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Improvement of *In vitro* Proliferation of Apple (*Malus domestica* Borkh.) by Enriched Nano Chelated Iron Fertilizer

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Abstract
Nano-fertilizers can increase value of products in agriculture. Iron plays many important and essential roles in plant growth and development as compared to other micronutrients. In the present study, effects of different levels of enriched nano chelated Iron fertilizer (25, 50, 100, and 200 mg l^{-1}) were investigated in comparison with the common Iron ($FeSO_4.7H_2O$) on *in vitro* proliferation of apple explants cultivar 'Gala' in the MS medium. The results showed that enriched nano chelated Iron increased growth and proliferation of apple in terms of number of nodes, shoots, and leaves, nodes and shoots length, leaf area, fresh and dry weights of shoot and chlorophyll index. The maximum growth and proliferation was observed at 100 mg l^{-1} of enriched nano chelated Iron, while in higher concentration (200 mg l^{-1}), the growth was decreased due to the Iron toxicity. According to the obtained results, 100 mg l^{-1} of enriched nano chelated Iron can be added for increasing growth on *in vitro* proliferation of 'Gala' apple cultivar. The present study is the first report of the effects of enriched nano chelated Iron fertilizer on growth and *in vitro* proliferation of apple that can be useful for *in vitro* culture of the plant.

Keywords: *In vitro* proliferation, Iron, *Malus domestica* Borkh, Nano-fertilizers.

Abbreviations: CHI, Chlorophyll index; **DW**, Dry weight; **FW,** Fresh weight; **INL**, Internode length, **LN**, Leaf number; **LA**, Leaf area; **MSHL,** Mean shoot length; **NN**, Node number; **RGR,** Relative growth rate; **SHL**, Shoot length; **SHN**, Shoot number.

Introduction

Apple is one of the most important fruits in temperate regions. Its global crop production is about 84.6 million tonnes in 2014 (FAO, 2017). Among the micronutrients, Iron (Fe) is a cofactor for approximately 140 enzymes that catalyze unique biochemical reactions (Brittenham, 1994). It also plays an important role in RNA synthesis and improves photosynthesis (Malakouti and Tehrani, 2005). Hence, Iron plays many essential roles in plant growth and development, including chlorophyll synthesis, thylakoid synthesis and chloroplast development (Miller et al., 1995; Hell and Stephan, 2003). Growth changings under Iron deficiency or toxicity affect various growth parameters such as root and shoot length,

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dry biomass accumulation, leaf number and area of different plant species under natural and laboratory conditions. A number of studies have tried to evaluate the effectiveness of Iron, its suboptimal and supra-optimal concentrations on relative growth rate and plant resistance (Snowden and Wheeler, 1993; Schmidt and Fühner, 1998; Dela-Guardia and Alcántara, 2002).

Different plant species including fruit trees, especially pome fruits (such as apples, pears and quinces), many trees and shrubs and ornamental plants are sensitive to factors that cause chlorosis (Shahabi and Malakouti, 2002; Kalbasi, 1995). It has been reported that lack of Iron causes yellowing of young leaves that leads to significant decrease of photosynthetic activity, resulting in decreased production of plant biomass (Briat et al., 2007).

Nanotechnology can increase the value of agricultural products and reduce envIronmental problems. Nanoparticles and powders due to extended specific surface area, more density of reactive areas, and increased reactivity of these areas on the particle surfaces, have high reactivity. These characteristics make the absorption of fertilizers that produced in nano scale easier (Mousavi and Rezaei, 2011). Nowadays nanotechnology has introduced new beneficial components and materials that help progress in ecological and environmental researches (Miller and Senjan, 2006). For example, the photo toxicity test of nano particles on *in vitro* culture was showed that nano Zn and ZnO particles have significant effects on explants' regeneration rate and inhibitions of bacterial and fungal contaminants (Helaly et al., 2014). Furthermore, the grown seedlings on MS culture medium are supplemented with uncoated nanoparticles of Iron oxide have highest rates of Iron uptake, lowest degree of chlorosis and increased vegetative growth characteristics such as fresh and dry weights and chlorophyll content when compared to MS medium without chelated Iron (Saeedi et al., 2016).

Iron as an essential element for plant growth and development is one of the important factors for horticultural plants. Recognition of Iron deficiency symptom on apple trees is a time consuming process. Consequently, application of nutrient on *in vitro* proliferation can be a model for comparison between conventional and nano fertilizers. On the other hand, production of explants with high quality and quantity without any diseases such as fungal and bacterial contaminants in fastest time on *in vitro* culture is extremely important. In this study the effects of nano chelated Iron fertilizer were investigated on *in vitro* proliferation of apple 'Gala' cultivar.

Materials and Methods

Plant Material

This experiment was carried out in the Tissue Culture Laboratory of the Department of Horticultural Sciences, Urmia University, Iran. The plant materials were on *in vitro* plantlets of apple cultivar 'Gala' which were obtained from Tissue Culture Laboratory of the mentioned Department.

Medium and Culture Conditions

For this experiment, nanoparticles of enriched chelated Iron were obtained from Sodour Ahrar Shargh Company (Iran). Enriched nano chelated Iron fertilizer characteristics for this research was Iron, 9% + Zinc1% + Magnesium1% purity and 10-20 nm particles size that was added to the Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

Iron was applied in two ways: (1) Explants subjected to 27.8 mg l^{-1} Iron ($FeSO_4.7H_2O$) treatment that is common in MS medium called control. (2) Common Ironin MS medium removed and explants subjected to enriched nano chelated Iron treatment in different concentrations (25, 50, 100 or 200 mg l^{-1}).

The explants (shoot at least with one node) were taken from *in vitro* explants, then were placed on MS (Control) and modified MS medium and supplemented

with 30 g l^{-1} sucrose, 4.44 µM L^{-1} BAP^1, 0.49 µML^{-1} IBA^2 and nano-Iron at 0, 25, 50, 100, and 200 mg l^{-1} concentrations and 7 g agar. Prior to adding agar, pH was adjusted to 5.8 then was autoclaved at 121 °C for 17 minutes with 100 kPa pressure. Explants cultured at 25±3 °C and 40 W m^{-2} of irradiation for 16-hr photoperiod.

Growth analysis

In this study the growth parameters like the number of nodes, shoots, and leaves were counted. In addition, shoot and internode length were measured by digital caliper (22855 NO: Z). Leaf area index was measured by leaf area meter (Model AM200, ADC Bio scientific Ltd., England). Also, chlorophyll index was measured with a chlorophyll meter (Konica Minolta 502, Japan) and expressed as SPAD index. Fresh weight of the explants was measured by a digital scale; dry weights were recorded after placing them in an oven with 70 °C for 48 h. Shoots were rated on the basis of their quality, considered as grade-I (branches that their lengths were more than 30 mm), grade-II (branches that their lengths were between 15-30 mm) and grade-III (branches that their lengths were less than 15 mm).

Data Analysis

The experiment was designed based on a completely randomized design (CRD) in five replications and five explants per replication. Data were analyzed using one-way analysis of variance (ANOVA). Polynomial regression analysis was performed also on the data to assess trends (Steel and Torrie, 1980) and mean comparisons were made using Duncan's multiple range test (DMRT) with software (SAS, v. 9.1, Cary, North Carolina, USA).

Results

According to the result of shoot elongation rate, the explants were classified to grade I, II and III. The results showed that shoot number increased with increasing enriched nano-Iron especially at 100 mg l^{-1} as compared to others in grade I and II (which branches length was longer than 15 mm). However, it didn't have any impact on shoot number in grade III (which the branches length was shorter than 15 mm) (Table 1). According to the results, the explant growth parameters were significantly increased with higher concentrations of enriched nano-Iron (Table 3).

Regression analysis showed an increasing trend in the growth rate by higher concentrations of enriched nano-Iron. So, with an increase in enriched nano-Iron concentrations the characteristics like the number of nodes, shoots, leaves, nodes length, shoots length and leaf area of apple explants increased in a quadratic trend (Table 3). In addition, the fresh and dry weights and chlorophyll index of explants were increased in a quadratic trend (Fig. 1 and 2). Moreover, visual assessment also indicated that the explants that treated by 100 mg l^{-1} enriched nano-Iron had better growth characteristics than the other treatments (Fig. 3).

Table1. Effects of enriched nano chelated Iron on shoot number in explants with the different branches length of 'Gala' apple cultivar under *in vitro* condition

Enriched nano chelated Iron (mg l^{-1})	Grade-I	Grade-II	Grade-III
Control	0.00	17.53	82.46
25	0.00	30.78	69.21
50	5.04	47.19	47.77
100	18.64	51.22	30.14
200	17.88	45.16	36.96

Grade-I (branches length is more than 30 mm), Grade-II (branches length is between 15-30 mm), Grade-III (branches length is less than 15 mm).

Table 2. F values of analysis of variance for the effects of enriched nano chelated Iron on different growth parameters of 'Gala' apple cultivar explants under *in vitro* condition.

F value									
NN	**SHN**	**LN**	**INL**	**SHL**	**MSHL**	**LA**	**CHI**	**FW**	**DW**
101.10**	137.84**	101.10**	41.79**	614.03**	60.60**	2.41**	6.14**	60.24**	35.42**

Abbreviations: node number (NN), shoot number (SHN), leaf number (LN), internode length (INL), shoot length (SHL), mean shoot length (MSHL), leaf area (LA), chlorophyll index (CHI), fresh weight (FW) and dry weight (DW); **: is significant at P<0.01.

Table 3. The effects of enriched nano chelated Iron on growth parameters of 'Gala' apple cultivar explants under *in vitro* condition.

Enriched nano chelated Iron mg l^{-1}	Node number	Shoot number	Leaf number	Internode length mm	Shoot length mm	Mean shoot length mm	Leaf area mm^2
Control	114.40	13.20	114.40	1.266	114.50	10.98	385.50
25	126.40	15.20	126.40	1.529	193.00	12.70	369.75
50	178.60	21.00	178.60	1.882	335.20	15.95	420.40
100	276.20	33.80	276.20	2.385	654.20	19.47	441.00
200	227.00	27.00	227.00	2.372	535.50	19.86	429.80
Trend	Q**	Q**	Q**	Q**	Q**	Q**	Q**

Q**: is significant quadratic regression at P<0.01.

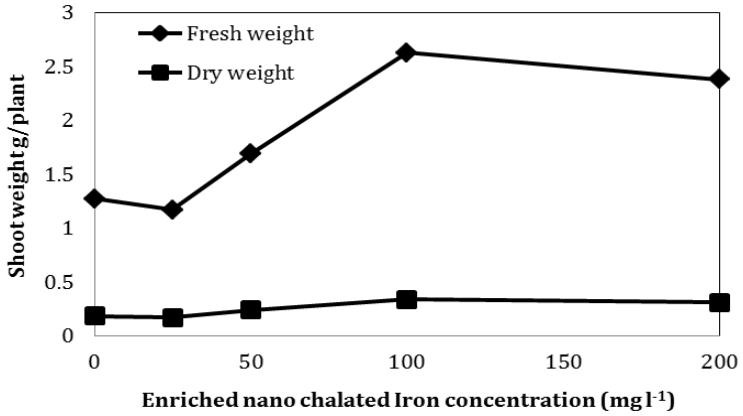

Fig. 1. Fresh and dry weights of apple explants as a function of enriched nano chelated Iron concentrations in the medium culture. The trends were significant quadratic regressions, fresh weight: y=1.009+0.0202x–0.05x2–7E, R2=0.85; dry weight: y=0.152+0.0023x–0.06x2–8E, R2=0.86**; ****P*<0.01.**

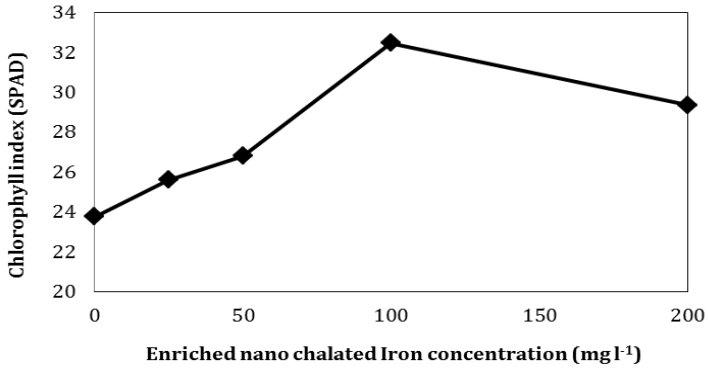

Fig. 2. Chlorophyll index as a function of enriched nano chelated Iron concentrations in the medium culture. The trend was a quadratic regression: y=22.96+0.1314x–0.0005x2; R2=0.89*; **P*<0.01.

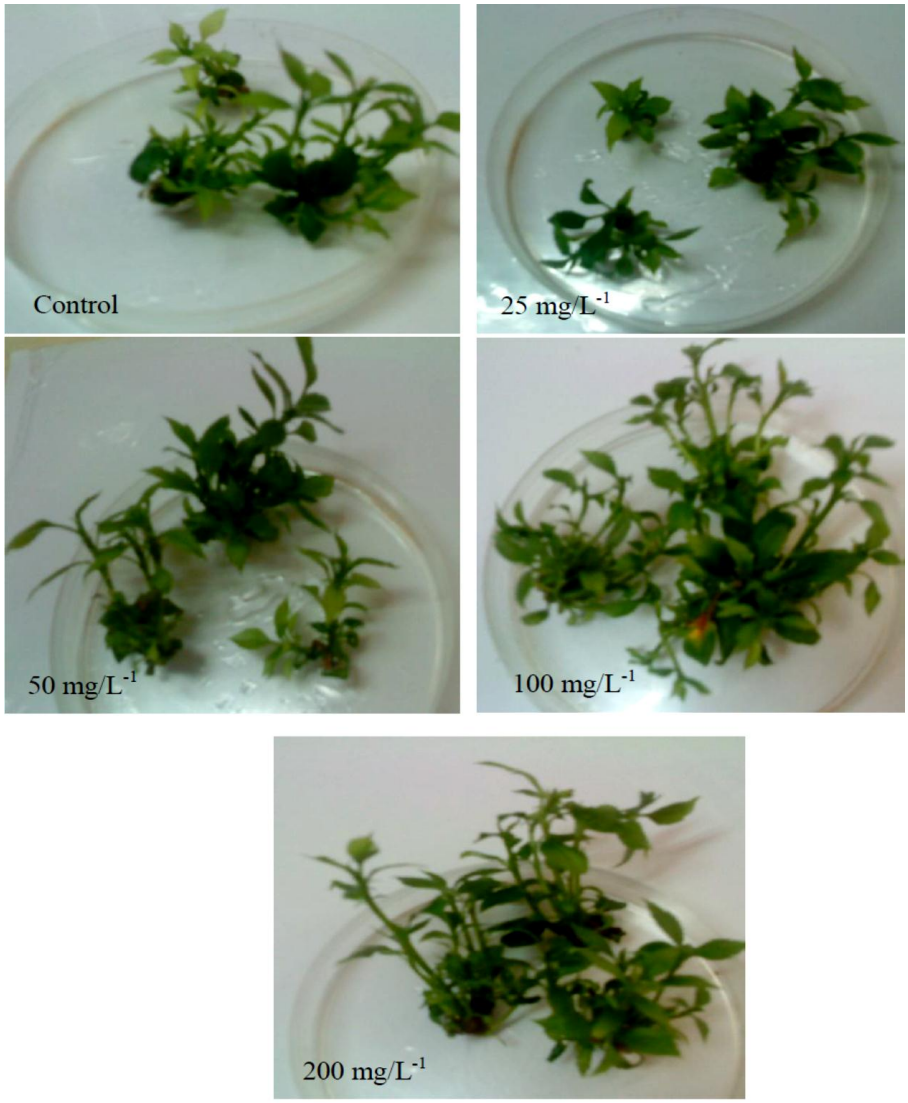

Fig. 3. Effects of different concentration of enriched nano chelated Iron on proliferation of 'Gala' apple explants in the MS medium.

The results showed that an addition of enriched nano-Iron to the concentration of about 100 mg l^{-1} in the MS medium was optimum for obtaining maximum vegetative growth and color for apple tissue culture.

Discussion

Fe-oxide nanoparticles are more effective than the chelate Iron under *in vitro* condition for decreasing the chlorosis induced by Iron deficiency. Therefore, Fe-chelate can be substitute by Fe-oxide nanoparticles on *in vitro* proliferation (Saeedi et al., 2016). According to Jamzad Fard et al. (2013), addition of nano-Fe oxide in VS medium significantly increased shoot length, shoot per explant, number leaves and rooting percentage on *in vitro* culture in *Rosa chinesis* Jacq. 'Minima'. Similarly, Jalali and Zargani (2014) reported that application of nano chelated Iron on *Lactuca sativa* L. varieties significantly increased root fresh weight, plant dry weight and chlorophyll compared to Fe-EDDHA, $FeSO_4$ and control. Helaly et al. (2014) researched the effects of conventional and nano particles on *in vitro* culture of banana in various concentrations. They observed that the highest percentage of somatic embryogenesis, excellent shooting, rooting and regenerated plantlets

were found in MS media supplemented with 100 mg l^{-1} nano Zn followed by nano ZnO. On the other hand, the microbial contaminants in banana on *in vitro* culture can be effectively eliminated by incorporation of nano Zn and nano ZnO particles on growth media at different concentrations (Helaly et al., 2014).

Feizi1 et al. (2013) has reported that applications of nanomaterial can promote plant germination earlier than control and improve the plant production. According to their results, the highest seedling weight was shown at 100 ppm nano-Iron dioxide which was 23.6% more than untreated control whereas bulk Fe_2O_3 increased seedling weight by only 8% in comparison to the control. In addition, higher concentrations of Iron-oxide exhibited phytotoxicity effects on seedling growth of wheat and Iron excess treatment generate oxidative stress in cells. But the ferro phase (Fe^{2+}) nano-particles not only may have a chemical but also a magnetic influence on the enzymatic structures implied in the different stages of the photosynthesis reactions (Feizi1 et al., 2013).

Raccuciua and Creanga (2007) have concluded that low concentrations of aqueous ferrofluid instigate the growth of young plants of maize whereas high concentration causes toxic effects or prevent it from growing. It appears that the Iron nanoparticles Fe_2O_3 will develop the process of seed germination like water and oxygen, which stimulates the growth of seedlings. Nadi et al. (2013) have showed that the highest and lowest grain yield belonged to 6 g l^{-1} nano-Iron and control, respectively. Increasing of nano-Iron concentration had positive and significant effects on grain yield, protein percent and chlorophyll content. Also, Kakiuchi and kobata (2008) have reported that essential element such as Iron fertilizers can affect the leaf area to be able to produce much more assimilates. Nazaran et al. (2012) have concluded that Iron application on sweet corn leaves affected number of seed and resulted in higher yield. Moreover, Sheykhbaglou et al. (2010) have showed that nano-Iron oxide had significant effects on dry pod weight; leaf plus dry pod, and yield. Also, they showed that application of nano Iron-oxide at 0.75 g l^{-1} had the highest effects on pod dry weight as compared to other treatments. Therefore, the use of Iron nano-particles caused higher pod number and leaf dry weight and finally it will increase total yield. To track dynamic changes in growth and PSII activity in pea plants developing under different Iron supply, ranging from complete deficiency to toxicity, Nenova (2006) reported that growth, photosynthetic pigment concentration and chlorophyll fluorescence parameters improved by adequate Iron supply. In another study, the effects of enriched nano chelated Iron fertilizer on fruit yield, number of fruits per plant, fruit length and fruit width were significant. In addition, there was a significant difference on number of branches per plant (Bozorgi, 2012). Also, the use of Iron-oxide nanoparticles has a major impact on peanut plant growth and also photosynthesis. Iron-oxide nanoparticles compared with other treatments such as organic matter and Iron citrate can facilitated the photosynthetic and Iron transporting in the peanut leaves (Liu et al., 2005). Abdzad-Gohari and Noorhosseini-Niyaki (2010) have reported that the effects of Iron foliar spraying on peanut caused maximum production with Iron foliar spraying treatment. Moreover, Zareie et al. (2011) have reported that the use of foliar spraying of Iron fertilizer (sulphate of Iron) had a significant effect on yield of safflower genotypes. Iron is needed for a lot of metabolism activity in plants (Motta et al., 2001). Thus, the plants to continue their optimum growth need plenty of Iron (Brown et al., 1972).

In this study the growth parameters was increased by increasing the concentration of enriched nano-Iron. The highest growth rate of shoots, dry and fresh weights, leaf

number, leaf area, etc. was increased in 100 mg l^{-1} nano-Iron concentration. This increase agreed with the results of Shahabi et al. (2005) which found that Iron deficiency in nutrient solution significantly reduced the fresh and dry weights, chlorophyll and dissolved sugar. Iron is an essential element in plants. It is used to increase crop production and improve its quality; therefore, deficiency or toxicity of this element will decrease performance (Shahabi et al., 2005). In another study, Wiersma (2007) has showed that in the condition of low to intermediate Iron deficiency, taking Iron chelate soil (Fe-EDDHA) in soybean planting time, reduced the symptoms of Iron deficiency in plants in the early of the growing season, but had no significant effect on performance and grain Fe concentration. This method is suitable for removing Iron deficiency but only effective for plants with mild symptoms of yellow leaves (chlorosis) or in a short period of time (Naeve, 2006).

Iron is an important element in higher plants. There are long-term studies on chlorophyll formation. In this study, increase in chlorophyll content was observed that was in agreement with Mak's results in 1973, he believed that accumulation of Iron in the chloroplasts and the activation of several enzymes like catalase, and cytochrome oxidase were considerably reduced with Iron deficiency (Mak, 1973). Iron plays an important role in the metabolism of nucleic acid, chloroplasts and chlorophyll synthesis, while, in the absence of Iron the leaves show chlorosis. Dhoke et al. (2013) have reported that foliar spray of nano-particle suspensions of ZnO, FeO and ZnFeCu oxides were able to affect development and growth processes of the mung plant (*Vigna radiata*). Increase in root and shoot lengths as well as biomass increase were recorded for nanoparticle treated plant as compared to the control. Moreover, Jahanara et al. (2013) have reported that application of nano-Iron oxide compared to the control treatment caused higher protein percentage, number of grains per pod and grain yield in *Phaseolus vulgaris* genotypes.

Conclusion

The results obtained from current study showed that enriched nano-Iron fertilizer is a useful tool for improving growth and proliferation of apple explants on *in vitro* conditions. The maximum growth and proliferation was observed at 100 mg l^{-1} enriched nano chelated Iron. In higher concentration (200 mg l^{-1}), the growth parameters were decreased due to the toxicity effects. According to the results, conventional Iron can be substitute by enriched nano chelated Iron for *in vitro* proliferation of apple. Therefore, 100 mg l^{-1} of enriched nano chelated Iron fertilizer can be added to the MS medium for increasing growth and proliferation of 'Gala' apple cultivar *in vitro*.

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