IN VITRO REGENERATION OF ORNAMENTAL SUNFLOWER (*HELIANTHUS ANNUUS* L.) FROM COTYLEDONS

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งานวิจัยนี้มีวัตถุประสงค์หลักเพื่อ 1) ตรวจสอบอิทธิพลของพันธุ์ สูตรอาหาร ทิศทางการวาง และอายุของใบเลี้ยงต่อการชักนำให้เกิดต้นใหม่ในทานตะวัน 2) เพื่อประเมินการปรับตัว และการเติบโตของต้นอ่อนพืชที่ได้จากการเพาะเลี้ยงในโรงเรือน และ 3) เพื่อตรวจสอบการเปลี่ยนแปลงลักษณะทางเซลล์วิทยาและทางเคมีของใบเลี้ยง ทำการทดลองโดยใช้ใบเลี้ยงจากต้นอ่อนทานตะวันสองพันธุ์ ได้แก่ พันธุ์ลูกผสมช่วงรุ่นที่ 1 (พันธุ์แปซิฟิก 22 x พราโดเรด) และพันธุ์พราโดเรดอายุ 0 1 และ 7 วัน แล้ววางด้านบนแผ่นใบเลี้ยง (อะดาเซียล) บนอาหารชักนำให้เกิดต้นจำนวน 4 สูตร (A1 - A4) เป็นเวลา 21 วัน นำส่วนยอดของต้นใหม่สูง 0.5-1.0 ซม ย้ายลงปลูกในอาหารชักนำให้เพิ่มจำนวนยอด จำนวน 5 สูตร (B1-B5) เป็นเวลา 21 วัน จากนั้นทำการย้ายส่วนยอดที่มีความสูง 1-1.5 ซม. มาเพาะเลี้ยงในอาหารชักนำให้เกิดรากจำนวน 10 สูตร (C1-C10) เป็นเวลา 21 วัน นำต้นอ่อนที่มีรากสมบูรณ์มาปลูกในกระถางพลาสติกบรรจุวัสดุปลูกจำนวน 3 สูตร (พีทมอส ทราย และพีทมอสผสมทรายอัตราส่วน 1 ต่อ 1) แล้ววางในโรงเรือนเป็นเวลา 14 วัน เพื่อประเมินอัตราการรอดชีวิต และการเติบโต นอกจากนี้ ทำการตัดตัวอย่างใบเลี้ยงจากต้นกล้าและใบเลี้ยงที่ได้จากการเพาะเลี้ยงบนสูตรอาหารชักนำต้นนาน 21 วัน เพื่อนำมาย้อมสีและวิเคราะห์ลักษณะทางเซลล์วิทยาและทางเคมี ผลการทดลองพบว่าความถี่การชักนำให้เกิดต้นขึ้นกับพันธุ์ชนิดสูตรอาหาร และอายุของใบเลี้ยง ใบเลี้ยงอายุ 1 วันตอบสนองต่ออาหารได้ดี (99.72%) มีร้อยละของยอดเพิ่มมากที่สุด (26.67%) จำนวนยอดสูงสุด (1.10 ยอด) และจำนวนรากเพิ่มมากที่สุด (17.78%) สูตรอาหารที่ประกอบด้วย BA 1 มก. ต่อลิตร (A3) ตอบสนองต่ออาหารได้ดีที่สุด (99.26 %) ร้อยละของการเกิดต้นมากที่สุด (30 %) และจำนวนต้นต่อชิ้นส่วนพืชมากที่สุด (1.15 ยอด/ชิ้นส่วนพืช) เมื่อนำส่วนยอดมาเพาะเลี้ยงในอาหารที่เติม BA 2 มก. ต่อลิตร พบว่าให้จำนวนต้น (3 ต้นต่อชิ้นส่วนพืช) และจำนวนใบมากที่สุด (5 ใบต่อต้น) เมื่อนำต้นอ่อนที่สมบูรณ์มาเลี้ยงในอาหารชักนำราก พบว่าอาหารที่เติม BA 1 มก. ต่อลิตร IAA 1 มก. ต่อลิตร และผงถ่าน ให้จำนวนรากต่อต้นสูงที่สุด (7.26 รากต่อต้น) เมื่อนำต้นทานตะวันที่สมบูรณ์มาปลูกในโรงเรือนพบว่าวัสดุปลูกที่ประกอบด้วยพีทมอสอย่างเดียวสามารถให้อัตราการรอดชีวิตสูงที่สุดร้อยละ 60 มีจำนวน 6 ใบ และสูง 7 ซม.

ผลการศึกษาการเปลี่ยนแปลงทางเซลล์วิทยาและทางเคมี พบว่าใบเลี้ยงจากต้นกล้า และใบเลี้ยงที่ผ่านการเพาะเลี้ยงในอาหารสังเคราะห์ ประกอบด้วยชั้นของเซลล์ ดังนี้ อะดาเซียล

พาเรงคิมา อะบาเซียล พาเรงคิมา โพรแคมเบียม เอพิเดอร์มิสด้านบนและเอพิเดอร์มิสด้านล่าง พบเม็ดแป้งกระจายอยู่ภายในเซลล์พาเรงคิมาเฉพาะใบเลี้ยงที่ได้จากต้นกล้าเท่านั้น ลักษณะทางเซลล์วิทยาของใบเลี้ยง พบว่าจำนวนเซลล์ พื้นที่ของเซลล์ และความหนาของใบเลี้ยงมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบทั้งสามปัจจัยได้แก่ พันธุ์ อายุใบเลี้ยงและสภาพการเพาะเลี้ยง ใบเลี้ยงอายุ 7 วันที่ผ่านการเพาะเลี้ยงบนอาหารมีจำนวนเซลล์น้อยที่สุด (13.33 เซลล์ต่อไมโครเมตร) แต่พื้นที่ของเซลล์และความหนาของใบเลี้ยงมีค่ามากที่สุด (6,894 ไมโครเมตร และ 1.22 มิลลิเมตร ตามลำดับ) สำหรับอาหารที่สะสมในใบเลี้ยง (โปรตีน ไขมันรวม น้ำตาลรวม และซูโครส) พบว่ามีความแตกต่างอย่างมีนัยสำคัญทางสถิติ โดยใบเลี้ยงที่ได้จากต้นกล้า 0 วัน จะพบปริมาณไขมัน น้ำตาลรวม และซูโครสมากที่สุด ในขณะที่ปริมาณโปรตีนพบน้อยสุด เมื่อนำใบเลี้ยงมาเพาะเลี้ยงบนอาหารสังเคราะห์พบว่าปริมาณโปรตีนมากกว่าและพบมากที่สุดในใบเลี้ยงอายุ 7 วัน โดยสรุป งานวิจัยนี้ได้ทราบปัจจัยที่มีอิทธิพลต่อการขยายพันธุ์ทานตะวันด้วยวิธีเพาะเลี้ยงใบเลี้ยงของต้นกล้า ได้ต้นใหม่ที่สมบูรณ์ และได้ทราบการเปลี่ยนแปลงทางเซลล์วิทยาและอาหารสะสมของใบเลี้ยงในหลอดทดลอง

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2560 ลายมือชื่ออาจารย์ที่ปรึกษา_____

KAMONPHON MONTATHONG : *IN VITRO* REGENERATION OF ORNAMENTAL SUNFLOWER (*HELIANTHUS ANNUUS* L.) FROM COTYLEDONS. THESIS ADVISOR : ASSOC. PROF. NOODUAN MUANGSAN, Ph.D. 97 PP.

CALLUS/ COTYLEDON/ CELL NUMBER/ TOTAL FAT/ SUNFLOWER

The main objectives of this research were; 1) to examine the effect of variety, media type and age of cotyledons on shoot induction of sunflower, 2) to evaluate acclimation and growth of regenerated plantlets in a greenhouse condition, and 3) to investigate cytological and chemical changes of cotyledons in seedlings and *in vitro* culture. Cotyledons of the F1 hybrid (Pacific 22 x Prado Red) and Prado Red varieties of 0, 1 and 7 days-old seedlings, as explants, were surface sterilized and placed on adaxial sides on 4 shoot induction media, A1-A4, for 21 days. Regenerated shoots with 0.5-1.5 cm in height were subcultured on 5 multiple shoot induction media, B1- B5, to increase the number of shoots for 21 days. The shoots with 1 -1 .5 cm in height were then subcultured on 10 root induction media, C1-C10, for 21 days. After that, complete plantlets were planted in plastic pots filled with sand, peat moss, and peat moss and sand with ratio 1:1 for 14 days and then were subjected for survival and growth measurement. Cotyledons of seedlings and *in vitro* culture were subjected to cytological and chemical analyses. The results showed that shoot induction frequency was affected by variety, media type and age of explants. Cotyledons of 1 day-old seedlings gave best response (99.72%), the highest percentage of shoot induction (26.67%), number of shoot per explant (1.10) and the root induction (17.78%). MS medium supplemented with 1 mg/l BA gave best response (99.26%), the highest

percentage shoot induction (30%) and number of shoot per explant (1.15). For shoot multiplication, the maximum shoot number (3.00) and number of leaves (5.60) were obtained on MS medium containing 2 mg/l BA. MS medium added with 1 mg/l BA, 1 mg/l IAA and charcoal powder gave the highest number of roots per plant (7.26). For acclimation and growth in a greenhouse, the peat moss gave the highest survival rate (60%) with 6 leaves and 7 cm in height.

Cross sections study revealed that cotyledon consisted of a layer of adaxial parenchyma, abaxial parenchyma, procambium, upper and lower epidermis. Starch granules only appeared within parenchymal cells of cotyledon seedlings. Cell number, cell area and cotyledon thickness were significantly different among conditions, varieties and ages. Cotyledons of 7 days-old seedlings after 21 days of culture had the lowest number of cells (13.33 cell/μm^2), but the highest cell area (6,894 μm^2) and cotyledon thickness (1.22 mm). For chemical analysis, there was a statistically significant difference among varieties, cotyledon ages and conditions. The 0 day-old cotyledons both in seedlings and *in vitro* condition showed the highest values of total fat, total sugar and sucrose, but the lowest value of protein content. In conclusion, this study has determined factors influencing sunflower propagation using cotyledons as explants *in vitro*, and successfully obtained complete plantlets, as well as indicated the changes of cytology and food reserve of cotyledon explants *in vitro*.

School of Biology Student's Signature _____

Academic Year 2017 Advisor's Signature _____

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LIST OF ABBREVIATIONS

ABA	abscisic acid
Aba	abaxial
Ada	adaxial
BA	6-benzylaminopurine
°C	degree Celsius
CH	casein hydrolysate
DI	deionized water
GA_3	gibberellic acid
g	gram
g/l	gram per liter
HCl	hydrochloric acid
h	hour
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IKI	iodine-potassium iodide
Kcal	kilocalorie
L	liter
L	length
µm	micrometer
µM	micromolar

LIST OF ABBREVIATIONS (Continued)

µg	microgram
µl	microliter
mg	milligram
mm	millimeter
ml	milliliter
mg/kg	milligram per kilogram
mg/l	milligram per liter
mg/ml	milligram per milliliter
M	molarity
mM	milimolar
min	minutes
MS	Murashige & Skoog medium
NAA	naphthalene acetic acid
N	normality
%	percentage
pH	potential of hydrogen ion
μm^2	square micrometer
v/v	volume by volume
w/v	weight by volume

CHAPTER I

INTRODUCTION

1.1 Background / Problems

Sunflower (*Helianthus annuus* L.) belongs to the Family Asteraceae. It is an annual dicotyledonous plant native to North America (Fozia, 2008). There are two types of sunflower varieties including oil variety and ornamental variety (Allah, 2015). The sunflower oil variety is one of the most important oil seed crops in the world. It is an important source of nutrients for health, used for chemical and industrial purposes due to its seeds containing a high concentration of essential fatty acids not synthesized by humans such as linoleic acid, vitamin E, proteins, and minerals such as potassium and calcium (Vaknin, 2008; Ogunwande, 2010). The ornamental variety sunflower are widely grown for decoration in home gardens and parks (Miklic et al., 2008). The ornamental sunflower has the global high demand for flower value worldwide due to the beauty of its inflorescence, the possibility of arrangements in pots or as cut flowers for bouquets and flower arrangements (Eurostat, 2015).

Propagation of sunflower can be mainly created through seeds which can possibly cause hybridization and variation in genotypes. Tissue culture technology is employed as an alternative way for reduction in genotype variation and for genetic improvement. Propagation through cotyledon culture depends on many factors such as genotype, culture medium, and the nature of the explant.

Therefore, this study aimed to investigate the factors that affects *in vitro* regeneration of ornamental sunflower from cotyledons.

1.2 Research objectives

1.2.1 To examine the effects of variety, media type and age of cotyledons on shoot regeneration of two sunflower varieties.

1.2.2 To evaluate acclimation and growth of regenerated plantlets.

1. 2. 3 To investigate cytological and chemical changes of cotyledons in germinating seeds and *in vitro* culture.

1.3 Research hypothesis

Tissue culture technique is one of efficient methods for sunflower propagation and genetic improvement. Many factors affect the success of sunflower tissue culture including varieties, media, age of explant and hormone, etc. It was hypothesized that 1) *in vitro* propagation efficiency of sunflower using cotyledons as explants would be affected by variety, media type and age of cotyledons, 2) acclimation and growth would be influenced by growing substrates, and 3) cell characteristics and chemical content composition of sunflower cotyledons explants would undergo changes cultured *in vitro*.

1.4 Scope and limitations

Two sunflower varieties: F1 hybrid (Prado Red x Pacific 22) and Prado Red were used as plant materials. Three affecting shoot regeneration, including variety, media type and age of cotyledons, were investigated. Moreover, survival rate and growth of regenerated plantlets were evaluated on various growing substrates.

For the study of cytological and chemical changes, the cotyledons from 0, 1 and 7 day-old germinating seeds and *in vitro* culture on MS media for 21 days were taken for analyses.

1.5 Expected results

The expected results from this study were:

1.5.1 Shoot regeneration will be affected by variety, media type and age of cotyledons.

1.5.2 Plantlets-derived from *in vitro* culture could survive and grow normally in greenhouse conditions.

1.5.3 Cytological and chemical changes of cotyledons in germinating seeds and *in vitro* culture will be obtained.

1.5.4 The well-developed and efficient protocol for *in vitro* propagation in sunflower will be beneficial for the future plant breeding especially in the steps of genetic transformation and mutagenesis.

CHAPTER II

LITERRATURE REVIEWS

2.1 Sunflower characteristics

Sunflower (*Helianthus annuus* L.) is one of the main oil crops in the world with an increasingly important economic value (Yang et al., 2012). The genus *Helianthus* comprises nearly 70 species with similar characteristics (Maiti et al., 2007). Sunflower is an annual plant. Its stem height depends on weather and environmental conditions. Root system consists of taproot and secondary roots. The leaves are large with rough surface and oval shape. Leaf arrangement is alternate in mature plant, while opposite in juvenile stage. The inflorescence consists of two florets including ray florets and disk florets. The petal ray florets have beautiful colors (Figure 2.1), while disk florets are black or brown color of tubular petal. Sunflower seeds are the achene fruits. They are grayish-green and covered by outer shell (pericarp) that appears black, white or striated depending on the variety (Hu et al., 2010).

2.2 Sunflower cultivation

The best cultivation of sunflower sow through a seed directly into the suitable soil, good climate and good farm management is required. Sunflower plants require a cool climate and watering during seed germination and seedling growth. The seed

before sowing must be treated with pesticide for protection from ants. Cultivation of sunflower seeds should be sown 70-75 cm apart in lines with a plant to plant spacing of 25-30 cm, and at 5-6 cm of depth in to soil. Sunflower seedlings highly require moisture, while adult plants should be watered twice daily after sowing and lodging should be avoided. Weed should be contorted in sunflower cultivation up to every 14 days after sowing (Extension and Training Office, 2016).

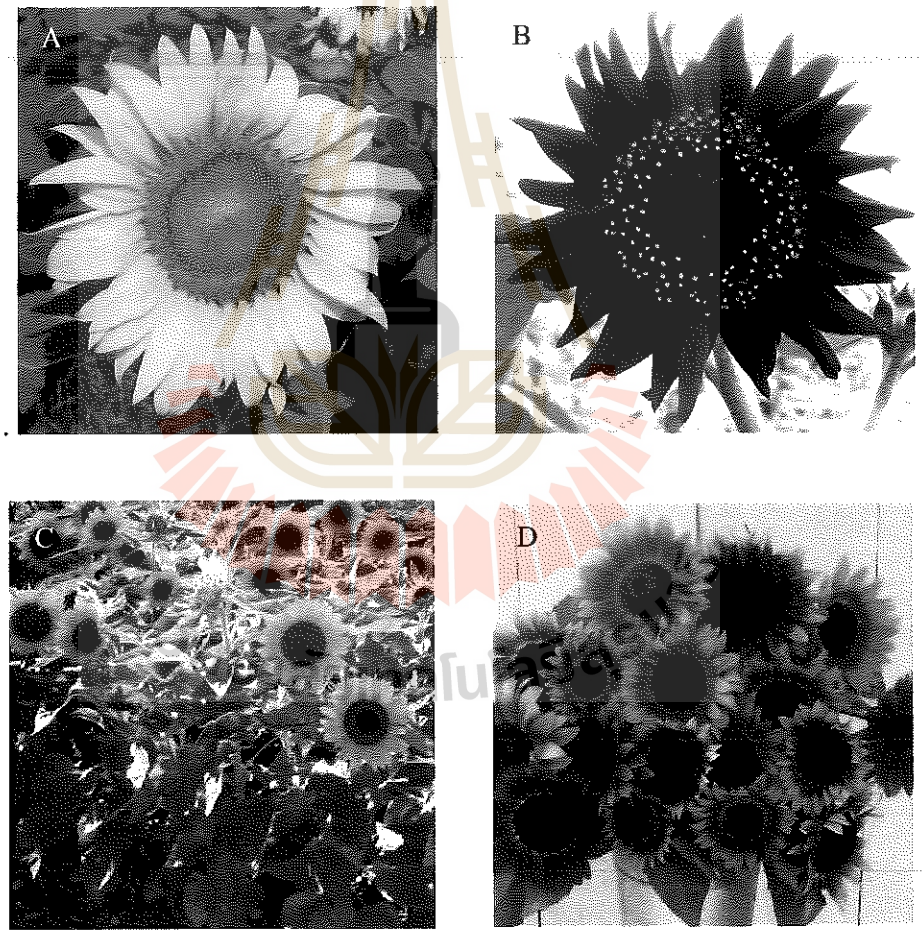

Figure 2.1 Sunflowers varieties. A) Pacific 22, B) Prado Red, C) Big smile, D) Autumn beauty (Johnnyseeds, 2017).

2.3 Developmental stages of sunflower

Sunflower is an annual crop. It emerges from seed until seed setting and follow several developmental stages. In the field, the average life cycle is about 125 days after sowing (Figure 2.2) and it depends on genetic variation. There are two developmental stages of sunflower (Schneiter and Miller, 1981). The first developmental stage starts with mature seed germination taking about 2-7 days, called vegetative stage (V). The vegetative stage is counted from the emergence of true leaves. The second stage is reproductive stage (R) starting from the blooming of inflorescence until the time the head turns black and die.

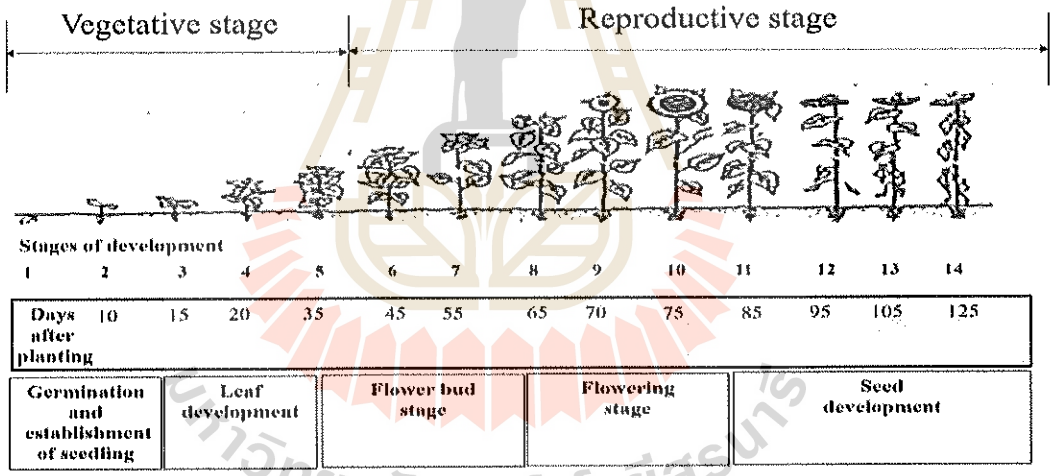

Figure 2.2 Developmental stages of sunflower (Schneiter and Miller, 1981).

2.3.1 Vegetative stage

Sunflower is annual plant, with two phases of development in their life cycle including a juvenile and adult phases (Jones, 2001). It relates growth of shoot direction. Juvenile phase starts from seed germination, which is the transition from the

embryonic seed to the embryonic seedling. This phase has important economic ramifications in many crops, including sunflower sprouts. The juvenile phase has high activities of plant growth. This phase requires high moisture in soil, nutrition and management of pest. The vegetative stage is determined by counting the number of true leaf at 4 mm in length of the plant such as v-1, v-2, and v-n (Schneiter and Miller, 1981).

2.3.2 Reproductive stage

Sunflower is a monoecious plant that reproduces sexually through both self and cross pollination. The reproductive development is divided into nine stages (R1-R9) based on the developmental inflorescence formation from its initial appearance through anthesis to physiological maturity of the seed as presented in Figure 2.3 (Shutterstock, 2017). The R1 stage is initial phase of reproductive stage, where the plant begins the bud formation at the top of apex of shoot, the next phases 2, 3 and 4 are the morphological changes in the elongated bud about 0.5 to more than 2 cm. R4 stage is starting of ray florets, while R5 stage the beginning of disk flowering of each inflorescence area. This R5 stage can be divided into sub stages such as R5.1, R5.2,... R5.9.

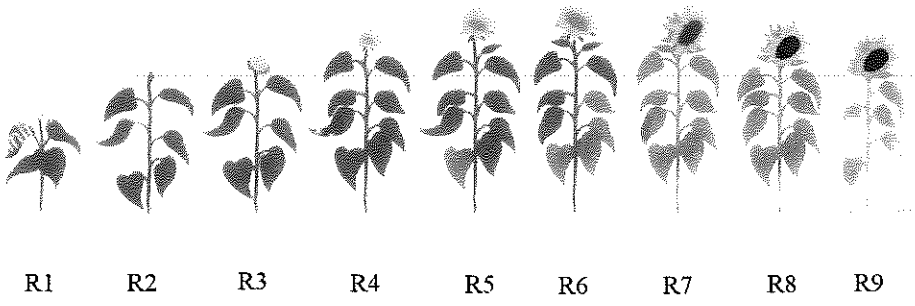

Figure 2.3 Reproductive stages of the life cycle in sunflower (Shutterstock, 2017).

2.3.2.1 Flower head

The inflorescence also known as flower head is large and its diameter is about 10 to 30 cm depending on the environment and genotype. Each inflorescence contains numerous florets. Two types of florets in inflorescence are ray florets and disk florets. The ray florets are outermost flower on the inflorescence of sunflower. They can occur in yellow, red, orange, or other colors, depending on the sunflower species or varieties. These florets are sterile and mark a beauty to the sunflower. The disk florets are located in the center of the inflorescence of sunflower. They are composed of either yellow or brown florets. These florets are fertile. The disk florets are complete flowers that produce a seed. A single flower head may have up to two thousand disk florets, each with the potential to develop into a seed. The disk florets are opened in sequence, beginning at the periphery of the disk and moving inward to the central of the inflorescence (Figure 2.4) (Percy, 1898).

Figure 2.4 Sunflower heads of (A) F1 hybrid (Prado Red × Pacific 22) and (B) Prado Red varieties (Photos taken by Montathong).

2.3.2.2 Seed and composition

The typical fruit of the sunflower family is achene form. The main anatomical parts of the mature seed are make-up of three basic parts including seed coat or hull, two large cotyledons, and embryo (plumule and hypocotyl-radicle axis) as shown in Figure 2.5A, B (Wolf, 1972). In sunflower seed, under the hull is the thin endosperm tissue surrounding the kernel part. The endosperm is covered by a single layer of aleurone cells. The cotyledons are source of nutrition during seed germination. Cotyledon is composed of closely packed mesophyll cells. The abaxial cotyledon (curve portion) consists of elongate palisade - like cells, which contain protein and lipid bodies, while the adaxial cotyledon (flat side) contains spongy-like cells (Zhang and Finer, 2015).

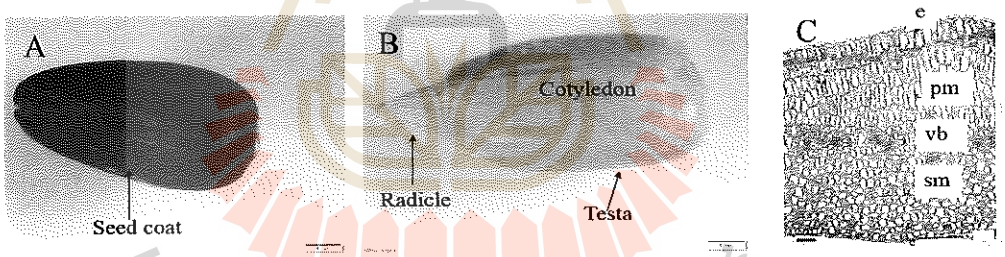

Figure 2.5 Morphology and anatomy of sunflower seed. A) Hulled sunflower seed, B) Unhulled sunflower seed, and C) Cotyledon cross section (Vega et al., 2007). E = epidermis, pm = palisade mesophyll, vb = vascular bundle, sm = spongy mesophyll. Bar = 1 mm.

The mature seed consists of fatty acids, proteins, vitamins and minerals (Cancalon, 1971). Unsaturated fatty acid is the main source of essential fatty acids such

as monounsaturated fatty acid (18.528 g), polyunsaturated fatty acid per 100 g of seeds (23.137 g), highly source of vitamin E (35.17 mg) and lower than 4.455 g saturated fatty acid (Phillips, 2000) as presented in Table 2.1. In case of seedling, cotyledons are functionally similar of true leave and synthesize carbohydrates. The anatomical cotyledon of seedling composes of upper epidermis, palisade mesophyll, spongy mesophyll, vascular bundle and lower epidermis. The cuticle covers both upper and lower epidermises (Figure 2.5C). The seed at different maturity stages is different in seed coat color, size and accumulation of food reserve (Torres and Martinez, 1990).

Table 2.1 Nutritional value per 100 g of sunflower seeds.

Nutrition	**value per 100 g**	
Energy	584	Kcal
Total fat	51.46	g
Saturated fat	4.455	g
Monounsaturated fat	18.528	g
Polyunsaturated fat	23.13	G
Protein	20.78	G
Vitamins		
Thiamine (B1)	1.48	Mg
Riboflavin (B2)	0.355	Mg
Niacin (B3)	8.335	Mg
Pantothenic acid	1.13	Mg
Vitamin B6	1.345	Mg

Table 2.1 (Continued)

Folate (B9)	227	Mg
Choline	55.1	Mg
Vitamin C	1.4	Mg
Vitamin E	35.17	Mg

Resource: United States Department of Agriculture Agricultural Research Service, 2016.

2.3.2.3 Seed germination mechanism

Seed germination is the process of the imbibition and radicle emergence. This process is started from water absorption of seed coat, and involves physical, chemical, and morphological changes in the embryo, cotyledon and testa including cell size and their food reserve (Nonogaki, 2006; Han and Yang, 2015). During seed maturation, the storage lipid forms oil body which is the formation of triacylglycerols surrounded by phospholipid (Huang, 1996). Lipid bodies or fats are the first sources of energy in the seed. The lipid body in sunflower seed is degraded by the metabolism during germination process as a carbon source (Feussner et al., 2001). In ground nut, the lipid content decreased drastically during seed germination (Offem et al., 1993), whereas some proteins, e.g. lipoxygenase (LOX) was synthesized during germination and degraded into lipid body (Rudolph, 2011). During cucumber seed germination, LOX was found in cytosol of mesophyll cells at 1 day and 4 days after culture (Feussner et al., 1996). In *Passiflora edulis* seeds, total fat levels decreased significantly over the first 10 days from sowing (Tozzia and Takaki, 2011). Proteins are the second source in

cotyledon seed of sunflower and they are accumulated in form of protein bodies (Millichip et al., 1996; Allen and Arnott, 1982). During sunflower seed germination, storage proteins began to decease within the first three days of growth.

Cotyledon-derived organ is the most important for the success of tissue culture process for shortage of sunflower life cycle. There are several processes related to this regeneration such as molecular mechanism, but much less in known about the biochemical changes with *in vitro* organogenesis (Vega et al., 2007). For cotyledon culture on artificial medium, proteins were decreasing within the first three days of growth while the proteins in the growing parts increased. They were hydrolyzed to peptides or amino acids for embryonic development (Aston, 1976). Four to five days of culture on MS medium supplemented with 1 μM BA, cotyledon was expanded as a result of imbibition, total protein levels remained high, while cotyledon cultured on MS medium supplemented with 10 M IAA did not show expansion (Allen et al., 1984).

Previous studies reported about protein body modification during sunflower seed germination. There are three patterns of protein change including: 1) intact protein body within 1 day after germination, 2) protein body fusion and coalescence which occurs within 2 days after germination, and 3) protein body hydrolysis which occurs within 7 days after culture (Allen and Arnott, 1982).

Starch and soluble sugar are the third source of food reserves in cotyledon and vary in quantity among different genotypes. They are accumulated for seed germination. Alencar et al. (2012) reported that the change of starch and soluble sugar may be increased during seed germination.

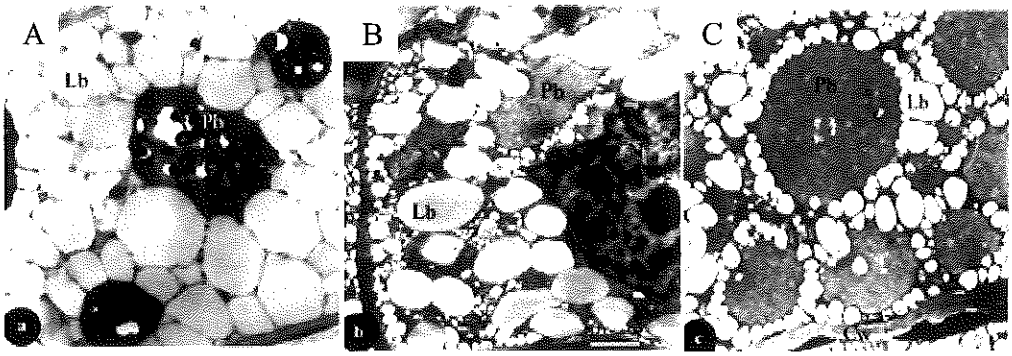

Figure 2.6 Cotyledon x-sections of cucumber species. A) prime cotyledon showing lipid body and protein body, B) 5 days old seedling, and C) 0 day old seedling (Allen and Arnott, 1982), Cw = cell wall, Lb = lipid body, Pb = protein body.

2.4 Sunflower propagation

Sunflower can be propagated by two main methods, seed propagation and *in vitro* propagation.

2.4.1 Seed propagation

The sunflower is an annual crop plant that is normally propagated by seeds. Seed propagation on the advantageous side is considerably cheap and quick (Wink, 1988). Sunflower plant can grow in a variety of soil types from sands to clay and a wide range of pH from 5.7 to over 8.0 (Seiler, 1997) and different climates. It is commonly grown in semi-arid and can tolerate both low and high temperatures. Optimum temperature for growth ranges from 21 to 26 °C (Putnam et al., 1990). In Thailand, sunflower harvesting is done in October when the moisture content is low (Laosuwan, 1997; Dedio, 2005).

2.4.2 *In vitro* propagation

One of the most important aspects of *in vitro* tissue culture is the capability to regenerate and propagate plantlets (Phillips and Hubstenberger, 2000). Plant tissue culture technique is used for growing isolated plant cells, tissue, and organs under sterile condition *in vitro* and in a controlled environment for regeneration and propagation of the entire plants. There are many types of plant cultures, namely callus, cell, protoplast, anther, meristem, embryo and organ cultures (Ahloowalia et al., 2004). Tissue culture is the major experimental system used for plant genetic engineering, for studying the regulation of growth and organized development through examination of structural, physiological, biochemical and molecular processes. Tissue culture has become an important part of the commercial propagation of many plants because of its advantages as a multiplication system. Several techniques for *in vitro* plant propagation have been devised, including the induction of axillary and adventitious shoots, the culture of isolated meristem and plant regeneration by organogenesis and somatic embryogenesis (Davey, 2010).

2.5 Factors affecting tissue culture success

2.5.1 Media

Culture media used for the *in vitro* cultivation of plant cells are composed of three basic components. Firstly, essential elements or mineral ions are supplied as a complex mixture of salts. Secondly, an organic supplement supplies vitamins and amino acid and thirdly, a source of carbon supplied as the sugar (Slater et al., 2003).

There are several basal media for sunflower tissue culture such as MS, N6 and B5. *In vitro* culture of sunflower can use both solid MS and liquid MS medium. Petitprez et al. (2005) used epidermal layers of sunflower cultured in liquid MS medium and found the number of embryogenic explants about 48%. Dagustu et al. (2010) reported that the mature seeds cultured on 1/2MS medium showed 67.33% of apical shoot meristem. Fischer et al. (1992) reported that cotyledons of sunflower regenerated compacts and green embryonic callus in L4 medium before regeneration of shoot.

2.5.2 Genotype

The major influence on sunflower tissue culture response appears to be genetic with culture requirements varying between species and cultivars (Slater et al., 2003). Different genotypes have effect on shoot regeneration *in vitro* such as seven different sunflower genotypes showed variation of shoot primordia between 25%-88.7% (Zhang and Finer, 2015; Sujatha et al., 2012). A significant variation for organogenesis using cotyledons as explants among 2 genotypes was found that shooting per 100 explants was about 35% in inbred line R-92 genotype (Azadi et al., 2002).

2.5.3 Plant hormones

Plant hormones are organic chemical compounds that are involved in the regulation of growth and development in plants. They are signal molecules that are produced within the plant organs or tissues and are active at very low concentrations. They may exist naturally and synthesized by humans or other living organisms. There are five major classes of plant hormones including auxins, cytokinins, gibberellins, abscisic acid and ethylene (Kumar et al., 2014).

Auxins are generally considered as compounds characterized by their ability to induce cell elongation and otherwise resemble indole-acetic acid (IAA), the first auxin isolated in physiological activity. The function of auxin hormones includes stimulating cell elongation, promoting root initiation and lateral root development, mediation of tropic responses such as bending in response to gravity and light, and regulation of pattern formation in embryogenesis (Slater et al., 2003). In efficient sunflower regenerations from cotyledon, the MS medium consisting of combination hormones as 0.5 mg/l of BA and 0.1 mg /l of NAA produced adventitious shoot about 2.5 shoots/explant (Inoka and Dahanayake, 2015). The root induction about 26.4% was obtained on MS medium with 3 mg/l of IBA (Elavazhagan et al., 2009). In addition, low concentration of IAA was used to improve roots formation (Baker et al., 1999). The first stage, plant regeneration could use media containing auxin hormone for callus induction (Witrzens et al., 1988).

Cytokinin hormones are involved primarily in cell growth and differentiation. They are synthesized mostly in the root apical meristems but also found in root cap cells, seed and tips of young inflorescences. Cytokinin activities include stimulating cell division and morphogenesis (shoot initiation/bud formation), promoting the growth of lateral buds, the release of apical dominance, leaf expansion, and slowing down the aging of some plant organs (Slater et al., 2003). In sunflower, cytokinin reconditioning of cotyledon of seed materials induced divisions in cotyledonary node cells, which contribute to shoot primordia (Zhang and Finer, 2015). The cotyledon cultured on MS medium with 4 mg/l of BA and 0.5 mg/l of IBA showed 46.2% of shoot induction (Elavazhagan et al., 2009), whereas the mature embryos cultured on MS medium with

1 mg/l of BA and 0.5 mg/l of NAA showed 44% of shoot regeneration and MS medium with 1 mg/l of 2, 4-D gave callus induction about 80-92% (Ozyigit et al., 2006).

Gibberellins (GA_3) are a large group classified on the basic of both structure and function. All gibberellins are derived from the *ent*- gibberellane skeleton. GA activities include stimulation of stem elongation by promoting cell division and α- amylase production for mobilization of starch reserves, breaking of seed dormancy to induce germination, and parthenocarpic fruit development (Slater et al., 2003). Several researchers reported the usage of GA_3 in regeneration medium to produce healthy shoots in sunflower and promotion of elongated pale green shoots in sunflower (Power et al., 1991; Fiore et al., 1997; Khalil1 et al., 2015). The solid MS medium supplemented with 0.6 mg/l of BA and 0.2 mg/l of GA_3 resulted in about 72.6% of callus induction (Thengane et al., 1994)., and the cotyledons cultured on MS medium with 2 mg/l of BA and 0.5 mg/l of GA_3 showed 36.4% of callus induction (Elavazhagan et al., 2009).

Abscisic acid is an isoprenoid compound formed by the cleavage of carotenoid precursors. It is more closely associated with responses to environmental stresses. Its involvement in seed development includes the following: promotion of synthesis of some seed storage proteins, acquisition of desiccation tolerance, and induction and maintenance of dormancy (Slater et al., 2003). There are reports showing that the hypocotyl explant of sunflower variety cultured on MS medium supplemented with 0.4 mg/l of BA, 0.2 mg/l of GA_3 and 0.2 mg/l of ABA had 58.2% of shoot regeneration (Narayanasamy, 2007).

Ethylene is a hydrocarbon gas and naturally occurring. Some plant will produce ethylene as fruit ripening begins. Ethylene activities include fruit ripening, losing of chlorophyll, cell division, cell expansion and responses to environmental stresses (Burg, 1973). In explant tissue culture of sunflower, ethylene gas caused failure of shoot induction (Abdoli et al., 2007).

2.5.4 Nature and developmental stage of explant

Developmental stage of explant is important factor affecting plant regeneration from *in vitro* culture. The difference of age of cotyledon seedling affected to some degree of plant regeneration. Chowdhur (2015) reported that 7 days-old cotyledon seedling had response about 89.84%, while 5 days-old cotyledon seedling died. Ozyigit et al. (2002) used 10 days-old cotyledon of sunflower seedling. They showed 40% shoot regeneration on MS medium supplemented with 1 mg/l of BA + 0.5 mg/l of NAA. Mohmand and Quraishi (1994) used 21 days-old seedlings for cotyledon explant culture. Azadi et al. (2002) used cotyledons of 2 days-old seedlings of three sunflower inbred lines. In addition, *in vitro* tissue culture methods in sunflower include anther culture, microspore culture, ovule culture, immature embryo. The stage of development of explant is important for regeneration new plant (Dagustu et al., 2012).

2.5.5 Explant type

There are many regeneration methods in sunflower propagation including auxiliary culture, young leave culture, young reproductive organ and cotyledon culture. Aurori et al. (2011) used embryonic axis from mature seed as the best regenerative explant rather than embryonic axis from germinated seedling. Cerian et al. (1992) used

half cotyledon explants of sunflower mature seed for regeneration of new shoot. Paterson and Everett (1985) reported embryogenesis of sunflower inbred from 12 days-old seedling for hypocotyl explants on a modified MS medium.

2.5.6 Age of cotyledon

Ages of cotyledon explant significantly affect *in vitro* shoot regeneration of sunflower. Two days-old seedling gave about 6.1 shoots per explant (Abdoli et al., 2007). Ten days-old seedling gave 90% of shoot induction (Ozyigit et al., 2007). Dagustu et al. (2010) used immature seed explants of 15 genotypes on MS medium and found that 10 days old of immature seed gave new shoot within 5-10 days after culture.

2.6 *In vitro* plant regeneration

Sunflower is a cross pollinating plant. Because the availability of mixed sex between males and females is not simultaneous. It makes sunflower breeding quite difficult for homozygous line production (Bayraktaroglu et al., 2011). In sunflower, plant regeneration could possibly be produced through either somatic embryogenesis or organogenesis (Liu et al., 2011).

2.6.1 Somatic embryogenesis

In somatic embryogenesis, embryo-like structures, which can develop into whole plant in a way analogous to zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells

without the production of an intervening callus, though common from some tissues (usually reproductive tissues such as the nucleus, styles or pollen) (Sarkar, 2009).

In indirect somatic embryogenesis, callus is first produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus (Slater et al., 2003). Somatic embryo of immature zygote of sunflower proliferated directly from the surface of the zygotic embryos and germinated after placement on a low sucrose medium (Finer, 1987).

2.6.2 Organogenesis

Organogenesis relies on the production of organs either directly from an explant or from a callus culture. It has been obtained from hypocotyls, young leaves, shoot apex, immature cotyledon, and mature cotyledon used as explant (Vega et al., 2007). There are three methods of plant regeneration via organogenesis. The first two methods depend on adventitious organs arising either from a callus culture or directly from an explant. Alternatively axillary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture. Organogenesis relies on the inherent plasticity of plant tissues and is regulated by altering the components of the medium (Slater et al., 2003). In sunflower, organogenesis was reported in several papers. Primary leaves of young seedling cultured in MS medium supplemented with 1.5 mg/l of BA gave an average of 12-19 shoot primordia per leaf explant (Zhang and Finer, 2015). Cotyledon regenerated shoot about 83.86% in RHA 6D-1 genotype (Sujatha et al., 2012). Cotyledon of 15 hybrids and wild type sunflowers showed shoot regeneration about 7 shoots and 6 shoots, respectively (Arda, 2004). The sunflower genotypes have been tested for their ability to produce shoot, root and plantlet

formation. (Bayraktaroglu et al., 2011). Cotyledons of mature seeds are frequent source of explant for organogenic regeneration. The directed organogenesis from cotyledons has been reported in sunflower species (Baker et al., 1999). Cotyledon cultured on MS supplemented with 5.71 μM of IAA, 9.3 μM of Kinetin, and 1.37 μM of glutamine, proximal part of cotyledon gave percentage of regeneration about 94%, while distal cotyledon gave percentage of shoot regeneration about 10% (Vega et al., 2007). Sunflower regeneration from cotyledons cultured on MS supplemented with 1% BAP and 1% NAA gave the highest shoot formation (10%), while hypocotyl explants produced the best callus formation about 100% (Bayraktaroglu et al., 2011).

CHAPTER III

MATERIALS AND METHODS

3.1 *In vitro* propagation of sunflower using cotyledons as explants

3.1.1 Plant material preparation

Sunflower seeds of two varieties: F1 hybrid (Prado Red x Pacific 22) and Prado Red were soaked in DI water and surface sterile with 20% Clorox for 4 h followed by five times rinses in sterile distilled water for 5 min and then washed in 70% (v/v) ethyl alcohol for 1 min. The sterile cotyledons at 0, 1, 7 days of age were cultured on the sterile paper containing in bottle and the materials were kept in tissue culture room under dark condition.

3.1.2 Effect of variety, media type and age of cotyledons on shoot induction

The cotyledons of sterile seeds from 0, 1 and 7 day-old germinating seeds were excised and placed with the adaxial side and cultured on MS medium containing various plant hormones (Table 3.1) and 30 g/l of sucrose. The medium was solidified with 2 g/l of gellan gum (Kelcogel, USA) and the pH was adjusted to 5.7-5.8 before autoclaving at 121 °C for 20 min with 1 N HCl and 1 N NaOH. All cultures were maintained at 25 ± 2 °C under a photoperiod of 16/8 h (day/night) for 21 days and sub-cultured every 3 weeks or until complete plantlets obtained.

Table 3.1 Cotyledon culture on various plant hormones in sunflower.

Media	Basal	Plant hormones					References
		Auxin (mg/l)		Cytokinin (mg/l)			
		IAA	NAA	2-iP	TDZ	BA	
A1	MS	0.5	-	2	0.1	-	Sujatha et al. (2012)
A2	MS	-	0.1	-	-	1	Mosharrat (2015)
A3	MS	-	-	-	-	1	This study
A4	MS	-	-	2	-	0.1	This study

Remark: BA = Benzylaminopurine, NAA = α- Naphthaleneacetic acid, IAA = Indole acetic acid, 2-iP = 6-γ-γ-Dimethylallylamino-purine, TDZ = Thidiazuron.

After 21 days of culture, the following six parameters were determined (shoot > 4 mm was counted) (Azadi et al., 2002).

$$\text{Percentage of response} = \frac{\text{No. of response} \times 100}{\text{Total of explants}}$$

Measurement of cotyledon response was done by observing any changes of cotyledons such as color change from pale white to green or dark green, cotyledon expansion, and regenerated callus or shoot, except explant death.

$$\text{Percentage of callus} = \frac{\text{No. of callus} \times 100}{\text{Total of explants}}$$

$$\text{Percentage of shoot induction} = \frac{\text{No. of shooting} \times 100}{\text{Total of explants}}$$

$$\text{Average number of shoots per explant} = \frac{\text{No. of shooting}}{\text{Explant}}$$

$$\text{Percentage of rooting regeneration} = \frac{\text{No. of rooting} \times 100}{\text{Total of explants}}$$

$$\text{Expansion rate (folds)} = \frac{\text{Width} \times \text{Length (after culture)}}{\text{Width} \times \text{Length (before culture)}}$$

The experimental design was done with factorial completely randomized design. Three factors were tested: four media types (A1, A2, A3 and A4), three ages of cotyledons (0, 1 and 7 days) and two varieties F1 hybrid and Prado Red. Each treatment consists of 10 explants per petri dish.

3.1.3 Effect of plant hormones and additives on shoot multiplication

Fifteen shoots derived from experiment 3.1.2 of two sunflower varieties: F1 hybrid and Prado Red were cut into 0.5-1.0 cm in size and cultured on MS medium containing different of plant hormones and additives (Table 3.2) and 30 g/l of sucrose. The medium was solidified with 2 g/l of gellan gum (Kelcogel, USA) and the pH was adjusted to 5.7-5.8 before autoclaving at 121 °C for 20 min with 1 N HCl and 1 N NaOH. All cultures were maintained at 25±2 °C under a photoperiod of 16/8 h (day/night) for 21 days and sub cultured every 3 weeks.

Table 3.2 MS medium supplemented with plant hormones and additives for shoot multiplication.

Media	Basal	Plant hormones (mg/l)		Additives (mg/l)	
		BA	TDZ	CH	$AgNO_3$
B1	MS	1	-	-	-
B2	MS	2	-	-	-
B3	MS	1	1	-	-
B4	MS	1	-	1	-
B5	MS	1	-	-	1

Remark: BA = Benzylaminopurine, CH = Casein hydrolysate, TDZ = Thidiazuron, $AgNO_3$ = Silver nitrate.

After 21 days of culture, the measurements were based on the formula below (Siwach et al., 2012).

$$\text{Number of shoots per explant} = \frac{\text{No. of shooting}}{\text{Explant}}$$

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

$$\text{Number of leaves per explant} = \frac{\text{No. of leaves}}{\text{Explant}}$$

The experimental data were analyzed by mean value that was taken from three replicates and significant mean differences were determined with Duncan's multiple

range tests (DMRT) at 0.05% level and analysis was done using IBM SPSS 23 software program (IBM SPSS Inc., USA).

3.1.4 Effect of plant hormones and charcoal on root induction

Fifteen shoot explants derived from experiment 3. 1. 3 of sunflower varieties: F1 hybrid and Prado Red were cut in size 1-1.5 cm and cultured on MS medium containing various plant hormones and charcoal (Table 3.3) and 25 g/l of sucrose. The medium was solidified with 2 g/l of gellan gum (Kelcogel, USA) and the pH was adjusted to 5.7-5.8 before autoclaving at 121 °C for 20 min with 1 N HCl and 1 N NaOH. All cultures were maintained at 25±2 °C under a photoperiod of 16/8 h (day/night) for 21 days and sub cultured every 3 weeks.

Table 3. 3 MS medium supplement with plant hormones and charcoal for root induction.

Media	**Basal**	**Plant hormones (mg/l)**			**Additive**
		BA	**NAA**	**IAA**	
C1	MS	-	-	-	
C2	MS	-	1	-	
C3	MS	-	-	1	No charcoal
C4	MS	1	1	-	
C5	MS	1	-	1	

Table 3.3 (Continued)

C6	MS	-	-	-	
C7	MS	-	1	-	
C8	MS	-	-	1	Charcoal
C9	MS	1	1	-	
C10	MS	1	-	1	

Remark: BA = Benzylaminopurine, NAA = α- Naphthaleneacetic acid, IAA = Indole acetic acid.

After 21 days of culture, the measurements of root induction were calculated following there formula (Siwach et al., 2012).

$$\text{Number of roots per explant} = \frac{\text{No. of rooting}}{\text{Explant}}$$

$$\text{Average length of roots} = \frac{\text{Root length}}{\text{Total of explant}}$$

The experimental data were analyzed by mean value that was taken from three replicates and significant mean differences were determined with Duncan's multiple range tests (DMRT) at 0.05% level and analysis was done using IBM SPSS 23 software program (IBM SPSS Inc., USA).

3.1.5 Effect of growing materials on acclimation and growth of sunflower plantlets

The complete plantlets were acclimated at room temperature for 5 days. After that healthy, stronger and full rooting of seedlings were transplanted to peat moss (100%), peat moss mix sand (1:1) and sand (100%) and grown to maturity. The survival rate, shoot height and number of leaves was recorded at 14 days after transplanting.

The experimental data were analyzed by mean value that was taken from three replicates and significant mean differences were determined with Duncan's multiple range tests (DMRT) at 0.05% level and analysis was done using IBM SPSS (v. 23).

3.2 Cytological and chemical changes of sunflower cotyledons in seedlings and *in vitro* culture

3.2.1 Cytological changes of sunflower cotyledons

The cotyledon explants from 0, 1 and 7 day-old germinating seeds were boiled at 80 ºC for 1 h in water bath, while cotyledons cultured on MS medium supplemented with 1 mg/l of BA for 21 days were directly sectioned. The explants were fine sectioned (2 μm in thickness) with a plant microtome (Automatic MT-3, NK System, Japan). The sliced sections were dehydrated by ethanol series (30, 50, 70 and 100%) for 10 min per each. The sections were fixed on microscope slides and stained with 0.05% (v/v) safranin O solution for cell wall indication. The images of sections were observed under an Olympus BX51 microscope (Olympus, Germany) and captured

using microscope digital camera DP73 (Olympus, Germany). The measurements including cell number, cell area and cotyledon thickness were counted under a light microscope.

The experimental data were analyzed by mean values that were taken from three replicates and significant mean differences were determined with Duncan's multiple range tests (DMRT) at 0.05% level and analysis was done using IBM SPSS v.23 software program (IBM SPSS Inc., USA).

3.2.2 Chemical change of sunflower cotyledons

3.2.2.1 Total protein

Total protein of cotyledons from 0, 1 and 7 day-old germinating seeds and cotyledon explants cultured on MS medium supplemented with 1 mg/l of BA for 21 days were measured. Total protein content was performed as demonstrated by Kjedahl method (Helrich, 1990). One g of the powder weight cotyledons was placed in digestion flask. 14 ml of concentrated H_2SO_4 and catalyst 2 tap (3.5g K_2SO_4 and 0.4 g $CuSO_4$) were added in the digestion flask. The samples were digested and placed on heating mantle and heated at 420 for 1 h. The samples were cool at room temperature. 70 ml of DI water were added in a receiving flash containing of mixed indicator (0.1 g of bromocresol green: 0.1 g of methyl red in 100 ml ethanol) and 50 ml of 40% NaOH (40 g NaOH in 100 ml of water). The samples were distilled under Foss/Kieltec 8400. To remove receiving flash wash tip of condenser and were titrated excess 0.1 N HCl until the solution were changed to purple or pink. To prepare a tube containing the above chemical except sample as blank. The calculation of total protein was done following this formula;

$$\text{Kjeldahl nitrogen (\%)} = \frac{(Vs - V_B) \times M \times 14.01}{W \times 10}$$

Crude protein (%) = % Kjeldahl N × F

Where Vs = volume (ml) of standard acid used to titrate a test

V_B = volume (ml) of standard acid used to titrate reagent blank

M = molarity of standard HCl

W = weight (g) of test portion or standard

1.4007 = atomic weight of nitrogen

10 = factor to convert mg/g to percent

F = the protein-nitrogen conversation factor for plant (5.30)

3.2.2.2 Total fat

Total fat of cotyledons from 0, 1 and 7 day-old germinating seeds and cotyledon explants cultured on MS medium supplemented with 1 mg/l of BA for 21 days were measured. Total fat content was done according to soxhlet method as described by Manirakiza et al. (2001). Bottles and lids were placed in the incubator at 105 ºC overnight to ensure that weight of bottle is stable. 0.5 g of the powder cotyledon sample were taken into extraction thimble and transferred into soxhlet (Foss tech 2050, Denmark). 10 ml petroleum ether were filled into the bottle and taken it on the heating mantle. The soxhlet was connected apparatus and turned on the water to cool them and then switch on the heating mantle. The sample was heated about 1 h (heat rate of 20 drop/min) and evaporated the solvent by using the vacuum condenser. The bottle

containing sample was incubated at 105 °C for 30 min and bottle was completely dry. After drying, the bottle was covered with lid to the desiccator for cooling. The bottle and dried content was reweighed. The calculation of total fat was done following this formula.

$$\text{Total fat (\%)} = \frac{(W_2 - W_1) \times 100}{S}$$

Where W1 = weight of empty flask (g)

W2 = weight of flask and extracted fat (g)

S = weight of sample (g)

3.2.2.3 Total sugar

Total sugar of cotyledons from 0, 1 and 7 day-old germinating seeds and cotyledon explants of cultured on MS medium supplemented with 1 mg/l of BA for 21 days were measured according to the phenol-sulfuric method described by Dubois et al. (1956). The cotyledon 100 mg of fresh weight was placed into a test tube and added 6 ml of 80% ethanol. A tube was taken into boiling water for 2 min and then was soaked in water bath for 2 h at 65 °C for sugar extraction.

100 µl plant- extracted solution was taken into a test tube and filled 500 µl of 5% phenol and 1 ml of sulfuric acid. The sample was mixed gently and set for 30 min. Spectrophotometric analysis (T80+UV/VIS spectrophotometer, USA) was carried out for total sugar at 490 nm. Total sugar content was calculated using glucose as the standard curve.

3.2.2.4 Sucrose content

Sucrose content of cotyledons s from 0, 1 and 7 day-old germinating seeds and cotyledon explant cultured on MS medium supplemented with 1 mg/l of BA for 21 days were measured according to the resorsinol-HCl method described by Robbins and Pharr (1987). 100 mg of fresh weigh cotyledon was placed into test tube, added 6 ml of 80% ethanol and taken a tube into boiling water for 2 min and then incubated in water bath for 2 h at 65 °C for sugar extraction.

500 µl plant-extracted solution were filled into test tube, added 100 µl of 0.2 N NaOH and taken a tube into water bath at 100 °C for 10 min to make decay fructose. 250 µl of 1% resorcinol and 750 µl of 30% HCl were added into the tube, and then were incubated at 80 °C for 10 min. The solution sample was analyzed in a UV spectrophotometer (T80+ UV/VIS spectrophotometer, USA) at 520 nm. Sucrose concentration was calculated using sucrose as the standard curve.

The experimental data were analyzed by mean values that were taken from three replicates and significant mean differences were determined with Duncan's multiple range tests (DMRT) at 0.05% level and analysis was done using IBM SPSS v. 23 software program (IBM SPSS Inc., USA).

CHAPTER IV

RESULTS AND DISCUSSION

The aims of this research were to determine the effect of variety, media type and age of cotyledons on plant regeneration of sunflower, to evaluate acclimation and growth of regenerated plantlets, and to investigate cytological and chemical changes in cotyledons of germinating seeds and *in vitro* culture.

4.1 *In vitro* propagation of sunflower using cotyledons as explants

Two sunflower varieties including F1 hybrid and Prado Red were used to examine the effects of variety, media type and age of cotyledons on regenerated plantlets through organogenesis.

4.1.1 Effect of variety, media type and age of cotyledons on shoot induction

The result of analysis of variances showed that three sources of variance (variety, age and media type) had significant effect on four parameters associated with regeneration. The percentage of callus induction, the percentage of shoot induction, number of shoot per explant, and the percentage of root induction. Their interaction was significant in some parameters the percentage of callus induction and the percentage of root induction. The percentage of response was not significant in all sources as presented in Table 4.1.

Table 4.1 Analysis of variance for variety, media type and age of cotyledons on shoot induction parameters in sunflower.

S.O.V	d.f.	Response (%)	Callus induction (%)	Shoot induction (%)	Number of shoot per explant	Root induction (%)
Variety (V)	1	7.57	1112.42*	845.01*	1.41*	312.54*
Age (A)	2	32.54	437.35*	2004.10*	2.30*	709.84*
Media type (M)	3	33.49	1692.58*	1899.69*	2.10*	259.83*
V * A	2	27.02	487.37*	75.13	0.36	450.51*
V * M	3	30.63	708.71*	9.26	0.38	173.41*
A * M	6	11.58	374.92*	337.87*	0.31	94.07*
V * A * M	6	15.48	251.66*	51.69	0.16	244.71*
Error	48	22.38	123.42	129.93	0.26	31.93*
C.V (%)		5.13	5.51	28.73	16.11	26.81

* = Significant at 0.05 probability level.

F1 hybrid variety at 1 day-old germinating seeds gave the highest response (99.72%), percentage of callus induction (51.11%) and percentage of root induction (17.78%), while Prado Red variety at 1 day-old germinating seeds had the highest percentage of directed shoot induction (26.67%) and number of shoot per explant (1.10 shoot per explant) as shown in Table 4.2. Regardless of variety, cotyledons from 1 day-old germinating seeds had the highest response callus induction, percentage of shoot induction, number shoot per explant and percentage root induction while cotyledons

from 7days- old germinating seeds showed the lowest values of all parameters as presented in Table 4.2.

Table 4.2 The mean values (±SD) of response, callus induction, shoot induction, number of shoots and root induction for *in vitro* cotyledon explants of two sunflower varieties.

Factors	Response (%)	
	F1 hybrid	Prado Red
0 day	95.56±2.43	98.06±0.64
1 day	99.72±0.27	98.06±0.96
7 days	96.39±1.76	97.5±0.60
F-test	ns	ns
Factors	Callus induction (%)	
	F1 hybrid	Prado Red
0 day	34.72±4.95b	37.25±3.54
1 day	51.11±6.39a	37.50±4.38
7 days	48.05±3.02ab	35.56±4.66
F-test	**	ns
Factors	Shoot induction (%)	
	F1 hybrid	Prado Red
0 day	3.89±2.49b	6.67±2.84b
1 day	18.06±5.32a	26.67±6.65a
7 days	3.61±2.82b	12.78±3.66b
F-test	**	**

Table 4.2 (Continued).

Factors	Number of shoots per explant	
	F1 hybrid	Prado Red
0 day	0.46±0.21b	0.58±0.21
1 day	0.94±0.13a	1.10±0.17
7 days	0.17±0.11b	0.73±0.16
F-test	**	ns
Factors	Root induction (%)	
	F1 hybrid	Prado Red
0 day	2.22±1.71b	3.33±2.78
1 day	17.78±4.81a	3.61±1.66
7 days	0b	0.56±0.56
F-test	**	ns

Mean followed by different letter significant at 0.05 probability level.

** = Significant at 0.05 probability level. ns = not significant.

The composition of media is one of the factors affecting the success of sunflower tissue culture. The A3 medium supplemented with 1 mg/l of BA showed the highest response about 99.26%, percentage of shoot induction about 30% and number shoot per explant (1.15 shoots per explant). In addition, the A1 medium supplemented with 2 mg/l of 2iP, 0.5 mg/l of IAA and 0.1 mg/l of TDZ had the highest percentage of callus induction about 65.93% and percentage of root induction about 13.70%. The A4 medium supplemented with 2 mg/l of 2iP and 0.1 mg/l of BA had the lowest percentage of shoot induction about 0.74% in F1 hybrid variety and 8.14% in Prado Red variety as shown in Table 4.3.

Table 4.3 The mean values (±SD) for response, callus induction, shoot induction, number of shoots and root induction of cotyledon sunflower exposed to various media types.

Factors	Response (%)	
	F1 hybrid	Prado Red
Medium		
A1	93.70±3.70	98.15±0.58ab
A2	98.15±1.12	96.67±0.96b
A3	99.26±0.48	99.26±0.48a
A4	97.78±0.96	97.41±1.07ab
F-test	ns	**
Factors	Callus induction (%)	
	F1 hybrid	Prado Red
Medium		
A1	65.93±5.46a	44.44±4.08a
A2	34.45±4.37b	39.70±4.95ab
A3	42.59±4.67b	27.41±4.15b
A4	35.56±3.23b	35.52±4.51ab
F-test	**	**
Factors	Shoot induction (%)	
	F1 hybrid	Prado Red
Medium		
A1	6.29±3.35b	14.82±4.01ab
A2	3.33±1.36b	8.51±4.27b
A3	23.70±6.83a	30.00±8.22a
A4	0.74±0.74b	8.14±3.81b
F-test	**	**

Table 4.3 (Continued).

Factors	Number of shoots per explants	
	F1 hybrid	Prado Red
Medium		
A1	0.39±0.20b	0.94±0.21ab
A2	0.44±0.18b	0.48±0.19b
A3	1.13±0.18a	1.15±0.16a
A4	0.11±0.11b	0.63±0.25ab
F-test	**	**
Factors	Root induction (%)	
	F1 hybrid	Prado Red
Medium		
A1	13.70±5.22a	1.85±1.25
A2	2.22±1.11ab	0
A3	10.74±5.84ab	5.18±3.64
A4	0b	2.96±2.04
F-test	**	ns

Mean followed by different letter significant at 0.05 probability level.

** = Significant at 0.05 probability level. ns = not significant.

Cotyledon explants turned green after 3-4 days of culture in both varieties. After 1 week in shoot induction medium, cotyledons started increasing their size for 1-3 folds (Figure 4.1) and the first sign of callus was observed approximately 7-21 days after culture. Almost of tested medium responded well and produced calli and regenerated shoots through organogenesis.

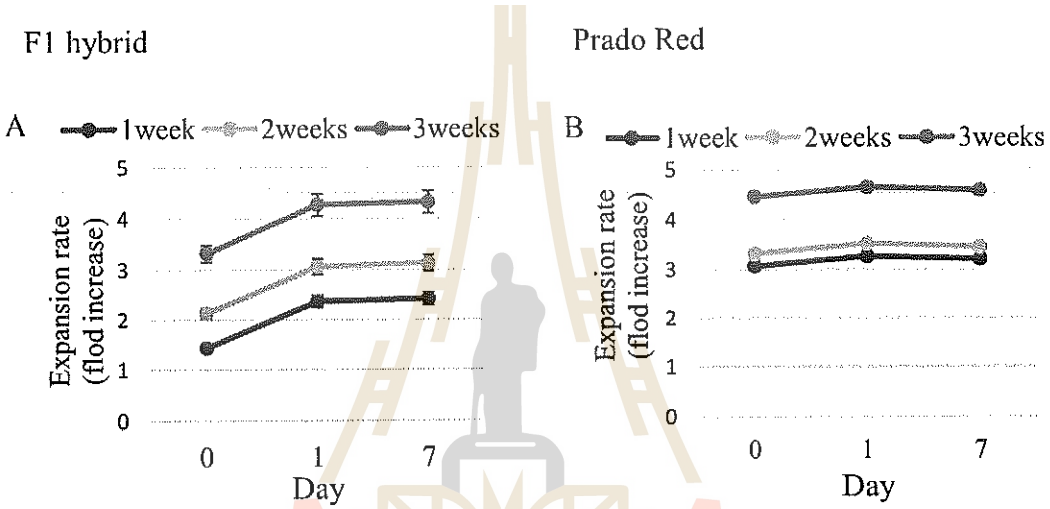

Figure 4.1 Cotyledon expansion rate of two sunflower varieties. A) F1 hybrid variety, B) Prado Red variety.

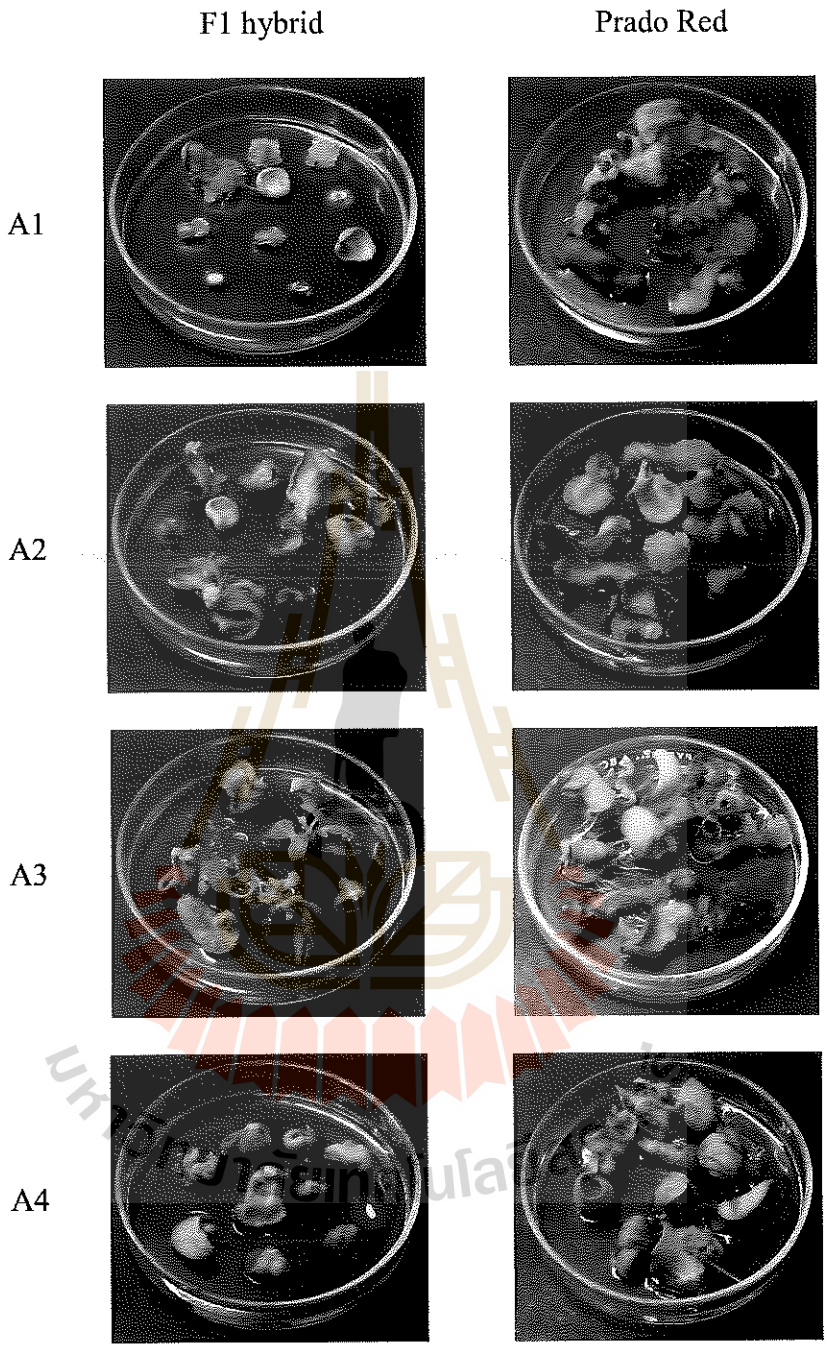

Figure 4.2 Cotyledon explants at 0 day-old seedlings of F1 hybrid and Prado Red with adaxial side on various shoot induction media (A1-A4).

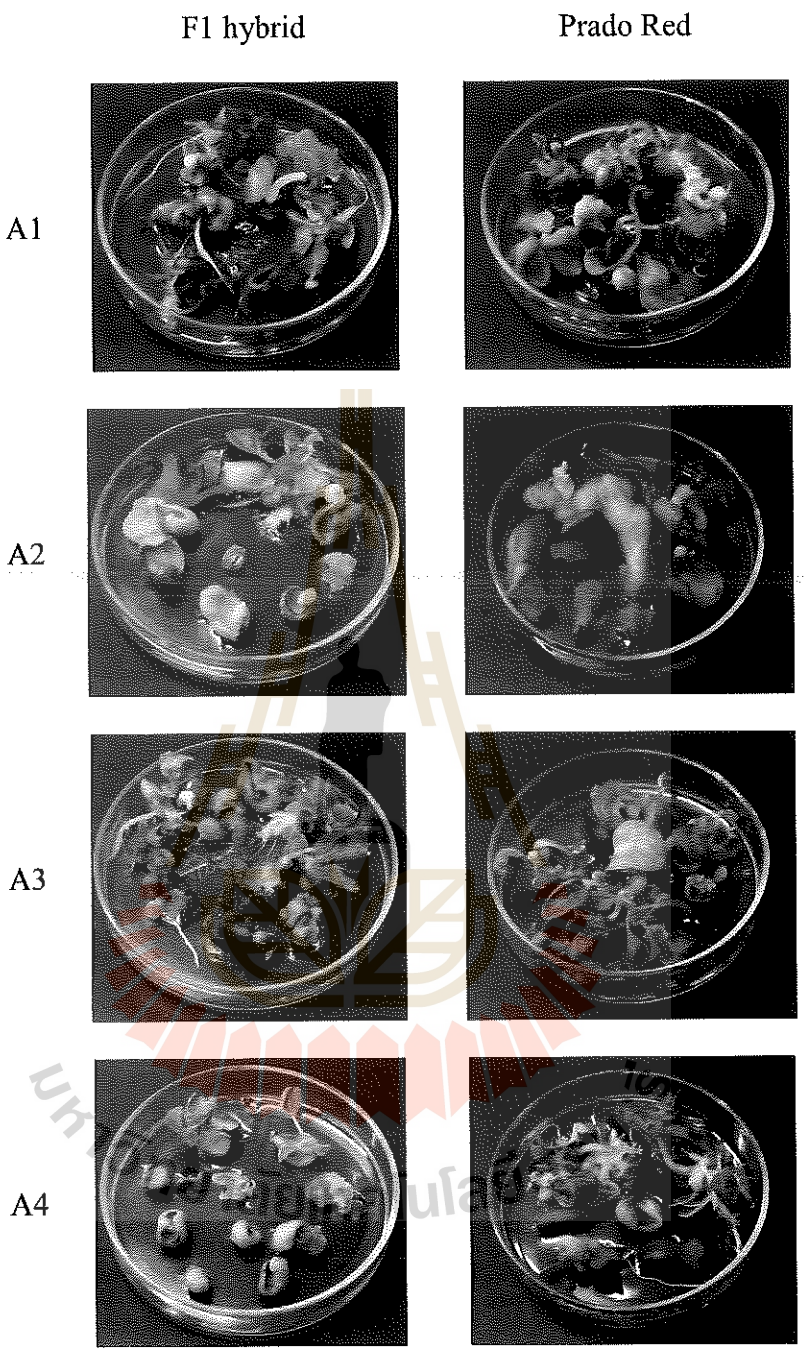

Figure 4.3 Cotyledon explants at 1 day-old seedlings of F1 hybrid and Prado Red with adaxial side on various shoot induction media (A1-A4).

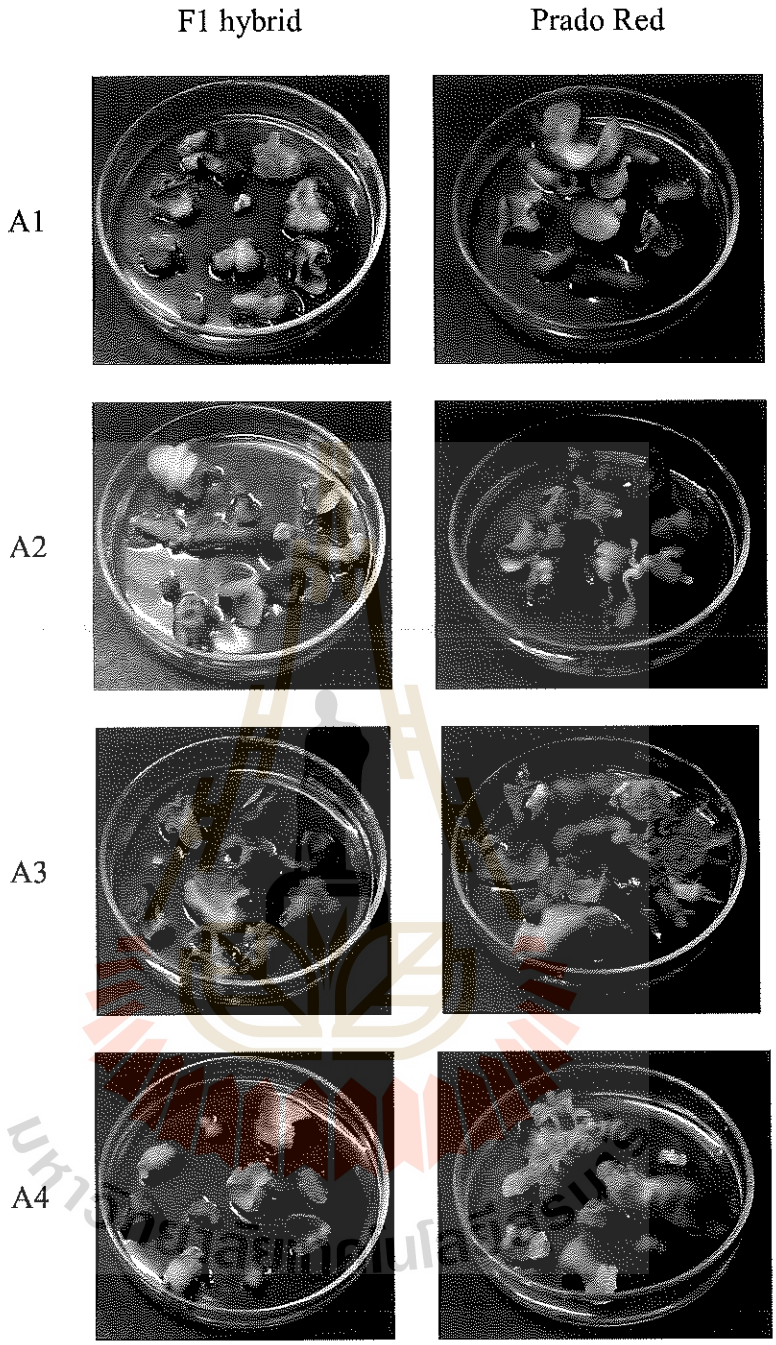

Figure 4.4 Cotyledon explants at 7 day-old seedlings of F1 hybrid and Prado Red with adaxial side on various shoot induction media (A1-A4).

The results of this thesis study were in agreement with most of the reports of Ozyigit et al. (2007) who reported that calli obtained from cotyledon explants of five different sunflower genotypes had the highest callus induction about 90% and gave shoot primordia about 18%. The variation in shoot induction percentages might be affected by different variety. Aurori et al. (2011) mentioned that the increase of age of cotyledon explants highly affected the capacity and developmental stage in sunflower organogenesis. Knittel et al. (1991) reported that the best regeneration was obtained from 4 to 10 day-old seedlings on different combinations of BA and NAA. They found that using 4 days-old cotyledons gave the best result. Chen and Chang (2002) reported that the adaxial-side-up orientation of *Oncidium* significantly promoted embryogenesis when compared with abaxial-side-up orientation. In addition, Knittel et al. (1991) reported the best shoot induction about 70% on adaxial side of sunflower cotyledons. Cytological studies have shown that the adaxial side has palisade density and the number of cells larger than that of the abaxial side, resulting in better shoot induction and response than the abaxial side. Ozyigit et al. (2002) reported that MS medium supplemented with various combinations of auxins and cytokinins affected the regenerated plantlets from cotyledon *in vitro* culture sunflower. Pugliesi et al. (1991) cultured cotyledons of 1 and 2 day-old seedlings on medium with 18.6 μM of Kinetin and 5.71 μM of IAA and obtained adventitious shoots originated with 4 – 10 buds per explant. Khalil et al. (2015) reported that 0.5 mg/l of NAA and 1.0 mg/l of BA induced the maximum shoot induction. However, my preliminary studies using cotyledon explants in *in vitro* culture, MS with 1 mg/l BA hormone had the best shoot induction so it was used in this study.

The percentage of shoot induction was positively correlated with number of shoot per explant (r = 0.681**) and percentage of root induction (r = 0.402**). The percentage of root induction was positively correlated with the percentage of callus induction (r = 0.436**) and number of shoot per explant (r = 0.344**) as presented in Table 4.4. The results of the relationship analysis, indicated that when the percentage of shoots increased, the number of shoots increased as well as the percentage of roots.

Table 4.4 Correlation analysis of shoot induction in two sunflower varieties.

Variations	Response (%)	Callus induction (%)	Shoot induction (%)	Number of shoot per explant
Callus induction (%)	-0.076			
Shoot induction (%)	0.166	0.020		
Number of shoot per explant	0.193	0.087	0.681**	
Root induction (%)	0.000	0.436**	0.402**	0.344**

4.1.2 Effect of plant hormones and additives on shoot multiplication

In vitro plantlets about 0.5-1.0 cm in height from experiment 4.1.1 were transferred into various shoot multiplication media. Analysis of variance for number of shoot per explant, length of shoot and number of leaves per explant parameters were significant in both varieties and media type, while their interaction between variety and media type was not significant ($p<0.05$) as shown at Table 4.5.

Table 4.5 Analysis of variances for shoot multiplication parameters of sunflower.

S.O.V	d.f.	Number of shoot per explant	Length of shoot (cm)	Number of leaves per explant
Variety (V)	1	0.006*	1.462*	52.806*
Media type (M)	4	12.556*	0.638*	82.790*
V*M	4	0.156	0.148	1.356
Error	140	1.137	0.252	2.206
%C.V		63.98	35.62	53.47

* = Significant at 0.05 probability level.

Plant hormones and additives promoted multiple shoots in regenerated media. Shoots derived from cotyledons of two varieties were induced into multiple shoots as presented in Table 4.6. Among various media types, the B2 medium containing 2 mg/l of BA had the best response for number of shoots per explant in F1 hybrid (3 shoots per explant) while Prado Red showed the maximum value about 2.8 shoots per explant.

MS medium supplemented with 1 mg/l of BA and 1 mg/l of $AgNO_3$ (B5) gave multiple shoot per explant about 2.06 shoots while MS medium supplemented with 1 mg/l of BA (B1) had 1.67 shoots per explant. B3 medium supplemented with 1 mg/l of BA and 1 mg/l of TDZ had shoot per explant about 1.33 shoots per explant and B4 medium supplemented with 1 mg/l of BA and 1 mg/l of CH had 1.27 shoots per explant The highest of shoot length was observed in F1 hybrid about 1.71 cm on B5 medium while Prado Red showed 1.6 cm on B2 medium. The maximum number of leaves was shown in F1 hybrid about 5 leaves/explant and Prado Red about 4 leaves/explant as presented in Table 4.6 and Figure 4.7.

Table 4.6 Mean (±SD) performance for organogenesis parameters in sunflower.

Factors	**Number of shoot per explant**	**Length of shoot (cm)**	**Number of leaves per explant**
F1 hybrid			
Media type			
B1	1.67±0.21b	1.53±0.11ab	5.40±0.25ab
B2	3.00±0.58a	1.66±0.07a	5.90±0.37a
B3	1.33±0.16b	1.62±0.12a	4.73±0.22b
B4	1.27±0.15b	1.28±0.05b	1.53±0.41c
B5	2.06±0.23b	1.71±0.16a	5.60±0.38ab
F-test	**	**	**

Table 4.6 (Continued).

Prado Red			
Media type			
B1	1.80±0.22b	1.35±0.15	3.80±0.47ab
B2	2.80±0.28a	1.60±0.08	4.90±0.36a
B3	1.33±0.25b	1.21±0.18	0.93±0.46c
B4	1.40±0.19b	1.24±0.14	3.13±0.31b
B5	1.93±0.22b	1.41±0.16	4.46±0.47a
F-test	**	ns	**

Mean followed by different letter significant at 0.05 probability level.

** = Significant at 0.05 probability level. ns = not significant.

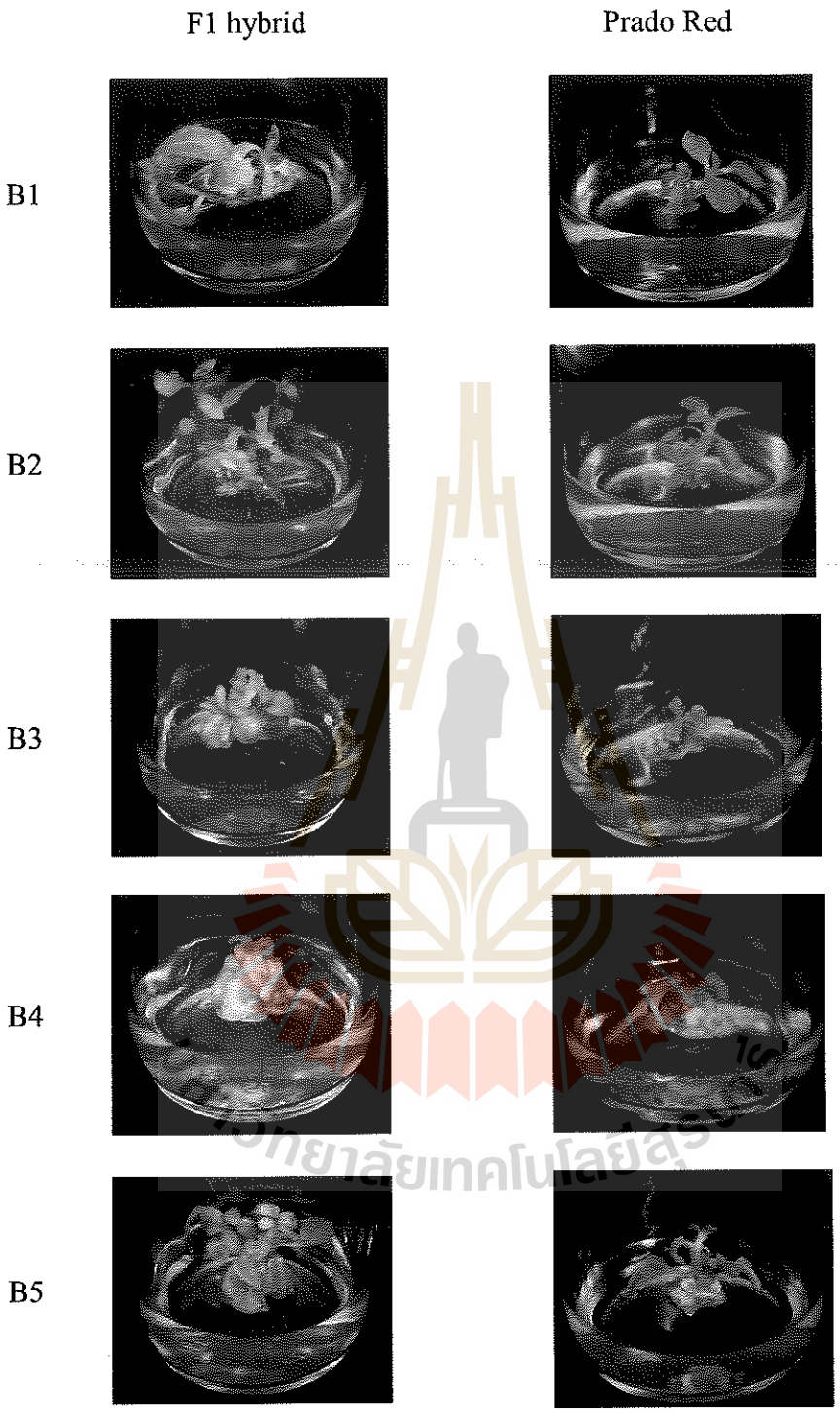

Figure 4.5 Shoot multiplication of F1 hybrid and Prado Red varieties on various shoot multiplication media (B1-B5) at 3 weeks of culture.

4.1.3 Effect of plant hormones and charcoal on root induction

Regenerated shoots about 1- 1. 5 cm in height were selected and transferred into rooting media. After 3 weeks of culture, number of root and length of root were recorded. Variety and media type showed significant effect on rooting, while their interaction between variety × media type × additive was not significant at $p \leq 0.05$ level (Table 4.7).

The highest number of root was produced on MS medium supplemented with 1 mg/ l of BA, 1 mg/l of IAA and charcoal about 7.26 roots per explant in Prado Red variety and 6. 2 root per explant in F1 hybrid variety. Rooting formation ranging from 1.13-7.26 roots per explant was generated within 7-12 days after culture. The developed roots were white pale and thin (Figure 4.6 and 4.7). The same combination of hormones without charcoal gave root ranging 0.8-4.06 roots per explant and development of roots occurred after 10 days of culture in the modified rooting medium. The maximum value of shoot length was about 4. 8 cm. in Prado Red and 3. 6 cm. in F1 hybrid as shown in Table 4.8. This result was similar to the report of Azadi et al. (2002) who reported that charcoal additive promoted root formation in cotyledon sunflower culture. In addition, Baker et al. (1999) used rooting medium containing 1. 0 g/l activated charcoal for improved rooting. The results showed that the mixture of charcoal has a beneficial effect on root growth. Because charcoal powder provides darkness which might help root development and also reduces phenolic compounds that may be toxic to plants.

Table 4.7 Analysis of variance for root induction parameters of sunflower.

S.O.V	d.f.	Number of root per explant	Length of root (cm)
Variety (V)	1	30.083*	28.607*
Medium (M)	4	225.986*	134.784*
Additive (A)	1	66.270*	5.216
V * M	4	7.966	2.235
V * A	1	1.203	5.344
M* A	4	26.270*	5.734
V * M * A	4	6.503	3.917
Error	280	4.074	3.681
%C.V		12.430	27.920

* = Significant at 0.05 probability level.

Table 4.8 The mean values (± SD) of root induction parameter of sunflower exposed to various medium.

Factors	Varieties	Medium	Number of root per explant	Length of root (cm)
No charcoal	F1 hybrid	C1	0c	0c
		C2	0.80±0.28bc	1.39±0.47bc
		C3	1.46±0.31b	2.64±0.42ab
		C4	2.13±0.32ab	3.21±0.43a
		C5	2.86±0.91a	3.26±0.81a
	Prado Red	C1	0c	0c
		C2	1.06±0.32c	2.05±2.34b
		C3	3.13±0.49ab	3.79±3.26a
		C4	2.80±0.51b	4.21±2.57a
		C5	4.06±0.53a	4.80±2.20a
F-test			**	**
Charcoal	F1 hybrid	C6	0d	0c
		C7	1.13±0.34d	2.16±0.64b
		C8	2.07±0.46bc	1.88±0.36b
		C9	3.20±0.63b	2.86±0.53ab
		C10	6.20±0.76a	3.60±0.48a
	Prado Red	C6	0d	0c
		C7	1.26±0.15cd	2.52±0.30b
		C8	4.40±0.60b	3.17±0.52ab
		C9	2.20±0.38c	2.74±0.44ab
		C10	7.26±1.26a	3.83±0.46a
F-test			**	**

Mean followed by different letter significant at 0.05 probability level.

** = Significant at 0.01 probability level.

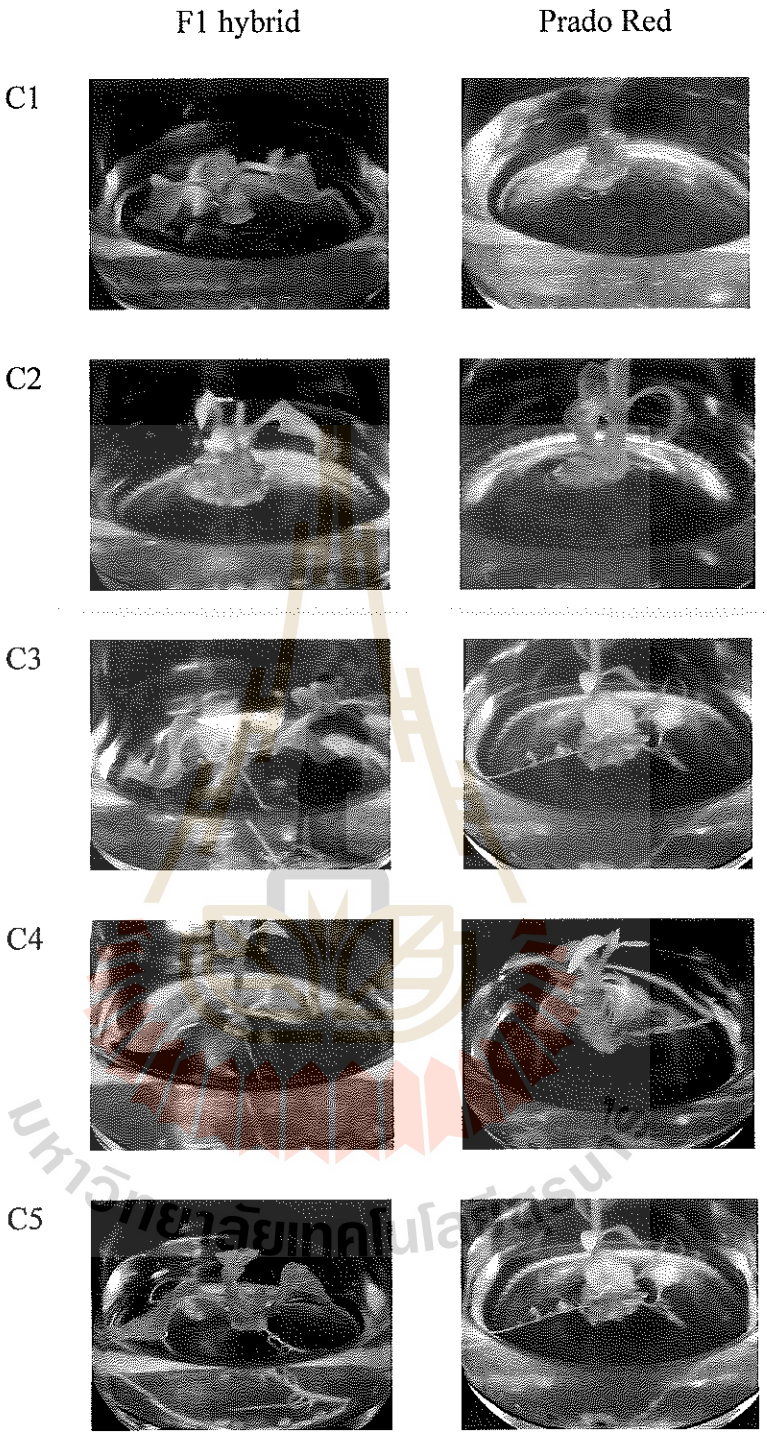

Figure 4.6 Root induction of F1 hybrid and Prado Red varieties on rooting media (C1-C5) without charcoal at 3 weeks of culture.

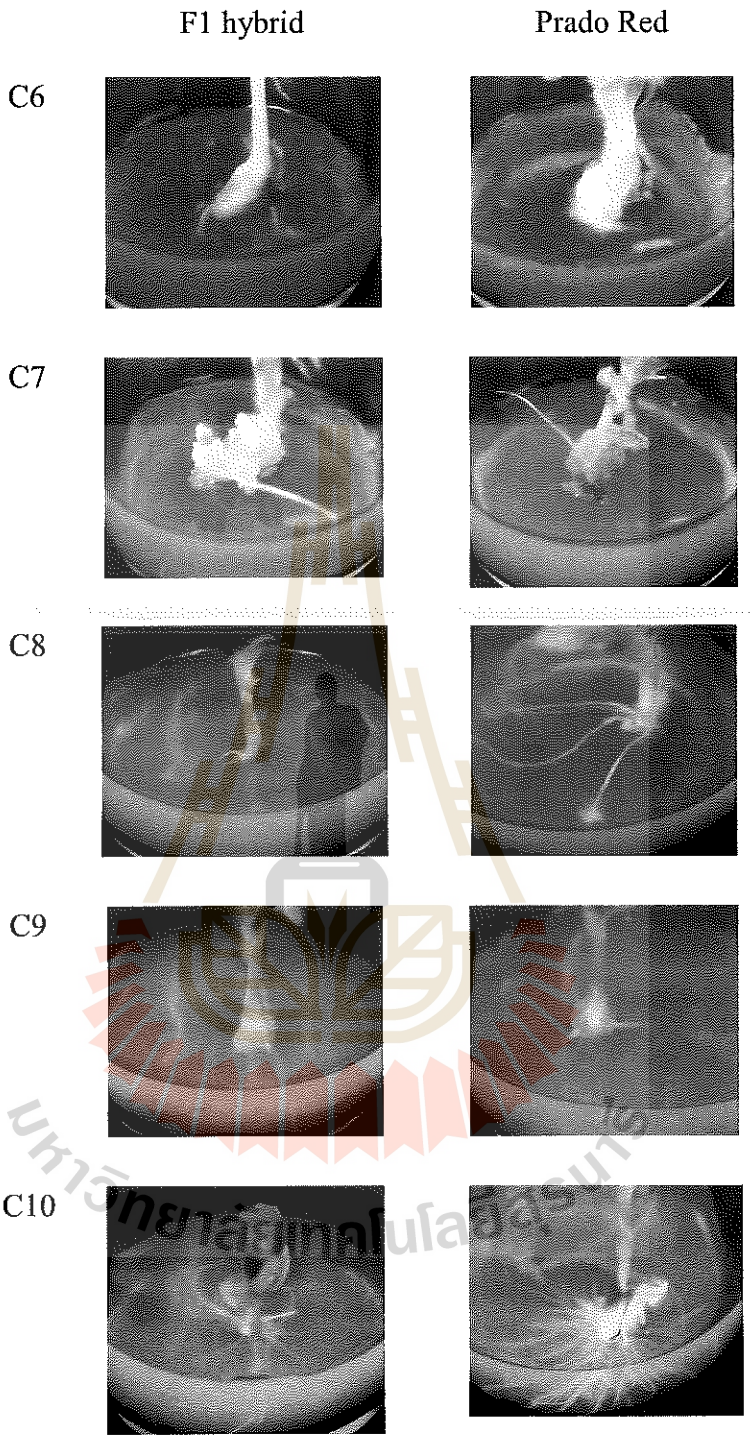

Figure 4.7 Root induction of F1 hybrid and Prado Red varieties on rooting media (C6-C10) supplemented with 0.5 mg/l of charcoal at 3 weeks of culture.

4.1.4 Effect of growing materials on acclimation and growth of sunflower plantlets

Complete plantlets with well- developed shoots and roots were acclimated for 5 days in room temperature. Complete sunflower plantlets were individually removed from the culture bottles into glass bottles containing autoclaved peat moss and sand compost mixture (ratio 1:1), peat moss (100%), and sand (100%) as shown in Figure 4.8. After 2 weeks, sunflower plantlets had 60% survival rate grown in peat moss with shoot length about 8.4 cm. in Prado Red variety and number of leaves about 6 leaves per explant in F1 hybrid variety while there were the lowest survival rate of 20% with 3.4 cm. of shoot length in Prado Red and number of leaves about 2.8 leaves per explant in F1 hybrid variety when were grown in sand as presented in Table 4.9.

In this experiment, peat moss was used instead of soil, because the peat moss has high airspace, keeps the moisture, and helps to retain nutrient Many factors affects acclimation and transplanting of sunflower. Chandra et al. (2010) reported that the survival rate of *in vitro* plantlets depended on many factors including light intensity, humidity, and carbohydrate concentration.

Table 4. 9 Effect of growing materials on acclimation and growth of sunflower plantlets.

Varieties	Growing materials	Survival rate (%)	Height (cm)	Number of leaves per explant
F1 hybrid	Peat moss	60a	7.20±1.03a	6.00±0.00a
	Sand	20c	3.60±1.29b	2.80±0.83c
	Peat moss mix sand (1:1)	40b	4.10±1.08b	4.00±1.22b
F-test		**	**	**
Prado Red	Peat moss	60a	8.40±1.19a	4.20±1.09
	Sand	20b	3.40±0.89b	3.20±0.83
	Peat moss mix sand (1:1)	20b	4.60±1.14b	4.40±0.54
F-test		**	**	ns

Mean followed by different letter significant at 0.05 probability level.

** = Significant at 0.05 probability level, ns = not significant. (n = 5 explants).

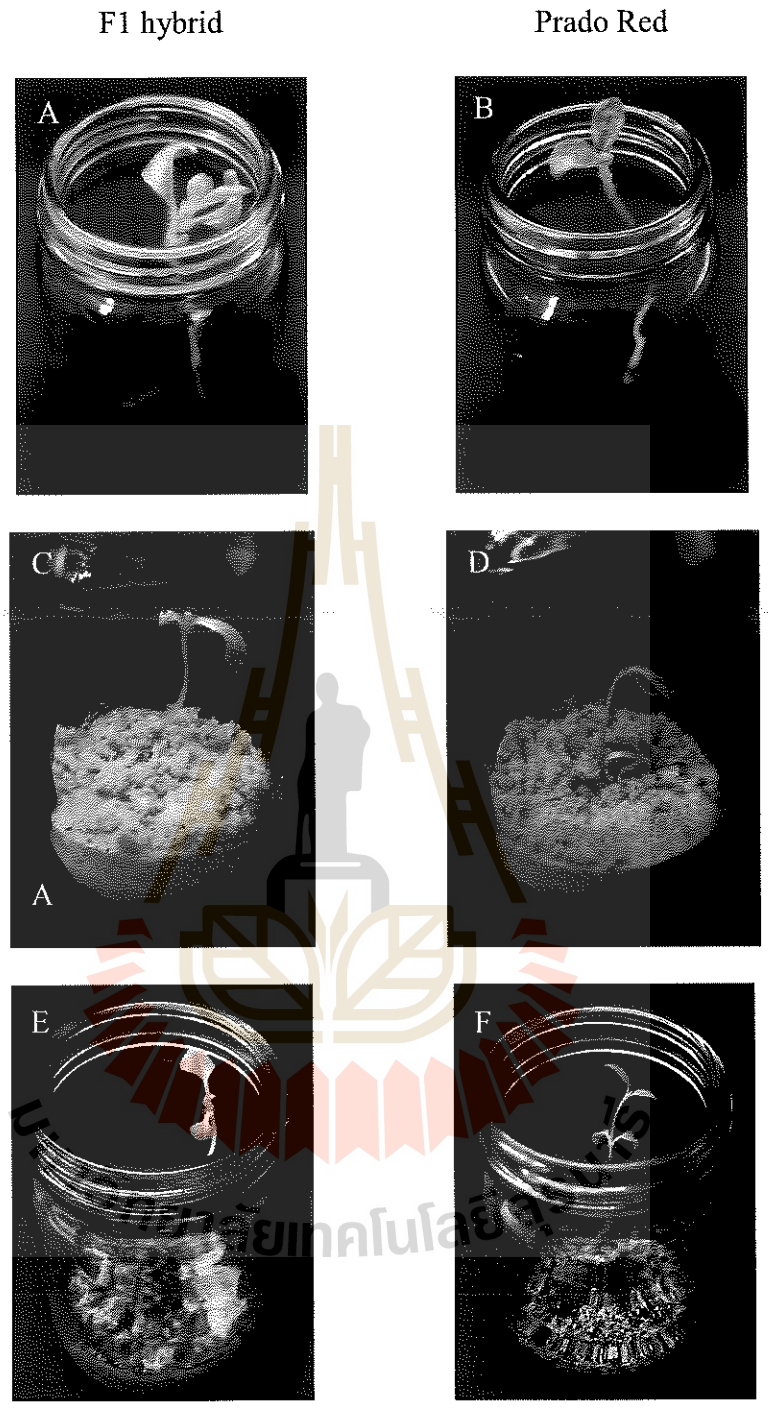

Figure 4.8 Sunflower plantlets of F1 hybrid and Prado Red varieties in peat moss (A and B), sand (C and D) and peat moss mix sand (1:1) (E and F) at 2 weeks after transplanting.

4.2 Cytological and chemical changes of sunflower cotyledons in germinating seeds and in *in vitro* culture

4.2.1 Cytological change of sunflower cotyledons

Sunflower seed is cylindrical or drop in shape and contains two cotyledons, endosperm, seed coat and embryonic axis. Cotyledon is a reserved material part for embryo of new plant. The result from cytological study showed that the cotyledons of sunflower mature seed comprised of several distinct tissue regions; food reserve parenchyma, procambium and epidermis. Food reserve parenchyma in cotyledon is important for monitoring of reserved storage change during the regeneration of new plantlets in medium. The storage parenchyma includes two types: adaxial parenchyma (rectangular shape) and abaxial parenchyma (spherical shape). The small vascular tissues of the cotyledon are scattered in the center of cotyledon. The outermost layer is covered by a layer of epidermal cells (Figure 4.9). During seed imbibition, physiological and morphological characters may be changed. Within 3-4 days after germination, a seedling appeared radicle, plumule and expanded cotyledons. At 7 days, adaxial parenchyma was differentiated into palisade parenchyma and abaxial parenchyma was differentiated into the spongy parenchyma. In addition, cell number, cell area and cotyledon thickness between the abaxial and adaxial regions were different in visualization.

For morphological characters, cotyledons of 0, 1 and 7 day-old germinating seeds enlarged 1-3 times and turned green within 2-3 days after culture. Calli and embryogenic calli were observed in both sunflower varieties within 21 days of culture. Cotyledons cultured on medium had the expansion rate about 4-5 folds.

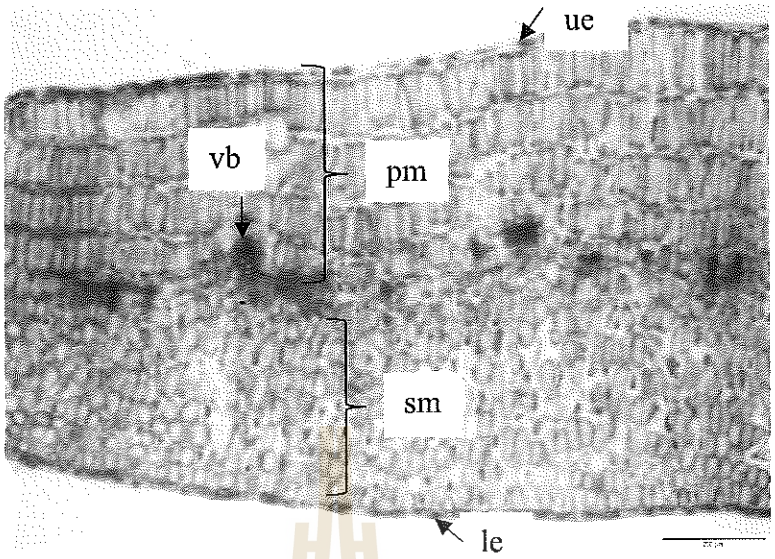

Figure 4.9 The anatomical of 7 days-old cotyledons from Prado Red variety. Le = lower epidermis, pm = palisade mesophyll, sm = spongy mesophyll, ue = upper epidermis, vb = vascular bundle (Bar = 50 μm).

Analysis of variance for cell number was significantly different in the condition, age, and the interaction between conditions × age. Cell area was significant in all parameters while the interaction between variety × age was not significant. Cotyledon thickness parameter was significant in condition and age of cotyledon as presented in Table 4.10.

In conclusion, the difference in condition and age of cotyledon factors influenced the change of cell number, while the difference in variety, the age of cotyledon and condition factors affected cell area change.

Table 4.10 Analysis of variance for cell number, cell area and cotyledon thickness of cytological changes.

S.O.V	d.f.	Cell number (cell/μm²)	Cell area (μm²)	Cotyledons thickness (mm)
Condition (C)	1	13417.361*	131017058.758*	0.065*
Variety (V)	1	17.361	1124501.181*	0.007
Age (A)	2	1694.694*	5455338.713*	0.180*
C * V	1	103.361	472122.442*	0.000
C * A	2	417.527*	688865.834*	0.001
V * A	2	89.194	25256.175	0.003
C * V * A	4	94.694	23096.105*	0.001
Error	24	29.306	25242.868	0.001
C.V (%)		27.55	23.85	11.76

* Means followed by the same letter are significant at 0.05 probability level.

In germinating seeds and *in vitro* culture conditions, the cell number per area decreased as age of cotyledons increased, ranging from 38.67 to 81.67 cells/μm² in Prado Red variety and 55.67 to 77.67 cells/μm² in F1 hybrid variety. Differences exist among varieties. Prado Red variety at 0 day-old germinating seeds had both the maximum cell number of 81.67 cell/μm² and the lowest in cell number about 38.67 cells/μm² in 7 days-old germinating seeds as shown in Table 4.11.

The cell area varied among germinating seeds (Figure 4.1A-C). In germinating seeds, the large cell area was observed in F1 hybrid with average cell area about 1,610 – 2,422 μm², while the cell area of the Prado Red were about 1,430 – 2,303 μm². In

addition, cell area of cotyledons cultured *in vitro* was larger than germinating seed condition (Figure 4.2D-F). The cell area of in *vitro* cotyledons from 7 day-old germinating seed F1 hybrid about 6,894.25 μm^2, while Prado Red gave the cell area about 6,509.91 μm^2 as shown in Table 4.11.

In vitro cotyledons had thickness larger than in germinating seed cotyledons. Similarly, thickness of 7 days-old cotyledons was higher than 0 day-old cotyledon. In germinating seeds, the cotyledon thickness in F1 hybrid variety was about 0.89 to 1.08 mm and Prado Red was about 0.94 to 1.17 mm. *In vitro* culture, F1 hybrid variety was about 0.87 to 1.66 mm and Prado Red was about 0.96 to 1.22 mm as shown in Table 4.11.

Fambrini (2006) reported that cotyledon anatomy of angiosperm was composed of adaxial and abaxial side, which are relatively leaf-like of seedling with distinct palisade and spongy parenchyma, epidermis layers and vascular cambium of each. The result of this study was similar to report of Lindstrom et al. (2005) who reported sunflower cotyledon composing of palisade and spongy parenchyma layers, storage reserves for seedling. Fedeli et al. (1971) reported that palisade and spongy parenchyma functionally accumulated lipid body. Lindstrom et al. (2005) found that cotyledon cell number of seeds in the middle, central and peripheral position of inflorescence showed no difference in cotyledon cell number in sunflower.

Prado Red variety has small seed when compared with F1 hybrid variety, which may relate with cell number, cell area and cotyledon thickness. Cotyledon organogenesis through *in vitro* culture was affected by several factors including gene hierarchies, gene expression, and hormone function (Chandler, 2008). The application

of cytokinin in this study caused great expansion of cotyledon, which was similar to the report of Munshi et al. (2007) and Sinha (2004) who reported that *in vitro* cotyledons had more cell expansion than the seedling condition which may be caused by cytokinin hormone.

Table 4.11 The mean values (±SD) for cell number, cell area and cotyledon thickness of sunflower cotyledons at different ages.

Condition	Variety	Age (day)	Cytological data		
			Cell number (cell/µm²)	Cell area (µm²)	Cotyledon thickness (mm)
In germinating seed	F1 hybrid	0	77.67±3.17a	1,610.43±40.35c	0.89±0.03b
		1	52.67±3.28b	1,792.49±13.09b	0.93±0.03b
		7	55.67±1.45b	2,422.96±74.69a	1.08±0.01a
	Prado Red	0	81.67±7.45a	1,430.87±39.62c	0.94±0.02c
		1	51.33±2.16b	1,718.02±99.19b	1.04±0.03b
		7	38.67±4.05b	2,303.68±64.50a	1.17±0.03a
F-test			**	**	**
In vitro	F1 hybrid	0	27.33±2.40a	5,259.16±40.35c	0.87±0.02c
		1	19.33±2.40b	5,805.86±112.80b	0.95±0.02b
		7	13.33±1.20b	6,894.25±137.89a	1.16±0.04a
	Prado Red	0	27.67±0.88a	4,578.44±150.85c	0.96±0.02c
		1	23.33±1.20b	5,123.38±73.27b	1.07±0.03b
		7	15.00±1.53c	6,509.91±128.58a	1.22±0.01a
F-test			**	**	**

Mean followed by the same letter are significant at 0.05 probability level.

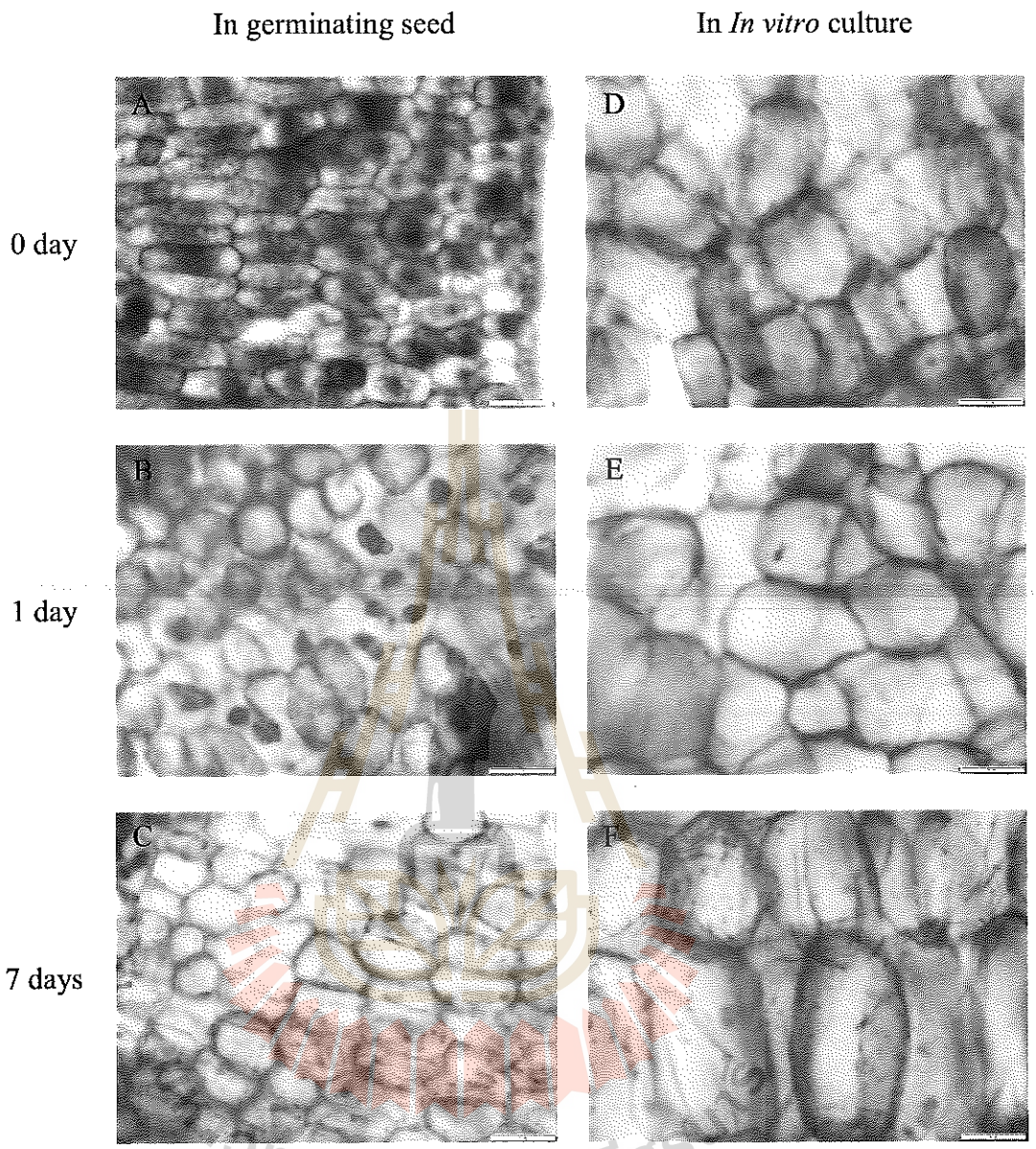

Figure 4.10 Transversal sections of cotyledon explants of F1 hybrid variety under a light microscope. Cotyledons of 0, 1 and 7 days-old germinating seed (A-C). 21 days cotyledons from *in vitro* culture of 0, 1 and 7 days- old germinating seed (D- F) (Bar = 50 μm).

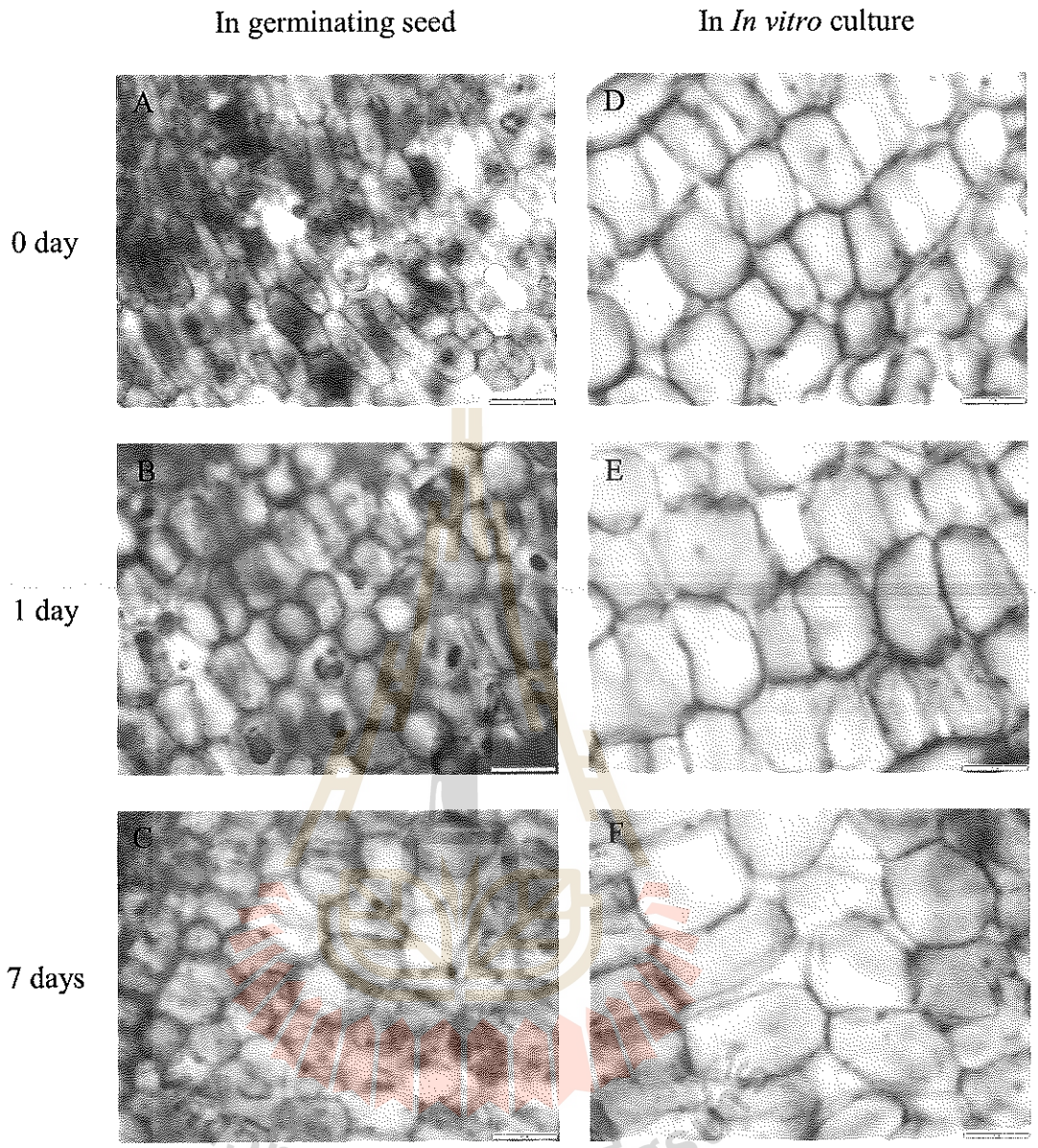

Figure 4.11 Transversal sections of cotyledon explants of F1 hybrid variety under a light microscope. Cotyledons of 0, 1 and 7 days-old germinating seed (A-C). 21 days cotyledons from *in vitro* culture of 0, 1 and 7 days- old germinating seed (D- F) (Bar = 50 μm).

4.2.2 Chemical change of sunflower cotyledons

A cotyledon is the food reserve tissue including carbohydrates, proteins, mineral, and lipid needed for seed germination. The factors affecting seed germination include water, air, temperature and food sources of the cotyledons (Hashim Abdel-Muttalib, 1933). The analytical results are summarized in Tables 4.12. The significant for all sources in the total protein and total sugar parameters. Total fat and sucrose were significant in condition, variety and age at p>0.05 level (Table 4.12).

Table 4.12 Analysis of variance for total protein, total fat, total sugar and sucrose of sunflower cotyledons.

S.O.V	d.f.	Total protein	Total fat	Total sugar	Sucrose
Condition (C)	1	862.792*	19841.070*	63.467*	977.812*
Variety(V)	1	114.632*	22.800*	3.610*	14.162*
Age (A)	2	12.454*	10.318*	22.922*	73.139*
C * V	1	78.322*	46.262*	49.140*	2.581
C * A	2	0.641*	0.288	3.871*	33.063*
V * A	2	0.166*	0.309	0.686*	3.323
C * V * A	2	0.776*	0.221	3.410*	0.943
Error	24	0.006	0.123	0.118	1.293
% C.V		18.30	20.21	26.43	28.14

* = Significant at 0.05 probability level.

Total fats were the most abundant reserve compounds in cotyledons at 0, 1 and 7 days-old germinating seeds, accounting for 54%, 53.19% and 52.66%, respectively.

Total fats decreased as the age of germinating seeds increased which was caused from degradation of lipid body (Figure 4.12 and 4.13). *In vitro* cotyledons had lower total fat than cotyledons in germinating seeds.

Table 4.13 The mean values (±SD) for chemical analysis exposed to the age of sunflower cotyledons.

Condition	Variety	Age (day)	Chemical analysis			
			Total protein (%)	Total fat (%)	Total sugar (%)	Sucrose (%)
Seedling	F1 hybrid	0	24.09±0.04c	54.00±0.35	10.41±0.28a	3.88±0.85a
		1	25.17±0.06b	53.19±0.28	9.73±0.04b	3.81±0.60a
		7	26.28±0.10a	52.66±0.49	7.03±0.05c	2.65±0.36b
	F-test		**	ns	**	**
	Prado Red	0	26.21±0.03a	53.96±0.03a	8.77±0.37a	4.37±1.84a
		1	25.93±0.02b	52.02±0.11b	5.09±0.15b	3.86±0.46a
		7	25.26±0.03c	51.84±0.12b	4.39±0.04b	2.54±0.15b
	F-test		**	**	**	**
In vitro	F1 hybrid	0	30.99±0.03c	4.98±0.04a	5.29±0.45a	1.41±0.31a
		1	31.96±0.05b	4.03±0.03b	3.59±0.09b	1.25±0.17ab
		7	33.11±0.04a	3.17±0.01c	3.30±0.02b	1.10±0.22b
	F-test		**	**	**	**
	Prado Red	0	36.97±0.04c	8.93±0.03a	6.63±0.04a	1.91±0.43a
		1	38.81±0.01b	7.95±0.05b	5.86±0.11b	1.49±0.02b
		7	39.84±0.02a	6.88±0.03c	5.10±0.01c	1.44±0.07b
	F-test		**	**	**	**

Mean followed by significant at 0.05 probability level. ns = not significant.

For starch granule analysis, a large number of starch granules were detected in cotyledons at 0 day-old germinating seeds in both varieties (Figure 4.12A and 4.13A). However, these granules were reduced in 1 day-old germinating seed (Figure 4.12B and 4.13B) and 7 days-old germinating seed (Figure 4.12C and 4.13C). *In vitro* cotyledons did not show starch granules at 0, 1 and 7 days-old germinating seed in both sunflower varieties as presented in Figure 4.12D-F and 4.13D-F.

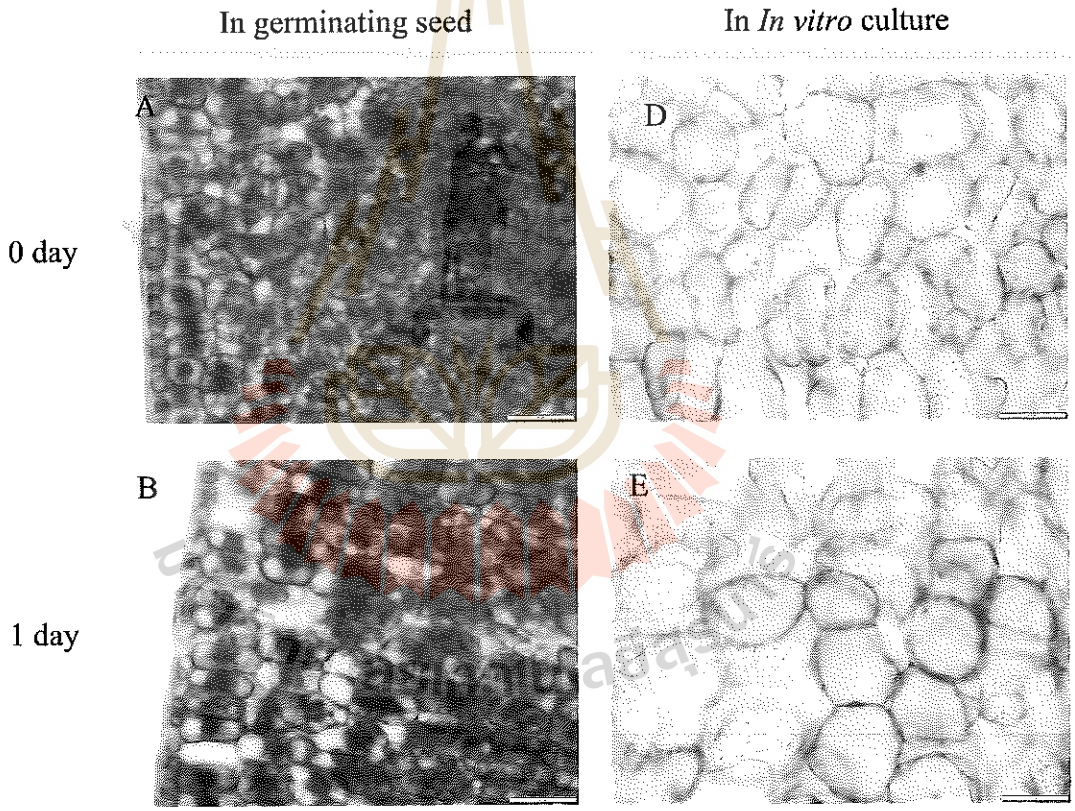

Figure 4.12 Starch granules of cotyledon explants of F1 hybrid variety under a light microscope. Cotyledons of 0, 1 and 7 days-old germinating seeds (A-C). 21 days cotyledons from *in vitro* cotyledons of 0 1 and 7 days-old germinating seeds (D-F) (Bar = 50 μm).

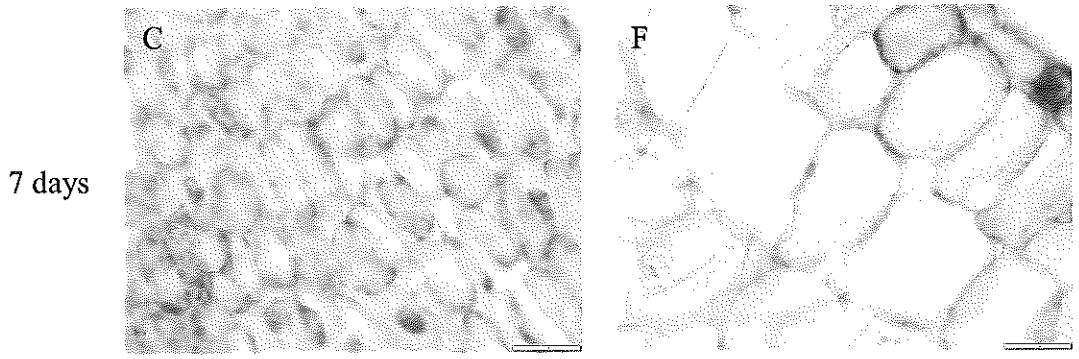

Figure 4.12 (Continued).

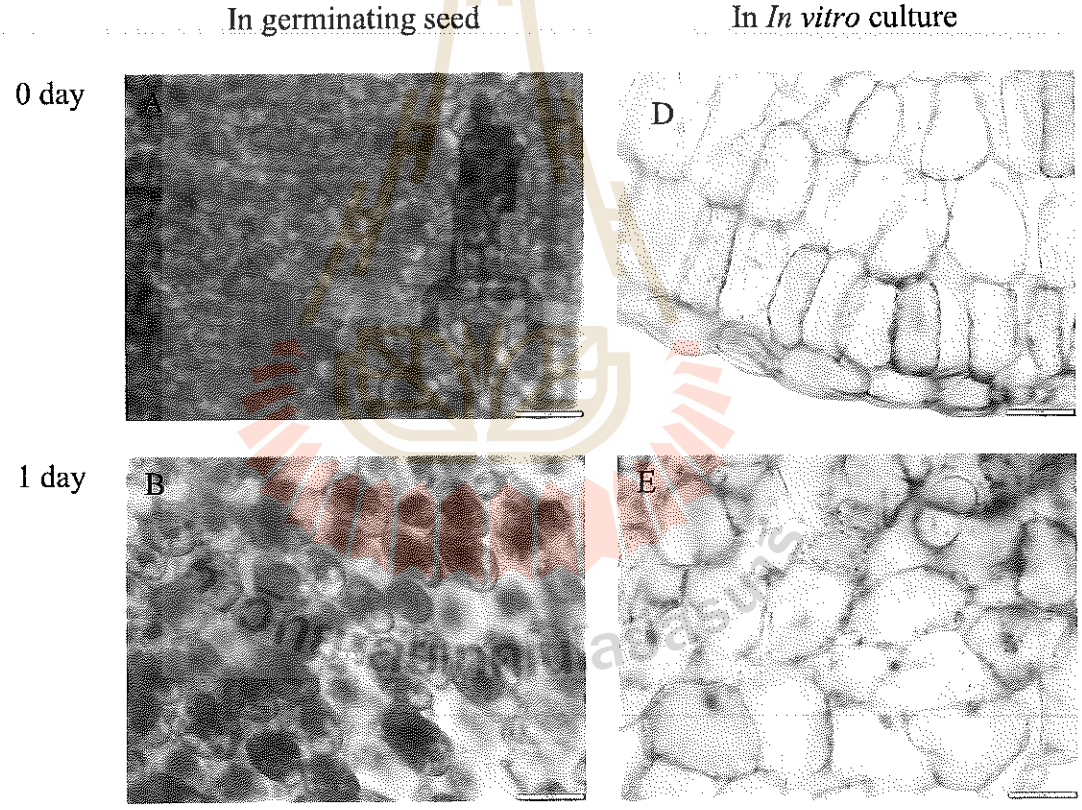

Figure 4.13 Starch granules of cotyledon explants of F1 hybrid variety under a light microscope. Cotyledons of 0, 1 and 7 days-old germinating seeds (A-C). 21 days cotyledons from *in vitro* cotyledons of 0 1 and 7 days-old germinating seeds (D-F) (Bar = 50 μm).

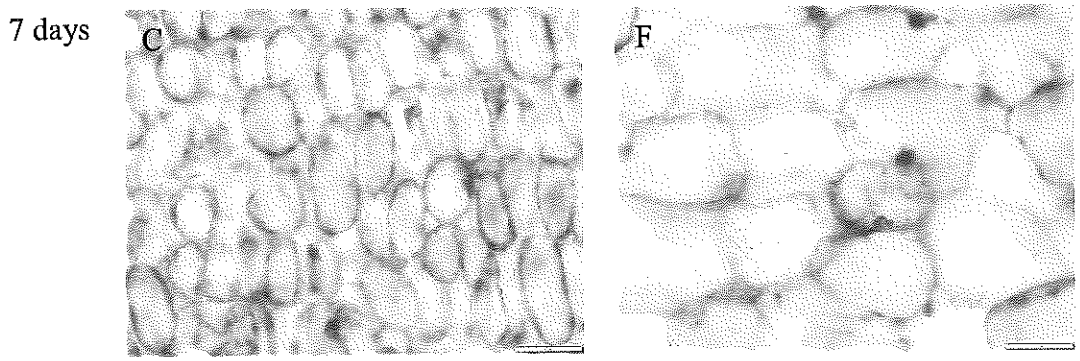

Figure 4.13 (Continued).

Proteins were the second most represented compounds in sunflower cotyledons both in germinating seeds and *in vitro* conditions. For both verities, total protein residues of the cotyledons ranged from 24.09% to 39.84% as presented in Table 4.13. The total protein content increased during seed germination in sunflower from 0 day-old germinating seeds about 24.09% to 7 days-old germinating seeds about 26.28% in F1 hybrid variety, which was opposite to Prado Red.

The total sugars or soluble sugars were the third source represented in sunflower seed. Changes in total sugar content in the cotyledons in germinating seeds and *in vitro* culture were observed in both sunflower varieties. In both varieties, the highest value of total sugar in cotyledons of 0 day-old germinating seeds while the lowest was observed in the cotyledons of 7 days-old germinating seeds F1 hybrid maximum total sugar 10.41% at 0 day-old germinating seeds and had the lowest 3.30% at 7 days-old germinating seeds as shown in Table 4.13.

The sucrose content in the germinating seeds and *in vitro* cotyledons were significant for variety, condition, age and the interaction between condition × age. In 0 day-old germinating seeds, both varieties had sucrose content higher than 7 days-old

germinating seeds in both conditions. *In vitro* culture cotyledons had the maximum of sucrose content about 1.41% at 0 day-old seedling and the lowest about 1.10% at 7 days-old seedling. Prado Red variety had high sucrose content then F1 hybrid variety as shown in Table 4.13.

This result agrees with the finding of Munshi et al. (2007) and Radic et al. (2009) who reported that sunflower seed had 36.82% of oil content and 23.61% of protein. Balasaraswathi and Sadasivam (1997) reported that total fat decreased significantly after 72 hours of sunflower seed germination. In sunflower oilseed species, its loss of oil reserve during germinating power was far more quickly (Lima et al., 2014). These results are similar with the finding of Alencar et al. (2012). Also, *in vitro* cotyledons of sunflower produced protein higher than cotyledons of germinating seeds which may be manipulated by changing nutrient composition, similar to finding of Morard and Henry (1998).

The percentage of shoot induction was positively correlated with cell area ($r = 0.569^{**}$), total protein (0.357^{*}), percentage of response ($r = 0.631^{**}$) and percentage of callus induction (0.498^{**}) while it had negative correlation with cell number ($r = -0.506^{**}$) as shown in Table 4.14. We first reported about correlation between cytology and food reserves in sunflower cotyledons. Correlation analysis of cytological, chemical change and shoot induction parameters indicated that increased protein content and cell size effect on shoot induction of sunflower.

Table 4.14 Correlation analysis between cytological features, chemical change and shoot induction parameters of cotyledon culture in sunflower.

Variable	Cell area	Cell number	Cotyledons thickness	Total protein	Total fat	Total sugar	Sucrose	Response (%)	Callus induction (%)	Shoots induction (%)
Cell number	-0.889**									
Cotyledons thickness	0.571**	-0.641**								
Total protein	0.254	-0.344*	0.359*							
Total fat	0.108	0.018	-0.134	-0.871**						
Total sugar	-0.239	0.377*	-0.513**	-0.501**	0.629**					
Sucrose	0.082	0.035	-0.337*	-0.782**	0.897**	0.755**				
Response (%)	0.947**	-0.844**	0.364*	0.325	0.034	-0.143	0.109			
Callus induction (%)	0.856**	-0.753**	0.305	0.063	0.194	-0.208	0.169	0.861**		
Shoots induction (%)	0.569**	-0.506**	0.312	0.357*	-0.095	-0.110	-0.013	0.631**	0.498**	
Roots induction (%)	0.301	-0.254	0.043	0.014	0.069	-0.094	0.174	0.345*	0.472**	0.483**

CHAPTER V

CONCLUSION

In vitro culture is one of the biotechnological tools for sunflower propagation. It is used to reduce lifecycle of the plants from normal method. In this thesis study, cotyledon at 0, 1 and 7 days-old germinating seeds of two sunflower varieties, namely F1 hybrid and Prado Red, were employed for cotyledon culture, cytological and chemical change studies. Summary of the study is as follows:

1. For shoot induction study of cotyledon culture, shoot induction efficiency was affected by variety, media type and age of explants. Prado Red variety gave higher shoot induction than F1 hybrid variety. Cotyledons from 1 day-old germinating seeds placed with adaxial side on MS medium supplemented with 1 mg/l BA gave the best response for shoot induction and the number of shoot per explant in both varieties.

2. For shoot multiplication study, MS medium containing 2 mg/l of BA (B2 medium) induced the greatest number shoots (3 shoots per explant) and number of leaves (5 leaves) in F1 hybrid variety while Prado Red variety had 2 shoots per explant and 4 leaves.

3. For root induction study, MS medium supplemented with combination of hormones and charcoal had higher root induction than without charcoal. The MS medium combined with 1 mg/l of BAP, 1 mg/l of IAA and charcoal also promoted rooting about 7 roots per explant and root length about 3 cm in Prado Red variety while 6 roots per explant and 2 cm in length in F1 hybrid.

4. For acclimation and growth study, peat moss was the most suitable growing material for sunflower plantlets when compared to other growing materials. It gave survival rate about 60% with shoot length about 7.2 cm and 6 leaves/explant.

5. For cytological study, the cell number was decreased depending on the age of cotyledons and conditions of culture. Prado Red variety had cell number more than F1 hybrid variety. The cell area and cotyledons thickness increased as the age of seedling increased for both varieties. Cell area of cotyledons cultured *in vitro* was larger than that of cotyledons in germinating seeds.

6. For chemical change study, total fats were the most abundant reserve in cotyledons of germinating seeds followed by proteins and carbohydrates. Total fats decreased as the age of germinating seeds increased which may be caused by the degradation of lipid body. Total fats, total sugar and sucrose of cotyledons at 0 day-old germinating seeds showed higher values than 7 days-old in both conditions, whereas proteins increased as the age increased that could be due to photosynthesis.

Future work

In this study, the complete sunflower plantlets using cotyledons as explants were successfully obtained, but acclimation of plantlets from *in vitro* condition to the ambient environment resulted in death of plantlets. Future research should focus on plant acclimation and growth. Several factors affecting survival rate and growth, including high humidity, light level, temperature, need to be investigated.

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APPENDICES

APPENDIX A

MURASHIGE AND SKOOG BASAL MEDIUM

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

Stock solution A: Macronutrients

NH_4NO_3	1,650	mg/l
KNO_3	1,900	mg/l
$CaCl_2.2H_2O$	440	mg/l
$MgSO_4.7H_2O$	370	mg/l
KH_2PO_4	170	mg/l

Preparation of stock solution A: each chemical component was dissolved in DI water then poured to a tank, to make a volume 1 liter. The solution was kept in a plastic container in the dark and stored at 4 - 7 °C.

Stock solution B: Micronutrients

KI	0.83	mg/l
H_3BO_3	6.2	mg/l
$MnSO_4.4H_2O$	22.3	mg/l
$ZnSO_4.7H_2O$	8.6	mg/l
$Na_2MoO_4.2H_2O$	0.25	mg/l
$CuSO_4.5H_2O$	0.025	mg/l

$CoCl_2.6H_2O$	0.025	mg/l

Preparation of stock solution B: each chemical component was dissolved in DI water then poured to a tank, to make a volume 1 liter. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

Stock solution C: Iron source

$FeSO_4.7H_2O$	27.85	mg/l
$Na_2.EDTA.2H_2O$	37.3	mg/l

Preparation of stock solution C: each chemical component was dissolved in DI water then poured to a tank, to make a volume 1 liter. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

Stock solution D: vitamins and organic

Myo inositol	100	mg/l
Nicotinic acid	0.5	mg/l
Pyridoxine HCl	0.5	mg/l
Thiamine HCl	0.5	mg/l
Glycine	2	mg/l

Preparation of stock solution C: each chemical component was dissolved in DI water then poured to a tank, to make a volume 1 liter. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

A.2 Plant hormones

100 mg/l of NAA

Preparation of stock hormone: the NAA was weighted in 0.01 g and then dissolved in 1-2 ml of absolute alcohol, mixed thoroughly until dissolved and brought volume up to 100 ml with DI water. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

100 mg/l of IAA

Preparation of stock hormone: the IAA was weighted in 0.01 g and then dissolved in 1-2 ml of absolute alcohol, mixed thoroughly until dissolved and brought volume up to 100 ml with DI water. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

100 mg/l of 2iP

Preparation of stock hormone: the 2iP was weighted in 0.01 g and then dissolved in 1-2 ml of DI water, mixed thoroughly until dissolved and brought volume up to 100 ml with DI water. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

100 mg/l of TDZ

Preparation of stock hormone: the TDZ was weighted in 0.01 g and then dissolved in 1-2 ml of absolute alcohol, mixed thoroughly until dissolved and brought volume up to 100 ml with DI water. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

100 mg/l of BA

Preparation of stock hormone: the BA was weighted in 0.01 g and then dissolved in 1-2 ml of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL with DI water. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

A.3 Additives

50,000 mg/l of casein hydrolysate (CH)

Preparation of stock additive: 50,000 mg of the CH was weighted and then dissolved in water, mixed thoroughly until dissolved and made up to a volume 250 ml. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

100 mg/l of silver nitrate ($AgNO_3$)

Preparation of stock additive: 100 mg of the $AgNO_3$ was weighted and then dissolved in water, mixed thoroughly until dissolved and made up to a volume 100 ml. The solution was kept in a plastic container in the dark and stored at 4-7 °C.

APPENDIX B

CHEMICAL REAGENTS

B.1 Chemical reagent solution for total protein extraction

- Kjedahl catalyst: mix 9 part of potassium sulphate (K_2SO_4) with 1 part of coppersulphate ($CuSO_4$)

- Sulfuric acid (H_2SO_4)

- 40% of NaOH solution

- 0.2 N HCl solution

- 4% H_3BO_3

- Indicator solution: Mix 100 ml of 0.1 % methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

B.2 Chemical reagent solution for total sugar and sucrose extraction

80% of ethanol

Preparation: diluted 80 ml of ethanol with distilled water make up a volume to 100 ml. The solution was kept in a glass container and stored at 4-7 °C.

5% of phenol

Preparation: dissolved 5 g of phenol in distilled water make up a volume to 100 ml. The solution was kept in a glass container and stored at 4-7 ºC.

0.2 N of NaOH

Preparation: dissolved 0. 8 g of phenol in distilled water make up a volume to 100 ml. The solution was kept in a glass container and stored at 4-7 ºC.

1% resorcinol

Preparation: dissolved 1 g of resorcinol in distilled water make up a volume to 100 ml. The solution was kept in a glass container and stored at 4-7 ºC.

30% of HCl

Preparation: diluted 30 ml of HCl with distilled water make up a volume to 100 ml. The solution was kept in a glass container and stored in fume hood.

APPENDIX C

CALIBRATION CURVES

C.1 Standard curves of glucose

Preparation: dissolved 1 g of glucose in distilled water make up a volume to 100 ml. the glucose solution at 100 µl was place into test tube. 500 µl of 5% phenol and 1 ml of sulfuric acid was added on each solution sample and then mixed gently and set for 30 min. following below the table.

Table C.1 Preparation of glucose stock solution.

No.	Standard (µg/ml)	Stock (2000 µg/ml	DI water (µl)	Total (µl)
1	0	0	2000	2000
2	50	50	1950	2000
3	80	80	1920	2000
4	200	200	1800	2000
5	400	400	1600	2000
6	600	600	1400	2000
7	800	800	1600	2000
8	1000	1000	1200	2000
9	2000	2000	0	2000

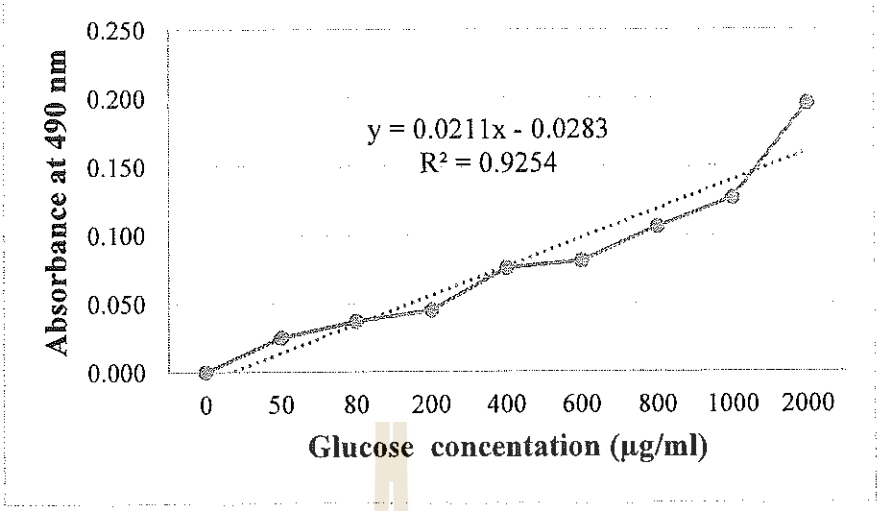

Figure C.1 Standard curve of glucose.

C.2 Standard curves of sucrose

Preparation: dissolved 1 g of sucrose in distilled water make up a volume to 100 ml. The sucrose sample at 500 µl was filled into test tube. Add 100 µl of 0.2 N NaOH. Take tube into water baht at 100 °C for 10 min to make decay fructose. Added 250 ml of 1 % resorcinol and 750 µl of 30 % HCl and then incubated at 80 °C for 10 min.

Table C.2 Preparation of sucrose stock solution.

No.	Standard (µg/ml)	Stock (2000 µg/ml)	DI water (µl)	Total (µl)
1	0	0	2000	2000
2	200	200	1800	2000
3	400	400	1600	2000
4	600	600	1400	2000
5	1000	1000	1000	2000

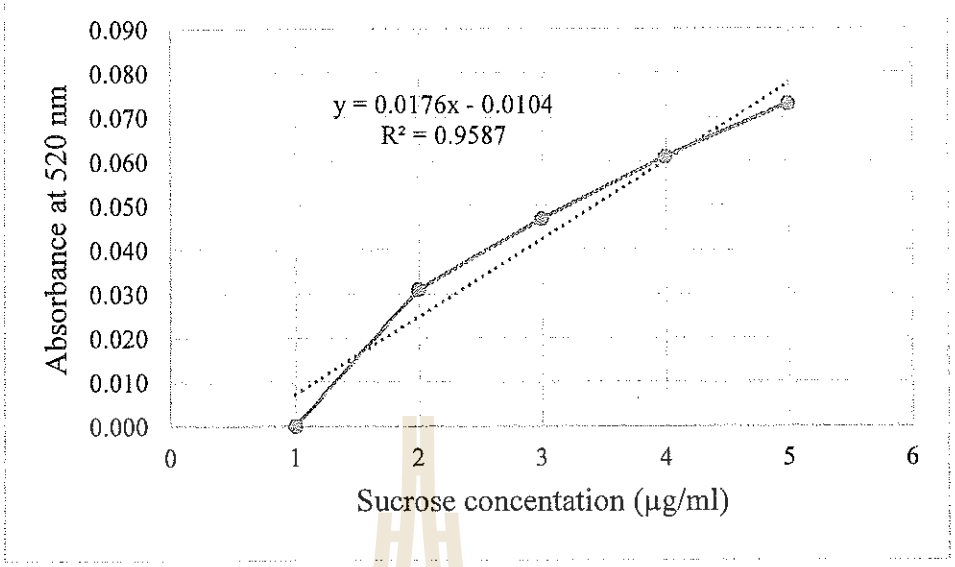

Figure C.2 Standard curve of sucrose.

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Montathong, K., Machikowa, T., and Muangsan, N. (2011). Effect of culture medium and cotyledons age on shoot induction of ornamental sunflower (*Helianthus annuus* L.) *in vitro*. **The 17th Flora of Thailand Conference, 21-25 August 2017, Krabi, Thailand**.

Award -

Grants and Fellowships Scholarship for Kittibundit at Suranaree University of Technology

Position and Place of Work -