INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 2, p. 460-467, 2015

RESEARCH PAPER OPEN ACCESS

In vitro screening of pea genotypes tolerant to iron deficiency based on physiological traits

Imran Ahmed, Ahmad Humayan Kabir*, Mohammad Farhadur Rahman, Mohammad Firoz Alam

Department of Botany, University of Rajshahi, Rajshahi 6205, Bangladesh

Key words: *In vitro* culture, Fe deficiency tolerance, peas, chlorophyll concentration, screening.

http://dx.doi.org/10.12692/ijb/6.2.460-467 Article published on January 29, 2015

Abstract

Screening of genotypes tolerant to Fe deficiency was performed in a number of Australian (Santi, Parfield, BC11, BC191, BC 17 and BC14) and Bangladeshi (BARI-1 and IPSA-2) genotypes based on different physiological parameters. Fe deficiency caused severe decrease in chlorophyll a and b concentrations in Parafield, BC17 and IPSA-2 grown on MS (Murashige and Skoog) media on *in vitro* conditions. In contrast, chlorophyll a and b concentrations were not significantly decreased in Santi, BC11, BC91, BC14 and BARI-1. Furthermore, number of leaves, shoot height and weight were significantly reduced in Parafield, BC17, BD14 and IPSA-2; whereas Santi, BC11, BC91 and BARI-1 did not show prominent decrease in the aforesaid growth parameters due to Fe deficiency. Again, Parafield, BC17 and IPSA-2 showed significant decrease in root length and root biomass under Fe deficiency. In contrast, these parameters were unchangeable in Santi, BC11, BC91, BC14 and BARI-1 in Fe shortage compared to controls. Based on these findings, tolerance to Fe deficiency in these pea genotypes can be categorized as: tolerant (Santi, BC11, BC91, BARI-1), intermediate (BC14) and sensitive (Parafield, BC17, IPSA-2). This study demonstrates the effectiveness of *in vitro* culture as an efficient method to screen Fe-efficient crop plants. Moreover, the ranking can be applied in plant breeding program and may have great advantage over conventional methods.

* **Corresponding Author:** Ahmad Humayan Kabir ✉ ahmad.kabir@ru.ac.bd

Introduction

Iron (Fe) deficiency induced leaf chlorosis is a widespread nutritional disorder in plants and can have serious consequences for agricultural production, causing a reduction in crop yields. Alkaline soils are regarded as potential inducers of Fe deficiency in plants even though the element might occur in high concentrations in the soil (Tangolar *et al.*, 2008). Fe is absorbed by soil particles in an insoluble form, which the plants are not capable of utilizing, and the soluble portion is usually insufficient (Lindsay, 1995). A high concentration of bicarbonate contributes to the soil alkalinity (Mengel *et al.*, 1984). Fe in interaction with other nutrients may become scarcely available to the plants (Prado, 2008). Based on the World Reference Base Soil Classification System, calcareous soil is classified under the reference soil group of Calcisols covering 800 million hectares worldwide, mainly found in South Asia, Australia, West Asia and North Africa under arid and semi-arid climates or Mediterranean climates (Srinivasarao *et al.*, 2006).

Plants have evolved a variety of mechanisms to increase Fe mobility and its uptake when Fe is deficient or unavailable in soil. These mechanisms are broadly categorized into two strategies in plants. Strategy I plants (belonging to dicots and non-graminaceous monocots) respond to Fe deficiency by inducing root ferric chelate reductase in the plasma membrane, releasing protons to acidify the rhizosphere soil, producing ethylene in roots and secreting organic acids or reductants such as phenolic compounds (Kabir *et al.*, 2012). As a dicot plant, pea plants follow the Strategy I mechanims (Kabir *et al.*, 2012, Kabir *et al.*, 2013) Strategy II mechanisms (in grasses) are involved in the formation of a complex with plant-borne high-affinity Fe(III) chelators (phytosiderophores) (Schmidt, 2003).

Field pea is one of the important legume vegetables and mostly grown for green pods and seeds. The green pods and immature seeds are rich in some important minerals i.e. calcium, phosphours and iron and vitamins and have a balanced amino acid composition. The crop becomes popular for its high nutritive value and good taste. It contains 13-35% protein, 20-50% starch, 4-10% sugar, 0.6- 1.5% fat and 2-4% minerals (Makasheva, 1983).

Pea plants are particularly susceptible to Fe deficiency. Growing Fe deficiency tolerant cultivars in Fe deficient soils could be economically preferable as it does not need application of any Fe compounds. However, selection of nutrient tolerant genotype is dependent on the suitable screening method. Therefore, genotypic differences in Fe-deficient plants on the basis of physiological and biochemical responses have long been the subjects of intensive studies. A large number of new field pea varieties with improved characteristics have been released in recent years in Australia. Nevertheless, very little is known towards the screening of Australian and Bangladeshi pea genotypes tolerant to Fe deficiency. Among the different screening method, *in vitro* screening facilitates rapid screening of large samples, aseptic culture condition and tightly controlled environments (Jimenez *et al.*, 2008, Lombardi, 2003a, Lombardi, 2003b, Makasheva, 1983, Tangolar *et al.*, 2008). *In vitro* screening through root culture, cell suspension, tissue or leaf culture, has been successfully used for nutrient efficiency studies other than Fe in *Brassica juncea* (Jain *et al.*, 1991), sugar beet (Larbi *et al.*, 2001), grapevine (Bavaresco *et al.*, 1993, Tangolar *et al.*, 2008), *Arabidopsis thaliana* (Cassin *et al.*, 2009, Wu *et al.*, 2002) and *Fragaria* sp. (Torun *et al.*, 2014). Despite the effectiveness and feasibility of *in vitro* screening, no reports are documented on the *in vitro* selection of pea genotypes tolerant to Fe deficiency.

Within the South Australia breeding program, a range of pea genotypes were derived from the crosses between Santi and Parafield and backcross of F1 hybrid either Santi (semi-leafless) or Parafield (conventional leaf) parents (Kabir *et al.*, 2012). However, these backcross genotypes were yet to screen for their Fe deficiency tolerance. Furthermore, peas are also popular in Bangladesh but propagation of pea severely affected by Fe deficiency in soil.

Thus, the present investigation was aimed at screening different pea genotypes mainly cultivated in Australia and Bangladesh. Further aim of this study was to establish the *in vitro* method for screening Fe deficiency genotypes where facilities and spaces are not available for field or hydroponic methods.

Materials and methods

Plant materials

Seeds of six Australian genotypes (Santi, Parafield, BC11, BC191, BC 17 and BC14) and two Bangladeshi genotypes (BARI-1 and IPSA-2) of *Pisum sativum* were collected from Dr. Jeff Paull, The University of Adelaide and Bangladesh Agricultural Research Institute, respectively.

Germination and culture conditions

Seeds were surface sterilized in 70% ethanol for 1 min and then washed in 5% sodium hypochlorite for 15 min. Seeds were then rinsed five times in sterile deionised water. Seeds were then germinated on moist filter paper wetted with deionised water placed on petridishes for one week in the dark at room temperature. After the roots started to germinate, only healthy and uniform seedlings were transferred to MS media (Murashige and Skoog 1962) supplemented with 1% sucrose, 0.5 g l^{-1} MES and 1% agar. Two different treatments were carried out: (a) control: MS media including Fe (b) treatment: MS media excluding Fe. The pH was adjusted to 6.0 by KOH/HCl just before autoclaving the medium at 121°C for 20 min. Plantlets were maintained in a climatic chamber at 24°C, under 55µmol $m^{-2}sec^{-1}$ PAR of light intensity and a 16/8 light/dark photoperiod and sub-cultured every 3 weeks.

Measurement of chlorophyll concentration

Chlorophyll (a and b) concentrations were measured according to the spectrophotometric method with some modifications (Wellburn, 1994). Briefly, the leaf samples were harvested and immediately dried in freezer. The leaf samples (50mg) were then grinded with mortar and pestle. About 8.0 ml of 96%-ethanol was then added and homogenized using vortex. The samples were placed in test tubes wrapped by aluminium foil and let them incubate at room temperature in an exhaust hood overnight. The next day, the samples were vortexed before measuring the absorbance of the extract at 470.0 nm, 648.6 nm and 664.2 nm

Measurement of morphological features

The number of leaves on each plant was counted three weeks after Fe deficiency was imposed. Whole shoot and root lengths were measured for each plant sample using a ruler. For measurement of fresh weight of root, roots were harvested and then wiped with clean tissue paper before measuring weight in electronic balance. Fresh weight of shoot was directly measured after harvesting. For measuring dry weight, roots and shoots were quickly rinsed in deionised water and then wiped with clean tissue paper. Root and shoot samples were then dried in an oven at 70°C for two days before dry weight was measured.

Statistical analysis

Statistical analyses (t-test) were performed using Genstat software (14th Edition). Significance was set at $P \leq 0.05$. Three replications of each sample were used for all experiments.

Results

Chlorophyll concentration

The concentration of chlorophyll a was significantly reduced in Parafield, BC17 and IPSA-2 under Fe deficiency compared to Fe sufficient plants (Figure 1). In contrast, no significant reduction in chlorophyll a concentration was observed in Santi, BC11, BC91, BC14 and BARI-1 due to Fe deficiency. Similar pattern was also observed for chlorophyll b except a significant decrease in chlorophyll b in BC14 under Fe deficiency compared to controls (Figure 2).

Number of leaves

Number of leaves was counted in all genotypes grown on both Fe sufficient and Fe deficient *in vitro* conditions. The number of leaves was not significantly reduced in Santi, BC11, BC91 and BARI-1 due to Fe deficiency compared to Fe sufficient controls (Table 1). In contrast, leaf number was

significantly reduced in Parafield, BC17, BD14 and IPSA-2 due to Fe deficiency compared to controls.

Shoot height

Alike leaf number, shoot height was also influenced by Fe deficiency. Shoot height in Santi, BC11, BC91 and BARI-1 was not significantly affected by Fe deficiency (Table 2). However, Fe deficiency caused significant decrease in shoot height in Parafield, BC17, BD14 and IPSA-2 compared to Fe sufficient plants.

Table 1. Number of leaves in different genotypes of field peas grown on Fe sufficient (Fe+) and Fe deficient (Fe-) *in vitro* culture. There were three replications for each sample. Data were taken on three weeks old plants.

Genotypes	Fe +	Fe -	t-test
Santi	11.0±1.4	10.0±1.0	*
BC11	10.3±0.5	9.3±1.5	*
BC91	10.6±5.0	8.3±3.5	*
BARI-1	11.4±3.0	10.6±1.1	*
Parafield	8.3±0.5	4.3±0.5	NS
BC17	10.3±1.5	7.0±1.0	NS
BC14	9.3±1.5	6.3±0.5	NS
IPSA-2	10.5±0.3	8.2±0.4	NS

* indicates statistically significant (P<0.05)

NS indicates statistically non-significant (P>0.05).

Fresh and dry weight of shoots

Fresh and dry weight of shoots was not significantly decreased in Santi, BC11, BC91, BC14 and BARI-1 under Fe deficiency compared to Fe sufficient plants. However, Fe deficiency caused significant decrease in shoot fresh and dry weight in Parafield, BC17 and IPSA-2 (Table 3).

Length of roots

Length of roots was not significantly decreased in Santi, BC11, BC91, BC14 and BARI-1 under Fe deficiency compared to the plants grown on Fe sufficient *in vitro* conditions. However, Parafield, BC17 and IPSA-2 were severely affected by Fe deficiency and their lengths of roots were significantly reduced under Fe deficiency (Table 4).

Table 2. Height of shoot (mm) in different genotypes of field peas grown on Fe sufficient (Fe+) and Fe deficient (Fe-) *in vitro* culture. There were three replications for each sample.

Genotypes	Fe +	Fe -	t-test
Santi	75.0±7.0	59.6±5.5	*
BC11	90.6±7.0	79.0±7.9	*
BC91	62.3±2.5	56.0±3.6	*
BARI-1	55.1±2.0	51.8±4.3	*
Parafield	41.0±3.0	33.6±2.0	NS
BC17	75.3±2.5	48.0±1.0	NS
BC14	40.5±0.7	31.0±3.6	NS
IPSA-2	65.9±1.2	59±2.1	NS

* indicates statistically significant (P<0.05)

NS indicates statistically non-significant (P>0.05).

Fresh and dry weight of roots

Like length of roots, fresh and dry weights of roots were also showed similar sensitivity to Fe deficiency (Table 5). Both fresh and dry weights of roots were not significantly decreased in Santi, BC11, BC91, BC14 and BARI-1 due to Fe deficiency compared to controls. Whereas, Fe deficiency caused significant decrease in both fresh and dry weights of roots in Parafield, BC17 and IPSA-2.

Table 3. Fresh weight and dry weight of shoot in different genotypes of field peas grown on Fe sufficient (Fe+) and Fe deficient (Fe-) *in vitro* culture. There were three replications for each sample. Data were taken on 3-week old plants.

Genotypes	Fresh weight (g)			Dry weight (g)		
	Fe +	Fe-	t-test	Fe +	Fe-	t-test
Santi	0.493±0.014	0.410±0.08	*	0.043±0.008	0.034±0.001	*
BC11	0.517±0.017	0.503±0.019	*	0.050±0.003	0.043±0.006	*
BC91	0.406±0.067	0.381±0.062	*	0.039±0.003	0.033±0.003	*
BARI-1	0.472±0.074	0.458±0.061	*	0.040±00.3	0.035±0.004	*
Parafield	0.371±0.035	0.265±0.019	NS	0.032±0.001	0.018±0.002	NS
BC17	0.376±0.017	0.244±0.005	NS	0.042±0.001	0.026±0.000	NS
BC14	0.326±0.148	0.168±0.056	*	0.034±0.018	0.020±0.006	*
IPSA-2	0.575±0.123	0.463±0.015	NS	0.050±0.003	0.038±0.002	NS

* indicates statistically significant (P<0.05)

NS indicates statistically non-significant (P>0.05)

Discussion

Screening of Fe-deficiency tolerant line has been mainly carried out *in vivo* by field tests and hydroponics culture experiments. Moreover, screening of the Australian and Bangladeshi pea genotypes for Fe deficiency was never extensively studied. The present study reveals the potentiality of Fe deficiency tolerance in a number of Australia and Bangladesh pea genotypes. The consistent results confirmed by different physiological parameters further pinpoint the efficiency of *in vitro* culture using MS media for Fe-efficient pea germplasm.

Table 4. Length of root (mm) in different genotypes of field peas grown on Fe sufficient (Fe+) and Fe deficient (Fe-) *in vitro* culture. There were three replications for each sample.

Genotypes	Fe +	Fe -	t-test
Santi	53.5±2.1	53.6±6.8	*
BC11	49.3±1.5	52.3±3.2	*
BC91	48.3±1.5	51.3±2.0	*
BARI-1	31.0±1.3	36.3±1.1	*
Parafield	36.6±2.0	32.3±1.5	NS
BC17	46.3±0.5	40.3±2.0	NS
BC14	47.5±2.1	47.1±1.0	*
IPSA-2	38±0.4	35±0.8	NS

* indicates statistically significant (P<0.05)

NS indicates statistically non-significant (P>0.05).

Chlorophyll concentrations in leaves of Santi and Parafield and their derivatives were studied in both Fe sufficient and Fe deficient *in vitro* conditions. Fe deficient plantlets grown *in vitro* showed the typical chlorosis within few days after the beginning of the experiments. Similar pattern of chlorophyll concentration was observed in all the genotypes except BC14. Results suggest that Santi, BC11, BC91 and BARI-1 are the Fe-deficiency tolerant line showing no significant reduction in chlorophyll a and b concentrations; whereas, Parafield, BC17 and IPSA-2 were found to be Fe-sensitive. BC14 showed

contrasting results in chlorophyll concentration and it may be attributed for their intermediate nature of tolerance to Fe deficiency. Tolerance and sensitivity of Santi and Parafield, respectively, were previously confirmed in both biochemical and molecular levels (Kabir *et al.*, 2012). Bangladeshi genotype, BARI-1 found to be highly tolerant to Fe deficiency that can be further used for pea breeding program.

Table 5. Fresh weight and dry weight of root (g) in different genotypes of field peas grown on Fe sufficient (Fe+) and Fe deficient (Fe-) *in vitro* culture. There were three replications for each sample. Data were taken on 3-week old plants.

Genotypes	Fresh weight			Dry weight		
	Fe +	Fe-	t-test	Fe +	Fe-	t-test
Santi	0.208±0.010	0.209±0.026	*	0.025±0.000	0.024±0. 215	*
BC11	0.209±0.301	0.203±0.012	*	0.0243±0.001	0.023±0.003	*
BC91	0.201±0.017	0.200±0.008	*	0.024±0.002	0. 667±0.001	*
BARI-1	0.201±0.017	0.220±0.010	*	0.024±0.004	0.026±0.006	*
Parafield	0.189±0.003	0.151±0.003	NS	0.025±0.000	0.016±0.002	NS
BC17	0.179±0.002	0.122±0.017	NS	0.023±0.0015	0.019±0.000	NS
BC14	0.188±0.004	0.183±0.002	*	0.023±0.000	0.022±0.001	*
IPSA-2	0.218±0.002	0.182±0.003	NS	0.027±0.000	0.028±0.001	NS

* indicates statistically significant (P<0.05)

NS indicates statistically non-significant (P>0.05).

Different growth parameters were severely affected by Fe-deficiency induced *in vitro* conditions. Results suggest that Parafield, BC17, BC14 and IPSA-2 are unable to tolerate Fe deficiency or in other words, they are not efficient to operate mechanisms conferring Fe deficiency tolerance. Inability of operating Strategy I mechanism in Parafield has been previously reported (Kabir *et al.*, 2012, Kabir *et al.*, 2013). In general, plants survive under Fe deficiency by operating a number of Fe-efficient mechanisms in roots. Santi, BC11, BC91, BC14 and BARI-1 were not significantly affected by *in vitro* induced Fe deficiency in their length and fresh and dry weights of roots. It suggests that Fe-efficient mechanisms are actively present in root systems that eventually let them continue normal growth and development. In contrast, these root parameters are negatively affected in Parafield, BC17 and IPSA-2 resulting stunned root and poor root biomass.

It is also evident that genotypic variation exits in both Australian and Bangladeshi genotypes in response to Fe deficiency. Taken as a whole, Santi, BC11, BC91 and BARI-1 are highly tolerant to Fe deficiency, showing normal chlorophyll synthesis and physiological growth. BC14 can be termed as intermediate genotype as this line shows both tolerance and sensitivity to Fe deficiency. Finally, Parafield, BC17 and IPSA-2 are highly sensitive and unable to survive or maintain normal growth and development under Fe deficiency.

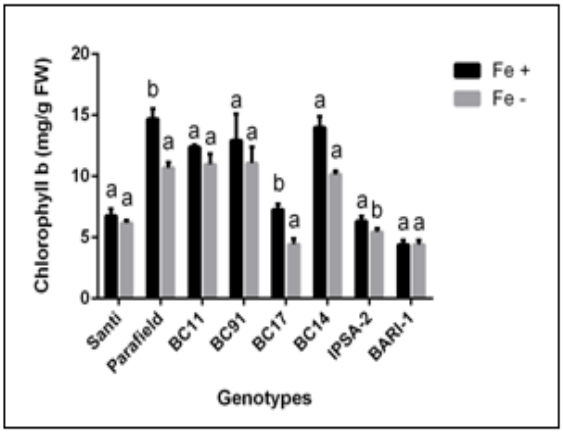

Fig. 1. Concentration of chlorophyll a in young leaves in a number of pea genotypes grown in Fe-sufficient (Fe+) and Fe-deficient (Fe-) *in vitro* culture. Different letters indicate significant differences between means ± SD of treatments (n = 3), comparisons were done for Fe + and Fe - conditions.

This study also confirms the efficiency of *in vitro* culture for screening pea genetic line for screening Fe or other mineral deficiency tolerant germplasm. This method overcomes the difficulty associated with the use of calcareous soils under field, greenhouse, and growth chamber conditions. Moreover, an *in vitro* system is easy to set up and it reduces time, space and cost associated with materials needed for glasshouse or hydroponic systems.

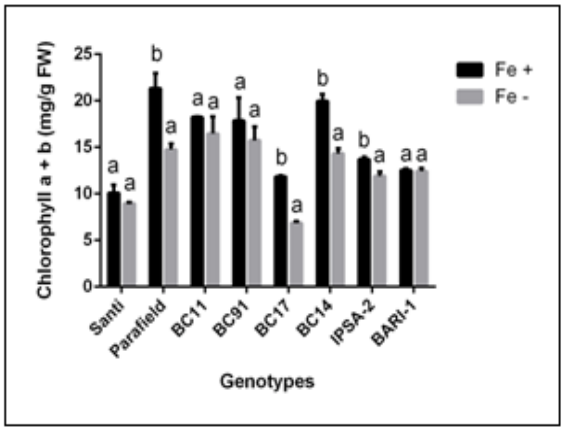

Fig. 2. Concentration of chlorophyll b in young leaves in a number of pea genotypes grown in Fe-sufficient (Fe+) and Fe-deficient (Fe-) *in vitro* culture. Different letters indicate significant differences between means ± SD of treatments (n = 3), comparisons were done for Fe + and Fe - conditions.

This paper explores a number of pea genotypes tolerant to Fe deficiency and the results can be used in pea breeding program. Results also enrich the knowledge for varietal characteristics of pea and can be used by farmers where Fe deficiency is a major obstacle for pea propagation. Efficiency of *in vitro* culture for the successful screening of plant genetic lines may also be followed by future scientists.

References

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

Cassin G, Mari S, Curie C, Briat JF, Czernic P. 2009. Increased sensitivity to iron deficiency in *Arabidopsis thaliana* overaccumulating nicotianamine. Journal of Experimental Botany **60(4),** 1249-59.

Jain S, Nainawatee HS, Jain RK, Chowdhury JB. 1991. Proline status of genetically stable salt-tolerant *Brassina juncea* L. somaclones and their parents cv. Prakash. Plant Cell Reports 684-687.

Jimenez S, Pinochet J, Abadia A, Moreno MA, Gogorcena Y. 2008. Tolerance response to iron chlorosis of prunus selections as rootstocks. Hort Science **43(2),** 304–309.

Kabir AH, Paltridge NG, Able AJ, Paull JG, Stangoulis JCR. 2012. Natural variation for Fe-efficiency is associated with upregulation of Strategy I mechanisms and enhanced citrate and ethylene synthesis in Pisum sativum L. Planta **235(6),** 1409-1419.

Kabir AH, Paltridge NG, Rossener U, Stangoulis JCR. 2013. Mechanisms associated with Fe-deficiency tolerance and signalling in shoots of Pisum sativum L. Physiologia Plantarum **147(3),** 381–395.

Larbi A, Morales F, Lopez-millan AF, Gogorcena Y, Abadia A, Moog PR, Abadia J. 2001. advance: reduction of Fe(III) chelates by mesophyll leaf disc of sugar beet. Multi-component origin and effects of Fe deficiency. Plant and Cell Physiology **42,** 94-105.

Lindsay WL. 1995. Chemical reactions in soils that affect iron availability to plants. A quantitative approach. In: Iron Nutrition in soils and plants. Ed. J. Abadia. Kluwer Academic Publishers, 7-14 p.

Lombardi L, Sebastiani L, Vitagliano C. 2003a. Physiological, biochemical, and molecular effects of *in vitro* induced iron deficiency in peach rootstock Mr.S 2=5. Journal of Plant Nutrition **26(10),** 2149–2163.

Lombardi L, Sebastiani L, Vitagliano C. 2003b. *In vitro* evaluation of iron-deficiency tolerance in an endemic putative apple rootstock. Research in Plant

Biology **2(6),** 23-29.

Makasheva R. 1983. Nutritional value in pea. Plenum press, New York, 605-640 p.

Mengel K, Breininger MT, Bubl W. 1984. Bicarbonate, the most important factor inducing iron clorosis in vine grapes on calcareous soil. Plant and Soil **81,** 333-334.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiologia Plantarum **15,** 473–497.

Prado RM. 2008. Nutrição de plantas. São Paulo: Unesp, 407.

Srinivasarao CH, Ganeshamurthy AN, Ali M, Venkateswar B. 2006. Phosphorus and micronutrient nutrition of chickpea genotypes in a multi-nutrient-deficient typic Ustochrept. Journal of Plant Nutrition **29,** 747–763.

Tangolar SG, Onlu 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 Cellular & Developmental Biology - Plant **44,** 233-237.

Torun AA, Kacar YA, Bicen B, Erdem N, SercE S. 2014. In vitro screening of octoploid *Fragaria chiloensis* and *Fragaria virginiana* genotypes against iron deficiency. Turkish Journal of Agriculture and Forestry **38,** 169-179.

Wu Z, Liang F, Hong B, Young JC, Sussman MR, Harper JF, Sze H. 2002. An endoplasmic reticulum-bound Ca^{2+}/Mn^{2+} pump, ECA1, supports plant growth and confers tolerance to Mn^{2+} stress. Physiologia Plantarum **130,** 128-137.

Schmidt W. 2003. Iron solutions: acquisition strategies and signaling pathways in plants. Trends in Plant Science **8(4),** 188-193.

Wellburn AR. 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal Plant Physiology **144,** 307-313.