Application of Silver and Molybdenum Nanoparticles in *In Vitro* Propagation of *Vanda tessellata* Orchids: Effects on Growth, Morphogenesis, and Contamination Control

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Abstract

The potential for nanotechnology to increase the efficiency of tissue culture and control contamination is significant. The study investigated the effects of silver nanoparticles (AgNPs) and molybdenum oxide nanoparticles (MoO_3 NPs) individually and in combination on *in vitro* culture of *Vanda tessellata*, focusing on contamination control, bud break, shoot multiplication, and root induction. The completely randomized design was applied to investigate the effects of different concentrations of AgNPs (0, 5, 10, 20 mg/L) and MoO_3 NPs (0, 1, 3, 5 mg/L) in MS medium. The results showed that the application of these nanoparticles can effectively reduce contamination and increase growth responses *in vitro*. The results also demonstrated that the interaction of 10 mg/L AgNPs and 3 mg/L MoO_3 NPs was the most favourable treatment for *Vanda tessellata in vitro* culture, as it recorded the lowest contamination (7%), the highest survival (91.67%), maximum bud break (12.68 days), maximum shoot multiplication (6.90 shoots/explant), maximum shoot length (4.25 cm), maximum number of leaves (11.80 leaves/explant), maximum fresh biomass (1.18 g), and maximum root induction (92%), while a hormetic effect was observed at 20 mg/L, as it recorded lower growth responses due to mild phytotoxicity. This indicates that the synergistic effect of silver and molybdenum oxide nanoparticles can effectively increase contamination resistance, growth responses, and morphogenic efficiency in *Vanda tessellata*. Therefore, this study provides valuable information to develop an efficient protocol for nanoparticle-assisted micropropagation of Vanda orchids.

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Introduction

Orchids represent one of the largest and most diverse families of flowering plants and *Vanda tessellata* is one of the most commercially cultivated genera due to the high demand of its ornamental and medicinal values [3]. Traditional method of orchid propagation using seed germination and vegetative division is slow, unreliable and affected by seasonality and susceptibility to diseases. Micropropagation using tissue culture technique has thus become an important tool for mass production of elite orchid cultivars [23, 15, 16]. While the micro-propagation protocol of many orchid species, including *Vanda tessellata*, has been developed and improved, there are some limitations of *in vitro* systems, which include microbial contamination, tissue necrosis and oxidative stress and may affect the scale and efficiency of the production of plantlets. Nanobiotechnology has now become a new approach to overcome the above-mentioned limitations of plant tissue culture system using engineered nanomaterials [24]. Among those, the most commonly studied nanomaterials are metal and metal oxide nanoparticles such as silver (Ag) and molybdenum oxide (MoO_3) for modifying the physiological responses of plants, improving nutrient availability and enhancing the stress tolerance of plants [22, 18].

Silver nanoparticles (AgNPs) have been extensively used and properties. When added to the appropriate concentration of and fungi, reduce the rate of contamination and stimulate the growth of shoots and roots by modulating the ethylene biosynthesis pathway and reactive oxygen species (ROS) [12, 5]. Some studies have demonstrated that AgNPs can influence the developmental process mediated by auxins and cytokinins and may act as abiotic elicitor to trigger signaling pathways that promote morphogenesis [11]. Although less common than AgNPs, molybdenum oxide nanoparticles (MoO_3 NPs) have attracted attention for their ability to regulate nutrient and to mitigate stress. Molybdenum (Mo) is a key component of molybdoenzymes such as nitrate reductase and nitrogenase involved in the nitrogen assimilation and redox metabolism [2]. Compared to bulk molybdenum compounds, the nanoform of molybdenum oxide may provide a larger surface area and higher solubility, which may result in a greater uptake and bioavailability of molybdenum [18]. Recent studies showed that MoO_3 NPs can increase seed germination, root elongation and antioxidant enzyme activity under normal and stressful conditions [7, 19]. Research on the effect of MoO_3 NPs in orchid systems is very limited.

Studies exploring the combined effects of AgNPs and MoO_3 NPs in plant tissue culture are also extremely rare, and the interactions of these two types of nanoparticles in this type of system are poorly understood. Since the potential of the two types of nanoparticles may be synergetic or antagonist, it is crucial to study the effects of the combination of these two types of nanoparticles, especially in complex morphogenic systems like orchids [22, 24].

Objectives of the Study

The purpose of this study was to identify the individual and interactive effects of silver and molybdenum oxide nanoparticles on the micropropagation of *Vanda tessellata* spp. More specifically, the study assessed the effects of the aforementioned nanoparticles on the control of contamination, initiation of bud break, shoot proliferation, and root induction, in controlled, *in vitro* conditions. The outcomes are expected to contribute to the development of a nanoparticle-integrated protocol for efficient, scalable, and contamination-resistant orchid micropropagation.

Research Methodology

Experimental Conditions

All experiments were performed in a laminar flow cabinet to help reduce potential microbial contamination of the tissues being cultured. The temperature in the culture room was controlled at 25±2°C with a 16-hour photoperiod to provide optimal conditions for growing *in vitro* plants. The cool white fluorescent tube lighting provided an average light intensity of 40±5 µmol m^{-2} s^{-1}, which is well within the optimal range that has been previously reported to promote photosynthesis while preventing photoinhibition during orchid micropropagation. The relative humidity level was set between 60-70% to mimic typical environmental conditions found in tropical regions where most orchids grow and to studied in plant sciences primarily because of them.
the culture medium, AgNPs can inhibit the growth of bacteria prevent desiccation. The experiments were conducted in three phases: establishment of cultures, shoot multiplication, and root induction.

Plant Material and Explant Preparation

Healthy, disease-free *Vanda tessellata* orchid plants grown under greenhouse conditions were selected as the plant material for these experiments. Nodal segments of 1.0 - 1.5 cm in length, with one axillary bud in an active developing condition, were excised from the mother plants in the early morning to maintain optimal turgor pressure and physiological stability. Subsequently, they were washed under running tap water with 1.0% v/v commercial detergent for 15 minutes to remove surface contaminants and dust particles. Surface sterilization of the segments was performed in aseptic conditions by immersing them in 70% ethanol for 30 seconds to remove air bubbles formed around the segments. Subsequently, they were immersed in 1.5% sodium hypochlorite solution for five minutes. Explants were sterilized again with a new solution of 1.5% sodium hypochlorite containing 2 – 3 drops of Tween-20, followed by rinsing them with sterile distilled water for five times. The cut ends of the segments were trimmed to remove oxidized tissues and to increase physiological activities before inoculation [15].

Nanoparticle Materials and Characterization

Silver Nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were purchased from Sigma-Aldrich (Merck, USA), product no. 730785. AgNPs were provided as an aqueous colloidal solution of polyvinylpyrrolidone (PVP) to maintain long-term colloidal stability. The size of the AgNPs was 20 nm, and the purity was $\geq$ 99.5%. AgNPs have already been established in plant biotechnology for their antimicrobial activity and have been shown to affect morphogenesis in plants via hormonal mimicry and the regulation of reactive oxygen species [24].

Molybdenum Oxide Nanoparticles (MoO_3 NPs)

Molybdenum (VI) oxide nanoparticles were obtained from US Research Nanomaterials Inc. (Product Code: US3340). The purity of the powder was 99.9%, and the nominal diameter of the MoO_3 nanoparticles was approximately 70 nm. Molybdenum oxide nanoparticles have been studied as a form of nutrient delivery to plants and are considered a readily available source of molybdenum, an essential micronutrient that plays an important role in nitrate reduction and nitrogen metabolism [25]. Their nanostructure provides enhanced uptake and can potentially stimulate better enzymatic response to nutrient availability *in vitro.*

Preparation of Nanoparticle Suspension

Stock solutions of both types of nanoparticles were prepared at a concentration of 1,000 mg/L using sterile, double-distilled water. Due to their dispersion nature, the AgNPs were simply diluted in sterile, double-distilled water. In contrast, the dry powder of the MoO_3 was mixed in a vortex, then treated with an ultrasonic probe sonicator at a frequency of 40 kHz, and an amplitude of 120W for 30 minutes to minimize particle aggregation and to achieve homogeneous dispersion. Prior to incorporation into the culture medium, the stock suspensions of the nanoparticles were filtered through 0.22 µm syringe filters to ensure sterility and stored at 4°C in sealed, autoclaved amber glass containers to avoid degradation due to light exposure and microbial contamination. Then, the nanoparticles were incorporated into the culture medium after it had been autoclaved, and after the medium had cooled to a temperature between 45-50°C, to prevent changes in the structure of the nanoparticles that

Composition of culture media

The basal medium used for all stages of the study consisted of Murashige and Skoog (MS) medium [14] that included 30 g/L sucrose as the primary carbon source and 7.5 g/L agar to solidify the medium. The pH of the medium was adjusted to 5.7±0.1 using 1 N NaOH or HCl before autoclaving the medium at 121°C for 20 min. Plant growth regulators (PGRs) were added to the MS medium in various combinations based on the stage of the culture.

MS medium was supplemented with 1.0 mg/L 6-benzylaminopurine (BAP) and 0.2 mg/L naphthaleneacetic acid (NAA) to promote shoot formation. Multiplication medium was supplemented with 2.0 mg/L BAP, 0.5 mg/L kinetin and 0.2 mg/L NAA to increase the rate of shoot proliferation. During the rooting phase, ½ strength MS medium was supplemented with 0.5 mg/L indole-3-butyric acid (IBA) to induce root primordia (Arditti, 2009) [3].

Experimental Design

The experimental design was set up as a 2-factor factorial, completely randomized design (CRD) to evaluate the individual and combine effects of silver nanoparticles (AgNPs) and molybdenum oxide nanoparticles (MoO_3 NPs) supplemented to the media. AgNPs were classified as Factor A (0, 5, 10 and 20 mg/L) and MoO_3 NPs were classified as Factor B (0, 1, 3 and 5 mg/L). Consequently, there were 16 total possible treatment combinations, one being the control (0 mg L for both nanoparticles). Ten explants per treatment were used with three replicates, thus using 480 explants in total. The culture vessels were randomly placed on the racks and rotated regularly to minimize any position bias due to light or temperature differences in the culture room.

Culture Establishment and Maintenance

The explants were first cultured on shoot initiation media with the different treatments of nanoparticles and were monitored for a period of four weeks, during which the explants were observed for signs of contamination, swelling, and bud formation. The explants that showed signs of survival and shoot induction were then transferred to multiplication media containing different combinations of nanoparticles and subcultured every four weeks for 12 weeks while collecting required data. For root formation, healthy shoots with a minimum length of 2.5 cm were cut and cultured on rooting media with the different treatments of nanoparticles. Data on root formation was recorded after four weeks.

Data Collection Parameters

In the culture initiation phase, percentage of contamination (%), percentage of explant survival (%), and the number of days to bud break were recorded. In the shoot multiplication phase, number of shoots per explant, length of shoots (cm), leaf number, and fresh biomass (g) were obtained. In the root induction phase, percentage of rooting (%), number of roots per shoot, and length of roots (cm) were recorded.

Statistical Analysis

Analysis of the data was performed using two-way Analysis of Variance (ANOVA) to determine the effect of AgNPs and MoO_3 NPs on all the studied parameters. When necessary, percentage values were arcsine transformed to ensure normality of the data. The means were compared using Tukey's Honestly Significant Difference (HSD) at a significance level of 0.05. All the statistical analysis was performed using R Studio (v4.2.0), to ensure the reproducibility and transparency of the study [27,8].

Results and Discussion

In this study, the individual and combined effects of silver nanoparticles (AgNPs) and molybdenum oxide nanoparticles (MoO_3 NPs) supplemented in various media of the important micropropagation phases, culture establishment, multiplication, and root induction of *Vanda tessellata* were investigated. Unlike previous studies that evaluated single nanoparticle applications, this research adopted a two-factor factorial approach to determine not only the independent effects but also the interactive and potentially synergistic effects of these nanomaterials supplemented in orchid tissue culture media. The experimental results presented in Tables 1, 2, and 3 not only show dose effects of nanoparticles, but also a combination effects of two nanoparticles (dual-nanoparticle strategy), which can improve existing techniques in orchid micropropagation.

Contamination Control and Culture Initiation

Table 1 shows the effects of silver nanoparticles (AgNPs) and molybdenum oxide nanoparticles (MoO_3 NPs) in the culture medium during the *in vitro* establishment phase of *Vanda tessellata,* along with reductions in microbial contamination. A significant reduction in percentage contamination was recorded with an increase in the concentration of AgNPs. The percentage contamination in the control treatment was 36.67%, whereas the percentage contamination decreased to 9.00% with the supplementation of 10 mg/ L AgNPs, and it decreased further to 7% with the combined use of 3 mg/ L MoO_3 NPs. This reduction in percentage contamination is attributed to the well-documented antimicrobial action of AgNPs, which includes disruption of the microbial cell membrane, inactivation of proteins, and ROS (reactive Oxygen Species)-induced microbial toxicity [17, 16]. Such a reduction in percentage contamination is also documented previously in plant tissue culture systems applied with *Valeriana officinalis* [1], and other ornamental crops treated with nanosilver [20]. The Ag^+ ions released from the silver nanoparticles react with the thiol groups of enzymes, resulting in the suppression of endogenous contamination, which is common in orchid tissue culture.

Table 1: Effects of silver nanoparticles (AgNPs) and molybdenum oxide nanoparticles (MoO_3 NPs) on contamination control and culture initiation of *Vanda tessellata* explants

Treatments	Contamination (%)		Survival (%)		Days to Bud Break	
MS+1.0 mg/L BAP + 0.2 mg/L NAA - Control (C)	36.67±5.77	a[1]	60.00±5.00	c	18.92±1.21	a
C + 1.0 mg/L MoO_3 NPs	35.00±5.00	a	61.67±5.77	c	18.43±1.34	a
C + 3.0 mg/L MoO_3 NPs	33.33±5.77	a	63.33±5.77	c	17.92±1.08	a
C + 5.0 mg/L MoO_3 NPs	34.00±5.00	a	62.67±5.13	c	18.15±1.26	a
C + 5.0 mg/L AgNPs	20.00±5.00	b	75.00±5.00	b	16.42±0.98	b
C + 5.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	18.33±5.77	b	77.00±5.29	b	15.78±0.91	bc
C + 5.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	17.00±5.00	b	78.67±4.51	b	15.12±0.84	c
C + 5.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	19.00±4.36	b	76.33±5.51	b	15.60±0.87	bc
C + 10.0 mg/L AgNPs	9.00±3.61	c	88.00±4.00	a	14.32±0.76	d
C + 10.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	8.33±2.89	c	89.33±4.16	a	13.75±0.70	d
C + 10.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	7.00±2.00	c	91.67±3.51	a	12.68±0.63	e
C + 10.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	8.00±2.65	c	90.00±3.61	a	13.10±0.68	d
C + 20.0 mg/L AgNPs	7.67±2.52	c	80.33±4.51	b	15.12±0.94	de
C + 20.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	7.00±2.00	c	82.00±4.00	b	14.85±0.82	e
C + 20.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	6.67±2.08	c	83.67±3.79	b	14.40±0.78	f
C + 20.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	7.33±2.52	c	78.00±4.58	b	15.95±0.93	De

Values are Mean±SD (n = 30 explants per treatment).

1. Means followed by the same letters in the same column are not significantly different at 5% level (Tukey's HSD)

The survival rate increased from 60% in the control to 91.67% with the combined treatment of 10 mg/ L AgNPs and 3 mg/ L MoO_3 NPs. The increase in survival rates can be attributed not only to the reduction of microbes, but also to the role of silver in the inhibition of ethylene perception. Silver ions, which act as antagonists of ethylene receptors, delay the process of senescence and browning of plant tissues in sealed vessels [4, 1]. This, in turn, increases the responsiveness of the plant tissue to cytokinins, resulting in quicker bud breaks. The time taken for bud breaks decreased significantly from 18.92 days in the control to 12.68 days with the optimal combined treatment. This can be attributed to the increase in nitrogen metabolism with the use of molybdenum-dependent enzymes, such as nitrate reductase [13]. Enhanced nitrogen assimilation promotes cell division and meristem activation in cultured tissues, a mechanism similarly described previously in molybdenum-supplemented tomato cultures [19].

At a concentration of 20 mg/L of AgNPs, the level of contamination remained low, while the survival rate dropped slightly, indicating a level of phytotoxicity. The dose-dependent toxicity of AgNPs, caused by the overproduction of ROS, leading to oxidative stress, has been well documented in previous studies [11, 26].

Shoot Multiplication

The plantlets that developed on the shoot initiation medium were transferred to the shoot multiplication medium containing different concentrations of the nanoparticles, as indicated in Table 2, which presents the effect of the nanoparticles on shoot multiplication.

Shoot proliferation parameters were significantly enhanced by supplementing nanoparticles to the media up to an optimal concentration. The highest values were recorded for shoot number (6.90 shoots per explant), shoot length (4.25 cm), leaf number (11.80), and fresh weight (1.18 g) when treated with 10 mg/ L AgNPs and 3 mg/ L MoO_3 NPs. The effect of silver nanoparticles on shoot organogenesis has been attributed to their capacity to stimulate endogenous hormone balance and prevent ethylene production [4,1]. An increase in shoot multiplication and biomass production has also been recorded when treating *Bacopa monnieri* with moderate doses of AgNPs [10]. Nanoparticles can also stimulate ROS signaling, which acts as a secondary messenger in cell division and differentiation [24]. MoO_3 NPs may have improved metabolic efficiency by optimizing molybdenum bioavailability, thus stimulating nitrate reductase activity and protein synthesis [13]. It was reported that metal oxide nanoparticles have also increased chlorophyll content and photosynthetic activity [18]. Stimulating biomass production in this way may have contributed to the significant increase in fresh weight recorded in this study.

Table 2: Effects of silver and molybdenum nanoparticles on shoot multiplication of *Vanda tessellata*

Treatments	Shoots per Explant		Shoot Length (cm)		Leaf Number		Fresh Weight (g)	
MS+2.0 mg/L BAP + 0.5 mg/L kinetin + 0.2 mg/L NAA - Control (C)	2.30±0.26	d[1]	2.61±0.21	d	4.20±0.58	d	0.42±0.04	d
C + 1.0 mg/L MoO_3 NPs	2.65±0.30	d	2.78±0.23	cd	4.85±0.60	d	0.46±0.05	d
C + 3.0 mg/L MoO_3 NPs	2.90±0.32	cd	2.95±0.24	c	5.30±0.63	cd	0.50±0.05	cd
C + 5.0 mg/L MoO_3 NPs	2.75±0.28	cd	2.85±0.22	c	5.05±0.59	cd	0.48±0.04	cd
C + 5.0 mg/L AgNPs	3.10±0.36	c	2.92±0.25	c	5.75±0.64	c	0.55±0.05	c
C + 5.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	3.80±0.38	bc	3.20±0.27	bc	6.90±0.68	bc	0.66±0.06	bc
C + 5.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	4.80±0.42	b	3.55±0.29	b	8.90±0.73	b	0.82±0.07	b
C + 5.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	4.40±0.40	bc	3.35±0.27	bc	7.80±0.70	bc	0.74±0.06	bc
C + 10.0 mg/L AgNPs	5.30±0.44	b	3.85±0.30	b	9.70±0.75	b	0.93±0.08	b
C + 10.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	6.10±0.45	a	4.05±0.31	a	10.90±0.79	a	1.05±0.09	a
C + 10.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	6.90±0.46	a	4.25±0.31	a	11.80±0.81	a	1.18±0.09	a
C + 10.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	6.40±0.44	a	4.10±0.30	a	11.10±0.78	a	1.10±0.08	a
C + 20.0 mg/L AgNPs	4.90±0.41	b	3.60±0.29	b	8.80±0.72	b	0.85±0.07	b
C + 20.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	5.20±0.42	b	3.75±0.30	b	9.20±0.74	b	0.89±0.07	b
C + 20.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	5.40±0.43	b	3.85±0.30	b	9.60±0.75	b	0.93±0.08	b
C + 20.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	4.20±0.40	bc	3.35±0.27	bc	7.40±0.69	bc	0.72±0.06	bc

Values are Mean±SD (n = 30 explants per treatment).

1. Means followed by the same letters in the same column are not significantly different at 5% level (Tukey's HSD)

Apart from the aforementioned mechanisms, silver nanoparticles have been proven to boost cytokinin sensitivity and meristematic potential in different *in vitro* systems. An increase in the frequency of shoot regeneration and biomass formation has also been observed in *Stevia rebaudiana* cell suspension systems treated with low concentrations of AgNPs [20]. The authors attributed this phenomenon to the improvement of redox balance and increased cell division activity in the treated cells. Similarly, it has been demonstrated that there is a potential of silver nanoparticles to stimulate shoot development and chlorophyll accumulation in *Brassica juncea*, further supporting the assumption that AgNPs can boost photosynthetic activity, in addition to shoot development and morphogenesis [21]. This assumption is supported by the increased leaf number and shoot length observed in the present investigation under the treatment containing 10 mg/L AgNP and 3 mg/L MoO_3 NP. Moreover, molybdenum has been linked with the improvement of nitrogen assimilation capacity and enzymatic activity associated with primary metabolism. emphasized the importance of molybdenum-containing enzymes, especially nitrate reductase, in the regulation of nitrogen-dependent growth processes have also been emphasised by previous studies [9]. Nanoscale MoO_3 might also increase its solubility and cellular uptake, which would further augment its metabolic benefits. The combined effect of hormone regulation induced by AgNPs and nutrient optimization induced by MoO_3 might be the reason for the significant improvement in shoot multiplication traits observed in the present study.

However, when the AgNPs were applied to the media at 20 mg/L, shoot multiplication activity was reduced when compared with that of 10 mg/ L AgNPs confirming the hormetic effect of nanoparticles. The biphasic effect has also been observed in soybean and rice when they were exposed to silver nanoparticles [11]. The over-accumulation of nanoparticles may cause damage to membrane integrity, increase oxidative stress above optimal

levels, and interfere with photosynthesis, which may limit morphogenic activity. Therefore, the superior response at moderate concentrations highlights the importance of optimization of nanoparticle dose *in vitro* propagation of orchids.

Root Induction and Development

Table 3 and Figure 1 show the effects of nanoparticles in rooting of *Vanda tessellata* plants transferred to rooting media.

The rooting responses also followed a trend similar to that of shoot multiplication. The percentage of rooting was significantly increased with the combined treatment of 10 mg/L AgNPs and 3 mg/L MoO_3, reaching 92% compared with 60% in the control.

Table 3: Effects of silver and molybdenum nanoparticles on root

Treatments	Rooting (%)		Roots per Shoot		Root Length (cm)	
½ MS+0.5 mg/L IBA - Control (C)	60.00±5.00	d	3.60±0.50	d	2.85±0.22	d
C + 1.0 mg/L MoO_3 NPs	63.00±4.58	cd	3.90±0.55	d	3.05±0.24	cd
C + 3.0 mg/L MoO_3 NPs	65.00±4.36	c	4.20±0.58	cd	3.25±0.26	c
C + 5.0 mg/L MoO_3 NPs	64.00±4.00	cd	4.00±0.54	cd	3.15±0.25	cd
C + 5.0 mg/L AgNPs	66.00±4.58	c	4.40±0.58	c	3.20±0.26	c
C + 5.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	72.00±4.36	bc	5.20±0.70	bc	3.70±0.28	bc
C + 5.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	78.00±4.00	b	6.50±0.76	b	4.35±0.32	b
C + 5.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	75.00±4.36	b	5.80±0.72	bc	3.95±0.30	bc
C + 10.0 mg/L AgNPs	85.00±3.79	a	7.40±0.79	b	4.85±0.35	b
C + 10.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	89.00±3.61	a	8.10±0.82	a	5.30±0.37	a
C + 10.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	92.00±3.61	a	8.80±0.83	a	5.85±0.38	a
C + 10.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	90.00±3.46	a	8.30±0.80	a	5.55±0.36	a
C + 20.0 mg/L AgNPs	80.00±4.00	b	6.70±0.75	b	4.50±0.33	b
C + 20.0 mg/L AgNPs + 1.0 mg/L MoO_3 NPs	82.00±3.79	b	7.00±0.78	b	4.70±0.34	b
C + 20.0 mg/L AgNPs + 3.0 mg/L MoO_3 NPs	83.00±3.61	b	7.30±0.79	b	4.85±0.35	b
C + 20.0 mg/L AgNPs + 5.0 mg/L MoO_3 NPs	75.00±4.36	b	5.70±0.72	bc	3.90±0.29	Bc

1. Means followed by the same letters in the same column are not significantly different at 5% level (Tukey's HSD).

Moreover, the number of roots per shoot (8.80) and the length of roots (5.85 cm) were also significantly enhanced compared with those of the control, revealing that the combined treatment of AgNPs and MoO_3 NPs also had a positive impact on the process of root formation and elongation. Silver nanoparticles can enhance the sensitivity of plant cells to auxins and increase the formation of lateral roots by inhibiting ethylene-induced suppression of root formation [1]. Ethylene build-up is a common limitation in the process of root elongation because of the closed environment of a culture vessel. The interference with ethylene precipitation, however, would have created a more conducive hormonal balance that favoured the process of root meristematic tissue activation. The increase in root biomass and root elongation with the supplementation of AgNPs, could also be attributed to the stimulation of cell division and the modulation of endogenous plant growth regulators [10]. The controlled level of reactive oxygen species (ROS), induced by a low concentration of nanoparticles, can also play a role in signalling, which is important for the process of adventitious root formation, where redox regulation coordinates auxin-responsive gene expression.

Molybdenum is crucial for nitrogen assimilation and redox control. Increased nitrate reductase activity is also beneficial for root meristem development and root elongation [13]. The increase in root length due to the application of MoO_3 NP can be explained as a consequence of enhanced nitrogen assimilation and amino acid synthesis, as these processes are vital for the proliferation of root cells. It was also reported that antioxidant enzyme activities, which contribute to root architecture and plant stress tolerance, are enhanced along with optimized nanoparticle concentrations [7]. Similarly, some of the published articles clearly indicated that nanoparticles containing metals can increase root system development due to enhanced nutrient uptake efficiency and modulation of oxidative balance [6]. The synergistic improvement observed under combined AgNP and MoO_3 NP treatment suggests complementary mechanisms where silver nanoparticles are known to modulating hormonal signalling and microbial suppression and molybdenum nanoparticles increase metabolic and enzyme activities. The enhanced root morphology due to nanoparticle application has been reported in wheat and other crops, where optimized nanoparticle concentrations have increased root surface area and biomass [18].

Nevertheless, increased levels (20 mg/L AgNPs) inhibited the process, most likely due to oxidative stress levels exceeding the beneficial signalling thresholds [26]. The detrimental effect of increased levels of nanoparticles on membrane integrity, mitochondrial function, and meristematic activity could be responsible for the reduction in the initiation of roots. This biphasic response also supports the hormetic model widely described in nanoparticle–plant interaction research.

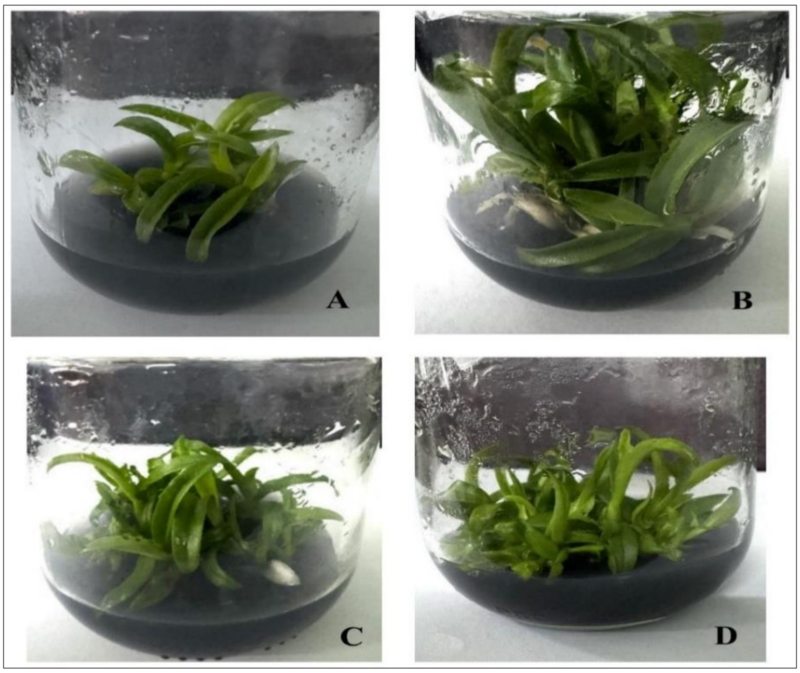

Fig 1: *Vanda tessellata* plants growing in rooting media at the 3rd week after transferring to the rooting media**.** A-Media with no nanoparticles (Control). B-Media supplemented with 10 mg/L AgNPs and 3 mg/L MoO_3 NPs. C-Media supplemented with 3 mg/L MoO_3 NPs. D-Media supplemented with 10 mg/L AgNPs.

Effects of AgNPs and MoO_3 NPs on Growth and Morphogenesis

This synergistic interaction between 10 mg/L of AgNPs and 3 mg/L of MoO_3 NPs indicates the involvement of complementary physiological and biochemical processes that cumulatively contributed to the improvement in *in vitro* performances of *Vanda tessellata* explants. Antimicrobial activity of silver nanoparticles might have reduced endogenous microbial contamination of explants, thereby increasing explant establishment and *in vitro* survival of explants [17]. Simultaneously, silver nanoparticles might have reduced the inhibitory effect of ethylene on morphogenesis of explants through the inhibition of ethylene perception, leading to increased shoot and root initiation and development [4]. Low concentrations of silver nanoparticles are also known to elicit optimal levels of ROS in plant cells. ROS act as signalling molecules to stimulate cell division and differentiation rather than inducing oxidative damage [24]. At the same time, molybdenum oxide nanoparticles might have increased nitrogen assimilation in explant cells through increased activity of molybdenum-dependent enzymes, leading to increased protein synthesis and meristematic growth [13]. Jointly, these interacting mechanisms, microbial suppression, hormonal modulation, redox signalling, and improved nutrient metabolism, lead to the significant improvements observed in culture initiation, shoot multiplication, and rooting of *in vitro* grown *Vanda tessellata* explants.

Conclusion

The present study reveals that the combined treatment of silver nanoparticles (AgNPs) and molybdenum oxide nanoparticles (MoO_3 NPs) significantly improve the *in vitro* propagation efficiency of *Vanda tessellata*. The findings of the study revealed that the combined treatment of silver and molybdenum oxide nanoparticles can work synergistically to improve the resistance to contamination, growth performance, and morphogenetic efficiency in *Vanda tessellata*. The combined treatment of 10 mg/L AgNPs and 3 mg/L MoO_3 NPs exhibited the optimal performance in reducing contamination, enhancing explant survival, promoting bud break, maximizing shoot proliferation, and improving rooting in *Vanda tessellata*. The enhancement of *in vitro* performance in *Vanda tessellata* can be attributed to the combined action of the nanoparticles, which includes the antimicrobial properties and ethylene-inhibiting capacity of AgNPs, ROS-mediated stimulation, and improved nitrogen metabolism through molybdenum-dependent enzymes provided by MoO_3 NPs., which can work together to ensure healthy establishment, active cell division, and improved organ development of the explants grown *in vitro*. The results of the study confirm the hormetic effect of nanoparticles at moderate concentrations, which induce growth and morphogenesis, while nanoparticles at higher concentrations exhibit mild phytotoxicity due to excessive oxidative stress. The results collectively suggest that there is an increased prospect for employing a synergistic dual nanoparticle approach for improving *in vitro* orchid propagation.

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