# EFFECT OF SOME MEDIA SUPPLEMENTS ON HYPERHYDRICITY IN SUNFLOWER (*HELIANTHUS ANNUUS* L.) *IN VITRO*

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A Thesis Submitted in Partial Fulfillment of the Requirements for the
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# ผลของสารเสริมบางชนิดต่อการฉ่ำน้ำของทานตะวันในหลอดทดลอง

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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อาการฉ่ำน้ำเป็นภาวะทางสัณฐานวิทยาและสรีรวิทยาที่พบได้บ่อยในการเพาะเลี้ยงเนื้อเยื่อพืช ปัญหาดังกล่าวอาจส่งผลให้เกิดความสูญเสียทางเศรษฐกิจ  พืชที่แสดงอาการฉ่ำน้ำมีลักษณะยอดหรือใบใส ชุ่มน้ำ โปร่งแสง และมีปริมาณน้ำสูง งานวิจัยนี้ประกอบด้วยการทดลองสองส่วน ส่วนแรกมีวัตถุประสงค์เพื่อศึกษาผลกระทบของสารเสริมในอาหารเพาะเลี้ยงเนื้อเยื่อ ประกอบไปด้วยซิลเวอร์ไนเตรท ไตรคลอโรอะซีเตท และเดกซ์แทรนซัลเฟต ต่อการเพาะเลี้ยงเนื้อเยื่อของทานตะวันพันธุ์ประดับภายใต้สภาวะการระบายอากาศ การทดลองส่วนที่สอง มีวัตถุประสงค์เพื่อศึกษาผลกระทบของสารเสริมซิลเวอร์ไนเตรท ไตรคลอโรอะซีเตท หรือการผสมกันของสารทั้งสองชนิด ต่อการเพาะเลี้ยงเนื้อเยื่อของทานตะวันพันธุ์น้ำมัน ในสภาวะที่มีการระบายอากาศและไม่มีการระบายอากาศ ผลการทดลองจากการศึกษาในส่วนแรกแสดงให้เห็นว่า ในกรณีส่วนใหญ่การเพิ่มความเข้มข้นของซิลเวอร์ไนเตรท ไตรคลอโรอะซีเตท และเดกซ์แทรนซัลเฟตไม่มีผลกระทบต่อการเกิดอาการฉ่ำน้ำ เมื่อเปรียบเทียบกับการทดลองชุดควบคุม ทั้งในระยะการชักนำการเกิดยอดและในระยะการยืดตัวของยอดเมื่อเพาะเลี้ยงภายใต้สภาวะระบายอากาศ พบว่าที่ความเข้มข้นของซิลเวอร์ไนเตรทต่ำ (1 มิลลิกรัม/ลิตร) ส่งผลให้จำนวนยอด อัตราการมีชีวิตรอด และความหนาแน่นของปากใบสูงที่สุดอย่างมีนัยสำคัญในระยะการยืดตัวของยอด เดกซ์แทรนซัลเฟตส่งผลต่อการลดเปอร์เซ็นต์การงอกของยอดพืชในการเพาะเลี้ยงเนื้อเยื่อทานตะวันพันธุ์ประดับในระยะการชักนำยอดอย่างมีนัยสำคัญ ความเป็นพิษของสารเสริมเป็นปัจจัยพิจารณาที่สำคัญ ความเข้มข้นของสารเสริมที่สูงเกินไปทำให้พืชมีการเจริญเติบโตและพัฒนาผิดปกติ รวมถึงมีการเจริญเติบโตที่ลดลงและมีอาการยอดฉ่ำน้ำ โดยเฉพาะอย่างยิ่งเมื่อได้รับซิลเวอร์ไนเตรทที่มีความเข้มข้นสูง การทดลองในส่วนที่สองในทานตะวันพันธุ์น้ำมันแสดงให้เห็นถึงผลกระทบของสารเสริมซิลเวอร์ไนเตรท ไตรคลอโรอะซีเตท และการผสมกันของสารทั้งสองชนิดต่อการเจริญเติบโตและการเกิดอาการฉ่ำน้ำของทานตะวันภายใต้สภาวะที่มีการระบายอากาศและไม่มีการระบายอากาศ ในระยะการชักนำให้เกิดยอด ประเภทของสารเสริมและสภาวะในการเพาะเลี้ยงส่วนมากไม่มีผลกระทบต่อการเจริญและการเกิดอาการฉ่ำน้ำ แต่พบว่ามีผลต่อความ

แตกต่างของความยาวยอดอย่างมีนัยสำคัญ โดยพืชที่เพาะเลี้ยงภายใต้สภาวะระบายอากาศสามารถเจริญเติบโตได้ดีกว่าพืชที่เพาะเลี้ยงในภาชนะปิด ส่วนในระยะการยืดตัวของยอดนั้น พบว่าไม่มีการเปลี่ยนเปลงของจำนวนยอดและการเกิดการฉ่ำน้ำจากระยะการชักนำการเกิดยอด แต่ผลการทดลองความยาวยอด จำนวนใบ และจำนวนของปากใบมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ โดยแสดงให้เห็นถึงผลกระทบของซิลเวอร์ไนเตรทและการระบายอากาศของภาชนะเพาะเลี้ยงในการส่งเสริมการเจริญเติบโตของทานตะวัน การเพิ่มซิลเวอร์ไนเตรท 1 มิลลิกรัม/ลิตร ส่งผลในการเพิ่มการเติบโต จำนวนใบ และความหนาแน่นของปากใบของทานตะวันพันธุ์น้ำมัน ในทางตรงกันข้าม ไตรคลอโรอะซีเตทมีความเป็นพิษต่อพืชและส่งผลยับยั้งการเจริญเติบโตและการพัฒนาของพืช ถึงแม้ว่าซิลเวอร์ไนเตรทจะมีผลในการเสริมการเจริญเติบโตของพืช การผสมสารเสริมซิลเวอร์ไนเตรทและไตรคลอโรอะซีเตทร่วมกันนั้นกลับพบว่าส่งผลกระทบด้านลบต่อพืชเนื่องจากยังมีคุณสมบัติความเป็นพิษของไตรคลอโรอะซีเตท อย่างไรก็ตาม ชนิดของสารเสริมและสภาวะการเพาะเลี้ยงที่ใช้ในการทดลองนี้ไม่ส่งผลให้เกิดความแตกต่างอย่างมีนัยสำคัญต่ออาการฉ่ำน้ำของทานตะวันพันธุ์น้ำมัน นอกจากนี้เมื่อเปรียบเทียบพืชที่เพาะเลี้ยงภายใต้รูปแบบสภาวะระบายอากาศทั้งสองแบบ พบว่าพืชที่เพาะเลี้ยงในสภาวะระบายอากาศมีการเจริญเติบโตสูงกว่าพืชที่เพาะเลี้ยงในภาชนะปิด การระบายอากาศทำให้เกิดการแลกเปลี่ยนหมุนเวียนอากาศภายในและภายนอกภาชนะเพาะเลี้ยงซึ่งส่งเสริมการเจริญเติบโตของพืช งานวิจัยนี้เป็นงานวิจัยแรกที่นำเสนอผลการทดลองของสารเสริมไตรคลอโรอะซีเตทและเดกซ์แทรนซัลเฟตต่อการเจริญเติบโตและการเกิดการฉ่ำน้ำของทานตะวันในหลอดทดลอง นอกจากนี้ยังเป็นงานวิจัยแรกที่รายงานผลกระทบของสารเสริมซิลเวอร์ไนเตรทและไตรคลอโรอะซีเตทต่อทานตะวันในหลอดทดลองภายใต้สภาวะการเพาะเลี้ยงแบบมีการระบายอากาศและไม่มีการระบายอากาศ โดยมีความคาดหวังว่าผลการทดลองที่ได้รับนี้จะเป็นประโยชน์ต่อการพัฒนาคุณภาพการเพาะเลี้ยงเนื้อเยื่อพืชต่อไปในอนาคต โดยสรุป มีหลายปัจจัยที่เกี่ยวข้องต่อการเกิดอาการฉ่ำน้ำในการเพาะเลี้ยงเนื้อเยื่อทานตะวัน การขยายพันธุ์พืชให้มีประสิทธิภาพในหลอดทดลองจำเป็นต้องปรับสภาวะการเพาะเลี้ยงให้เหมาะสม

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา ธาดา แซ่มสูงเนิน

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Keywords: dextran sulfate, hyperhydricity, silver nitrate, sunflower, trichloroacetate

Hyperhydricity (HH) is a morphological and physiological condition common in plant tissue culture. The problem can potentially result in economic losses for the plant micropropagation economy. Hyperhydric shoots and leaves have a glassy, water-soaked appearance, a transparent aspect, and a high-water content. This research included two parts. The first part of the study aimed to examine the effect of several media additions, including silver nitrate, trichloroacetate (TCA), and dextran sulfate, on ornamental sunflower tissue culture under ventilation condition. The second part aimed to investigate the impact of media supplemented with silver nitrate, TCA, or their combination on the tissue culture of oil seed sunflower in ventilated and non-ventilated conditions. The results on ornamental genotype showed that, in most cases, the addition of silver nitrate, TCA, and dextran sulfate showed no differential impact on the HH occurrence as compared to the control in both shoot induction and shoot elongation phases when cultured explants under ventilation. The lowest concentration of silver nitrate (1 mg/L) resulted significantly in the highest number of shoots per explant, survival rate, and stomata density at the elongation phase. Dextran sulfate significantly reduced the shoot regeneration percentage in sunflower tissue culture at the shoot induction phase. The toxicity of the supplements is an important consideration. The addition of the supplements at excessively high concentrations caused the plants to develop abnormally with decreased growth and produced HH shoots, especially when receiving high concentrations of silver nitrate. In addition, the second experiment of the oil seed genotype showed the effect of silver nitrate, TCA, and their combination on plant growth and hyperhydricity under ventilation and non-ventilation conditions. During the shoot induction phase, the supplements and culture conditions mainly had no impact on plant regeneration and hyperhydricity but had a significant result on shoot length, the ventilated plants had better growth than non-

ventilated ones. At the shoot elongation phase, there were no changes in the regeneration and hyperhydricity percentages compared to the induction phase. The findings of high shoot length and number of leaves per shoot showed the significant impact of silver nitrate and culture ventilation on sunflower growth improvement. Adding 1 mg/L silver nitrate improved shoot regeneration, leaf number, and stomata density of oil seed genotype. In contrast, TCA seemed toxic to the plant and reduced plant growth and development. Even though silver nitrate had shown to improve plant growth, combining two supplements appeared to cause an adverse effect on the plant since the toxic property of TCA still affected the plant. However, neither supplement types nor culture conditions had a significance influence on HH of the oil seed sunflower genotype among all media tested. Comparing between ventilation and non-ventilation plants, the vented plants had significantly higher growth than another. The vented culture vessels brought in fresh air and exchanged the old gas and balanced the internal air space that aided plant transpiration. This is the first study to report the effect of TCA and dextran sulfate on *in vitro* sunflower. It also provided the first finding of the impact of silver nitrate and TCA on *in vitro* sunflower under different culture conditions, including vented and non-vented, which are expected to benefit the development of plant tissue culture quality in the future. Overall, many factors affect HH in sunflower tissue culture, and the culture conditions for efficient micropropagation should be optimized.



School of Biology Student's Signature ธาดา ยอดมณีวงษ์

Academic Year 2021 Advisor's Signature

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| | |
|---|---|
| 2-iP | $N^6$-[2-Isopentenyl] adenine |
| BA | $N^6$-Benzyladenine |
| cm | Centimeter |
| °C | Degree Celsius |
| DMRT | Duncan's Multiple Range Test |
| hr | Hour |
| HH | Hyperhydricity |
| HCl | Hydrogen chloride |
| L | Liter |
| mg | Milligram |
| mm | Millimeter |
| $mm^2$ | Square millimeter |
| µM | Micromolar |
| µm | Micrometer |
| MS | Murashige & Skoog medium |
| NAA | α-Naphthaleneacetic acid |
| NaOH | Sodium hydroxide |
| pH | Potential of hydrogen ion |
| TCA | Trichloroacetate |

# CHAPTER I

# INTRODUCTION

## 1.1 Background/Problems

Sunflower (*Helianthus annuus* L.) is an oilseed crop native to North America, belonging to the Asteraceae (Compositae) family (Heiser, 1978). Endemic people have grown and cultivated sunflowers since 3000 B.C. for food, medicinal purposes, and ornamental plants (Selmeczikovzcs, 1975; Putt, 1978). Depending on proposes, there are three types: oilseed, confectionery, and ornamental sunflowers (Jocic et al., 2015). Sunflowers are an essential oilseed crop on the global market (Vassilevska-Ivanova et al., 2014). Sunflowers have many applications in modern society. Consumers have used sunflower seeds for making bread, snacks, bird feed, medicines, paints, varnishes, soaps, candles, cosmetics, and a biodiesel component. The stem cellulose is also used in paper production (Davey and Jan, 2010). Globally, 50 million tons of sunflower seeds were 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). Oil sunflower seed production in Europe increased from 2008 to 2018, with 10.4 to 11.8 million tons produced in 2018 (Popescu et al., 2019). Major companies utilize sunflower oil for replacing other artificial hydrogenated oils in their products (Tarrago-Trani et al., 2006). Sunflower oil is high in polyunsaturated fats and thus is becoming more common as a healthy replacement (Grompone, 2020).

People can grow sunflowers worldwide as ornamental plants because of their high resilience to harsh weather conditions (Kiani et al., 2007; Kaya et al., 2012). As ornamental plants, their beauty and vividness are unparalleled, making them popular in the global flowering business (Jocic et al., 2015; Mladenović et al., 2016). Sunflower is used for decoration as cut flowers, potted plants, or garnishes in the garden (Jocic et al., 2015). It must have a strong stem and last long in a vase or a cut flower. In comparison, potted plant characters have a shorter stem height, small leaves, and a short period to bloom. Moreover, the sunflower for garden decoration usually has a

temperature resistance ability and a vigorous plant habit (Kaya et al., 2012). The development of new cultivars offers a wide range of flower colors, flower shapes, and stem types to fulfill the ornamental flower market's high demand.

The sunflower went through the breeding and genetic selection process and gained the desired genotypes through two main methods, including conventional breeding and in vitro technologies (Davey and Jan, 2010). The original breeding studies have resulted in cultivars with improved agronomic characteristics, such as high fat, pests, and disease resistance (Faure et al., 2002; Seiler et al., 2008; Vassilevska-Ivanova et al., 2014). In another way, biotechnology in tissue culture is a powerful technique used for fast propagation and germ-free plantlet production. It is used to improve plant breeding in the agricultural industry and biological research practice. This technique is necessary to develop genetic variation, for example, oil and protein qualities, high tolerance to stress conditions (drought, salt, etc.), and flower appearance variety of cultivated sunflowers (Dagustu, 2018). Successful sunflower regeneration highly depends on the genotype, the explant type, the culture media, concentration and the type of growth regulators, and the culture conditions. Nonetheless, plant regeneration has different problems, such as precocious flowering, poor rooting, and hyperhydricity (HH) (Nestares et al., 1996; Baker et al., 1999).

Hyperhydricity is a morphological and physiological disorder in plant tissue culture (Pâques, 1991). Hyperhydric shoots showed an abnormal shoot formation, translucent shoot appearance, water accumulation in tissues, and reduced cell wall lignin. 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 generated by apoplast waterlogging, which causes hypoxia and severe oxidative damage. The confined atmosphere of culture vessels, high relative humidity, poor gaseous exchange, and ethylene accumulation can lead to hyperhydricity problems (Kevers et al., 2004). Ethylene, a plant hormone, also could stimulate shoot hyperhydration, decreased chlorophyll contents, and tissue necrosis (Iqbal et al., 2017; Gao et al., 2018). Plantlets with hyperhydricity symptoms poorly survive when transferred to an *ex vitro* environment (Gaspar et al., 1995; Sen and Alikamanoglu, 2013).

Hyperhydricity can be restrained by controlling the ethylene level using silver nitrate (Gaspar, 1986) or silver nanoparticles (AgNPs) (Sreelekshmi and Siril, 2021) as an inhibitor of ethylene activity. The addition of cobalt chloride stimulates shoot regeneration and strongly prevents ethylene production (Chraibi et al., 1992). De Klerk and Pramanik (2017) used trichloroacetate (TCA), an inhibitor of wax biosynthesis, to prevent the development of hyperhydricity since TCA increases plant transpiration rate and reduces waterlogged in plantlets. Dextran sulfate is an antivitrification agent (AVA) that could reduce shoot hyperhydricity in Eucalyptus species (Nairn and Furneaux, 1997). Besides, ventilation of the vessels could reduce the gaseous ethylene level by enhancing the gas exchange in culture vessels and could reduce shoot hyperhydricity (Lai et al., 2005).

Mayor et al. (2003) reported the use of silver nitrate in preventing HH in sunflower. However, no reports are available on TCA and dextran sulfate in sunflower tissue culture. Therefore, this study aimed to evaluate the effect of different media supplements (silver nitrate, TCA, and dextran sulfate) on hyperhydricity occurrence and regeneration efficiency in sunflower tissue culture of two sunflower genotypes. A combined impact of silver nitrate and TCA with ventilation and non-ventilation treatment was further evaluated to understand the effect of anti-hyperhydricity supplements on sunflower HH.

## 1.2 Research objectives

1.2.1 To investigate the effects of 3 media supplements; silver nitrate, trichloroacetate (TCA), and dextran sulfate on shoot regeneration, shoot length, and hyperhydricity of *in vitro* ornamental sunflower at shoot induction phase.

1.2.2 To investigate the effects of 3 media supplements; silver nitrate, TCA, and dextran sulfate on shoot regeneration, shoot length, hyperhydricity, relative water content, and stomata number of *in vitro* ornamental sunflower at shoot elongation phase.

1.2.3 To investigate the effects of 2 media supplements; silver nitrate, TCA, or their combination on shoot regeneration, shoot length, and hyperhydricity of *in vitro*

oil seed sunflower under ventilation and non-ventilation condition at shoot induction phase.

1.2.4 To investigate the effects of 2 media supplements; silver nitrate, TCA, or their combination on shoot regeneration, shoot length, hyperhydricity, relative water content, and stomata number of *in vitro* oil seed sunflower under ventilation and non-ventilation condition at shoot elongation phase.

## 1.3 Research questions

1.3.1 Do media supplements (silver nitrate, TCA, or dextran sulfate) and variable concentration of supplement levels (1, 2, and 3) affect the average number of shoots per explant, average shoot length, and the proportion of plants with hyperhydricity at shoot induction phase?

1.3.2 Do media supplements (silver nitrate, TCA, or dextran sulfate) and variable concentration of supplement levels (1, 2, and 3) affect the average number of shoots per explant, average shoot length, the proportion of plants with hyperhydricity, the water content, and the number of stomata of explants at shoot elongation phase?

1.3.3 Do media supplements (silver nitrate, TCA, or their combination) under ventilation or non-ventilation condition affect the average number of shoots per explant, average shoot length, and the proportion of plants with hyperhydricity at shoot induction phase?

1.3.4 Do media supplements (silver nitrate, TCA, or their combination) under ventilation or non-ventilation condition affect the average number of shoots per explant, average shoot length, the proportion of plants with hyperhydricity, the water content, and the number of stomata of explants at shoot elongation phase?

## 1.4 Research hypotheses

1.4.1 If media supplement type and concentration impact shoot number, shoot length, and hyperhydricity at 3 weeks for sunflower explants, then at least one group is expected to significantly differ in mean shoot number, shoot length, and percent hyperhydricity based on either concentration or media supplement type.

1.4.2 If media supplement type and concentration impact shoot number, shoot length, hyperhydricity, water content, and the number of stomata at 5 weeks for sunflower explants, then at least one group is expected to significantly differ in mean shoot number, shoot length, hyperhydricity, water content, and the number of stomata based on either concentration or media supplement type.

1.4.3 If media supplement type and culture condition impact shoot number, shoot length, and hyperhydricity at 3 weeks for sunflower explants, then at least one group is expected to significantly differ in mean shoot number, shoot length, and percent hyperhydricity based on either media supplement type or culture condition.

1.4.4 If media supplement type and culture condition impact shoot number, shoot length, hyperhydricity, water content, and the number of stomata at 5 weeks for sunflower explants, then at least one group is expected to significantly differ in mean shoot number, shoot length, hyperhydricity, water content, and the number of stomata based on either media supplement type or culture condition.

## 1.5 Scope and limitation of the study

In this research, seeds of two sunflower genotypes: Suranaree 473 (S473) or Autumn Beauty x Moulin Rouge hybrid, were used as explant samples. Factors including media supplements (silver nitrate, TCA, or dextran sulfate), the variable concentration of supplement levels, and culture condition (ventilation or non-ventilation) were determined if these affect shoot regeneration, hyperhydricity, relative water content, and stomata number of *in vitro* sunflower.

## 1.6 Expected results

1.6.1 The most suitable media supplement type and concentration that give the lowest hyperhydricity and water content, and the highest shoot number, shoot length, and stomata number of *in vitro* sunflower were obtained.

1.6.2 The most suitable media supplement type and the culture condition that give the lowest hyperhydricity and water content, and the highest shoot number, shoot length, and stomata number of *in vitro* sunflower were obtained.

1.6.3 The data on the changes in the anatomical structures and physiological characteristics of hyperhydric sunflower plantlets, which will clarify the possible mechanism of hyperhydricity and provide possible ways to prevent hyperhydricity in sunflowers, were investigated.

1.6.4 The findings of this study might be utilized to enhance the sunflower tissue culture process in the future.

# CHAPTER II

# LITERATURE REVIEW

## 2.1 Sunflower

Sunflower (*Helianthus annuus* L.) is the fourth most important oilseed plant globally (together with palm, soy, and rapeseed). It was first domesticated by the indigenous people of North America (Kaya et al., 2012). The sunflower is a member of the Asteraceae family. Other common members of this family include lettuce, chrysanthemum, and marigold. The *Helianthus* genus has 53 species (14 annuals and 39 perennials) (Schilling, 2006; Stebbins et al., 2013). The sunflower (*H. annuus*) has 17 chromosomes. *Helianthus* means "flower of the sun": from the Greek root "helios", meaning the sun, "anthus", meaning flower. Globally, people cultivate *H. annuus* most frequently (Putt, 1997). At present, the food industry uses sunflowers mainly for oils, but some people grow them as ornamentals for their spectacular size and large flower heads. Sunflower seeds contain a healthy oil with high mono- and polyunsaturated fatty acids and vitamin E contents (Kaya et al., 2012). So, sunflowers play a role in many aspects of the global economy, in the ornamental flower market, and play a critical role as the main oil for food processing.

The sunflower is an herbaceous annual. The plant has a hairy erected stem (1-4.5 m), usually unbranched— but some ornamental sunflowers have multiple stems. Sunflowers have a tap root system with abundant secondary roots. The hairy rough leaves are large (5-30 cm), heart-shaped, petiolated, and arranged in opposing pairs that alternate up the stem. The inflorescence or floral head is large (10-30 cm diameters with 250-1500 tubular flowers). The disk flowers are brown, yellow, or purple. The petal-like ray florets (at the outer part of the floral head) are yellow, orange, or red. The florets attract insect pollinators, such as beetles, butterflies, bees, and flies (Nderitu et al., 2008). The fruit is a single-seeded achene (Figure 2.1).

Sunflowers begin to bloom 55-75 days after planting, depending on the variety. After the pollination and seed development stage, the flower head droops and turns brown. Seed harvesting occurs about 120 days after sowing. Sunflowers commonly bloom during summer and early fall, with the middle of summer being the peak season in temperate regions (Seiler, 1997; Davey and Jan, 2010). As an annual, the entire lifecycle of a sunflower can complete in less than a single year which lends itself to many uses.

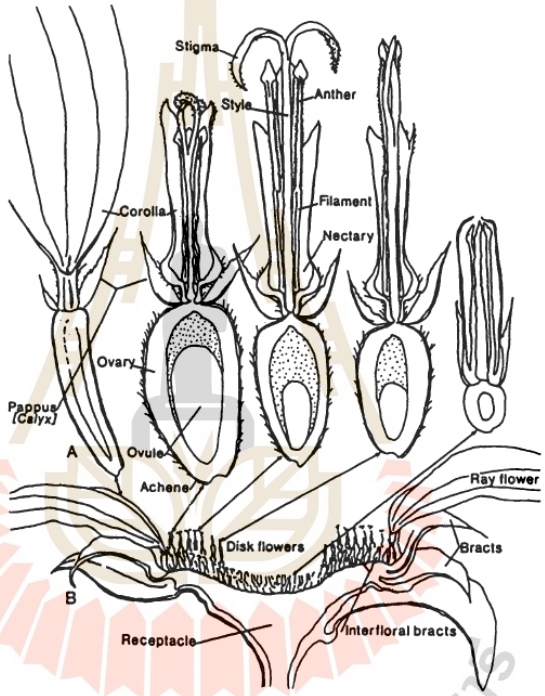


**Figure 2.1** Sunflower flower head consists of ray florets and disc florets (Seiler, 1997).

## 2.2 Sunflower use

Indigenous peoples, such as the Aztecs of Mesoamerica (a historical region and cultural area in southern North America and much of Central America), cultivated the sunflower as early as 2300 BC by utilizing it as food, medicine, and ceremonial body paint (Lentz et al., 2008). Natives ground sunflower seeds into flour for meals (Heiser et al., 1969). Sunflower hulls and petals, in contrast, became dyes for clothing (Yusuf et al., 2014). At the same time, petals and pollen were present as face paint—the oil from sunflower seeds aided cooking and hair treatment (Heiser et al., 1969). The stalks and stem fibers had value as paper. Indigenous people believed sunflowers could treat snake bites, warts and even expel worms from the body. Some American tribes used the sunflower as a symbol of the solar god. So, the entire sunflower, from stem to flower, was used by native communities as far back as ancient Mesoamerica.

There are three main modern sunflower types: oilseed, confectionery, and ornamental (Jocic et al., 2015). Humans consume about 90% of all produced sunflower oil; only 10% goes to biodiesel and industrial applications (Jocic et al., 2015). The industry usually processes seeds into edible oils, but some remain as whole seeds (a protein crop) while the rest becomes feed for domestic animals. Both high oil content sunflowers and sunflowers without oil exist in the market. Low-oil-containing flowers are usually for whole consumption (confectionary). Other low-oil sunflowers are ornamental, usually either seedless or have tiny seeds.

Oil sunflower seeds are all black, while the confection sunflower seeds have white stripes or can be colorful and are significantly larger than the oil-type seeds. The confectionary seed also has a thicker hull that is easy to detach from the kernel (González-Pérez and Vereijken, 2007). Confectionery sunflower seeds contain less than 30% oil, lower cadmium, higher protein, oleic acid, and high vitamin E content (Lofgren, 1997; Jovanović et al., 1998). Confectionery sunflower seeds are present in the pet feed market, bakeries, and snack products (Hofland and Kadrmas, 1989).

Although oilseed sunflowers dominate the market, ornamentals are still popular worldwide (Jocic et al., 2015). Large ornate flowers make the sunflower both vivid and beautiful. The first ornamental sunflower varieties had yellow flowers and were very high (over 2 meters tall). Some of these varieties are still present in the

market, such as the Mammoth Russian and Russian Giant (Castaño, 2018; Thompson and Morgan, 2020). These tall-growing sunflowers produce a single, enormous flower at the top of the plant. These varieties have been on the market for over 130 years, and their demand is still high (Jocic et al., 2015). Pleasing plant appearance, number of flowers, stem traits, and the length of flowering are the goals of ornamental sunflower breeding. Current ornamental sunflower breeding guidelines depend upon the target use of the flower, which could be ornamental hybrids for cut flowers, ornamental hybrids for gardens or parks, and ornamental hybrids with short stems for growth pots (Miklič et al., 2008). The different cultivars can range from giant flowers suitable for bouquets to small flowers better for boutonnieres. There are many ornamental sunflower cultivars on the market today, such as Autumn Beauty, Big Smile, Ring of Fire, Sunbright, Sunrich Lemon, and Teddy Bear (Johnny's, 2022) (Figure 2.2).

**Figure 2.2** Example of sunflower cultivars, from left to right, Autumn Beauty, Ring of Fire, Sunrich Lemon, and Teddy Bear (Johnny's, 2022).

## 2.3 Sunflower breeding and propagation

Cultivating sunflowers has traditionally consisted of breeding and, more recently, biotechnology (Dagustu, 2018). Conventional breeding improves observable agronomic characteristics: fat content, pest, and disease resistance. Biotechnology targets desired genetic traits using plant tissue culture.

### 2.3.1 Conventional breeding

Breeders (in the early 16th century) selected and cross-pollinated sunflowers to improve agronomic traits (Seiler et al., 2017): shorter time to maturity, larger seeds, higher/greater oil content, seed yields, and stronger resistance to frost, diseases, and insects (Davey and Jan, 2010). Creating such new hybrid lines is time-intensive—seven generations, up to three years.

They used the cross-pollinating technique to breed sunflowers by applying pollen from one flower to the pistil of another flower. Before pollinating, the breeders must manually remove mature stamens to avoid sunflower self-pollination. (Encheva and Christov, 2006). After transferring male pollen, they wrap the flower heads with a paper bag to prevent further pollination. They collect the floral head after 60 days from covering when all the seeds have matured. Then they evaluate the hybrid seed characteristics by cultivation and observation of visible traits.

Crossbreeding relies on planting and harvesting. Planting and harvesting have specific requirements: beneficial climate conditions with little rainfall in the breeding season, hot days and cool nights, a proper production field irrigation system, and no plant diseases. The best environmental conditions for producing sunflower seeds are low rainfall and low daytime humidity (Davey and Jan, 2010).

Traditional sunflower breeding for new hybrids improvement also has the obstacles: a gene pool limitation in the species, time-consuming, genetic purity loss (when other cultivars grow nearby), and poor seed quality (from poor storage conditions) (Durante et al., 2002; Davey and Jan, 2010). However, plant breeding by biotechnology in plant tissue culture could provide the newly preferred traits to the plant without those problems (Larkin and Scowcroft, 1981).

### 2.3.2 Sunflower breeding and micropropagation using tissue culture applications

Plant tissue culture is the *in vitro* aseptic culture of plant cells, tissues, organs, or entire plants under controlled environmental and nutritional conditions. Researchers and scientists use it in plant science, forestry, and horticulture. Micropropagation (a tissue culture method) produces abundant clonal plantlets from a single explant in a short time within a limited space. These techniques facilitate plant

breeding and propagate whole plants from modified plant cells (Hussain et al., 2012). Tissue culture can also save species by sterilizing endangered plants with environmentally contracted bacterial or viral infections and safely propagating and storing plantlets. Growing plants in botanical gardens, farms, or reserves expose them to infection risk. So tissue culture is a viable conservation alternative, and is valuable to breeders, and helps maintain sterile conditions.

Breeders use biotechnology in plant tissue culture to breed and improve sunflower characteristics (e.g., salinity tolerance, drought tolerance, and disease resistance). Nevertheless, different plant species require specific methods to grow effectively (Dagustu, 2018). Sunflower plantlets have narrow tolerance ranges to environmental conditions (plant hormones, media, either minerals or carbon sources, even light) *in vitro*, making them difficult to culture (Nichterlein and Horn, 2005; Davey and Jan, 2010; Moghaddasi, 2011). The goal of sunflower tissue culture is to develop sufficient plantlet growing conditions.

Sunflower tissue culture research began in the 1940s (Hildebrandt et al., 1946). The first researchers used sunflower tumor cells in tissue culture but could only regenerate roots successfully (White and Braun, 1941). At present, regeneration protocols for sunflower have been successful for various explants, such as immature embryos, mature embryos, meristems, shoot tips, embryonic axes, leaves, roots and stems, anthers, ovaries, hypocotyls, protoplasts, and cotyledons (Dagustu, 2018). Mature sunflower seed cotyledons are the most common because they are widely available (annual production). Using cotyledons as explants showed the most assuring results compared with other explant types (Sujatha et al., 2012; Zhang and Finer, 2015). Further, experimental manipulations are simple: using the seeds from different genotypes allows for direct comparisons of plants between genotypes; it is challenging to control characteristics of other explant types, and the culture and the methods easily apply to a wide range of sunflower genotypes (Baker et al., 1999).

Tissue culture technology to improve target sunflower genetic traits has a wide range of techniques: organogenesis (adventitious organs develop from undifferentiated cell mass); somatic embryogenesis (a process that grows plant embryo from a single somatic cell); interspecific hybridization (cross between plants in two

different species); embryo culture (culture of isolated immature or mature embryos); haploid production (a technique that the produce haploid plants *in vitro*); somaclonal variation (genetic variation of plants regenerated from somatic cells cultured *in vitro*); and protoplast culture (a process that culture protoplast, a cell without a cell wall, *in vitro*) (Dagustu, 2018).

The sunflower's regenerative success by tissue culture is highly variable and related to the genotype effects, explant types, specific media components, seedlings ages, and hormone concentrations in the culture medium. The culture vessels' lighting and micro-environmental conditions can hinder sunflower callus induction and shoot regeneration (Punia and Bohorova, 1992; Dağstü, 2002).

Although many methods and techniques have successfully bred and regenerated sunflowers, methods applicable to most sunflower genotypes are still lacking. To deal with the highly inflexible nature of sunflower species, we must develop a suitable in vitro regeneration system (Radonic et al., 2015). Furthermore, we must also address other challenges that clog sunflower tissue culture success: precocious flowering (flowering before maturing); long culture duration; abnormal morphogenesis; difficulty rooting; callus proliferation that inhibits shoot and root development; and hyperhydricity (Witrzens et al., 1988; Freyssinet and Freyssinet, 1988; Knittel et al., 1991; Nestares et al., 1996; Baker et al., 1999). Future studies should optimize tissue culture protocols in sunflower regeneration and breeding to mitigate costly or time-intensive problems during cultivation.

## 2.4 Hyperhydricity

Bidney and Scelonge (1997) reported that organogenesis and somatic embryogenesis successfully regenerated sunflowers. Although, there is still a significant problem in regenerating sunflowers—hyperhydricity. Vitrification was the previous name for hyperhydricity (Gribble, 1999; Lai et al., 2005), a physiological malformation influencing plant growth under tissue culture conditions (Debergh et al., 1992). This malformation is affiliated with excessive hydration of plant tissue, chlorophyll, and lignin deficiency. Hyperhydric plantlets cannot survive after transplanting to *ex vitro* conditions (Kevers et al., 2004). The hyperhydric plants fail to acclimatize by up to 60-

percent, limiting the tissue culture's success (Paques and Boxus, 1985a). The unusual conditions during *in vitro* cultures: low light intensity, high relative humidity, excess sugar and nutrients can cause plant hyperhydricity (Pospóšilová et al., 1999).

**2.4.1 Hyperhydric aspects**

According to Fauguel et al. (2008), hyperhydricity occurs when a plant exhibits atypical symptoms during *in vitro* cultivation. They considered *in vitro* leaf and stem characteristics differing from those of *ex vitro* plants as precursors to hyperhydricity. The hyperhydric plants appear glassy, water-soaked, translucent, succulent, or fleshy with brittle, thickened, and malformed stem and-or leaves; they also have delayed growth rates. These symptoms range from mild to severe. So, they classified four observable intensity levels of abnormal hyperhydric morphogenesis.

Seiler (1997) defined *in vitro* non-hyperhydric sunflower plantlets as having similar morphological characteristics to the *ex vitro* plants. However, when the plant is hyperhydric, it can have numerous possible symptoms. Fauguel et al. (2008) classified the hyperhydric sunflower macroscopic features during the regeneration stage into four categories according to different abnormalities, including

1. Translucent-transparent shoots and light green humid aspect leaves.
2. Thickened shoots with short internodes and thick stems.
3. Twisted shoots with rolled leaves so that only the lower epidermis is visible.
4. At the highest HH level, succulent shoots, deformed aspect, fleshy, easily breakable, and excessive hydration in all tissues.

The summary of the symptom category and severity is in Table 2.1.

**Table 2.1** Symptom category and severity of hyperhydric plants.

| Symptom Category | Level 1 | Level 2 | Level 3 | Level 4 |
|---|---|---|---|---|
| shoot translucence | low | moderate | high | high |
| shoot thickness | - | moderate | moderate | high |
| shoot twisting | - | - | moderate | high |
| leaf curling | - | - | high | high |
| shoot succulence | - | - | moderate | high |

Anatomic features of ordinary sunflower shoots regenerated *in vitro* include the single layer of epidermis on both leaf surfaces. The leaves have abundant chloroplasts with 1-2 rows of palisade mesophyll cells over spongy parenchyma and some intercellular spaces. Leaf vascular bundles are collateral with well-lignified vessels. The stem has a single epidermis layer with few stomata. The cortex contained numerous vascular bundles.

In contrast, the features of hyperhydric plantlets and thickened shoot leaves have a single layer of epidermis cells, loose and disorganized mesophyll cells, and an increase in cellular volume. The leaf vascular bundles are also disorganized with hypolignified condition (lack of lignin). Leaves of severe succulent shoots showed a thin palisade tissue or lacked it together. Palisade and spongy parenchyma are unorganized and complicated to distinguish (Figure 2.3). Stems of hyperhydric shoots have few vascular bundles. The vascular system lacks lignin and a typical arrangement. Cortical and pith parenchyma are overgrowth, and the cells have thin and non-rigid cell walls. The stem diameter can be double as large as the normal stem. Some hyperhydric and succulent shoots regenerated *in vitro* do not present an evident stem (Pâques, 1991; Fauguel et al., 2008).

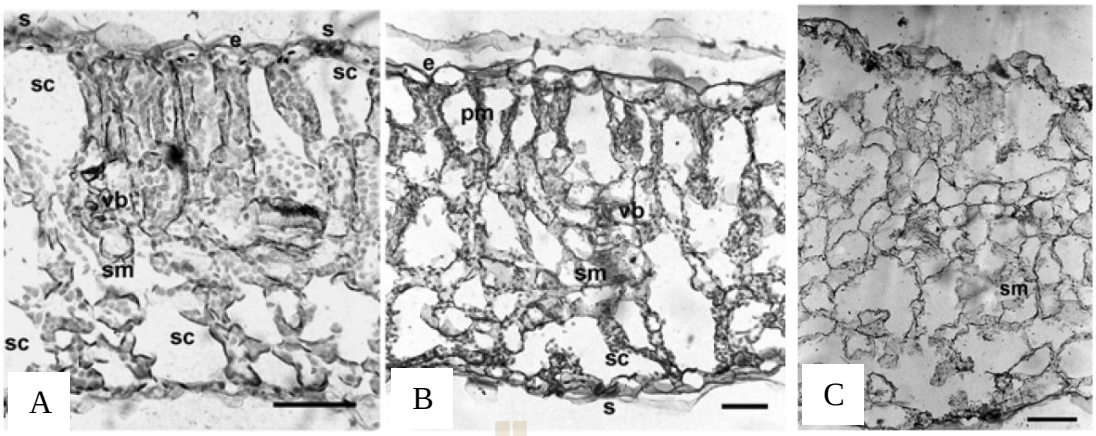


**Figure 2.3** Transverse section of sunflower leaves. (A) Seedling leaves, (B) Normal leaves regenerated *in vitro*, (C) Hyperhydric leaves. e= epidermis, pm= palisade mesophyll, s= stomata, sc= subestomatal cavity, sm= spongy mesophyll, vb= vascular bundle. (Fauguel et al., 2008).

The hyperhydricity causes an effect on the epidermis of hyperhydric leaves by reducing the stomata number and surface area and disrupting their function (Pâques, 1991). The studies on blueberry (*Vaccinium* spp.) (Gao et al., 2018), *Arabidopsis* seedlings (de Klerk and Pramanik, 2017), and *Dianthus chinensis* L. (Sreelekshmi and Siril, 2021) revealed that the hyperhydric explants have fewer stomata number and stoma area compared to normal explants. However, presently, there is no report about the effect of hyperhydricity on stomata number and area in the sunflower plant.

**2.4.2 Causes of hyperhydricity in plant tissue culture**

The morphological abnormalities observed previously in hyperhydric plants are a consequence of physiological abnormalities (Kevers et al., 1988). There are two main kinds of plant hyperhydricity. The first one is a passive phenomenon. This phenomenon could result from a passive water infiltration inside the plant tissue (Paques and Boxus, 1985b). Rojas-Martínez et al. (2010) demonstrated that hyperhydric disorder results from the stressful conditions brought by waterlogging of the plant apoplast, leading to hypoxia and severe oxidative stress. The second one is an active phenomenon that could result from strong biochemical metabolic disturbances (Zimmerman and Cobb, 1989).

The main causes of hyperhydricity in plant tissue culture include a high salt concentration in the plant medium that results in plant oxidative stress, the type of explants used in the culture, the concentrations of plant hormone in the medium, low light intensity in the culture room, high relative humidity and gas accumulation in the atmosphere of the container, the length of time using in plant cultures and subcultures, the number of subcultures, concentration and type of gelling agent, and high ammonium concentration in the medium. Additionally, the liquid culture medium or the medium with a low concentration of gelling agent also causes hyperhydricity (Bhatia and Sharma, 2015).

**2.4.3 Remedies**

Hyperhydricity can be avoided and reversed if the symptom is not too severe. However, curing the disease will exchange in decreasing the proliferation rate. The decrease of relative humidity and the decrease of temperature in the culture container, the decrease of cytokinins and ammonium ions concentrations, and the increase of the gelling agent concentration could reduce not only the hyperhydricity rate but also affect the regeneration rate (Pâques, 1991; Bhatia and Sharma, 2015).

However, several previous research seems successful in the hyperhydricity reduction in plant tissue culture. Mayor et al. (2003) used silver nitrate ($AgNO_3$) as an ethylene inhibitor to reduce the hyperhydricity rate of sunflower tissue culture. Adding silver nitrate into plant media could reduce the hyperhydric shoot formation. However, at the higher silver nitrate concentration, even though the hyperhydricity rate is decreased, the average number of shoots per explant and explant regeneration proportion are decreased. Vinoth and Ravindhran (2015) used silver nitrate to relieve the hyperhydricity in watermelon (*Citrullus lanatus* Thunb.).

Not only that it can prevent hyperhydricity, but silver nitrate also has an additional ability. Gao et al. (2017) use silver nitrate supplements in plant media to reverse hyperhydricity symptoms to normal in pink (*Dianthus chinensis* L.). The study from Sreelekshmi and Siril (2021) also successfully restore hyperhydric *D. chinensis* L. to its regular aspect using silver nitrate and cobalt chloride. So silver nitrate can be widely used in different plant species.

High ethylene and carbon dioxide ($CO_2$) accumulation occur in the culture container because of hermetically sealed containers during the incubation process. Excessive ethylene production is the response of the plant to the stress from *in vitro* culturing. This response could lead to abnormal stomatal closure, causing water accumulation in the tissues and hyperhydricity, but the ventilation of culture vessels can solve the water accumulation problem (Lai et al., 2005). Moreover, enhancing the cuticular transpiration of *in vitro* plantlets can resolve the unusual stomatal closure and water accumulation in plant tissue. Adding trichloroacetate (TCA, an inhibitor of wax biosynthesis) to culture media could prevent the development of hyperhydricity in *Arabidopsis* seedlings. It also increased the permeability of leaves for water and increased air in the intercellular spaces of explant tissue (de Klerk and Pramanik, 2017).

Mackay and Kitto (1986) tested the effects of cytokinin types and their concentrations on the hyperhydricity of *in vitro Pyrus pyrifolia* shoots. This study revealed that both the type of cytokinin hormone and concentration affected the rate of hyperhydricity. Reducing and adjusting the hormone concentration to an appropriate level can reduce the rate of hyperhydric illness. The work also showed that even though the plant is the same species, the different cultivar has different responses and sensitivity to the disease.

Gelling agent type and its concentrations also affect hyperhydricity. The previous work in *Prunus avium* (Franck et al., 2004) and *Arabidopsis thaliana* (de Klerk and Pramanik, 2017) showed that culturing the plant in the media using gelrite as a gelling agent causes a higher percent of hyperhydricity than using agar. The high agar concentration (0.8%) gave a healthier plant with a lower hyperhydricity percentage compared to the low concentration (0.4%) in *in vitro* shoot organogenesis of sunflower (Abdoli et al., 2007).

Agar is a gelling polysaccharide that occurs naturally in certain red seaweeds (particularly in *Gracilaria*, *Gelidium*, and *Pterocladia* species). In some commercial agars and some seaweeds, there is a substance that has an anti-vitrification effect. The antivitrification agent (AVA) is a sulfated polysaccharide that can reduce plant hyperhydricity. Dextran sulfate is one of the AVA types commercially available in a pure form. It has the ability as an anti-vitrification agent and has low toxicity to

plants. Culturing *Eucalyptus* species on a media containing dextran sulfate 0.27% can reduce shoot hyperhydricity by 37% lower than the control that using only 0.25% gelrite (Nairn and Furneaux, 1997). However, more basic studies are necessary to optimize the remedies and better understand hyperhydricity in plant tissue culture.

**2.4.4 Ethylene, reactive oxygen species and hyperhydricity**

Ethylene ($C_2H_4$) is a plant hormone that affects plants' growth, differentiation, and senescence (Reid, 1995). Agriculturists have used it for fruit ripening and promoting flowering. It is produced by mechanical and stress effects, such as rubbing, increased pressure, pathogenic microorganisms, waterlogging, and drought. Wounding during explanting or subculture is the leading cause of ethylene production in plant tissue culture (Reid, 1995; Kumar et al., 1998). Cultured plant tissues produce ethylene and accumulate it in gaseous form (Thomas and Murashige, 1979). The ethylene quantity varies with the growth stage, growth regulators, the plant species, and the closure type (Dalton and Street, 1976; Hughes, 1981).

An oxidant is a chemical species that removes electrons from another reactant in a chemical reaction. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation (degradation of lipids) or oxidizing DNA or proteins that cause damage and mutations and possibly lead to cancer. Reactive oxygen species (ROS) is one of the oxidant agents that contain oxygen, such as peroxides, superoxide, and hydroxyl radicals. It is highly reactive (quickly reacts with other compounds) and can damage lipid, DNA, RNA, and proteins, contributing to cell aging and death (Zhang et al., 2016).

ROS formulation exists during the mitochondrial oxidative metabolism and when the cell responds to xenobiotics (an unnaturally chemical substance within an organism), cytokines (small proteins in cell signaling), and bacterial invasion. Oxidative stress occurs when plants have excess ROS or oxidants over the cell's capability to handle them (Baxter et al., 2014).

Hyperhydricity is a consequence of plant response to stress when culture explants in an unsuitable *in vitro* environment. Ethylene (a stress-related plant hormone) and ROS play critical roles in the plant stress response regulation (Song et al., 2014; Yin et al., 2015; Sun et al., 2016). ROS influences ethylene metabolism (Li et

al., 2015), while ethylene can amplify ROS accumulation (Wi et al., 2010). Ethylene influences *in vitro* morphogenesis and the development of tissue cultured plants (Lieberman, 1979; Hall and Smith, 1995). Excessive ethylene accumulation during micropropagation is generally associated with hyperhydricity (Kevers and Gaspar, 1985; Fal et al., 1999; Franck et al., 2004). Gaspar (1986) suggested that the ethylene level affected hyperhydricity. in addition, Gao et al. (2017), Sreelekshmi and Siril (2021) also reported that hyperhydric plantlets had increased ethylene levels.

Eliminating excessive ethylene can effectively prevent or reduce hyperhydricity by applying the methods such as ventilation systems in the culture vessels (Majada et al., 1997; Zobayed et al., 2001), using ethylene absorbent (Dimasi-Theriou et al., 1993; Sarkar et al., 2002) and using ethylene biosynthesis inhibitors (Lakshmanan et al., 1997). Adding silver nitrate to the culture media can control ethylene. Silver ion (Ag+) blocks the ethylene activity by binding to ethylene receptors involved in ethylene signaling. Thereby inhibiting ethylene signal transduction and its activity, reducing hyperhydricity and then enhancing shoot regeneration in many plant species (Songstad et al., 1988; Hyde and Phillips, 1996; Eapen and George, 1997; Qin et al., 2005).

Plant media with cobalt chloride can stimulate shoot regeneration and strongly inhibits ethylene production (Latche et al., 1991). Cobalt inhibits ethylene synthesis by suppressing ACC synthase (an enzyme that creates ethylene precursor), shown in Figure 2.4 (Kumar et al., 2009). So, both silver nitrate and cobalt chloride can block ethylene activity, prevent hyperhydricity, and promote successful shoot development in plant tissue culture.

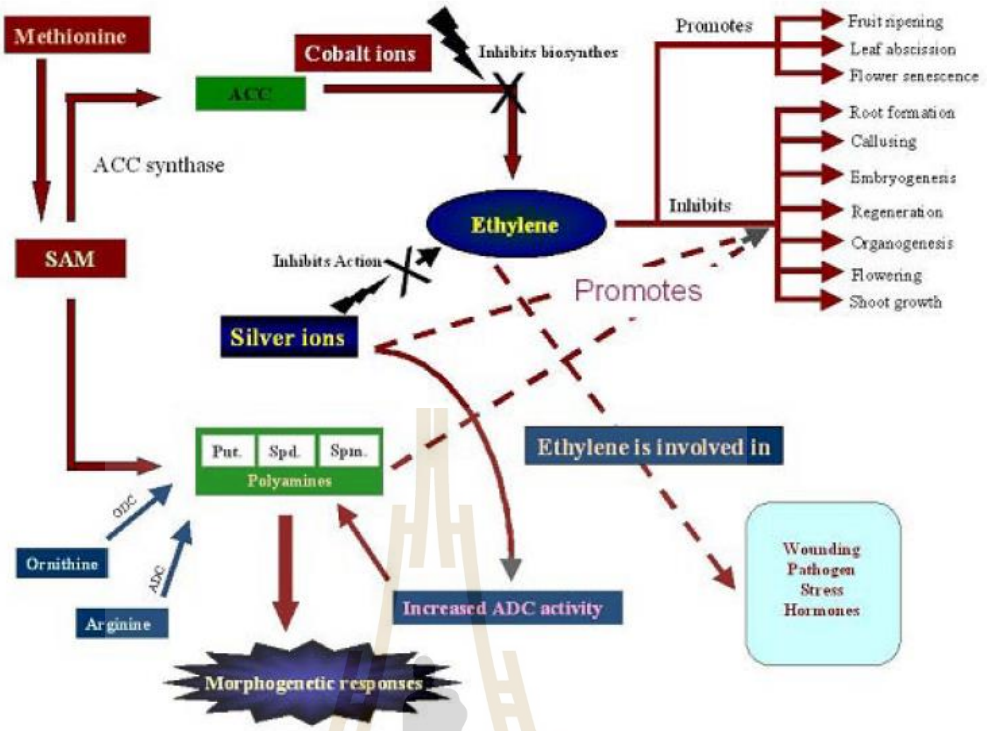


**Figure 2.4** Ethylene biosynthesis and its influences on various physiological processes (Kumar et al., 2009).

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Explant materials

### 3.1.1 Sunflower genotypes

In this study, plant materials were sunflower seeds. Seeds used in this study included seeds from oilseed representative, Suranaree 473 (S473) (Figure 3.1A) genotype received from SUT farm. Moreover, F1 hybrid seeds of ornamental sunflower, Autumn Beauty x Moulin Rouge hybrid (Figure 3.1B) from Johnny's Selected Seeds, USA.

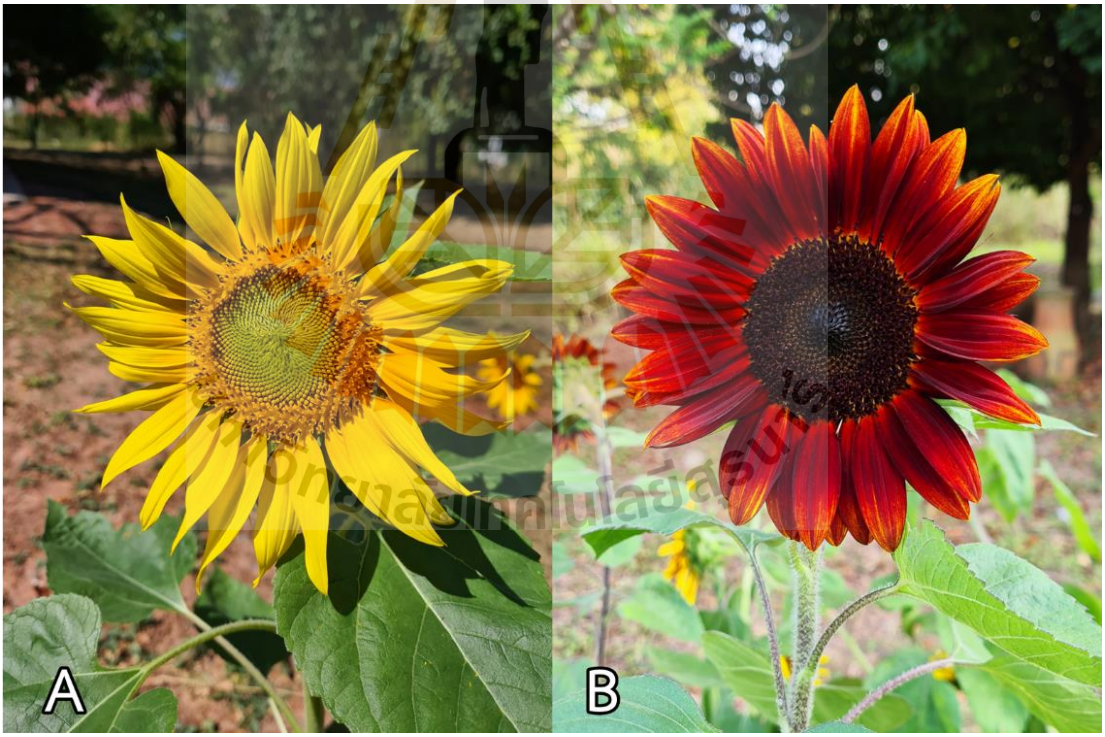


**Figure 3.1** The inflorescence of sunflowers. (A) The inflorescence of S473 genotype. (B) The inflorescence Autumn Beauty x Moulin Rouge hybrid genotype.

### 3.1.2 Explant material preparation

S473 or F1 hybrid seeds of ornamental sunflower (Autumn Beauty x Moulin Rouge hybrid) were first sterilized with sodium hypochlorite (1.8%, v/v) for 30 minutes. Then seeds were rinsed with sterile distilled water three times in a laminar flow hood. The seed hulls were removed, and dehulled seeds were disinfected with a 3% hydrogen peroxide solution for 30 seconds. After that, seeds were placed on wet sterile tissue paper on a Petri dish plate. These seeds were kept in the tissue culture room under dark conditions and at 25±2 ℃ for 48 hours before using as explant materials in the experiment part I or II.

Before cultured explants on the media, germinated seeds were cut to remove the radicle and cotyledons (Figure 3.2A). Obtained explants, about 3 mm, carrying the meristem and a base of cotyledon, as shown in Figure 3.2B, were used for the experiments.

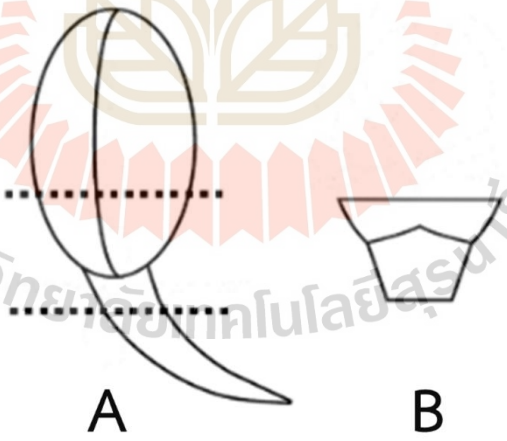


**Figure 3.2** Explant material preparation. (A) Germinated seed: lines represent the cuts performed to remove the radicle and cotyledons. (B) Explant with meristem and cotyledon base.

## 3.2 Experiment I: Effect of media supplements on hyperhydricity and shoot regeneration of *in vitro* ornamental sunflower

### 3.2.1 Effect of media supplements on hyperhydricity, shoot regeneration, and shoot length of *in vitro* sunflower at shoot induction phase

Explants (Figure 3.2B) derived from seeds of F1 hybrid ornamental sunflower (Autumn Beauty x Moulin Rouge hybrid) were used in experiment I.

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 various concentrations of silver nitrate (1, 2, and 4 mg/L), TCA (100, 200, and 300 mg/L) or dextran sulfate (10, 20, and 30 mg/L). Supplements were not equal in concentrations due to their toxicity. Total 10 media were tested, including A1 to A10 (Table 3.1). 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 minutes.

**Table 3.1** Explants cultured on various media supplements: silver nitrate, TCA, or dextran sulfate, and three supplement levels at shoot induction phase.

| Media | Basal | Supplements | Level | Concentration (mg/L) | References |
|---|---|---|---|---|---|
| A1 | MS + BA 2 mg/L | - | - | - | (Montathong, 2017) |
| A2 | | Silver nitrate | 1 | 1 | (Mayor et al., 2003) |
| A3 | | | 2 | 2 | |
| A4 | | | 3 | 4 | |
| A5 | | TCA | 1 | 100 | (de Klerk and Pramanik, 2017) |
| A6 | | | 2 | 200 | |
| A7 | | | 3 | 300 | |
| A8 | | Dextran sulfate | 1 | 10 | (Nairn and Furneaux, 1997) |
| A9 | | | 2 | 20 | |
| A10 | | | 3 | 30 | |

The culture vessels were 16 oz glass bottles (12 cm × 7 cm) with a vented plastic cap. The cap has a 1 cm diameter punched hole with a 0.2-micron filter for ventilation. So, the experiment part I was conducted under ventilation conditions for all treatments. Each bottle contained 50 mL of medium and 4 explants (Figure 3.3). Ten treatments in total included 12 explants per replicate, and 4 replicates were investigated. Culture conditions were standardized with equal light intensity (Panasonic FL40SS-D/36, 36W, 2600 lumens), 16/8-hour (day/night) photoperiod, 25±2 °C for all treatments.

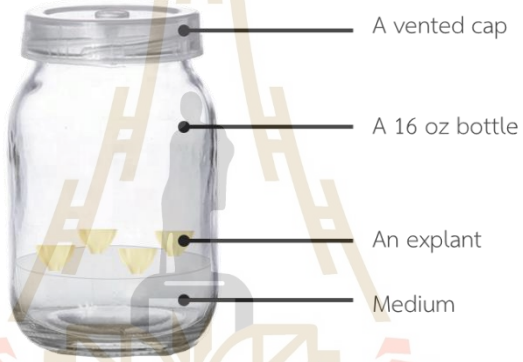


**Figure 3.3** A culture vessel diagram with explants and plant medium.

Explants were completely randomly selected to place into one of the possible media treatments. Cotyledon explants were grown for 3 weeks, and then HH was determined.

Hyperhydricity in this study is defined as explants with thick translucent shoots, humid leaf aspects, and/or curled leaves, transparent, lighter green color, and a thicker stem than normal explants (Fauguel et al., 2008).

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

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

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

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

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

All explants were transferred to the shoot elongation medium after all data have been collected.

**3.2.2 Effect of media supplements on hyperhydricity, shoot regeneration, shoot length, water content, and stomata number of *in vitro* ornamental sunflower at shoot elongation phase**

The whole and unmodified explants from the shoot induction phase (section 3.2.1) were transferred from SIM to shoot elongation medium (SEM) for further multiplication and shoot elongation, using the same supplement types and concentrations as in the previous phase. The basal of SEM was MS medium containing 2 mg/L N6-[2-Isopentenyl] adenine (2-iP) and 1 mg/L BA (Sujatha et al., 2012). A total of 10 media comprised B1 to B10 (Table 3.2). Each culture bottle contained 50 mL of medium and 3 explants. The culture conditions were the same as in the shoot induction phase.

Explants were grown for 2 weeks, and then HH percentage, percentage of regeneration, number of shoots per explant, and average shoot length were determined and counted using the same criteria as stated in the experiment section 3.2.1.

For water content measurements, 10 explants from each treatment were randomly selected as samples. The explant samples were weighed using the analytical balance, and the initial fresh weight was recorded. The samples were then dried using a hot air oven at 80 ℃ for 3 days. Thereafter, the dry weights were measured. The formula for calculating the water content is:

$$\text{Water content (\%) (w/w)} = \frac{\text{Fresh weight - Dry weight}}{\text{Fresh weight}} \times 100$$

**Table 3.2** Explants cultured on various media supplements: silver nitrate, TCA, or dextran sulfate, and three supplement levels at shoot elongation phase.

| Media | Basal | Supplements | Level | Concentration (mg/L) | References |
|---|---|---|---|---|---|
| B1 | MS+ 2-iP 2 mg/L + BA 1 mg/L | - | - | - | (Sujatha et al., 2012) |
| B2 | | Silver nitrate | 1 | 1 | (Mayor et al., 2003) |
| B3 | | | 2 | 2 | |
| B4 | | | 3 | 4 | |
| B5 | | TCA | 1 | 100 | (de Klerk and Pramanik, 2017) |
| B6 | | | 2 | 200 | |
| B7 | | | 3 | 300 | |
| B8 | | Dextran sulfate | 1 | 10 | (Nairn and Furneaux, 1997) |
| B9 | | | 2 | 20 | |
| B10 | | | 3 | 30 | |

The number of stomata was examined on 10 randomly selected leaf samples within the treatment. The abaxial epidermis of the leaves (ventral side) was peeled off and then stained with toluidine blue dye. The epidermis was observed under a light microscope (Olympus CH-2, Japan). Images were captured using a compatible microscope camera (Moticam X3 Plus, USA). Stomata density was measured using a digital ruler in Motic Images Plus 3.0 digital microscope/camera software. Finally, the mean of the stomata number was calculated in the form of stomata per square millimeter ($mm^2$).

### 3.2.3 Rooting and acclimatization

After 2 weeks of culture on SEM, elongated shoots from the best performance medium were transferred to half-strength MS medium supplemented with 0.5 mg/L α-Naphthaleneacetic acid (NAA) and 200 mg/L charcoal for rooting, modified from Sujatha et al. (2012). 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, the healthy completed healthy plantlets were transplanted into the soil and growing to maturity. The plants that survived after being transplanted into the soil at 3 weeks were tallied.

### 3.2.4 Data analysis

A completely randomized design (CRD) of experiments was used in this study. The experimental data were analyzed by mean value that was taken from four replicates and significant mean differences were determined with Duncan's multiple range tests (DMRT) test level at 0.05 % and analysis was done using IBM SPSS (v. 25.0) program. Each treatment consisted of 12 explants per replicate with 4 replicates.

## 3.3 Experiment II: Effect of media supplements and ventilation on hyperhydricity and shoot regeneration of *in vitro* oil seed sunflower

### 3.3.1 Effect of media supplements and ventilation on hyperhydricity, shoot regeneration, and shoot length of *in vitro* oil seed sunflower at shoot induction phase

Explants derived from seeds of S473 genotype were used in experiment II. Plant media were prepared using MS media containing 2 mg/L BA and 30 g/L sucrose as the basal of SIM. The SIM was supplemented with either 1 mg/L silver nitrate, 100 mg/L TCA, or 1 mg/L silver nitrate + 100 mg/L TCA for the supplement combination test. SIM with supplements were divided into two sets of culture conditions. Ventilation and non-ventilation conditions were applied using the normal plastic cap and the vented cap. Total 8 media were tested, including C1 to C4V (Table 3.3). 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 minutes.

**Table 3.3** Explants cultured on various media supplements: silver nitrate, TCA, or silver nitrate + TCA, and two ventilation conditions.

| Media | Basal | Conditions | Supplements |
|---|---|---|---|
| C1 | MS + BA 2 mg/L | Non ventilation | - |
| C2 | | | Silver nitrate 1 mg/L |
| C3 | | | TCA 100 mg/L |
| C4 | | | Silver nitrate 1 mg/L + TCA 100 mg/L |
| C1V | | Ventilation | - |
| C2V | | | Silver nitrate 1 mg/L |
| C3V | | | TCA 100 mg/L |
| C4V | | | Silver nitrate 1 mg/L + TCA 100 mg/L |

The culture vessels were 16 oz glass bottles 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-hour (day/night) photoperiod, 25±2 °C for all treatments.

Explants were completely randomly selected to place into one of the possible media treatments. Cotyledon explants were grown for 3 weeks, and then HH was determined. Hyperhydricity in this study is defined as stated in experiment part I. All shoots (explant stems with true leaves) greater than 5 mm were measured in length and counted by eyes. Percentage of hyperhydric shoots, percentage of regeneration (explants with shoots), the average number of shoots per explant, and average shoot length were calculated and averaged over three replicates.

All explants were kept growing in the same culture after all data had been collected. It is to be noted that this experiment had no subculture or transfer of explants along with both shoot induction and elongation phase.

**3.3.2 Effect of media supplements and ventilation on hyperhydricity, shoot regeneration, shoot length, water content, and stomata number of *in vitro* oil seed sunflower at shoot elongation phase**

Explants from the shoot induction phase (section 3.3.1) were continuously cultured on the same SIM for two weeks as the shoot elongation phase for further multiplication and shoot elongation. Then HH percentage, percentage of regeneration, number of shoots per explant, and average shoot length were determined and counted using the same criteria as stated in the first experiment.

For water content measurements, 20 explants from each treatment were randomly selected as samples. The explant samples were weighed using the analytical balance, and the initial fresh weight was recorded. The samples were then dried using a hot air oven at 80 °C for 3 days. Thereafter, the dry weights were measured, and water content was calculated.

The number of stomata was examined on 20 randomly selected leaf samples within the treatment. The abaxial epidermis of the leaves was peeled off and then stained with toluidine blue dye. The epidermis was observed under a light microscope (Olympus CH-2, Japan). Images were captured using a compatible microscope camera (Moticam X3 Plus, USA). Stomata density was measured using a digital ruler in Motic Images Plus 3.0 digital microscope/camera software. Finally, the mean of stomata number per square millimeter ($mm^2$) was calculated.

**3.3.3 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 being transplanted into the soil at 3 weeks were counted.

### 3.3.4 Data analysis

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

# CHAPTER IV
# RESULTS AND DISCUSSION

## 4.1 Experiment I: Effect of media supplements on hyperhydricity and shoot regeneration of *in vitro* ornamental sunflower

### 4.1.1 Effect of media supplements on hyperhydricity, shoot regeneration, and shoot length of *in vitro* ornamental sunflower at shoot induction phase

Explants increased in size on Shoot induction medium (SIM) supplemented with various concentrations of silver nitrate (1, 2, and 4 mg/L), TCA (100, 200, and 300 mg/L), or dextran sulfate (10, 20, and 30 mg/L). After 3 weeks, several shoots and their leaves developed (Figure 4.1). Explants from the control medium had long and thin shoots with long internodes. Leaves were normally developed with smooth leaf edges but were relatively small, and some still were not expanded yet. The hypocotyl stumps were increased, and callus formed on the base of explants (Figure 4.1A).

Media A2-A4 were supplemented with silver nitrate (Figure 4.1B-4.1D). Explants from the A2 medium with low silver nitrate concentration had a wider stem and larger leaves compared to other media. Leaves were larger and had curled leaf edges. Increasing silver nitrate concentration resulted in abnormal leaf formation. The color of the shoots and leaves was slightly translucent, along with deformed leaf development, showing twisted and asymmetrical leaves which is one characteristic of hyperhydricity. Some explants did not develop shoot, whereas other exhibited hyperhydric aspect throughout explant with a thick, short, brittle, and clear pale green color stem (Figure 4.1D).

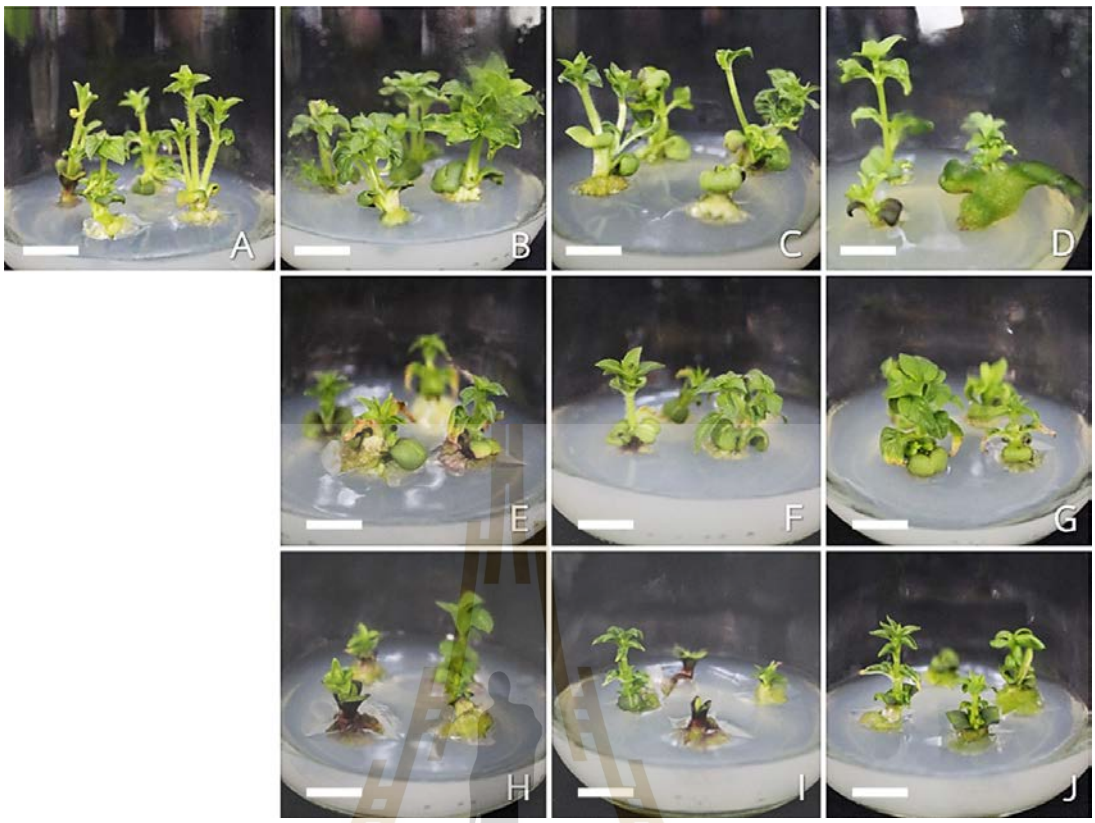


**Figure 4.1** Ornamental sunflower regeneration from meristem and cotyledon base explants on shoot induction medium. (A) Explants cultured for 3 weeks on MS medium + 2 mg/L BA as SIM. (B-D) Explants cultured for 3 weeks on SIM + 1, 2, or 4 mg/L silver nitrate respectively. (E-G) Explants cultured for 3 weeks on SIM + 100, 200, or 300 mg/L TCA respectively. (H-J) Explants cultured for 3 weeks on SIM + 10, 20, or 30 mg/L dextran sulfate respectively. Bars = 1 cm.

Explants from media A5-A7 with trichloroacetate (TCA) showed a larger hypocotyl stump than other treatments. The stump was enlarged and exposed in light green callus formation at the explant base, where it touched the surface of the media. Forming and growing without hyperhydric aspect, shoots and leaves had standard green color but had dry and yellow color at distal part of leaves. This symptom may be caused by extra water loss in the plant cells (Figure 4.1E-4.1G).

Explants from media with dextran sulfate are in Figure 4.1H-4.1J. Cotyledon bases were increased and produced calli with yellow and green colors. Leaves were smaller than other treatments and curled from the lateral edge. Some explants did not develop shoot but only had the enlargement of the explant base. Explants grown

with minor hyperhydric symptoms showed clear shoot form and no hyperhydric aspect on leaves.

**Table 4.1** Effect of media supplements on shoot regeneration, number of shoots per explant, shoot length, hyperhydric shoots, and callus formation of *in vitro* ornamental sunflower at shoot induction phase.

| Media | Supplements | Shoot regenera-tion (%) | Number of shoots per explant (mean±SE) | Mean shoot length (mm) (mean±SE) | Hyperhydric shoots (%) |
|---|---|---|---|---|---|
| A1 | - | 83.33$^{a}$ | 1.78±0.37$^{ab}$ | 12.24±0.76$^{a}$ | 16.70$^{abc}$ |
| A2 | Silver nitrate 1 mg/L | 95.83$^{a}$ | 2.15±0.25$^{a}$ | 11.95±0.97$^{ab}$ | 17.96$^{abc}$ |
| A3 | Silver nitrate 2 mg/L | 89.58$^{a}$ | 1.38±0.44$^{bcd}$ | 11.09±1.32$^{ab}$ | 31.01$^{ab}$ |
| A4 | Silver nitrate 4 mg/L | 87.50$^{a}$ | 1.58±0.21$^{bc}$ | 11.48±1.76$^{ab}$ | 37.32$^{a}$ |
| A5 | TCA 100 mg/L | 85.42$^{a}$ | 1.60±0.20$^{bc}$ | 10.72±1.31$^{abc}$ | 6.51$^{bc}$ |
| A6 | TCA 200 mg/L | 75.00$^{a}$ | 1.33±0.11$^{bcd}$ | 9.44±2.25$^{bc}$ | 5.98$^{bc}$ |
| A7 | TCA 300 mg/L | 91.67$^{a}$ | 1.66±0.18$^{bc}$ | 10.57±0.84$^{abc}$ | 1.39$^{c}$ |
| A8 | Dextran sulfate 10 mg/L | 52.08$^{b}$ | 1.18±0.48$^{cd}$ | 8.23±0.65$^{c}$ | 28.21$^{abc}$ |
| A9 | Dextran sulfate 20 mg/L | 45.83$^{b}$ | 1.29±0.39$^{bcd}$ | 9.74±2.81$^{abc}$ | 31.02$^{ab}$ |
| A10 | Dextran sulfate 30 mg/L | 35.42$^{b}$ | 1.08±0.17$^{d}$ | 8.38±1.36$^{c}$ | 36.46$^{a}$ |

Means in columns followed by different letters are significantly different according to DMRT at $P$ = 0.05.

Table 4.1 shows the effect of types and concentrations of supplements on sunflower responses. The percentage of HH at the shoot induction phase in A1-A10 media ranged from 1.39% to 37.32%, with no significant difference between the treatments and the control. The A7 medium added with 300 mg/L TCA gave the lowest HH percentage (1.39%), whereas the media with 4 mg/L silver nitrate gave the highest HH percentage (37.32%). Hyperhydric shoots from A3 and A4 media appeared to have a translucent, light green color of shoot tips with abnormal leaf formation. So, the

hyperhydric symptom was not too severe. When silver nitrate and TCA were added to the SIM medium, the percentage of shoot regeneration, number of shoots per explant, and shoot length were not statistically significant compared to the control, except for the shoot length of the A6 medium. The percentage of shoot regeneration was considerably reduced in the dextran sulfate-added media, with the lowest achieved in the A10 medium with 30 mg/L dextran sulfate, as shown in Figure 4.1H-4.1J. Some explants from dextran sulfate were not developing shoot. In addition, as compared to the control, the medium with 10 mg/L and 30 mg/L dextran sulfate resulted in a significantly reduced number of shoots per explant and shoot length. The highest number of shoots per explant (2.15±0.25) was achieved on an A2 medium supplemented with 1 mg/L silver nitrate. However, it was not considerably significantly greater than the control, despite the control having the most extended shoot length (12.24±0.76 mm).

Dextran sulfate, a sulfated polysaccharide, has an anti-vitrification property by antioxidant activity that could relieve plant stress and HH. Nairn and Furneaux (1997) stated that some gelling agents have other materials which can be toxic to growing plants. It is possible that dextran sulfate used in this study has the toxic materials, when used with an agar gelling agent, contributed to the imbalance between anti-vitrification and toxicity effects that resulted in low regeneration efficiency and affected in undersized shoots of sunflower tissue culture.

In this experiment, silver nitrate treatments showed no significant difference in HH when cultured for 3 weeks compared to the control. Hence, this result does not agree with the report presented by Mayor et al. (2003), who found a reduced HH percentage in sunflower when using silver nitrate at 5.0-7.5 µM (0.9-1.3 mg/L) as a supplement after 5 weeks of culture. The concentration varied in this experiment (1-4 mg/L) may be too high for plants and could cause the negative effect that resulted in abnormality of shoot and leaf formation and stunted growth. As shown in Figure 4.1C-4.1D, explants exhibited malformed developed shoots and leaves compared with explants from no supplement and low silver nitrate media.

The amount of toxicity may differ depending on the plant species or type and the age of the explant. The supplement concentration and period of culture are

also necessary. Khan et al. (2019) reported the toxicity of a high concentration of silver nitrate on Pearl Millet (*Pennisetum glaucum* L.) seedlings which caused plant oxidative stress and reduced plant growth. Karimi and Mohsenzadeh (2017) reported the same result of the toxicity on seedlings of common wheat (*Triticum aestivum*). Seedling growth cultured under high silver nitrate concentration (10 and 100 mg/L) had lower seedling weight and reduced chlorophyll content. They stimulated stress response enzymes, which caused negative aspects in plants. So, silver nitrate at 2-4 mg/L may be toxic to sunflower seedlings caused of those negative appearances.

On the other hand, TCA treatment resulted in a lower HH percentage than silver nitrate. TCA may inhibit wax synthesis and help plants eliminate excess water caused by the reduction of HH and expressed as yellow and dry leaf edges in explants within the TCA treatments, indicating that plants may lose water easier (de Klerk and Pramanik, 2017).

**4.1.2 Effect of media supplements on hyperhydricity, shoot regeneration, shoot length, water content, and stomata number of *in vitro* ornamental sunflower at shoot elongation phase**

Explants from the shoot induction phase were transferred from SIM to shoot elongation medium (SEM) using the same supplement types and concentrations as in the previous phase. The basal of SEM was MS medium containing 2 mg/L N6-[2-Isopentenyl] adenine (2-iP) and 1 mg/L BA (Sujatha et al., 2012). A total of 10 media comprised B1 to B10. After 2 weeks of culture on SEM medium for further shoot multiplication and elongation, explant shoots were elongated and had more expanded leaves (Figure 4.2).

Explants from the control medium (B1) usually had long and slim stems grown up from the tissue of cotyledons. Leaves were green and heart-shaped with smooth leaves and edges. The base of explants enlarged from the cut area had tight, solid, and had brownish-green color tissue. Explants usually had standard color with no sign of hyperhydric aspects such as translucent shoot or leaves (Figure 4.2A).

In contrast, explants grown on SEM supplemented with silver nitrate (B2-B4) appeared to have a different appearance. At low silver nitrate concentration (1 mg/L), stems were slightly wilder, and the distance of the whorl of the two first primary

leaves was shorter than from the control. Leaves were large and had smooth leave edges. Some leaves were developed differently with curled leaves, incomplete leaf tips, and asymmetrical formations. The color of the shoots and leaves were the same as from the control with no hyperhydric aspect. The color was opaque green, with no translucent and grassy looks in any part of the explant (Figure 4.2B). Increasing silver nitrate concentration to 2-4 mg/L caused abnormal shoot formation (Figure 4.2C-4.2D). Explants had thicker shoots than other explants, and shoot tips were dry and dead. The leaves were not heart-shaped like those in the control group. A dead brown area of leaf nodes was between asymmetrical aberrant leaves. Cotyledon's base comprises compact cells with a dark brown-green hue and a water-soaked appearance.

TCA supplemented media were used with explants that show in Figure 4.2E-4.2G. Regular shoots developed without a hyperhydric aspect. The length from cotyledons to the first leaf whorl was shorter than the controlled explants. Leaves were usually fully expanded without curling or fluttering edges. Some shoots started to produce tiny flower buds in the first week of culture on SEM (Figure 4.2G). The tips of the leaves usually changed color from green to yellow, and drying out, and showed the light brown dead zone on the leaf blade. It is worth noting that plants cultured on media B5-B7 usually had large light green callus formation that developed from hypocotyl stomp compared to other treatments.

Figure 4.2H-4.2J shows explants cultured on B8-B10 media supplemented with 10-30 mg/L dextran sulfate, respectively. Explants had shoot developed with some dying and dry shoot tips. Leaves shapes were elongated and differed from other treatments. The tips of the leaves were slightly yellow and dry. Curled leaves rolled from the lateral edge along with translucent glassy leaves shown in dark green were formed when increased the dextran sulfate concentration (Figure 4.2J). Tiny flower buds started to form in the first week of cultured on SEM. Some explants produced calli that hardly distinguished from the part of the cotyledon's base—the callus color widely from light green to dark greenish-brown.

Table 4.2 shows the mean values for parameters of *in vitro* sunflower at the shoot elongation phase. For the results, the hyperhydric shoots ranged from 1.56% to 37.28%, with no significant difference compared with the control, except for the B3

medium with 2 mg/L silver nitrate. Hyperhydric shoots differed in morphology and severeness depending on the type and concentrations of added supplements (Figure 4.3).

The control medium (B1) gave 5.21% HH shoots. Hyperhydric shoots in the control medium had low severity with normal shoots and leaf development. However, some parts of the leaves expressed a translucent and water-soaked appearance as a symptom of HH (Figure 4.3A). The lowest frequency of hyperhydric shoots was 1.56% found in the B7 medium containing 300 mg/L TCA. Hyperhydric shoots rarely occurred in TCA treatments, but when it had HH, the symptom was quite severe, which can be noticed from the shoot appearances. The HH shoots were short, and leaves were rolled and elongated with dark green color and water-soaked looks (Figure 4.3B). In addition, the highest percentage was 37.28% in the B3 medium with 2 mg/L silver nitrate, followed by 14.30% HH in the B4 medium with 4 mg/L silver nitrate. Hyperhydric explant from silver nitrate supplement media had thickened shoot that was not clearly separated. Some leaves exhibited HH symptoms with malformed aspects and dark green translucent color. Compared to HH from other media, HH leaves of silver nitrate treated media had coiled leaves attached to the stem and could not see the petiole (Figure 4.3C). Media with dextran sulfate resulted in 7.81-9.29% HH, which did not significantly differ from the control. The hyperhydric shoots were short and had normal leaf shape but showed a translucent and dark green color (Figure 4.3D). So, the HH could occur within all types of supplemented media, but the plant responded and showed different physical characteristics.

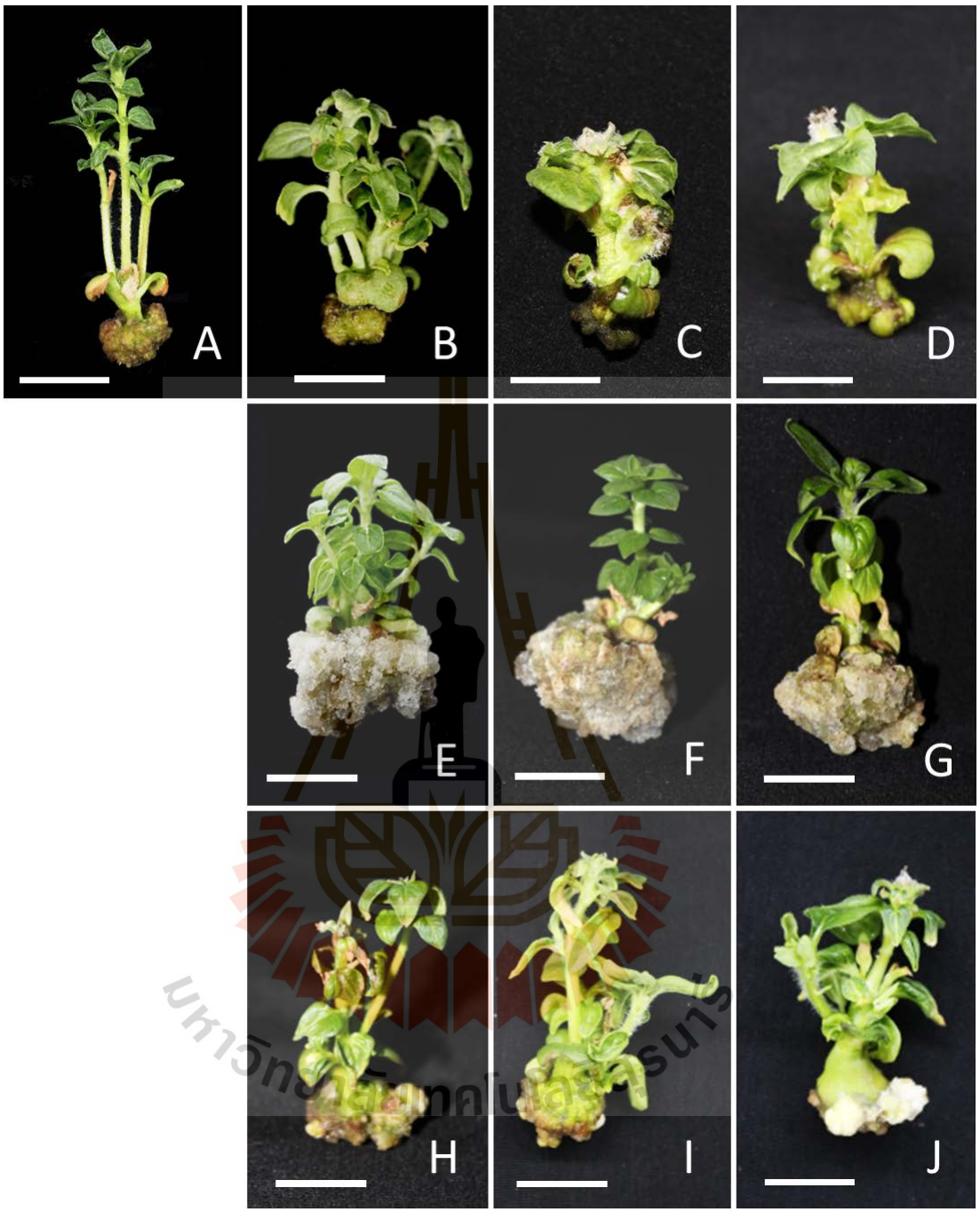


**Figure 4.2** Sunflower explants cultured on shoot elongation medium (SEM). (A) Explants cultured for 2 weeks on MS medium containing 2 mg/L 2-iP + 1 mg/L BA as SEM. (B-D) Explants cultured for 2 weeks on SEM + 1, 2, or 4 mg/L silver nitrate respectively. (E-G) Explants cultured for 2 weeks on SEM + 100, 200, and 300 mg/L TCA respectively. (H-J) Explants cultured for 2 weeks on SEM + 10, 20, and 30 mg/L dextran sulfate respectively. Bars = 1 cm.

**Table 4.2** Effect of media supplements on shoot regeneration, number of shoots per explant, shoot length, and hyperhydric shoots of *in vitro* ornamental sunflower at shoot elongation phase.

| Media | Supplements | Shoot regene-ration (%) | Number of shoots per explant (mean±SE) | Mean shoot length (mm) (mean±SE) | Hyper-hydric shoots (%) | Survival rate (%) |
|---|---|---|---|---|---|---|
| B1 | - | 70.83$^{abc}$ | 1.54±0.60$^{bcd}$ | 20.50±2.02$^{ab}$ | 5.21$^{b}$ | 66.67$^{c}$ |
| B2 | Silver nitrate 1 mg/L | 83.33$^{a}$ | 2.20±0.38$^{a}$ | 19.52±1.34$^{abc}$ | 3.89$^{b}$ | 100.00$^{a}$ |
| B3 | Silver nitrate 2 mg/L | 79.17$^{ab}$ | 2.02±0.28$^{ab}$ | 15.92±2.33$^{cd}$ | 37.28$^{a}$ | 93.75$^{ab}$ |
| B4 | Silver nitrate 4 mg/L | 79.17$^{ab}$ | 1.54±0.24$^{bcd}$ | 17.44±3.67$^{bcd}$ | 14.30$^{b}$ | 81.25$^{bc}$ |
| B5 | TCA 100 mg/L | 83.33$^{a}$ | 1.83±0.33$^{abc}$ | 21.20±2.60$^{a}$ | 2.39$^{b}$ | 93.75$^{ab}$ |
| B6 | TCA 200 mg/L | 68.75$^{abc}$ | 1.42±0.28$^{cd}$ | 18.96±1.34$^{ab}$ | 7.50$^{b}$ | 93.75$^{ab}$ |
| B7 | TCA 300 mg/L | 81.25$^{a}$ | 1.80±0.14$^{abc}$ | 21.15±2.33$^{a}$ | 1.56$^{b}$ | 93.75$^{ab}$ |
| B8 | Dextran sulfate 10 mg/L | 56.25$^{bc}$ | 1.57±0.18$^{bcd}$ | 14.25±1.23$^{d}$ | 7.81$^{b}$ | 79.17$^{bc}$ |
| B9 | Dextran sulfate 20 mg/L | 47.92$^{c}$ | 1.39±0.31$^{cd}$ | 15.85±2.26$^{cd}$ | 9.29$^{b}$ | 87.50$^{ab}$ |
| B10 | Dextran sulfate 30 mg/L | 64.58$^{abc}$ | 1.23±0.10$^{d}$ | 18.20±2.50$^{abc}$ | 8.17$^{b}$ | 93.75$^{ab}$ |

Means in columns followed by different letters are significantly different according to DMRT at $P$ = 0.05.

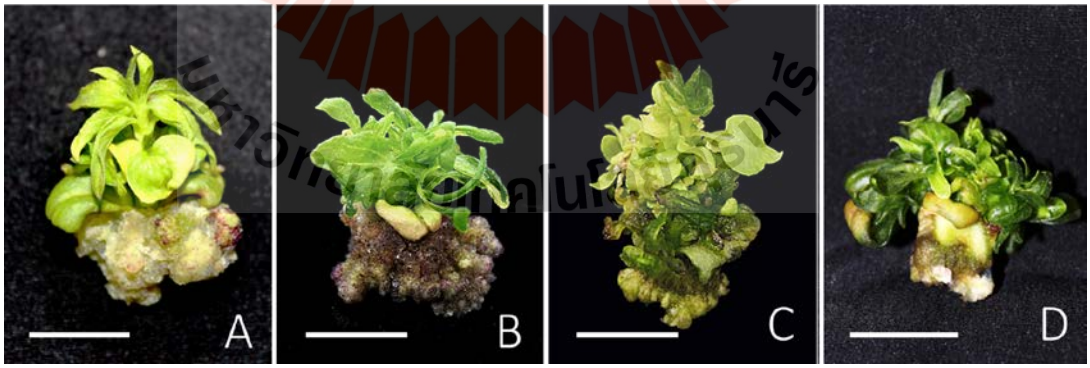


**Figure 4.3** Hyperhydric explants from different media. (A) SEM with no supplement. (B) SEM + 200 mg/L TCA. (C) SEM + 4 mg/L silver nitrate. (D) SEM + 30 mg/L dextran sulfate. Bars = 1 cm.

Moreover, when silver nitrate, TCA, and dextran sulfate were added to the SEM medium, the percentage of shoot regeneration was not statistically significant

compared to the control. The maximum number of shoots per explants (2.20±038) and survival rate (100%) were found with 1 mg/L silver nitrate, which differed significantly from the control, indicating a positive effect of this compound at low concentration. Conversely, increasing the level of silver nitrate to 4 mg/L significantly reduced the number of shoots per explant (1.54±0.24) and survival rate (81.25%), showing the negative effect of silver nitrate at higher concentration. In addition, a higher concentration of silver nitrate in the medium could be toxic to the plants and result in shoot malformation and harsh hyperhydricity. These findings do not agree with the report from Mayor et al. (2003), who showed that an increase in silver nitrate concentration reduced HH percentage in sunflower. The variations in the findings could be due to starting explants, sunflower variety differences, and concentrations of the supplement. Since that study varied silver nitrate concentrations at 2.5, 5.0, and 7.5 µM (0.42, 0.84, and 1.27 mg/L, respectively), the low concentrations maybe not be toxic to sunflower. At low concentration, 0.42 mg/L, shoot number was increased, and HH was decreased compared with control. Increasing silver nitrate to 1.27 mg/L reduced regeneration capacity, shoot number, and HH in sunflower tissue culture. In the current study, the concentrations at 1, 2 and 4 mg/L may be high for sunflower and caused a negative effect on the regeneration, explants morphology, and survival rate throughout the experiment.

Additionally, silver nitrate toxicity has been reported on wheat and *Brassica* seedlings. The high concentration could induce plant stress and inhibit plant growth, resulting in more significant yield loss, abnormal development, and a low survival rate (Karimi and Mohsenzadeh, 2017; Vishwakarma et al., 2017). However, the toxicity level is highly dependent on plant genotypes, explant type, the concentration of supplement, and the duration of the cultivation. Based on the regeneration results, this study has confirmed that toxicity tolerance to silver nitrate of *in vitro* ornamental sunflower starts at 2-4 mg/L in silver nitrate concentrations.

Media supplemented with TCA (B5-B7) exhibited low HH frequencies (1.56-7.50%) but was not significantly different among concentration levels and the control (5.21%), indicating that it had no impact on reducing HH of sunflower. A previous report by de Klerk and Pramanik (2017) showed that HH percentage in *Arabidopsis* seedlings

decreased with increased TCA concentrations. The authors used gelrite as the gelling agent to induce HH for over 90% before reducing it using TCA.

The addition of dextran sulfate to media (B8-B10) increased HH percentages and shoot regeneration and the number of shoots per explant, but it was not significantly different from the control. These results suggested that dextran sulfate did not help reduce HH in sunflower and did not improve the regeneration capacity of sunflower cotyledon explants. These consequences do not agree with the results reported by Nairn and Furneaux (1997), who demonstrated the successful reduction of HH in Eucalyptus species from 80% to 47%. Perhaps the amount given to the culture medium in this study was not appropriate to inhibit ethylene production. There are no other results of the effect of dextran sulfate on other growth parameters of *in vitro* plants. Therefore, the study effect of this substance on plant growth is not widely available.

The water content of regenerated plants grown in the B3- B6 medium was significantly higher than that of the control. The B2 medium with 1 mg/L silver nitrate added had low water content, similar to the control. Water content also increased when cultured explants on media with TCA (B5-B7). Media B8-B10 were not significantly different from the control, indicating that it does not affect changes in the water level in the cells (Figure 4.4).

Hyperhydric plant loses their ability to sustain normal metabolism. One of them is to maintain a water balance. The water imbalance makes the plant unable to remove water from the cells. Hyperhydric aspects of plants are water-soaked appearance and have excess water accumulating in the tissue, and then it is along with high water content. This study found high HH percentages in explants from B3 and B4 media with high water content. The study of water content in hyperhydric sunflower has never been tested before. However, the results were consistent with that from Gao et al. (2017) in hyperhydric pink (*Dianthus chinensis* L.) and in *Arabidopsis* seedlings (de Klerk and Pramanik, 2017), which confirmed that hyperhydric plantlets had higher water content than the normal explants.

Media B5-B7 with TCA, on the other hand, gave high water content, although the HH percentage was low. The results were inconsistent with the previous

study of TCA by de Klerk and Pramanik (2017); this may be because the present study used the hole explant as in the water content experiment. Explants from TCA treatments had large callus lumps formed at the explant base. Since callus is an unmodified cluster of cells, the cell and tissue component, such cell wall will not form in a thick layer as plant organ tissue. Callus cells may have more water content than organ cells, resulting in the high water content of explants from TCA treatment.

Agriculturists used TCA as one of the herbicides to control grass weeds in some crops. Therefore, it is a phytotoxic chemical to plants that can cause chlorosis and reduce plant growth and mineral components in plant tissue. The toxicity level may differ depending on plant species and TCA concentration (Theodore and Leroy, 1954). It can inhibit of wax synthesis in the plant. Waxes are very long-chain lipid molecules forming at the outer surface of the plant cell wall. It acts as a barrier to water loss and protects plants from pathogens and radiation. TCA inhibits the incorporation of $^{14}$C-acetate in $C_{32}$ chains (Dewey et al., 1962). The reduction of wax synthesis may enhance the transpiration of water by the cell and cause cell division more easily, thus making high callus proliferation in explants cultured on TCA treatments. Hence cell multiplication and differentiation in plants, whether developing into callus or organs, are highly dependent on plant hormone action. The effect of TCA on the functioning of plant hormones is still not well known.

Large stomata number in plant leaves could assist the plants in eliminating the excess water in plant cells and increasing air space in the tissue, therefore preventing HH. Stomata from control (Figure 4.5A) had narrower stomata opening than stomata from B1 medium (Figure 4.5B), indicating the effect of silver nitrate (at low concentration) on stomata functioning of *in vitro* sunflower. On the contrary, stomata were fully closed on the epidermis of HH leaf retrieved from B4 medium with 4 mg/L silver nitrate (Figure 4.5C). Abnormal stomata opening indicates a loss of the stomata's ability to exchange water, air, and respiration efficiency of the hyperhydric plant.

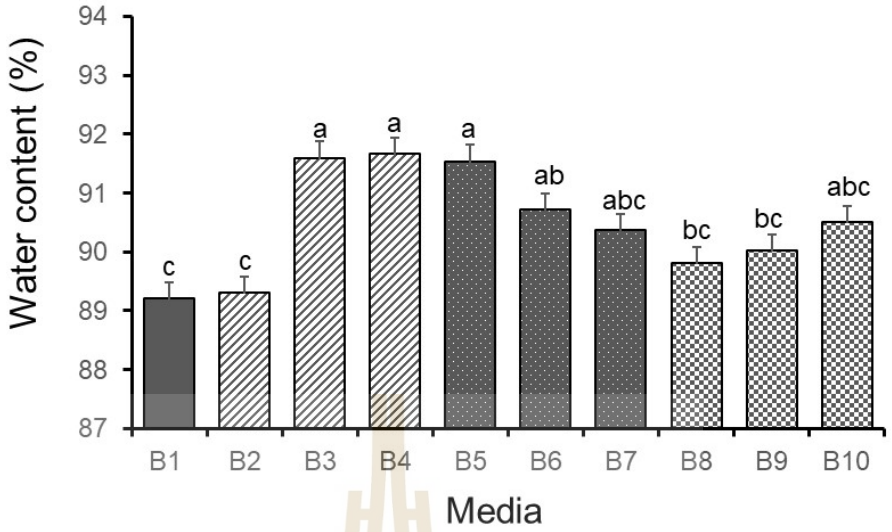


**Figure 4.4** Effect of media supplements on water content of *in vitro* ornamental sunflower. Different letters indicate significant difference between means according to DMRT at $P$ = 0.05.

Stomata number per area of explants grown in media with 1 mg/L silver nitrate was significantly increased (469.09±105.09 per $mm^2$) as compared to the control explant (322.21±47.67 per $mm^2$) (Figure 4.6). Higher concentrations of silver nitrate showed no significant change compared to the control. The low concentration of silver nitrate (1 mg/L) was a suitable level to reduce the risk of hyperhydricity and induce stomata density. The concentrations at 2 and 4 mg/L were too high and may be toxic to growing plants. Gao et al. (2017) reported that silver nitrate could increase stomata density in HH plantlets of pink (*Dianthus chinensis* L.). In this study, the additional low amount of silver nitrate (1 mg/L) could induce stomata density in sunflower tissue culture, reducing the risk of hyperhydricity. A report from Mayor et al. (2003) showed that a higher concentration of silver nitrate could reduce HH but harm sunflower shoot regeneration. Moreover, silver nitrate toxicity at high concentrations has been reported on wheat and *Brassica* seedlings (Karimi and Mohsenzadeh, 2017; Vishwakama et al., 2017)

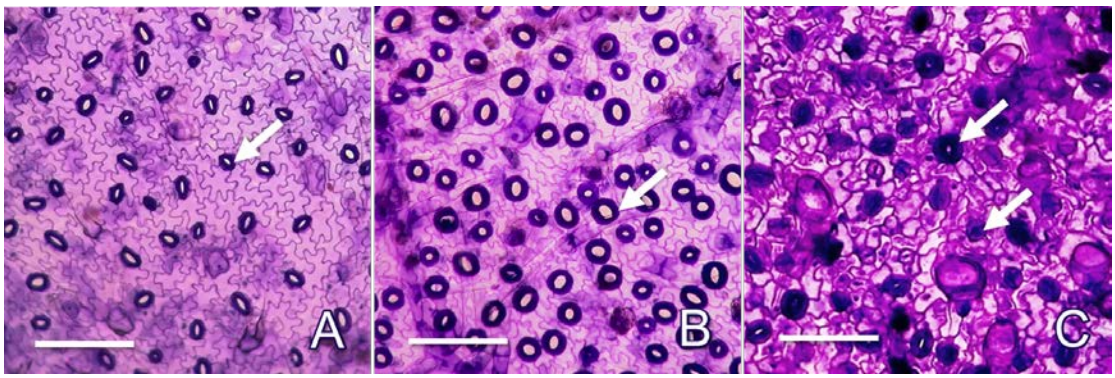


**Figure 4.5** Microscopic images of the abaxial epidermis of leaf. (A) Epidermis of normal leaf of explant cultured on SEM without supplement. (B) Epidermis of leaf of explant cultured on SEM + 1 mg/L silver nitrate. (C) Epidermis of hyperhydric leaf of explant cultured on SEM + 4 mg/L silver nitrate. Arrows indicate stomata. Bars = 100 µm.

However, a significant increase in stomata density was also found at a high concentration of TCA (300 mg/L). TCA can reduce the wax synthesis in the plant. Less wax forming on the leaf epidermis allows gas and water exchange better and makes plant growth better and healthier.

Stomata density of explants from dextran sulfate was reduced compared to the control but only significantly reduced at 20 mg/L of concentration. The reduction may be caused by the toxicity of dextran sulfate when together with agar. Although Nairn and Furneaux (1997) studied the effect of dextran sulfate in HH plant regeneration, the effect on other plant growth parameters such as stomata density is still inadequate and questionable. On the other hand, Dextran sulfate has been widely employed in animal models of murine colitis, a chronic digestive illness marked by inflammation of the colon's inner lining. It is assumed that its toxic capacity causes mucosal damage and inflammation by first having a direct toxic impact on epithelial cells, leading to cytokine-mediated cytotoxicity (Kunes and Kvetina, 2016).

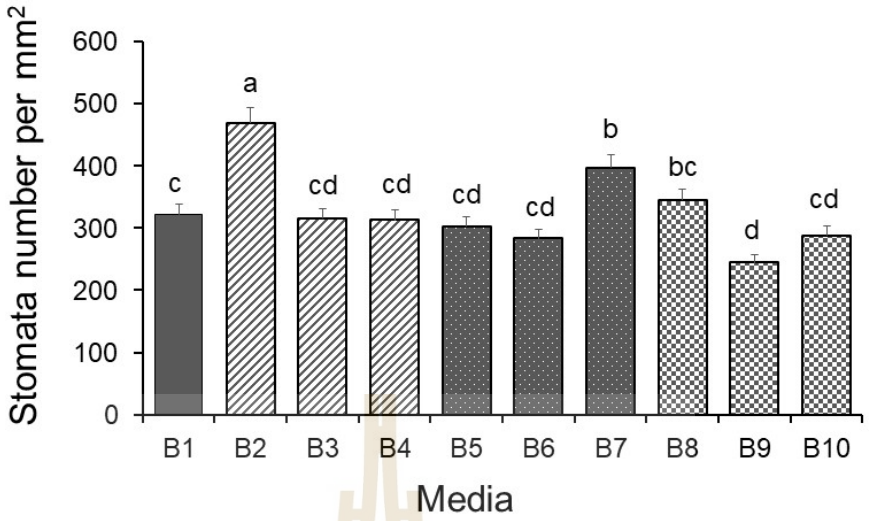


**Figure 4.6** Effect of media supplements on stomata density of *in vitro* ornamental sunflower. Different letters indicate significant difference between means according to DMRT at $P$ = 0.05.

### 4.1.3 Rooting and acclimatization of *in vitro* ornamental sunflower

From the shoot elongation phase results, explants from B2 medium with 1 mg/L silver nitrate gave the most satisfactory overall results. It exhibited the highest percent shoot regeneration (83.33%), highest number of shoots per explant (2.20±0.38), low percent HH shoots (3.89%), a hundred percent of survival rate, appropriate percentage of water content, and highest stomata density compared to other treatments. Then 16 healthy explants within the treatments were cut for 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 transplant (Figure 4.7A). After 2 weeks, roots formed and expanded over media (Figure 4.7B). All shoot explants in the RIM medium in this study achieved 100% rooting. Then complete plantlets with shoots and roots were acclimated at room temperature for 2 weeks before being transplanted to soil.

During the acclimatized period, some rooted plants dried up and eventually died (Figure 4.7C). The sufficient light during this period caused the plant to lengthen its shoot, and dried-up leaves with flowers developed (Figure 4.7D). The

problems that occurred during this period caused the failure of the acclimatization plant. There were 10 out of 16 plantlets survived after 2 weeks of acclimatization. After 3 weeks of transplant, 50% (8 from 16) of regenerated plantlets were successfully acclimatized and survived (Figure 4.7E).

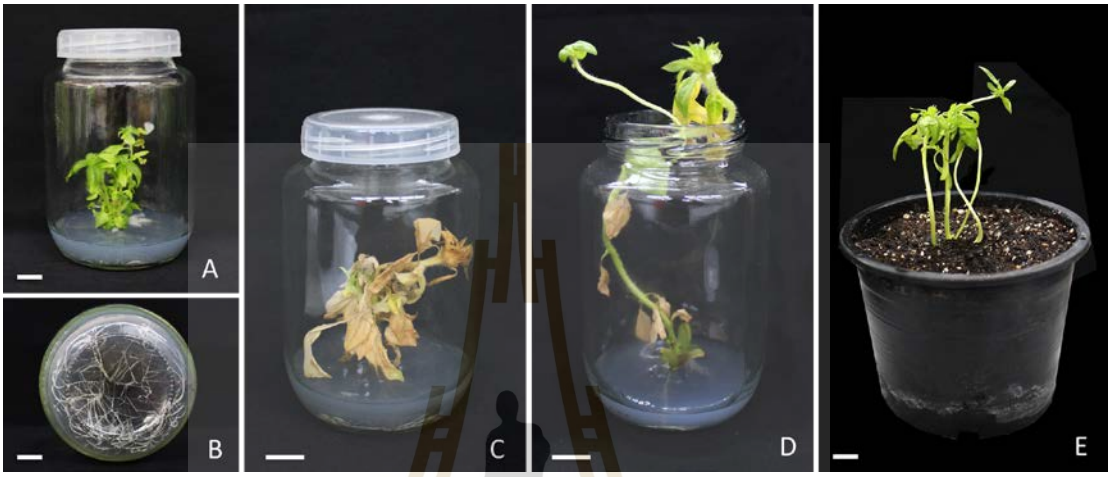


**Figure 4.7** Root induction and acclimatization of *in vitro* ornamental sunflower. (A) Rooting of the shoots on half-strength MS medium + 0.5 mg/L NAA and 200 mg/L charcoal for 2 weeks. (B) Roots forming and expanding over media. (C) Died plantlets at acclimatized period. (D) Elongated shoots with precocious flowering plantlet at acclimatization period. (F) Acclimatized plantlet at 3 weeks after transplanting to the soil. Bars = 1 cm.

## 4.2 Experiment II: Effect of media supplements and ventilation on hyperhydricity and shoot regeneration of *in vitro* oil seed sunflower

### 4.2.1 Effect of media supplements and ventilation on hyperhydricity, shoot regeneration, and shoot length of *in vitro* oil seed sunflower at shoot induction phase

From the previous experiment, the use of the supplements at high concentrations may cause plant toxicity and adverse effects. Therefore, in this experiment, media supplements that had a positive effect at low concentrations (silver nitrate and TCA) were selected to study their potentials on hyperhydricity for another sunflower genotype, an oil seed variety. Moreover, the differences in culture conditions may have a high effect on HH symptoms. The following experiments were applied into two culture conditions, including the addition of no ventilation and ventilation conditions for more effective results. Explants of oil seed sunflower genotype Suranaree 473 (S473) were grown on shoot induction media (SIM) supplemented with either 1 mg/L silver nitrate, 100 mg/L TCA, or their combination. The additional experimental treatments were conducted with the culture condition applied, including no ventilation (C1-C4) and ventilation (C1V-C4V) conditions.

After 3 weeks, shoots and leaves were formed with different media supplements and condition aspects. Explants from SIM with no supplement as a control had different shoots forming than no ventilation (C1) and ventilation (C1V). A hyperhydric 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 have uneven, rough, and serrated edges (Figure 4.8A). Explants from the C1V medium under ventilation are shown in Figure 4.8B. 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. No hyperhydric shoots were found under the ventilation condition.

Media C2 and C2V were MS medium supplemented with 1 mg/L silver nitrate. Shoots from C2 with no ventilation had light green shoots with regular green

leaves but with a rough leaf surface like those from C1. Some leaves are not fully expanded, and the leaf tips curled inward from the lateral margin and were yellow or brown at the leaf tips. Not much difference from non-ventilated medium, explants on C2V medium formed light green shoots and opaque green leaves. Some leaves, especially those near the medium surface, had uneven leaf surfaces and yellowish and brown scorch at the tips of the leaves, similar to leaves from C1. Compared to the ventilated medium, the leaves were flat, not jagged, and the plant showed no signs of succulence as the shoots were clear or any translucent leaves (Figure 4.8D).

Explants from media C3 and C3V with TCA are shown in Figure 4.8E-4.8F. The plants from C3 produced relatively short shoots compared to other media. The leaves were fully expanded, and the leaves were light green to yellow. The leaf surfaces were uneven, and the edges were slightly distorted. Some leaves were incompletely developed, but there were no signs of HH. Unlike the non-ventilated medium, the shoots from C3V were straight. The leaves were darker green and expanded normally with flat and smooth leaf blades. Some leaves, especially the first true leaves, are often irregularly shaped with a warped shape and dry yellow tips (Figure 4.8F).

When the silver nitrate and TCA supplements were used together, the explants in the media C4 and C4V seemed stunted. The leaves were yellowish-green, and the leaf surfaces were not smooth. Leaves usually had dry brown parts at the tips of the leaves. 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 (Figure 4.8G). In the ventilated medium, the characteristics of shoots and leaves were not significantly different from non-ventilated except for a slightly darker green color. Shoots from C4V often had precocious flower buds even after only three weeks of cultivation (Figure 4.8H).

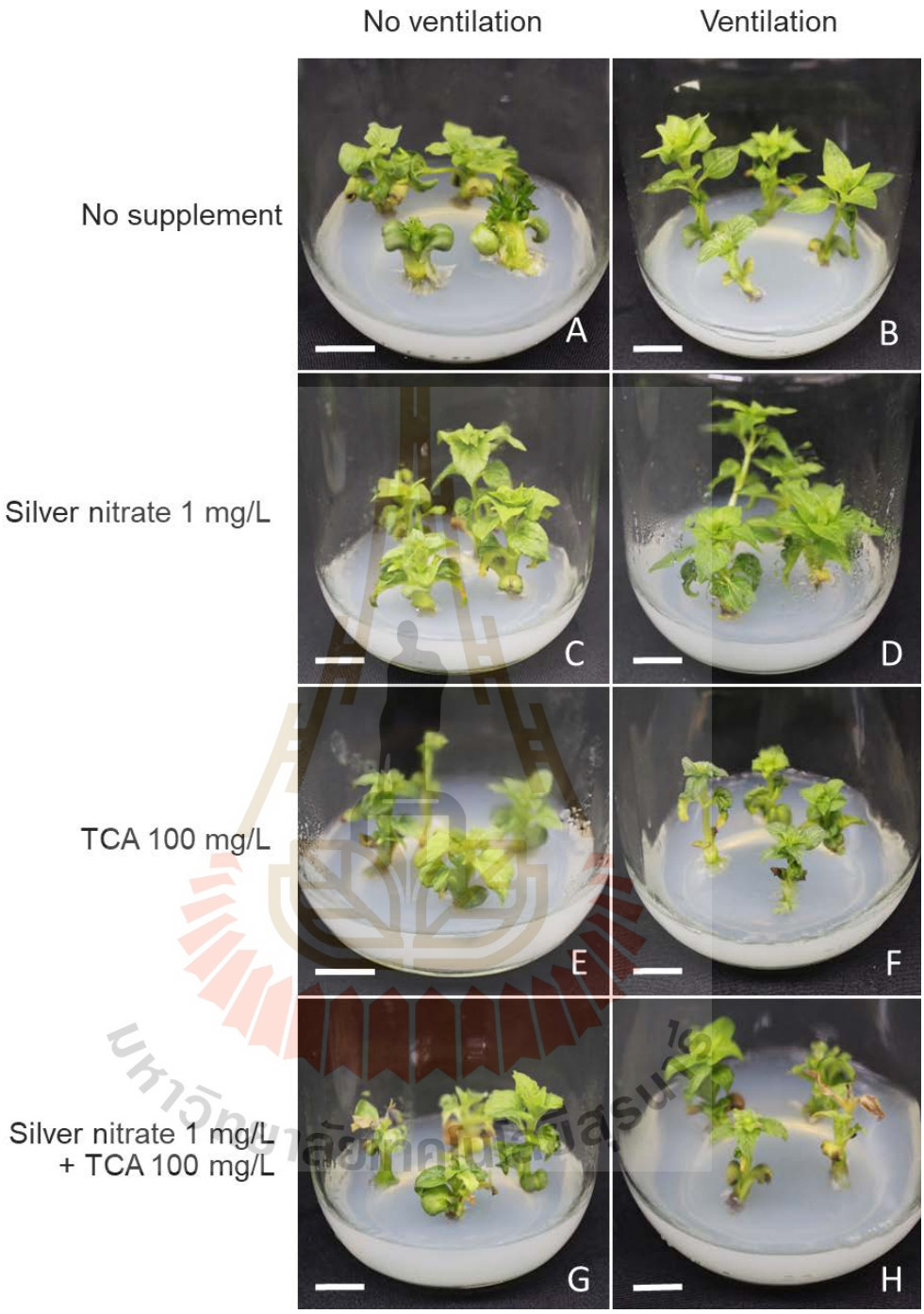


**Figure 4.8** Oil seed sunflower regeneration from meristem and cotyledon base explants on shoot induction medium with no ventilation and ventilation condition applied. (A-B) Explants cultured for 3 weeks on MS medium + 2 mg/L BA as SIM. (C-D) Explants cultured for 3 weeks on SIM + 1 mg/L silver nitrate. (E-F) Explants cultured for 3 weeks on SIM + 100mg/L TCA. (G-H) Explants cultured for 3 weeks on SIM + 1 mg/L silver nitrate + 100 mg/L TCA. Bars = 1 cm.

**Table 4.3** Effect of media supplements on shoot regeneration, number of shoots per explant, shoot length, and hyperhydric shoots of *in vitro* oil seed sunflower at shoot induction phase.

| Media | Supplements | Shoot regeneration (%) | Number of shoots per explant (mean±SE) | Mean shoot length (mm) (mean±SE) | Hyperhydric shoots (%) |
|---|---|---|---|---|---|
| C1 | No supplement | 100[a] | 1.13±0.46[a] | 12.73 ±4.05[c] | 1.67[ab] |
| C1V | | 100[a] | 1.18±0.56[a] | 19.13±7.10[a] | 0.00[b] |
| C2 | Silver nitrate 1 mg/L | 100[a] | 1.18±0.56[a] | 15.97±4.79[b] | 0.00[b] |
| C2V | | 100[a] | 1.07±0.31[a] | 16.73±5.72[b] | 0.00[b] |
| C3 | TCA 100 mg/L | 100[a] | 1.03±0.26[a] | 11.02±3.32[cd] | 5.00[a] |
| C3V | | 100[a] | 1.13±0.39[a] | 12.63±4.14[c] | 0.00[b] |
| C4 | Silver nitrate 1 mg/L | 100[a] | 1.02±0.13[a] | 9.63±2.05[d] | 1.67[ab] |
| C4V | + TCA 100 mg/L | 100[a] | 1.12±0.37[a] | 12.62±4.31[c] | 0.00[b] |

Means in columns followed by different letters are significantly different according to DMRT at $P = 0.05$.

Table 4.3 shows the effect of media supplements on shoot regeneration, the number of shoots per explant, shoot length, and hyperhydric shoots of *in vitro* oil seed sunflower at the shoot induction phase. Sunflower in this experiment had 100% of shoot regeneration in all supplement types and conditions.

The average shoots per explants were 1.02-1.18, which is not statistically different among the treatments. 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 C1V medium without supplement under ventilation conditions. The shortest shoot length was from the C4 medium supplemented with silver nitrate and TCA combination. The C4 medium also had the lowest non-significant average shoot number per explants with the occurrence of hyperhydric shoot (Table 4.3).

The emergence of hyperhydric shoots occurred at a shallow randomness rate, with only 1.67-5.00% in the non-ventilated control, TCA added, and the combined medium; however, the average number of shoots is not significantly different. When

comparing the media with the same supplement but with different aero conditions, the average shoot length in all media with ventilation was longer than those cultured in non-ventilated media. In addition, hyperhydric shoots were found to occur in all non-ventilated media.

From the results, the additives did not affect the average number of shoots of sunflowers *in vitro*. However, the growth parameters, shoot length, and appearances showed that each substance had different effects. Silver nitrate did not induce HH at all in both culture conditions. In addition, the explants were normal, which indicated the positive effects of this substance on plant growth. Some previous studies have improved plant quality and plant quantity in tissue culture using silver nitrate. It has shown to be a highly effective ethylene inhibitor and is commonly used in plant tissue culture. Silver ion-mediated reactions appear to be engaged in ethylene pathways and serve an essential role in regulating physiological processes such as morphogenesis. So, the low concentration added silver nitrate in this study had assisted in plant growth with normal and healthy plant morphogenesis, which seems to be consistent with the experiment of Beyer (1975) that increased shoot growth of *Pisum sativum* using silver nitrate. Also, as in the regeneration and shoot organogenesis of sunflower (*Helianthus annuus*) (Chraibi et al., 1991). Shoot length, shoot number, and flowering of *Cichorium intybus* exposed to silver nitrate were improved too (Bais et al., 2001). Nevertheless, excessive concentrations can also adversely affect the growth factors. The result depends on the duration of cultivation and the type of plant.

TCA was used as a plant herbicide to kill weeds. The remaining of this acid in the environment is also hazardous to creatures such as plants, algae, and aquatic or surface animals. Plants exposed to TCA reduce their growth and may cause leaf wilting. The pigment was destroyed and is not resistant to the environment. Moreover, if the animal gets it, it can cause tissue burns, which can be fatal due to the acidity (Lewis, Wolfinger, and Barta, 2004). Since the report from de Klerk and Pramanik (2017) showed the reduction of HH in Arabidopsis, this experiment found that the addition of TCA did not affect sunflower HH under these culturing conditions. However, the addition of TCA at 100 mg/L alone or the combination with silver nitrate seemed to impair the sunflower growth for this experiment, indicating that although the substance

appears to enhance plant growth, when used together they may not always work together.

When ventilation was applied, plant growth was increased, and the explants' appearances looked healthier, with a greener color and fully expanded leaves with smooth flat leaf blades. The results of this experiment seemed to be consistent with Majada, Tadeo, Fal, and Sanchez-Tames (2000) in the ventilated condition on carnation that modified the anatomical characteristics of shoots and leaves. The ventilated plant had a thicker cell wall, decreasing cell size and intracellular space. Also, there were more photosynthetic and supportive tissues. The culture vessel's ventilation also helps avoid physiological and anatomical abnormalities, including the inability to photosynthesize, low chlorophyll content, the non-functional stomata, and abnormal xylem parenchyma development of *in vitro* Persian walnut plants (Hassankhah et al., 2014). However, the definitive results of the effect of ventilation on plant growth also depended on the duration of cultivation as different plants have different growth periods. Therefore, follow-up is needed from this third week in the shoot induction phase to clarify this effect.

**4.2.2 Effect of media supplements and ventilation on hyperhydricity, shoot regeneration, shoot length, water content, and stomata number of *in vitro* oil seed sunflower at shoot elongation phase**

At this experimental phase, all explants from the shoot induction phase were cultured further in the same media and the same culture vessels for an additional two weeks as a shoot elongation phase. In this experiment, none of the explants were subcultured, because the purpose of this experiment was to see the effect of ventilation conditions. If the culture vessels had opened, the air in the bottle would be assumed to be vented, and non-ventilated results would have been distorted. Therefore, the explants were cultured in the same media for 5 weeks from the shoot induction phase of 3 weeks and an extended shoot elongation phase of 2 weeks. After five weeks, the experimental results were measured and calculated.

The explants from the C1 medium (Figure 4.9A) had stunted shoots with short internodes. The leaves were green with a yellow tint and distorted leaf blades, and the leaf tips were yellowish-brown. In comparison, the shoots from C1V were taller, leaves were green, and leaf blades were relatively smooth. The leaves' color was opaque green with no signs of succulence, but some leaves showed a non-expanding appearance and had yellow color (Figure 4.9B).

In the C2 medium with silver nitrate (Figure 4.9C), the explants had tall, elongated shoots and many leaves. Leaf blades were heart-shaped, smooth surfaces. The edges of the leaves were smooth, without twisting and fluttering appearances. Some leaves, especially the first paired leaves, often withered and died, turning brown. Some shoots produced small flower buds from the first week of this phase. When the ventilation was applied in C2V, the morphological characteristics of the explants were not significantly different from C2, except that the color of the leaves was slightly darker green together, with no signs of withering in leaves.

Plants cultured in C3 medium supplemented with TCA had short shoots and relatively few leaves. The leaves were oval and elongated and differed from the control and the silver nitrate supplements. The surfaces of the leaf plates were smooth, but the leaves tended to wrap and roll down, and had a dark green color but showed no signs of transparency. The explants often had large, light green callus

formations at the explant bases (Figure 4.9E). When ventilation was applied, the shoots had 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 (Figure 4.9F).

The silver nitrate and TCA combination gave the regenerated explants with a hybrid appearance appearing as a shared physical feature. Explants from the C4 medium had short shoots, slightly distorted leaves, yellow at the end of the leaves, and a large callus lump at the base of the explants (Figure 4.9G). When cultured under ventilation, the shoots were slightly longer. The leaves were green and yellow at the tips of the leaves, along with uneven leaf a dry appearance in some leaves. There were basal calluses produced at the base of explants and other media with TCA added (Figure 4.9H).

Applying a ventilated culture system appeared to influence the growth and the morphological changes of *in vitro* sunflowers, which overall increased the length of the shoots and the appearance of different leaf colors and leaf surfaces.

Regenerated explants showed the hyperhydric symptoms differently, depending on the supplement they received (Figure 4.10). The HH shoot from the non-ventilated control medium had a succulent short shoot and short internodes. The leaves were smaller than the normal ones and were spherical compared to the normal ones, which were heart-shaped. 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 silver nitrate exhibited only clear leaves, and a dark green color presented about 10 percent of the whole explant as the HH symptoms, indicating that the level of severity is 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. The leaves were deformed, twisted, and elongated into abnormal shapes, which indicated that the level of severeness was high.

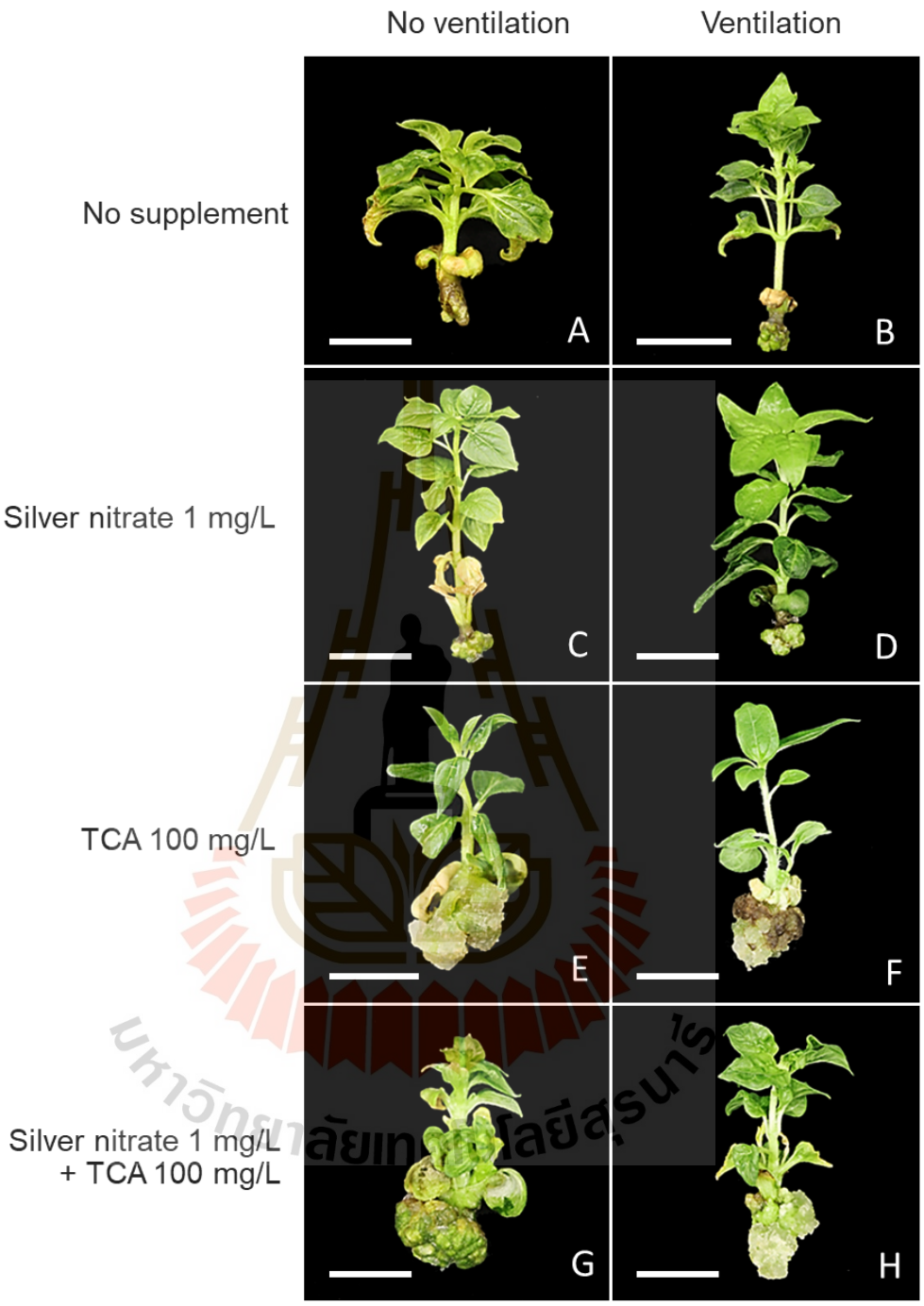


**Figure 4.9** Oil seed sunflower explants cultured on media at shoot elongation phase under no ventilation and ventilation condition for 2 weeks. (A-B) Explants cultured for 5 weeks on MS medium + 2 mg/L BA. (C-D) Explants cultured for 5 weeks on SIM + 1 mg/L silver nitrate. (E-F) Explants cultured for 3 weeks on SIM + 100mg/L TCA. (G-H) Explants cultured for 5 weeks on SIM + 1 mg/L silver nitrate + 100 mg/L TCA. Bars = 1 cm.


**Figure 4.10** Hyperhydric explants from different media under no ventilation condition at the elongation phase. (A) SIM with no supplement. (B) SIM + 1 mg/L silver nitrate. (C) SIM + 100 mg/L TCA. Bars = 1 cm.

**Table 4.4** Effect of media supplements on shoot regeneration, number of shoots per explant, hyperhydric shoots, and survival rate of *in vitro* oil seed sunflower at shoot elongation phase.

| Media | Supplements | Shoot regeneration (%) | Number of shoots per explant (mean±SE) | Hyper-hydric shoots (%) | Survival rate (%) |
|---|---|---|---|---|---|
| C1 | No supplement | 100[a] | 1.13±0.46[a] | 1.67[a] | 86.67[ab] |
| C1V | | 100[a] | 1.18±0.56[a] | 0.00[a] | 95.00[a] |
| C2 | Silver nitrate 1 mg/L | 100[a] | 1.18±0.56[a] | 1.67[a] | 96.67[a] |
| C2V | | 100[a] | 1.07±0.31[a] | 0.00[a] | 96.67[a] |
| C3 | TCA 100 mg/L | 100[a] | 1.03±0.26[a] | 1.67[a] | 78.33[b] |
| C3V | | 100[a] | 1.13±0.39[a] | 0.00[a] | 91.67[a] |
| C4 | Silver nitrate 1 mg/L + | 100[a] | 1.02±0.13[a] | 0.00[a] | 86.67[ab] |
| C4V | TCA 100 mg/L | 100[a] | 1.12±0.37[a] | 0.00[a] | 85.00[ab] |

Means in columns followed by different letters are significantly different according to DMRT at $P = 0.05$.

Table 4.4 shows the effect of media supplements on shoot regeneration, the number of shoots per explant, hyperhydric shoots, and survival rate of *in vitro* oil seed sunflower S473 at the shoot elongation phase. The *in vitro* sunflower regeneration percentage is 100% in all treatments.

As for the number of shoots per explant over a five-week of cultivation, no additional shoot formation was found from 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.

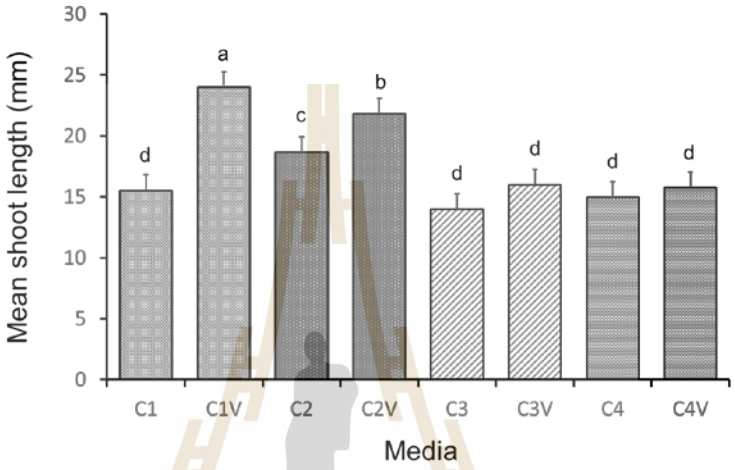


**Figure 4.11** Effect of media supplements and culture conditions on mean shoot length of *in vitro* oil seed sunflower at shoot elongation phase. Different letters indicate significant difference between means according to DMRT at *P* = 0.05.

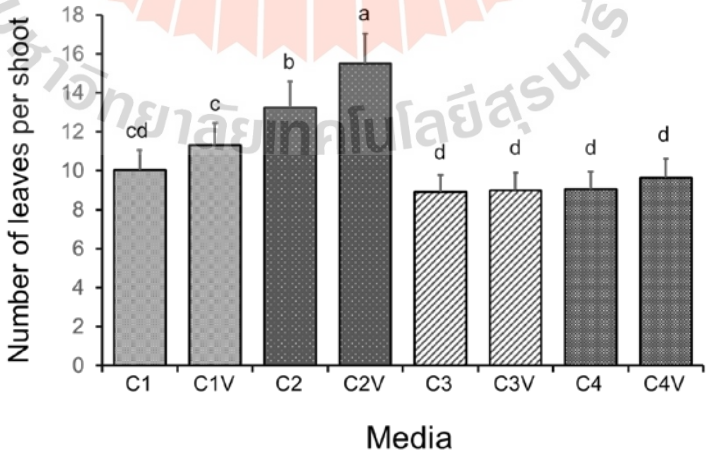


**Figure 4.12** Effect of media supplements and culture conditions on number of leaves per shoot of *in vitro* oil seed sunflower at shoot elongation phase. Different letters indicate significant difference between means according to DMRT at *P* = 0.05.

The length of the shoots and the number of leaves per shoot were also counted and measured. The average shoot lengths ranged from 13.97-24.00 mm (Figure 4.11), with the longest shoots obtained on C1V medium followed by C2V medium, respectively, and the shortest mean shoot length was obtained from C3 non-ventilated. In addition, it was found that media with TCA added, under any conditions, gave the low shoot heights as low as the non-ventilated control medium with the same significant level.

Explants cultivation under the ventilated conditions increased the average shoot length than in closed culture vessels. There was a significant increase in the control medium and the silver nitrate medium. On the other hand, leaf number per shoot ranged from 8.90-15.50. Silver nitrate-treated plants showed a significant 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 (Figure 4.12).

The percentage of HH shoots occurred at 1.67% in the non-ventilated media only, including C1, C2, and C3. 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 of *in vitro* sunflowers when cultured in the 16 oz bottle under non-ventilated and ventilated conditions.

Survival rates were varied, with the C2 and C2V media having the highest survival rate at 96.67%, with no significant difference from the control (86.67%). The highest plant mortality was obtained from C3 medium with TCA added without ventilation, with a drop in survival to 78.33%. The cultivation under ventilation conditions elevated plant survival to a higher percentage in the control and TCA media. In silver nitrate supplemented media (C2 and C4), the survival rate was not significantly different in both ventilation conditions.

The average plant water content showed that plants treated with TCA had a higher percentage of water content than other media. The explants produced large calluses, with the highest percentage from the C4 medium (91.32%). In contrast, the control medium and the silver nitrate medium had significantly less water content,

with C1 having 89.96% and C2 having 90.46%, respectively, and slightly higher than C1V and C2V with ventilation but not statistically different (Figure 4.13).

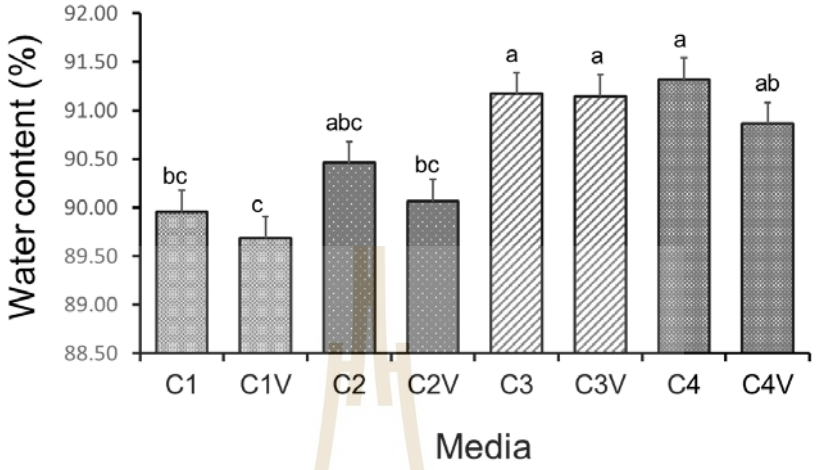


**Figure 4.13** Effect of media supplements and culture conditions on water content of *in vitro* oil seed sunflower. Different letters indicate significant difference between means according to DMRT at $P$ = 0.05.

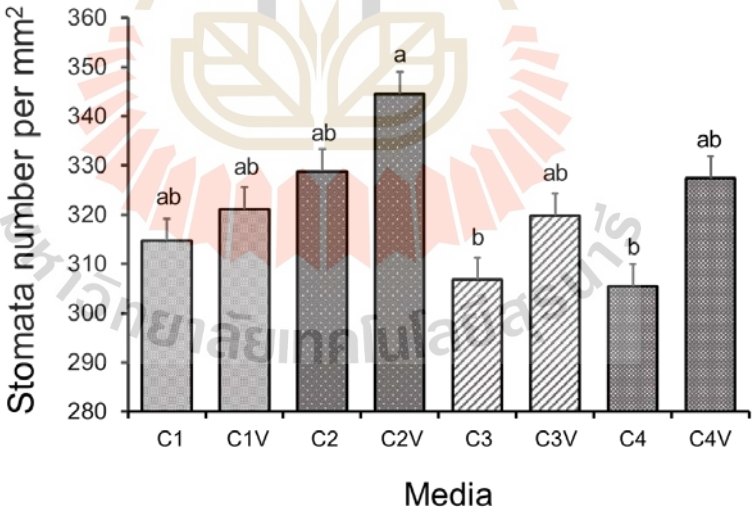


**Figure 4.14** Effect of media supplements and culture conditions on stomata density of *in vitro* oil seed sunflower. Different letters indicate significant difference between means according to DMRT at $P$ = 0.05.

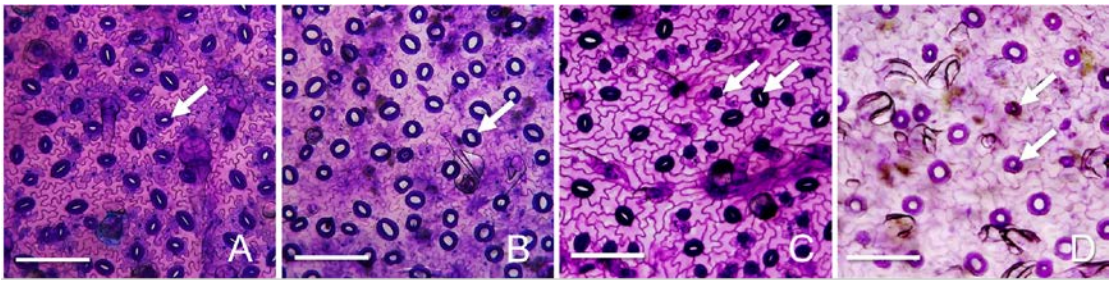


**Figure 4.15** Microscopic images of the abaxial epidermis of oil seed sunflower leaf. (A) Epidermis of normal leaf of explant cultured on ventilated SIM without supplement. (B) Epidermis of leaf of explant cultured on SIM + 1 mg/L silver nitrate under ventilation. (C) Epidermis of leaf of explant cultured 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.

At five weeks, the number of stomata from the *in vitro* sunflower's leaf surface was measured and calculated (Figure 4.14). Stomata from different treatments showed different shapes and the opening characteristics of guard cells (Figure 4.15). The stomata density was from 305.45-344.52 per square millimeter. The most significant number was found in the C2V medium with ventilated and silver nitrate added, corresponding to the highest leaf number per shoot, indicating the positive effect of applying silver nitrate and ventilation. The use of silver nitrate in the media also significantly increased the number of stomata in a non-vented medium. In contrast, TCA use was found to slightly decrease the number of stomata but not significantly differ from the control.

As the results, the experimental supplements, including silver nitrate, TCA, or its combination, did not affect the formation of HH shoots of *in vitro* oil seed sunflower under the addressed conditions. Silver nitrate seemed to enhance plant growth in this experiment, although the number of shoots did not differ significantly in each treatment. However, plants treated with silver nitrate at low concentrations of 1 mg/L showed an increase in leaf number and stomata number compared to the control and TCA media.

Tissue culture usually involves culturing plants under limited conditions. Plant growth nutrients, minerals, humidity, temperature, light, and plant hormone are

critical factors for growth, and stress factors affect seedling quality. In addition, the cut process of explant for the culture that causes wound is an additional factor causing stress on plants (Pérez-Clemente and Gómez-Cadenas, 2012).

The ethylene hormone responsible for plants' stress response will play an essential role when the stated factors stress plants. This hormone will cause the plant to reduce its growth, reduce chlorophyll production, and affect the closing of the stomata because plant cellular functions may be disturbed and malfunction. Acceleration of the cell aging led to the plant's death. Therefore, if the activity level of ethylene is at the appropriate level, plants will be able to grow normally and vigorously (Morgan and Drew, 1997).

Silver nitrate is widely used as an ethylene inhibitor in tissue culture and improves plant growth. The increase in plant growth is due to the silver ion binding properties to the ethylene receptor. It inactivates the receptor protein that receives the signal from the ethylene hormone, resulting in the plant being unresponsive to ethylene (Kumar et al., 2009). When the ethylene cannot function, plants grow better and can prevent HH shoots.

Silver nitrate has also 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 silver nitrate in banana (*Musa acuminata* L.). It was used to enhance *in vitro* growth, where concentrations up to 10 mg/L were found to increase the number of shoots and the size of the leaves. But in this current experiment on sunflower, even the 2 mg/L concentration showed that the plants responded negatively, such as a decrease in the number of shoots and stomata. More severe HH symptoms also indicated the toxicity of silver nitrate at low concentrations. 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 makes plants respond by the inhibition of plant growth, reduction in plant biomass and transpiration, caused oxidative stress, decreased chlorophyll contents, induced cell death, and DNA damage through generation of ROS, and damage to the cell morphology and its structural features. However, both positive and negative effects of silver 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).

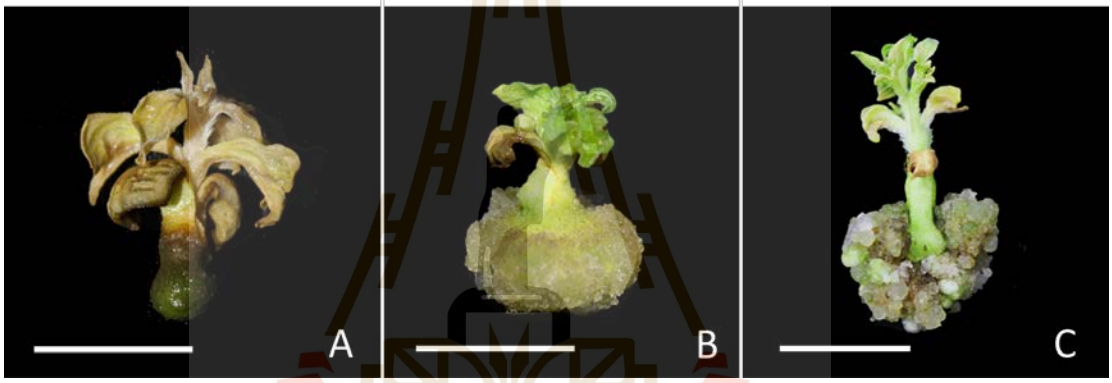


**Figure 4.16** Effect of TCA toxicity on the morphology of regenerated explants. (A-B) SIM + 100 mg/L TCA. (C) SIM + 1mg/L silver nitrare + 100 mg/L TCA. Bars = 1 cm.

TCA is widely used as an herbicide for weeds. It has properties that are selectively toxic to some plants. This property is used to remove weeds from crops in sugar beet, sugar cane, alfalfa, peas, and other crops. TCA causes phytotoxic symptoms such as leaf withering and root and stem growth suppression. The foliage temporarily becomes dark green, and then chlorosis is manifested. Weeds treated die after 3-4 weeks. TCA treatment alters the permeability of cell membranes and lowers leaf wax release. TCA is slightly toxic to mammals. The free acid is extremely corrosive, and prolonged contact with the sodium salt results in burns (Fedtke and Duke, 2005).

In the present experiment, at a concentration of 100 mg/L, TCA appeared to be toxic to sunflower resulting in negative consequences. The shoots were dry up and almost dying 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 (Figure 4.16).

This experiment employed two separate ventilation trials, with the regular bottle cap without holes and vented cap holes for ventilation. In this study, the culture conditions did not affect the HH production of *in vitro* sunflower. The indifference might be because the culture vessel (16 oz glass bottle) used in the experiment was large enough for plant growth. There was adequate air 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 the effect of container height on HH shoot formation of *in vitro Salvia santolinifolia* that the higher vessel magnitude caused a lower percentage of HH shoots and improved shoot number and shoot length. The small and non-ventilated culture containers reduce aeration in the vessel, which may have resulted in excessive humidity in the culture container and enhanced water absorption by the cells. The container's high humidity may also hinder wax production on the leaves, resulting in poor transpiration and HH development, causing the tissues to seem 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, Murphy, Leifert, and Lumsden (2000) reported that the ventilation of culture vessels could reduce ethylene in the air space and improved the growth and development of *Delphinium in vitro*.

When silver nitrate, which can suppress ethylene activity, was combined with ventilation in this present experiment, it was discovered that it might boost 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 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 et al., 1983).

### 4.2.3 Rooting and acclimatization of *in vitro* oil seed sunflower

The shoot elongation phase results show that explants from C2V vented medium with 1 mg/L silver nitrate gave the optimum overall results. It presented the 100% shoot regeneration with 1.07±0.31 shoots per explant, a completely non-HH shoots formation, a highest 96.67% survival rate, the appropriate percentage of water content, and the highest stomata density compared to other treatments.

Then 20 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 transplant. After 2 weeks, roots formed and expanded over media (Figure 4.17A-4.17B). All shoot explants in the RIM medium in this study achieved 100% rooting. Then complete plantlets with shoots and roots were acclimated at room temperature for 2 weeks before being transplanted to soil.

The problem encountered at this stage was that the plants formed flowers before they reached maturity (precocious flowering) (Figure 4.17C), 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 are difficult to predict (Figure 4.17D).

In addition, in the 2 weeks of the acclimatized period after root induction, the plants had elongated stems spreading across the bottle (Figure 4.17E). 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 dying during the acclimatized period. Only 4 of the 20 plants survived at three weeks after being transplanted (Figure 4.17F). According to this result, it is expected that the conditions utilized in the acclimatization of *in vitro* sunflower will be improved in the future.

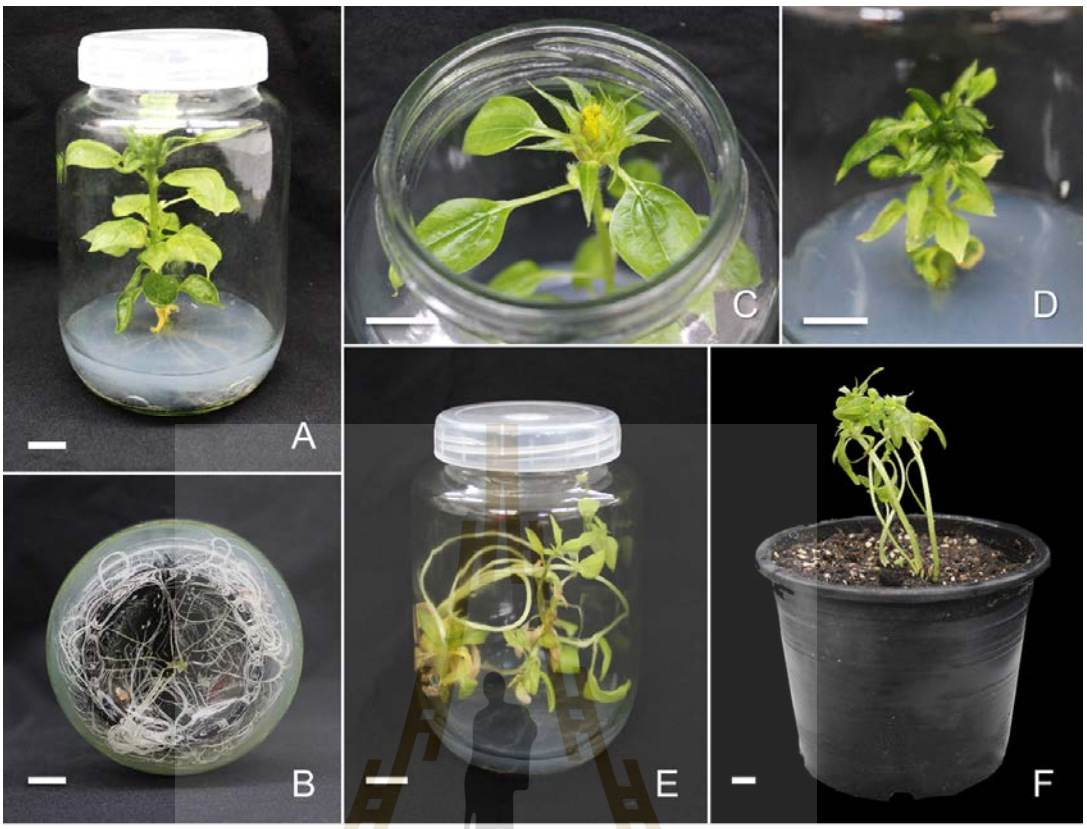


**Figure 4.17** Rooting and acclimatization of *in vitro* oil seed sunflower. (A) Rooting of the shoots on half-strength MS medium + 0.5 mg/L NAA and 200 mg/L charcoal for 2 weeks. (B) Roots forming and expanding over media. (C) Precocious flowering after 2 weeks of cultured on root induction medium. (D) Hyperhydric leaves formation at rooting phase. (E) Elongated-shoot plantlet at acclimatization period. (F) Acclimatized plantlet at 3 weeks after transplanting to soil. Bars = 1 cm.

## 4.3 Effect of plant genotype on regeneration and hyperhydricity of *in vitro* sunflower

Plant tissue culture requires external factors, including proper supply of nutrients, pH medium, adequate temperature, and proper gaseous and liquid environment for the growth and success of the process, and the genetics of plants, whether species or variety, are also an essential factor.

As the results from sections 4.1 and 4.2, the experimental explants in section 4.1 were ornamental sunflower, and in section 4.2 were oil seed sunflower. Due to the different experimental units and the experimental design, the explanation does not refer to comparative statistical results.

Plant characteristics, number of shoots, and response were different, with ornamental varieties having different responses to hormones and producing different shoots from oil varieties. Although the physical characteristics of seedlings growing in the soil of both genotypes have a single stem,the ornamentals seemed to produce more shoots per explant *in vitro*. In addition, the shoot length and the average number of stomata were different in the same treated media and condition (Figure 4.18).

Some previous studies showed that the sunflower genotype affected the growth of shoot and callus regeneration (Ozyigit, Gozukirmizi, and Semiz, 2007; Sujatha et al., 2012), the success in genetic transformation (Escandon and Hahne, 1991), and the reduced capacity on hyperhydric shoots (Mayor et al., 2003).

This demonstrates that genetics significantly impacts plant development, even if they are of the same species or differ in variety. Therefore, the future search will need to account for the genetic influence aspect.


**Figure 4.18** Morphology of two different explant genotypes from shoot elongation phase, ornamental sunflowers (left column) and oil seed sunflowers (right column). (A-B) Explants from control media at shoot. (C-D) Explants from media supplemented with 1 mg/L silver nitrate. (E-F) Explants from media supplemented with 100 mg/L TCA. Bars = 1 cm.

# CHAPTER V
# CONCLUSIONS

In the first experiment, the effects of three media supplements, including silver nitrate, trichloroacetate (TCA), and dextran sulfate on shoot regeneration, shoot length, and hyperhydricity of *in vitro* ornamental sunflower at the shoot induction phase were investigated.

The present work showed that, in the shoot induction phase under ventilation conditions, in most cases, the addition of silver nitrate, TCA, and dextran sulfate showed no differential impact on the HH occurrence as compared to the control. The addition of low silver nitrate concentration (1 mg/L) to the medium has been shown to promote the percentage of shoot regeneration, the number of shoots per explant, and the survival rate. In contrast, dextran sulfate reduced the regeneration rate in tissue-cultured ornamental sunflower.

At the shoot elongation phase, the addition of low silver nitrate concentration showed a positive on the *in vitro* sunflower, which improved the shoot regeneration, the number of shoots, survival rate, and stomata number, and did not promote HH shoots. In contrast, high concentrations seemed toxic to the plants showing negative results of reducing the number of shoots, shoot length, survival rate, and increasing HH shoots. The effects of TCA and dextran sulfate on *in vitro* sunflower were first investigated. The results showed that TCA and dextran sulfate did not promote the HH in sunflower. The toxicity of dextran sulfate may cause a lower regeneration percentage and a reduction of shoot number and shoot length in ornamental sunflower.

In the second experiment, the effects of 2 media supplements, including silver nitrate, TCA, and their combination on shoot regeneration, shoot length, hyperhydricity, relative water content, and stomata number of *in vitro* oil seed sunflower under ventilated and non-ventilated conditions of oil seed sunflower (S473) were investigated.

In most cases, the addition of a low concentration of silver nitrate or TCA had no impact on the shoot regeneration, the number of shoots, and hyperhydricity. TCA seemed to have toxicity to the plant even at low concentrations as the reduction of shoot growth. Ventilation mostly improved plant growth in all media. The regeneration percentages were higher than the ornamental sunflower due to plant genotype and seed quality.

The results of the shoot elongation phase showed that media supplement types and ventilation conditions had no impact on the shoot regeneration, the number of shoots, and hyperhydricity of *in vitro* oil seed sunflower. However, there was an impact on the morphology of regenerated explants. Explants with ventilation applied had a healthier appearance compared to non-ventilated explants. In other words, the ventilation improved the growth of sunflower *in vitro*. The addition of low concentration silver nitrate to the culture media has been shown to help promote healthy plant growth. When the silver nitrate was used together with ventilation, the plants grew even better, with the highest leaves per explant and highest stomata density. TCA, in contrast, had toxicity to plants. The media with TCA added (TCA alone and the combination) had reduced plant growth. In addition, the high volume of culture vessels used in this study seemed to have a positive effect on HH regeneration in tissue-cultured sunflower, in which the percent HH was low over the experiment when plants were cultured in large vessels.

In the root induction phase, plants could form roots well. But the problematic part is the process of acclimatized conditions. Both genotypes of sunflower had similar problems. Most plants died or showed abnormal long shoots during this period due to improper conditions; therefore, it needs to be improved by taking care of plants closely, providing enough light for the plants, and maintaining proper humidity and temperature.

This study is the first study demonstrating the influence of TCA and dextran sulfate on the growth and hyperhydricity of *in vitro* sunflower. This work showed that these compounds are toxic to plants and cause more adverse effects on plant growth. The results are expected to be useful in future applications for other plant tissue cultures.

In conclusion, several factors affect HH in sunflower tissue culture, such as genotypes, ventilation condition, media supplements, therefore the culture conditions for successful micropropagation should be optimized.

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# APPENDICES

# APPENDIX A

# MEDIUM AND SUPPLEMENTS

## A.1 Murashige and Skoog Basal Medium

**Table A.1** Murashige and Skoog basal medium (MS) (Murashige and Skoog, 1962).

| Components | mg/L |
|---|---|
| Ammonium nitrate | 1,650.0 |
| Boric acid | 6.20 |
| Calcium chloride (anhydrous) | 332.20 |
| Cobalt chloride hexahydrate | 0.0250 |
| Cupric sulfate pentahydrate | 0.0250 |
| Disodium EDTA dihydrate | 37.260 |
| Ferrous sulfate heptahydrate | 27.80 |
| Glycine | 2.0 |
| Magnesium sulfate (anhydrous) | 180.70 |
| Manganese sulfate monohydrate | 16.90 |
| *myo*-Inositol | 100.0 |
| Nicotinic acid | 0.50 |
| Potassium iodide | 0.830 |
| Potassium nitrate | 1,900.0 |
| Potassium phosphate monobasic | 170.0 |
| Pyridoxine hydrochloride | 0.50 |
| Sodium molybdate dihydrate | 0.250 |
| Thiamine hydrochloride | 0.10 |
| Zinc sulfate heptahydrate | 8.60 |

Remark: 4.4 g of powder are required to prepare 1 L of medium.

Sugar 30 g/L, Agar 8 g/L, Adjusted pH at 5.6-5.8.

### A.2 Plant hormones 100 mg/L of $N^6$-Benzyladenine (BA)

Preparation of stock: the BA is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at 4 °C.

### A.3 Plant hormones 100 mg/L of $N^6$-[2-Isopentenyl] adenine (2-iP)

Preparation of stock: the 2-iP is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at 4 °C.

### A.4 Plant hormones 100 mg/L of α-Naphthaleneacetic acid (NAA)

Preparation of stock: the NAA is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at 4 °C.

### A.5 Supplement 100 mg/L of Silver nitrate

Preparation of stock: the silver nitrate is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at 4 °C.

### A.6 Supplement 100 mg/L of Trichloroacetate (TCA)

Preparation of stock: the TCA is weighted at 1 g and then dissolved in 1-2 mL of distilled water, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at 4 °C.

### A.7 Supplement 100 mg/L of Dextran sulfate

Preparation of stock: the dextran sulfate is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at 4 °C.

# APPENDIX B

# DATA OF EXPERIMENT I - II

## B.1 Data of ornamental sunflower

**Table B.1** Data of growth of ornamental sunflower at shoot induction phase.

| Media | Supplements | Shoot regeneration (%) | Number of shoots per explant (mean±SE) | Mean shoot length (mm) (mean±SE) | Hyperhydric shoots (%) |
|---|---|---|---|---|---|
| A1 | - | 83.33 | 1.78±0.37 | 12.24±0.76 | 16.70 |
| A2 | Silver nitrate 1 mg/L | 95.83 | 2.15±0.25 | 11.95±0.97 | 17.96 |
| A3 | Silver nitrate 2 mg/L | 89.58 | 1.38±0.44 | 11.09±1.32 | 31.01 |
| A4 | Silver nitrate 4 mg/L | 87.50 | 1.58±0.21 | 11.48±1.76 | 37.32 |
| A5 | TCA 100 mg/L | 85.42 | 1.60±0.20 | 10.72±1.31 | 6.51 |
| A6 | TCA 200 mg/L | 75.00 | 1.33±0.11 | 9.44±2.25 | 5.98 |
| A7 | TCA 300 mg/L | 91.67 | 1.66±0.18 | 10.57±0.84 | 1.39 |
| A8 | Dextran sulfate 10 mg/L | 52.08 | 1.18±0.48 | 8.23±0.65 | 28.21 |
| A9 | Dextran sulfate 20 mg/L | 45.83 | 1.29±0.39 | 9.74±2.81 | 31.02 |
| A10 | Dextran sulfate 30 mg/L | 35.42 | 1.08±0.17 | 8.38±1.36 | 36.46 |

Mean values of 12 explants per replicate, 4 replicates.

**Table B.2** Data of growth of ornamental sunflower at shoot elongation phase (01).

| Media | Supplements | Shoot regeneration frequency (%) | Number of shoots per explant (mean±SE) | Mean shoot length (mm) (mean±SE) | Hyperhydric shoots (%) |
|---|---|---|---|---|---|
| B1 | - | 70.83 | 1.54±0.60 | 20.50±2.02 | 5.21 |
| B2 | Silver nitrate 1 mg/L | 83.33 | 2.20±0.38 | 19.52±1.34 | 3.89 |
| B3 | Silver nitrate 2 mg/L | 79.17 | 2.02±0.28 | 15.92±2.33 | 37.28 |
| B4 | Silver nitrate 4 mg/L | 79.17 | 1.54±0.24 | 17.44±3.67 | 14.30 |
| B5 | TCA 100 mg/L | 83.33 | 1.83±0.33 | 21.20±2.60 | 2.39 |
| B6 | TCA 200 mg/L | 68.75 | 1.42±0.28 | 18.96±1.34 | 7.50 |
| B7 | TCA 300 mg/L | 81.25 | 1.80±0.14 | 21.15±2.33 | 1.56 |
| B8 | Dextran sulfate 10 mg/L | 56.25 | 1.57±0.18 | 14.25±1.23 | 7.81 |
| B9 | Dextran sulfate 20 mg/L | 47.92 | 1.39±0.31 | 15.85±2.26 | 9.29 |
| B10 | Dextran sulfate 30 mg/L | 64.58 | 1.23±0.17 | 18.20±2.50 | 8.17 |

Mean values of 12 explants per replicate, 4 replicates.

**Table B.3** Data of growth of ornamental sunflower at shoot elongation phase (02).

| Media | Supplements | Explants fresh weight (mg) (mean±SE) | Explants dry weight (mg) (mean±SE) | Water content (%) | Stomata number per $mm^2$ |
|---|---|---|---|---|---|
| B1 | - | 1318.82±720.02 | 136.49±61.47 | 89.22 | 322.21±47.67 |
| B2 | Silver nitrate 1 mg/L | 1176.64±327.30 | 123.48±33.81 | 89.32 | 469.09±105.09 |
| B3 | Silver nitrate 2 mg/L | 1194.77±602.57 | 95.30±37.99 | 91.60 | 315.79±70.26 |
| B4 | Silver nitrate 4 mg/L | 1507.24±830.52 | 121.94±61.59 | 91.66 | 312.60±85.04 |
| B5 | TCA 100 mg/L | 3301.48±771.40 | 276.67±59.50 | 91.54 | 302.56±108.10 |
| B6 | TCA 200 mg/L | 3290.43±2618.55 | 258.58±117.76 | 90.73 | 283.39±34.61 |
| B7 | TCA 300 mg/L | 2440.02±1124.66 | 226.70±83.75 | 90.36 | 397.20±64.79 |
| B8 | Dextran sulfate 10 mg/L | 1512.79±773.40 | 150.13±69.38 | 89.81 | 344.85±48.05 |
| B9 | Dextran sulfate 20 mg/L | 1365.64±592.46 | 130.76±37.22 | 90.03 | 244.87±29.96 |
| B10 | Dextran sulfate 30 mg/L | 1659.14±671.00 | 150.85±47.79 | 90.51 | 288.16±64.33 |

Mean values of 10 explants from 4 replicates.

## B.2 Data of oil seed sunflower

**Table B.4** Data of growth of oil seed sunflower at shoot induction phase.

| Media | Supplements | Shoot regeneration (%) | Number of shoots per explant (mean±SE) | Mean shoot length (mm) (mean±SE) | Hyperhydric shoots (%) |
|---|---|---|---|---|---|
| C1 | No supplement | 100 | 1.13±0.46 | 12.73 ±4.05 | 1.67 |
| C1V | | 100 | 1.18±0.56 | 19.13±7.10 | 0.00 |
| C2 | Silver nitrate 1 mg/L | 100 | 1.18±0.56 | 15.97±4.79 | 0.00 |
| C2V | | 100 | 1.07±0.31 | 16.73±5.72 | 0.00 |
| C3 | TCA 100 mg/L | 100 | 1.03±0.26 | 11.02±3.32 | 5.00 |
| C3V | | 100 | 1.13±0.39 | 12.63±4.14 | 0.00 |
| C4 | Silver nitrate 1 mg/L | 100 | 1.02±0.13 | 9.63±2.05 | 1.67 |
| C4V | + TCA 100 mg/L | 100 | 1.12±0.37 | 12.62±4.31 | 0.00 |

Mean values of 20 explants per replicate, 3 replicates.

**Table B.5** Data of growth of oil seed sunflower at shoot elongation phase (01).

| Media | Supplements | Shoot regeneration (%) | Number of shoots per explant (mean±SE) | Mean shoot length (mm) (mean±SE) | Hyper-hydric shoots (%) | Survival rate (%) |
|---|---|---|---|---|---|---|
| C1 | No supplement | 100 | 1.13±0.46 | 15.52±4.05 | 1.67 | 86.67 |
| C1V | | 100 | 1.18±0.56 | 24.00±7.10 | 0.00 | 95.00 |
| C2 | Silver nitrate 1 mg/L | 100 | 1.18±0.56 | 18.65±4.79 | 1.67 | 96.67 |
| C2V | | 100 | 1.07±0.31 | 21.82±5.72 | 0.00 | 96.67 |
| C3 | TCA 100 mg/L | 100 | 1.03±0.26 | 13.97±3.32 | 1.67 | 78.33 |
| C3V | | 100 | 1.13±0.39 | 16.00±4.14 | 0.00 | 91.67 |
| C4 | Silver nitrate 1 mg/L + | 100 | 1.02±0.13 | 14.95±2.05 | 0.00 | 86.67 |
| C4V | TCA 100 mg/L | 100 | 1.12±0.37 | 15.77±4.31 | 0.00 | 85.00 |

Mean values of 20 explants per replicate, 3 replicates.

**Table B.6** Data of growth of oil seed sunflower at shoot elongation phase (02).

| Media | Supplements | Number of leaves per shoot | Explants fresh weight (mg) (mean±SE) | Explants dry weight (mg) (mean±SE) | Water content (%) | Stomata number per $mm^2$ |
|---|---|---|---|---|---|---|
| C1 | No supplement | 10.05 | 2.96±0.76 | 0.30±0.11 | 89.96 | 314.65±67.25 |
| C1V | | 11.30 | 3.33±0.82 | 0.34±0.10 | 89.68 | 321.11±31.52 |
| C2 | Silver nitrate 1 mg/L | 13.25 | 3.43±0.63 | 0.33±0.09 | 90.46 | 328.78±37.85 |
| C2V | | 15.50 | 3.17±0.74 | 0.32±0.10 | 90.07 | 344.52±47.02 |
| C3 | TCA 100 mg/L | 8.90 | 3.83±0.75 | 0.34±0.11 | 91.17 | 306.81±35.90 |
| C3V | | 9.00 | 4.24±0.71 | 0.38±0.11 | 91.14 | 319.83±33.09 |
| C4 | Silver nitrate 1 mg/L + | 9.05 | 3.40±0.91 | 0.29±0.08 | 91.32 | 305.45±31.64 |
| C4V | TCA 100 mg/L | 9.65 | 3.62±1.09 | 0.33±0.11 | 90.86 | 327.40±35.04 |

Mean values of 20 explants from 3 replicates.

# CURRICULUM VITAE

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2021. **Amsungnoen T**, and Muangsan N. Effect of silver nitrate, trichloroacetate, and dextran sulfate on hyperhydricity and shoot regeneration of *in vitro* sunflower. In *the Proceedings of The SUT International Virtual Conference on Science and Technology 2021 (IVCST 2021)* (pp. 365-371). Nakhon Ratchasima, Thailand.

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