light-green-colored leaves. Both MM.106 and *M. baccata* genotypes illustrated deficiency symptoms earlier than Gami-Almasi at 14 and 21 days after treatment. Furthermore, MM.106 was more tolerant to iron deficiency than *M. baccata*, as it was expected according to the literature (Fig 1A and Fig 2). These results revealed that visual chlorosis can be used as an index in discriminating and screening tolerant and sensitive cultivars in *in vitro* conditions.

Gami-Almasi in vitro shoots indicated deficiency symptoms 14 days after treatments. The treatments containing 9 and 18 mg/l, in third time range, had no difference with the control (Fig 1B).

Inspection of 36mg/l treatment's effect on MM.106 genotype, showed no striking difference with control. However, 14 days after treatment, a remarkable increase in the appearance of symptoms was observed and this finally led to light green-colored leaves. Treatments 0, 9 and 18 mg/l did not indicate any prominent difference prior to the day of 14. After 21 days, the 0 mg/l treatment resulted in more severe symptoms (Fig 1C). In 0mg/l treatment, severe damage to chlorophyll and photosystem II proteins as well as fully consumption of plant iron content can explain this difference. In some cases, plants may pursue growth reduction strategy against environmental stresses and through this manage nutritional elements. On the other hand, in such conditions the possibility of iron transmission from old leaves to young ones could not be ignored.

In *M. baccata* genotype the deficiency symptoms were observed in shoots as diminishing green color of the leaves in both 18 and 38 mg/l treatments with no significant difference. In 9 mg/l treatment, up to the day 14, there was a direct relationship between time and chlorosis appearance. Nevertheless, thereafter, a remarkable increase in symptoms severity was observed. These results can be attributed to the primary rapid growth of shoots and iron fully unloading as well as high iron dilution in plant (Fig 1D).

The results of this study indicated that visual chlorosis index can be successfully used for selection of both sensitive and tolerant fruit tree cultivars in *in vitro* condition as declared by other researchers (Tangolar et al. 2008, Lombardi et al. 2003). Visual chlorosis index is severely affected by iron source and cultivar (Norvell and Adam 2006). Although this index is mostly observational and has low accuracy in comparison to chemical evaluations, it has good correlation with leaf chlorophyll content and cultivar resistance.

Analysis of variance for leaf chlorophyll index showed a significant difference between genotypes and treatments at $p<0.01$ and $p<0.05$, respectively. Interaction of genotypes and treatments was not significant (Table 2). Comparing treatment means revealed that Gami-Almasi genotype had the highest chlorophyll index, while *M. baccata* having the lowest (Fig 3A). Also the control treatment affected differently the measured attributes (Fig 3B). Tangolar et al. (2008) showed that leaf chlorophyll content has a positive relationship with increasing level of iron in culture medium. Lombardi et al. (2003) used this index to inspect the resistance of the peach rootstock Mr.S 2/5 to iron deficiency.

M. baccata illustrated the highest leaf area and green weight increment located in a group and Gami-Almasi with the lowest leaf area and green weight increment ranked last. The leaf area and green weight of MM.106 genotype was intermediate and located in the middle group ($p<0.05$) (Fig

3C-3D); while the effect of iron treatments was not significant on these traits (Table 2).

Norvell and Adams (2006) conducted an experiment on *Glycine max* and reported that young leaves were not developed under iron deficiency conditions, especially in sensitive cultivars. Furthermore, Dolcet-Sanjuan et al. (1992) in an experiment focusing on iron deficiency treatments upon quince and pear reported the green weight increase of seedling during the application of treatments was significant in pear while not in quince.

Statistical analysis for dry weight showed that genotype and iron treatment sources of variation were not significant. This was in agreement with the results of Tangolar et al. (2008) on iron deficiency treatment in different grape cultivars. However, Lombardi et al. (2003) reported that iron deficiency treatment decreased dry weight as much as 62% in Mr.S 2/5 peach rootstock 10 days after treatment. This paradox can be attributed to different mechanisms of iron deficiency tolerance in diverse plant species.

Both MM.106 and *M. baccata* genotypes illustrated deficiency symptoms earlier than Gami-Almasi, while MM.106 was more tolerant to iron deficiency than *M. baccata* as it was expected according to the literature. In other words, Gami-Almasi exhibited remarkably better tolerance than the other two genotypes to iron deficiency and can be considered as a valuable candidate to be used as an apple rootstock in calcareous soils.

In conclusion, the visual chlorosis index can be used as a useful parameter for discriminating and screening tolerant cultivars in *in vitro* conditions in a shorter time. Moreover, the *in vitro* system can be a useful approach to study the physiological, biochemical, and molecular alterations induced by iron deficiency even in the absence of root system. In particular, the time, the space and the needed amount of vegetative material can be drastically reduced which may alleviate these typical difficulties commonly encountered in fruit tree studies.

Acknowledgements
This research was supported by a grant from the University of Tabriz to HM for his MSc. Program.

References:

Bavaresco L, Fregoni M, Gambi E. 1993. In vitro method to screen grapevine genotypes for tolerance to lime-induced chlorosis. Vitis. 32: 145–148.

Dolcet-San Juan R, Mok D W S, Mok M. 1992. Characterization and in vitro selection for iron efficiency in Pyrus and Cydonia. In Vitro Cell. Dev. Biol.-Plant. 28: 25–29.

Fairbanks D J. 2000. Development of genetic resistance to iron-deficiency chlorosis in soybean. J. Plant Nutr. 23: 1903–1913.

Krouma A, Gharsalli M, Abdelly C. 2003. Differences in response to iron deficiency among some lines of common bean. J. Plant Nutr. 26: 2295-2305.

Lombardi L, Sebastiani L, Vitagliano C. 2003. Physiological, biochemical and molecular effects of in vitro induced iron deficiency in peach rootstock Mr.S 2/5. J. Plant Nutr. 26: 2149–2163.

Marino G, Beghelli S, Rombola A D , Cabrini L. 2000. In vitro performance at high culture pH and in vivo responses to Fe deficiency of leaf-derived quince BA 29 (Cydonia oblonga) somaclones regenerated at variable medium pH. J. Hortic. Sci. Biotechnol. 75 (4): 433–440.

Marschner H, Romheld V.1994. Strategies of plants for acquisition of iron. Plant and Soil. 165: 261–27.

Muleo R, Cinelli F, Viti R. 1995. Application of tissue culture on quince rootstock in iron-limiting conditions. J. Plant Nutr. 18 : 91-103.

Norvell W, Adams M. 2006. Screening soybean cultivars for resistance to iron-deficiency chlorosis in culture solutions containing magnesium or sodium bicarbonate. J. Plant Nutr. 29: 1855-1867.

Romera F J, Alcantara E, Delaguardia M D. 1991. Characterization of the tolerance to iron chlorosis in different peach rootstocks grown in nutrient solution. II. Iron-stress response mechanisms. Plant and Soil. 130: 121–125.

Scandalios J G. 1990. Response of plant antioxidant defence genes to environmental stress. Adv. Genet. 28: 1–41.

Serpil G, unlu G, Tangolar S, DaSgan Y, Yilmaz N. 2008. Use of in vitro method to evaluate some grapevine varieties for tolerance and susceptibility to sodium bicarbonate-induced chlorosis. In Vitro Cell Dev. Biol.-Plant. 44: 233–237.

Soldatini G F, Tognini M, Castagna A, Baldan B, Ranieri A. 2000. Alteration in thylakoid membrane composition induced by iron starvation in sunflower plants. J. Plant Nutr. 23: 1717–1732.

Tagliavini M, Abadia J, Rombola A D, Abadia A, Tsipouridis C, Marangoni B. 2000. Agronomic means for the control of iron deficiency chlorosis in deciduous fruit trees. J. Plant Nutr. 23: 2007–2022.

Tangolar S G. 2008. Use of in vitro method to evaluate some grapevine varieties for tolerance and susceptibility to sodium bicarbonate-induced chlorosis. In Vitro Cell Dev. Biol.-Plant. 44: 233-237.

Terry N, Abadıa J. 1986. Function of iron in chloroplast. J. Plant Nutr. 9: 609–646.

Wallace A, lunt O R. 1960. iron chlorosis in plants, a review. J. Am. Soc. Hortic. Sci. 75: 819-841.