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Innovative Nanoparticle-Assisted Strategies for In Vitro Micropropagation of *Hibiscus sabdariffa* L.

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Abstract

The objective of this study was to investigate the capacity of zinc oxide nanoparticles (ZnO-NPs) and silver nanoparticles (Ag-NPs) to enhance efficiency under in vitro micropropagation of roselle (*Hibiscus sabdariffa L.*). Murashige and Skoog (MS) media was used to cultivate shoot tips as well as nodal segments, and appropriate plant growth regulators were added. ZnO-NPs applied at concentrations 0, 10, 20 and 30 mgL^{-1}, whereas Ag-NPs were used at 0, 5, 10 and 15 mgL^{-1}. The study evaluated growth indices during the shoot multiplication phase, rooting features, survival rate after acclimatization, and a few biochemical properties such as chlorophyll, anthocyanin, and flavonoid levels. The study findings revealed that nanoparticle treatments markedly enhanced shoot proliferation, root formation, and the success of acclimatization as compared to the control. For most of the investigated parameters, the usage of ZnO-NPs at 20 mgL^{-1} and

Ag-NPs at 10 mgL^{-1} produced the best results. Furthermore, nanoparticle treatments resulted in an increased synthesis of secondary metabolites, thus indicating a better physiological and metabolic status of the plants. These results will shed light on the promising application of nanoparticle-based approaches as efficient tools for the improvement of micropropagation protocols of Hibiscus sabdariffa and other medicinal plants.

Keywords: Hibiscus sabdariffa, Roselle، Zinc oxide nanoparticles, silver nanoparticles, in Vitro Micropropagation

Introduction

Hibiscus sabdariffa L. is an economically and medicinally important plant with wide distribution in tropical and subtropical regions that is cultivated for its calyces, which are rich in antioxidant anthocyanins, flavonoids, and other phenolic compounds that have been shown to exert beneficial effects. Such bioactive metabolites play a vital role in the nutritional, pharmaceutical and industrial worth of the crop (Broadley et al., 2007; Mousavi et al., 2013).

However, poor multiplication rates, genetic diversity, and vulnerability to biotic and abiotic stimuli impede the large-scale production of uniform planting material in traditional *H. sabdariffa L*. propagation.

A popular method for quickly propagating horticultural and medicinal plants by clonal propagation (in vitro) is micropropagation. Murashige and Skoog (MS) culture medium is still the most commonly used basal medium because of its balanced macro- and micronutrient composition, which encourages effective shoot regeneration and root development and produces a very helpful formulation for plant material adaptation (Murashige and Skoog, 1962; George et al., 2008). However, standard tissue culture techniques suffer from low regeneration efficiency and microbial contamination, as well as physiological disorders and limited secondary metabolite production especially in the case of medicinal plants.

Utilizing metal and metal oxide nanoparticles to optimize plant tissue culture systems has been made possible

by recently discovered nanotechnology. Large surface area, high reactivity (solubility), and regulated release behavior are some of the special physicochemical characteristics of nanoparticles that allow them to efficiently interact with plant cells and tissues (Tripathi et al., 2017; Rai & Shekhawat, 2014). Furthermore, a number of studies have shown that nanoparticles can operate as nano-elicitors, and that applying them in the right amounts can enhance metabolic processes, enzymatic activity, nutritional absorption, and morphogenetic responses (Ghasemi et al., 2019).

ZnO nanoparticles have garnered interest due to the significance of zinc in plant metabolism, photosynthesis, enzyme activation, and hormone control. Similar studies claimed that the concentration-dependent treatment of ZnO-NPs enhanced shoot proliferation, chlorophyll content, and overall growth parameters in vitro (Faizan et al., 2021). Recent studies validated that ZnO-NPs optimize photosynthetic capacity and metabolic control, consequently stimulating enhanced shoot development and physiological robustness in cultured plants (Zhang et al., 2024; Kumar et al., 2025).

Because of its antibacterial qualities and capacity to lessen ethylene buildup in closed culture vessels, silver nanoparticles (Ag-NPs) have also been thoroughly studied in plant tissue culture. Nanoparticles are usually used to increase the growth and regeneration capacity of plants, such as in increasing shoot multiplication or rooting efficiency, as well as improving survival during acclimatization by reducing contamination and promoting cells differentiation (Rai et al., 2018; Sarmast et al., 2015). Moreover, Ag-NPs can regulate physiological responses and affect the biosynthesis of secondary metabolites under low and controlled application (Zafar et al., 2016).

Recent investigations have put the spotlight on nanoparticles as stimulators of secondary metabolites in medicinal plants when applied across nanotechnology and plant tissue culture systems (Calabrese et al. 2023). By activating metabolic pathways involved in phenolic biosynthesis, nano-integrated culture systems were shown to substantially promote de novo callus

growth (Iqbal et al., 2024) and the accumulation of bioactive compounds, e.g., curcuminoids. Furthermore, the capacity of nanoparticles to improve plant physiological performance while maintaining cellular viability and reducing phytotoxicity has been the focus of green and sustainable nanotechnology initiatives (Mishra et al., 2024).

While there is an increasing literature focusing on the use of nanoparticle-assisted micropropagation, knowledge about the co-administration of ZnO-NPs and Ag-NPs in terms of their effects on in vitro propagation and secondary metabolite accumulation in Hibiscus sabdariffa is still scarce. In order to provide a scientific foundation for enhancing nano-particle assisted micropropagation protocols in medicinal plants, the current study intends to evaluate the impact of various concentrations of zinc oxide and silver nanoparticles on shoot proliferation, rooting characteristics, acclimatization success, and biochemical parameters of H. sabdariffa in vitro.

Materials and Methods

Plant Material and Explant Preparation

The seeds of Roselle (Hibiscus sabdariffa L.) were acquired from al-zuhairi office for seeds-Babylon and germinated in a controlled environment to obtain uniform seedlings. Disease-free seedlings were used to obtain shoot tips and nodal segments as explants for in vitro culture. Before being sterilized, samples were washed under running tap water for 20 to 30 minutes to get rid of surface dirt.

Surface Sterilization

In a cabinet with laminar airflow, aseptic explant sterilization was carried out. After soaking both explants in 70% ethanol for 30 seconds and rinsing them with sterile distilled water, they were treated for disinfection for 10 to 15 minutes using 4% sodium hypochlorite (NaOCl) and two drops of Tween-20. To remove sterilant residues and prevent phytotoxicity, explants were washed three times with sterile distilled water following treatment.

Culture Medium and Conditions

Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962), a standard culture medium containing 30 g L^{-1} sucrose and 7 g L^{-1} agar, was supplemented with cytokines. The medium was autoclaved for 20 minutes at 121°C and 1.5 bar after the pH was adjusted to 5.7 ± 0.1. Cultures were cultivated at 25 ± 2°C in a growth chamber under a photoperiod of 16 h light / 8 h dark with light intensity of 2000–3000 lux.

Plant Growth Regulators and Nanoparticle Treatments

The MS medium was supplemented with either IBA (1.0 mgL^{-1}) for rooting or BAP (1.0 mgL^{-1}) for shoot proliferation. Silver nanoparticles (Ag-NPs) and zinc oxide nanoparticles (ZnO-NP) were introduced to the medium at varying quantities in a dose-dependent fashion: Ag-NPs at 0, 5, 10 and 15 mg L^{-1} and ZnO-NPs at 0, 10, 20 and 30 mg L^{-1}. Moreover, combination treatments were formed with the optimal doses of every nanoparticle, as determined by preliminary tests and literature findings (Faizan et al., 2021; Sarmast et al., 2015).

Preparation of Nanoparticle Solutions

The nanoparticles were dispersed in sterile distilled water to create stock solutions (100 mg L^{-1}), which were then sonicated for 20–30 minutes to produce a homogeneous suspension free of aggregation. Before being added to cooled culture medium (~45–50°C), solutions were filter-sterilized using 0.22 μm membrane filters.

Parameters Measured

The measured parameters included shoot number, shoot length, stem thickness, chlorophyll content and contamination rate during shoot multiplication. Root number, root length, and number of lateral roots are parameters of the rooting stage. Parameters such as survival rate, anthocyanin content and flavonoid concentration post acclimatization were measured.

Experimental Design and Statistical Analysis

Three replicates of each treatment were used in the completely randomized design (CRD) trial. Each replicate consisted of five explants cultured individually. This experimental design provided a mechanism for statistically

evaluating treatment effects under uniform environmental conditions (Gomez & Gomez, 1984). SAS software was used to do a one-way analysis of variance (ANOVA) on all gathered data. The least significant difference (LSD) test was used to assess significant differences between treatment averages at $p < 0.05$. Data were presented in tables and figures to illustrate nanoparticle effects on the measured traits.

1. Shoot Multiplication

Shoot multiplication of *Hibiscus sabdariffa L.* cultures was significantly influenced by the application of silver nanoparticles (Ag-NPs) and zinc oxide nanoparticles (ZnO-NPs). The concentrations that produced the greatest number of shoots, shoot length, stem thickness, and chlorophyll content were 20 mg L^{-1} for ZnO-NPs and 10 mg L^{-1} for Ag-NPs (Table 1).

Results and Discussion

Table 1. Effect of ZnO and Ag nanoparticles on shoot multiplication of *Hibiscus sabdariffa L.*

Nanoparticle Concentration (mg L^{-1})	Shoot multiplication (%)	Number of shoots	Shoot length (cm)	Stem thickness (mm)	Chlorophyll (mg/g FW)
Control	70.2	2.1	3.4	1.2	1.45
10 ZnO-NPs	78.6	2.8	4.2	1.5	1.88
20 ZnO-NPs	90.4	4.3	6.1	2.1	2.45
30 ZnO-NPs	82.1	3.1	4.7	1.7	2.01
5 Ag-NPs	80.3	2.9	4.4	1.6	1.92
10 Ag-NPs	92.6	4.6	6.4	2.2	2.58
15 Ag-NPs	79.5	3.0	4.3	1.6	1.89

Zinc is one of the most crucial elements involved in enzyme activation, chlorophyll production, and auxin-mediated cell division, which may be the

primary cause of the enhanced shoot proliferation observed in connection with the usage of zinc oxide nanoparticles (Broadley et al., 2007; Faizan et al., 2021).

On the other hand, silver nanoparticles drastically lowered the level of microbial contamination, thus indirectly resulting in shoot elongation and multiplication. Interestingly, at higher concentrations, the nanoparticles (ZnO 30 mg L^{-1} and Ag 15 mg L^{-1}) slightly reduced performance, probably because of the nanoparticle, induced generation of reactive oxygen species inside the cells which led to the disruption of cellular integrity (Tripathi et al., 2017).

According to the most recent research, zinc oxide nanoparticles pose a potential to improve photosynthesis and chlorophyll content in C3 plants, which is in line with the results showing that the plants treated with zinc oxide nanoparticles were able to grow better (Hao Chen et al., 2024).

2. Rooting Stage

Both nanoparticles significantly enhanced root growth. The maximum rooting percentage, root number, length, and secondary roots were formed by ZnO-NPs at 20 mg L^{-1} and Ag-NPs at 10 mg L^{-1} (Table 2).

Table 2. Effect of ZnO and Ag nanoparticles on rooting of Hibiscus sabdariffa L.

Nanoparticle Concentration (mg L^{-1})	Rooting (%)	Number of roots	Secondary roots	Root length (cm)
Control	65.8	3.2	1.1	2.6
10 ZnO-NPs	74.5	4.5	2.0	3.8
20 ZnO-NPs	88.3	7.2	4.6	6.1
30 ZnO-NPs	76.9	5.0	2.7	4.1
5 Ag-NPs	72.6	4.1	1.9	3.6
10 Ag-NPs	90.7	7.5	4.9	6.4
15 Ag-NPs	75.2	4.8	2.5	4.0

Zinc nanoparticles stimulate root growth through the increased production of auxin and stimulation of meristematic cells. On the other hand, silver nanoparticles mainly help rooting through the reduction of microbial contamination of the culture media (Mousavi et al., 2013; Sarmast et al., 2015). The drop in root growth at the highest doses reveals that the plants start experiencing stress due to the nanoparticles, thus the dosage of nanoparticles needs to be optimized to get the best results.

3. Survival and Secondary Metabolites

The survival percentage after acclimatization and the accumulation of anthocyanins and flavonoids were positively influenced by the nanoparticle treatments (Table 3). The combination of ZnO 20 mg L^{-1} and Ag 10 mg L^{-1} yielded the highest survival and metabolite accumulation.

Table 3. Effect of ZnO and Ag nanoparticles on survival and secondary metabolites of Hibiscus sabdariffa L. after acclimatization.

Nanoparticle Concentration (mg L^{-1})	Survival (%)	Anthocyanins (mg/100g)	Flavonoids (mg QE/g)
Control	60.4	18.2	21.5
10 ZnO-NPs	72.6	22.8	27.3
20 ZnO-NPs	88.9	31.6	36.8
30 ZnO-NPs	75.3	25.1	29.4
5 Ag-NPs	70.8	24.5	28.6
10 Ag-NPs	91.5	34.2	38.9
15 Ag-NPs	73.9	26.3	30.1

The better survival and higher secondary metabolite content may be due to better root and shoot growth and less microbial stress. ZnO-NPs help enzymatic pathways making phenolic compounds, while Ag-NPs lower microbial contamination, thus creating conditions for good plant physiology (Ghasemi et al., 2019; Rai & Shekhawat, 2014). These findings imply that nanoparticles

can boost micropropagation efficiency and bioactive chemical synthesis at the same time. The use of nanoparticle, mediated culture systems has led to increased callus growth and secondary metabolite production in several species, including turmeric (Curcuma longa) (Iqbal et al., 2024).

Conclusions

The current research revealed that utilizing zinc oxide nanoparticles (ZnO-NPs) and silver nanoparticles (Ag-NPs) is an effective and promising approach to increase micropropagation. The outcome of the experiment showed that nanoparticles effective and promising approach after transplanting better than plants not treated with nanoparticles.

From the different concentrations that were tested, ZnO-NPs at 20 mg L and Ag-NPs at 10 mg L were the ones that most consistently led to the best results in most of the morphological and physiological characteristics measured, such as the number of shoots, length and thickness of shoots, content of chlorophyll, development of roots, and formation of secondary roots. These changes are an indication of a better physiological condition and higher metabolic activity of plantlets grown in vitro.

Besides that, the use of nanoparticles also had a positive effect on the accumulation and composition of secondary metabolites, especially anthocyanins and flavonoids, which are the main components giving medicine and nutrition value to Hibiscus sabdariffa. The increase in the production of metabolites indicates that nanoparticles may be stimulating the metabolic pathways related to phenolic biosynthesis while also supporting the health of the cells if used in suitable amounts.

The study's findings have shown that nanoparticle-assisted micropropagation is a highly promising method for producing high-quality, consistent, and physiologically robust Hibiscus sabdariffa planting material in large quantities. For the sustainable application of nanotechnology in plant tissue culture systems, it is recommended that future studies focus on the long-term consequences, environmental safety, and molecular

processes of nanoparticle plant interactions.

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