Exposure of *Paulownia* Seedlings to Silver Nitrate Improves Growth Parameters via Stimulation of Mild Oxidative Stress

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Summary

In the present study we investigated the morpho-physiological and biochemical responses of microclonally produced seedlings of *Paulownia* Pao Tong Z07 under exposure to silver nitrate ($AgNO_3$) in various concentrations (1, 10, 50 mg L^{-1}) in the growth media. The exposure to $AgNO_3$ at all studied concentrations resulted in a significant enhancement in seedling growth, as evidenced by notable increments in shoot length, stem mass and leaf mass. Seedlings treated with $AgNO_3$ showed higher catalase activities, ascorbate peroxidase, and ascorbate oxidase. At the same time, the activities of superoxide dismutase and glutathione-*S*-transferase, the concentrations of low molecular mass thiols and carotenoids dropped in plants exposed to $AgNO_3$. The levels of chlorophylls *a* and *b* were unaffected by any silver nitrate concentration. The results suggest that silver nitrate, at concentrations of 10 and 50 mg L^{-1}, has modulating effects on the antioxidant defense of *Paulownia*.

Key words

micropropagation, silver nitrate, *Paulownia*, pigments, ROS, antioxidant enzymes

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Introduction

Silver (Ag) is a naturally occurring element in the earth, typically found in relatively low concentrations. However, due to its diverse applications, Ag can be released into the environment in various chemical forms or compounds (Purcell and Peters, 1998). The environmental damages of silver vary depending on its concentration and chemical state (Etris, 2001; Purcell and Peters, 1998).

Silver ions (Ag^+), commonly employed in the form of silver nitrate ($AgNO_3$), have been shown to impact plant embryogenesis, influencing bud, root and shoot development, which are essential prerequisites for successful organogenesis (Bais et al., 2000; Bais et al., 2001). However, the precise mechanisms underlying $AgNO_3$ effects on plants remain elusive. Limited evidence suggests that $AgNO_3$ may disrupt pathways of ethylene uptake and production. Kumar and colleagues (2009) demonstrated that Ag^+ can specifically inhibit the effects of exogenously administered ethylene in classical reactions such as wilting, senescence and stunted plant growth. Ouma and colleagues (2004) showed that $AgNO_3$ restrained ethylene biosynthesis, prompting the regeneration of multiple shoots from cotyledon and hypocotyl explants in cotton. Labraba and Araus (1991) reported an enhanced wheat seed yield with the application of $AgNO_3$ spraying. A study on kenaf (*Hibiscus* sp.) and cotton (*Gossypium hirsutum* L.) revealed that introducing $AgNO_3$ to the initiation medium augmented the formation of shoot buds on hypocotyl explants (Ouma et al., 2004).

Intracellular concentration is a determinant of the overall outcome of Ag^+ exposure. Seif Sahandi et al. (2011) reported the potential cumulative effect of silver in plant shoots. Previous studies have demonstrated Ag^+ uptake by cells through Cu^+ transport systems (Fortin and Campbell, 2000). Transcriptomic and proteomic analyses have revealed aquaporin water channels (AQP) as potential transporters of silver ions across the plasma membrane (Bienert et al., 2007; Maurel et al., 2015). Moreover, Ag^+ up-regulates the expression of cytoplasmic copper metallochaperone Atx1, responsible for Cu^+ transport to proteins for their optimal function. Transporters such as P-type ATPases on organelle membranes are also subject to regulation. These findings imply that intracellular Ag^+ distribution within cellular compartments is mediated through Cu^+ chaperones (Shikanai et al., 2003; Abdel-Ghany et al., 2005).

The toxicity of $AgNO_3$ is associated with heightened metal accumulation, resulting in an excessive generation of reactive oxygen species (ROS) (Barceló and Poschenrieder, 2004). Studies of the impact of $AgNO_3$ on plants have indicated the appearance of diverse toxicity symptoms, such as chlorosis, root system darkening, diminished growth and other alterations, which are caused by elevated ROS production in roots and shoots followed by excessive oxidative damage (Pokhrel and Dubey, 2013). The augmented damage observed in $AgNO_3$-treated roots hints at the potential occurrence of suboptimal metal compound translocation, resulting in its pronounced retention within roots and, consequently, exacerbating the damage. This process intensifies the production of superoxide anion radicals ($O_2^{\bullet-}$), followed by the formation of hydrogen peroxide (H_2O_2), which correlates with the inactivation of mitochondrial electron transport in plants (Jimenez et al., 1997).

As potent oxidizing agents, ROS can cause oxidative damage to membrane lipids, affecting membrane integrity and prompting electrolyte leakage (Anjum et al., 2013). ROS generation within root apical cells also modulates root growth responses under stress. The plant immune system deploys ROS accumulation within plant cells to combat foreign pathogens. These complex mechanisms of interaction and their significant role in the pragmatic realm of agriculture have sparked a growing interest in studying the adaptive response to oxidative stress in plant organisms (Lushchak, 2011).

Paulownia, a genus of fast-growing plants, can accumulate significant amounts of various metal ions. *Paulownia* Pao Tong Z07 originates from the intricate hybridization of three distinct *Paulownia* species, namely *P. tomentosa*, *P. fortunei*, and *P. kawakamii*. This particular super-hybrid is characterized by its exceptional disease resistance and robust adaptability to diverse environmental stresses encompassing heat, drought and cold resilience, withstanding temperatures down to -33 °C. Notably, this variety holds the distinction of being the most frost-resistant among all the available *Paulownia* varieties. Given these unique characteristics, it emerges as an optimal choice for cultivation in Europe, particularly within regions prone to substantial temperature fluctuations and severe winter frosts. In view of these considerations, we deliberately selected the *Paulownia* Pao Tong Z07 variety for our study. Thus, this study is designed to investigate the impact of $AgNO_3$ on growth, pigment concentrations, indices of oxidative stress, and activities of antioxidant enzymes in *Paulownia* seedlings.

Material and Methods

Reagents

Phenylmethylsulfonyl fluoride (PMSF), cumene hydroperoxide, ethylenediamine-tetraacetic acid (EDTA), xylenol orange, 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and ferrous sulphate were obtained from Sigma-Aldrich Corporation (USA. All other reagents were received from local suppliers (Ukraine) and they were of analytical grade.

Plant Material and Cultural Conditions

The effect of different concentrations of silver nitrate on plant growth and biochemical parameters was studied on *Paulownia* seedlings (*Paulownia* Z07 Pao Tong). *Paulownia* seedlings, previously established *in vitro* in our laboratory as microclonal culture, were transferred to glass jars with 25 mL of Murashige and Skoog (MS) medium containing 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, 6.0 g L^{-1} agar, 0.5 mg L^{-1} of 6-benzyl amino purine (BA) and 0.02 mg L^{-1} indole-3-acetic acid (Murashige and Skoog, 1962). To investigate the effects of $AgNO_3$ on *Paulownia* shoot explants, one of the following concentrations of $AgNO_3$ was added: 0 (control), 1, 10, or 50 mg L^{-1}. The media were adjusted to pH 5.8 by adding 1 N NaOH prior to the inclusion of agar and autoclaved for 30 min at 121 °C. The shoot explants were maintained for 40 days in a growth room at 25 ± 2 °C, under a photoperiod of 16 hours of light and 8 hours of darkness, with a light intensity of 50 µmol m^{-2} s^{-1}, and a relative humidity of 95 - 99%. The experiments were carried out in four independent replicates with three samples in each (n = 12).

Morphometric Plant Analysis

Ten cultivated *in vitro Paulownia* seedlings from each experimental group were randomly selected for the evaluation of growth characteristics after 40 days of cultivation. The plants were washed with sterile distilled water, and morphometric measurements such as shoot length, stem mass, and leaf mass were taken. The length of the aerial part of the plant was measured using a ruler, from the base of the stalk to the last expanded leaf. The fresh mass of shoots was evaluated using a precision balance (Husak et al., 2020). For the analysis of biochemical parameters, all leaves from each plant were collected and frozen in liquid nitrogen.

Quantification Pigments

For pigment extraction, fresh leaves were homogenized with ice-cold 96% ethanol (1:10, w:v) in the presence of 10 mg mL^{-1} $CaCO_3$ to avoid pheophytinization. The homogenates were centrifuged at 8000×g for 10 min at 4 °C. The supernatants were collected, and the pellets were re-extracted three times with 1 ml ice-cold 96% ethanol, then combined extracts were used for spectrophotometric measurement of pigment concentration. Specific absorption coefficients for chlorophyll *a*, chlorophyll *b*, and carotenoids were used (Lichtenthaler, 1987). A molecular mass of 570 was used to calculate the carotenoid concentrations. Anthocyanin content was measured after acidifying the extract with concentrated HCl to a final concentration of 1%. The anthocyanin concentration was determined spectrophotometrically at 530 nm using an absorption coefficient of 30 mM^{-1} cm^{-1} (Gitelson et al., 2001; Stambulska and Luschak, 2013; Husak et al., 2020).

Determination of Oxidative Stress Indices

The xylenol orange method measured lipid peroxides concentration (LOOH) (Evans et al. 1999). Frozen leaf samples were homogenized (1:20, w: v) in 96% ice-cold ethanol and centrifuged at 8000×g for 15 min at 4 °C. The supernatants were used for assay as described previously (Lushchak et al., 2005). The concentration of LOOH was expressed as nanomoles of cumene hydroperoxide equivalents per gram wet mass (nmol gwm^{-1}) of plant material.

The contents of carbonyl groups of proteins (CP) were determined as described previously (Lushchak et al., 2005). The frozen leaves of *Paulownia* seedlings were homogenized (1:10, w:v) in medium (50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM PMSF) and centrifuged at 15,000×g for 15 min at +4 °C. Supernatants were removed and 0.25 mL aliquots were mixed with 0.25 ml of 40% trichloroacetic acid (TCA) (final TCA concentration 20%) and centrifuged at 5000×g for 5 min at +20 °C. Protein carbonyl content was measured in the resulting pellets by reaction with 2,4-dinitrophenyl hydrazine, leading to the formation of dinitrophenylhydrazones. The amount of CP was assayed spectrophotometrically at 370 nm using a coefficient of molar extinction 22×10^3 M^{-1} cm^{-1}. Values are expressed as nanomoles of CP per milligram of protein.

Concentrations of free thiols were assayed spectrophotometrically with 5,5'-dithiobis (2-nitrobenzoic acid) at wavelength 412 nm using supernatants prepared as above for the CP assays. The total thiol concentration was measured as described previously (Lushchak and Bagnyukova, 2006). For evaluation of concentrations of low-molecular-mass thiols (L-SH), aliquots of supernatants were mixed with trichloroacetic acid (TCA) to reach a final TCA concentration of 10%. The mixture was centrifuged at 15,000×g, for 5 min to remove pelleted protein. Final supernatants were used for the assay. Thiol concentrations were expressed as micromoles of SH-groups per gram wet weight of plant material. The high-molecular-mass thiol content (HSH) was calculated by subtracting the LSH concentration from the total thiol concentration (Mosiichuk et al., 2015).

Assay of Enzyme Activities and Protein Concentration

The supernatants prepared for the CP and thiol assays were used to measure enzyme activities.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using a method that involves the enzyme's ability to compete with nitroblue tetrazolium for superoxide anion radicals generated from the photochemical reduction of water in the presence of riboflavin. The optical density was recorded at 560 nm and quantified as SOD activity in conventional units (U) mg $protein^{-1}$, with 1 U causing 50% inhibition of the change color of the mixture reaction (Beyer and Fridovich, 1987).

Catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically at 240 nm using a coefficient of molar extinction 39.4 M^{-1} cm^{-1} (Aebi, 1984).

Ascorbate oxidase (AO, EC 1.10.3.3) activity was measured as described by Diallinas et al. (1997). The enzyme activity was assayed spectrophotometrically by measuring the decrease in absorbance at 265 nm wavelength due to ascorbate oxidation using a coefficient of molar extinction 14,000 M^{-1} cm^{-1} (Diallinas et al., 1997; Semchuk et al., 2009).

The activity of ascorbate peroxidase (APx, EC 1.11.1.11) was determined by following the decrease of absorbance at 290 nm. The extinction coefficient 2800 M^{-1} cm^{-1} for ascorbate was used (Aebi, 1984; Semchuk et al., 2009).

Glutathione-*S*-transferase (GST, EC 2.5.1.18) activity was assayed spectrophotometrically at 340 nm by monitoring the formation of an adduct between reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) (Lushchak et al., 2005).

One unit (U) of CAT, AO, APx, and GST activities is defined as the amount of enzyme consuming 1 μmol of substrate or generating 1 μmol of product per minute. Activities were expressed as international units (or milliunits) per milligram soluble protein (U mg $protein^{-1}$ or mU mg $protein^{-1}$).

The protein concentration was determined with Coomassie brilliant blue G-250 according to the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

Statistical Analysis

Data are presented as means ± S.E.M. Shovene's and Dixon's Q tests were used for identification and rejection of outliers. Shapiro-Wilk test was conducted to assess the normality of the data distribution. Levene's test was conducted to assess the homogeneity of variances across the groups. Statistical analysis was performed using Mynova computer program (version 1.3) with ANOVA followed by a Dunnett's test. A probability value of $P < 0.05$ was considered statistically significant.

Results and Discussion

Effect of $AgNO_3$ on Morphometric Characteristics of Plants

In this study, we revealed that varying concentrations of $AgNO_3$ improved morphometric parameters, such as shoot length, stem and leaf mass as shown in Figure 1.

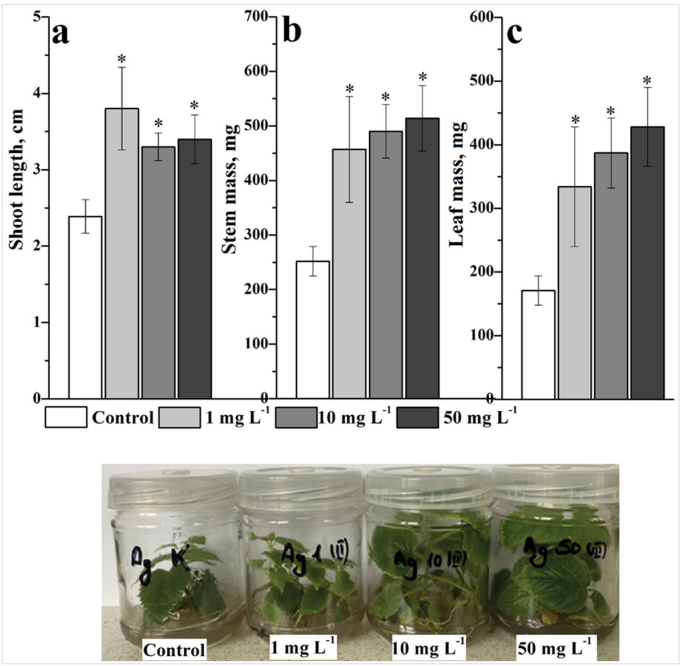

Figure 1. The effects of $AgNO_3$ (1, 10, 50 mg L^{-1}) on shoot length (a), stem mass (b) and leaf mass (c) of *iv vitro Paulownia* seedlings cultivated in MS medium during 40 days

Note: Data are presented as means ± S.E.M, n = 12.

*Significantly different from the control (without $AgNO_3$) group of plants ($P < 0.05$) according to ANOVA followed by Dunnett's test

In particular, we observed a statistically significant elevation in shoot length across all experimental groups compared to the control group. At $AgNO_3$ concentrations of 1, 10, and 50 mg L^{-1} in the cultivation medium, the length of *Paulownia* shoots was by 59%, 38%, and 42% higher, respectively, as compared with the control plants. Stem mass in plants cultivated with different $AgNO_3$ concentrations was 1.8-2.0-times higher than in the control group (Fig. 1b). Similarly, leaf mass displayed 2.0-2.5-fold increase in plants grown on $AgNO_3$-supplemented media (Fig. 1c). Supplementation of the growth medium, lacking growth regulators, with $AgNO_3$ has been demonstrated to exert a regenerative impact on explants and induce the emergence of axillary shoots in *Arachis hypogaea* L. (Ozodogru et al., 2005). In our study, the phenomenon in question was not observed. Additionally, explants cultured on hormone-free MS medium, both with and without the addition of silver nitrate, exhibited negligible growth (data not shown). Moreover, silver nitrate has been evidenced to positively influence shoot elongation and mitigate callus proliferation at the basal tips of shoots in *Cichorium intybus* L. (Bais et al., 2000) and *Vanilla planifolia* Andr. (Giridhar et al., 2001; Ozodogru et al., 2005). Silver nitrate utilization *in vitro* culture conditions has also been reported to enhance the growth and development of potato explants (Homaee and Ehsanpour, 2015) and elicit different effects on diverse growth parameters in arabica and robusta coffee (Giridhar et al., 2003). This phenomenon might be attributed to $AgNO_3$ inhibitory effects on the production of ethylene, which is known to be a regulator of the growth of different plant tissues and organs (Evans and Batty, 1994). Therefore, the introduction of silver ions into the growth medium could potentially mitigate the impact of ethylene and improve the regeneration of *Paulownia* seedlings in this study.

Effect of $AgNO_3$ on the Concentrations of Pigments

In our experiments, we measured the levels of Chl *a* and Chl *b* and found that exposure to $AgNO_3$ had minimal effects on pigment content in leaves. No significant changes were observed in Chl *a* and Chl *b* levels in *Paulownia* seedlings exposed to silver nitrate at any of the three concentrations used (Fig. 2a, b). However, explants treated with $AgNO_3$ demonstrated a dose-dependent decline in carotenoid concentrations compared to the control (Fig. 2c). Carotenoid content in leaves of *Paulownia* seedlings treated by 10 and 50 mg L^{-1} $AgNO_3$ was 2.1- and 1.6-fold lower than in the control (5.95 ± 0.85 μmol gwm^{-1}), respectively, while no significant difference was observed between control plants and those treated by 1 mg L^{-1} $AgNO_3$. Heavy metal ions have been shown to exert adverse effects on many physiological processes in plants, including photosynthesis. Photosynthetic pigments have been identified as potential biomarkers for stress induced by heavy metal ions (Kumar et al., 2012). Prior studies have demonstrated that the accumulation of silver ions in leaves disrupts the thylakoid membrane structure and reduces chlorophyll (Chl) levels, thereby inhibiting plant growth in *Arabidopsis thaliana* (Quian et al., 2013). *Lycopersicon esculentum* plants exposed to 10, 20, or 30 mg L^{-1} $AgNO_3$ exhibited a significant reduction in Chl *b* levels, highlighting the adverse impact of $AgNO_3$ on this parameter (Noori et al., 2020). Similarly, *Physcomitrella patens* exposed to Ag^+ also displayed diminished levels of Chl *b* and *a*, along with an imbalanced profile of essential elements in the leafy gametophytes (Liang et al., 2018).

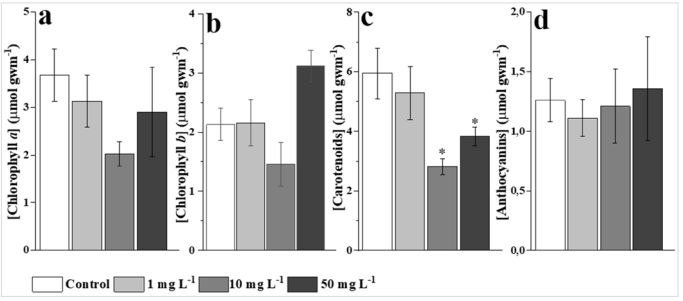

Figure 2. Concentrations of chlorophyll *a* (a), chlorophyll *b* (b), carotenoids (c) and anthocyanins (d) in *Paulownia* seedlings, exposed to different concentrations of $AgNO_3$

Note: Data are presented as means ± S.E.M, n = 8

*Significantly different from the control (without $AgNO_3$) group of plants ($P < 0.05$) according to ANOVA followed by Dunnett's test

Our experiments found no substantial alterations in anthocyanin content within explants treated with silver nitrate (Fig. 2d). Anthocyanins serve as natural antioxidants, offering

protection to plants against a spectrum of biotic and abiotic stresses (Gould, 2004; Syu et al., 2014; Yan and Chen, 2019). These stressors encompass cold temperatures, drought, UV radiation, exposure to diverse pathogens and heavy metals. Research suggests that anthocyanins exhibit efficacy in scavenging ROS within plant cells (Hatier and Gould, 2009). Previous research has indicated an elevation in anthocyanin levels within the leaves of tomato plants exposed to 10–20 mg L^{-1} $AgNO_3$ and in *Arabidopsis thaliana* plants subjected to Ag^+ treatment (Quian et al., 2013; Noori et al., 2020).

The Effect of $AgNO_3$ on Stress Markers

The concentration of lipid peroxides in the control group of plants was 4.83 ± 0.77 nmol gwm^{-1}. The exposure to $AgNO_3$ at all used concentrations did not cause statistically significant changes in LOOH concentration in *Paulownia* seedlings suggesting that oxidative modification of lipids did not occur (Table 1).

Lipid peroxidation, a phenomenon typically occurring under stress conditions, disturbs the structure of lipid bilayer in cellular membranes, affecting the fluidity, bilayer thickness, permeability, and other membrane properties due to oxidative damage of lipids and membrane proteins (Mbarki et al., 2018). It has been shown that $AgNO_3$ has phytotoxic effects and leads to a gradual increase in the content of malonic dialdehyde with increasing concentrations of $AgNO_3$ and exposure time in *Lemna* sp. (Iannelli et al., 2022), *Pennisetum glaucum* L. (Khan et al., 2019), *Lycopersicon esculentum* L. (Noori et al., 2020), and other plants. Our experiments revealed unaltered levels of carbonylated proteins (CP) in *Paulownia* seedlings exposed to $AgNO_3$ at all concentrations used (Table 1). In plants, metal-induced stress often results in augmented ROS production followed by protein damage. The primary oxidative protein modification involves the formation of additional carbonyl groups, leading to protein inactivation and loss of function (Mano et al., 2019). Earlier investigations indicated that $AgNO_3$ treatment induced ROS generation and increased carbonyl content in tobacco plants (Štefanić et al., 2018).

The levels of intracellular thiol-containing compounds can also be influenced by ROS (Lushchak and Bagnyukova, 2006). The content of sulfhydryl groups in proteins (H-SH) remained unchanged in *Paulownia* seedlings even after treatment with 1.0, 10, and 50 mg L^{-1} $AgNO_3$ for 40 days (Table 1). Low-molecular-mass thiols (LSH) constitute a group of highly reactive compounds crucial for maintaining cellular redox homeostasis. They are involved in plant responses to almost all stress factors, as well as in the regulation of cellular metabolism. Glutathione (GSH), the most abundant LSH in plants, plays a pivotal role in redox and regulatory functions (Pivato et al., 2014; Das and Roychoudhury, 2014). GSH is known to bind metals and interact with xenobiotics owing to its sulfhydryl group, thereby regulating enzymatic activity and stress-responsive gene expression (Noctor et al., 2012). Metal ions can deplete the pool of reduced glutathione, leading to disruption of redox balance. Our study found that levels of low-molecular-mass thiols in *Paulownia* seedlings exposed to 1, 10, and 50 mg L^{-1} $AgNO_3$ were 3.3-, 3.7-, and 2.5-fold lower, respectively, than those in the control group (Fig. 5a). Thus, our findings suggest that silver nitrate might induce mild oxidative stress in *Paulownia*. Upon entry into plants, silver ions can interact with cellular compounds, disrupting their structure and function. We propose that the primary mechanisms through which Ag^+ depletes the levels of reduced glutathione in *Paulownia* include glutathione conjugation with electrophiles and inhibition of glutathione regeneration from the oxidized state (i.e., GSSG, oxidized glutathione). The cellular GSH pool is replenished either through the *de novo* synthesis or through conversion of GSSG to GSH by glutathione reductase (Das and Roychoudhury, 2014).

Effect of $AgNO_3$ on Antioxidant Enzyme Activity

Metals can exert inhibition on various intracellular processes by impacting the activities of numerous enzymes (Kumar and Trivedi, 2018; Dobritcsz et al., 2020).

In this work, SOD activity was lower in seedlings treated with silver nitrate at all concentrations in contrast to the control value (23.6 ± 0.4 U mg $protein^{-1}$) (Fig 3a). Particularly, SOD activity in explants of plants treated by 1, 10, and 50 mg L^{-1} $AgNO_3$, was 65%, 53%, and 25% of the control value, respectively (Fig. 3a). Superoxide dismutase (SOD) is an antioxidant enzyme that protects cells against the superoxide anion radical by converting it to less toxic hydrogen peroxide (Berwal and Ram, 2018). Notably, Cu, Zn-SOD is localized in the cytosol and is also present in the plant apoplast (Berwal and Ram, 2018). The lower SOD activity in $AgNO_3$-treated cells could potentially result from the ability of silver ions to displace copper ions from the enzyme's active site, as demonstrated for other copper-containing proteins (Sigfridsson, 1998).

In *Paulownia* seedlings, catalase activity was 6.8- and 2.6-fold higher upon exposure to silver nitrate concentrations of 1 and 10 mg L^{-1}, respectively, in comparison to the control value (12.6 ± 4.4 U mg $protein^{-1}$) (Fig. 3b).

Table 1. Concentrations of stress markers in *Paulownia* seedlings grown in control conditions or exposed to 1, 10, and 50 mg L^{-1} of $AgNO_3$ for 40 days

Parameters	Plant group			
	Control	1.0 mg L^{-1}	10 mg L^{-1}	50 mg L^{-1}
LOOH (nmol gwm^{-1})	4.83 ± 0.77	3.76 ± 0.68ns	6.52 ± 1.97 ns	4.30 ± 0.41 ns
CP (nmol mg $protein^{-1}$)	67.8 ± 7.9	73.1 ± 9.1 ns	84.4 ± 17.8 ns	76.7 ± 20.9 ns
HSH (μmol gwm^{-1})	0.830 ± 0.132	0.669 ± 0.043 ns	0.948 ± 0.144 ns	1.02 ± 0.12 ns

Note: Data are presented as means ± S.E.M, n = 8; ns – not different from the control value

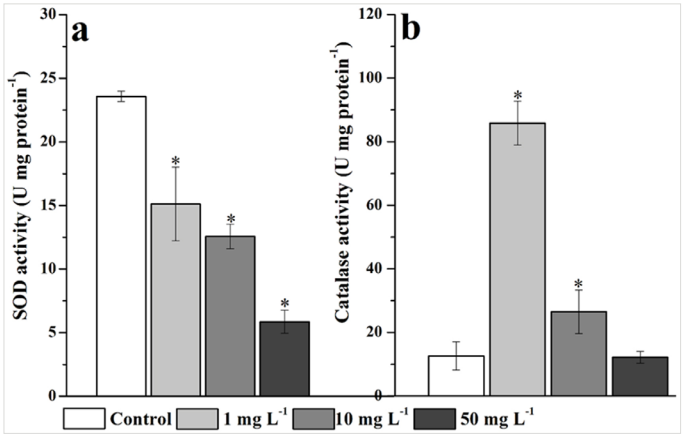

Figure 3. The activities of superoxide dismutase (SOD, U mg protein^{-1}) (a) and catalase (CAT, U mg protein^{-1}) (b) in *Paulownia* seedling, exposed to 1, 10, and 50 mg L^{-1} of $AgNO_3$ for 40 days

Note: Data are presented as means ± S.E.M, n = 8

*Significantly different from the control (without $AgNO_3$) group of plants ($P < 0.05$) according to ANOVA followed by Dunnett's test

Previously, Homaee and Ehsanpour (2016) reported an elevation of catalase activity by silver ions that was linked to enhanced hydrogen peroxide generation. Hence, the sharp increase in catalase activity in $AgNO_3$-treated *Paulownia* seedlings should be interpreted as an adaptive response mechanism to counteract $AgNO_3$-mediated ROS production. Under stress conditions, cells often up-regulate catalase expression to counteract escalating hydrogen peroxide levels. Conversely, lower catalase activity or expression can be associated with pathological processes within cells (Glorieux and Calderon, 2017).

As depicted in Fig. 4b, the APx activity was 2.7-fold higher in plants treated with $AgNO_3$ at 1 mg L^{-1} compared to the control value (1.24 ± 0.23 U mg protein^{-1}) (Fig. 4b). Ascorbate peroxidase reduces hydrogen peroxide water by utilizing ascorbate as a specific electron donor (Takeda et al., 1998).

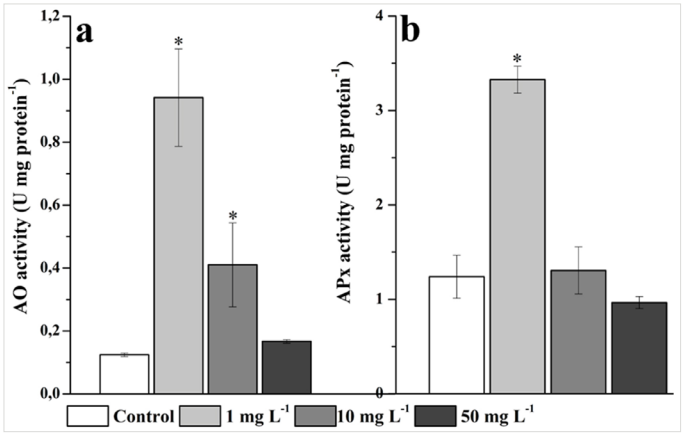

Figure 4. The activities of ascorbate oxidase (AO, U mg protein^{-1}) (a) and ascorbate peroxidase (APx, U mg protein^{-1}) (b) in *Paulownia* seedlings, exposed to control conditions or 1, 10, and 50 mg L^{-1} of $AgNO_3$ for 40 days

Note: Data are presented as means ± S.E.M, n = 8

*Significantly different from the control (without $AgNO_3$) group of plants ($P < 0.05$) using ANOVA followed by Dunnett's test

Ascorbate peroxidase (APx) is distributed in various subcellular compartments, including chloroplasts, cytosol, and membrane-associated microbodies. Earlier, a substantial elevation in APx activity was also found in potatoes under the influence of silver ions (Homaee and Ehsanpour, 2016).

Figure 4a illustrates that ascorbate oxidase (AO) activities in *Paulownia* seedlings subjected to 1 and 10 mg L^{-1} silver nitrate concentrations were respectively 7.5-fold and 3.0-fold higher compared to the control (0.124 ± 0.006 U mg protein^{-1}) (Fig. 4a). Ascorbate oxidase is an enzyme catalyzing the conversion of ascorbate to monodehydroascorbate while concurrently reducing oxygen to water. This enzyme plays a pivotal role in plant growth, development, signal transduction, and stress tolerance (Stevens et al., 2018).

Figure 5b illustrates a significant reduction in glutathione S-transferase (GST) activity in all experimental groups of *Paulownia* seedlings in comparison to the control (32.8 ± 5.6 mU mg protein^{-1}). Treatment with $AgNO_3$ in concentrations of 1, 10, and 50 mg L^{-1}, suppressed GST activity by 2.3-, 1.5-, and 2.3-fold, respectively, compared to the control group. Among the cellular antioxidant defense system, GST activity operates as a bifunctional enzyme, encompassing both glutathione S-transferase and glutathione peroxidase functions (Dobritcsz et al., 2020). An emerging body of evidence suggests that metals at toxic levels can impact GST either through a direct enzyme inhibition or indirectly by diminishing glutathione concentrations (Kumar and Trivedi, 2018; Dobritcsz et al., 2020).

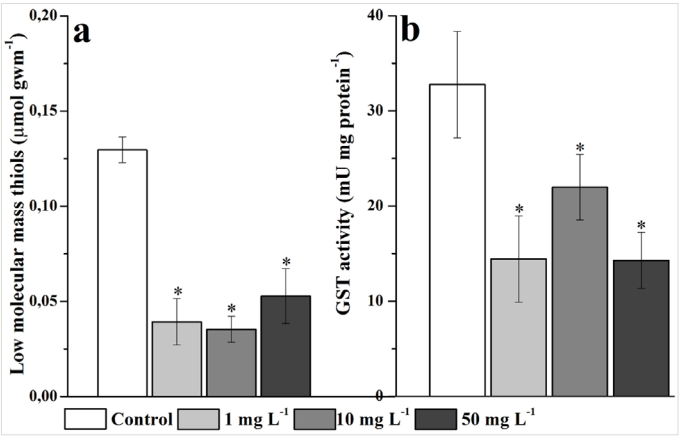

Figure 5. The concentrations of low molecular mass thiols (μmol gwm^{-1}) (a) and the activity of glutathione-*S*-transferase (GST, mU mg protein^{-1}) (b) in plants, exposed to control conditions or 1, 10 and 50 mg L^{-1} of silver nitrate for 40 days

Note: Data are presented as means ± S.E.M, n = 8

*Significantly different from the control (without $AgNO_3$) group of plants ($P < 0.05$) according ANOVA followed by Dunnett's test.

Hypothetical Mechanism of Silver Nitrate Action

In this study, we investigated the role of silver ions as a potent regulator of morphogenesis and an inducer of oxidative stress in *Paulownia*. Our research demonstrates the stimulating physiological effects of Ag^+ in the form of $AgNO_3$ on the growth of plants in culture *in vitro*. Nonetheless, the molecular

mechanisms underlying the influence of Ag^+ on plant growth remain insufficiently elucidated. It is established that Ag^+ possesses the capacity to counteract the ethylene action on plant growth (Kumar et al., 2009). Ethylene is a phytohormone, which controls a broad spectrum of plant developmental processes, such as inhibition of root formation, regeneration, organogenesis, shoot growth, and other phenomena (Kumar et al., 2009; Street and Schaller, 2016). This phytohormone is recognized by a family of five copper-containing, membrane-localized receptors: Ethylene Response 1 (ETR1), ETR2, Ethylene Response Sensor 1 (ERS1), ERS2, and Ethylene Insensitive 4 (EIN4) (McDaniel and Binder, 2012; Schaller and Binder, 2017). Ethylene binding to its receptors leads to the deactivation of the Constitutive Triple Response 1 (CTR1, negative regulator) kinase, a negative regulator. This deactivation results in the dephosphorylation and activation Ethylene-Insensitive 2 (EIN2), a positive regulator. EIN2 then triggers primary transcription factors EIN3/EILs, inducing comprehensive ethylene responses (Qiao et al., 2009; Tao et al., 2015). Given the similarities in size and oxidation state between silver and copper ions, along with their capability to form complexes with ethylene (Tao et al., 2015), Ag^+ may effectively displace essential copper cofactors within the ethylene signal (Binder et al., 2007; McDaniel and Binder, 2012). Consequently, our observations revealed an accelerated growth and development of *Paulownia* under the influence of silver ions (Fig. 6). In parallel, numerous studies have also documented improved plant growth on $AgNO_3$-supplemented mediums (Fuentes et al., 2000; Misra and Datta, 2001; Giridhar et al., 2003).

With the interference of silver ions disrupting receptor structure via displacing copper in their structure, the binding of ethylene is curtailed, possibly resulting in elevated intracellular ethylene concentrations. This elevation can potentially activate NADPH oxidases (NOXs), such as AtRbohF (Desikan et al., 2006). NOXs are transmembrane proteins pivotal in transferring electrons across membranes, catalyzing the reduction of oxygen into $O_2^{\bullet-}$ (Bedard and Krause, 2007; Panday et al., 2015). These superoxide anions may be released into the apoplast, where they are subsequently transformed into H_2O_2 by SOD (Wang et al., 2018). H_2O_2 can then traverse the cellular membrane via aquaporin water channels (AQP), participating in downstream signaling cascades (Bienert et al., 2007; Miller et al., 2010; Maurel et al., 2015). The plasma membrane NOX stands as the primary source of H_2O_2 in plant cells. In response to elevated H_2O_2 levels, plant cells may activate enzymes like catalase, ascorbate peroxidase, and ascorbate oxidase in the leaves of *Paulownia* (Fig. 6). Reduced glutathione (GSH) contains cysteine (Cys) that exists as a thiolate anion ($G–S^-$) being susceptible to oxidation (Cremers and Jakob, 2013; Holmström and Finkel, 2014). H_2O_2 molecules can oxidize Cys residues in glutathione to form anions sulfenic (G–SOH), sulfinic ($G–SO_2H$), or sulfonic ($G–SO_3H$) acids (Fig. 6). Therefore, lowering of LSH content in our research may result from oxidation of reduced glutathione or free Cys (Fig. 6).

Further, silver ions have capability of substituting Cu^+ ions in copper-containing proteins, including Cu, Zn-SOD, and plastocyanin (Palacios et al., 2003; Veronesi et al., 2015). This substitution inactivates these proteins. This inactivation mechanism might account for the observed decrease in SOD activity across all experimental concentrations of $AgNO_3$, as compared to the control group (Fig. 3a).

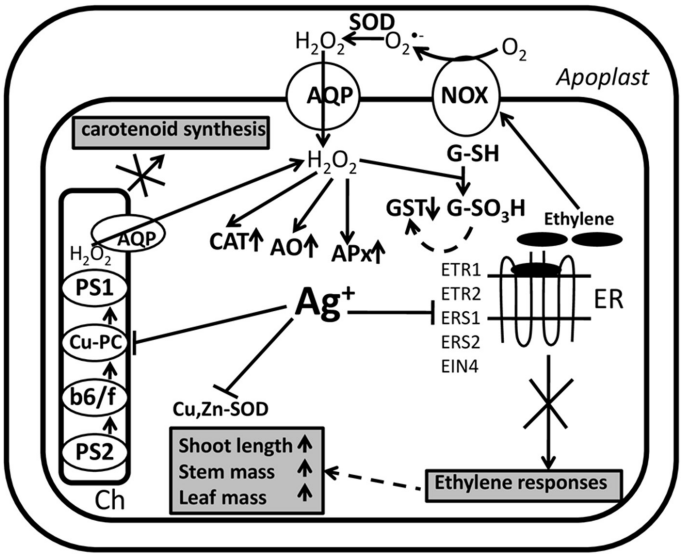

Figure 6. Hypothetical representation of mechanisms responsible for silver-induced stress in plants

Note: Ethylene, a phytohormone, regulates various plant developmental processes. It interacts with copper-containing receptors (ETR1, ETR2, ERS1, ERS2, EIN4) to initiate a signaling cascade. This involves deactivating CTR1, and activating EIN2, which triggers transcription factors EIN3/EILs for ethylene responses. Silver ions disrupt this process by substituting copper ions in receptors, hindering ethylene binding. These anions are converted into H_2O_2 by superoxide dismutase or non-enzymatically, participating in downstream signaling. Elevated H_2O_2 levels trigger enzymes like catalase (CAT), ascorbate peroxidase (APx) and ascorbate oxidase (AO), impacting glutathione (G-SH) oxidation and glutathione-*S*-transferase (GST) activity. Silver ions also substitute copper in proteins like superoxide dismutase (Cu, Zn-SOD), and plastocyanin (Cu-PC), reducing their function. Plastocyanin's disruption slows the photosynthetic electron transfer chain, generating ROS and decreasing carotenoid content. ER, endoplasmic reticulum; Ch, chloroplast; PS1, photosystem 1; PS2, photosystem 2; *b6/f*, cytochrome *b6/f*.

Plastocyanin (PC) is a soluble copper-containing protein in the thylakoid lumen that facilitates electron transfer from cytochrome *b6/f* to photosystem I (PSI) in the chloroplast photosynthetic electron-transfer (ET) chain (Katoh, 2003; Höhner et al., 2020). Ag^+ competes with Cu^+ for PC, potentially disrupting or deactivating the ET chain (Sujak, 2005; Jansson and Hansson, 2008). This disruption leads to the generation of ROS, including H_2O_2, which can readily permeate the chloroplast membrane through AQP. Malfunctioning of the ET chain may contribute to the decline in carotenoid content due to the inhibition of their synthesis (Fig. 2c).

Conclusions

Results showed that $AgNO_3$ treatment enhanced growth of *Paulownia* seedlings, which could possibly be due to inhibitory effects of silver ion on ethylene perception. From the results, it can be concluded that $AgNO_3$ at concentrations of 1-50 mg L^{-1} improved the shoot length, stem mass and leaf mass, thereby enhancing the growth and development of *Paulownia* seedlings under *in vitro* culture conditions. However, based on biochemical responses of treated seedlings on carotenoids and low molecular mass thiols contents, and superoxide dismutase, catalase, ascorbate oxidase, ascorbate peroxidase and Glutathione-*S*-transferase activities, it can be considered that phytotoxicity of $AgNO_3$ at 1-50 mg L^{-1} is likely due to induction of mild oxidative

stress. Furthermore, a dose-dependent effect of silver nitrate on superoxide dismutase activity was observed. Specifically, an inverse relationship was established; as the concentration of silver nitrate increased, the activity of superoxide dismutase decreased. This phenomenon underscores the potential inhibitory impact of higher concentrations of silver nitrate on the enzymatic function of superoxide dismutase.

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CRediT Authorship Contribution Statement

Viktor Husak: conceived the project and supervised the work. **Uliana Ya. Stambulska**: investigated, performed some of the experiments, analyzed the data and drafted the manuscript. **Angelika M. Pitukh:** performed most of the experiments. **Volodymyr I. Lushchak:** contributed to the editing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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