

ALLEVIATIVE EFFECTS OF CHITOSAN AND NANOSILVER ON *Solanum pimpinellifolium* UNDER HEAVY METAL STRESS *IN VITRO*

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Abstract

This study aimed to investigate the potential mitigation of copper stress in tomato plants (*Solanum pimpinellifolium* L0566) through the addition of chitosan (CH) or nanosilver (nAg) to Murashige and Skoog (MS) medium under *in vitro* conditions. Various growth parameters; the proline, malondialdehyde (MDA), total polyphenol and mineral contents; and CIE $L^*a^*b^*$ colour parameters were evaluated. After the multiplication stage, the explants were transferred to MS medium (control); MS + 20 ppm chitosan with a molecular weight of 3.33 kDa ($CH_{3.33}$); MS + 6 mg l⁻¹ nAg; MS + 100 μM l⁻¹ copper sulfate ($CuSO_4$); MS + 20 ppm $CH_{3.33}$ + 100 μM l⁻¹ $CuSO_4$; or MS + 6 mg l⁻¹ nAg + 100 μM l⁻¹ $CuSO_4$. The results indicated that while $CuSO_4$ or $CuSO_4$ -nAg solutions inhibited growth traits, $CH_{3.33}$ stimulated growth, particularly shoot production, and plants treated with $CH_{3.33}$ exhibited better developed roots and a higher fresh mass. Additionally, $CH_{3.33}$ alleviated the negative effects of $CuSO_4$ on the proline, MDA and total polyphenol contents in tomato plants. Moreover, tomato explants exhibited greener leaves, while those treated with nAg and $CuSO_4$ showed decreased colour values. $CH_{3.33}$ or nAg positively influenced the mineral content of tomato leaves under heavy metal stress. This study underscores the complex interactions between growth medium components on tomato plant growth, physiology and the mineral content, highlighting the potential of chitosan in mitigating heavy metal stress in tomato plants.

Keywords: tomato, chitosan, *in vitro*, nanoparticle, copper

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1. Introduction

The expansion of industrial production activities has led to an alarming increase in environmental pollution from wastewater containing heavy metals. These metals, particularly prevalent in developing countries, are disposed of directly or indirectly into the environment in significant quantities. Due to their non-biodegradable nature, heavy metals tend to accumulate in living organisms, where they create a significant risk of toxicity. When copper (Cu) is present slightly above the optimal tissue levels, it can become toxic to a plant [1, 2]. However, heavy metals as trace elements can play an important role in various oxidation and reduction reactions. For example, Cu acts as a crucial cofactor for various enzymes associated with the oxidative stress response, such as catalase, superoxide dismutase, peroxidase, cytochrome *c* oxidase, ferroxidases, monoamine oxidase and dopamine β -monooxygenase [3, 4]. Heavy metals accumulated in excessive amounts in tissues cause oxidative stress, which contributes to the release of oxygen free radicals. Due to their strong attraction to sulfur-containing peptides and proteins, free radicals reduce enzymatic and non-enzymatic antioxidant levels [5]. Reactive oxygen species (ROS) can potentially cause massive oxidation of membrane proteins and lipids, resulting in increased malondialdehyde (MDA), a by-product of lipid peroxidation [1]. Consistently, following exposure to Cu, there was a notable accumulation of MDA [6].

New solutions are being investigated to alleviate the detrimental impacts of environmental stresses on plants, including the application of biologically active substances and nanotechnology. As a relatively modern field of science, nanotechnology offers various applications in agriculture. The use of nanoparticles in agriculture provides for the elimination of nutrient deficiencies in plant tissues and enhances tolerance to stress, thus contributing to improved growth and increased yield [7]. Many authors [8, 9] have highlighted that nanosilver (nAg) improves seed germination, plant growth and photosynthetic efficiency, and reduces microbial growth on plant bodies. An alternative to the use of synthetic materials to remove heavy metals from the environment is biopolymers, including chitosan, which is widely available. Chitosan is a non-toxic, natural elicitor and biodegradable compound of natural origin, obtained by enzymatic deacetylation of chitin [10, 11]. This biocompatible biopolymer has a variety of applications in agriculture. It improves both qualitative and quantitative crop characteristics by facilitating nutrient uptake by plants [12].

In addition, it stimulates seed germination as well as shoot and root growth [13] and influences numerous metabolic and physiological processes. As a consequence, plants treated with chitosan may show increased resistance to environmental stresses [14].

The tomato, *Solanum pimpinellifolium*, is a widely favoured vegetable within the Solanaceae family. Ranked as the world's second most significant vegetable crop, it is cultivated in nearly every country. Renowned for its taste, nutritional benefits and simple cultivation process, the tomato holds a pivotal place in human diets [15, 16]. Due to the important role of the Solanaceae family in agronomic and ornamental crops, the objective of the present study was to evaluate the effectiveness of chitosan with a molecular weight of 3.33 kDa ($\text{CH}_{3.33}$) and nAg in alleviating the harmful effects of Cu stress on tomato plants under *in vitro* conditions. This study tested the hypothesis that chitosan and nAg alleviate Cu stress by enhancing growth parameters, reducing stress markers and improving mineral content and leaf colouration. Chitosan is expected to have a more pronounced beneficial effect compared with nAg.

2. Materials and Methods

2.1. Plant Materials

S. pimpinellifolium (L0566) seeds were obtained from the Tomato Genetics Resource Centre (University of California, Davis). They were disinfected by soaking in a 70% ethanol solution for 30 s, washed twice with sterile deionised water and submerged in 7% sodium hypochlorite (NaOCl) for 10 min. Then, the seeds were rinsed 3×5 min with sterile and deionised water under a laminar flow hood.

2.2. Medium and Culture Conditions

Sterilised tomato seeds were cultured individually in glass tubes (35×110 mm) containing 15 ml of initiation Murashige and Skoog (MS) medium [17] without plant growth regulators for 4 weeks. Explants were sub-cultured three times. After that, a shoot with auxiliary buds was transferred to a 300-ml flask with 30 ml of MS medium containing 20 ppm $\text{CH}_{3.33}$; MS medium containing 6 mg l^{-1} nAg (nanopowder < 100 nm particle size, Sigma-Aldrich, Germany); MS containing 100 $\mu\text{l l}^{-1}$ CuSO_4 ; or MS containing 100 $\mu\text{l l}^{-1}$ CuSO_4 , 20 ppm $\text{CH}_{3.33}$ and MS + 100 $\mu\text{l l}^{-1}$ CuSO_4 + 6 mg l^{-1} nAg. MS medium without plant growth regulators served as the control. Each combination included 32 shoots (4 explants per flask and 8 replicates).

Following a 35-day incubation period, the explants were carefully removed and cleaned with deionised distilled water. The following parameters were assessed: shoot and root length [cm]; the number of new shoots per explant; the fresh mass [cm]; the proline, MDA and total polyphenol (TP) contents; the macroelement (phosphorus [P], potassium [K], calcium [Ca] and magnesium [Mg]) and microelement (iron [Fe], zinc [Zn], manganese [Mn] and Cu) levels; and CIE $L^*a^*b^*$ colour.

All media contained 30 g L^{-1} sucrose (Chempur, Poland) and 100 mg L^{-1} myo-inositol (Duchefa, the Netherlands) and were solidified using 8 g L^{-1} agar (Biocorp, Poland). The pH was adjusted to 5.8 prior to autoclaving at 121°C and 0.1 MPa. Cultures were incubated in a growth room at $25 \pm 2^\circ\text{C}$ under a 16-h photoperiod, with a photosynthetic flux density (PPFD) of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by Narva (Germany) emitting cool white daylight, and maintained at 60%–70% humidity.

2.3. Biochemical Analysis

The proline content of fresh tomato leaves was determined following the method outlined by Bates et al. [18], utilising a spectrophotometer set to 520 nm; it is expressed as micromoles per gram fresh weight. The MDA content was assessed according to Sudhakar et al. [19] based on the absorbance at 600, 532 and 450 nm. The mineral content in dried plant material was evaluated as per the Polish Standard (IUNG 1972) using certified reagents from Chempur and Merck (Germany). The P, K, Ca and Mg contents were measured following wet mineralisation in sulfuric acid (H_2SO_4 , 96%) and perchloric acid (HClO_4 , 70%). The Cu, Zn, Mn and Fe contents were measured following mineralisation in nitric acid (HNO_3 , 65%) and HClO_4 (70%). The K content was measured via atomic emission spectrometry. The Mg, Ca, Cu, Zn, Mn and Fe contents were measured using flame atomic absorption spectroscopy utilising a iCE 3000 Series instrument (Thermo Fisher Scientific, UK). P was assessed using the colourimetric method on a Specol 221 apparatus (Carl Zeiss, Germany) [20]. The TP content was determined spectrophotometrically by employing the Folin–Ciocalteu reagent. The standard curve, calculated using gallic acid as a standard, was measured spectrophotometrically at 700 nm and is expressed as milligrams of gallic acid equivalents (GAE) per gram of plant material.

2.4. Leaf Pigment Estimation

Leaves from the middle part of the shoot were evaluated using a CM-700d spectrophotometer (Konica Minolta, Japan). Measurements were conducted in the CIE $L^*a^*b^*$ system [21], employing the 10° observer type and the D65 illuminant. Colour readings were taken in triplicate for each experimental combination.

2.5. Statistical Analysis

Statistical analyses were conducted using Statistica 13.0 (StatSoft, Poland). The homogeneity of variance and normality of the distribution of the data were determined. Because the data showed a normal distribution, they were submitted to analysis of variance (ANOVA) followed by Tukey's post hoc test. A p value < 0.05 indicated a statistically significant difference. The relationships between macro- and microelements were analysed using agglomerative cluster analysis and classified into hierarchical groups using Ward's method.

3. Results and Discussion

Excessive amounts of heavy metals significantly inhibit plant growth due to physiological changes caused by oxidative stress. Cu toxicity can lead to severe ultrastructural damage, thus affecting key biochemical transformation processes in plants. This study was conducted to analyse the possibility of mitigating Cu stress in tomato plants by adding $\text{CH}_{3,33}$ or nAg to the medium under *in vitro* conditions. Table 1 provides the mean shoot and root length, the number of new shoots per plant and the fresh mass.

Table 1. The effects of different medium compositions on the morphological traits of *Solanum pimpinellifolium* (L0566) plants under *in vitro* condition after 35 days of culture (n = 32 shoots per treatment).

Medium	Shoot length [cm]	Number of new shoots per plant	Root length [cm]	Fresh mass [g]
MS (control)	11.30 ^b	1.6 ^{abc}	8.55 ^{ab}	1.71 ^b
MS + 20 ppm $\text{CH}_{3,33}$	12.95 ^b	2.3 ^c	11.25 ^a	1.98 ^b
MS + 6 mg l ⁻¹ nAg	12.65 ^b	1.5 ^{abc}	6.25 ^b	1.63 ^b
MS + 100 μM l ⁻¹ CuSO_4	11.35 ^b	1.1 ^a	9.35 ^b	1.41 ^{ab}
MS + 100 μM l ⁻¹ CuSO_4 + 20 ppm $\text{CH}_{3,33}$	10.95 ^b	2.1 ^{ab}	6.05 ^b	1.54 ^{ab}
MS + 100 μM l ⁻¹ CuSO_4 + 6 mg l ⁻¹ nAg	5.15 ^a	1.3 ^{bc}	12.30 ^a	0.6 ^b

Note. In each column, means followed by a different superscript letter differ significantly according to Tukey's test (p < 0.05). Abbreviations: $\text{CH}_{3,33}$, chitosan with a molecular weight of 3.33 kDa; CuSO_4 , copper sulfate; MS, Murashige and Skoog; nAg, nanosilver.

There were almost no significant differences between explants grown on different media, except the explants grown on MS containing $100 \mu\text{M l}^{-1} \text{CuSO}_4$ and $6 \text{ mg l}^{-1} \text{nAg}$ were 55% smaller compared with the control (Figure 1a). The explants grown on MS medium containing $20 \text{ ppm CH}_{3,33}$ or MS medium containing $100 \mu\text{M l}^{-1} \text{CuSO}_4$ and $20 \text{ ppm CH}_{3,33}$ showed the highest number of new shoots per plant (144% and 131% of the control, respectively). In contrast, the number of new shoots in the explants grown on MS medium containing $6 \text{ mg l}^{-1} \text{nAg}$ was the same as the control (1.6). Only the addition of CuSO_4 or CuSO_4 and nAg to the MS medium had an inhibitory effect on the number of new shoots, with a 31% and 19% reduction, respectively, compared with the control. However, the explants grown on MS medium containing $100 \mu\text{M l}^{-1} \text{CuSO}_4$ and $6 \text{ mg l}^{-1} \text{nAg}$ had longer roots compared with plants grown on the other media. The exception was explants grown on MS medium containing $20 \text{ ppm CH}_{3,33}$, which had a root length of 11.25 cm . Moreover, the roots of these plants were thicker and developed more adventitious roots (Figure 1b). In contrast, explants grown on MS medium containing $100 \mu\text{M l}^{-1} \text{CuSO}_4$ and $20 \text{ ppm CH}_{3,33}$ showed shorter roots (70% of control). There was a significant decrease in the fresh weight of plants grown on MS medium containing $100 \mu\text{M l}^{-1} \text{CuSO}_4$. However, the explants grown on MS medium containing $20 \text{ ppm CH}_{3,33}$ had the highest fresh mass (116% of control).

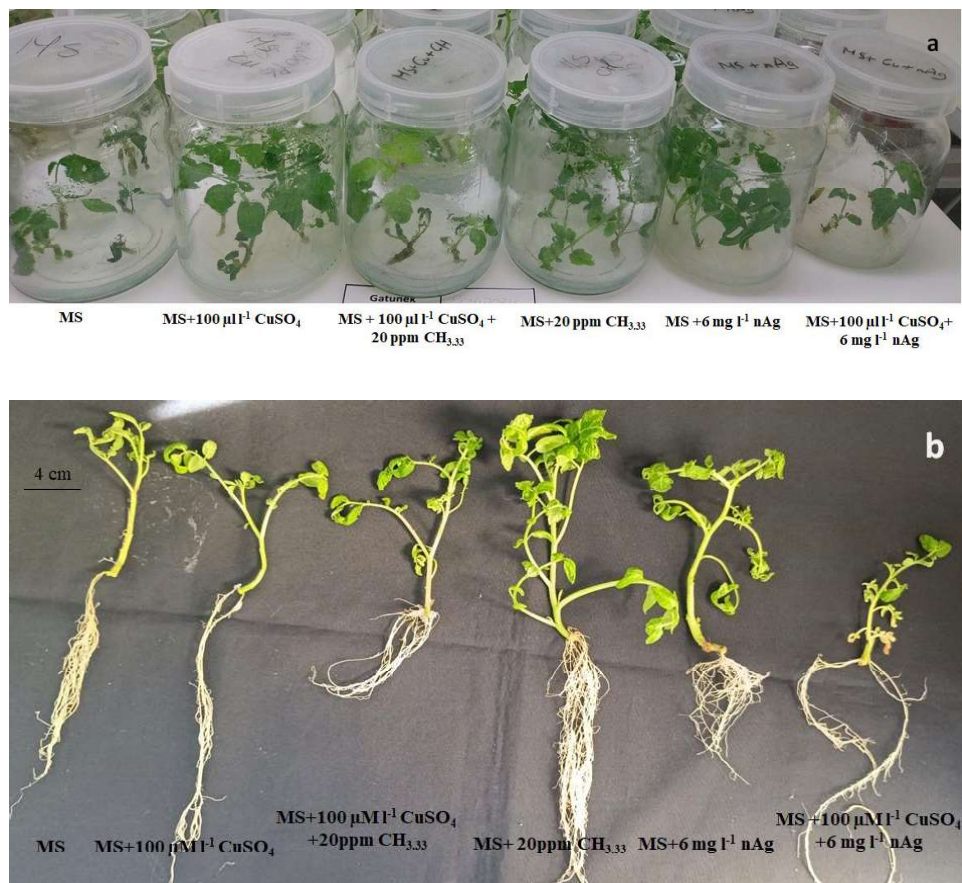


Figure 1. The photographs show the effects of chitosan ($\text{CH}_{3,33}$), nanosilver (nAg) and copper sulfate (CuSO_4) in the Murashige and Skoog (MS) medium on the growth of tomato explants (a) *in vitro* and (b) after 35 days of culture.

The results are consistent with the study by Ernst et al. [22], who observed that the roots of higher plants have some barrier to prevent the translocation of heavy metals to the shoot apices. Hence, there is likely a tolerance mechanism that acts in the root cells. Rostami and Shahsavari [23] demonstrated the impact of nAg on the morphological traits of the explants. Their findings revealed that the addition of 6.0 mg l⁻¹ nAg in MS medium reduced the growth of *Olea europea* L. plants. Krupa-Małkiewicz et al. [8] showed that the barley cultivar Eunova seedlings with the longest roots grew on MS medium containing 4 or 8 mg l⁻¹ nAg.

The positive effect of chitosan in alleviating environmental stress in plants has been described by Pongprayonn et al. [24] in rice, Jabeen and Ahmad [25] in safflower and sunflower and Krupa-Małkiewicz and Smolik [26] in *Petunia × atkinsiana* D. Don under salinity stress. The survival of plants growing under environmental stress conditions depends on its effect on biochemical transformation processes. This process depends on the degree of accumulation of inorganic ions and/or organic compounds [26]. According to Jabeen and Ahmad [25] and Krupa-Małkiewicz and Smolik [26], one of the first symptoms of the effect of stress on plants is an increase in the proline, MDA and TP contents in plant tissue. In the present study, compared with the control, the proline, MDA and TP contents in tomato plants increased by 87%, 82% and 84%, respectively, after application of 100 μM l⁻¹ CuSO₄ (Table 2). The addition of 6 mg l⁻¹ nAg to MS also increased the proline (39% of control), MDA (32% of control) and the TP (12% of control) contents.

Table 2. The effects of chitosan, nanosilver (nAg) and heavy metal treatment on the proline, malondialdehyde (MDA) and total polyphenol contents in *Solanum pimpinellifolium* (L0566) leaves.

Medium	Proline [μmol g ⁻¹]	MDA [nmol g ⁻¹]	Total polyphenol [GAE mg g ⁻¹]
MS (control)	6.1 ^a	22.4 ^a	7.4 ^a
MS+ 20ppm CH _{3,33}	6.3 ^a	21.0 ^a	7.0 ^a
MS+6 mg l ⁻¹ nAg	8.5 ^b	29.5 ^b	8.3 ^b
MS+100 μM l ⁻¹ CuSO ₄	11.40 ^d	40.7 ^d	13.6 ^c
MS+100 μM l ⁻¹ CuSO ₄ + 20 ppm CH _{3,33}	9.0 ^{bc}	32.2 ^{bc}	11.2 ^d
MS+100 μM l ⁻¹ CuSO ₄ + 6 mg l ⁻¹ nAg	9.6 ^c	35.8 ^c	9.4 ^c

Note. In each column, means followed by a different superscript letter differ significantly according to Tukey's test ($p < 0.05$). Abbreviations: CH_{3,33}, chitosan with a molecular weight of 3.33 kDa; CuSO₄, copper sulfate; MS, Murashige and Skoog.

While the addition of 20 ppm CH_{3,33} or 6 mg l⁻¹ nAg alleviated the negative effect of CuSO₄, the former induced the most pronounced decrease in the proline and MDA contents (by 21% and 21%, respectively, compared with the plants treated with 100 μM l⁻¹ CuSO₄). In contrast, the addition of 6 mg l⁻¹ nAg reduced the TP content by 31%, more than the 18% reduction due to the addition of 20 ppm CH_{3,33} compared with the plants treated with 100 μM l⁻¹ CuSO₄. The findings align with those of Mahdavi and Rahimi [14]

and Krupa-Malkiewicz et al. [27], who validated the role of chitosan in regulating plant responses to various abiotic stresses. Additionally, Jabeen and Ahmad [25] highlighted chitosan's efficacy as a biostimulant in mitigating severe stress by reducing enzyme activity through ROS scavenging. Conversely, Sumalia et al. [7] suggested the utility of nanocomposites as agents to remove toxic substances.

The mineral content of plant tissues plays a crucial role in the process of proper growth and development of plants [8]. In the present study, CH_{3,33}, nAg and CuSO₄ had distinct effects on the mineral content of tomato leaves (Table 3). The addition of 100 μM l⁻¹ CuSO₄ to the MS medium decreased most of the minerals compared with the control. The addition of CH_{3,33} or nAg to the MS medium had a minimal effect on the levels of these elements. On the other hand, the addition of CH or nAg to the MS medium containing CuSO₄ had a positive influence on the mineral content of tomato leaves. The results are consistent with what Kahromi and Khara [20, 21] reported, namely that chitosan had a positive effect on the total content of most macronutrients in *Dracocephalum kotschyi*. In contrast, Krupa-Malkiewicz et al. [8] reported that the addition of 6 mg l⁻¹ nAg to the MS medium increased the content of most of minerals (N, Mg, Zn, Cu and P) in the leaves of spring barley.

Table 3. The influence of chitosan, nanosilver (nAg) and heavy metal (CuSO₄) treatment on the mineral content of *Solanum pimpinellifolium* (L0566) leaves.

	MS (control)	MS + 100 μM l ⁻¹ CuSO ₄	MS + 20 ppm CH _{3,33}	MS + 6 mg l ⁻¹ nAg	MS + 100 μM l ⁻¹ CuSO ₄ + 20 ppm CH _{3,33}	MS + 100 μM l ⁻¹ CuSO ₄ + 6 mg l ⁻¹ nAg
[g 100 g⁻¹]						
P	0.52 ^{bc}	0.44 ^a	0.57 ^d	0.50 ^b	0.55 ^{cd}	0.42 ^a
K	4.11 ^c	4.37 ^e	3.88 ^a	4.05 ^{bc}	3.92 ^{ab}	4.20 ^d
Ca	3.87 ^c	3.36 ^a	4.12 ^e	3.95 ^{cd}	4.04 ^{de}	3.59 ^b
Mg	0.46 ^{bc}	0.51 ^d	0.41 ^a	0.48 ^{bc}	0.45 ^b	0.49 ^{cd}
[mg 1000 g⁻¹]						
Fe	242 ^{cd}	144 ^a	255 ^d	206 ^b	230 ^c	158 ^a
Zn	27.4 ^c	35.5 ^e	22.6 ^a	25.0 ^b	24.8 ^b	30.0 ^d
Mn	26.5 ^e	12.4 ^a	28.9 ^f	18.3 ^c	22.5 ^d	14.7 ^b
Cu	5.03 ^a	11.19 ^e	5.27 ^a	7.84 ^c	6.18 ^b	9.42 ^d

Note. In each column, means followed by a different superscript letter differ significantly according to Tukey's test ($p < 0.05$). Abbreviations: CH_{3,33}, chitosan with a molecular weight of 3.33 kDa; CuSO₄, copper sulfate; MS, Murashige and Skoog.

A cluster analysis using Ward's method facilitated the identification of three distinct groups of media with comparable impacts on the micro- and macroelements present in tomato leaves (Figure 2). Group (a) – MS; MS + 20 ppm CH_{3,33}; and MS + 100 μM l⁻¹ CuSO₄

+ 20 ppm $\text{CH}_{3,33}$ – resulted in the highest mineral content. Group (c) – MS + $100 \mu\text{M l}^{-1}$ CuSO_4 and MS + $100 \mu\text{M l}^{-1}$ CuSO_4 + 6 mg l^{-1} nAg – led to the lowest mineral content. Finally, group (b) – MS + 6 mg l^{-1} nAg – led to an intermediate mineral content.

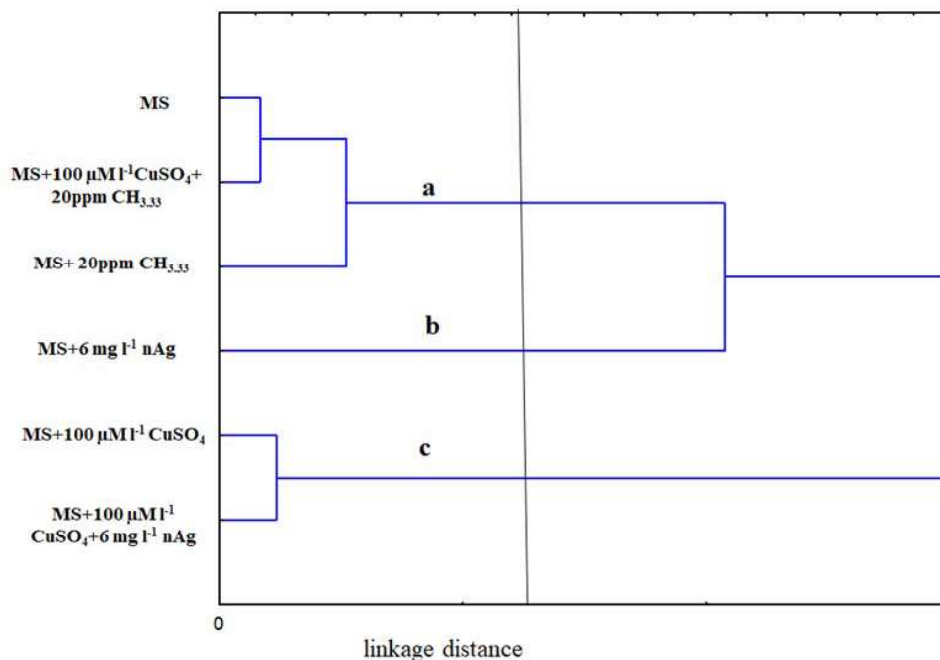


Figure 2. Dendrogram of cluster analysis of the micro- and microelements in *Solanum pimpinellifolium* (L0566) leaves grown on different medium *in vitro*. Abbreviations: $\text{CH}_{3,33}$, chitosan with a molecular weight of 3.33 kDa; CuSO_4 , copper sulfate; MS, Murashige and Skoog; nAg, nanosilver.

The condition of a plant can be assessed by measuring the colour of its leaves [10, 28, 29]. In the present study, the control tomato leaves were the brightest, as determined by L^* . The addition of CuSO_4 and $\text{CH}_{3,33}$ to the MS medium significantly decreased L^* (by 15%) compared with the control (Figure 3a). Otherwise, there were no significant differences between the treatment groups regarding brightness. The leaf surface colour defined by a^* indicates the location along the axis between green and red. The explants grown on MS medium alone or MS medium containing $\text{CH}_{3,33}$ had greener leaves (Figure 3b). In contrast, the explants grown on MS medium containing nAg and CuSO_4 showed a significant decrease in a^* (56% of control). There was a similar relationship for b^* , which indicates the blue/yellow colour. The leaves from the control explants and explants grown in MS medium containing $\text{CH}_{3,33}$ had the highest b^* ; they were more yellow compared with the other treatments (Figure 3b). The leaves from the explants grown in MS medium containing CuSO_4 and nAg had the lowest b^* (24% of control) and were bluer in colour. This change is probably due to a stress response, metal ion interaction or oxidative stress induced by nAg and Cu. These factors may interfere with the synthesis of assimilatory pigments, leading to the observed colour change [8].

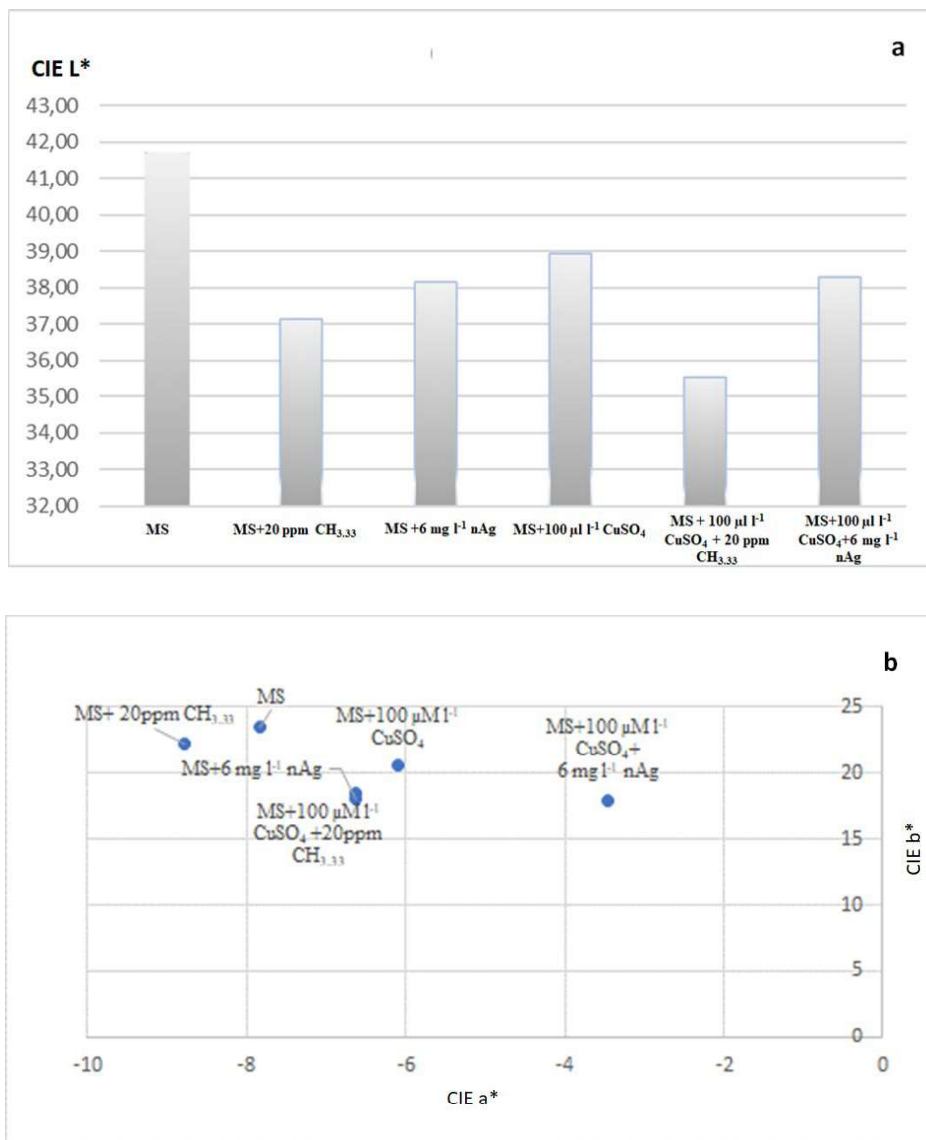


Figure 3. The effects of the medium compositions on the colour of *Solanum pimpinellifolium* (L0566) leaves (n = 32 shoots per treatment), based on the CIE L*a*b* system – (a) L*, the lightness coefficient and (b) a* (green colour) and b* (yellow colour) – at the end of the experiment. Abbreviations: CH_{3,33}, chitosan with a molecular weight of 3.33 kDa; CuSO₄, copper sulfate; MS, Murashige and Skoog; nAg, nanosilver

4. Conclusions

The results highlight the complex interactions between heavy metals, nAg and chitosan on tomato plant growth, physiology and the mineral content, and provide valuable information for further research into optimising tomato plant growth and stress response.

The addition of chitosan to MS medium alleviated some of the harmful effects of heavy metal stress on tomato explants *in vitro* by enhancing their antioxidant defence systems. Specifically, chitosan significantly increased the fresh mass and decreased the proline and MDA contents, indicating a potential reduction in stress responses in tomato plants. Additionally, chitosan improved plant growth and health by stimulating the production of growth-promoting hormones and enhancing nutrient uptake. The effects of nAg appear to be more nuanced compared with chitosan. The addition of nAg to the growth medium did not seem to have a significant impact on shoot growth or fresh mass production, but it did influence root development in tomato plants. Furthermore, the addition of nAg increased the proline, MDA and TP contents, although to a lesser extent than CuSO₄. This suggests that nAg may induce some level of a stress response in tomato plants.

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