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ORIGINAL ARTICLE

Silver compounds regulate leaf drop and improve in vitro regeneration from mature tissues of Australian finger lime (*Citrus australasica*)

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Abstract

Finger lime is a small-leaved citrus native to Australia that is becoming increasingly popular worldwide. Finger lime is monoembryonic and cannot be propagated true to type from seeds. Initial studies to develop a tissue culture protocol for the mass production of elite lines being developed in our breeding program were unsuccessful due to complete leaf abscission during the culture establishment phase. In this study, silver-containing compounds such as silver thiosulfate (STS), silver nitrate ($AgNO_3$), and silver nanoparticles (AgNPs) were applied at three concentrations (20, 40, and 60 µM) to manage ethylene biosynthesis in finger lime tissue cultures. The Murashige and Skoog (MS) medium supplemented with 2.2 µM BAP and 60 µM silver thiosulfate was the best medium for culture establishment. The same level of STS with 0.70 µM GA_3 produced the largest number of shoots and prevented leaf abscission. Micropropagated shoots were successfully rooted in half-strength MS medium supplemented with 0.10 µM NAA. The addition of 60 µM silver thiosulfate (STS) to the MS medium resulted in efficient micropropagation of the finger lime explants and controlled leaf abscission.

Key message

The study evaluates the efficacy of various silver-containing compounds on their ability to effectively control ethylene production and allow for the development of an efficient tissue culture protocol for Finger lime.

Keywords Microcitrus · Finger lime · Adventitious regeneration · Ethylene · Silver thiosulfate · Silver nitrate · Silver nanoparticles

Introduction

Finger lime (*Citrus australasica* F. Muell) is a native Australian species. Previously classified in the genus *Microcitrus* (Swingle 1915), this cultivar was recently reclassified into the *Citrus* genus based on revised taxonomic studies (Mabberley 1998). Finger limes are compact thorny understory shrubs or small trees (Delort and Yuan 2018). In recent years, the demand for the fruit has grown exponentially due to increased usage of the fruit pulp (Rennie 2017). Commercially grown finger lime cultivars are seedling selections collected from the forests of eastern Australia (New South Wales and Queensland) (Rennie 2017). Most of the finger lime fruit available in the international markets are also produced in those Australian states. However, the demand for the fruit in the United States market has led to an increase in commercial finger lime cultivation in the United States (Karp 2009). Locally available varieties are either pale pink or whitish in color, but newer varieties are being developed in California and Florida to satisfy the demand for red pulp cultivars. To prevent the spread of graft-transmissible pathogens, all citrus budwood used for propagating trees in the USA have to be obtained from an approved source (Vidalakis et al. 2010). This is advantageous in preventing disease spread but can lead to a shortage of available material for

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propagating newly released or experimental germplasm. In turn, this can hamper large scale experimental field trials or hinder grower demand. Since finger limes are monoembryonic (Delort and Yuan 2018) and contain only a single zygotic embryo, propagation via the seed is not an option.

Tissue culture has been widely utilized for the rapid propagation of citrus germplasm (Carimi and De Pasquale 2003). However, the establishment of an efficient in vitro regeneration protocol is crucial for micropropagation success. In finger lime, a suitable micropropagation technique could provide a method to rapidly produce plant materials, but currently, there are no tissue culture protocols for this species.

Initial experiments to develop a simple tissue culture for rapid finger lime production failed due to excessive leaf drop caused by enhanced ethylene production in the in vitro culture vessels. Subsequent studies developed an efficient micropropagation protocol of the finger lime, as reported in this paper. Here, we report the results of using different silver ions forms—silver thiosulfate (STS), silver nitrate ($AgNO_3$), and silver nanoparticles (AgNPs)—as ethylene inhibitor agents to control leaf abscission and for efficient tissue culture. We also investigate the effect of the basal media, plant growth regulators, and solidification agents on shoot induction and multiplication.

Materials and methods

Plant material and explant preparation

The primary explants of this study were from mature trees of an experimental finger lime accession (KB-08) being developed by the citrus improvement team at the University of Florida and grown in a screenhouse. Nodal segments 10–20 mm in length with an axillary bud were excised. Explants were surface sterilized in a 1.2% Sodium hypochlorite solution (20% (v/v) Clorox® bleach) with two drops of Tween-20 for 15 min and rinsed four times in sterile double distilled water.

Effect of basal media and BAP concentrations on in vitro establishment

The sterilized explants were trimmed and cultured on three different basal media: MS (Murashige and Skoog 1962), C2D (Chee and Pool 1987), and DKW (Driver and Kuniyuki 1984). The explants were also supplemented with four levels of 6-benzylaminopurine (BAP) (0, 2.2, 4.4, 6.6 µM), 0.05 µM α-naphthaleneacetic acid (NAA), and 2.5 mM of 2-(*N*-morpholino) ethanesulfonic acid (MES). For culture establishment, the explants were placed in 25 × 100 mm Petri dishes containing 30 ml medium, and for multiplication and elongation, the cultures were transferred to magenta boxes. Each experiment consisted of three replicates for each treatment. Each replicate represented six containers; three explants were cultured in each container. The media were solidified with 3 g L^{-1} phytagel, pH 5.8. All of the chemicals and tissue culture media were obtained from Phyto Technology Laboratories, Shawnee Mission, KS, USA, and the phytagel obtained from Sigma-Aldrich, St. Louis, MO, USA. The cultures were maintained under a 16/8 h light/dark regime at 25 ± 2 °C. The shoot induction percentage was calculated after 2 weeks, and the leaf abscission percentage was calculated after 4 weeks as:

$$\text{Shoot induction } \% = \frac{\text{Number of the proliferated explants}}{\text{Total number of explants}}$$

$$\text{Leaf abscission frequency } \% = \frac{\text{Number of shoots with leaf drop}}{\text{Total number of the regenerated shoots}}$$

Biosynthesis of silver nanoparticles using citrus callus cultures

Callus cultures were initiated from immature ovules of 'Valencia' sweet orange, according to Grosser and Gmitter (1990)–One-year-old rapidly proliferating callus was used in this study. Silver nanoparticles were produced as in Ponarulselvam et al. (2012), with several modifications. Twenty grams of fresh weight callus was extracted in 100 mL of methanol and boiled for 6 min. The extract was filtered and used for the biosynthesis of silver nanoparticles. Ten milliliters of the callus extraction was mixed with 90 mL of 1 mM silver nitrate ($AgNO_3$) in a 250 mL Erlenmeyer flask. The reaction mixture was adjusted to pH 7.5 and heated to 60 °C for 10 min in complete darkness. The mixture was then placed on a shaker (100 rpm) at room temperature for 24 h. The nanoparticles were initially monitored by ultraviolet–visible spectroscopy using a Biochrom libra UV–Visible spectrophotometer, as outlined by Gurunathan (2019). The UV–Vis analysis of the reduction reaction of silver was measured between 200 and 800 nm. The nanoparticle solution was subsequently dropped on a carbon-coated copper grid and dried at ambient conditions before analysis. The transmission electron microscopy (TEM) characterization of the nanoparticles was carried out using a Morgagni 268 transmission electron microscope.

Ethylene measurements

After 2 months of in vitro culture in the shoot multiplication medium, the ethylene content was measured in the

headspace of the Petri dishes using an F-900 portable ethylene analyzer (Felix instruments, WA, USA). The Petri dish lid containing the explants was punctured with a sterile needle and covered with two layers of 3M™ Micropore™ Surgical Tape (The 3M Company, Maplewood, MN) and wrapped with parafilm to avoid ethylene loss. Five milliliters of headspace gas were removed with a sterile syringe and injected into the machine. The same volume was pulled from uncultured media as a control, and the data were recorded as ppm. Three replicates were tested for each treatment, and each vessel contained 3 plants.

Effect of the silver compounds on culture establishment

The explants were initially cultured on MS medium containing 2.2 µM BAP and different compositions of the three silver sources: silver thiosulfate (STS), silver nitrate ($AgNO_3$), and silver nanoparticles (AgNPs) at 0, 20, 40, or 60 µM. The media was solidified using 8 g L^{-1} Agar or 3 g L^{-1} phytagel. After 1 month, the leaf abscission percentage, shoot number, and shoot lengths were recorded. To test shoot multiplication, the explants were transferred to an MS medium supplemented with 2.22 µM BAP and 20, 40, or 60 µM STS. Gibberellic acid (GA_3) at 0, 0.70, or 1.40 µM was also added to the media to investigate its effect on shoot elongation. The silver compounds and gibberellic acid (GA_3) were added after the media had cooled to 50 °C. Silver thiosulfate was prepared fresh; a 10 mM stock solution was prepared by mixing a 100 mM sodium thiosulfate solution ($Na_2S_2O_3$) with a 100 mM silver nitrate solution ($AgNO_3$) in a molar ratio of 1:4 between silver and thiosulfate (Navarro-García et al. 2016). The number of shoots formed were recorded after 4 weeks following transfer to the treatment medium.

Root induction and micrografting to rootstocks

The cultured explants were grown under a light intensity of 2500 lx with a 16-h photoperiod at 25 ± 2 °C. To confirm our results, the micropropagated shoots were subcultured twice on the recommended medium and the shoot number was calculated. Adventitious shoots, 2–3 cm in height, were separated and individually cultured on MS media without BAP or GA_3 for 1 week. The shoots were then transferred to ½ MS medium supplemented with 0.1 µM NAA and 60 µM STS or micrografted onto Carrizo citrange rootstocks for further development, as outlined by Navarro (1992).

Experimental design and data analysis

The experimental data were analyzed with an analysis of variance (ANOVA) test, followed by the student's *t*-test to compare the different treatments. The combinations of basal media and BAP and STS and GA_3 were designed as a factorial experiments. The effect of the silver compounds was determined using one-way ANOVA. All the analyses were conducted in JMP Pro 14 and the statistical significance was established at $p < 0.05$.

Results and discussion

In this study, we developed an efficient protocol for the rapid micropropagation of mature finger lime tissues, a monoembryonic citrus cultivar. The development of a tissue culture

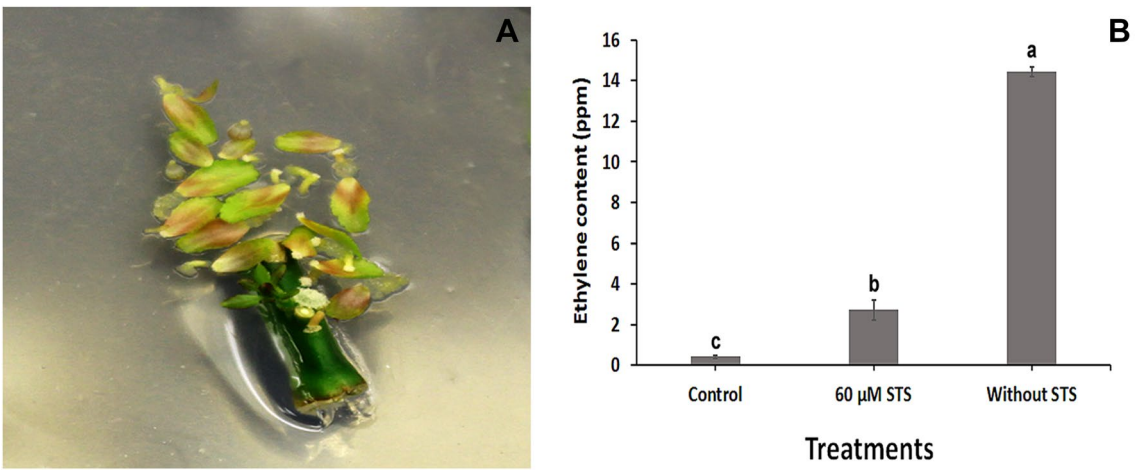

Fig. 1 **a** Leaf abscission in the tissue cultured shoots without the addition of ethylene suppressing silver compounds. **b** Ethylene content in the head space of the tissue culture vessels containing the finger lime shoots with and without the addition of silver thiosulfate. Control indicate the amount of ethylene in headspace of tissue culture vessels without any plant samples. Means followed by the same letter were not different at $\alpha = 0.05$ using student's *t* test

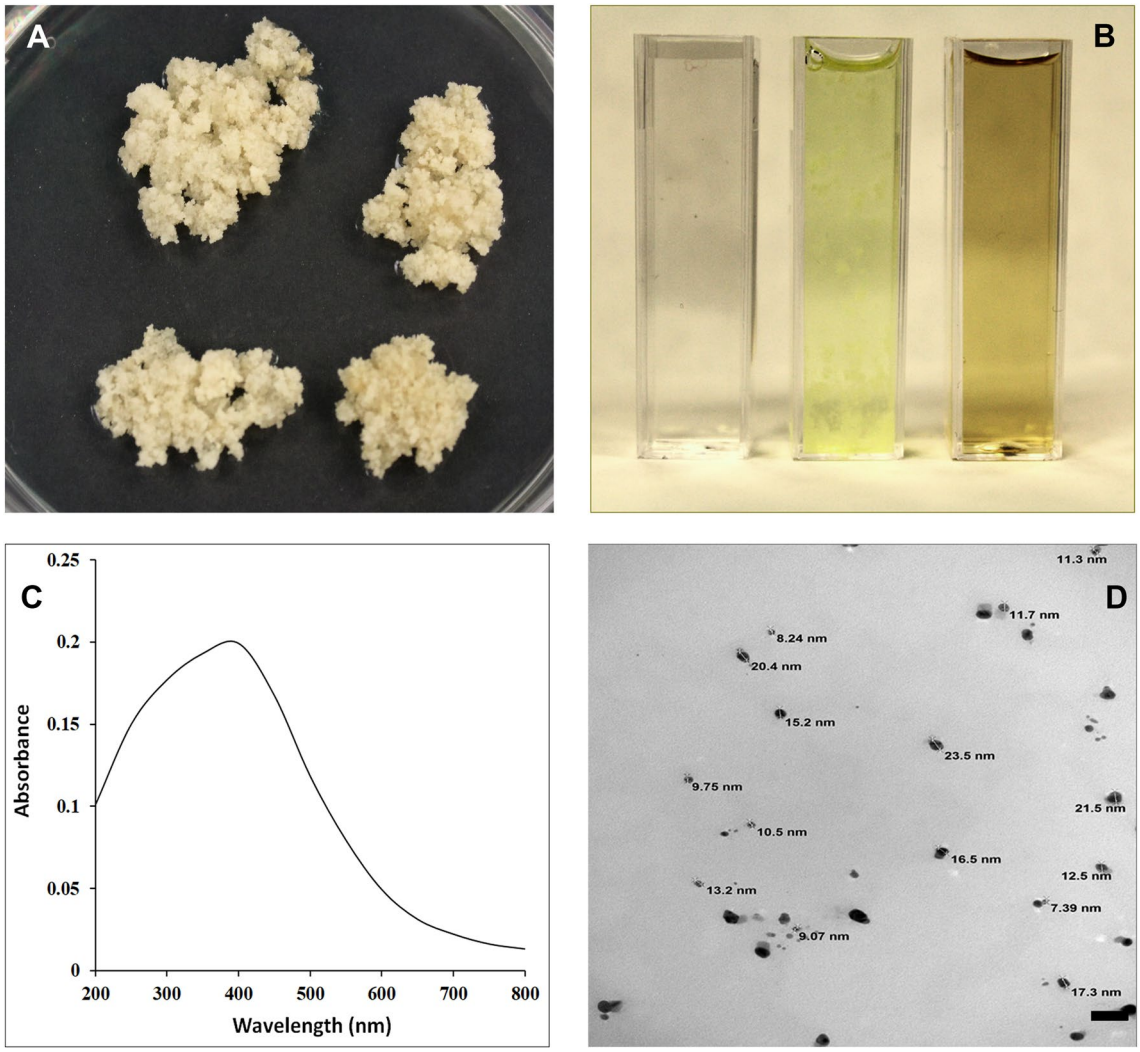

Fig. 2 Silver nanoparticle biosynthesis using citrus callus. **a** 'Valencia' sweet orange citrus cells; **b** Progressive color change following the synthesis of AgNPs. Cuvette on the left is the $AgNO_3$ solution, middle cuvette is the color following callus extraction and right cuvette is the color of the reaction mixture after 24 h of exposure to $AgNO_3$ indicating the formation of AgNPs; **c** UV–Vis spectra graph showing the plasmon resonance peak of AgNPs; **d** TEM image of AgNPs biosynthesized using the citrus callus extracts

protocol allowed for the mass production of a large population of finger lime shoots. The initial experiments to establish a simple tissue culture protocol were not successful, hampered mainly by excessive leaf abscission and subsequent mortality of the in vitro cultures (Fig. 1a). These are classic symptoms of excessive ethylene production in vitro, and high ethylene levels were detected in the cultures suffering from leaf drop (14.4 ppm; Fig. 1b). It is well know that all plants produce ethylene in vitro (Iqbal et al. 2017). However, excess ethylene inhibits cell division (Constabel et al. 1977) and high levels of ethylene results in leaf abscission (Jackson and Osborne 1970). Ethylene biosynthesis can be controlled by two mechanisms, (1) blocking the enzymes in its biosynthetic pathway or (2) blocking the ethylene receptors. Silver ions have been reported to inhibit the physiological action of ethylene and to control ethylene production (Beyer 1976). Silver ion donors such as silver nitrate and silver thiosulfate have been used in numerous studies to control ethylene biosynthesis (Kumar et al. 1998; Mohiuddin et al. 1997; Purnhauser et al. 1987). More recently, silver nanoparticles have also been used due to their physical and chemical properties and their easy uptake and mobility into plant cells (Sarmast and Salehi 2016).

Biosynthesis of silver nanoparticles

In addition to their role as an antimicrobial compounds (Spinoso-Castillo et al. 2017), AgNPs are known to enhance shoot

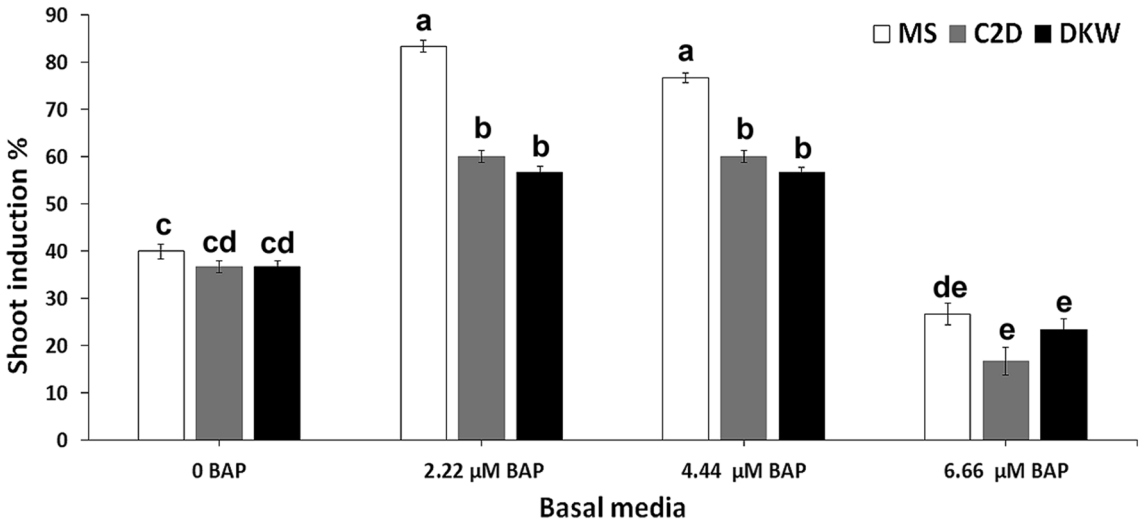

Fig. 3 Effect of basal media on shoot induction percentage from stem explants of finger lime after 2 weeks. Bars represented as mean ± standard error. Shoots induction % was counted after 2 weeks. Means followed by the same letter were not different at α = 0.05 using student's *t* test. BAP—N6-Benzyl aminopurine, MS—Murashige and Skoog (1962) based medium, C2D—Chee and Pool (1987) based medium and DKW—Driver and Kuniyuki (1984) based medium

Table 1 Effect of silver thiosulfate (STS), silver nitrate ($AgNO_3$), silver nanoparticles (AgNPs) on leaves abscission of finger lime nodal segments after 4 weeks

Treatment	Leaf abscission % (Agar)	Leaf abscission % (Phytagel)
Control	100 ± 0.00a*	100 ± 0.00a
STS 20 µM	0 ± 0.00d	0 ± 0.00c
STS 40 µM	0 ± 0.00d	0 ± 0.00c
STS 60 µM	0 ± 0.00d	0 ± 0.00c
AgNO3 20 µM	8 ± 4.89cd	4 ± 4.00bc
AgNO3 40 µM	8 ± 4.89cd	4 ± 4.00bc
AgNO3 60 µM	24 ± 7.48b	4 ± 4.00bc
AgNPs 20 µM	4 ± 4.00cd	4 ± 4.00bc
AgNPs 40 µM	8 ± 4.89cd	8 ± 4.89bc
AgNPs 60 µM	12 ± 4.89c	12 ± 4.89b

*Means followed by the same letter were not different at α = 0.05 using student's *t* test. Data were collected after 4 weeks. The experiment was designed as five replications per treatment and six explants per replication

STS silver thiosulfate, *$AgNO_3$* silver nitrate, *AgNPs* Silver nanoparticles

regeneration in in vitro systems (Saha and Dutta Gupta 2018). The biosynthesis of AgNPs using citrus callus (Fig. 2a) caused a distinct color change. In the beginning, the color of the citrus callus and aqueous $AgNO_3$ mixture was yellow (Fig. 2b). However, after stirring for 6 min at 60 °C and pH 7.5, followed by 24 h incubation at room temperature, the color changed to brown (Fig. 2b). This color change is an indication of successful AgNP synthesis and could be due to the surface stimulation of the plasmon resonance phenomenon of the silver metal (Alsammarraie et al. 2018; Shameli et al. 2014). Citrus callus tissues contain bioactive compounds (Jiménez et al. 2001) and enhanced the reduction of the silver ions during exposure to the callus tissue extraction. The AgNPs were characterized using ultraviolet–visible spectroscopy and TEM. UV–Visible absorption spectroscopy revealed the presence of an absorbance peak at ~420 nm, indicating the formation of AgNPs (Fig. 2c); other studies have also reported that a UV–Visible absorption band between 420 and 460 nm indicates the surface plasmon resonance of AgNPs (Gurunathan 2019; Jiménez et al. 2001; Swamy et al. 2015). The nanoparticles used in our study ranged in size from 5 to 25 nm (Fig. 2d).

Effect of the basal media and BAP concentrations on in vitro culture establishment

The primary focus of our study was to produce a suitable protocol for the micropropagation of finger limes using mature tissue explants. We investigated the best basal media and BAP concentrations for shoot induction using three different media (MS, C2D, and DKW) and four concentrations of BAP (0, 2.2, 4.4, and 6.6 µM). The results from all the treatments are presented in Fig. 3. The mature nodal segments induced shoots within 2 weeks. The explants cultured on MS medium supplemented with 2.2 µM BAP recorded the highest shoot induction, followed by C2D and DKW. There were no statistical differences between the 2.2 µM and 4.4 µM BAP treatments for any of the tissue culture media evaluated in this study. Therefore, all further experiments were carried out using the MS medium with 2.2 µM BAP.

Table 2 Effect of silver thiosulfate (STS), silver nitrate ($AgNO_3$), silver nanoparticles (AgNPs) on shoots number and shoot length in the cultural establishment stage

Treatments	Agar		Phytagel	
	Shoots number	Shoots length (cm)	Shoots number	Shoots length (cm)
Control	0.00 ± 0.00d*	0.00 ± 0.00f	0.00 ± 0.00f	0.00 ± 0.00d
STS at 20 µM	2.0 ± 0.00ab	1.58 ± 0.03bc	2.0 ± 0.00bc	1.42 ± 0.03ab
STS at 40 µM	2.2 ± 0.20a	1.68 ± 0.03b	2.2 ± 0.20abc	1.46 ± 0.05ab
STS at 60 µM	2.4 ± 0.24a	1.94 ± 0.09a	2.4 ± 0.24ab	1.50 ± 0.04a
AgNO3 at 20 µM	1.6 ± 0.24bc	1.16 ± 0.05e	2.0 ± 0.00bc	1.44 ± 0.02ab
AgNO3 at 40 µM	1.4 ± 0.24c	1.28 ± 0.8de	2.6 ± 0.24a	1.46 ± 0.04ab
AgNO3 at 60 µM	1.2 ± 0.20c	1.14 ± 0.04e	1.8 ± 0.20cd	1.48 ± 0.03ab
AgNPs at 20 µM	1.4 ± 0.24c	1.44 ± 0.03cd	1.8 ± 0.20cd	1.40 ± 0.03ab
AgNPs at 40 µM	2.0 ± 0.00ab	1.28 ± 0.03de	1.4 ± 0.24de	1.38 ± 0.03b
AgNPs at 60 µM	2.0 ± 0.00ab	1.26 ± 0.09e	1.0 ± 0.00e	1.20 ± 0.03c

*Means followed by the same letter were not different at α = 0.05 using student's *t* test. Data were collected after 4 weeks. The experiment was designed as five replications per treatment and six explants per replication

STS silver thiosulfate, *$AgNO_3$* silver nitrate, *AgNPs* Silver nanoparticles

Table 3 Effect of silver thiosulfate (STS) in combination with gibberellic acid (GA_3) on shoots multiplication and elongation

Treatments		Shoots number	Shoots length (cm)
STS (µM)	GA_3 (µM)		
0	0	0.00 ± 0.00d*	0.00 ± 0.00f
0	0.70	0.33 ± 0.47d	1.33 ± 0.00e
0	1.40	0.67 ± 0.00d	1.43 ± 0.00e
20	0	2.67 ± 0.27bc	1.47 ± 0.09b
40	0	2.67 ± 0.33bc	2.27 ± 0.20cd
60	0	3.33 ± 0.23ab	2.00 ± 0.00e
20	0.70	2.00 ± 0.00c	2.77 ± 0.28b
40	0.70	3.33 ± 0.27ab	2.60 ± 0.19bc
60	0.70	4.00 ± 0.00a	3.53 ± 0.02a
20	1.40	3.33 ± 0.27ab	1.25 ± 0.03e
40	1.40	3.67 ± 0.27a	1.27 ± 0.02e
60	1.40	3.67 ± 0.23a	1.28 ± 0.02e

*Means followed by the same letter were not different at α = 0.05 using student's *t* test. Data were collected after 4 weeks. The experiment was designed as three replications per treatment and six explants per replication

STS silver thiosulfate, *GA_3* gibberellic acid

This concentration has also been identified as a suitable condition for other mature citrus tissue-derived cultures, e.g., sweet orange (Oliveira et al. 2010) and 'Rangpur' lime (Almeida et al. 2002).

Effect of the silver compounds on in vitro culture proliferation

To test the effect of the silver formulations on leaf abscission control, aseptic explants were cultured in MS medium with 2.2 µM BAP and supplemented with STS, $AgNO_3$, and AgNPs at three concentrations (20, 40, and 60 µM). Our results indicated that the STS treatments were effective at preventing leaf abscission (Supplementary Figs. S1 and S2). There was no leaf drop recorded in the finger lime shoots cultured on any of the STS treatments used in this study (Table 1). The explants in the medium supplemented with 20 µM silver nanoparticles and solidified with either agar or phytagel had 4% leaf abscission. There was 8% leaf abscission with the same concentration of $AgNO_3$ in the agar medium. There was a significant decrease in the ethylene concentration when 20 µM STS was supplemented to the agar medium (2.72 ppm). Similar results were observed in the other media supplemented with different levels of $AgNO_3$ and AgNPs (results not shown). The gaseous phase accumulation inside plant tissue culture vessels has been well studied—ethylene (C_2H_4) is the most common gas in sealed vessels (Gould and Murashige 1985; Thomas and Murashige 1979). Various environmental conditions such as temperature and light can also promote enhanced ethylene biosynthesis in plant tissues (LaRue and Gamborg 1971; Yu et al. 1980). Ethylene can function as a promoter or inhibitor of plant growth depending on the species (De Proft et al. 1985) and in vitro finger limes are highly sensitive to ethylene with leaf abscission and subsequently mortality of the shoots. The over-accumulation of ethylene commonly induces the senescence and abscission of shoot culture leaves (Righetti et al. 1990). STS is an active compound, providing Ag^+ ions to the ethylene inhibition sites (Steinitz et al. 2010), and is superior to $AgNO_3$ in inducing roots in *Corymbia maculata* (Steinitz et al. 2010). The breakdown of the silver thiosulfate complex is probably important before it interferes with the ethylene binding sites (Veen and Overbeek 1989). Additionally, the presence of

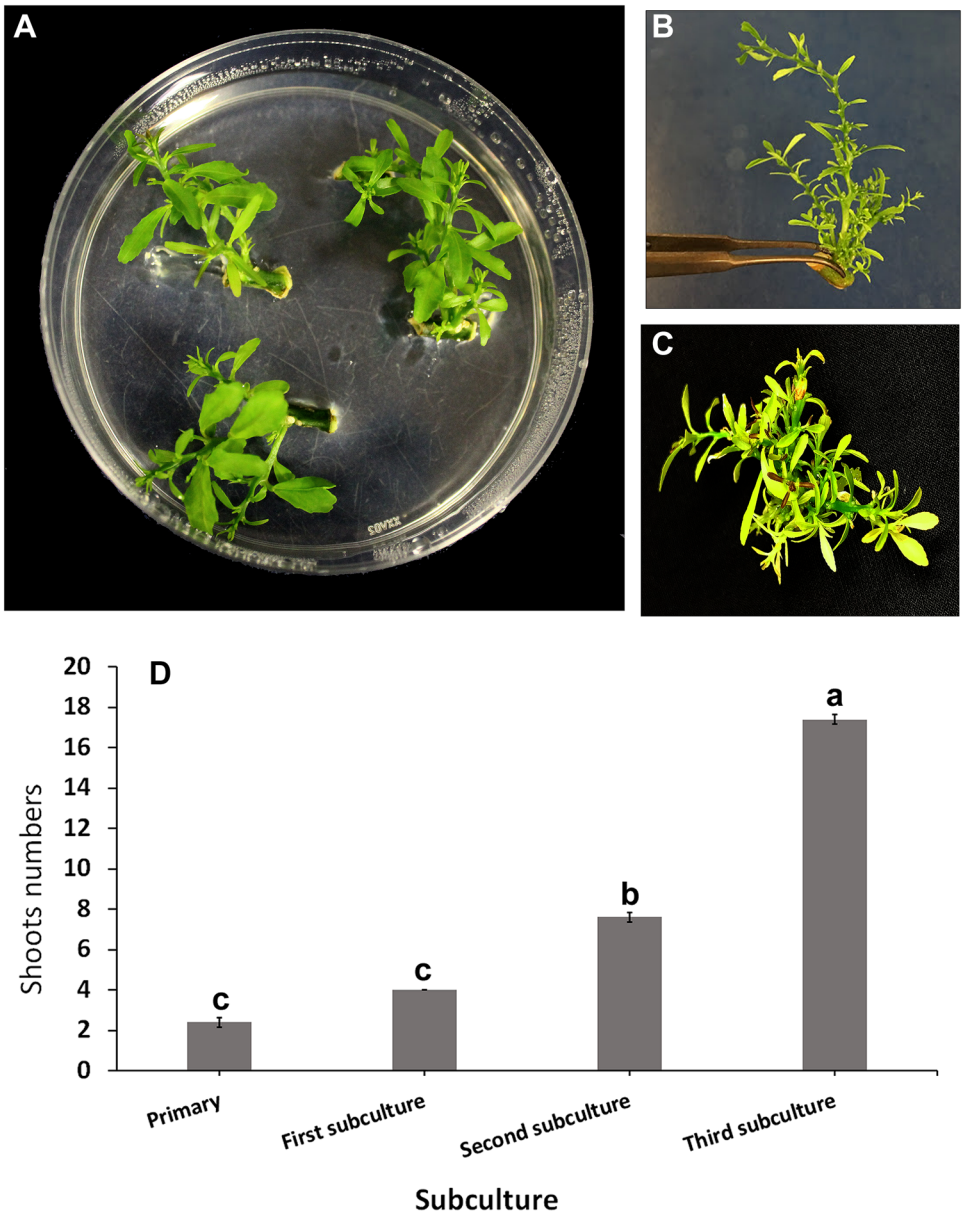

Fig. 4 Micropropagation of finger lime through nodal segments. **a** In vitro shoot regeneration from nodal segment on MS medium supplemented with 60 µM STS after 4 weeks; **b** close of one shoot clump after the second subculture after 2 months; C. shoot multiplication after three subcultures with 4 months. D. Shoot multiplication rate of in vitro finger lime explants. 60 µM STS was added to all subcultures. The primary and first subculture did not contain any GA_3 while 0.7 µM GA_3 was added to the second and third subcultures. Shoots numbers were calculated in each stage after 1 month of culture initiation. Means followed by the same letter were not different at $\alpha = 0.05$ using student's *t* test

thiosulfate S^2O^{3-} ions enhances silver uptake and alleviates the phytotoxicity of Ag^+ ions (Fortin and Campbell 2001). Thiosulfate ions are also an active, mobile of silver (Biddington 1992). AgNPs have been produced using the callus cells of many plant species such as *Aloe vera* (Chandran et al. 2006), basil (Ahmad et al. 2010), papaya (Mude et al. 2009), and persimmon (Song and Kim 2008). This report is the first to produce AgNPs using citrus callus cells. AgNPs have a large surface area and interact with other particles in the medium to increase efficiency (Ingle et al. 2008). In our study, the AgNPs were also effective at controlling leaf abscission. Similar to STS, $AgNO_3$ can also inhibit ethylene from binding to the cell during division (Mohiuddin et al. 1997) and at the lowest concentration, they did not statistically differ from the AgNPs in their inhibition of leaf abscission.

The utilization of agar or phytagel to solidify the media did not affect the leaf abscission rates in most treatments. The notable exception was the 60 µM $AgNO_3$ treatment, where phytagel proved superior. We also observed a color change when $AgNO_3$ was incorporated into the medium, indicating a positive reaction between the medium and $AgNO_3$ (Supplementary Fig. S3). Other groups have also documented coloration changes with $AgNO_3$ supplementation. This is primarily due to the Ag^+ ions forming precipitates in the tissue culture medium (Blum et al. 1987; Shevchenko et al. 1996; Tsai and Frasch 1982). The presence of Cl^- ions in the agar and other chloride sources in

Fig. 5 **a** Micrografted finger lime shoot on Carrizo root stock. **b** Root induction from a shoot cultured on ½ MS medium supplemented with 0.1 µM NAA and 60 µM STS

the media may have reacted with $AgNO_3$ and caused silver chloride precipitation (Steinitz et al. 2010).

Effect of the silver compounds on in vitro growth

We investigated the effect of three different silver compounds on different plant growth parameters. The highest shoot number (2.40 shoots/explant) and shoot length (1.94 cm) was recorded in the 60 µM silver thiosulfate treatment group (Table 2). The shoot number and shoot length of the STS group (60 µM) was superior to $AgNO_3$ and AgNPs in the agar solidified media, but in the phytagel medium, STS and $AgNO_3$ were similar. STS addition to the media generally enhanced the regeneration percentage of two citrus cultivars, Verna 51 and Fino 49 (Navarro-García et al. 2016). Also, when $AgNO_3$ was used in the in vitro culture of mature citrus internodal stem segments, significantly more buds were produced than in the control group (Marutani-Hert et al. 2011). Our results agree with these studies—we also observed the beneficial effects of adding STS or $AgNO_3$ to our tissue culture media. The AgNPs were superior to the $AgNO_3$ treatments in the agar-based medium but not in the phytagel media. Though not observed in our study, AgNPs can be detrimental to plant growth and regeneration, especially when used at higher concentrations, as was observed during the in vitro propagation of *Campomanesia rufa* (Timoteo et al. 2019).

GA_3 is known to increase shoot length in in vitro citrus cultures (Kotsias and Roussos 2001; Pérez-Tornero et al. 2010). From our results, we inferred that STS is the best chemical to prevent ethylene damage and leaf abscission in mature tissue-derived explants of finger lime. However, the shoot length remained less than 2 cm in the best treatment (Table 2). Thus, we set up a factorial experiment to test the impact of three concentrations of STS with two concentrations of GA_3 (Table 3). An average of 4 shoots with an average shoot length of 3.53 cm was recorded in the 60 µM STS, 0.70 µM GA_3 treatment group (Supplementary Fig. S4). Higher GA_3 levels (1.4 µM) did not influence shoot numbers but did adversely affect shoot length (length decreased in the 1.4 µM group). There was complete defoliation in the medium without STS. Several earlier studies have also found that STS enhances the regeneration rate in several genera such as *Citrus* (Navarro-García et al. 2016), *Echinacea* (Chae and Park 2012), *Gloxinia* (Chae et al. 2012), *Passiflora* (Dias et al. 2010), and *Prunus* (Petri and Scorza 2010). A single finger lime bud cultured in an agar solidified MS medium supplemented 2.2 µM BAP, 60 µM STS, and 0.7 µM GA_3 produced, on average, 17.4 shoots after three subcultures (Fig. 4).

Rooting of the established in vitro cultures

Two to three cm long shoots were excised and either placed in vitro in a rooting medium similar to that described by Grosser and Gmitter (1990) or micrografted onto Carrizo rootstocks as outlined by Navarro (1992). We obtained 80% rooting among the cuttings placed in vitro and 90% of the shoots that were micrografted survived the process. Thus, either method for further development was deemed suitable (Fig. 5). When propagated through cuttings using a mist bed, the rooted finger lime cuttings do not demonstrate apical dormancy. A similar trend was also observed in our in vitro rooted explants. Within a year however a strong dominant bud takes over resulting in the formation of the upper growth of the tree. This trend is also expected to be observed in plants obtained from our in vitro shoots.

Conclusion

An ethylene inhibitor was required to establish finger lime in vitro cultures, as ethylene influenced both culture establishment and shoot induction from mature tissues. The addition of silver compounds improved shoot growth and prevented leaf abscission, and the Murashige and Skoog (MS) medium supplemented with 2.2 µM BAP was optimal for shoot induction. The addition of 0.7 µM GA_3 with 60 µM STS promoted shoot elongation in vitro.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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