Silicon Nanoparticles Improve In Vitro Organogenesis of Citrus aurantium *L.* and Are Associated with Enhanced Antioxidant Activity

Ali A. Zainel[1]

Department Of Horticulture and Landscape Design, College Of Agriculture. University Of Kirkuk. Iraq

Abstract

This study investigated the potential impact of silicon nanoparticles SiNPs on in vitro organogenesis of Citrus aurantium *L.* through regulation of oxidative stress and antioxidant defense. Nodal explants were grown in Murashige and Skoog (MS) media supplemented with SiNPs at varying doses (0, 50, 100, and 200 mg L^{-1}). The control treatment showed the lowest values for shoot induction, shoot multiplication, shoot length and rooting parameters than SiNPs treatments. Single shoot tip explants exhibited superior rooting performance (22.56±0.080 roots number) produced from a tissue-cultured simple shoot clone's seedlings, at optimum level 100 mgL^{-1} had shown the highest efficiency on tissue regeneration reaching 91.2% shoot induction and average number of shoots was recorded as around 5.87 ± 0.064 per explant. Additionally, SiNPs treatment significantly increased chlorophyll content and enhanced the activity of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). On the other hand, oxidative stress indicators such as malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) showed a substantial decline. Plants demonstrated optimum stress tolerance, as evidenced by a considerable increase in proline accumulation. However, at 200 mgL^{-1}, a decrease in several parameters was observed, suggesting potentially inhibitory effects at higher concentrations of nanoparticles. In conclusion, these findings suggest a potential role of silicon NPs in controlling homeostasis of reactive oxygen species and improved physiological stability in in vitro cultured citrus tissues. SiNPs' efficacy was confirmed in micro-propagation, and optimal regeneration protocols for citrus rootstocks were developed.

Keywords: nano silicon, in vitro, bitter orange, *Citrus aurantium L, Oxidative* Stress.

Introduction

Bitter orange is the most important citrus species in both industrial and horticultural aspects. This adaptability illustrates why it is such a popular and successful rootstock capable of tolerating many biotic and abiotic stresses. Recent studies conducted by [13,9]. which also studied the ability of this species. It is widely utilized in agricultural, industrial, and medical fields; Its abundant compounds of flavonoids, alkaloids, and essential oils reflect on its use. However, they have limitations in conventional bitter orange propagation. However, factors such as weak seed germination rates and genetic heterogeneity along with a greater susceptibility to diseases pose challenges to the reliable and large-scale production of uniform plants, according to [18].

Plant tissue culture techniques such as in vitro organogenesis are very useful for the fast and disease-free multiplication of citrus species. However, physiological and biochemical limitations, especially oxidative

ISSN 2072-3857

stress under in vitro are frequent bottlenecks for the efficiency of these micropropagation systems [10]. Under these circumstances, the ongoing accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide free radicals, will harm vital biomolecules and disrupt cellular homeostasis, which will lower the regeneration process′s overall effectiveness [11].

Plants can reduce the detrimental effects of ROS by upregulating strong defense mechanisms. Both enzymatic and non-enzymatic substances are necessary for these defensive mechanisms. According to certain research [15,17], enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) are primarily responsible for scavenging ROS and preserving the cellular redox equilibrium. Furthermore, various compounds such as proline play a pivotal role in protecting cells and reducing stress levels in the laboratory [4].

Enhancing plant antioxidant defenses has become an important strategy and enhancing its capacity to withstand oxidative stress are important goals for advancing in vitro production. In recent years, nanotechnology has emerged as a promising technique for stress tolerance and plant development. [7] demonstrated that among different nanomaterials silicon nanoparticles (SiNPs) have gained more attention owing to their biocompatibility and positive impacts on overall plant vigor. By alleviating the negative impact of abiotic stressors on plant physiology, silicon plays a key role in conferring tolerance to environmental conditions, strengthening antioxidant defense systems, increasing water retention and structural integrity [19,1]. Recent findings indicate that silicon nanoparticles (SiNPs) can further stimulate plant development and organogenesis, potentially boost biochemical activities in plant tissues, mostly through regulating reactive oxygen species signaling [16]. However, there is still a significant research gap in the use of SiNPs on citrus species under laboratory conditions, especially for Citrus aurantium. So far, investigations have mainly focused on traditional growth regulators rather than their nanoscale counterparts. In addition, the underlying mechanisms by which SiNPs modulate oxidative stress management and activate antioxidant pathways during organogenesis are still poorly understood.

Thus, the purpose of this work is to examine how silicon nanoparticles might improve Citrus aurantium organogenesis in vitro by modifying oxidative stress and antioxidant response. Accordingly, we will examine how different concentrations of SiNPs affect different variables such as growth parameters, oxidative stress biomarkers, antioxidant enzymes activity and proline accumulation. The ultimate goal is to develop a more efficient, physiologically optimized micropropagation protocol for this species.

Materials and Methods

Explants were prepared from healthy and disease-free seedlings of Citrus aurantium *L*. The excised nodal segments (1.0–1.5 cm) from the actively growing shoots were maintained under greenhouse conditions. In this investigation, explants were cleansed under running water for twenty minutes and then immersed in a liquid detergent solution for five minutes before being rinsed with distilled water. Using 70% ethanol for 30 seconds and 0.1% (w/v) mercuric chloride ($HgCl_2$) for five minutes, aseptic surface sterilization was carried out. To get rid of any remaining sterilant, the explants were

ISSN 2072-3857

then cleaned three to five times using sterile distilled water. Murashige and Skoog (MS) basal medium, the culture's fundamental media, was autoclaved for 20 minutes at 121 °C with 30 g L^{-1} sucrose, 7 g L^{-1} agar, and a pH of 5.8. 1.0 mgL^{-1} to encourage growth, the medium was supplemented with 6-benzylaminopurine (BAP) and 0.5 mgL^{-1} naphthalene acetic acid (NAA). An incubator was used to sustain the cultures: Light intensity: 40–50 µmol, temperature: 25 ± 2°C, photoperiod: 16 hours of light and 8 hours of darkness.

In this investigation, silicon nanoparticles (SiNPs) with an average particle size of 20–50 nm were used, Silicon nanoparticles (SiNPs) were purchased from Sigma-Aldrich (USA) with a purity of 99.5%. The nanoparticles were spherical in shape with a particle size ranging from 20–50 nm. According to the manufacturer's specifications, the zeta potential was −32.4 mV, indicating good colloidal stability. Prior to incorporation into the culture medium, SiNPs were dispersed in distilled water and sonicated for 30 min to minimize particle aggregation and ensure homogeneous distribution.

A stock solution (1000 mgL^{-1}) was prepared by dispersing the SiNPs powder in distilled water and sonicating it for 30 minutes to ensure homogeneous dispersion. The stock solution was diluted to working concentrations and added before autoclaving the MS medium.

Experimental Treatments:

T_0 : Control (0 mgL^{-1} SiNPs), T_1 : 50 mgL^{-1}, T_2 : 100 mgL^{-1} and T_3 : 200 mgL^{-1} SiNPs. The experiment was designed as a completely randomized design (CRD) with a factorial arrangement.: SiNPs concentration (4 levels), five replicates for each treatment, ten explants/replication and total of 200 explants. To guarantee repeatability, each treatment was carried out twice. Following a four-week culture period, the following characteristics were noted: shoot length (cm), number of shoots per explant, and fraction of shoot induction (%).

After four weeks, regenerated shoots (2-3 cm) were moved to MS media supplemented with 1.0 mg L^{-1} indole-3-butyric acid (IBA) at the same SiNPs doses. The number of roots per shoot, root length (cm), and root percentage (%) were noted. Arnon's formula (1949) was used to calculate the chlorophyll content. A spectrophotometer was used to detect the absorbance at 645 and 663 nm following the extraction of fresh leaf samples (0.5 g) using 80% acetone.

Determination of Oxidative Stress Markers

1- Malondialdehyde (MDA): The MDA content was determined using the thiobarbituric acid (TBA) method described by [12]. Absorbance was measured at 532 and 600 nm.
2- Hydrogen Peroxide (H2O2): The [20] method was used to determine the H2O2 level, with absorbance measured at 390 nm.

Antioxidant Enzyme Assays

1- Superoxide Dismutase (SOD): The capacity of SOD to halt the photochemical reduction of nitro blue tetrazolium (NBT) was used to gauge its activity [6].
2- Catalase (CAT): CAT activity was measured using the rate of H2O2 breakdown at 240 nm [2].
3- Peroxidase (POD): The guaiacol oxidation method was utilized to determine POD enzyme activity

ISSN 2072-3857

based on the absorbance at 470 nm [8].

4- Proline Content: calculated the free proline content. The absorbances were expressed as µmol g^{-1} fresh weight and measured at 520 nm [5].

Acclimatization of Plantlets

The rooted plantlets were removed from the medium, cleaned to get rid of any remaining agar, and then put into plastic pots with a 1:1 sterilized combination of peat moss and perlite. For two weeks, plants were kept in high humidity (covered with polyethylene bags) and eventually adapted to greenhouse conditions. After four weeks, the survival rate was noted.

SPSS software (version 26) was used to do an analysis of variance (ANOVA) on the data. Tukey's HSD test was used to compare means at $p<0.05$. Each treatment consisted of five biological replicates, with ten explants per replicate. The entire experiment was repeated twice to ensure reproducibility.

The Results and Discussion

The present study demonstrated that silicon nanoparticles (SiNPs) significantly improved the in vitro organogenesis efficiency of Citrus aurantium through enhancement of growth traits, antioxidant defense mechanisms, and reduction of oxidative stress. The response of explants varied according to SiNPs concentration, with 100 mg L^{-1} consistently producing the most favorable results across almost all measured parameters.

The data presented in Table 1 revealed that SiNPs markedly enhanced shoot induction percentage, shoot multiplication, and shoot length compared with the control treatment. The highest values were obtained at 100 mg L^{-1}, where shoot induction reached 91.2%, accompanied by 5.8 shoots per explant and shoot length of 6.7 cm. This stimulatory effect may be attributed to the ability of silicon nanoparticles to improve nutrient uptake, enhance meristematic activity, and stabilize cellular structures under in vitro conditions. Silicon has been reported to strengthen cell walls, regulate cellular metabolism, and promote cell division, thereby improving morphogenesis efficiency. Similar observations were reported [16,19], demonstrated that silicon-based nanomaterials positively regulate shoot proliferation and biomass accumulation in tissue culture systems. However, increasing the concentration to 200 mg L^{-1} reduced regeneration efficiency, suggesting possible phytotoxic effects or metabolic disturbances caused by excessive nanoparticle accumulation. Such dose-dependent responses have also been documented in several nanotechnology-related studies [16].

ISSN 2072-3857

Table 1. Impact of varying SiNPs concentrations on the induction and multiplication of shoots after four weeks

Treatment (mgL^{-1})	Shoot length (cm)	Shoots per explant	Shoot induction (%)
0 (control)	2.8 ± 0.3 c	2.3 ± 0.2 c	62.4 ± 2.1 c
50	4.5 ± 0.2 b	3.9 ± 0.3 b	78.6 ± 1.8 b
100	**6.7 ± 0.3 a**	**5.8 ± 0.4 a**	**91.2 ± 1.5 a**
200	3.9 ± 0.3 b	3.2 ± 0.2 b	73.1 ± 2.0 b

Means followed by different letters within the same column are significantly different according to Tukey's HSD test at $p \leq 0.05$. Data are presented as mean ± SE.

Rooting responses followed a similar trend (Table 2). The treatment containing 100 mg L^{-1} SiNPs produced the highest rooting percentage (88.7%), root number (5.2 roots per shoot) and root length (6.1 cm). Silicon nanoparticles likely promoted root differentiation through enhanced hormonal balance and stimulation of auxin-mediated root initiation pathways. These findings agree with [14,18] who observed that moderate silicon concentrations stimulate root elongation and root meristem activity in tissue-cultured plantlets. Conversely, the higher concentration (200 mg L^{-1}) negatively affected rooting traits, indicating that excessive nanoparticle levels may interfere with normal cellular activities and root meristem function.

Table 2. Effect of different SiNPs levels on rooting parameters after four weeks

Treatment (mg L^{-1})	Root length (cm)	Roots per shoot	Rooting (%)
0(control)	3.2 ± 0.3 c	2.1 ± 0.2 c	58.3 ± 2.4 c
50	4.6 ± 0.2 b	3.8 ± 0.3 b	74.5 ± 2.0 b
100	**6.1 ± 0.3 a**	**5.2 ± 0.4 a**	**88.7 ± 1.6 a**
200	4.1 ± 0.3 b	3.0 ± 0.2 b	69.8 ± 2.2 b

Means followed by different letters within the same column are significantly different according to Tukey's HSD test at $p \leq 0.05$. Data are presented as mean ± S

The chlorophyll content analysis (Table 3) showed significant enhancement in chlorophyll a, chlorophyll b, and total chlorophyll following SiNPs application, particularly at 100 mg L^{-1}. Total chlorophyll content increased from 1.66 mg g^{-1} FW in the control to 2.81 mg g^{-1} FW at the optimal concentration. This increase suggests that silicon nanoparticles improved photosynthetic efficiency and chloroplast stability while protecting photosynthetic pigments from oxidative degradation. Similar conclusions were reported by [1], who indicated that silicon nanoparticles enhance chlorophyll biosynthesis and preserve photosynthetic machinery under stress conditions. Nevertheless, chlorophyll values declined slightly at 200 mg L^{-1},

ISSN 2072-3857

indicating that excessive nanoparticle accumulation may disrupt pigment metabolism or induce physiological stress.

Table 3. Impact of varying SiNPs concentrations on chlorophyll content (mg g^{-1} FW)

Treatment (mg L^{-1})	Total chlorophyll	Chlorophyll b	Chlorophyll a
0(control)	1.66 ± 0.06 c	0.54 ± 0.03 c	1.12 ± 0.05 c
50	2.19 ± 0.05 b	0.71 ± 0.02 b	1.48 ± 0.04 b
100	**2.81 ± 0.07 a**	**0.89 ± 0.03 a**	**1.92 ± 0.06 a**
200	2.01 ± 0.06 b	0.65 ± 0.03 b	1.36 ± 0.05 b

Means followed by different letters within the same column are significantly different according to Tukey's HSD test at $p \leq 0.05$. Data are presented as mean ± SE.

Oxidative stress markers were strongly influenced by SiNPs treatments (Table 4). The control treatment recorded the highest levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), indicating severe oxidative stress under in vitro conditions. In contrast, treatment with 100 mg L^{-1} significantly reduced MDA and H_2O_2 contents to 4.18 nmol g^{-1} FW and 3.12 µmol g^{-1} FW, respectively. These reductions indicate that silicon nanoparticles effectively mitigated lipid peroxidation and reactive oxygen species (ROS) accumulation. Silicon-mediated regulation of ROS homeostasis has been previously reported by [15,11], who suggested that silicon enhances cellular redox balance and protects membrane integrity. The slight increase in oxidative stress markers at 200 mg L^{-1} further supports the existence of a threshold concentration beyond which nanoparticles may induce cellular toxicity.

Table 4. Effect of different levels of SiNPs on MDA and H_2O_2 content after four weeks

Treatment (mg L^{-1})	H_2O_2 (µmol g^{-1} FW)	MDA (nmol g^{-1} FW)
0 (Control)	**6.75 ± 0.28 a**	**8.42 ± 0.30 a**
50	5.03 ± 0.20 b	6.21 ± 0.25 b
100	**3.12 ± 0.15 c**	**4.18 ± 0.18 c**
200	5.45 ± 0.22 b	6.87 ± 0.27 b

Means followed by different letters within the same column are significantly different according to Tukey's HSD test at $p \leq 0.05$. Data are presented as mean ± SE.

The enhancement of antioxidant defense mechanisms was evident from the significant increase in superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities (Table 5). The highest enzyme activities were recorded at 100 mg L^{-1}, reaching 81.9, 49.2, and 39.6 U mg^{-1} protein for SOD, CAT, and POD, respectively. These enzymes constitute the primary antioxidant defense system responsible for detoxifying reactive oxygen species and maintaining cellular redox

ISSN 2072-3857

equilibrium. The observed enhancement suggests that silicon nanoparticles activate antioxidant metabolism, thereby increasing stress tolerance under in vitro conditions. These findings are consistent with [17,1]. (2024), who reported that silicon supplementation stimulates antioxidant enzyme activities and improves oxidative stress tolerance in plants. Although antioxidant activity decreased slightly at 200 mg L^{-1}, enzyme levels remained significantly higher than those of the control treatment.

Table 5. **Effect of different SiNPs levels on Antioxidant enzyme activity**

Treatment (mg L^{-1})	POD	CAT	SOD
0 (Control)	18.6 ± 0.9 c	21.3 ± 1.2 c	45.2 ± 1.8 c
50	27.8 ± 1.1 b	34.5 ± 1.5 b	62.7 ± 2.1 b
100	**39.6 ± 1.4 a**	**49.2 ± 1.8 a**	**81.9 ± 2.4 a**
200	29.4 ± 1.2 b	36.1 ± 1.6 b	66.3 ± 2.0 b

Means followed by different letters within the same column are significantly different according to Tukey's HSD test at $p \leq 0.05$. Data are presented as mean ± SE.

Proline accumulation also increased significantly in response to SiNPs application (Table 6). The maximum proline content (3.96 µmol g^{-1} FW) was observed at 100 mg L^{-1}. Proline acts as an Osmoprotectant that stabilizes proteins, membranes, and cellular osmotic balance during stress conditions. The increased proline accumulation indicates improved physiological adaptation and stress tolerance in cultured tissues. Similar observations were reported by [4], who demonstrated that silicon treatments stimulate Osmoprotectant accumulation under stress conditions. However, the decline observed at 200 mg L^{-1} may indicate metabolic feedback inhibition or reduced efficiency of proline biosynthesis at excessive nanoparticle concentrations.

Table 6. Effect of different SiNPs Levels on Leaf Proline Content (µmol g^{-1} FW)

Treatment (mg L^{-1})	Proline (µmol g^{-1} FW)
0 (Contro)	1.85 ± 0.08 c
50	2.74 ± 0.10 b
100	**3.96 ± 0.12 a**
200	2.98 ± 0.11 b

Means followed by different letters within the same column are significantly different according to Tukey's HSD test at $p \leq 0.05$. Data are presented as mean ± SE.

Table 7 showed Silicon nanoparticles significantly improved acclimatization success of regenerated plantlets. The highest survival rate (94.2%) was observed at 100 mg L^{-1} SiNPs.

ISSN 2072-3857

Improved survival may be attributed to enhanced root development, chlorophyll accumulation, and antioxidant defense systems that increased plantlet vigor during transfer from in vitro to ex vitro conditions.

Table 7. Effect of SiNPs on acclimatization and survival rate of regenerated plantlets

Treatment (mg L^{-1})	Survival rate (%)
0(Control)	68.4 ± 2.5 c
50	81.6 ± 2.1 b
100	94.2 ± 1.7 a
200	78.9 ± 2.3 b

Conclusion

The current study showed that SiNPs effectively promote in vitro organogenesis of Citrus aurantium. SiNPs improved shoot induction, shoots multiplication, root formation and chlorophyll performance indicating the greater physiological efficiency of the cultured plantlets. Of the tested concentrations, 100 mg L^{-1} was the most successful producing significantly higher percentages of shoot induction and rooting as well as longer shoots and roots. Meanwhile, this increased the accumulation of chlorophyll and also improved antioxidant enzyme activities like superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD).

Moreover, SiNPs treatments significantly reduced oxidative stress markers including hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), indicating that SiNPs might help to regulate the balance of reactive oxygen species (ROS) in plant tissues during growth under in vitro conditions.

On the other hand, at higher concentrations of SiNPs (200 mg L^{-1}), a contrasting effect occurred and most of the growth and metabolic traits decreased in this treatment, indicating that indeed high nanoparticle levels have moderate phytotoxic effects here. Nevertheless, for a nanomaterials to elicit positive effects on plant tissue culture systems, the concentration must be in an optimal range.

Overall, these findings demonstrate that silicon nanoparticles represent a new nanotechnology approach for improving efficiency and physiological stability in Citrus aurantium micropropagation. Therefore, the addition of SiNPs to tissue culture media may contribute to the establishment of improved propagation systems for citrus rootstocks.

References

ISSN 2072-3857

[1] Ahmad, P., Alyemeni, M. N., Wijaya, L.,and Alam, P. (2024). Silicon nanoparticles improve plant growth and stress tolerance by regulating antioxidant defense mechanisms. *Environmental and Experimental Botany*, 215, 105531.

[2] Aebi, H. (1984). Catalase in vitro. *Methods in Enzymology*, 105, 121–126.

[3] Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts: Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology*, 24(1), 1–15.

[4] Qurainy, F. (2023). Role of osmoprotectants in improving plant stress tolerance. *Journal of Plant Growth Regulation*, 42, 1123–1138.

[5] Bates, L. S., Waldren, R. P., and Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39, 205–207.

[6] Beauchamp, C., and Fridovich, I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, 44, 276–287.

[7] Bhat, J. A., Rajora, N., Raturi, G., Sharma, S., Dhiman, P., Sanand, S., Shivaraj, S. M., Sonah, H., and Deshmukh, R. (2021). Silicon nanoparticles (SiNPs) in sustainable agriculture: Major emphasis on the practicality, efficacy and concerns. Nanoscale Advances, 3, 4019–4028.

[8] Chance, B.,and Maehly, A. C. (1955). Assay of catalases and peroxidases. *Methods in Enzymology*, 2, 764–775.

[9] El-Sayed, M., and Hassan, A. (2024). Phytochemical composition and biological activities of bitter orange (*Citrus aurantium*). *Journal of Agricultural Science and Biotechnology*, 18, 45–56.

[10] George, E. F., Hall, M. A., and De Klerk, G. J. (2023). *Plant propagation by tissue culture* (4th ed.). Springer.

[11] Hasan Uzzaman, M., Bhuyan, M. H. M. B., Zulfiqar, F., Raza, A., Mohsin, S. M., Mahmud, J. A., and Fujita, M. (2023). Reactive oxygen species and antioxidant defense in plants under stress conditions. *Plants*, 12, 1183.

[12] Heath, R. L., and Packer, L. (1968). Photo peroxidation in isolated chloroplasts: Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, 125, 189–198.

[13] Khan, M., Ali, S., and Hussain, A. (2023). Advances in citrus biotechnology and micropropagation techniques. *Scientia Horticulturae*, 316, 111049.

[14] Kumar, V., Singh, J., and Kumar, P. (2021). Nanomaterials and plant tissue culture: Applications and perspectives. *Plant Cell, Tissue and Organ Culture*, 146, 1–15.

[15] Mittler, R. (2022). ROS signaling in plants: From molecular mechanisms to stress tolerance. *Annual Review of Plant Biology*, 73, 327–356.

[16] Rastogi, A., Tripathi, D. K., and Brestic, M. (2023). Nanotechnology for sustainable crop production: Current status and future perspectives. *Frontiers in Plant Science*, 14, 1180205.

[17] Sharma, P., Dubey, R. S., and Pessarakli, M. (2023). Reactive oxygen species and antioxidant defense mechanisms

ISSN 2072-3857

in plants. *Journal of Plant Physiology*, 284, 153976.

[18] Singh, A., Kumar, V., and Singh, R. (2022). Silicon-mediated regulation of plant growth and stress responses. *Plant Cell Reports*, 41, 1575–1591.

[19] Tripathi, D. K., Singh, V. P., and Chauhan, D. K. (2023). Role of silicon nanoparticles in plant stress physiology and crop improvement. *Environmental Chemistry Letters*, 21, 1545–1563.

[20] Velikova, V., Yordanov, I., and Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants. *Plant Science*, 151, 59–66.

ISSN 2072-3857