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Full Length Article

Effect of Silver Nitrate, Trichloroacetate, and Culture Ventilation on Hyperhydricity and Shoot Regeneration of Sunflower *In Vitro*

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Abstract

Hyperhydricity (HH) is a morphological and physiological condition widespread in plant tissue culture that can potentially result in economic losses for the plant micropropagation economy. This study was aimed at investigating the effects of various media supplements; silver nitrate ($AgNO_3$), trichloroaetate (TCA), and their combinations on tissue culture of sunflower under ventilation and non-ventilation conditions. The results showed that during the shoot induction phase, plant regeneration and HH percentages were unaffected by supplements and culture conditions, but shoot length was significantly affected. Ventilated plants grew better than non-ventilated ones. At the shoot elongation phase, there were no changes in the regeneration and HH percentages compared to the induction phase. High shoot length and number of leaves per shoot showed a significant relationship between $AgNO_3$ and culture ventilation, which improved sunflower growth. The shoot regeneration, leaf number, and stomatal density were all increased by the addition of 1 mg/L $AgNO_3$. In contrast, TCA seemed toxic to the plant and reduced plant growth and development. Even though $AgNO_3$ enhanced plant growth, combining two supplements harmed the plants since TCA's toxic effects dominated. However, across all investigated conditions, neither supplement types nor culture conditions had a significant impact on the HH of sunflower, despite prior claims on HH. The ventilated plants had noticeably higher growth, and this is expected to help raise the quality of plant tissue culture further. © 2023 Friends Science Publishers

Keywords: Growth; *Helianthus annuus*; Hyperhydricity; Plant tissue culture; Stomatal density

Introduction

Sunflower (*Helianthus annuus* L.) is an essential oilseed crop that belongs to the Asteraceae family. Globally, 50 million tons of sunflower seed was produced in 2019, averaging 7,000 million USD in profits. Most sunflower oilseed production comes from European nations like Ukraine and Russia (Havrysh *et al*. 2020). Sunflower oil is high in polyunsaturated fats and thus is becoming more common as a healthy replacement. As an ornamental plant, sunflowers are unsurpassed in their beauty and vibrancy, making them popular worldwide. The development of new cultivars offers a high product yield and great oil quality for oil seed sunflower, while a wide range of flower colors and shapes for the ornamentals fulfill the sunflower market's high demand (Kaya *et al*. 2012).

The sunflower underwent breeding and genetic selection. Two main techniques, conventional breeding and *in vitro* technologies, have been used to produce genotypes with desired traits (Davey and Jan 2010). Due to the initial breeding studies, cultivars with better agronomic characteristics such as high fat content, pest resistance etc. were created (Vassilevska-Ivanova *et al*. 2014). Tissue culture technology is a powerful technique for rapid propagation and virus-free production of plantlets. It is used to improve plant breeding in agribusiness and biological research practices. This method is required to produce the genetic variation in sunflowers cultivars that are better tolerant of stress conditions (drought, salt, etc.) and flower aesthetic variety (Dagustu 2018). The genotype, the explant type, the culture media, the concentration, the type of growth regulators, and the culture conditions altogether serve a key role in the success of sunflower regeneration. However, there are many issues with plant regeneration, including precocious flowering, inadequate germination, and HH of tissue (Nestares *et al.* 1996).

The HH is a morphological and physiological disorder in plant tissue culture (Pâques 1991). Shoots from HH plants have unusual shoot growth, a translucent appearance, an accumulation of water in the tissues, and less lignin in the cell walls. The condition affected many plant species, including herbaceous, woody, and succulent plants such as carnation, eggplant, cabbage, sunflower, apple and aloe (Gao *et al.* 2017). It results from stressful circumstances

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generated by apoplast waterlogging, which causes hypoxia and severe oxidative damage. HH issues can be caused by the limited environment of culture containers, excessive relative humidity, poor gaseous exchange, and ethylene buildup (Kevers *et al.* 2004). Ethylene, a plant hormone, also could stimulate shoot HH, decrease chlorophyll contents, and cause tissue necrosis (Iqbal *et al.* 2017; Gao *et al.* 2018). Plantlets with HH symptoms poorly survive when transferred to an *ex vitro* environment (Gaspar *et al.* 1995; Sen and Alikamanoglu 2013).

The HH can be restrained by controlling the ethylene level using silver nitrate ($AgNO_3$) or silver nanoparticles (AgNPs) as an inhibitor of ethylene activity (Gaspar 1986; Sreelekshmi and Siril 2021). The addition of cobalt chloride stimulates shoot regeneration and strongly prevents ethylene production (Chraibi *et al.* 1992). Klerk and Pramanik (2017) used trichloroacetate (TCA), as an inhibitor of wax biosynthesis, to prevent the development of HH since TCA increases plant transpiration rate and reduces waterlogged in plantlets. Additionally, by increasing gas exchange in culture vessels, the vessel ventilation could lower the gaseous ethylene level and shoot HH (Lai *et al*. 2005).

The silver ion has been used to prevent or restore HH in many plants, such as sunflower (Mayor *et al*. 2003), blueberry (Gao *et al*. 2018), watermelon (Vinoth and Ravindhran 2015), and pinks *Dianthus chinensis* L. (Sreelekshmi and Siril 2021). However, there was a decrease in plant growth with increasing concentrations because high concentrations of silver inhibit plant growth due to its toxicity to plants. However, the optimal concentrations may differ depending on the plant's nature and species. For example, Tamimi (2015) studied the effect of $AgNO_3$ in banana (*Musa acuminata* L.). It was used to enhance *in vitro* growth, with concentrations at up to 10 mg/L increasing the number of shoots and the size of the leaves. In addition, the toxicity of silver was studied in several plants such as squash (*Cucurbita pepo*) (Musante and White 2012), rocket (*Eruca sativa*) (Vannini *et al*. 2013), tobacco (Štefanić *et al*. 2018), and tomato (Noori *et al*. 2020). It was found that silver ion inhibited plant growth, reduced plant biomass and transpiration, caused oxidative stress, decreased chlorophyll contents, induced cell death, and DNA damage through generation of ROS, and damaged the cell morphology and its structural features. However, silver ion has both positive and negative effects on plant growth and development. These contradictory results indicate that the complexity of plants' responses to silver is also dependent on the plant system used (species, tissue, organ, developmental stage, etc.) and experimental methodology, such as the media type and culturing time (Yan and Chen 2019).

TCA is widely used as an herbicide for weeds. TCA treatment alters the permeability of cell membranes and lowers leaf wax release. This was demonstrated in Arabidopsis plants, where 1 mM TCA strongly reduced the amount of wax and strongly increased the permeability of leaves for water, resulting in fully prevented HH (De Klerk and Pramanik 2017).

According to Mayor *et al.* (2003), $AgNO_3$ is used to minimize HH in sunflower. However, there are no studies on TCA and using $AgNO_3$ in combination with sunflower tissue culture. Consequently, the objective of this research was to assess the impact of various media supplements ($AgNO_3$, TCA, and their combination) on the occurrence of HH and the effectiveness of regeneration in sunflower tissue culture. To better understand the influence of anti-HH supplements on HH in sunflower, the combined effects of $AgNO_3$ and TCA with ventilation and non-ventilation treatment were assessed.

Materials and Methods

Plant materials

Plant materials used in this study were sunflower genotype, Suranaree 473 (S473), received from SUT farm, Suranaree University of Technology, Thailand. Seeds were firstly sterilized with sodium hypochlorite (1.8%, v/v) for 30 min and rinsed with sterile distilled water 3 times in a laminar flow hood. Seed hull was removed and dehulled seeds were cleaned with 3% hydrogen peroxide solution for 30 sec. After that, seeds were placed on moist sterile tissue paper in a Petri dish plate. These seeds were kept in the tissue culture room under dark condition and 25±2°C for 2 days before using as explant materials in the first experiment. Before cultured explants on the media, germinated seeds were cut to remove the radicle and cotyledons. Obtained explants, about 3 mm, carrying the meristem and a base of cotyledon were used for the experiments.

Effect of media supplements and ventilation on HH and sunflower growth at shoot induction phase

Plant media were prepared by using Murashige and Skoog (MS) (Murashige and Skoog 1962) containing 2 mg/L BA and 30 g/L sucrose as the basal of shoot induction medium (SIM). The SIM was supplemented with either 1 mg/L $AgNO_3$, 100 mg/L TCA, or 1 mg/L $AgNO_3$ + 100 mg/L TCA for the supplement combination test (Mayor *et al.* 2003; De Klerk and Pramanik 2017). SIM with supplements were divided into two sets of culture conditions. Ventilation (using the vented plastic cap) and non-ventilation (using the normal plastic cap) conditions were applied. The vented cap had 1 cm diameter punched hole with 0.2 μm filter for ventilation. A total of eight media were tested, including A1 to A4V. Media were solidified with 8 g/L of agar, and the pH was adjusted to 5.7±0.1 (with 1 N HCl or 1 N NaOH) before autoclaving at 121°C for 20 min.

The culture vessels were 480 mL glass bottles (12 cm × 7 cm) with a normal plastic cap or vented plastic cap, depending on the experimental treatment. Each bottle contained 50 mL of medium and 4 explants. Eight

treatments in total included 20 explants per replicate, and 3 replicates (for a total of 480 explants) were investigated. Culture conditions were standardized with equal light intensity (Panasonic FL40SS-D/36, 36W, 2600 lumens), 16/8-h (day/night) photoperiod, 25±2°C for all treatments. Explants were randomly selected to place into one of the possible media treatments. Cotyledon explants were grown for 3 weeks in shoot induction phase, and then HH was determined.

All shoots (explant stems with true leaves) greater than 5 mm were measured in length and counted by eyes. The percentage of HH shoots, regeneration percentage (explants with shoots), the average number of shoots per explant, and average shoot length were calculated and averaged in triplicates using the formulas below.

$$\text{HH shoots (\%)} = \frac{\text{No. of HH shoot}}{\text{Total of shoots}} \times 100$$

$$\text{Shoot regeneration (\%)} = \frac{\text{No. of explant with shoot}}{\text{Total of explants}} \times 100$$

$$\text{Average number of shoots per explant} = \frac{\text{No. of shoots}}{\text{Total of explants}}$$

$$\text{Average shoot length} = \frac{\text{Total of shoot length}}{\text{Total of explants}}$$

After the data were collected, all the explants were continued to grow in the same culture. It is to be noted that this experiment had no subculture or transfer of explants along with both shoot induction and elongation phase.

Effect of media supplements and ventilation on HH, shoot regeneration, shoot length, water content, and stomatal density of *in vitro* in sunflower at shoot elongation phase

Explants from the shoot induction phase were continuously cultured on the same SIM for two weeks as the shoot elongation phase for further multiplication and shoot elongation. Then HH percentage, regeneration percentage, number of shoots per explant, and average shoot length were determined as stated in the first experiment. Twenty explants from each treatment were taken at random as samples to measure the water content. The analytical balance was used to weigh the explant samples, and the initial fresh weight was collected. The samples were then dried for three days in a hot air oven at 80°C. The water content was determined after taking dry weights. Twenty randomly chosen leaf samples from the treatment were used to quantify the stomatal density. The abaxial epidermis of the leaves was peeled off and then stained with toluidine blue. The epidermis was observed under a light microscope (Olympus CH-2, Japan). Images were captured using a microscope camera (Moticam X3 Plus, USA). Stomatal density was measured using a digital ruler in Motic Images Plus 3.0 digital microscope/camera software. Finally, the mean number of stomata per square millimeter (mm^2) was calculated.

Rooting and acclimatization

After 2 weeks of culture in the elongation phase, elongated shoots from the best performance medium were transferred to root induction medium modified from Sujatha *et al.* (2012), a half-strength MS medium supplemented with 0.5 mg/L NAA and 200 mg/L charcoal for rooting. The percentage of root induction was calculated after 2 weeks. Complete plantlets with shoots and roots were acclimated at room temperature for 2 weeks. After that, complete healthy plantlets were transplanted into the soil and grown to maturity. The plants that survived after transplantation into the soil at 3 weeks were counted.

Statistical analysis

A completely randomized design (CRD) of experiments was used in this study. The mean value from three replicates was used to examine the experimental data, and significant mean differences were determined using Duncan's multiple range tests (DMRT) with a test level of 0.05%. The analysis was done using IBM SPSS (v. 25.0) program. Each treatment consisted of 20 explants per replicate with 3 replicates.

Results

Effect of media supplements and ventilation on HH, shoot regeneration, and shoot length of *in vitro* sunflower at shoot induction phase

After 3 weeks, shoots and leaves were formed with different media supplements and condition aspects. Explants from SIM without supplements (control) produced more shoots than no ventilation (A1) and ventilation (A1V). A HH shoot developed with a glassy and dark green leaves color, and short internode and stem from no ventilation. Other normal shoots had no translucent color, but some shoots were not fully developed. Leaves from the normal shoot expressed ordinary green color with no symptoms of leaves turning yellow or brown. However, leaves are usually not fully expanded and uneven, rough, and serrated edges (Fig. 1A). Explants from the A1V medium are shown in Fig. 1B. Long shoots developed with fully expanded opaque green leaves. The leaf blades were smooth, not rough, the margins were straight, and the leaves were not curled like those from non-ventilated. No HH shoots were found under the ventilation condition.

Media A2 and A2V were MS medium supplemented with 1 mg/L $AgNO_3$. Color and appearance of shoots were like those from A1 and A1V (Fig. 1C–1D. Explants on A2V medium formed light green shoots and opaque green leaves with no signs of HH. Explants from media A3 and A3V with TCA (Fig. 1E–F) produced relatively short shoots compared to other media. The leaves were fully expanded, with light green to yellow and some incompletely developed.

When the $AgNO_3$ and TCA supplements were used together (A4 and A4V), the explants seemed stunted. The leaves were yellowish green, and usually had dry brown parts at the tips of the leaves (Fig. 1G). Some shoots showed signs of wilting, and some tissue was dead. The succulent aspect found on the leaves caused some parts to turn clear without the formation of unusual leaves or shoots, indicating that the HH symptoms are of low severity (Fig. 1G–1H).

Sunflower in this experiment had 100% of shoot regeneration in all supplement types and conditions (Table 1). The average shoots per explants were 1.02–1.18 ($P>0.05$). The mean shoot length, in contrast, slightly had a high gap among the treatments from 9.63–19.13 mm. The highest mean shoot length was obtained from the A1V medium without supplement under ventilation conditions. The shortest shoot was measured from the A4 medium supplemented with $AgNO_3$ and TCA combination. The A4 medium also had the lowest non-significant average shoot number per explant with HH shoot (Table 1).

The emergence of HH shoots occurred at a shallow randomness rate, with only 1.67–5.00% in the non-ventilated control, TCA added, and combined medium, the average number of shoots was not significantly different. When comparing the media with the same supplement but with different ventilation conditions, the average shoot length in all media with ventilation was longer than those cultured in non-ventilated media. In addition, HH shoots were found in all non-ventilated media.

Effect of media supplements and ventilation on HH, shoot regeneration, shoot length, water content, and stomatal density of *in vitro* sunflower at shoot elongation phase

The explants from A1 medium had stunted shoots with short internodes (Fig. 2A). The leaves were green with a yellow tint and distorted leaf blades, and the leaf tips were yellowish-brown. In comparison, the shoots from A1V were taller, leaves were green, and leaf blades were relatively smooth. The leaf color was opaque green with no signs of succulence, but some leaves showed a non-expanding appearance and had yellow color (Fig. 2B).

In the A2 medium with $AgNO_3$ the explants had tall, elongated shoots and many leaves (Fig. 2C). Leaf blades were smooth, without twisting and fluttering appearances. When the ventilation was applied in A2V, the morphological characteristics of the explants were not significantly different from A2, except that the color of the leaves was slightly darker green together, with no signs of withering in leaves (Fig. 2D).

Plants cultured on A3 medium supplemented with TCA produced short shoots and relatively few leaves. The leaves differed from the control and the $AgNO_3$ supplements. The leaves tended to wrap and roll down and were dark green but showed no signs of transparency. The explants often had large, light green callus formations at the explant bases (Fig. 2E). When ventilation was provided, the shoots increased in length, and the trichomes around the stems were visible. The leaf curling was reduced to a more spreading leaf. Explants still often had callus formation at the base of the explants (Fig. 2F).

The $AgNO_3$ and TCA combination from the A4 medium gave the regenerated explants with a hybrid appearance as a shared physical feature, with short shoots, slightly distorted leaves, yellow at the end of the leaves, and a large callus lump at the base of the explants (Fig. 2G). When cultured under ventilation, the shoots were slightly longer. The leaves were yellow at the tips of the leaves, with a dry appearance in some leaves, and calluses were produced at the base of explants (Fig. 2H).

Ventilated culture system influenced the growth and the morphological changes of *in vitro* sunflowers, which led to increased length of the shoots and the appearance of different leaf colors and surfaces. Regenerated explants showed the HH symptoms differently, depending on the supplement they received. The HH shoot from the non-ventilated control medium had a succulent short shoot with short internodes. The leaves were smaller than the normal ones and were spherical compared to the normal heart-shaped ones. The leaves were also translucent with dark green color, and the callus was formed at the base of the plant. In contrast, the HH shoot from the non-vented medium with $AgNO_3$ exhibited only clear leaves, and a dark green color presented about 10% of the whole explant with HH symptoms, indicating that the level of severity was at a very low level.

The stem development was not clearly visible on the part of the HH shoot from a non-vented TCA medium. The leaves were transparent and had light green to dark green color. The leaves were deformed, twisted, and elongated into abnormal shapes, which indicated that the level of severeness was high. Table 2 shows the effect of media supplements on shoot regeneration, the number of shoots per explant, HH shoots, and survival rate of *in vitro* sunflower S473 at the shoot elongation phase. The *in vitro* sunflower regeneration percentage was 100% in all treatments. As for the number of shoots per explant over a five-week of cultivation, no additional shoot formed during the three-week shoot induction phase. There was only the elongation of the shoots, and the development of the leaves changed. The shoot number ranged from 1.02 to 1.18 for all treatments with no significant difference.

The average shoot lengths ranged from 13.97–24.00 mm with the longest shoots obtained on A1V medium, and the shortest mean shoot length was obtained from A3 non-ventilated (Table 2). In addition, it was found that TCA added media under any conditions gave the low shoot heights as low as the non-ventilated control medium with the same significant level ($P\leq0.05$). Explants under the ventilated conditions increased the average shoot length than in closed culture vessels. There was a significant increase in the control media and the $AgNO_3$ medium. On

Table 1: Effect of media supplements on shoot regeneration, number of shoots per explant, shoot length, and HH shoots of *in vitro* sunflower at shoot induction phase

Media	Supplements	Shoot regeneration (%)	Number of shoots per explant (mean ± SE)	Mean shoot length (mm) (mean ± SE)	Hyperhydric shoots (%)	Survival rate (%)
A1	No supplement	100	1.13±0.46	15.52±4.05^{d}	1.67^{ab}	100
A1V		100	1.18±0.56	24.00±7.10^{a}	0.00^{b}	100
A2	Silver nitrate 1 mg/L	100	1.18±0.56	18.65±4.79^{c}	0.00^{b}	100
A2V		100	1.07±0.31	21.82±5.72^{b}	0.00^{b}	100
A3	TCA 100 mg/L	100	1.03±0.26	13.97±3.32^{d}	5.00^{a}	100
A3V		100	1.13±0.39	16.00±4.14^{d}	0.00^{b}	100
A4	$AgNO_3$ + TCA	100	1.02±0.13	14.95±2.05^{d}	1.67^{ab}	100
A4V		100	1.12±0.37	15.77±4.31^{d}	0.00^{b}	100

Means in columns followed by the same letters are not significantly different according to DMRT at $P \leq 0.05$. V=ventilation

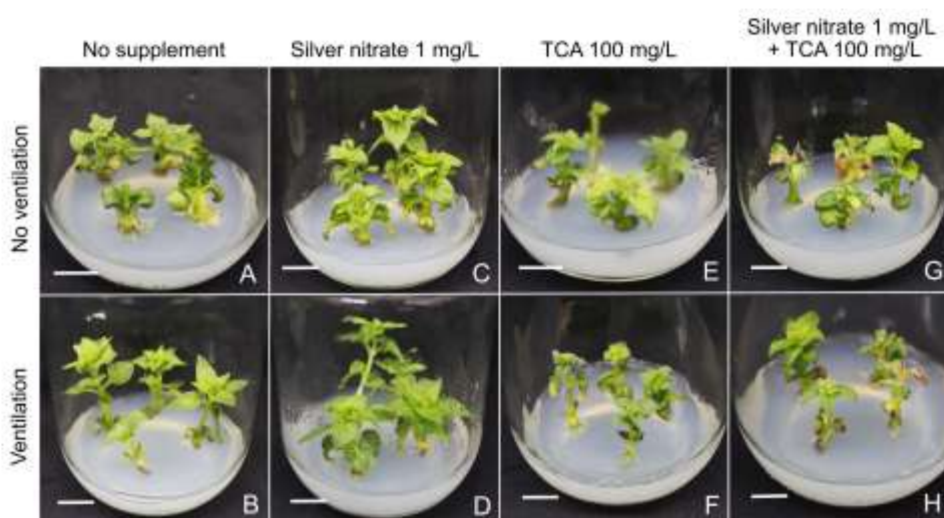

Fig. 1: Sunflower regeneration from meristem and cotyledon base explants on shoot induction medium with no ventilation and ventilation condition applied. (A–B) Explants cultured for 3 weeks on MS medium + 2 mg/L BA as SIM. (C–D) Explants cultured for 3 weeks on SIM + 1 mg/L silver nitrate. (E–F) Explants cultured for 3 weeks on SIM + 100 mg/L TCA. (G–H) Explants cultured for 3 weeks on SIM + 1 mg/L silver nitrate + 100 mg/L TCA. Bars = 1 cm

the other hand, leaf number per shoot ranged from 8.90–15.50. $AgNO_3$-treated plants showed a significant ($P \leq 0.05$) increase in leaf number compared to control, and the cultivating in ventilation conditions increased the number of leaves. As for the media containing TCA, the number of leaves did not increase in any medium and conditions (Fig. 3B).

The percentage of HH shoots was 1.67% in the non-ventilated media only, including A1, A2, and A3. No HH explants formed on the ventilated media at all. However, this difference in HH percentage was not statistically significantly different. Therefore, the supplements and their combinations did not affect the HH shoots formation in *in vitro* condition when cultured in the 480 mL bottle under non-ventilated and ventilated conditions (Table 2). Survival rates varied markedly, A2 and A2V media exhibited the highest survival rate (96.67%), with no significant difference from the control (86.67%). A highest plant mortality was obtained from A3 medium with TCA added without ventilation, with a drop in survival up to 78.33%. The cultivation under ventilation conditions improved plant survival percentage in the control and TCA media. In $AgNO_3$ supplemented media (A2 and A4), the survival rate was not significantly different in both ventilation conditions (Table 2).

The average plant water content showed that plants treated with TCA had higher water content than the other media. The explants produced large calluses, with the highest percentage from the A4 medium (91.32%). In contrast, the control medium and the $AgNO_3$ medium had significantly less water content, with A1 having 89.96% and A2 having 90.46%, respectively, and slightly higher than A1V and A2V with ventilation but not statistically different (Fig. 3C).

After five weeks, the number of stomata from the *in vitro* sunflower's leaf surface was measured (Fig. 3D). Stomata from different treatments showed different shapes and the opening characteristics of guard cells (Fig. 4). The stomatal density was from 305.45–344.52 mm^2. The most significant number was found in the A2V medium with

Table 2: Effect of media supplements on shoot regeneration, number of shoots per explant, HH shoots, and survival rate of *in vitro* sunflower at shoot elongation phase

Media	Supplements	Shoot regeneration (%)	Number of shoots per explant (mean±SE)	Mean shoot length (mm) (mean±SE)	Hyper-hydric shoots (%)	Survival rate (%)
A1	No supplement	100	1.13±0.46	15.52±4.05^{d}	1.67	86.67^{ab}
A1V		100	1.18±0.56	24.00±7.10^{a}	0.00	95.00^{a}
A2	Silver nitrate 1 mg/L	100	1.18±0.56	18.65±4.79^{c}	1.67	96.67^{a}
A2V		100	1.07±0.31	21.82±5.72^{b}	0.00	96.67^{a}
A3	TCA 100 mg/L	100	1.03±0.26	13.97±3.32^{d}	1.67	78.33^{b}
A3V		100	1.13±0.39	16.00±4.14^{d}	0.00	91.67^{a}
A4	Silver nitrate 1 mg/L + TCA 100 mg/L	100	1.02±0.13	14.95±2.05^{d}	0.00	86.67^{ab}
A4V		100	1.12±0.37	15.77±4.31^{d}	0.00	85.00^{ab}

Means in columns followed by the same letters are not significantly different according to DMRT at $P \leq 0.05$. V=ventilation

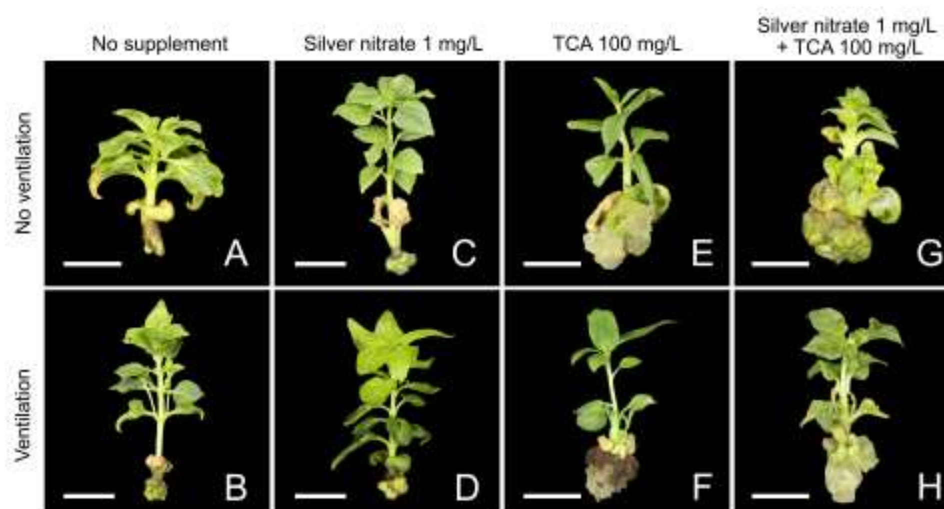

Fig. 2: Sunflower explants cultured on media at shoot elongation phase under no ventilation and ventilation condition for 2 weeks. (A–B) Explants cultured for 5 weeks on MS medium + 2 mg/L BA. (C–D) Explants cultured for 5 weeks on SIM + 1 mg/L silver nitrate. (E–F) Explants cultured for 3 weeks on SIM + 100 mg/L TCA. (G–H) Explants cultured for 5 weeks on SIM + 1 mg/L silver nitrate + 100 mg/L TCA. Bars = 1 cm

ventilated and $AgNO_3$ added. This corresponded to the highest leaf number per shoot, indicating the positive effect of applying $AgNO_3$ and ventilation. The use of $AgNO_3$ in the media also significantly increased the number of stomata in a non-vented medium. In contrast, TCA use slightly decreased the number of stomata but not significantly differ from the control.

Rooting and acclimatization of *in vitro* sunflower

Explants from A2V medium with 1 mg/L $AgNO_3$ produced the best results during the shoot elongation phase. When compared to other treatments, it exhibited 100% shoot regeneration with 1.07±0.31 shoots per explant, entirely non-HH shoot development, the highest 96.67% survival rate, with 90.07% water content, and the highest stomatal density (Fig. 3).

Twenty healthy explants within the treatments were cut to separate shoots and the base of the cotyledons. Cut shoots were transferred to root induction medium (RIM), a half-strength MS medium supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA) and 200 mg/L charcoal for rooting. Shoots cultured on RIM started forming roots 1 week after transplantation. After 2 weeks, roots formed and expanded over media (Fig. 5A–5B). All shoot explants in the RIM medium in this study indicated 100% rooting. Complete plantlets with shoots and roots were acclimated at room temperature for 2 weeks before being transplanted to soil. Only 20% plants survived at three weeks after transplantation.

Discussion

Results showed that the supplements, including $AgNO_3$, TCA, or its combination, did not affect the formation of HH shoots of *in vitro* sunflower under the experimental conditions. $AgNO_3$ enhanced plant growth in this experiment, although the number of shoots did not differ significantly in each treatment. However, plants treated with $AgNO_3$ at 1 mg/L showed an increase in leaf number and

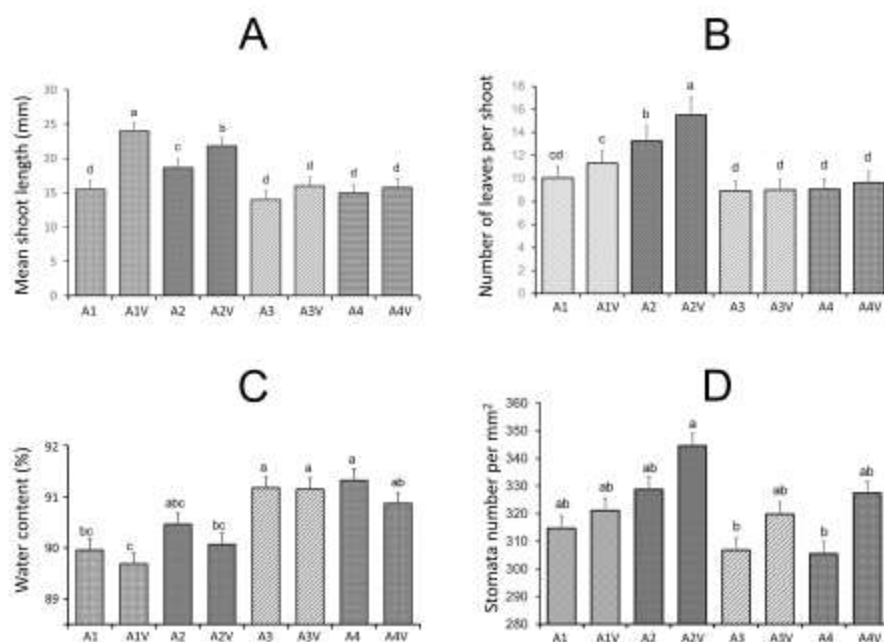

Fig. 3: Effect of media supplements and culture conditions on *in vitro* sunflower at shoot elongation phase. (A) Mean shoot length. (B) Number of leaves per shoot. (C) Water content. (D) Stomatal density. Different letters indicate significant difference between means according to DMRT at $P \leq 0.05$. V=ventilation

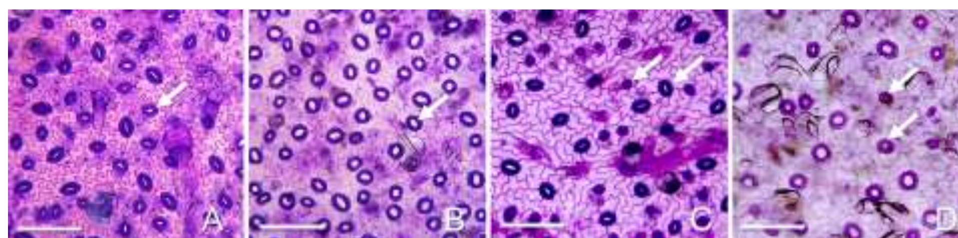

Fig. 4: Microscopic images of the abaxial epidermis of sunflower leaf. (A) Epidermis of normal leaf of explant cultured on ventilated SIM without supplement. (B) Epidermis of leaf of explant cultured on SIM + 1 mg/L silver nitrate under ventilation. (C) Epidermis of leaf of explant cultured on SIM + 100 mg/L TCA under ventilation. (D) Epidermis of leaf of explant cultured on SIM + 1 mg/L silver nitrate + 100 mg/L TCA under ventilation. Arrows indicate stomata. Bars = 100 µm

stomatal density compared to the control and TCA media. Tissue culture usually involves culturing plants under confined conditions. Plant growth nutrients, minerals, humidity, temperature, light and plant hormone are critical factors for growth, and stress factors affect seedling quality. In addition, wounding during explant cut process for culture is an additional factor causing stress on plants (Pérez-Clemente and Gómez-Cadenas 2012).

In the present experiment, at a concentration of 100 mg/L, TCA was toxic to sunflower resulting in negative consequences (Fig. 6A, 6B). The shoots dried up and almost died due to the inability to maintain water or inhibited growth and exhibited a chlorosis appearance. The plants were also unable to develop shoots properly and showed a light green color due to chlorophyll deficiency caused by TCA toxicity (Fig. 6). The culture conditions did not affect the HH production in *in vitro* cultured sunflower (Table 2). The indifference might be because the culture vessel (480 mL glass bottle) used in the experiment was large enough for plant growth. There was adequate space for air and growth for plants to develop without crowding appropriately so that the plants were not stressed till this HH occurred.

Jan *et al.* (2021) reported in *Salvia santolinifolia* that the higher vessel magnitude caused a lower HH shoots and improved shoot number and shoot length. The small and non-ventilated culture containers reduce aeration in the vessel, which resulted in excessive humidity in the culture container and enhanced water absorption by the cells. High humidity in the container may also hinder wax production on the leaves, resulting in poor transpiration and HH

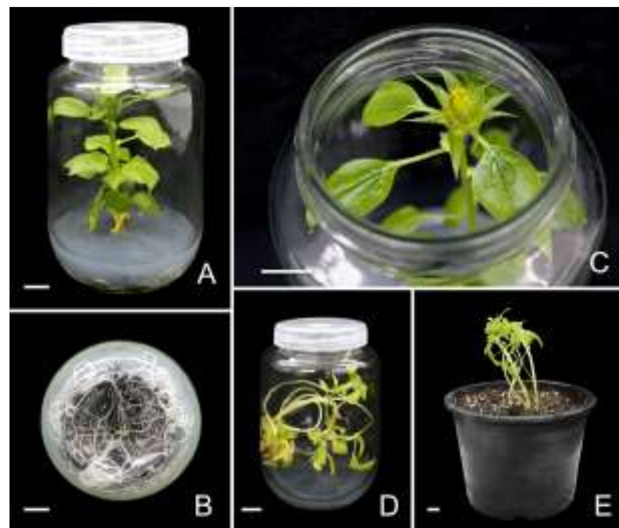

Fig. 5: Rooting and acclimatization of *in vitro* sunflower. (A) Rooting of the shoots on half-strength MS medium + 0.5 mg/L NAA and 200 mg/L charcoal for 2 weeks. (B) Roots forming and expanding over media. (C) Precocious flowering after 2 weeks of cultured on root induction medium. (D) Elongated-shoot plantlet at acclimatization period. (E) Acclimatized plantlet at 3 weeks after transplanting to soil. Bars = 1 cm

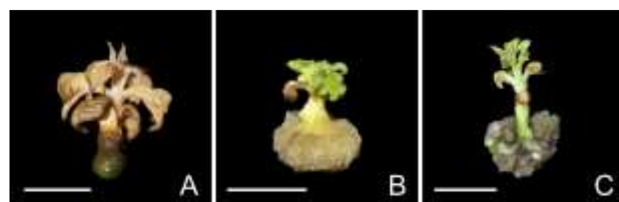

Fig. 6: Effect of TCA toxicity on the morphology of regenerated explants. (A–B) SIM + 100 mg/L TCA. (C) SIM + 1 mg/L silver nitrare + 100 mg/L TCA. Bars = 1 cm

development, thus causing the tissues to be transparent. The ventilation can, therefore, reduce ethylene levels, a gas hormone that tends to accumulate in the headspace in bottles and can cause problems for plant growth and HH. Santamaria *et al.* (2000) reported that the ventilation of culture vessels could reduce ethylene in the air space and improve the growth and development of *Delphinium in vitro*.

When $AgNO_3$, which can suppress ethylene activity, was combined with ventilation in this present experiment, it was revealed that it boosted the efficiency in minimizing the role of ethylene on the sunflower. The plant growth was higher in ventilated and non-ventilated media than in other media. Nevertheless, normal shoot morphogenesis necessitates high vessel volume, and low humidity in the culture containers, which may have aided the process. The size of the culture vessel, ventilation, closure types, and climatic conditions of the culture room all impact HH (Lai *et al.* 2005). High relative humidity above the cultures may increase HH development (Wardle and Short 1983).

At the root induction stage, the problem encountered was that the plants formed flowers before they reached maturity i.e., precocious flowering (Fig. 5C), which is not good for plant tissue culture. This may be because the life cycle of sunflowers, which are annual herbaceous plants, is short (50–70 days from seed to flowering). So, the time spent in this experiment may be too much for growing *in vitro* sunflower. It was also found that the plants showed succulent leaf growth even though the selected plants were healthy, indicating that the problem of HH shoots is difficult to predict. In addition, in the two weeks of the acclimatized period after root induction, the plants had elongated stems spreading across the bottle (Fig. 5D). It may be a problem with inadequate light exposure to plants while combined with the hormone auxin used in root induction medium contributing to elongated plant growth. These problems resulted in 80% of the plants' death during the acclimatized period. So, it is expected that the conditions utilized in the acclimatization of *in vitro* sunflower need to be improved in the future.

Conclusion

Low concentration of $AgNO_3$ or TCA had no impact on the shoot regeneration, the number of shoots, and HH. Adding a low concentration $AgNO_3$ to the culture media helped promote healthy plant growth. The plants grew significantly better when the $AgNO_3$ was combined with ventilation, having the most leaves per explant and stomatal density. TCA caused toxicity to the plant even at low concentrations by reducing shoot growth. Ventilation mostly improved plant growth in all media. It is crucial to optimize the culture conditions for efficient micropropagation because a variety of factors, including genotypes, ventilation conditions and medium supplements impact HH in sunflower tissue culture.

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Author Contributions

TA carried out research work, data analysis, and wrote original draft of the manuscript, NM supervised the work, and technically improved the final manuscript.

Conflicts of Interest

The authors have no conflict of interest.

Data Availability

Data is available on a fair request to the corresponding author.

Ethics Approval

This work does not require ethics approval.

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