Ornamental Horticulture
ISSN 2447-536X | HTTPS://ORNAMENTALHORTICULTURE.EMNUVENS.COM.BR/RBHO
SBFPO Sociedade Brasileira de Floricultura e Plantas Ornamentais

SCIENTIFIC ARTICLE

# Silver nanoparticles eliminate hyperhydricity in micropropagated Lavender

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**Abstract**

It is challenging to plant lavender outside of suitable conditions. This economically important plant requires optimal conditions to germinate and produce a high yield. To increase the quality of this plant, tissue culture is utilized. The most problematic aspect of lavender micropropagation is that cytokinin (CK) causes hyperhydricity (HH) during the shooting process. Consequently, this study was conducted to resolve HH in micropropagated lavender plantlets. Different concentrations of silver nanoparticles (AgNPs) were applied in conjunction with 1.0 mg $L^{-1}$ 6-benzylaminopurine (BA). Then, the performance of HH, growth and development, as well as total phenolic (TPC) and total flavonoid (TFC) content, were evaluated. The application of 20 mg $L^{-1}$ of AgNPs was found to be the optimal method for halting HH. Although shoot proliferation was lower than in BA-supplemented media-grown plants, adding this concentration of AgNPs improved shoot and root quality. An increase in secondary metabolites and antioxidant activity may have caused the remedy of HH.

**Keywords:** AgNPs, *Lavandula angustifolia* Mill., tissue culture, vitrification.

**Resumo**

**Nanoparticulas de prata eliminam hiperhidricidade em Lavanda micropropagada**

É um grande desafio o cultivo de lavanda fora das condições adequadas. Essa planta, de grande importância econômica, requer condições ótimas para germinar e produzir rendimentos elevados. Sendo o uso da técnica de cultura de tecidos vegetais uma possibilidade de melhorar a qualidade da planta. No entanto, um dos principais problemas da micropropagação da lavanda é a ocorrência de hiperhidricidade (HH) devido à presença da citocinina (CK) durante o processo de cultivo *in vitro*. Dessa forma, objetivou-se realizar um estudo para determinar soluções para a HH em plântulas de lavanda micropropagadas. Diferentes concentrações de nanopartículas de prata (AgNPs) associados a 1,0 mg de $L^{-1}$ de 6-benzilaminopurina (BA) foram aplicadas e avaliou-se o desempenho da HH, o crescimento e o desenvolvimento, bem como o teor de fenólicos totais (TPC) e flavonóides totais (TFC). A aplicação de 20 mg $L^{-1}$ de AgNPs foi considerada a concentração ideal para interromper a ocorrência de HH. Embora a proliferação de brotos tenha sido inferior àquela observada em plantas cultivadas em meio suplementado com BA, a adição dessa concentração de AgNPs melhorou a qualidade dos brotos e das raízes. Acredita-se que o aumento de metabólitos secundários e da atividade antioxidante possa ter contribuído para a resolução da HH.

**Palavras-chave**: AgNPs, cultura de tecidos, *Lavandula angustifolia* Mill., vitrificação

## Introduction

True lavender, also known as *Lavandula angustifolia* Mill., is a valuable economic plant native to the Mediterranean region. This plant is widely employed in culinary, fragrance, cosmetic, and medicinal applications (Sharma et al., 2020). It contains active secondary metabolites such as α-pinene, borneol, camphene, camphor, linalool, and linalyl acetate 1,8-cineole, which have antioxidant, antibacterial, anti-inflammatory, wound healing, and anticancer, properties (Rani and Sharma, 2022). The extract could be derived from essential oils or organic material. Additionally, lavender is widely used as an ornamental plant. Nevertheless, environmental factors can make growing lavender difficult and time-consuming. To germinate, the seed requires a dormancy break, which affects the quality and quantity of the plant (Caser et al., 2022).

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*https://doi.org/10.1590/2447-536X.v29i3.2565*
*Received Nov 1st, 2022 | Accepted July 12, 2023 | Available online Aug 15, 2023*

*Editor: Fernanda Carlota Nery*

The tissue culture technique is implemented to solve the production problem for lavender propagation. Various plant materials, including seed, seedlings, and explants, can be utilized to complete the procedure in this plant (Li et al., 2019). Each of the four steps of lavender micropropagation-germination, multiplication, rooting, and acclimatization requires a distinct *in vitro* media composition. Murashige and Skoog (MS) media containing multiple plant growth regulators is commonly utilized (Koefender et al., 2021). Gibberellic acid ($GA_3$) can stimulate germination (Szekely-Varga et al., 2021). Consequently, CK can optimize multiplication. During the rooting process, plant hormones are not required. To help the plants get used to their new environment, they are moved to a greenhouse with the right substrate (Li et al., 2019; Koefender et al., 2021).

Although CK improves shooting, it increases hyperhydricity (HH) in *in vitro* lavender plantlets during multiplication (Koefender et al., 2021). The translucent plant, HH, poses a significant problem in plant micropropagation. The underlying mechanism of this issue remains unclear. HH can be triggered by internal factors such as hormonal levels (Gao et al., 2022) or external factors like growth and media conditions (Vlachou et al., 2019). The cause of this disorder's phenotype has been the subject of multiple studies (Kemat et al., 2021; Gao et al., 2022). HH inducements consist of abnormalities in water uptake and osmotic stress, deprivation of cell wall lignification (Kemat et al., 2021), excessive ethylene content, elevated reactive oxygen species (ROS) levels and oxidative stress (Gao et al., 2022).

Reducing plantlet water absorption appears to be the most cost-effective and straightforward method for combating HH. Numerous previous studies have confirmed the efficacy of water absorption adjustment in a variety of plants, including adjusting the gelling composition and venting the culture in *Cycladenia humilis* Benth. (Pence et al., 2020), increasing agar concentration in *Salvia santolinifolia* Boiss. culture media (Jan et al., 2021), and adding sodium chloride to *Agave sisalana* Perr. culture media (Nikam et al., 2019).

Recently, AgNPs have been utilized to reduce plant water uptake. It is involved in stomatal conductance in *Triticum aestivum* L. (Petruș-Vancea, 2018). In *Lactuca sativa* L., it can dose-dependently reduce transpiration rate and stomatal conductance without causing toxic effects (Torrent et al., 2020). Additionally, AgNPs induce cell wall thickening in *Pisium sativum* L., which is associated with decreased water absorption (Tripathi and Pandey-Rai, 2021). Depending on the species, AgNPs have various effects on plants. Therefore, AgNPs have beneficial effects on lavender when utilized at the suitable concentration. In addition to increasing polyphenol content and antioxidant properties, they stimulate shoot multiplication and growth (Jadczak et al., 2019).

This study demonstrates the HH-related effects of AgNPs on lavender based on their ability to reduce water uptake in several plants and their positive effects on lavender's antioxidants, growth, and development. This research will contribute new information to the remedy of the HH-induced physiological disorder.

## Material and Methods

### Plant Materials and Growth Conditions

*L. angustifolia* Mill. seeds (Mr Fothergill's, The United Kingdom) were soaked in a 50 mL commercial sodium hypochlorite (NaOCl) solution (The Clorox Company, The United States of America). The 50 mL solution contained 15% (v $v^{-1}$) NaOCl solution, two drops of Tween20, and one drop of hydrogen peroxide ($H_2O_2$). The solution was mixed thoroughly for 20 min. The seeds were then transferred to a 10% (v $v^{-1}$) NaOCl solution and shaken for an additional 20 min in a laminar airflow chamber. The seeds were then washed three times with distilled water separated by one minute. On a sterile solid Murashige and Skoog (MS) medium containing 3% sucrose (w/v), decontaminated seeds were placed. The growth conditions were 25 °C, a 16 hour photoperiod, and 0.67 µmol $s^{-1}$ $m^{-2}$light integral from 40 watt fluorescent light bulbs (Philips, The Netherlands). In the step of shoot multiplication, identical seedlings were chosen for the study four weeks after germination.

### Treatments with silver nanoparticles

This study included six treatments: MS medium (Sigma-Aldrich, USA) without plant growth regulator (MS), MS medium supplemented with 1,0 mg $L^{-1}$ BA (Sigma-Aldrich, USA), MS medium supplemented with 1,0 mg $L^{-1}$ BA and 20, 40, 60, and 80 mg $L^{-1}$ AgNPs (Sigma-Aldrich, USA; 20 Ag, 40 Ag, 60 Ag, and 80 Ag, respectively). The average diameter (TEM) of AgNPs was 20±4 nm based on the manufacturer's specifications. There were seven replications per treatment, with one seedling transferred to each culture bottle. The growth condition was identical to the previous point. After four weeks of cultivation, the percentage of HH and necrosis, the longest shoot and root length, the number of shoots and roots, and the number of leaves per shoot were investigated.

### The determination of total chlorophyll content

The obtained plantlets were thoroughly washed with distilled water. Following this, 10 ml of acetone was added to 500 mg of the dissected material. The mixtures were incubated in the dark at room temperature for 12 h before being centrifuged at 10,000 rpm for 5 min. The supernatants were collected and stored at -20 °C until needed. After that, the spectrophotometric method (Lichtenthaler and Buschmann, 2001) was used to figure out how much total chlorophyll was in the extracts.

### Identification of total phenolic and flavonoid content

For extraction, plantlets were thoroughly washed with distilled water and dried in a hot air oven at 50 °C for two days. With a mortar and pestle, the dried plantlets were ground. A hundred milligram sample was combined with 10 ml of methanol at a concentration of 80% (v $v^{-1}$). The extracts were shaken repeatedly at room temperature for 12 h, followed by 10 min of sonication and 10 min of centrifugation at 8,000 rpm. The supernatants were collected and stored at 4 °C until use. The Folin-Ciocalteu (FC) method (Chaiyana et al., 2017) and the aluminum

chloride colorimetric assay (Do et al., 2014) were used, respectively, for the determination of total phenolic (TPC) and flavonoid content (TFC). Ascorbic acid was chosen as the standard for identification purposes.

**Evaluation of antioxidant activities**

The same batch of extracts was used for antioxidant, TPC, and TFC assays. The antioxidant activity was determined using the ferric reducing antioxidant power (FRAP) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assays (Chaiyana et al., 2017). Ascorbic acid serves as the standard for both analyses.

**Statistical analysis**

All experiments were carried out with a completely random design. Seven replications were conducted per treatment. Three replicates were performed for the evaluation of antioxidant activity. The one-way ANOVA and the Tukey test (Assaad et al., 2014) were used to find out if there was a statistically significant difference between the mean values of each experiment.

## Results

**Effects of AgNPs on hyperhydricity, growth, and development**

AgNPs influenced the lavender plantlet's HH, growth, and development (Figure 1). The variation in plant characteristics between treatments demonstrated this. MS was the only treatment where stem branching was not observed (Figure 1a). The BA treatment clearly illustrated the greatest stem multiplication and elongation (Figure 1b), in comparison to the other treatments. Additionally, the translucent stem was unique to BA. Regarding stem length, 80 Ag appeared to be the shortest (Figure 1f). Likewise, leaf characteristics varied between treatments.

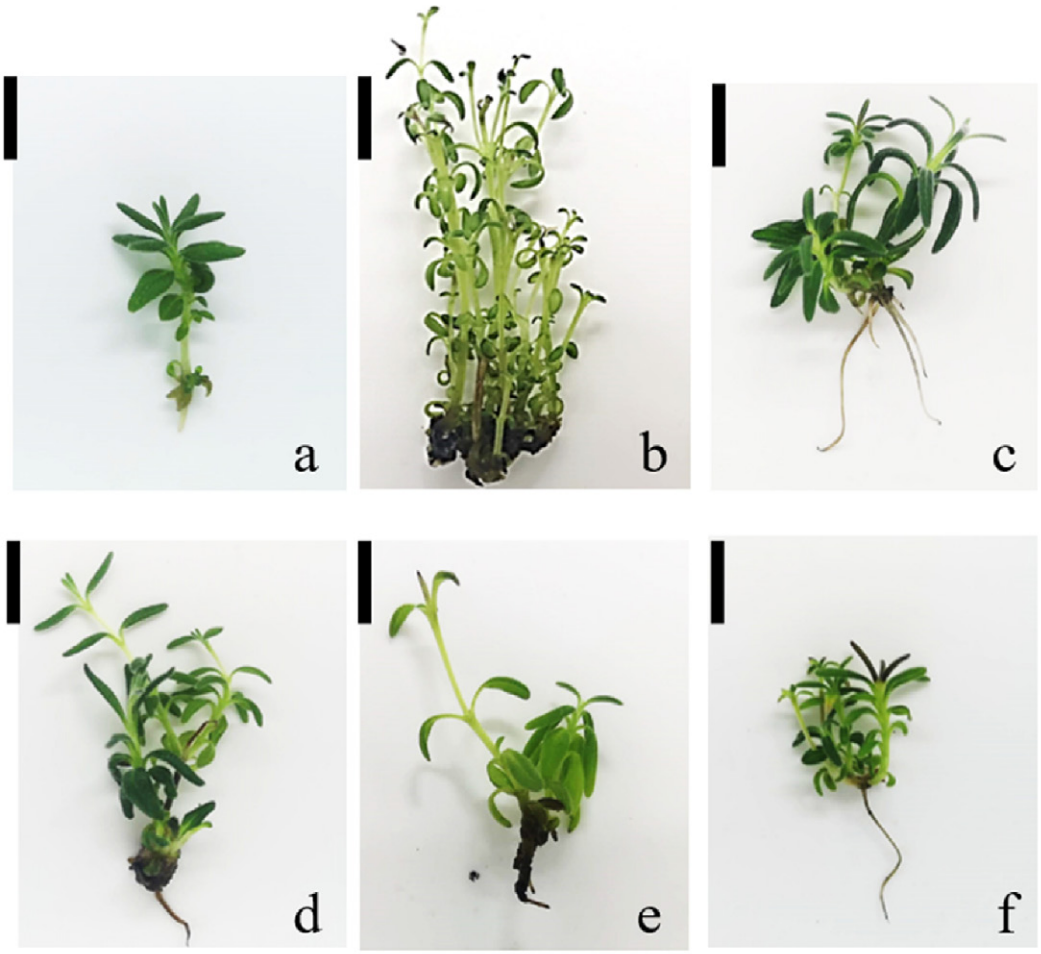


**Figure 1.** Variation in plant characteristics among treatments with AgNPs after four weeks of culture. MS (a), BA (b), 20 Ag (c), 40 Ag (d), 60 Ag (e), and 80 Ag (f) were grown with a 16-hour photoperiod at a temperature of 25 °C. The black line indicates a 1 cm scale.

The statistical differences between each treatment are presented (Table 1). MS medium alone produced normal plants devoid of HH, whereas the addition of BA strongly induced this abnormal physiology in plants. However, the HH could be fully recovered through the application of AgNPs to the medium. In addition to plant HH, growth and development were also altered. The percentage of necrosis (leaf burn) reveals the quality of the leaf. The highest levels of necrosis were found in BA (22.2% ± 0.718%) and 80 Ag (21.9% ± 0.355%). The percentages of 60 Ag and 40 Ag were the second and third, respectively. The MS and 20 Ag showed the lowest percentage of necrosis. The total chlorophyll content was also one of the indicators of plant health. MS and 20 Ag exhibited the greatest amount, followed by 40 Ag and 80 Ag. Simultaneously, the chlorophyll content of 60 Ag did not differ significantly from the highest and second highest levels. BA had the lowest chlorophyll content.

**Table 1** The effect of AgNPs on hyperhydricity, necrosis, and total chlorophyll content in micropropagated lavender.

| Name | Treatment (mg $L^{-1}$) | | % Hyperhydricity shoot (HS) | % Necrosis | Total chlorophyll (µg $g^{-1}$ FW) |
|---|---|---|---|---|---|
| | BA | AgNPs | | | |
| MS | - | - | $0 \pm 0^{b}$ | $0 \pm 0^{d}$ | $23.2 \pm 0.48^{a}$ |
| BA | 1 | - | $99.6 \pm 0.29^{a}$ | $22.2 \pm 0.72^{a}$ | $9.66 \pm 0.37^{d}$ |
| 20 Ag | 1 | 20 | $0 \pm 0^{b}$ | $0 \pm 0^{d}$ | $24.4 \pm 0.35^{a}$ |
| 40 Ag | 1 | 40 | $0 \pm 0^{b}$ | $11.7 \pm 0.12^{c}$ | $19.4 \pm 0.48^{b}$ |
| 60 Ag | 1 | 60 | $0 \pm 0^{b}$ | $14.2 \pm 0.1^{b}$ | $17.8 \pm 0.26^{bc}$ |
| 80 Ag | 1 | 80 | $0 \pm 0^{b}$ | $21.9 \pm 0.35^{a}$ | $17 \pm 0.32^{c}$ |

Values are means ± SEM, n = 7 per treatment group. Means in a row without a common superscript letter differ ($P<0.05$) as analyzed by one-way ANOVA and the TUKEY test.

The BA treatment resulted in the highest number of shoots (7 ± 0.488). The 20 Ag and 40 Ag provided the second highest quantity of shoots, followed by the 60 Ag and 80 Ag (Figure 2a). Plants grown in MS medium without supplementation only produced a single shoot, indicating that branching did not occur. The leaf count per stem was intriguing (Figure 2c) and the 20 Ag resulted in the highest value (19.5 ± 0.265). The BA and 20 Ag did not differ significantly and had the second highest number of leaves. In addition, the MS did not differ statistically between the first and second leaf number treatments. The 60 Ag and 80 Ag results were the lowest.

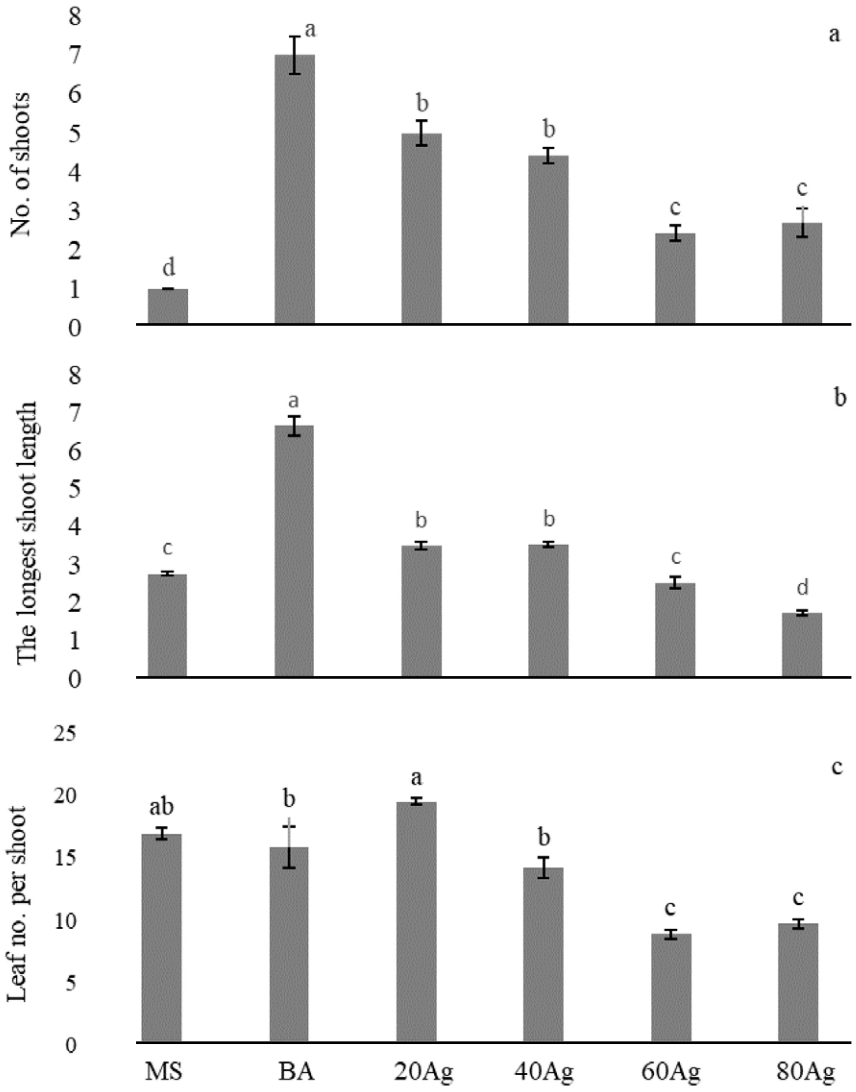


**Figure 2.** The effects of AgNPs on the growth and development of shoots after four weeks of cultivation. The number of shoots (a), the length (cm) of the longest shoot (b), and the number of leaves per shoot (c) are provided. The error bars represent the standard error of the mean calculated from seven replicates at each data point. Different superscript letters on each bar reflect the statistical difference between treatments ($P<0.05$).

In the 20 Ag treatment, the greatest number of roots (5.29 ± 0.286) were observed. The 60 Ag treatment yielded the second-highest root number, followed by the 40 Ag, 60 Ag, and MS (Figure 3a). Interestingly, no root was presented in BA that produced the biggest number of shoots (Figures 2a and 3a). The length of the shoot and root represented their quality. Despite the absence of root development, BA had the longest shoot (6.66 ± 0.256 cm). The second and third longest shoots were presented in 20 Ag and 40 Ag, and MS and 60 Ag, respectively. In 80 Ag, the shortest shoot appeared (Figure 2b). The longest roots were produced by 20 Ag and 80 Ag, followed by 40 Ag and 60 Ag, MS, and BA, in that order (Figure 3b).

**AgNPs stimulate antioxidant activity in a dose-dependent manner.**

By treating lavender with AgNPs, the TPC and TFC were increased (Figure 4). Both phenolic and flavonoid amounts were presented in the same manner. The highest value was obtained by 20 Ag followed by 40 Ag, 60 Ag, MS, 80 Ag, and BA, respectively.

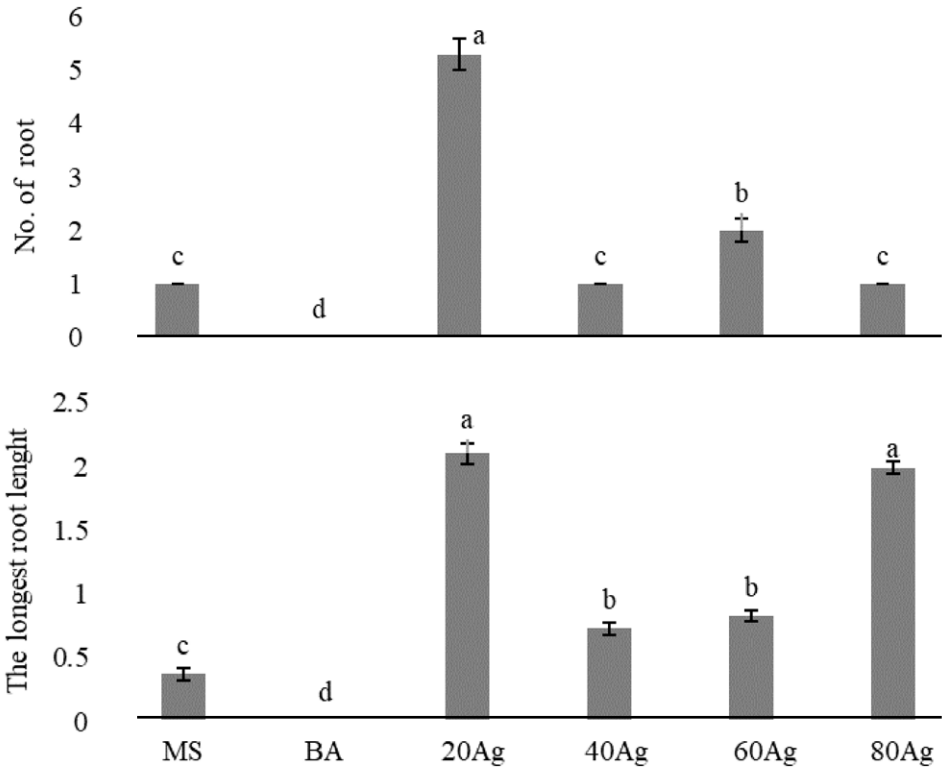


**Figure 3.** The effects of AgNPs on the growth and development of roots after four weeks of cultivation. The number of roots (a) and the length (cm) of the longest root (b) are provided. The error bars represent the standard error of the mean calculated from seven replicates at each data point. Different superscript letters on each bar reflect the statistical difference between treatments (P<0.05).

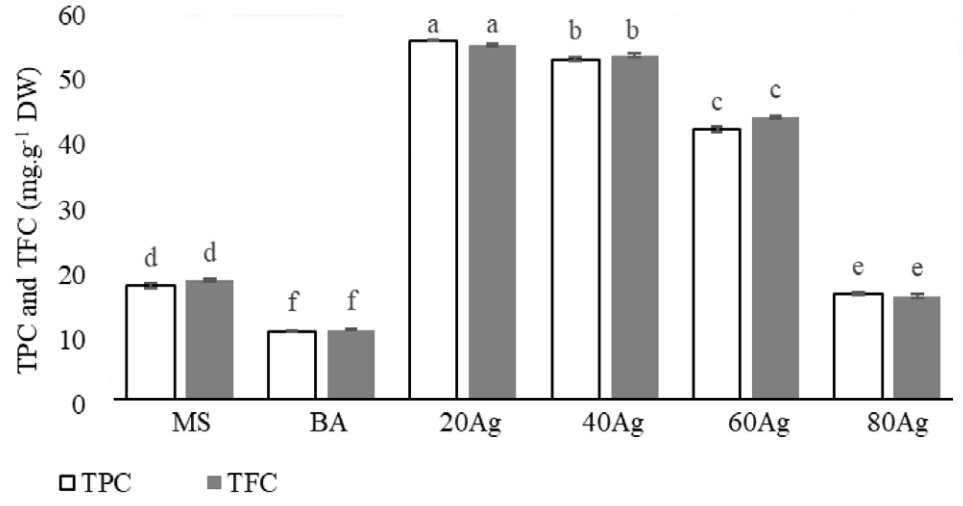


**Figure 4.** Total phenolic (TPC) and flavonoid (TFC) content after four weeks of culture for each treatment. The error bars show the standard error of the mean based on seven replicates for each data point. Different superscript letters on each bar (different colours for independent analyses) represent the statistical difference between treatments ($p < 0.05$).

The antioxidant property generated slightly different results in two separate assessments. Antioxidant activity as measured by the DPPH assay followed the same pattern for TPC and TFC (Figure 5a). The 20 Ag treatment exhibited the highest percentage of inhibition activity (53.1 ± 0.404), followed by 40 Ag (50.8 ± 0.293), 60 Ag (44.5 ± 0.379), MS (42.8 ± 0.0922), 80 Ag (32.6 ± 0.231), and BA (32.6 ± 0.153), respectively. Despite the minor variation in the FRAP assay, Still, 20 Ag and BA had the highest and lowest antioxidant values, respectively (Figure 5b). The pattern of reducing power from highest to lowest value began with 20 Ag (72.1 ± 0.17) and continued with 40 Ag (70.9 ± 0.22). There was no statistical difference between 60 Ag (66.1 ± 0.419) and MS (65.1 ± 0.138) for third place. The value from 80 Ag (61.3 ± 0.284) followed, and BA (58 ± 0.158) was the last position.

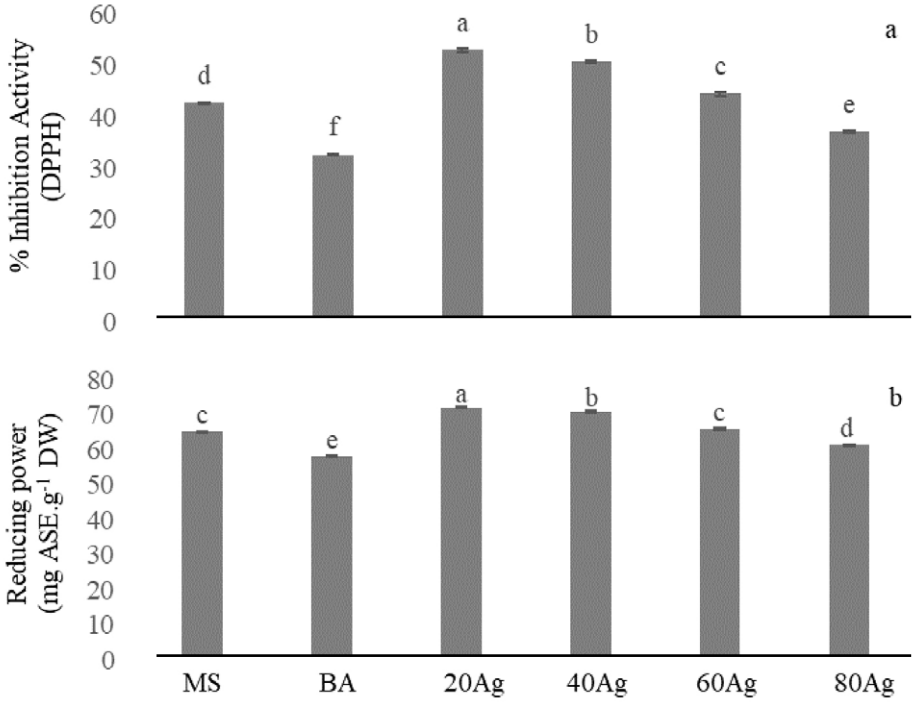


**Figure 5.** The effect of AgNPs on the antioxidant properties of lavender plantlets. The results of the DPPH (a) and FRAP (b) antioxidant assays are shown. The error bars illustrate the standard error of the mean for each data point based on seven replicates. Different superscript letters show the statistical difference between treatments ($P<0.05$) for each bar.

## Discussion

Based on the results of HH, growth, and development, BA-treated plants could only benefit the shoots. In addition to the highest level of HH, deficiencies in root development, leaf quality, and chlorophyll also occurred. Although the number and length of shoots were the greatest, the quality of the plants was inadequate. CK stimulates cell division, it is widely employed in the plant tissue culture industry for the induction of shoot growth (Foo et al., 2018). CK application, however, can induces HH in plants (Ivanova and Van Staden, 2011).CK is also found to inhibit root development. It has the opposite effect of melatonin, the root stimulant (Wang et al., 2022). Also, CK can break epicotyl dormancy (root development provider) (Zhang et al., 2022). In addition, CK has an antagonistic effect on auxin at specific concentrations, which inhibits root growth (Kurepa et al., 2019). Therefore, adding BA to lavender growing medium will undoubtedly inhibit root development. Although BA had an acceptable number of leaves per shoot, it had the lowest quality leaves compared to other plants. It contained the highest level of necrosis and the lowest level of chlorophyll. The mechanism of plant necrosis remains unknown. A possible cause could be a mineral imbalance (Silva et al., 2020). Consequently, the necrosis in BA-treated lavender may have resulted from a lack of roots, which may have affected mineral absorption. Interestingly, BA can decrease or increase necrosis depending on the type of plant (Silva et al., 2020). In this study, the necrosis may be related to HH because resolving the HH issue can also reduce necrosis in lavender, as also reported by Machado et al. (2014).

HH is related to chlorophyll content in addition to leaf damage because it stimulates chloroplast degradation (Petruş-Vancea, 2018). Therefore, the BA-induced hyperhydric lavender contains the least chlorophyll. AgNPs could alleviate the negative symptoms caused by BA. Clearly, with HH, any treatment containing AgNPs could mitigate this symptom. The remedy yields the same result as previous research on plants such as *Stevia rebaudiana*

Bert. (Salem, 2020), *Gerbera jamesonii* L. (Tung et al., 2022), and *Dianthus chinensis* L. (Sreelekshmi et al., 2022). This may be due to AgNPs' osmotic balancing, ethylene inhibition, and mineral retention properties in plants.

In addition to HH reversion, AgNPs and CK facilitated the growth and development of the shoot and root. Although the number of shoots in AgNPs-added samples was lower than in BA-treated sample alone, it was still greater than in MS media. It is suggested that AgNPs together with BA can also promote the proliferation of shoots in a dose-dependent manner. Even though the shoot proliferation is lower than BA, the plant's quality in terms of leaf and root characteristics is better. Interestingly, the beneficial effect of AgNPs on lavender's growth and development is also discovered in a previous study. The culture media containing AgNPs enhanced shoot and root development and increased plant biomass (Jadczak et al., 2019). This similar result is also presented in an additional study involving *Solanum lycopersicum* L. (Guzmán-Báez et al., 2021). This response is a consequence of AgNPs growth-promoting properties. In addition, the increase in total chlorophyll content in lavender caused by AgNPs is consistent with research conducted on *Chrysanthemum grandiflorum* Ramat. (Tymoszuk and Kulus, 2020), *Zea mays* L. (Sehnal et al., 2020), and *Oryza sativa* L. (Gupta et al., 2018). The result can be explained by AgNPs' ability to increase chlorophyll activity.

All positive outcomes from AgNPs were presented in a dose-dependent manner. The lowest concentration of AgNPs used (20 mg $L^{-1}$) provided the best performance, while increasing the concentration diminished the effects. The reason for this is that excessive silver concentrations are toxic to plants (Kaveh et al., 2013).

Phenolic and flavonoid content of each treatment was correlated with its antioxidant capacity. Intriguingly, these findings also demonstrated a dose-dependent relationship similar to that found in growth and development. Compared to MS alone, adding BA decreased TPC and TFC production, which may be one of the reasons why HH developed. Although current research regarding the association between TPC, TFC, and HH in plants is still unclear, a previous study on *Scutellaria* species showed that TFC and HH levels were antagonistic (Tascan et al., 2010). Consequently, the amount of TPC and TFC in this study may be related to HH recovery. When AgNPs were added to the BA-supplemented medium, the production of TPC and TFC was greatly increased. Accordingly, the antioxidant activity enhanced, and HH was subsequently resolved. Therefore, this study provides results that confirm the effect of AgNPs on lavender's antioxidant properties. AgNPs activated antioxidant enzymes such as ascorbate peroxidase (APX) and superoxide dismutase (SOD) in lavender in a past analysis (Jadczak et al., 2020). Several plants, including *Lactuca sativa* L. (Wu et al., 2020), *Helianthus annuus* L. (Batool et al., 2021), and *Oryza sativa* L. (Sultana et al., 2021), were found to be activated by AgNPs.

The results of this and previous studies can explain the potential mechanism by which AgNPs inhibit HH. It triggers antioxidants to eliminate reactive oxygen species (ROS). Elimination of ROS is advantageous for the cure of HH because an excessive amount of ROS can cause cell damage, leading to abnormal water intake and HH (Gao et al., 2022). In addition, ROS can stimulate ethylene production in plants, and it is well-known that ethylene influences HH (Gao et al., 2022).

## Conclusions

BA can induce shoot growth in true lavender, but it does not promote root development or normal growth. This hormone induces HH strongly, which is detrimental to the plant's survival. Together with BA, AgNPs can be applied to the growing medium to completely resolve this issue. Based on the performance of lavender plantlets in each treatment, 20 mg $L^{-1}$ AgNPs is the optimal concentration for HH recovery. This concentration promotes the greatest growth and development, as well as the total phenolic and flavonoid content, which is associated with an increase in antioxidant activity. Correspondingly, the lowest leaf necrosis is displayed by this treatment. Overall, AgNPs may stimulate antioxidant activity in lavender by scavenging ROS, the HH stimulator. This groundbreaking discovery could enhance the micropropagation of true lavender in the future.

**Acknowledgements**

This work was supported in part by the annual internal research grant from the Faculty of Agricultural production at Maejo University.

**Author Contribution**

PA: carried out the experiment, data analysis, and wrote the manuscript with support from PN and AT PN: helped supervise the project. AT: contributed to the micropropagation and the interpretation of the results. All authors discussed the results and contributed to the final manuscript.

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