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Study of Shoot Tip Necrosis Problems of Fegra Fig (*Ficus palmata* Forssk.) In Vitro in Saudi Arabia

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Abstract. **This work studied the micropropagation of fegra fig (*F. palmata* Forssk.) during which we experienced the incidence of shoot-tip necrosis (STN). STN was evident during the shoot elongation stage, which was regenerated on Murashige and Skoog (MS) medium supplemented with 2 mg/L 6-benzylaminopurine. To alleviate SNT, we conducted a series of experiments and supplemented the medium with calcium chloride (40, 80, and 120 mg/L), ascorbic acid (50, 100, and 150 mg/L), silver nitrate (1, 2, and 3 mg/L), and boric acid (9.3, 12.4, and 15.5 mg/L). Results showed that all the treatments controlled STN at varying levels, and supplementation of medium with 3 mg/L silver nitrate reduced the incidence of STN from 80% to 24%. The regenerated shoots were rooted on the same medium with incubation of cultures in the dark for 3 weeks and subsequent 4 weeks of incubation under 16/8-hour light/dark photoperiod. The growth parameters (number of shoots and roots, length of the main shoot and root, fresh and dry weights), photosynthetic pigments (chlorophylls and carotenoids), and relative water content of plantlets were restored with the application of 3 mg/L silver nitrate to the medium. Incubation of cultures initially in the dark followed by 16/8-hour light incubation facilitated axillary shoot elongation. On the basis of our findings, it is recommended to culture the regenerated axillary shoots of fegra fig onto MS medium containing 3% sucrose, 1.5 mg/L activated charcoal, and 3 mg/L silver nitrate to manage STN effectively.**

Ficus is an important genus distributed in tropical, semitropical, and temperate regions of the world which some ones species yields edible fruits (Badgujar et al. 2014). Fegra fig (*Ficus palmata* Forssk.) is commonly distributed in plains to high altitudes in India, Nepal, Bangladesh, Myanmar, and Thailand (Bhatt et al. 2010). It is also cultivated in different tropical and temperate regions. The young developing fruits and fronds are used as vegetables (https://research.iitmandi.ac.in/botanical/Plant_description/Ficus%20palmata.pdf). The edible fruits are rich in nutrients. Phytochemical analysis of fruits revealed that they are rich in phenolics, flavonoids, and several bioactive constituents (Tewari et al. 2021). The conventional propagation techniques of *Ficus* species such as hardwood and semi-hardwood cuttings, grafting, and air-layering methods have been studied (Shamsuddin et al. 2021). Cuttings from 1- or 2-year-old shoots to produce new trees have been the most common practice for the propagation of fig trees (Aljane and Nahdi 2014; Danthu et al. 2002; Shamsuddin et al. 2021). Plant tissue culture methods have been studied with various species and cultivars of *Ficus* and used for large-scale propagation in several species (Dessoky et al. 2016; Ling et al. 2022; Sahraroo et al. 2019; Sriskanda et al. 2021). Several explant, medium, and physical factors such as size and type of explant, culture medium growth regulators, carbon source, growth additives, light, and temperature, among others, are affecting the successful regeneration of plants.

One of the physiological disorders during micropropagation of plants is shoot-tip necrosis (STN), which arises in shoots or plantlets and results in death of the shoot tip (Teixeira da Silva et al. 2020). This condition can spread from the tip to base of shoots and affect the emergence of axillary shoots from buds lower down the stem and is due to cessation of apical dominance. STN can occur at any stage of micropropagation from shoot multiplication or rooting (Teixeira da Silva et al. 2020). STN was reported to be due to varied factors of medium including plant growth regulators (increase or decrease auxin/cytokinin or combination of PGRs), nutrient deficiency especially varied minerals such as calcium/boron and others), medium type (semisolid/liquid) and composition, sugar source, salt strength, aeration, and frequency of sub-culturing (Jain et al. 2009; Kataeva et al. 1991; Raven 1977; Surakshitha et al. 2019). Varied conditions such as high relative humidity and poor ventilation within the culture vessel, higher ethylene production, and restricted transpiration have also been reported to hasten the incidence of STN (Teixeira da Silva et al. 2020; Williams 1993).

STN has been reported during the propagation of many plant species, especially fruit-yielding plants including apricots (Perez-Tornero and Burgos 2000), grapes (Surakshitha et al. 2019; Thomas 2000), pear (Grigoriadou et al. 2000), and raspberry (Wu et al. 2009). Bayoudh et al. (2015) noted a significant interaction between the shoot tip size, the *F. carica* varieties and the establishment media concerning STN rate. The majority of explants were susceptible to necrosis on all culture media, and the highest rate of necrosis (78.33%) was recorded with ‘Bither Abiadh’ on Murashige and Skoog (MS) medium containing 0.2 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L naphthalene acetic acid, 0.1 mg/L kinetin, and 90 mg/L phloroglucinol. Several theories have been tested to alleviate STN problems during micropropagation including frequent subculture (Alderson et al. 1987; Surakshitha et al. 2019). Supplementation of silver nitrate/$AgNO_3$ (Martin 2002), gibberellic acid/GA_3 (Valles and Boxus 1987), fructose (Kulkarni and D’Souza 2000), calcium (McCown and Sellmer 1987), benzyladenine (BA), and calcium chloride/$CaCl_2$ + BA (Piagnani et al. 1996; Vieitez et al. 1989) to the medium, dipping of shoot tips in BA before transferring (Perez-Tornero and Burgos 2000), and improving ventilation of culture vessel (Thomas 2000). Such methods have improved STN problems with varied success. In the present study, we have initiated micropropagation of fegra fig and experienced STN problems during the shoot elongation and rooting stages. Therefore, we supplemented the nutrient medium with calcium chloride, ascorbic acid, silver nitrate, and boric acid at different concentrations and tested their impact in alleviating the STN during the stages of shoot elongation rooting of fegra fig.

Materials and Methods

Study location and experimental design. This study was conducted in the plant tissue culture laboratory, at the College of Food and Agriculture Sciences, King Saud University. The experiments were arranged in a completely randomized design.

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Plant material and description of experiments. In vitro axillary shoots of fegra fig were multiplied onto MS medium (Murashige and Skoog, 1962) containing 3% sucrose, and supplemented with 2 mg/L BAP (Fig. 1A). The pH of the medium was adjusted to 5.8 before autoclaving (121 °C and 1.2 $kg \cdot cm^{-2}$ for 15 min), and the medium was gelled with 0.8% (w/v) agar-agar. The cultures were then incubated at 25 ± 2 °C under 16/8-h light/dark photoperiod provided with 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density (PPFD) provided by cool white fluorescent tubes; PPFD was measured using a luminous intensity meter (Testo 545; Testo, Melrose, MA, USA). After 6 weeks of incubation, the regenerated axillary shoots of fegra fig were transferred onto MS medium containing 3% sucrose and 1.5 mg/L activated charcoal without plant growth regulators and incubated for 3 weeks under dark conditions for their elongation (Fig. 1B) followed by 4 weeks under light conditions. A majority of the elongated shoots (80%) exhibited STN upon their transfer to light conditions (Fig. 1D and E).

Treatments to alleviate STN during the elongation stage. To alleviate STN, microshoots of fegra fig (10–15 mm in length with pair of young leaves) were inoculated to Magenta culture vessels (six shoots per culture vessel) containing 60 mL MS medium with 3% sucrose and 1.5 mg/L activated charcoal, and supplemented with various concentrations of calcium chloride (0, 40, 80, and 120 mg/L), ascorbic acid (0, 50, 100, and 150 mg/L), silver nitrate (0, 1, 2, and 3 mg/L) or boric acid (0, 9.3, 12.4, and 15.5 mg/L). The pH of the medium was adjusted to 5.8 before autoclaving (121 °C and 1.2 $kg \cdot cm^{-2}$ for 15 min) and the medium was gelled with 0.8% (w/v) agar-agar. There were four replicates in each treatment, and each replicate was represented by a Magenta vessel containing six explants. The cultures were incubated for 3 weeks in the dark and at 25 ± 2 °C. Subsequently, cultures were incubated at 25 ± 2 °C under 16/8-h light/dark photoperiod provided with 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD provided by cool white fluorescent lights.

Measurements of morphological parameters. After 4 weeks of light incubation, the percentage of necrotic shoots, number of shoots per explant, shoot length, number of roots, root length, fresh weight, and dry weights were recorded.

Measurements of leaf pigment content. Fresh leaves (0.1 g) of fegra fig axillary shoots treated with silver nitrate (3 mg/L), which gave the best result in controlling STN, as well as the control treatment (with or without silver nitrate of healthy and necrotic shoots), were placed in a glass test tube containing 10 mL of 80% acetone. The test tubes were placed in a refrigerator at 4 °C under dark conditions for 48 h. After checking the turbidity of the extract, the absorbances of chlorophyll a, chlorophyll b, and carotenoids were measured at wavelengths of 663.2, 646.8, and 470.0 nm, respectively, using a spectrophotometer (T60 ultraviolet/Visible Spectrophotometer; PG Instruments Ltd., Lutterworth, UK). The quantities of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids in the leaves were calculated according to (Smart 1974) using the following formula:

$$\text{Chlorophyll a } mg \cdot g^{-1} = [(12.7 \times O.D\ 663.2) - (2.69 \times O.D\ 646.8)] \times V/1000 \times FW$$

$$\text{Chlorophyll b } mg \cdot g^{-1} = [(22.9 \times O.D.\ 646.8) - (4.68 \times O.D\ 663.2)$$

$$\text{Total chlorophyll } mg \cdot g^{-1} = [(20.2 \times O.D\ 646.8) + (8.02 \times O.D.\ 663.2)] \times V/1000 \times FW$$

$$\text{Carotenoids } mg \cdot g^{-1} = [O.D\ 470 + (0.114 \times O.D\ 663.2)] \times (0.638 \times O.D\ 646.8)$$

where O.D = the optical density of the extract at the indicated wavelength, V = the volume of the extract (mL), and FW = the fresh leaf weight (g).

Measurement of leaf relative water content. Leaf relative water content (RWC) was determined using leaf disks (1 cm) for the samples (with or without silver nitrate of healthy and necrotic shoots) and calculated as follows:

$$(Wfresh - Wdry)/(Wturgid - Wdry) \times 100$$

where Wfresh is the weight of the freshly harvested sample, Wturgid is the turgid weight after saturating the sample with distilled water for 24 h at 4 °C, and Wdry is the oven-dry weight of the sample dried at 70 °C for 48 h (Weatherley 1950).

Leaf electrolyte leakage. Leaf samples (with or without silver nitrate of healthy and necrotic shoots) were incubated in vials containing 100 mL of distilled water for 24 h, and the primary electrolyte leakage (EL; EC1) was recorded using the multiparameter bench meter. The vials were then placed in an autoclave at 121 °C and 1.2 atm for 20 min. After being allowed to cool, the secondary EL (EC2) was recorded. Leaf EL was calculated as follows:

$$(EC1 - EC0)/(EC2 - EC0) \times 100$$

where EC0 is the electrical conductivity of distilled water (Sairam 1994).

Determination of total phenolic content. The content of total phenolics was determined in the leaf methanolic extract of fegra fig (with or without silver nitrate of healthy and necrotic shoots) by a colorimetric method according to a technique established by (Singleton et al. 1999) with modifications. One hundred microliters of methanolic extracts were mixed with 2.5 mL deionized water, followed by the addition of 0.1 mL (2N) Folin–Ciocalteu reagent. They were mixed well and allowed to stand for 6 min before 0.5 mL of a 20% sodium carbonate solution was added. The color was developed after 30 min at room temperature. The absorbance of the mixtures was measured using an ultraviolet (UV)-visible spectrophotometer at a wavelength of 765 nm, and the

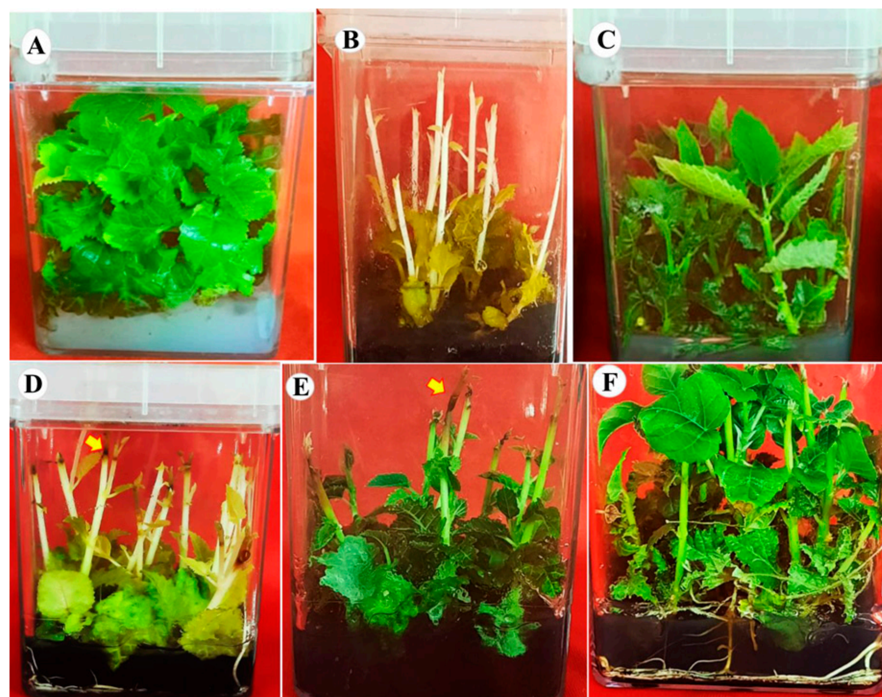

Fig. 1. Occurrence of shoot tip necrosis during elongation stage of fegra fig microshoots. (**A**) Multiple axillary shoots formed onto Murashige and Skoog medium supplemented with 2 mg/L benzylaminopurine after 6 weeks of culture. (**B**) Elongated shoots after 3 weeks dark incubation. (**C**) Normal elongated shoots. (**D** and **E**) Shoots exhibiting shoot-tip necrosis (STN; arrow) upon their transfer to light incubation after 1 and 4 weeks, respectively. (**F**) Alleviation of STN with 3 mg/L silver nitrate treatment after 4 weeks light incubation.

total phenolic content in the leaf samples was calculated from the calibration curve (y = 0.0021x + 0.0245) using gallic acid as the reference standard. The results were expressed in terms of milligrams of gallic acid equivalents (GAE) per gram dry weight of the extract.

Determination of total flavonoid content. Total flavonoid content was determined in fegra fig leaves (with or without silver nitrate of healthy and necrotic shoots) by a colorimetric method according to (Ordonez et al. 2006). One milliliter of leaf extract (1 mg/mL) and 1.0 mL of 2% aluminum chloride solution were placed in a test tube. After 30 min of incubation at room temperature, the absorbance was measured immediately at 420 nm using a UV-visible spectrophotometer. Using a quercetin solution (50–800 μg/mL) to generate a standard curve (R^2 = 0.9996) the standard solution was prepared. The flavonoids in the extracts were expressed as quercetin (mg/g dry weight [DW]) using the calibration curve equation (y = 0.0011x + 0.0928), where y was the absorbance and x was the quercetin equivalent concentration (mg/g).

Determination of total tannins. The total tannins in fegra fig leaf extracts (with or without silver nitrate of healthy and necrotic shoots) were determined using the method reported by Kavitha Chandran and Indira (2016) with modifications. One hundred microliters of the extract (1 mg/mL) was mixed with 200 μL of Folin–Ciocalteu reagent, 1.5 mL of ultrapure water, and 200 μL of 35% sodium carbonate solution, the mixture was thoroughly shaken after that, with an incubation period of thirty minutes at room temperature in the dark. The absorbance of the samples was measured at a wavelength of 700 nm using a ultraviolet-visible spectrophotometer. The total tannin content in the leaf extracts was determined by using tannic acid as a reference standard and the calibration curve (y = 0.0052x + 0.0021). where y denoted absorbance and x denoted the tannic acid equivalent concentration (mg/g).

Statistical analysis. Data expressed as percentages were arcsine transformed before analysis. All data were subjected to analysis of variance and Tukey's multiple range tests using the SAS Program (Version 9.13; SAS Institute, Cary, NC, USA).

Results

Shoot tip necrosis. During micropropagation of fegra fig on MS medium supplemented with 2 mg/L BAP, we observed optimal shoot proliferation, and no STN event cultures were maintained under dark conditions; however, when the cultures were transferred to the light and dark photoperiod, we observed growth and elongation of shoots. Nevertheless, STN was invariably observed in 80% of shoots (Fig. 1D and E). The necrotic shoots progressively extended from the tip to the bottom of the shoots, which leads to the death of the shoot during the multiplication and rooting stages. We tested the effect of supplementation of calcium chloride (40, 80, and 120 mg/L), ascorbic acid (50, 100, and 150 mg/L), silver nitrate (1, 2, and 3 mg/L), and boric acid (9.3, 12.4, and 15.5 mg/L), and the results are presented in Fig. 2 and Table 1. Supplementation of calcium chloride reduced the STN considerably, and the percentage of STN was 39.98% with supplementation of 120 mg/L of calcium chloride. The addition of boric acid to the nutrient medium also reduced STN, with 46.66% of shoots exhibiting STN. Augmentation of ascorbic acid was also beneficial; with the addition of 150 mg/L, STN was reduced from 80% to 56.68%. Supplementation of mercuric chloride was highly beneficial when compared with all the treatments and the addition of 3 mg/L reduced the STN from 80% to 23.34% (Fig. 2). Therefore, in subsequent shoot elongation and rooting experiments, we supplemented the nutrient medium with 3 mg/L mercuric chloride kept the cultures initially in the dark for 3 weeks, and subsequently incubated the cultures in the 16 h light and 8 h dark photoperiod, ascorbic acid (50, 100, and 150 mg/L), silver nitrate (1, 2, and 3 mg/L), and boric acid (9.3, 12.4, and 15.5 mg/L on number of shoots per explant, length of shoots, number of roots per explant, and length of the main root are presented in Table 1.

Shoot morphological characteristics. Silver nitrate treatment was superior compared with all other treatments, and the number of shoots, length of the main shoot, number of roots, and length of the main root was 1.9 per explant, 11.3 cm, 2.1 roots/explant, and 5.4 cm, respectively, with the addition of 3 mg/L of silver nitrate (Table 1). The treatment of silver nitrate was statistically significant as per Tukey's test. The influence of argumentation of varied factors on shoot, root fresh, and dry biomass data are presented in Table 2. Although there was much variability in the biomass data, 3% silver nitrate was found to be highly superior for the alleviation of STN in shoot growth and rooting of shoots.

Leaf pigment contents, RWC, and EL. We estimated the impact of STN on the accumulation of chlorophyll a, chlorophyll b, total chlorophylls, carotenoids, RWC, and EL in the leaves of healthy (Fig. 1C), STN (Fig. 1E) and plants grown with the treatment of 3 mg/L silver nitrate (Fig. 1F), and data are presented in Fig. 3. The healthy plants that did not have STN had 2.33 mg/g FW; 1.47 mg/g FW; 3.84 mg/g FW; and 1.5 mg/g FW of chlorophyll a, chlorophyll b, total chlorophyll content, and carotenoids, respectively. The photosynthetic pigments were drastically reduced in STN leaves, with concentrations of 1.23, 0.08, 1.34, and 0.40 mg/g FW, respectively (Fig. 3). The plants that were treated with 3 mg/L silver nitrate showed recovery in the biosynthesis of photosynthetic pigments and contained 1.81, 0.79, 2.63, 0.82 mg/g FW of chlorophyll a, chlorophyll b, total chlorophylls, and carotenoids. The RWC (68.39% vs. 76.19% control plants) and EL (17.87% vs. 14.09%) were high in plants that developed STN (Fig. 3). The plants that were grown with the supplementations of silver nitrate recovered with relative water content (72.57%) and electrolyte leakage (14.94%) (Fig. 3).

Shoots phytochemical composition. Quantification of total phenolics, flavonoids, and total tannins in microshoots of healthy plants, necrotic, and silver-nitrate-treated cultures is presented in Table 3. The results reveal that the STN microshoots possessed 91.93 mg GAE/g DW of phenolics, 57.63 mg QE/g DW flavonoids, and 26.33 mg TAE/g DW tannins, and the amount of phenolics, flavonoids, and tannins was reduced with healthy microshoots. However, with the silver-nitrate-treated (3 mg/L) shoots, there was a recovery in the accumulation of phenolics (51.93 mg GAE/g DW), flavonoids (47.17 mg QE/g DW), and tannins (19.78 mg TAE/g DW).

Discussion

Micropropagation of plants using in vitro techniques can lead to several problems, such as hyperhydricity or STN. These phenomena may lead to the regeneration of unhealthy plants and to death of regenerated plants. STN leads to the browning terminal portion of shoots, which resembles symptoms of mineral deficiency and leads to the death of the shoots or plantlets. STN was recorded both during shoot establishment and multiplication of Tunisian local varieties of *Ficus carica*—namely, 'Zidi', 'Soltani', 'Bither Abiadh', and 'Assafri' (Bayoudh et al. 2015). Additionally, STN was recorded in micropropagation of *Pistacia vera* (Abousalim and Mantell 1994;

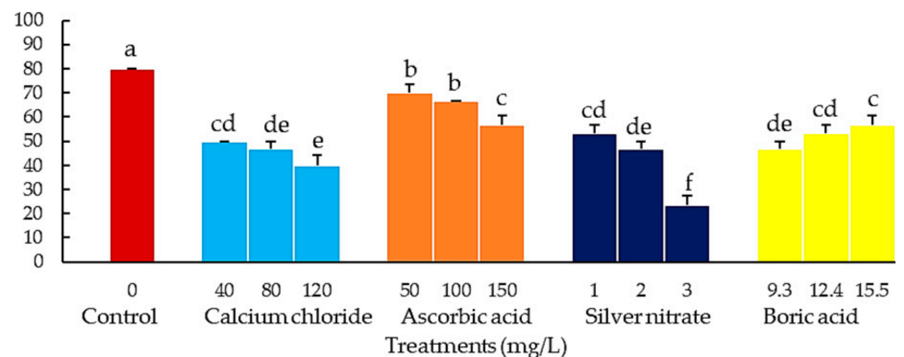

Fig. 2. Percentage of fegra fig microshoots exhibiting shoot tip necrosis in response to calcium chloride, ascorbic acid, silver nitrate, and boric acid treatments during elongation stage (3 weeks under dark followed by 4 weeks incubation under light conditions).

Table 1. Effect of calcium chloride, ascorbic acid, silver nitrate, and boric acid on shoot tip necrosis and growth characteristics of fegra fig microshoots grown on Murashige and Skoog medium with 1.5 mg/L activated charcoal (3 weeks under dark followed by 4 weeks incubation under light conditions).

Treatments	Conc. (mg/L)	No. of shoots/explant	Length of the main shoot (cm)	No. of roots/explant	Length of the main root (cm)
Control	0	2.0 bc	9.4 de	4.0 a	8.4 cde
Calcium chloride	40	1.7 ef	10.2 bcd	3.8 ab	7.4 fg
	80	1.7 ef	11.0 abc	3.4 cd	11.2 a
	120	1.8 def	11.7 a	3.2 d	11.6 a
Ascorbic acid	50	1.9 cde	9.2 de	3.6 bc	6.8 g
	100	1.6 f	9.3 de	2.6 e	7.8 ef
	150	1.6 f	9.8 cde	2.5 e	8.2 def
Silver nitrate	1	2.7 a	7.7 f	3.2 d	5.6 h
	2	2.1 b	9.4 de	2.6 e	5.8 h
	3	1.9 bcd	11.3 ab	2.1 f	5.4 h
Boric acid	9.3	1.8 def	9.4 de	3.4 cd	10.0 b
	12.4	1.8 def	8.8 ef	3.4 cd	9.2 bc
	15.5	2.6 a	9.8 cde	2.6 e	8.8 cd
P values					
Treatment		<0.0001***	0.0001***	<0.0001***	<0.0001***
Concentration		0.001***	0.0001***	<0.0001***	<0.0001***
Treatment × Concentration		<0.0001***	0.015*	<0.0001***	<0.0001***

Values in each column followed by the same letter are not significantly different at $P \leq 0.05$ according to Tukey's multiple range test. *, ***Significant at $P \leq 0.05$ and $P \leq 0.001$, respectively. 1 cm = 0.3937-inch; 1 g = 0.0353 oz.

Barghchi and Alderson 1996), *Castanea sativa* (Piagnani et al. 1996; Xing et al. 1997), *Quercus* spp. (Vieitez et al. 1989), *Vitis vinifera* cultivars (Surakshitha et al. 2019; Thomas 2000), and numerous other plants. The reasons for the development of STN during micropropagation are not well understood; however, mineral deficiency of elements such as calcium or boron; concentrations of auxins or cytokinins, or a combination of both; high humidity; ethylene production and accumulation during the growth of propagules inside the culture vessel; choice of nutrient medium; and genotype-specific responses are some causes responsible for STN (reviewed by Teixeira da Silva et al. 2020). Therefore, supplementation of nutrient medium with calcium and boron, antioxidants, and silver nitrate; altering the concentration of plant growth regulators and culture conditions such as humidity; and aeration are some of the measures to overcome STN during in vitro propagation (Teixeira da Silva et al. 2020). For example, it was suggested that the cultures in half-strength MS media supplemented with 1 mg/L BAP, 180.18 mg/L of calcium, and 1.08 mg/L of boron with 2 weeks subculture interval in the initial subculture phase to alleviate the incidences of STN grape cv. Red Globe (Surakshitha et al. 2019). Similarly, supplementation of calcium and boron, reducing the concentration of benzyl adenine in the nutrient medium, and frequent subculturing are the measures followed for overcoming STN problems during micropropagation of *Pistacia vera* (Abousalim and Mantell 1994) and *Vitis vinifera* (Surakshitha et al. 2019).

In the current studies, we adopted supplementation of MS medium with supplementation of calcium chloride (40, 80, and 120 mg/L), ascorbic acid (50, 100, and 150 mg/L), silver nitrate (1, 2, and 3 mg/L), and boric acid (9.3, 12.4, and 15.5 mg/L) and assessed incidence of STN and evaluated growth, biomass, accumulation of chlorophyll pigments, RWC, and EL during shoot elongation and rooting of fegra fig plantlets. Supplementation of calcium chloride at 120 mg/L reduced STN by 40%, and this treatment was also beneficial in enhancing the growth of shoots and roots (Fig. 1 and Table 1). Similarly, Piagnani et al. (1996) and Abousalim and Mantell (1994) achieved excellent results in overcoming the STN with in vitro chestnut and pistachio cultures, respectively. We also experimented with the addition of boric acid to alleviate the STN abnormalities in fegra fig in vitro cultures; again, the addition of 9.3 mg/L of boric acid reduced STN by 34% and also encouraged growth recovery of shoots and roots (Fig. 1 and Table 1). These results are in agreement with earlier reports in which the addition of boric acid to the cultures alleviated STN in *Pisticia vera* (Abousalim and Mantell 1994), *Pisticia* hybrid UCB1 (Nezami et al. 2015), and *Harpagophytum*

Table 2. Effect of variance concentrations of calcium chloride, ascorbic acid, silver nitrate, and boric acid on microshoots growth characteristics of fegra fig microshoots grown on Murashige and Skoog medium with 1.5 mg/L activated charcoal (3 weeks under dark followed by 4 weeks incubation under light conditions).

		Fresh wt (g)			Dry wt (g)		
Treatments	Conc. (mg/L)	Shoot	Root	Total	Shoot	Root	Total
Control	0	0.837 ab	0.093 de	0.930 bcd	0.118 abc	0.011 b	0.129 abcd
Calcium chloride	40	0.677 d	0.087 de	0.764 ef	0.118 abc	0.011 b	0.129 abcd
	80	0.760 bcd	0.190 b	0.950 abcd	0.128 ab	0.020 a	0.149 a
	120	0.831 ab	0.245 a	1.076 a	0.125 ab	0.021 a	0.146 a
Ascorbic acid	50	0.810 abc	0.116 d	0.926 bcd	0.121 abc	0.011 b	0.132 abcd
	100	0.771 bcd	0.069 e	0.840 def	0.105 cd	0.007 bc	0.112 de
	150	0.920 a	0.081 e	1.001 abc	0.136 a	0.011 b	0.147 a
Silver nitrate	1	0.814 abc	0.083 de	0.898 bcde	0.122 abc	0.011 b	0.133 abc
	2	0.767 bcd	0.071 e	0.838 def	0.111 bcd	0.009 b	0.121 cde
	3	0.697 cd	0.027 f	0.724 f	0.098 d	0.004 c	0.101 e
Boric acid	9.3	0.691 cd	0.179 bc	0.870 cde	0.109 bcd	0.021 a	0.130 abcd
	12.4	0.690 cd	0.087 de	0.777 ef	0.116 bcd	0.009 b	0.125 bcd
	15.5	0.887 ab	0.153 c	1.041 ab	0.124 abc	0.017 a	0.141 ab
P values							
Treatment		0.045	<0.0001***	0.048*	0.209^{NS}	<0.0001***	0.008**
Concentration		0.029	0.063^{NS}	0.016*	0.599^{NS}	0.193^{NS}	0.425^{NS}
Treatment × concentration		0.025	<0.0001***	<0.0001***	0.036 *	<0.0001***	0.003**

Values followed by the same letter in each column are not significantly different at $P \leq 0.05$ according to Tukey's multiple range test. NS, *, **, ***Not significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. 1 g = 0.0353 oz.

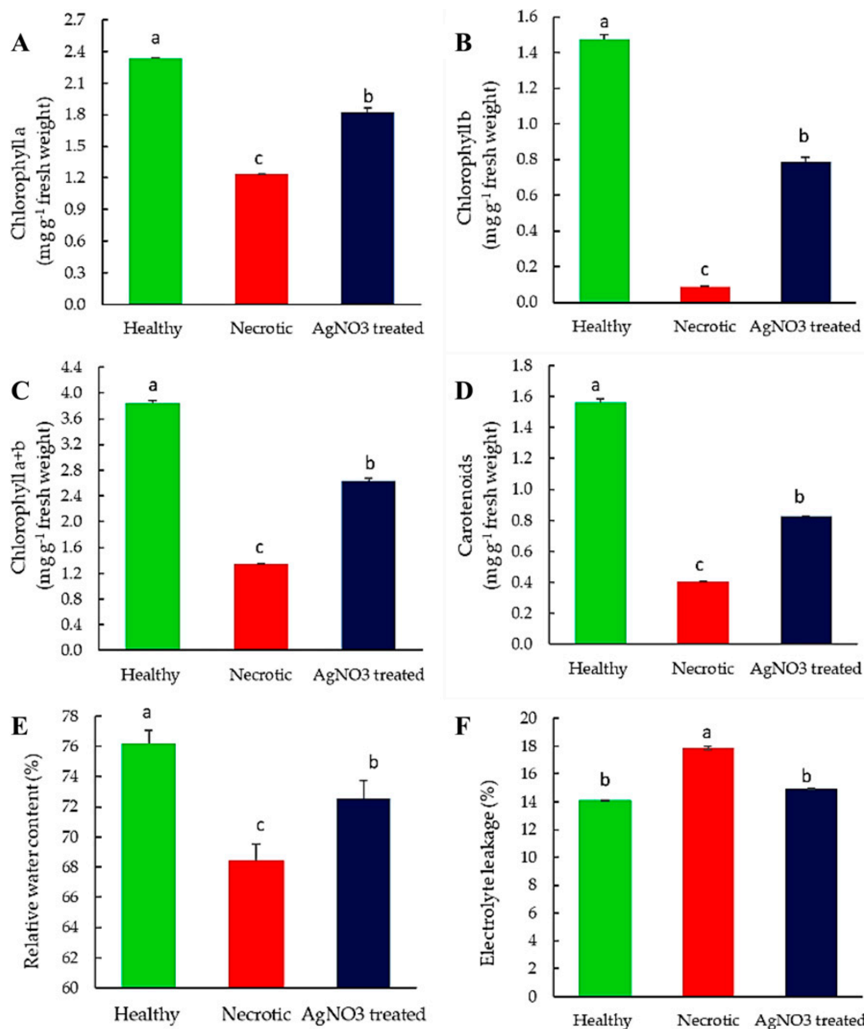

Fig. 3. (**A**) Chlorophyll a, (**B**) chlorophyll b, (**C**) chlorophyll a+b, (**D**) carotenoids, (**E**) relative water content, and (**F**) electrolyte leakage of healthy, necrotic and $AgNO_3$ treated (3 mg/L) microshoots of fegra fig during elongation stage (3 weeks under dark followed by 4 weeks incubation under light conditions). Different letters within a set of values in each figure denote significant differences at $P \leq 0.05$ according to Tukey's multiple range test.

procumbens (Liskova et al. 2016) tissue cultures. Supplementation of ascorbic acid to the fegra fig in vitro cultures led to recovery of STN by 14% and restoration of shoot and root growth in the cultures. Ascorbic acid is a multifunction molecule that acts as an enzyme cofactor, detoxifies reactive oxygens species generation, regulates stress responses, and controls the cell cycle activities and cell division in plants (Gallie 2013). Amalia et al. (2014) reported reduction of STN of *Rubus idaeus* shoots when 50 or 100 mg/L of ascorbic acid was used. The reduction of STN was also recorded in *Pterocarpus marsupium* (Jaiswal et al. 2013) and *Jatropha curcus* (Misra et al. 2010) tissue cultures. In our experiments, we observed that supplementation of silver nitrate was highly useful in the reduction of STN, and with supplementation of 3 mg/L silver nitrate, we observed a 57% reduction of STN. Supplementation of silver nitrate also favored shoot and root growth and recovered the synthesis of photosynthetic pigments (chlorophylls and carotenoids), physiological conditions of leaves (i.e., RWC and EL) from the leaves. Ag^+ ions in plant tissue culture medium were reported to prevent varied ethylene-induced plant responses, including growth inhibition, and senescence (Beyer et al. 1984). Therefore, supplementation of silver nitrate in the plant culture medium effectively reduced the incidences of STN in *Quercus rubra* (Vieitez et al. 2009) and *Rosa hybrida* (Park et al. 2016). The role of silver nitrate in modulating plant growth and development was probably mediated by the regulation of polyamines, ethylene- and calcium-mediated pathways as interpreted by Kumar et al. (2009). Our results indicate that STN microshoots have high phenolic content compared with healthy microshoots of fegra fig. It has been reported that a sufficient calcium supplement impedes STN by suppressing the accumulation of phenolic compounds and hence programmed cell death (PCD) (Teixeira da Silva et al. 2020). According to Gaspar et al. (2002), PCD is triggered when oxidative stress is no longer controlled. Demidchik (2015) noted that PCD manifests the death signal in response to H_2O_2 and other reactive oxygen species. Beckman (2000) additionally postulated that specialized cells trigger cell suberization and lignification due to the build-up of phenolic compounds, consequently promoting PCD.

In conclusion, a large number of fegra fig axillary shoots exhibited symptoms of STN during the elongation stage. The supplementation of 120 mg/L of calcium chloride, 15.5 mg/L boric acid, 150 mg/L ascorbic acid, and 3 mg/L silver nitrate were useful in alleviating STN optimally. These results corroborate the earlier reports on the role of calcium, boron ions, and ascorbic acid (antioxidant) in the reduction of STN events during in vitro propagation. However, silver nitrate was found to be superior in reducing the incidence of STN in regenerated shoots. It is recommended to incorporate silver nitrate (3 mg/L) in the nutrient medium to overcome STN in *F. palmata* axillary shoots. Our results show that shoot necrosis events also affect secondary metabolite biosynthesis.

Table 3. Total phenolics, flavonoids, and tannins contents of fegra fig microshoots grown on MS medium with 1.5 mg/L activated charcoal (3 weeks under dark followed by 4 weeks incubation under light conditions).

Plant materials	Total phenolics (mg GAE/g DW)	Total flavonoids (mg QE/g DW)	Total tannins (mg TAE/g DW)
Healthy	37.19 c	42.98 c	17.37 c
Necrotic	91.93 a	57.63 a	26.33 a
$AgNo_3$ treated (3 mg/L)	51.93 b	47.17 b	19.78 b
P value	<0.0001***	<0.0001***	<0.0001***

Values followed by the same letter in each column are not significantly different at $P \leq 0.05$ according to Tukey's multiple range test. ***Significantly different at $P \leq 0.001$.

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