IRON NANOPARTICLES ON GROWTH AND ACCLIMATIZATION OF *Chrysanthemum morifolium* Ramat. cv. "Jimba" IN DIFFERENT CULTURE SYSTEMS

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Received: 26.11.2019
Accepted: 27.3.2020

SUMMARY

In plant tissue culture, iron nanoparticles (FeNPs) was one of the first types of nano to be used in plants. Previous reports have identified the effect of FeNPs on many different plant species. In this study, FeNPs was used to replace Fe-EDTA in MS (Murashige, Skoog, 1962) medium to assess their effects on growth, chlorophyll (a, b and a+b) accumulation, antioxidant activity of ascorbate peroxidase (APX) and superoxide dismutase (SOD) enzymes, and acclimatization in greenhouse conditions in different culture systems (*in vitro* solid, *in vitro* hydroponic and microponic culture). The obtained results show that FeNPs added to MS medium was higher growth, chlorophyll (a, b and a+b) content, antioxidant activity of SOD and APX enzymes than Fe-EDTA in MS medium as control treatment. The effect of FeNPs are differences between culture systems. *In vitro* solid and microponic culture systems, the optimal concentration is 75 mM FeNPs and *in vitro* hydroponic culture system is 100 mM FeNPs. The optimal activity of the antioxidant enzyme SOD (35.04 U.mg⁻¹ prot) obtained in the roots of cultured plants in microponic culture system; meanwhile, the optimal activity of the antioxidant enzyme APX (2.11 μmol.min⁻¹.mg⁻¹ prot) obtained in leaves cultured in solid culture system. The plantlets derived from MS medium added FeNPs were transfered into greenhouse conditions, the microponic cultivated plants supplemented with FeNPs at a concentration of 100 mM gave the highest survival rate (94.67%). The results of this study showed that FeNPs can replace Fe-EDTA salt in MS medium, and iron deficiency in culture media will reduce chlorophyll content.

Keywords: Ascorbate peroxidase, Chrysanthemum, in vitro culture, iron nanoparticles, microponic, superoxide dismutase.

INTRODUCTION

The culture systems differ not only from the ingredients in the culture medium but also different conditions such as medium status, aeration or sterility, etc. Depending on certain conditions, uptake of nutrients will also be affected. There have been several reports on the use of different culture systems in investigation of nanoparticles on plants. Kumari *et al.* (2009) cultivated onion plants on liquid media showed that silver nanoparticles have the ability to seriously affect cell division, other studies have shown that liquid medium has the ability to help increase the absorption of plant growth regulators, and dissolve nutrients better in solid medium (Gurel *et al.*, 1998; Klimaszewska *et al.*, 2000). Aeration is also a factor that greatly affects the ability to absorb...
nutrients from the roots. However, there are currently no studies on the uptake of nanoparticles in aerated and non-aerated culture.

As a micronutrient in plant tissue culture, FeNPs was one of the first types of nano to be used in plants. Previous reports have identified the effect of FeNPs on many different plant species. FeNPs have been reported to significantly increase yield on soybean crops (Roghayyeh et al., 2010), Faba beans (Nadi et al., 2013), and ginger (Ganesan, Lazer, 2016). Libralato et al. (2006) studied the effects of FeNPs compared to other forms of iron, which showed that FeNPs were significantly effective in biomass growth in Lepidium sativum, Sinapis alba and Sorghum saccharatum and could be replaced Fe-EDTA in medium. Recent studies on the addition of FeNPs as a nutritional component in in vitro culture have also reported initial results. Soad et al. (2016) analyzed the effectiveness of FeNPs compared to other iron forms in minimizing chlorosis and increasing growth of in vitro Volkamer lemon. However, the results of research on FeNPs are still limited, especially in different culture system. In this study, the effect of FeNPs on plant growth in different culture systems (in vitro solid, in vitro hydroponic and microponic) was investigated as well as the acclimatization at the greenhouse.

MATERIALS AND METHODS

Plant material

Chrysanthemum shoots (Chrysanthemum morifolium Ramat. cv. "Jimba") in 1-month-old, disease-free in vitro originating from Japan, was available at the Department of Molecular Biology and Plant Breeding (Tay Nguyen Institute for Scientific Research).

Nanoparticles solution

FeNPs solution (20 - 60 nm) was made by dehydration method using precursor FeSO₄·7H₂O. Sodium borohydride was used as a reducing agent and carboxymethyl cellulose as a stabilizer (Chau et al., 2008).

Effect of FeNPs on the growth of Chrysanthemum in culture systems

Chrysanthemum shoots (2.0 cm) with 2 pairs of leaves were cultured on modified MS medium that removed Fe-EDTA, and added different concentrations of FeNPs (0, 25, 50, 75, 100 and 200 mM corresponds to Fe0, Fe¼, Fe½, Fe¾, Fe¹ and Fe2). MS medium with 100 mM FeSO₄·7H₂O (FeMS) was used as the control.

The culture systems in this study include:

**In vitro solid culture:** 250 mL glass bottle contained 40 mL of MS medium was supplemented with 30 g/L sucrose and 8 g/L agar.

**In vitro hydroponic culture:** 250 mL glass bottle contained 40 mL of MS medium was supplemented with 30 g/L sucrose (Nhut et al., 2005). The medium after preparation was adjusted to pH = 5.8. In vitro solid and in vitro hydroponic culture systems were autoclaved at 121°C, 1 atm for 30 min.

**Microponic culture:** A circular plastic box (500 mL) with 8.5 cm in height, 12 cm in top diameter and 9 cm in bottom diameter (Dai Dong Tien, Vietnam) was contained 40 mL of ½ MS medium (a half mineral) (Tung et al., 2018).

Absorption spectrum and chlorophyll content analysis

SOD activity was determined according to Beyer and Fridovich (1987). The specific SOD activity was expressed as enzyme units per mg soluble protein (U.mg⁻¹ prot).

APX activity was measured following the H₂O₂-dependent oxidation of ascorbate in a reaction mixture composed of 50 mM K-phosphate buffer (pH 7.0), 1 mM EDTA, 0.5 mM ascorbate, and 0.1 mM H₂O₂. The decrease in absorbance was recorded at 290 nm (Miyak, Asada, 1992). The specific APX activity was expressed as μmol ascorbate per min mg soluble protein (μmol.min⁻¹.mg⁻¹ prot).

The activities of enzymes were measured in 10 explants and triplicate, at 30°C, using a
Shimadzu UV-160 Spectrophotometer (Shimadzu UV-160, Kyoto, Japan).

Content of chlorophyll a and chlorophyll b were determined based on maximum absorption spectrophotometer of chlorophyll a (662 nm) and chlorophyll b (645 nm) using UV-2900 spectrophotometer machine (Lichtentaler, Wellburn, 1985).

Chlorophyll a = (11.75*A_{662} − 2.35*A_{645}) \, (\mu g/g)

Chlorophyll b = (18.61*A_{645} − 3.96*A_{662}) \, (\mu g/g)

**Culture conditions**

*In vitro:* The culture condition was set up fluorescent light with photoperiod of 16 h/day, intensity of 45 \, \mu mol.m^{-2}.s^{-1}, relative humidity 50 - 60% at 25 ± 2°C.

*Ex vitro:* Experimental greenhouse conditions with daytime temperatures of 27 ± 2°C, night temperatures of 14 ± 2°C, humidity of 70 - 80%, light coverage of 50%, the medium was humus added to a plastic pot with a mouth diameter of 10 cm, a bottom of 5 cm, and a height of 7 cm.

**Data processing**

The experiments were repeated 3 times. All data were processed by MicroSoft Excel 2010 and SPSS 16.0 statistical analysis software by Duncan test method with \( \alpha = 0.05 \) (Duncan, 1955).

**RESULTS AND DISCUSSION**

**In vitro solid culture**

The effect of FeNPs on the growth of shoots cultured on solid medium was recorded after 4 weeks of culture (Table 1 and Fig. 1, 2). Results showed that plant height, number of leaves, number of roots did not have a clear difference between the treatments. However, the fresh weight, dry weight, root length, chlorophyll content index (a, b and a+b) were different (Table 1 and Fig. 1).

In particular, the addition of Fe\(^{2+}\) (75 mM FeNPs) on MS medium resulted in an increase in fresh weight (1.45 g), dry weight (0.16 g), chlorophyll a (26.67 \, \mu g/g), chlorophyll b (12.49 \, \mu g/g) and chlorophyll a+b (39.16 \, \mu g/g) were the best compared to other treatments (Table 1 and Fig. 1). In the treatment without FeNPs (Fe0), the lowest chlorophyll (a, b and a + b) content was observed (17.12 \, \mu g/g, 8.22 \, \mu g/g, and 25.34 \, \mu g/g, respectively), lack of pigment, light green and small leaves. FeNPs concentration exceeded Fe1 (100 mM), most of the indicators such as plant height, fresh weight, dry weight and chlorophyll content tended to decrease again (Table 1 and Fig. 1).

**Table 1.** Effect of FeNPs on *Chrysanthemum* growth in *in vitro* solid culture after 4 weeks of culture.

| Treatment | Plant height (cm) | No. of leaves | No. of root | Root length (cm) | Fresh weight (g) | Dry weight (g) |
|-----------|------------------|--------------|-------------|------------------|-----------------|---------------|
| FeMS      | 4.34b*           | 13.33ab      | 13.67ab     | 0.33c            | 0.70c           | 0.09b         |
| Fe0       | 4.83ab           | 11.33ab      | 12.33ab     | 0.61a            | 1.01bc          | 0.11b         |
| Fe\(^{2+}\) | 4.21b           | 11.00ab      | 11.00b      | 0.40b            | 0.81bc          | 0.09b         |
| Fe\(^{1+}\) | 4.22b           | 11.67ab      | 13.00ab     | 0.38bc           | 0.79bc          | 0.08b         |
| Fe\(^{3+}\) | 5.02a           | 14.67a       | 15.67a      | 0.43b            | 1.45a           | 0.16a         |
| Fe1       | 5.17a            | 11.00b       | 12.33ab     | 0.42b            | 1.08b           | 0.13b         |
| Fe2       | 4.53ab           | 12.33ab      | 13.33ab     | 0.36bc           | 0.94bc          | 0.10b         |

* Different letters (a, b, …) in the same column represent statistically significant differences at \( \alpha = 0.05 \) (Duncan’s test).
This result shows that iron plays an important role in the growth and synthesis of chlorophyll. Iron not only participates in chlorophyll biosynthesis by being a constituent of proteins [4Fe - 4S], FX, FA, FB (PsbA, PsbB, PsbC) in photosystem I but also participates in proteins Cyt b559 (PsbE, PsbF), Cyt b6 f complex, Rieske [2Fe - 2S] protein (PetC), Cyt f (PetA), etc. with a key role in electronic transport (Mamyandi et al., 2009). In addition, iron is the substrate that forms the active center of the protein glutamyl-tRNA reductase, which contributes to the synthesis of 5-aminolevulinic acid as a precursor to chlorophyll (Kumari et al., 2009).

The chlorophyll content in Fe-EDTA and FeNPs treatments were higher than those in the non-nanoparticles (Fe0) treatment. The concentration of Fe¾ (75 mM) is more effective when using Fe-EDTA (100 mM), showing that FeNPs are more effective than iron in the form of ions. FeNPs have a higher surface penetration and interaction efficiency than iron in the form of Fe-EDTA, thus optimizing the effect of iron in culture media. This result was consistent with the study of Lopez-Moreno et al. (2010), FeNPs were significantly effective in biomass growth in L. sativum, S. alba and S. saccharatum and could be substituted for Fe-EDTA. Plantlets in MS medium supplemented with FeNPs showed a significant increase in fresh weight, dry weight, chlorophyll content compared to the control (Syu et al., 2014).

**In vitro hydroponic culture**

In vitro hydroponic culture, Fe1 (100 mM) supplemented in MS medium showed the best effect on plant height (7.51 cm), fresh weight (1.49 g), and dry weight (0.16 g) compared to other treatments (Table 2 and Fig. 4).

Especially, the chlorophyll (a, b and a+b) content (14.67 µg/g, 6.68 µg/g and 21.35 µg/g) was the lowest in Fe0 treatment and the highest in Fe1 (100 mM) supplementation were 29.85 µg/g, 14.39 µg/g and 44.24 µg/g (Fig. 3). This demonstrated the important role of iron in the pigmentation of Chrysanthemum, and the addition of FeNPs was more effective than the ionic form in the same concentration.
The results also showed that the root length was inversely proportional to the concentration of replacement FeNPs in the culture medium. All treatments with FeNPs showed lower root length than the FeMS (Fe-EDTA in MS medium). Liquid conditions cause the roots to release some of the oxygen in the respiration, which oxidizes the metal layer around the root area and forms an insoluble metal oxide layer, which precipitates in the surrounding area. Around the roots reduces the ability to absorb other nutrients and inhibits root growth (Hinsinger, 2001). The phenomenon of FeNPs inhibiting root growth is also observed in rice (Dariosh, Akihiro, 2014), and sunflower (Musante, White, 2012). In this study, replacing Fe-EDTA with FeNPs (100 mM) gave the best growth but FeNPs made roots shortened in in vitro hydroponic.

![Figure 2. Effect of FeNPs on Chrysanthemum growth in in vitro solid culture after 4 weeks of culture.](image)

**Microponic culture**

Results of cultivation in microponic with Fe – EDTA replacement with FeNPs at different concentrations are shown in Table 3, and Figure 5, 6. In this study, the results showed that plant height, number of leaves and number of roots wasn’t significant difference; however, dry weight, fresh weight and root length, chlorophyll were the best in Fe³⁄₄ (75 mM) treatment.

Cultivation of plants in a microponic condition, which inhibits the extension of roots in liquid medium, has been overcome.
The root length achieved in Fe½ was 3.4 times longer than in the same treatment in the same liquid medium (Table 2 and 3). This result is explained by the better nutrient uptake combined with photoautotrophic conditions in microponic condition (open systems) that promote root growth, causing them to grow longer while culture in closed in vitro condition. Experiments show that the most suitable concentration of FeNPs to replace Fe-EDTA in microponic condition is Fe¾.

Table 2. Effect of FeNPs on Chrysanthemum growth in in vitro hydroponic culture after 4 weeks of culture.

| Treatment | Plant height (cm) | No. of leaves | No. of root | Root length (cm) | Fresh weight (g) | Dry weight (g) |
|-----------|-------------------|---------------|-------------|------------------|-----------------|---------------|
| FeMS      | 7.17ab*           | 13.00a        | 16.00a      | 1.60a            | 1.22b           | 0.13ab        |
| Fe0       | 5.97d             | 12.33a        | 11.67b      | 1.41ab           | 0.76c           | 0.09c         |
| Fe1/4     | 6.63c             | 14.00a        | 16.67a      | 1.45ab           | 0.68d           | 0.07cd        |
| Fe1/2     | 6.45c             | 13.00a        | 14.33ab     | 1.41b            | 0.96b           | 0.11b         |
| Fe3/4     | 7.23ab            | 13.00a        | 15.33a      | 1.29b            | 1.36b           | 0.14ab        |
| Fe1       | 7.51a             | 11.67ab       | 10.00d      | 1.13bc           | 1.49a           | 0.16a         |
| Fe2       | 5.48d             | 9.00b         | 11.00cd     | 1.25bc           | 0.68e           | 0.06d         |

* Different letters (a, b, ...) in the same column represent statistically significant differences at $\alpha = 0.05$ (Duncan’s test).

Figure 3. Effect of FeNPs on chlorophyll accumulation of 4-week Chrysanthemum in in vitro hydroponic culture.
Figure 4. Effect of FeNPs on *Chrysanthemum* growth in *in vitro* hydroponic culture after 4 weeks of culture.

Table 3. Effect of FeNPs on *Chrysanthemum* growth in microponic culture after 4 weeks of culture.

| Treatment | Plant height (cm) | No. of leaves | No. of root | Root length (cm) | Fresh weight (g) | Dry weight (g) |
|-----------|-------------------|---------------|-------------|------------------|------------------|---------------|
| FeMS      | 4.52bc*           | 6.67b         | 18.67a      | 2.50d            | 0.64bc           | 0.07d         |
| Fe0       | 4.34c             | 10.00a        | 16.00a      | 3.37c            | 0.59c            | 0.06e         |
| Fe1/4     | 5.05b             | 8.00b         | 16.67a      | 3.24c            | 0.73ab           | 0.10ab        |
| Fe1/2     | 5.31a             | 8.33ab        | 18.00a      | 4.83a            | 0.83a            | 0.11ab        |
| Fe1/4     | 5.26a             | 7.67b         | 18.33a      | 4.01ab           | 0.80ab           | 0.10ab        |
| Fe1       | 5.15ab            | 8.00b         | 17.67a      | 3.44c            | 0.73ab           | 0.08bc        |
| Fe2       | 4.53bc            | 7.67b         | 17.67a      | 2.83cd           | 0.70b            | 0.08bc        |

* Different letters (a, b, ...) in the same column represent statistically significant differences at $\alpha = 0.05$ (Duncan’s test).
Figure 5. Effect of FeNPs on chlorophyll accumulation of 4-week *Chrysanthemum* in microponic culture.

|            | FeMS | Fe0  | Fe1/4 | Fe1/2 | Fe3/4 | Fe1  | Fe2  |
|------------|------|------|-------|-------|-------|------|------|
| Chl a      | 24.21| 15.33| 21.58 | 24.73 | 29.98 | 25.93| 24.87|
| Chl b      | 11.18| 9.66 | 12.89 | 13.10 | 15.33 | 13.55| 12.67|
| Chl a+b    | 35.39| 24.99| 34.47 | 37.83 | 45.31 | 39.48| 37.54|

Figure 6. Effect of FeNPs on *Chrysanthemum* growth in microponic culture after 4 weeks of culture.
So far, studies on microroponic have been limited. Micropopnic systems have many advantages over traditional breeding systems because of inheriting the advantages of micropropagation and culture systems (Hahn et al., 1996, 1998, 2000; Nhut et al., 2005; Tung et al., 2018). In a micropopnic system, it only takes 2 weeks for the plant to transfer the plants to the greenhouse. This explains why the growth parameters of plants in micropopnic systems are lower than in other culture systems. Plantlets cultured in a micropopnic system (an open system, plantlets grown in non-sterile conditions) are easily adapted to external conditions because the plants are trained in both rooting processes and adapt during culture.

**The activities of enzymes**

The results of antioxidant activity analysis are presented in Figure 7 and 8. In general, the addition of FeNPs to the culture media showed that the SOD and APX enzymes in the roots and leaves of the plants were always highly active, while in the control treatments supplemented with Fe-EDTA, the enzymes were low change. For the control treatment using Fe-EDTA, the content of the antioxidant enzymes [SOD (7.68 and 7.47 U.mg⁻¹ prot) and APX (0.36 and 0.32 μmol.min⁻¹.mg⁻¹ prot)] in the roots and leaves were low (Figure 7 and 8). The activity of both SOD and APX enzymes of cultured plants in different systems (FeNPs) increased.

In micropopnic culture with Fe₂⁄₃, SOD enzyme activity in roots (35.04 U.mg⁻¹ prot) is higher than other culture systems and 4.5 times higher than the control (FeMS). Meanwhile, the addition of FeNPs to culture media in culture systems increased approximately 2 times the activity of the SOD enzyme compared to the control. APX (2.11 μmol.min⁻¹.mg⁻¹ prot) enzyme activity in leaves increased by 6.5 times compared to the control (0.36 μmol.min⁻¹.mg⁻¹ prot) in solid culture systems.

![Figure 7. Effect of FeNPs on antioxidant activity of superoxide dismutase.](image-url)
A number of previous studies have mainly assessed the effects of silver nanoparticles in increasing the activity of enzymes SOD, APX, CAT, etc. (Sharma et al., 2012; Nair, Chung, 2014). In this study, FeNPs with different concentrations (75 - 100 mM) plays a role in increasing the activity of SOD and APX enzymes in different culture systems. With the function of breaking down $H_2O_2$ to $H_2O$ and $O_2$, the activity of APX and SOD increases, reducing the amount of $H_2O_2$ which is toxic to the roots. Thus, the increase of SOD, APX activity at 75 - 100 mM FeNPS shows that the plant’s antioxidant ability increases, reduces oxidative radicals of $O_2$ and $H_2O_2$ in plants, contributing to increased resistance tolerance of plants in culture medium.

**Figure 8.** Effect of FeNPs on antioxidant activity of ascorbate peroxidase.

**Figure 9.** Acclimatization of *Chrysanthemum* grown in different systems in the greenhouse after 8 weeks.
Acclimatization in the greenhouse

The effectiveness of FeNPs supplementation in culture systems was assessed in the greenhouse after 8 weeks of acclimation (Fig. 9 and 10). The recorded results showed that the highest survival rate (94.67%) of microponic (Fe¾ – M) was higher than in in vitro solid (Fe¾ – S) and hydroponic (Fe1 – H) systems (84.00% and 75.67%, respectively). The microponic system is a well-ventilated, reduced nutrient system that helps plants get accustomed to the ex vitro conditions, increasing the nursery survival rate.

CONCLUSION

The results of this study showed that FeNPs can replace Fe-EDTA in MS medium. Iron deficiency in culture media will reduce chlorophyll content. The FeNPs concentrations replaced Fe– EDTA in media of in vitro solid, in vitro hydroponic and microponic culture systems were 75 mM, 100 mM and 75 mM, respectively.

Acknowledgement: This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under Grant number 106.01-2019.301 and Tay Nguyen Institute for Scientific Research under Grant number 30/QĐ-NCKHTN.

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**Journal of Biotechnology** 18(2): 307-319, 2020

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**ÁNH HƯỞNG CỦA NANO SÁT LÊN SỨ SINH TRƯ окол VÀ THÍCH NGHI CÁY CÚC (Chrysanthemum morifolium Ramat. cv. "Jimba") TRONG CÁC HỆ THÔNG NUÔI CÁY KHÁC NHAU**

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**TÓM TẮT**

Trong nuôi cây mô tế bào thực vật, sát là một trong những nano kim loại được sử dụng đầu tiên trên cây trồng. Trong nghiên cứu này, nano sát (FeNPs) được sử dụng để thay thế nano Fe-EDTA trong môi trường nuôi cây MS nhằm đánh giá ảnh hưởng của chúng lên sự sinh trưởng, khả năng tích lũy chlorophyll (a, b và a+b), hoạt tính các enzyme chống oxy hóa SOD, APX và khả năng thích nghi ở điều kiện ướt ẩm trong các hệ thống nuôi cây khác nhau (nuôi cây in vitro môi trường rắn, thủy canh in vitro và vi thủy canh). Kết quả nhận được cho thấy, bổ sung FeNPs vào môi trường nuôi cây MS cho hiệu quả sinh trưởng, tích lũy chlorophyll, hoạt tính các enzyme chống oxy hóa SOD và APX tốt hơn so với bổ sung nano Fe-EDTA. Hiệu quả tác động của FeNPs có sự khác biệt giữa các hệ thống nuôi cây khác nhau. Trong hệ thống nuôi cây in vitro môi trường rắn và vi thủy canh năng độ tối ưu là 75 mM và hệ thống thủy canh in vitro là 100 mM FeNPs. Hoạt tính của enzyme chống oxy hóa SOD (35,04 U.mg⁻¹.prot) tối ưu nhất thuần trong hệ cây nuôi trong hệ thống vi thủy canh; trong khi đó, hoạt tính của enzyme chống oxy hóa APX (2,11 μmol.min⁻¹.mg⁻¹.prot) tối ưu nhất thuần trong là của cây nuôi cây trong môi trường rắn. Khi chuyển cây ra điều kiện ướt ẩm, cây có nguồn gốc nuôi cây vi thủy canh bổ sung FeNPs ở nồng độ 100 mM cho tỷ lệ sống sót cao nhất (94,7%). Kết quả của nghiên cứu này cho thấy FeNPs có thể thay thế được ion sát Fe-EDTA trong môi trường MS và thiếu sít trong môi trường nuôi cây sẽ làm giảm hàm lượng chlorophyll.

**Từ khóa**: Ascorbate peroxidase, cây, nuôi cây in vitro, nano sát, superoxide dismutase.