

SILVER NITRATE: A CATALYST FOR UNPRECEDENTED IN VITRO DEVELOPMENT OF *SPATHIPHYLLUM WALLISII*, AN IMPORTANT ORNAMENTAL PLANT

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ABSTRACT

The ornamental plant *Spathiphyllum wallisii* is becoming increasingly popular worldwide, and tissue culture is regarded as a practical option for its massive production. Following the multiplication phase and successive subcultures for an extended period, some morphological changes in shoots were observed in *Spathiphyllum* explants, primarily a reduction in leaf area. For the first time, we report the application of silver nitrate to enhance *Spathiphyllum* shoot and root quality. The Murashige and Skoog (MS) medium, with 30 g·L⁻¹ of sucrose and 100 mg·L⁻¹ of ascorbic acid, was supplemented with different concentrations of AgNO₃. The addition of AgNO₃ improved shoot development and leaf expansion by increasing their length and width. Specifically, at the 2.0–4.0 mg·L⁻¹ range, silver nitrate successfully induced the highest leaf area (1.7 cm²), while stem elongation was promoted with the application of 3.0 mg·L⁻¹ of AgNO₃. Additionally, optimal leaf numbers of new shoots were observed at 1 mg·L⁻¹. Furthermore, when used at an optimal concentration, AgNO₃ significantly boosted rhizogenesis, increasing the number (9.2 roots), length (5.8 cm), and diameter (1 mm) of roots per explant. After the plantlets were transferred to pots, they acclimatized successfully with a 100% survival rate. This finding underscores the potential of silver nitrate to optimize the growth and rooting of *Spathiphyllum* shoots, paving the way for a more efficient micropropagation process.

Key words: ethylene inhibitor, leaf area, micropropagation, rhizogenesis, shoot elongation

INTRODUCTION

The Araceae family contains plants with economic importance, comprising about 3600 species and 144 genera (Boyce & Croat 2014). *Spathiphyllum* ranks among the most known ornamental genus of the Araceae family, and it is commonly used for decorating interiors in public and private properties (Henny et al. 2008). It has become prominent in the foliar plant sector because of its attractive deep green leaves, striking white flowers, and tolerance to interior environments (Henny & Chen 2003; Chen et al. 2005). Moreover, *Spathiphyllum* is known for its ability to purify the air of certain gaseous pollutants such as formaldehyde, trichloroethylene, and benzene, which was proven in a NASA study (Wolverton et al. 1989).

Although conventional *Spathiphyllum* propagation may not be commercially viable to meet the increasing demand for planting material (Kumar & Singh 2016), micropropagation can be an alternative. Typically, direct organogenesis from axillary buds is the primary strategy used for mass multiplication of some commercial *Spathiphyllum* species and cultivars (Ramirez-Malagon et al. 2001; Bandyopadhyay et al. 2011; García et al. 2015).

The use of closed containers in the micropropagation technique leads to the accumulation of ethylene due to limited gas exchange between the interior and exterior of the container (Matthys et al. 1995). This accumulated ethylene produced by the explants in tightly sealed vessels hurts plant growth, differentiation, and morphology. Furthermore, it induces some disorders in generated plants, such as excessive

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stem elongation (Cardoso 2019), complete leaf abscission (Mahmoud et al. 2020), senescence of leaves (Sirisom & Te-Chato 2012), and inhibition of new shoots regeneration (Al Ramadan et al. 2021).

Silver nitrate has been demonstrated as an efficient ethylene inhibitor agent, and it is commonly used in micropropagation because of its availability, solubility, stability, and specificity (Kumar et al. 2009). The potential of silver nitrate to inhibit ethylene action was proved in previous studies by its effect on suppressing excessive stem elongation (Cardoso 2019), promoting leaf growth (Giridhar et al. 2003; Tahoori et al. 2018), maximizing shoot production (Drisya Ravi et al. 2019; Kaur & Kumar 2020), increasing in leaf chlorophyll content (Tamimi 2015), and reducing leaf abscission (Drisya Ravi et al. 2019; Mahmoud et al. 2020).

Our investigation revealed some anomalies on *Spathiphyllum* shoots that were subcultured in a medium without plant growth regulators (PGRs) within tightly sealed vessels. Despite numerous studies reporting the beneficial effects of silver nitrate, to our knowledge no existing report or previous studies have investigated the effect of ethylene or ethylene inhibitors on the micropropagation of *S. wallisii*. Therefore, the primary goal of our research is to examine the effect of ethylene and ethylene inhibitors, which will allow obtaining valuable information on their potential impact on the micropropagation of *S. wallisii*. Additionally, we aim to enhance shoot quality before the acclimatization stage by using silver nitrate for the first time on *Spathiphyllum*.

MATERIALS AND METHODS

Establishment of *S. wallisii* shoots under *in vitro* conditions

Shoots were collected from mature, healthy *S. wallisii* 'Bellini' plants in fully flowering period, maintained under greenhouse conditions. The collected shoots, 30 cm high, were washed under running tap water. Leaves and roots were removed, and the shoot tips were washed for 10 min with tap water and dipped in 1% of Tween-20 for 15 min. Under the laminar flow hood, the explants were submerged in 80%

(v/v) ethanol for 1 minute and in 0.2% of mercuric chloride (HgCl_2) for 20 minutes. Finally, the shoot tips were thoroughly rinsed three to four times with sterile distilled water and trimmed to 1 cm.

The disinfected explants were transferred to tubes (2.5×15 cm) containing 16 ml of MS (Murashige & Skoog 1962) with a complete set of macro- and micronutrients and Murashige and Skoog vitamins (Sigma-Aldrich), $30 \text{ g} \cdot \text{L}^{-1}$ of sucrose and $100 \text{ mg} \cdot \text{L}^{-1}$ of ascorbic acid with the addition of $2 \text{ mg} \cdot \text{L}^{-1}$ of BAP (6-benzylaminopurine, Sigma-Aldrich), and $0.5 \text{ mg} \cdot \text{L}^{-1}$ of IAA (indole-3-acetic acid, Fluka Bio-Chemika). The pH of the medium was adjusted to 5.8 with sodium hydroxide and then $2.5 \text{ g} \cdot \text{L}^{-1}$ of Phytigel® (Sigma-Aldrich, Germany) was added as a gelling agent. The glass test tubes containing culture medium were then autoclaved at 121°C and 1 hPa pressure for 20 min. The cultured explants were incubated at $23 \pm 2^\circ\text{C}$ with 16-h light/8-h dark photoperiod using fluorescent lamps with $35\text{--}45 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ photosynthetic photon flux density (PPFD) (Philips, EcoFit LEDtube, China).

Initial multiplication of *S. wallisii*

Explants from the initiation phase were subjected to subsequent multiplication until the desired number of explants was obtained. Multiple shoots were obtained after 3 subcultures (6 weeks each) in the same medium with $2 \text{ mg} \cdot \text{L}^{-1}$ of BAP and $0.5 \text{ mg} \cdot \text{L}^{-1}$ of IAA. During the multiplication phase, the culture conditions were the same as described above.

Testing different concentrations of AgNO_3 to enhance shoot and root quality

In glass jars with tightly sealed metal caps, multiple subcultures were performed after the multiplication phase in a medium without PGR. Altered shoots (shoots exhibiting negative morphological changes, specifically being thinner and having smaller leaves than normal) with 3 cm of height were used as explants to evaluate the effect of AgNO_3 . This experiment was performed with seven concentrations of AgNO_3 (0, 0.5, 1, 2, 3, 4, and $5 \text{ mg} \cdot \text{L}^{-1}$) added to the same culture medium without PGR before autoclaving. After inoculation, the explants in sealed vessels were incubated in the phytochamber at $23 \pm 2^\circ\text{C}$ with a 16-h photoperiod and $35\text{--}45 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PPFD generated by white fluorescent lamps for 2 months.

To evaluate the effect of AgNO_3 on *Spathiphyllum* plantlets, different morphological parameters were selected based on their positive response to AgNO_3 . After 60 days, the height of the plantlets, the number, length, and width of leaves, the number of new shoots, and the number of leaves on newly emerged shoots were measured. To calculate leaf area, we followed the formula $\text{width} \times \text{length} \times 0.75$, as described by Rybiński and Garczyński (2004), in which the length and width measurements were obtained from the longest and widest sections of the leaf, respectively, using a grid count.

After two months, the number, size, and diameter of roots were determined. Root size was measured from root base to tip, and root diameter was measured using standard measuring paper.

The experiment was organized in a randomized complete design (CRD) with three replicates and ten explants. Averages were analyzed using analysis of variance (ANOVA), and means were compared using Duncan's multiple range test (IBM SPSS Statistics ver. 24) at $p \leq 0.05$.

Acclimatization of plantlets

The plantlets were removed from the medium and washed under running tap water before acclimatization. Then, the plantlets were transferred to pots 7 centimeters in diameter, containing a combination of peat moss (Kekkilä Professional) and coconut fiber (COFLOR) in a ratio of 3 : 1 (v/v). They were kept in the hardening chamber where the temperature was set at $24 \pm 3^\circ\text{C}$, with a PPFD of $35\text{--}45 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The plantlets were kept closed in high humidity and watered every two weeks. After one month, the plants were opened and watered once a week. The survival percentage was counted after eight weeks.

RESULTS

Improvement of *Spathiphyllum* shoot quality using AgNO_3

Our findings revealed a significant variability of the assessed parameters due to silver nitrate concentrations. Shoots exposed to 3.0 and 4.0 $\text{mg}\cdot\text{L}^{-1}$ of AgNO_3 were the longest (4.7 and 4.5 cm, respectively) compared to shoots inoculated in media with 5.0 $\text{mg}\cdot\text{L}^{-1}$ and 0–2 $\text{mg}\cdot\text{L}^{-1}$ of AgNO_3 (Fig. 1A). The addition of AgNO_3 at a concentration of 3.0 $\text{mg}\cdot\text{L}^{-1}$ promoted the leaf area (Fig. 2A–B, 3).

The results showed a significant effect of AgNO_3 on leaf size. A gradual increase in concentration from 0 to 5 $\text{mg}\cdot\text{L}^{-1}$ gradually increased the leaf length (from 1.5 to 2.1 cm) and leaf width (from 0.6 to 1.1 cm) at AgNO_3 concentrations of 2.0, 3.0, or 4.0 $\text{mg}\cdot\text{L}^{-1}$ of (Fig. 3A). Consequently, the above concentrations increased the largest leaf area (1.7 cm^2) (Fig. 2B, 3B). At 1.0 $\text{mg}\cdot\text{L}^{-1}$ AgNO_3 concentration, a notable proliferation rate, similar to that at 3.0 $\text{mg}\cdot\text{L}^{-1}$, and a significant number of leaves were observed (Fig. 4A–B). However, concentrations of 4.0 $\text{mg}\cdot\text{L}^{-1}$ and 5.0 $\text{mg}\cdot\text{L}^{-1}$ resulted in the lowest number of new shoots and leaves.

Effect of AgNO_3 concentration on rooting

All explants developed roots on the stem base, but AgNO_3 affected root quality. The highest number of roots formed on media containing 1.0 and 0.5 $\text{mg}\cdot\text{L}^{-1}$ (9.2 and 8.6 roots per explant, respectively). The lowest root number was recorded on shoots grown without and with 2 $\text{mg}\cdot\text{L}^{-1}$ AgNO_3 (4.4 and 5.1, respectively). Roots in media containing 0.5–4 $\text{mg}\cdot\text{L}^{-1}$ of AgNO_3 were over 5 cm long. The roots in the control and with a silver nitrate concentration of 5 $\text{mg}\cdot\text{L}^{-1}$ were 3.8 and 4.0 cm long. Additionally, a gradual increase in root diameter was observed with increasing silver nitrate concentration from 0 to 5 $\text{mg}\cdot\text{L}^{-1}$ (Table 1).

In general, the use of silver nitrate has practical implications for the *in vitro* rooting of *Spathiphyllum*. By increasing the number, size, and diameter of roots, it can significantly improve the efficiency of plant propagation. Moreover, simultaneous shoot growth and rooting after the multiplication phase on the same medium shortened the production cycle.

Effect of AgNO_3 on acclimatization

Larger leaves were crucial for promoting robust rhizogenesis. Shoots with well-developed roots are important not only for immediate survival and growth, but also for facilitating the healthy development of plantlets during the acclimatization phase. It is worth mentioning that after two months, the *ex vitro* acclimatization was successful after transferring the plantlets to pots, reaching 100% survival of plantlets treated with different concentrations of AgNO_3 , including the control. Plantlets treated with 1, 2, and 3 $\text{mg}\cdot\text{L}^{-1}$ AgNO_3 were well acclimatized (Fig. 2D), with those treated with 3 $\text{mg}\cdot\text{L}^{-1}$ showing more expanded leaves. The remaining concentrations did not allow shoot formation for longer three months.

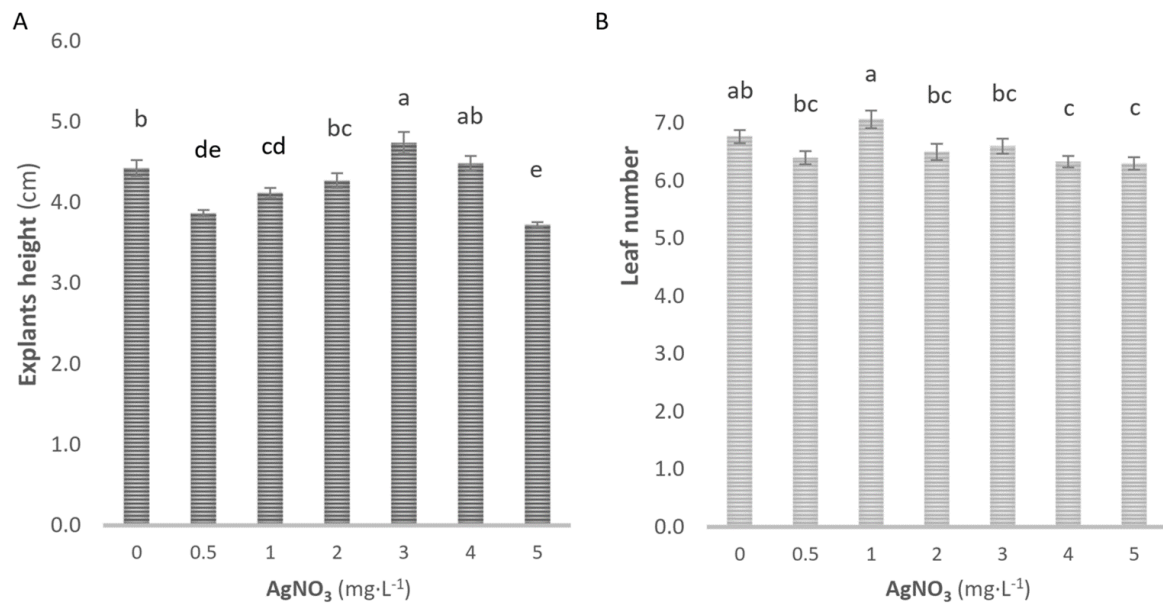


Figure 1. Effect of different AgNO_3 concentrations on the height of explants (A) and the number of leaves (B) of *Spathiphyllum wallisii*

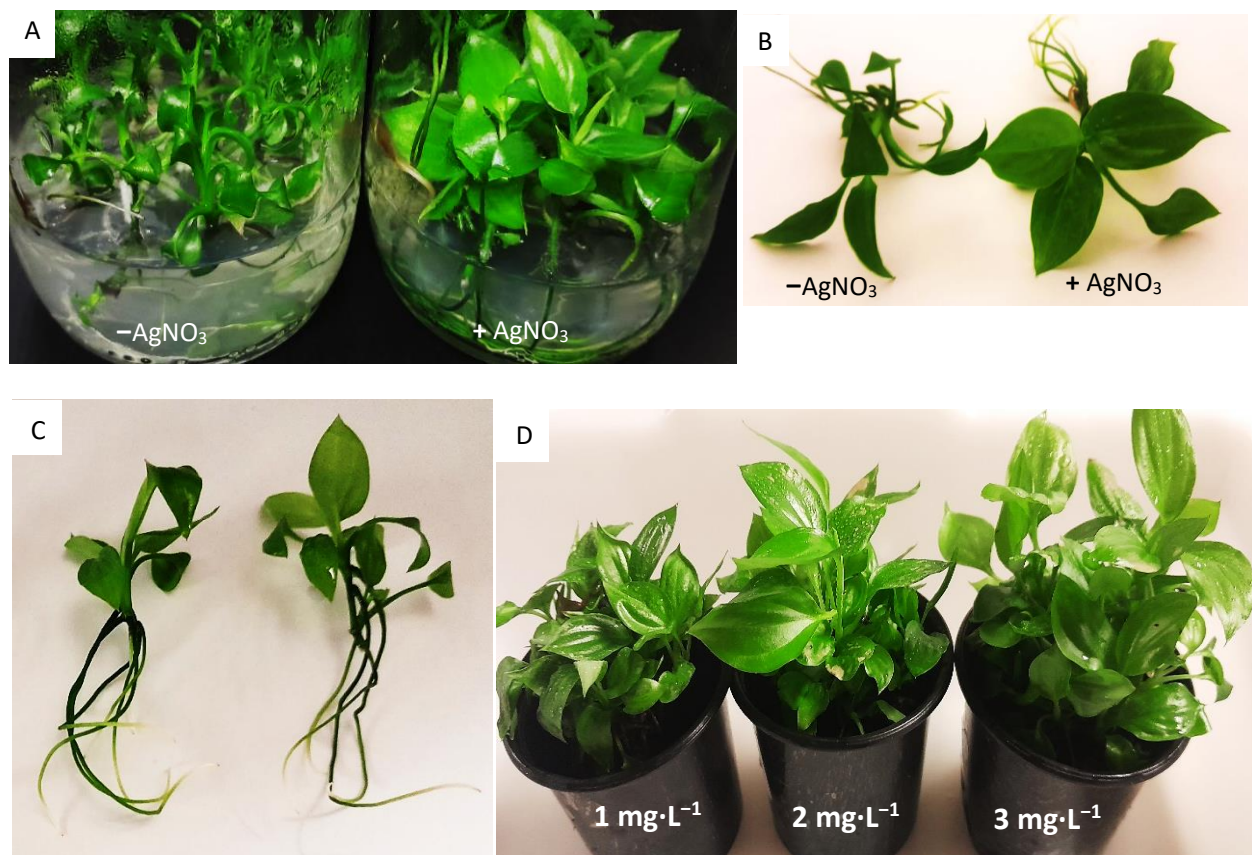


Figure 2. Effect of $3.0 \text{ mg}\cdot\text{L}^{-1}$ AgNO_3 on different *in vitro* phases of *Spathiphyllum wallisii* after two months (A–C) and acclimatization of plantlets grown on media with 1–3 $\text{mg}\cdot\text{L}^{-1}$ silver nitrate added (D)

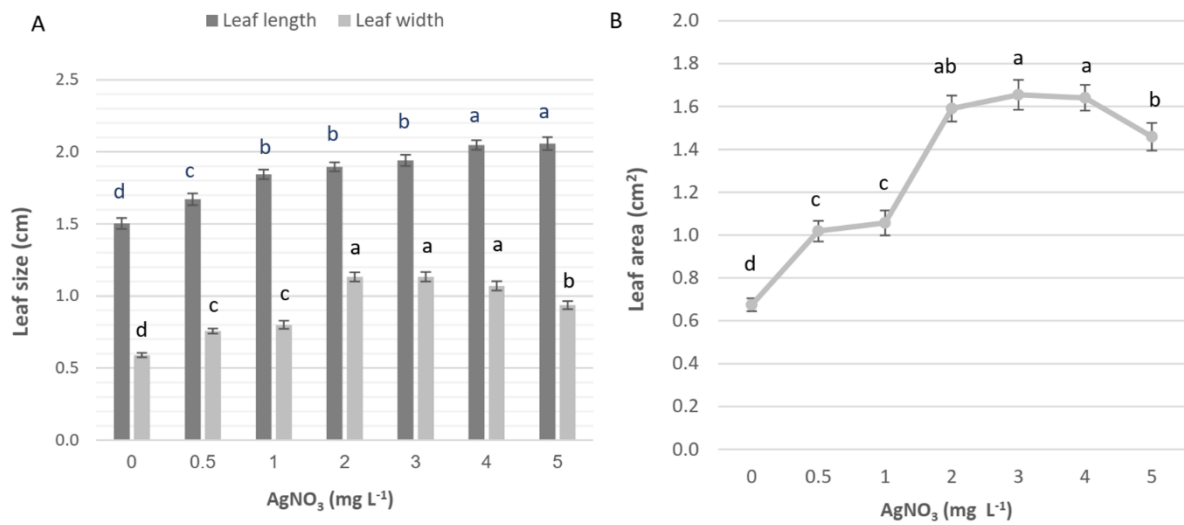


Figure 3. Effect of different AgNO_3 concentrations on leaf size (length and width) (A) and leaf area (B) of *Spathiphyllum wallisii*. Values are represented as means $\pm$ standard error; Mean values with different letter(s) indicate a significant difference between treatments according to Duncan's multiple range test ($p \leq 0.05$).

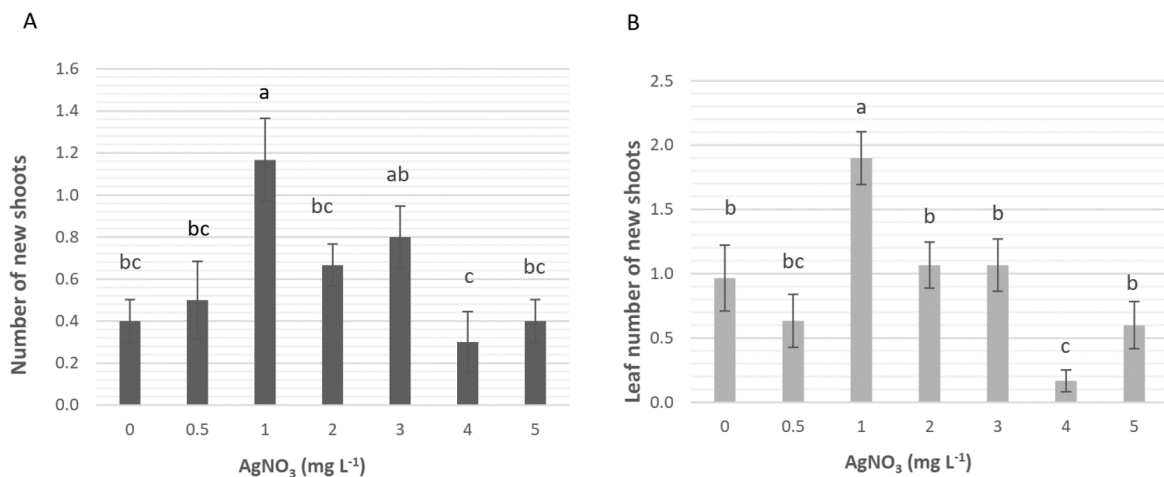


Figure 4. Effect of different AgNO_3 concentrations on the number of newly emerged shoots (A) and the number of leaves on newly emerged shoots (B) of *Spathiphyllum wallisii*. Values are represented as means $\pm$ standard error of 30 cultured explants; Mean values with different letter(s) indicate a significant difference between treatments according to Duncan's multiple range test ($p \leq 0.05$).

Table 1. The effect of different AgNO_3 concentrations on the *in vitro* rooting of *Spathiphyllum wallisii*

AgNO_3 ($\text{mg} \cdot \text{L}^{-1}$)	Root number	Root length (cm)	Root diameter (mm)
0	4.4 ± 0.2 ^d	3.8 ± 0.1 ^c	0.5 ± 0.0 ^d
0.5	8.6 ± 0.3 ^a	5.4 ± 0.2 ^{ab}	0.9 ± 0.0 ^c
1	9.2 ± 0.5 ^a	5.1 ± 0.2 ^b	0.9 ± 0.0 ^b
2	5.1 ± 0.3 ^{dc}	5.8 ± 0.2 ^a	1.0 ± 0.0 ^a
3	6.7 ± 0.4 ^b	5.7 ± 0.1 ^a	1.0 ± 0.0 ^a
4	6.0 ± 0.2 ^{bc}	5.6 ± 0.2 ^{ab}	1.0 ± 0.0 ^a
5	6.7 ± 0.3 ^b	4.0 ± 0.1 ^c	1.0 ± 0.0 ^a

Values are represented as means $\pm$ standard error; Mean values within column with different letter(s) indicate a significant difference between treatments according to Duncan's multiple range test ($p \leq 0.05$).

DISCUSSION

Most plants propagated by tissue culture accumulate the ethylene in closed and semi-closed cultivation systems, which may affect the growth of explants. The effect of this accumulated ethylene on plant growth varied greatly depending on plant sensitivity. To our knowledge, this study represents the first report about morphological changes in *Spathiphyllum* development that occurred in tightly sealed vessels. These changes included growth inhibition, as evidenced by the reduced leaf size. In comparison, Cardoso (2019) observed different symptoms on multiplied explants of *Anthurium* cultivars Alabama and Dakota, which showed an apparent stem elongation in medium without AgNO_3 .

The physiological response to ethylene in plants could be suppressed by mild antagonists such as CO_2 and potent antagonists such as silver compounds, which have been used in different applications in tissue culture (Kumar et al. 2009). Ag^+ ions prevent various explant reactions due to their ability to influence ethylene activity. Some reports mentioned the increase in plantlets' height using silver nitrate as an ethylene inhibitor, while the opposite was found in other experiments. In the current study, we observed that silver nitrate promoted the augmentation of *Spathiphyllum* plantlet height, which varied depending on the concentration. Samiei et al. (2021) showed that AgNO_3 stimulated shoot elongation in rose six times compared with the control. Conversely, silver nitrate caused shorter stems in *Anthurium* and *Solanum tuberosum* (Alva Ticona & Oropeza 2013; Cardoso 2019).

However, in our experiment, silver nitrate exhibited little effect on the propagation rate and the number of leaves in new regenerated shoots. Similarly, Sirisom and Te-Chato (2012) suggested that silver nitrate could enhance the number of shoots in the *Hevea brasiliensis* *in vitro* culture.

Moreover, we successfully obtained well-established plantlets with 1.7 cm^2 of leaf area by treating *Spathiphyllum* with $3 \text{ mg} \cdot \text{L}^{-1}$ of silver nitrate.

This was similar to the results of Tamimi (2015) who found the same reaction in banana tissue culture. Ethylene inhibitor was effective in some species at low concentration such as $0.5 \text{ mg} \cdot \text{L}^{-1}$ for regeneration of *Alternanthera sessilis* plants (Sowmya et al. 2020), and $1 \text{ mg} \cdot \text{L}^{-1}$ for production of maximum number of shoots and roots in *Lantana camara* (Tien et al. 2019). High quality and growth vigor were obtained in the *Rosa canina* at a silver nitrate concentration of $100 \text{ mg} \cdot \text{L}^{-1}$ (Samiei et al. 2021). However, Vidyalakshmi et al. (2017) found that high concentration of silver nitrate ($200 \text{ mg} \cdot \text{L}^{-1}$) was toxic to *Musa* explants. Based on previous findings, we conclude that the effectiveness of AgNO_3 in *in vitro* culture depends on the concentration and genotype of the plant. Therefore, it is crucial to determine the exact concentration of AgNO_3 for *in vitro* cultivation of *Spathiphyllum wallisii*. Our research demonstrated that $3 \text{ mg} \cdot \text{L}^{-1}$ was the optimal concentration to produce shoots with the highest stems and biggest leaves.

In our experiment, regenerated shoots were able to take root with and without the addition of silver nitrate and plant growth regulators. However, at some concentrations AgNO_3 increased root quality, supporting acclimatization and accelerating plantlet growth. These findings are from the experiment by Tamimi (2015) on *Musa acuminata*. However, the effect of AgNO_3 on rooting depends on the genotype, as found in cherry (CAB-6P and Gisela 6) (Sarropoulou et al. 2016).

In the case of *Spathiphyllum*, we show that silver nitrate at concentrations of $2\text{--}4 \text{ mg} \cdot \text{L}^{-1}$ significantly increases leaf area, improving shoot quality and inducing rapid root induction per shoot for acclimatization. Moreover, the use of silver nitrate was found to enhance rooting of *Spathiphyllum* with simultaneous shoot growth, without subculture of multiplied shoots on a rooting medium, which helps reduce the costs of micropropagated plantlets. All rooted plantlets in AgNO_3 medium were successfully acclimatized with 100% survival rate. Despite these promising results, questions remain about the long-term effects of silver nitrate following acclimatization. Therefore, future studies on this topic are recommended.

CONCLUSION

Silver nitrate at different concentrations significantly affected the growth and development of *Spathiphyllum* shoots and roots. In particular, AgNO_3 at $3.0 \text{ mg}\cdot\text{L}^{-1}$ significantly promoted stem elongation, while $1.0 \text{ mg}\cdot\text{L}^{-1}$ increased the number of leaves on new shoots and the rate of proliferation. Larger leaves were formed at increased silver nitrate concentrations, especially at $2\text{--}4 \text{ mg}\cdot\text{L}^{-1}$. The highest number of roots was recorded at 0.5 and $1.0 \text{ mg}\cdot\text{L}^{-1}$. The longest roots were observed at $0.5\text{--}4.0 \text{ mg}\cdot\text{L}^{-1}$. All plantlets, including those treated with AgNO_3 , showed 100% survival during acclimatization, demonstrating successful adaptation to *ex vitro* conditions.

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Conflict of interest

The authors have no relevant financial or nonfinancial interest to report.

Compliance with ethical standards

The authors report no conflicts of interest, nor did they perform any experiments on humans or animals during the preparation of this paper.

Data availability

This published article contains data collected or analyzed during this investigation. Further inquiries can be sent to the corresponding author.

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