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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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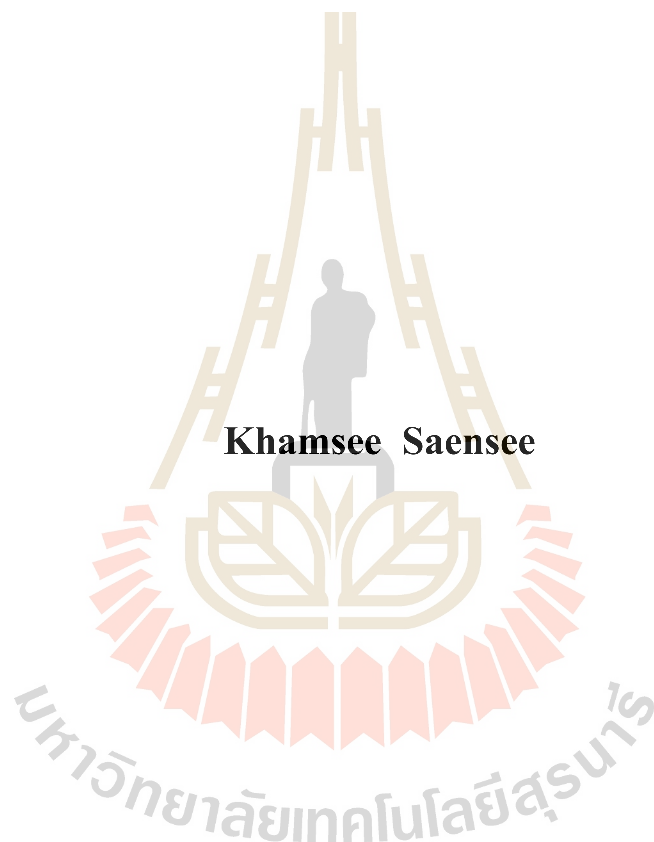
FACTORS AFFECTING DOUBLED HAPLOID PRODUCTION IN SUNFLOWER



Khamsee Saensee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

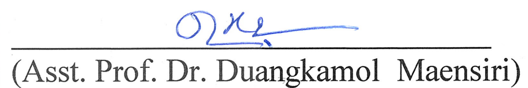
Suranaree University of Technology

Academic Year 2017

FACTORS AFFECTING DOUBLED HAPLOID PRODUCTION IN SUNFLOWER

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee



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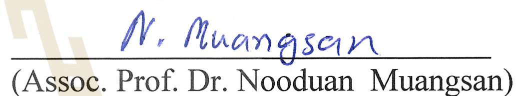

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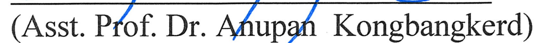

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คำสี แสนสี : ปัจจัยที่มีผลต่อการผลิตต้นดับเบิลแฮพพลอยด์ในทานตะวัน (FACTORS AFFECTING DOUBLED HAPLOID PRODUCTION IN SUNFLOWER) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.หนูเดือน เมืองแสน, 125 หน้า.

งานวิจัยนี้มีเป้าหมายเพื่อผลิตต้นดับเบิลแฮพพลอยด์ในทานตะวัน และมีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างลักษณะของดอกย่อยและลักษณะของไมโครสปอร์กับพัฒนาการของไมโครสปอร์ เพื่อศึกษาผลของพันธุ์ วงดอก และอาหารสังเคราะห์ต่อการผลิตพืชแฮพลอยด์ด้วยวิธีการเพาะเลี้ยงอับเรณู และ เพื่อศึกษาผลของการใช้สารโคลชิซินต่อการเพิ่มชุดโครโมโซมในเอ็มบริโอจินิคแคลลัส ในการศึกษาวิจัยนี้ใช้ดอกย่อย 3 วงจากจานดอกระยะ R5.1 ของทานตะวัน 3 พันธุ์ ได้แก่ พันธุ์สุรนารี 473 แปซิฟิค 22 และพันธุ์พราโด เร็ด ทำการศึกษาความสัมพันธ์ระหว่างลักษณะดอกย่อยลักษณะของไมโครสปอร์กับระยะพัฒนาการของไมโครสปอร์ โดยใช้กล้องจุลทรรศน์แบบสเตอริโอ กล้องจุลทรรศน์แบบใช้แสง (LM) กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน (TEM) และกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) สำหรับการศึกษาผลของพันธุ์ วงดอกย่อย และอาหารสังเคราะห์ต่อการผลิตพืชแฮพลอยด์ด้วยวิธีเพาะเลี้ยงอับเรณู ทำการทดลองโดยนำอับเรณูมาเพาะเลี้ยงในอาหารสังเคราะห์จำนวน 4 สูตรเพื่อชักนำให้เกิดแคลลัสนาน 30 วัน แล้วย้ายไปยังอาหารชักนำให้เกิดต้น ด้วยสูตรอาหารจำนวน 4 สูตรนาน 14 วัน นำเอ็มบริโอจินิคแคลลัสมาแช่ในสารละลายโคลซิซินความเข้มข้น 300 และ 600 ไมโครโมลาร์ เป็นเวลา 3 และ 6 ชม. เพื่อเพิ่มชุดโครโมโซมแล้วย้ายลงในอาหารชักนำให้เกิดต้นที่เหมาะสมนาน 14 วัน และทำการตรวจสอบชุดโครโมโซมด้วยเครื่องโฟลว์ ไซโตเมทรี ผลการศึกษาพบว่าลักษณะสัณฐานวิทยาดอกย่อยมีความแตกต่างอย่างมีนัยสำคัญระหว่างพันธุ์และวงดอกย่อย จำนวนไมโครสปอร์ต่อดอกอยู่ระหว่าง 14,694 - 28,106 เม็ด และความมีชีวิตของละอองเรณูมีค่าร้อยละ 90.81 - 99.33 ทั้งขนาดดอกย่อยและขนาดของอับเรณูมีความสัมพันธ์เชิงบวกกับจำนวนไมโครสปอร์ต่อดอกและความมีชีวิตของละอองเรณู โดยพันธุ์แปซิฟิก 22 มีลักษณะสัณฐานวิทยาของดอกดีสุด ระยะไมโครสปอร์จะมีความต่างกันอย่างมีนัยสำคัญในแต่ละพันธุ์และวงดอกย่อย วงดอกย่อยนอกสุดมีร้อยละของไมโครสปอร์ที่มีนิวเคลียสเดียวระยะกลางถึงปลายสูงกว่าดอกย่อยวงใน สำหรับการศึกษาการชักนำให้เกิดแคลลัสโดยการเพาะเลี้ยงอับเรณู พบว่า อาหารเพาะเลี้ยงมีผลอย่างมีนัยสำคัญต่อการเจริญของแคลลัส ร้อยละการเกิดแคลลัส และร้อยละการเกิดเอ็มบริโอจินิคแคลลัส อาหารเพาะเลี้ยงสูตร MS (A2) ที่ประกอบด้วยฮอร์โมน เอ็นเอเอ (NAA) 2 มก./ล. บีเอพี (BAP) 1 มก./ล. และ น้ำมะพร้าว 10% ชักนำให้เกิดแคลลัสสูงถึงร้อยละ 65.48 และให้เอ็มบริโอจินิคแคลลัสมากที่สุดร้อยละ 21.88 สำหรับการศึกษาการชักนำต้น พบว่าเอ็มบริโอจินิค

แคลลัสตอบสนองได้ดีในอาหารสูตร MS (S4) ที่เติม บีเอพี 2 มก./ล. เคซีนไฮโดรไลเสท 500 มก./ล. และ ผงถ่าน 0.2% บางแคลลัสสามารถพัฒนาเป็นต้น หรือราก แต่ไม่สามารถพัฒนาไปเป็นต้นที่สมบูรณ์ได้ ความเข้มข้นและเวลาในการได้รับสารละลายโคลชิซินมีผลต่อการรอดชีวิตของแคลลัสและการเจริญของแคลลัสอย่างมีนัยสำคัญ โดยสรุป งานวิจัยนี้ได้ศึกษาปัจจัยที่มีผลต่อการเพิ่มประสิทธิภาพการเพาะเลี้ยงอับเรณูของทานตะวันสามพันธุ์ อย่างไรก็ตาม เนื่องจากการชักนำให้เกิดยอดจากทานตะวันได้ต่ำและการเกิดต้นใหม่ยังไม่สมบูรณ์ ดังนั้นในอนาคตควรมุ่งการศึกษาเพิ่มเติมไปที่การเกิดต้นโดยการเพิ่มฮอร์โมน สารเสริม หรือตัวยับยั้งอื่น

สาขาวิชาชีววิทยา

ปีการศึกษา 2560

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KHAMSEE SAENSEE : FACTORS AFFECTING DOUBLED HAPLOID PRODUCTION IN SUNFLOWER. THESIS ADVISOR : ASSOC. PROF. NOODUAN MUANGSAN, Ph.D. 125 PP.

ANTHER CULTURE/ CALLUS/ DOUBLED HAPLOID/ SUNFLOWER/ UNINUCLEATE MICROSPORE

The goal of this research was to produce doubled haploid (DH) plants of sunflower and specific objectives were to determine the correlation between floret characteristics, microspore features and microspore developmental stages in three sunflower varieties, to investigate the effects of varieties, floret whorl, and culture medium on haploid plant production via anther culture, and to investigate the effect of colchicine treatment on chromosome doubling of embryogenic calli-derived from anther. Three outermost unopened disk florets of the flower head at the R5.1 reproductive stage of three sunflower varieties including S473, Pacific 22 and Prado Red were used in this study. Determination of flower bud characters and microspore features were carried out using stereomicroscope, light microscope (LM), transmission electron microscope (TEM) and scanning electron microscope (SEM). For haploid plant production via anther culture, anthers were cultured on various callus induction media for 30 days, and the anther-derived embryogenic calli were subsequencely induced into shoot on induction medium for 14 days. Then, the embryogenic calli were immerged in 100 and 300 μM of colchicine solution for 3 and 6 hours for chromosome doubling. The polyploidy level was determined with flow cytometry. The results showed that there was significant variation of floret characters among sunflower varieties and their floret whorls. Microspore per floret was in the

range of 28,106-14,694 grains and pollen viability ranged between 90.81%-99.33%. Both disk length and anther length parameters of floret maintained positive correlation with the percentage of microspore per flower and pollen viability. Among three varieties, Pacific 22 has the best morphological characters of floret. The microspore stages were significantly different in frequency among varieties and whorls. The outer disk floret had the higher percentage of mid-to-late uninucleate stage than the inner florets. For callus induction study via anther culture, it was found that culture medium significantly affected callus growth parameters, the percentage of callus, and the percentage of embryogenic calli. MS medium (A2) supplemented with 2 mg/l NAA, 1 mg/l BAP and 10% (v/v) coconut water induced the highest percentage of calli for all three varieties with the highest frequency of 65.48%. For shoot induction study, embryogenic calli gave the best response on MS medium (S4) supplemented with 2 mg/l BAP, 500 mg/l CH and 0.2% activated charcoal. Some embryogenic calli could develop into shoot or root but not a complete plant. Colchicine concentrations and treatment durations had significantly affected the survival rate of callus and callus growth. In conclusion, several factors affecting in vitro anther culture of three sunflower varieties were investigated and optimized. Future investigations will need to focus on the shoot induction through addition of other plant hormones, additives or inhibitors since shoot production from sunflower plants is very low and plant regeneration is problematic.

School of Biology

Academic Year 2017

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Khamsee Saensee

CONTENTS

Page

ABSTRACT IN THAI I

ABSTRACT IN ENGLISH III

ACKNOWLEDGEMENTS V

CONTENTS VI

LIST OF TABLES XII

LIST OF FIGURES XIV

LIST OF ABBREVIATIONS XVI

CHAPTER

I INTRODUCTION 1

1.1 Background/Problem 1

1.2 Research objectives 3

1.3 Research hypothesis 3

1.4 Scope and limitations of the study 4

1.5 Expected results 4

II LITERATURE REVIEW 6

2.1 Sunflower characteristics 6

2.2 Growth stage of sunflower 9

2.3 Sunflower classification 12

2.4 Reproductive flowers 13

2.4.1 Stamen development 14

CONTENTS (Continued)

Page

2.4.2 Pistil development 18

2.5 Sunflower breeding 18

2.5.1 Conventional breeding.. 20

2.5.2 Modern plant breeding 20

2.6 Anther culture techniques.. 22

2.7 Factors affecting the success of anther culture.. 24

2.8 Haploid in sunflower and chromosome doubling technique..................... 29

2.9 Polyploidy level determination 31

III MATERIALS AND METHODS.. 32

3.1 Correlation between morphological characters of floret bud,

microspore features and microspore developmental stage 32

3.1.1 Plant materials and plant growth 32

3.1.2 Measurement of disk floret bud and anther size 33

3.1.3 Determination of microspore viability and production 33

3.1.4 Observation of anther structure using LM 34

3.1.5 Determination of microspore features 35

3.1.6 Determination of microspore developmental stage.......................... 35

3.1.6.1 Sample preparation for microscope analysis (both

light microscope and scanning electron microscope 35

3.1.6.2 Determination of microspore developmental stage using

light microscope (LM) 36

CONTENTS (Continued)

Page

3.1.7 Ultrastructure of microspore and its surface morphology 36

3.1.7.1 Determination of ultrastructure of microspore using transmission electron microscope (TEM)........................... 36

3.1.7.2 Determination of surface morphology of microspore using scanning electron microscope (SEM) 36

3.1.8 Statistical analysis 37

3.2 Effects of varieties, floret whorl, and culture medium on haploid plant production via anther culture.. 37

3.2.1 Callus induction on various medium through anther culture 37

3.2.2 Shoot and root regeneration of embryogenic callus derived from anthers... 39

3.2.3 Statistical analysis 40

3.3 Effect of colchicine treatment on chromosome doubling of embryogenic calli-derived from anthers 40

3.3.1 Effect of concentration and timing condition of colchicine treatment on growth and survival rate of embryogenic calli........... 40

3.3.2 Determination of ploidy level using flow cytometry 41

3.3.3 Statistical analysis 42

IV **RESULTS AND DISCUSSION**.. 43

4.1 Correlation between morphological characteristics of floret bud, microspore feature and microspore developmental stage 43

มหาวิทยาลัยเทคโนโลยีสุรนารี

CONTENTS (Continued)

Page

4.1.1 Morphological characteristics of floret bud 43

4.1.2 Determination of microspore production per floret and pollen viability 47

4.1.3 Anther structure and microspore features 51

4.1.4 Determination of microspore developmental stages 57

4.1.5 Ultrastructure of microspore and its surface morphology................ 63

4.2 Effects of varieties, floret whorl, and culture medium on haploid plant production via anther culture... 69

4.2.1 Callus induction on various medium through anther culture 69

4.2.2 Shoot and root regeneration of embryogenic callus derived from anther 80

4.3 Effect of colchicine treatment on chromosome doubling of anther-derived callus.. 84

V CONCLUSIONS... 91

REFERENCES.. 95

APPENDICES 117

APPENDIX A MURASHIGE AND SKOOG'S MEDIUM 118

APPENDIX B CHEMICAL REAGENTS 121

CURRICULUM VITAE.. 125

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF TABLES

Table **Page**

2.1 Sunflower growth stages. 11

3.1 Sunflower varieties and their origin for the experimental study 33

3.2 Callus induction 39

3.3 Shoot induction medium. 40

4.1 Analysis of variances for morphological characteristics in three sunflower varieties 44

4.2 The mean values for disk floret features in three sunflower varieties 46

4.3 Correlation of floret bud features of three sunflower varieties 51

4.4 Analysis of variance for microspore features and microspore developmental stage in three sunflower varieties 53

4.5 The mean values for microspore developmental stage and morphology of microspore in three sunflower varieties 55

4.6 Correlation analysis of variance between morphological disk floret bud and microspore developmental stage in three sunflower varieties 61

4.7 Analysis of variances of callus growth parameters and the percentage of callus induction and the percentage of embryogenic calli in three sunflower varieties 70

4.8 Comparison between the mean values (± SD) and callus induction characteristics in three sunflower varieties 73

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF TABLES (Continued)

Table **Page**

4.9 Correlation analysis of variances of callus growth parameters in three sunflower varieties 78

4.10 Analysis of variance of anther-derived callus parameters on regeneration medium in three sunflowers varieties.. 81

4.11 The mean values (±SD) of callus growth characteristics on various shoot induction medium in three sunflower varieties 82

4.12 Analysis of variance of morphological characteristics of embryogenic callus-derived from anther treated with colchicine in three sunflower varieties 84

4.13 Comparison of morphological characteristics and ploidy level of embryogenic calli in three sunflower varieties 87

4.14 The ploidy level of the treated embryogenic calli with colchicine in three sunflower varieties 90

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF FIGURES

Figure **Page**

2.1 Vertical section of sunflower head 7
2.2 Composition of sunflower florets 8
2.3 Parts of sunflower seed 8
2.4 A schematic representation of sunflower developmental stages. 10
2.5 Ornamental sunflower varieties with multiple small capitulum 12
2.6 Cultivated sunflower with a single large capitulum 13
2.7 Sunflower stamen 14
2.8 Illustration of microsporogenesis in sunflower 16
2.9 Pollen measurement technique and terminology 17
2.10 Sunflower ovule 18
2.11 Comparison sunflower between traditional breeding and modern breeding 19
2.12 Haploid plant production pathway 24
2.13 Schematic diagram of haploid production procedures 29
4.1 Sunflower heads at the R5.1 reproductive stage and dissected disk florets into whorls. 45
4.2 Images of microspore in three sunflower varieties 48
4.3 Images of IKI stained microspores viability in three sunflower varieties 50
4.4 Thin anther sections from floret whorls in three sunflower varieties. 52

LIST OF FIGURES (Continued)

Figure **Page**

4.5 Light microscope images of microspore in three sunflower varieties. 56

4.6 Microspores stained with toluidine blue O (TBO) under light three sunflower microscope in varieties 59

4.7 TEM micrographs of microspore wall in three sunflower varieties. 64

4.8 SEM images of microspore surface in three sunflowers varieties.... 67

4.9 Anther-derived calli from different whorls of S473 variety on various callus induction medium 75

4.10 Anther-derived calli from different whorls of Pacific 22 variety on various callus induction medium 76

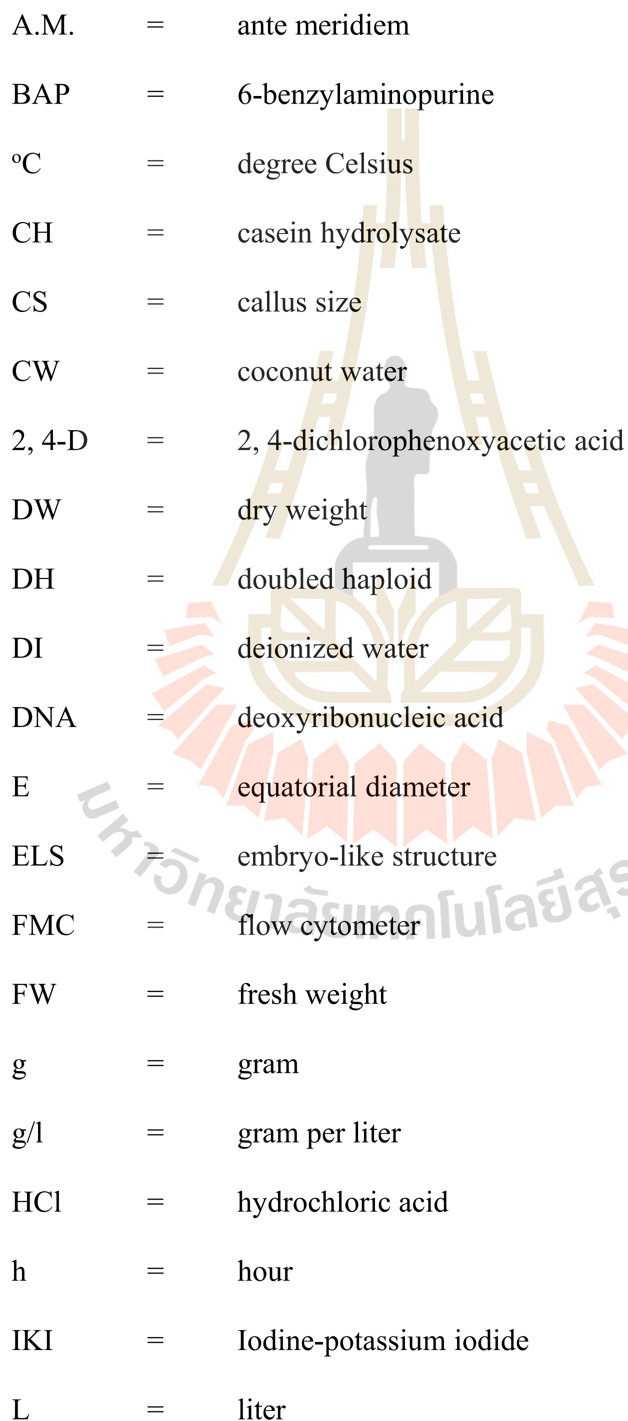
4.11 Anther-derived calli from different floret whorls of Prado Red variety on various callus induction medium 77

4.12 Embryogenic calli with embryo, shoot and root organogenesis on shoot induction medium in three sunflower varieties. 83

4.13 Images of organogenesis in the treated embryogenic calli with colchicine.... 89

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS



A.M.	=	ante meridiem
BAP	=	6-benzylaminopurine
^{o}C	=	degree Celsius
CH	=	casein hydrolysate
CS	=	callus size
CW	=	coconut water
2, 4-D	=	2, 4-dichlorophenoxyacetic acid
DW	=	dry weight
DH	=	doubled haploid
DI	=	deionized water
DNA	=	deoxyribonucleic acid
E	=	equatorial diameter
ELS	=	embryo-like structure
FMC	=	flow cytometer
FW	=	fresh weight
g	=	gram
g/l	=	gram per liter
HCl	=	hydrochloric acid
h	=	hour
IKI	=	Iodine-potassium iodide
L	=	liter

LIST OF ABBREVIATIONS (Continued)

%	=	percentage
L	=	length
µm	=	micrometer
µM	=	micromolar
µg	=	microgram
µl	=	microliter
mm	=	millimeter
ml	=	milliliter
mg/l	=	milligram per liter
mg/ml	=	milligram per milliliter
mg/kg	=	milligram per kilogram
M	=	molarity
MMC	=	microspore mother cell
mM	=	milimolar
min	=	minutes
MS	=	Murashige & Skoog medium
N	=	normality
NAA	=	naphthaleneacetic acid
P	=	polar axis
PI	=	propidium iodide
PC	=	percentage of callus induction
P/E	=	polar axis per equatorial diameter

LIST OF ABBREVIATIONS (Continued)

pH	=	potential of hydrogen ion
rpm	=	revolutions per minute
SEM	=	scanning electron microscope
TEM	=	transmission electron microscope
VE	=	vegetative stage
v/v	=	volume by volume
W	=	width
w/v	=	weight by volume

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CHAPTER I

INTRODUCTION

1.1 Background/Problem

Sunflower (*Helianthus annuus* L.) belonging to Asteraceae family is one of the world largest oilseed crops after soybean, rapeseed and cottonseed (Foreign Agricultural Service, 2017). It is traditionally appreciated as a high-quality commodity. Sunflower seeds are an important source of healthy edible oil of great commercial importance. Oilseed has many applications, both as food and raw materials for industry. Sunflower oil is a major source of mono- and polyunsaturated fatty acids as well as vitamin E, the major source of antioxidants in human nutrition. It has dietary antioxidants for reduction of cancer risk by minimizing DNA damage (Singh et al., 2007). Moreover, Manning et al. (2013) reported that sunflower oil decreased postprandial lipemia and triacylglycerol-rich after single mixed meals in healthy animals.

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Sunflower is a commercial control plant in Thailand. Currently, seed import is likely to increase every year, such as 131.61 tons in 2013, 209.34 tons in 2014, and 469.67 tons in 2015 (Thasta, 2016). There are several factors leading to increase seed import, such as the reduced crop area, flooding, and drought. Moreover, the production was not sufficient for domestic consumption in the form of oil and meal (Office of Agricultural Economics, 2013). Most sunflower cultivation areas are in the central areas such as Lop Buri, Saraburi, Nakhon Sawan and Phetchabun.

Sunflower cultivation in these areas is used to extract oil and promote tourism. The popular varieties are hybrids, which have to be imported. They are expensive for subsistence farmers and sometimes are not available when needed. Development of new sunflower varieties is required to meet the demand for farmers and to maintain yields under environmental and topographic conditions of Thailand.

The key to sunflower breeding programs is to increase seed yield, which is closely associated with height, head diameter, a number of seeds per head, improved oil quality and resistance to different stresses (Kaya et al., 2012). The sunflower is a highly cross-pollinated crop and a breeding program of this crop involves several breeding cycles. Doubled haploids (DHs) technology has the potential to greatly shorten the time needed to produce completely homozygous lines compared to conventional breeding (Dagustu et al., 2012). Anther culture is one of the very popular methods for production of haploids through culturing anthers or microspores on artificial culture medium. It allows novel allele combinations, particularly ones involving recessive characters, to be assessed in intact plants. The haploid duplicates the chromosome complement in order to obtain homozygous diploids (Baenziger and DePauw, 2009). In pollen derived plants, duplication of chromosomes may occur spontaneously in cultures, but due to the small percentage of such double-haploids, it is necessary to double the haploids by colchicine treatment. Haploid induction through anther culture depends on many factors such as genotype, physiology of the donor plant, culture medium, culture density, microspore developmental stage and environment of culture condition (Bhojwani and Bhatnagar, 2009).

Therefore, the main goal of this study was to produce doubled haploid (DH) sunflower plants.

1.2 Research objectives

The main objective of this research was to produce doubled haploid (DH) sunflower plants that may be used as parental materials for future breeding programs. Specific objectives were as below;

1.2.1 To determine correlation between morphological characteristics of floret bud, microspore features and microspore developmental stage

1.2.2 To investigate the effects of varieties, floret whorl, and culture medium on haploid plant production via anther culture

1.2.3 To investigate the effect of colchicine treatment on chromosome doubling of embryo-like structure (ELS) derived embryos from anthers

1.3 Research hypothesis

The production of doubled haploid (DH) plants through anther culture is one of popular methods for the production of homozygous lines. The success of this method depends on several factors such as the developmental stage of microspore, the composition of nutrient, plant genotypes, and others. The experimental hypotheses are as follows;

1.3.1 Microspore development stage in term of the percentage of uninucleate microspore stage varies among floret whorls of the flower bud at the R5.1 reproductive stage, and it correlates with floret characteristics such as floret size and/or anther size that could be used as a morphological marker for microspore developmental stage.

1.3.2 Microspore viability and production varies among sunflower varieties and it relates with the efficiency of anther culture.

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1.3.3 Culture medium, varieties, and floret whorl affect haploid plant production via anther culture in term of the percentage of callus induction and the percentage of embryonic callus induction.

1.3.4 Chromosome doubling in anther-derived embryonic callus is affected by colchicine concentration and duration time.

1.4 Scope and limitations of the study

In anther culture study, three sunflower varieties were selected including; S473 (synthetic variety), Prado Red (hybrid variety), and Pacific 22 (hybrid variety). The microspore developmental stages were identified using a light microscope (LM) and electron microscope (TEM and SEM). Colchicine is used as a duplication inducing reagent at different concentrations and time conditions. The percentage of uninucleate microspore stage, the percentage of callus induction, the percentage of embryonic callus induction, the percentage of plant regeneration, and the percentage of chromosome doubling were determined.

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1.5 Expected results

The expected results from this study were:

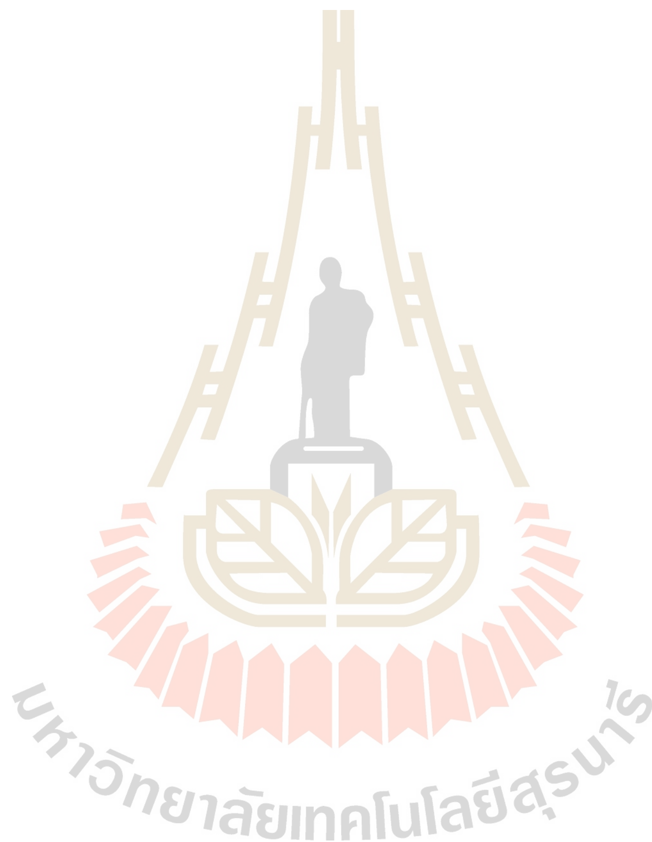
1.5.1 Microspore developmental stages of sunflower were identified and may differ among floret whorls of the R5.1 reproductive stage.

1.5.2 Morphological markers for microspore developmental stage base on floret characteristics are obtained such as floret size and/or anther size.

1.5.3 Identification and optimization of factors affecting anther-derived regenerated DH plants.

1.5.4 The optimum colchicine concentration and timing for chromosome doubling in anther-derived embryonic callus.

1.5.5 Doubled haploid sunflower plants via anther culture that could serve as parental materials for breeding in the future.



CHAPTER II

LITERATURE REVIEW

2.1 Sunflower characteristics

Sunflower (*Helianthus annuus* L.), family Asteraceae, is now a valuable oilseed crop throughout the world. Sunflower seeds are the harvested crop plants as a source of essential oil and feed for animals (U.S. Department of Agriculture, 2016). In 2015-2016, sunflower seed production was around 40.9 million metric tons and accounted around 7.57% of the total of the world after soybean and rapeseed (Food and Agricultural Organization of the United Nations, 2016). Sunflower seeds are highly nutritious because they contain about 43.21% of unsaturated oils (C18:1), which is higher than soybean oil and palm, vitamin E (36.33 mg), α-tocopherol (425.67 mg/kg oil) and protein (20.06 g), depending on the genotypes (Radic et al., 2008; U.S. Department of Agriculture, 2017). Types of sunflowers classified according to their use can be divided into two types of oil extraction and ornamental varieties. The oil extraction variety is characterized by a large inflorescence and a single head. While the ornamental varieties have more than one branch, the flowers are small and the ray petals have several color shades such as red or cream (Beard, 1981). The important part of sunflowers is a head that produces the seeds composed of oil to use. The head is composed of multiple whorls of disk florets surrounded by a single whorl of ray floret (Figure 2.1). The ray flower characters have a strap-like petal and make up the yellow circle at the edge of the head, zygomorphic flower, which is sterile (Figure

2.2A). The disk floret characters have a tubular corolla, actinomorphic flower, and are fertile (Figure 2.2B, C). Sunflower leaves are a simple, rough surface, cordate shape, with three main veins, and alternately arranged on the stem. The achene, fruit of sunflower that consists of a single seed, often is called the kernel, and the adhering pericarp that usually is called the hull. The seed coat consists of the ovary wall and testa fuse together (Figure 2.3).

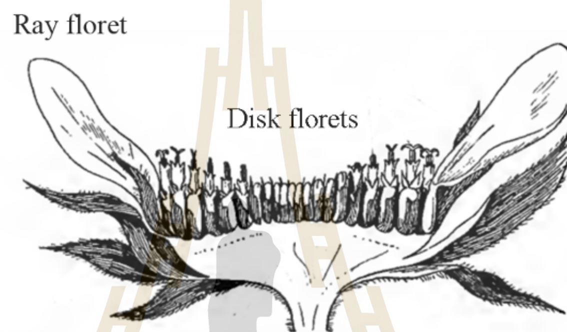


Figure 2.1 Vertical section of sunflower head (Percy, 1898).

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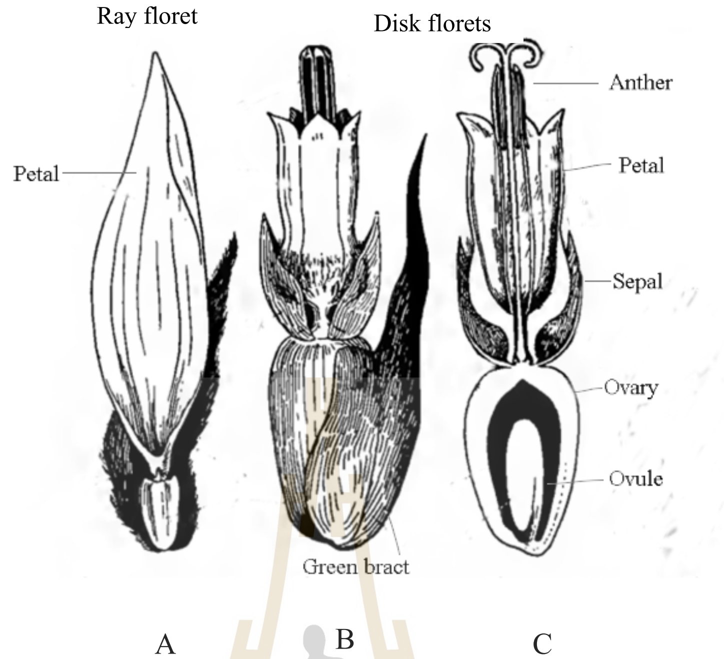

Figure 2.2 Composition of sunflower florets. A) Ray floret, B) Closed disk floret, and C) Opened disk floret (Percy, 1898).

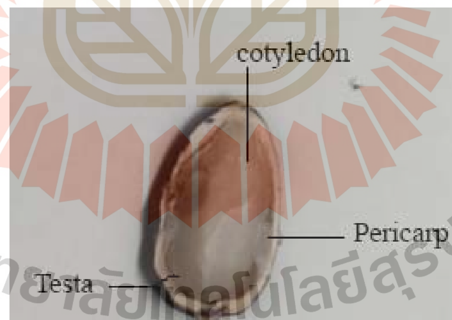

Figure 2.3 Parts of sunflower seed.

The sunflower is cultivated primarily for its seeds, which yields one of the world's most important sources of edible oil. Sunflower oil is considered as premium oil because of high levels of unsaturated fatty acids, lack of trans fat and high oxidative stability (Mekki et al., 2002). The sunflower oil is used for cooking and margarine production. Moreover, the leftover meat after oil extraction is a valuable

animal feed. In contrast, ornamental sunflowers are cultivated worldwide. The development of ornamental sunflower for floricultural work has led to using them in home garden decoration, in pots or like a cut flower for vase and bouquets for weddings (Mladenovic et al., 2016). The aims of ornamental development are desirable plant architecture, the color of ray and disk flowers and flowering period (Miklic et al., 2008). The popular ornamental variety is red color shading such as Prado Red hybrid developed by Pan American (Kalb, 2009) (Figure 2.3).

2.2 Growth stage of sunflower

Plant development progresses through a distinct phase of vegetative phases, followed by a reproductive phase and eventually seeds set and senescence (Huijser and Schmid, 2011). Sunflower is an annual plant and takes around 125 days to grow from a seed to seed set, depending on the variety and environmental condition (Dagustu et al., 2012). The developmental stage of sunflower is divided into vegetative (V) and reproductive (R) stages. The early vegetative stage begins when the seed has emerged and occurred the cotyledon, seed germination and adult vegetative. There is increased activity, such as photosynthesis and nutrient accumulation, during the reproductive stage. As the seedling continues to grow, the true leaves have occurred and continued development. The vegetative stage is determined by counting of true leaves. The reproductive stage begins at the terminal bud forms a miniature floral, the inflorescence opens, and both ray and disk florets are blooming activity until the end of seed setting (Figure 2.4) (Schneiter and Miller, 1981). A representative of each reproductive stage with the letter R and a corresponding number will help identify the specific stage during this period; for

example, R-5.1 means disk floret bloom at 10 percent of the area appears in the heads (Table 1).

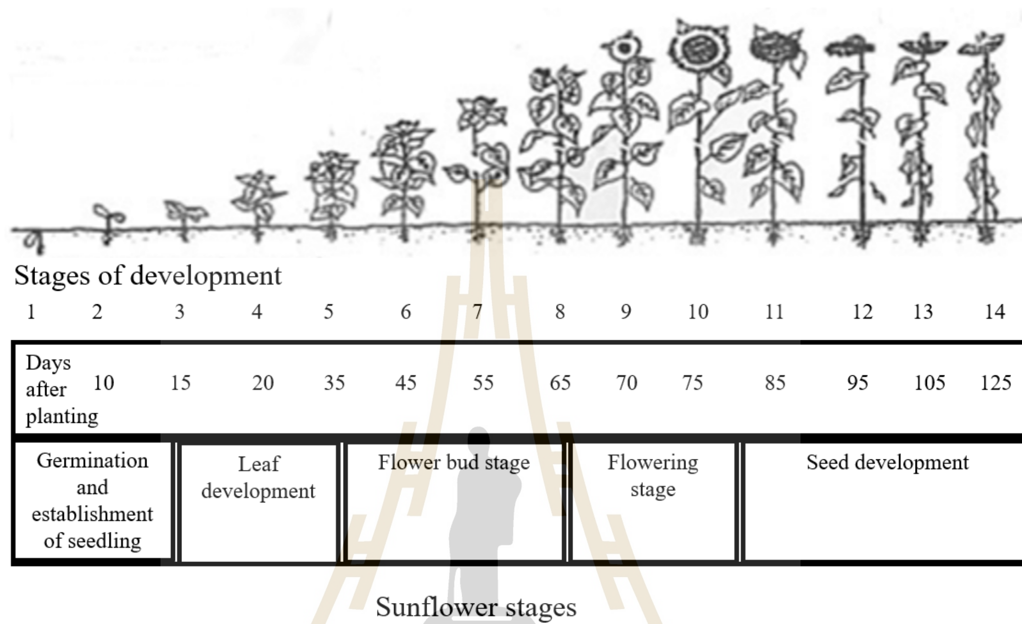

Figure 2.4 A schematic representation of sunflower developmental stages. (Agriculture Environmental Affairs and Rural Department, 2016).

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Table 2.1 Sunflower growth stages.

Stages	Description
VE	Seedling has emerged and the first leaf beyond the cotyledons is less than 4 cm long
V (number) (i.e. V-1, V-2, V-3, etc.)	These are determined by counting the number of true leaves at least 4 cm in length beginning as V-1, V-2, V-3, V-4, etc. If senescence of the lower leaves has occurred count leaf scars (excluding those where the cotyledons were attached) to determine the proper stage
R-1	The terminal bud forms a miniature floral head rather than a cluster of leaves. When viewed from directly above the immature bracts form a many-pointed star-like appearance
R-2	The immature bud elongates 0.5 to 2.0 cm above the nearest leaf attached to the stem. Disregard leaves attached directly to the back of the bud
R-3	The immature bud elongates more than 2.0 cm above the nearest leaf
R-4	The inflorescence begins to open. When viewed from directly above, immature ray flowers are visible
R-5 (decimal) (i.e. R-5.1, R-5.2, R-5.3, R-5.4, R-5.5 through R-5.9, etc.)	This stage is the beginning of flowering. The stage can be divided into substages dependent upon the percent of the head area (disk flowers) that has completed or is in flowering. Ex. R-5.3 (30%), R-5.8 (80%) etc
R-6	Flowering is complete and the ray flowers are wilting
R-7	The back of the head has started to turn a pale yellow color
R-8	The back of the head is yellow but the bracts remain green
R-9	The bracts become yellow and brown. This stage is regarded as physiological maturity

Schneiter and Miller (1981).

2.3 Sunflower classification

Sunflowers have many varieties, such as wild and domestic varieties. They are grown for seed and oilseed extraction, ornamental garden flower and food for animals.

Sunflower can be classified by flowering branches into two types (Emino and Hamilton, 2003). The first, the wild variety is typically characterized by many branches, small head and relatively small achene, and initially cultivated popularity as a garden ornament. It has been a source of genes for cytoplasmic male sterile and fertility restorer and pathogen resistance. Some varieties can be also found on the market include Prado Red (Figure 2.5). The second, the domesticated variety is typically characterized a single large capitulum, large achene, single stem and higher seed oil content than wild forms and they are continually developed for oilseed type and confectionery types (Figure 2.6) (Cronn et al., 1997).

Figure 2.5 Ornamental sunflower varieties with multiple small capitulums.

Figure 2.6 Cultivated sunflower with a single large capitulum.

2.4 Sunflower reproduction

The cultivated sunflower is an annual crop plant that is only propagated by seeds which can hybridize spontaneously with wild varieties (Burke et al., 2002). Sunflower reproduction is sexual through the method of pollination. It is also known as crossbreeding, in contrast, inbred line breeding is self-pollination. The disk florets exhibit protandry that is the male gametes mature before the female gamete (Smart et al., 1994). Self-pollination variety is not adding pollinator, while cross-pollination is adding pollinator like a bee. The large flower is mainly characteristic of this plant, and it consists of the outermost whorl defined by ray floret. It is generally yellow ray florets. They are sterile because they remnants of aborted stamens and empty ovaries and the petal of ray floret can be yellow, red, orange, or other colors (Knowles, 1978); while the inner whorl one called disk florets that they are a perfect flower. The bloom of the disk florets starts from the outside moving to the center of the head. The perfected disk flower is the important propagation of sunflower. It consists of stamen

and pistil parts. Both sections act to remove the genetic traits of parents to the next generation.

2.4.1 Stamen development

The male reproductive organ is produced in the stamen, which consists of anthers that produce pollen. The androecium consists of five fused stamen unit of their anthers, which form a tube around the style. The stamen unit separated filaments attached to the base is located inside the tubular of each disk floret (Figure 2.7). The receptive surfaces of the stigma are in close contact in the bud stage before the opened flower. Sunflower filaments consist of parenchyma cell around a vascular bundle which may be amphicribral. The vascular bundle ends blindly either on the anther base or in the connective tissue between the two anther halves.

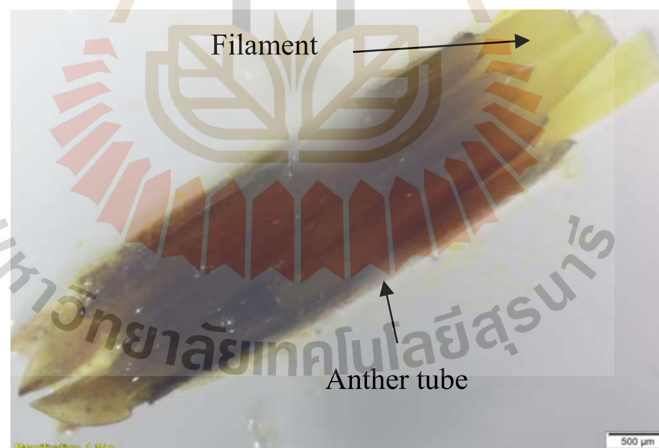

Figure 2.7 Sunflower stamen.

Male reproduction, pollen occur in pollen sac (microsporangia) of anther, and it can be divided into two major processes as microsporogenesis and microgametogenesis (Bedinger, 1992). Each anther consists of four layers; from the exterior: the epidermis, endothecium, middle layer and tapetum (Gotelli et al., 2008;

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Nakshima and Hosokawa, 1974). The microspore mother cell (MMC) consisting of tapetum cells are large and often binucleate (Figure 2.8A). They provide an excellent target control of fertile genes (Mariaini et al., 1992). Initially, in meiosis stage I, the callose (β-1-1-glucan) forms asymmetrically around each MMC, leaving many gaps through massive cytoplasmic channels form between the neighboring MMCs (Gotelli et al., 2008). In the microspore tetrad stage, MMCs undergo simultaneous meiosis forming tetrad microspores (Figure 2.8B) with a tetrahedral arrangement and the exine is beginning to develop and is surrounded by a thick callosic wall (Hsu et al., 2013). Four uninucleate-microspores are released from microspore tetrad, and each has a dense cytoplasm, inconspicuous vacuole and a membrane is formed covering the outer tangential wall of the tepetum cell and in touch with the middle layer (Figure 2.8C). Toward the end of pollen development, the periplasmodium is lost and its contents are deposited on the surface of pollen in the form of sporopollenin (Shivanna and Sawhney, 1997). While the initial of microgametogenesis, pollen nuclei were divided into large vegetative nuclei and small generative nuclei (Figure 2.8D). During pre-anthesis, the generative nuclei produce two sperm cells (Figure 2.8E).

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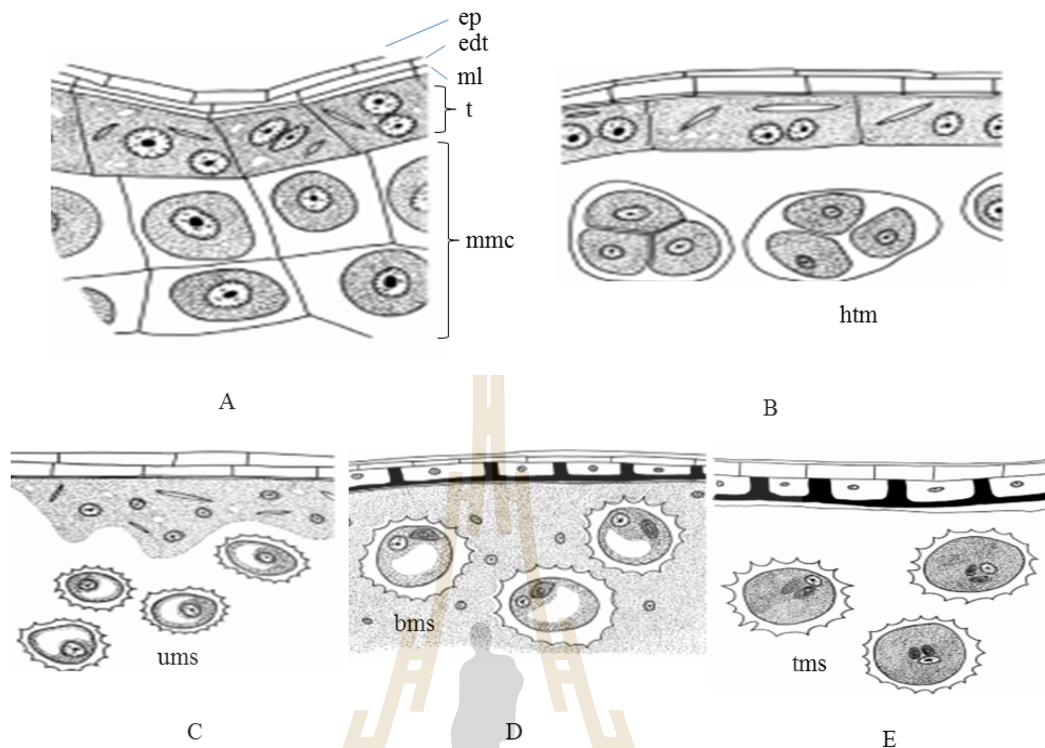

Figure 2.8 Illustration of microsporogenesis in sunflower. A) Tapetum layer of the anther, B) Tetrahedral microspore, C) Released microspores, D) Two nuclei microspore, and E) Three nuclei pollen grain. (bms = bi nucleate pollen, ep = epidermis, edt = endothecial layer, htm = tetrahedral microspore, ml = middle layer, mmc = microspore mother cell, ums = uninucleate microspore, t = tapetum, tms = tri nucleate pollen (Gotelli et al., 2008).

As demonstrated by several researchers such as Klimko et al. (2000); Wortley et al. (2007); Coutinho and Dinis (2007); Coutinho et al. (2012); and Wortley et al (2012), the morphology of pollen such as the structure of the pollen exine using a light microscope (LM), transmission electron microscope (TEM) and scanning electron microscope (SEM) has been studied. Knowledge of morphology of pollen is a great importance to understand the plant systematics of the Asteraceae. LM is used

to examine pollen identification. TEM reveals the complex structure of pollen cell wall, while SEM examines the pollen wall architecture for morphology and taxonomy (Jone and Bryant Jr, 2007).

Pollen grain morphology describes in the terms of shape, size, aperture, and ornamentation. The pollen shape refers to the ratio of P/E, for example, P/E ratio is more than 2, it is defined to perprorate shape and P/E ratio is 1-0.88, it is called prolate-spheroidal. Pollen size refers to the polar diameter (P) and equatorial diameter (E). Generally, pollen size ranges from 4 - 245 µm. Erdtman (1952) classified pollen size based on the longest axis of pollen. If it is 10-25 µm, it is called small grain. If it is 25-50 µm, it is called medium size grain. If it is 50-100 µm, it is called large grain. The pollen aperture in *Helainthus annuus* L. occurs as tricolporate aperture. The ornamentation of pollen comprises spines is called echinate pollen grain as presented in Figure 2.9 (Punt et al., 2007).

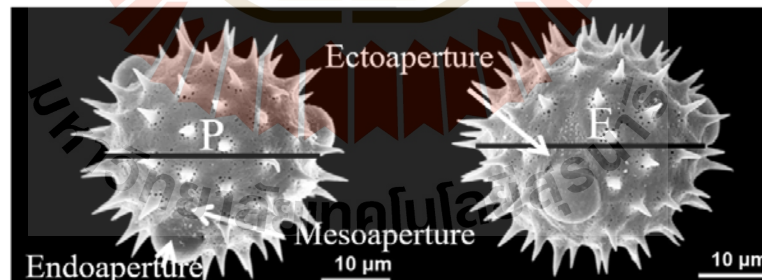

Figure 2.9 Pollen measurement technique and terminology. E = equatorial axis, P= polar axis (https://www.paldat.org, 2016).

2.4.2 Pistil development

Eggs are developed from the megaspore mother cell (MMC) through meiotic division and then develop into seeds after being pollinated. In sunflower, the MMCs contains within the ovule which it is located at the base of the ovary. The ovule is covered with ovary and it is attached to the placenta. The ovule consists of a thin tissue layers called integument, embryo sac, nucellus, and chalaza. The nucellus cell is a thin layer located between embryo sac and integument. The vascular bundle passes through the placenta and chalaza (Figure 2.10) (Voronova, 2013).

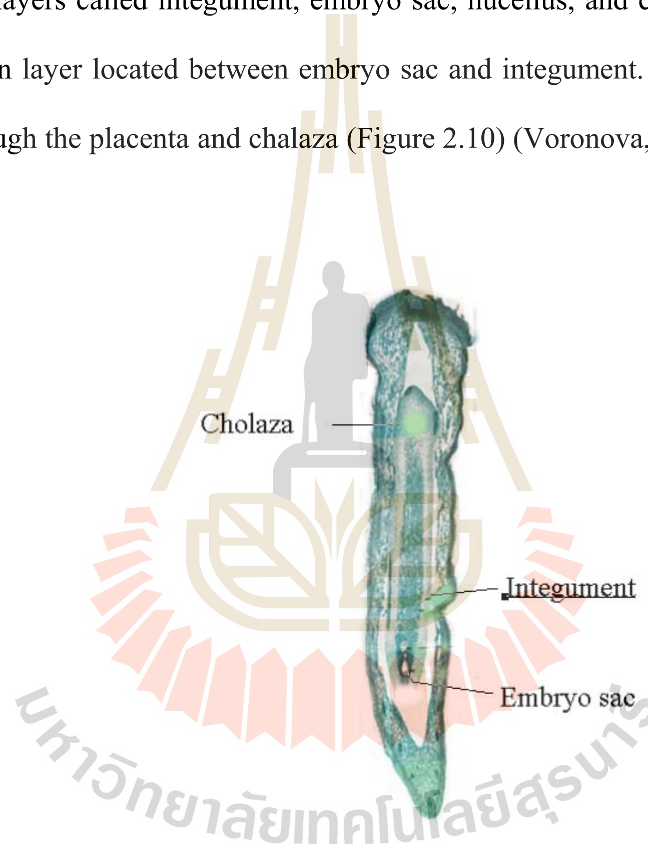

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Figure 2.10 Sunflower ovule (Voronova, 2013).

2.5 Sunflower breeding

The core of plant breeding is the selection of the best type among a variety of new generation, in terms of agronomic traits and it can be defined as the application of techniques for exploiting the genetic potential of plants. In addition, the sunflower

breeders emphasize developing new varieties that improve some characteristics such as high seed yield, high oil content and resistance in different stresses (Kaya, 2010). The challenge in plant breeding depends on the ability to identify promising parents, to combine the desirable attributes through hybridization, and select effectively among segregating populations (Breseghello and Coelho, 2013).

The earliest method in sunflower breeding knowledge as conventional breeding has been the selection of the best progenies of a random sample of the population. The plant breeding is still under development, with plant biotechnology and molecular biology recently added as new tools called the modern plant breeding (Stoskopf et al., 1993). It has allowed more precise and rapid sunflower crop better than varieties and they are very fast through target plant breeding. The desired gene may be added to a strand of new varieties. In contrast, conventional breeding may combine many genes from parental plant to be transferred to new varieties (Figure 2.11).

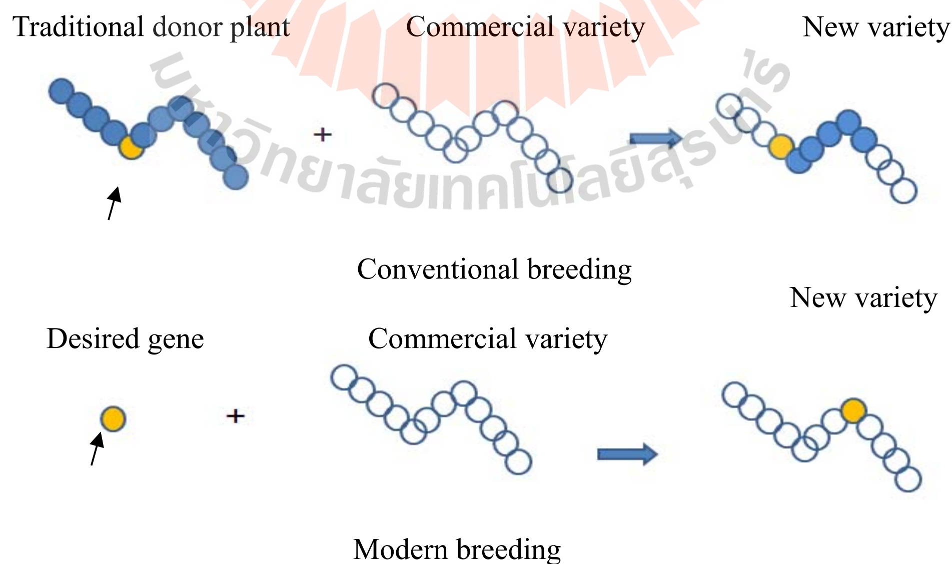

Figure 2.11 Comparison between traditional breeding and modern breeding (Breseghello and Coelho, 2013).

2.5.1 Conventional breeding

Conventional plant breeding is the process of using a good characteristic parental plant to create new offspring plants which may be share characteristic from each parents and can be considered as the manipulation of the combination of chromosomes (Va de Wiel et al., 2010). It has been very successful, but are now slowly in yield. The conventional breeding crosses between the different plants through pollination process or the self-pollination. In general, consist of three main procedures to manipulate plant chromosome combination. First, plants of a given population, which show desired traits can be selected and used for further breeding and cultivation, a process is called pure line selection. Second, desired traits found in different plant lines can be combined together to obtain plants which exhibit both traits simultaneously, and a method termed hybridization. Heterosis, a phenomenon of increased vigor, is obtained by hybridization of inbred lines. Third, polyploidy (increased number of chromosome sets) can contribute to crop improvement. Finally, the new genetic variability can be introduced through spontaneous or artificially induced mutations (Jan and Seiler, 2007).

2.5.2 Modern plant breeding

Biotechnology has provided tools for plant improvement that desired gene is transferred to a good variety (Figure 2.11). It is being developed to be done more precisely, quickly and cheaply (Kumar et al., 2009). The development of a new sunflower genotype was previously done using modern breeding. Sandu et al. (1999) applied the genetic method for studying a genetic control of branching stems in sunflower. They found *b1* gene, which controls the branching stems in plants. Yue et al. (2008) observed that the lemon gene (*yf1*) controlled the color of petal of ray floret.

1) Mutation breeding

Instead of relying only on the introduction of genetic variability from the wild species gene pool or from other cultivars, an alternative is the introduction of mutations induced by chemicals or radiation. The obtained mutants are tested and further selected for desired traits. The site of the mutation cannot be controlled when chemicals or radiation are used as agents of mutagenesis. Because the great majority of mutants carries undesirable traits, this method has not been widely used in breeding programs. Diseases are the major problem of yield reduction in sunflower such as Alternaria leaf spot. The chemical mutation as ethyl methanesulfonate (EMS) was applied to sunflower to create new offspring which is resistant to Alternaria leaf spot (de Oliveria et al., 2004). Cvejic et al. (2014) were successful in sunflower mutation, and found that application of mutagens caused changes in fatty acid composition in sunflower oil.

2) Precision breeding

Plant breeding method is a new tool for improving the genetics of plant in which it transfers only genetic sequences of testing and known expression into existing outstanding cultivars. The precision plant breeding can be divided into two types as molecular marker-assisted selection (MAS) and genetic engineering (Sriwatanapongse et al., 2007). The application of precision breeding was done in sunflower variety because *Helicoverpa armigera* caused of yield loss in sunflower seed as much as 30% (Çaliskan and Dangol, 2016). Seed yield of sunflower loss is also caused by Charcoal rot disease due to some fungi species. The transgenic gene method is applied using OXO gene transfer into hypocotyl of sunflower with *Agrobacterium* mediated transformation (Hu et al., 2003).

3) Polyploid breeding

Polyploid is a genetic condition of organisms which a cell has multiple sets of chromosomes in excess of the diploid number and it is the major of adaptation and speciation from nature (Ramsey and Schemske, 1998). The polyploidy can arise spontaneously in nature and has occurred frequently in any organism, such as some angiosperms about 50-70%, which include many crop plants. Polyploids may be classified based on their chromosomal composition into either euploids or aneuploids. Euploids constitute the majority of polyploids (Acquaah, 2007). In sunflower, polyploids are induced by colchicine, which causes diploid microspore mother cells to divide into tetraploid plant species (Gautam and Kuman, 2013).

2.6 Anther culture techniques

Anthers are made to divide and grow into callus or embryonic callus in either a liquid medium or on solid medium. Pollen-containing anthers are removed from an unopened flower bud and then put in a culture medium; some microspores survive and develop into tissue or organism. If embryonic tissue develops, it is put in a medium favorable for the shoot and root development. If it is callus tissue, it is put in a solution of hormones, it will be induced to differentiate and developed shoot and root tissue, then generated into new plants.

Anther culture, one of the very popular methods for production of haploids, can be done through culturing anthers or microspores on artificial culture medium. Each MMC in the anther produces 4 microspores or pollen grains. Thus an anther with microspores can be cultured on artificial medium for raising into haploid plants. Generally, haploid plant production is one way to shorten the time in this process. It allows novel allele combinations, particularly ones involving recessive characters, to

be assessed in intact plants. Haploid production through anther culture has been referred as androgenesis (Guha and Maheshwari, 1966). Haploids have been obtained in more than 150 species in to 23 families of angiosperms (Maluszynski et al., 2007).

Doubled haploid breeding methods involve making haploid tissues or plants (n) from heterozygous parents and doubling the chromosomes in order to obtain diploid plants (2n), which is referred to as doubled haploids as presented in Figure 2.12 (Baenziger, 1996). After chromosome doubling every gene is homozygous, and therefore each doubled haploid plant is considered to be homozygous. Today, doubled haploid cultivars are the favored choice of many breeders and are often used as parental materials for F1 hybrid seed production. Doubled haploid techniques can greatly reduce the time needed to obtain stable resistant lines suitable for future breeding efforts on many crops. In sunflower, for example, by using doubled haploid technology, the time required to produce inbred lines is reduced from six or more generations of self-pollination to two (Trigiano and Gray, 2011).

The potential of sunflower haploidization was first tested by Bohorova et al. (1985). Development of haploid plants in sunflower has been attempted through gynogenesis (Gelebart and San, 1987), anther culture (androgenesis) (Gurel et al., 1991; Zhong et al., 1994; Vijaya Priya et al., 2003) and microspore culture (Counans and Zhong, 1995). Anther culture in sunflower still needs considerable improvement, as the sunflower is proved to be very recalcitrant in anther culture (Mezzarobba and Jonard, 1986), and exhibited poor reproducibility and doubtful ploidy status (Saji and Sujatha, 1998). As in other species, anther culture response of sunflower is strongly affected by physical, nutritional, physiological and genetic factors (Gurel et al., 1991).

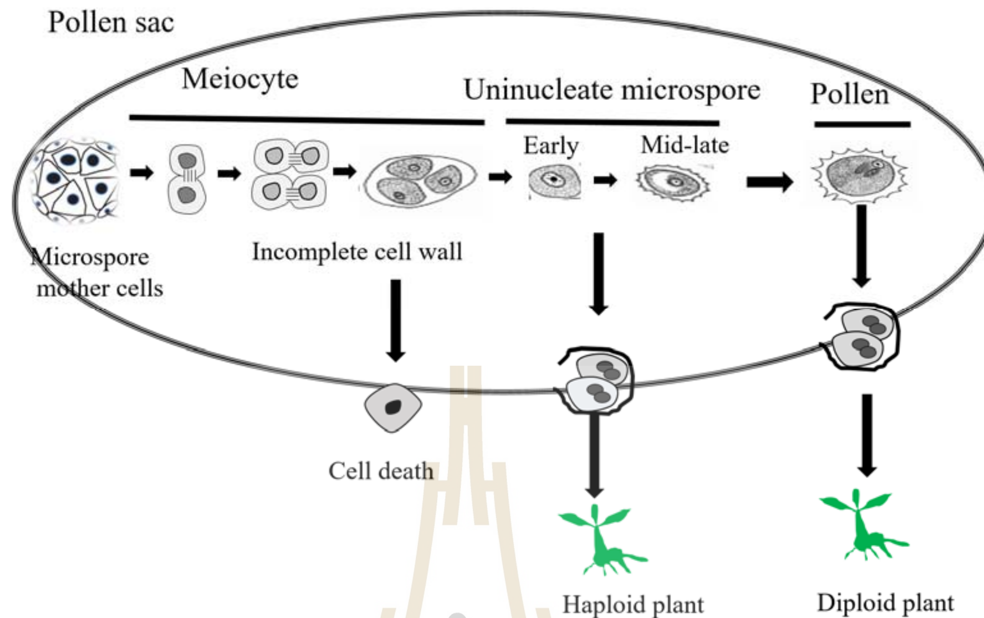

Figure 2.12 Haploid plant production pathway (Seguí-Simarro and Nuez, 2006).

2.7 Factors affecting the success of anther culture

The success of doubling haploid from anther culture or microspore culture in sunflower depends on many factors.

a) Physiological status of donor plants

The selection of starting plant material for anther culture in sunflower is very importance. The donor plants should have good healthy growth with disease and pest free and they should get the desired lighting, water, and fertilizer. For some of the donor species, the number of pollen per anther is important for the success of haploid production (Schrammeijer et al., 1990). Plants starved of nitrogen may give more responsive anthers compared to those that are well fed with nitrogenous fertilizers. Therefore, only materials grown under controlled environmental conditions should be used. Application of pesticide should be avoided for 3-4 weeks preceding sampling. The anther section from wheat or barley is grown during October to December

provided an excellent anther response (Datta, 2005). While rice plant grown during the summer season has provided the best anther culture (Park et al., 2013).

Pollen viability is one of physiological donor plant factor for anther culture. Pollen viability implies the ability of pollen complete post-pollination. Pollen viability is important for anther culture or pollen biological works. With stored pollen or sterilized pollen before culture also, its viability needs to be monitored after storage (Shivanna and Rangaswamy, 1992). Machikowa et al. (2012) reported viability in sunflower hybrid cv. Pacific 77 to be from 89.80% to 99.00% and synthetic variety cv. S473 from 55.67% to 96.93%.

b) Genotype of donor plants

The success of *in vitro* anther culture depends mainly on the plant genotypes (Nurhidayah et al., 1993). Haploid plant production via androgenesis has been very limited or nonexistent in many plant species. Furthermore, within a species, differences exist as to the ability to produce haploid plants. In sunflower, some genotypes produce haploids at a much higher rate than do others (Trigiano and Gray, 2011). Moreover, anther culture of sunflowers at the same stage and with the same combination of growth regulators, callus production, and plant regeneration capacity depend essentially on genotypes (Ozyigit et al., 2007; Sujatha and Prabakaran, 2006). In potato, microsporogenesis has also been influenced greatly by genotype of donor plants (Jacobsen and Sopory, 1978).

Interspecific sunflower is donor plant factor which is imperative to know the extent of anther culture method. There are several works about interspecific hybrid sunflower through anther culture. For example, Todorova et al. (1997) cultured six F1 hybrids between cultivation sunflower and wild sunflower, and they reported 500 shooting formed in *Helianthus mollis* x *Helianthus annuus* L. Nurhidayah et al.

(1993) reported 97 shooting formed from sunflower and wild sunflower. Vijaya Priya et al. (2003) reported 34.0% callus proliferation in *Helianthus annuus* L x *Helianthus occidentalis*.

c) Stage of microspore development at the time of culture

The stage of microspores within an anther at the moment of isolation is of vital importance for haploid induction. Most plant species, the productive anthers contain the uninucleate stage of microspores midway between release from the tetrad and the first pollen grain mitosis (Sujatha and Prabakaran, 2006; Sunderland and Dunwell, 1977). The anther of maize containing early to bicellular pollen is suitable for anther culture (Jahne and Lorz, 1995). The first cell division is a small generative nucleus and a large vegetative nucleus arise can be identified by the Feulgen-staining, to reveal the cell nuclei (Pierik, 1997). Several researches used sizeflower head as a morphological marker for sunflower anther culture such as flower head diameter 1.5 to 2.0 cm (Vijaya Priya et al., 2003), R5.1 and R5.2 reproductive stages (Phaosang et al., 2003; Krudnak et al., 2013).

Morphology of microspore is important for anther culture or microspore culture. When microspores place on the medium, they require nutrients from the synthetic medium through the aperture. The pollen wall is divided into a set of layers. The outermost is sexine layer, nexine and intine, respectively. In sunflower, the term of colporate aperture is used to describe a pollen grain that has compound aperture consisting ectoaperture and endoaperture. The ectoaperture is formed on sexine, while endoaperture is formed on nexine (El-Ghazaly and Anderberg, 1995).

d) Nutritional factors

Various basal media with modified components have been established and studied for anther cultures. The list of media suitable for a wide range of species is

constantly expanding; however, most media are specific for a few or even only one genotype (Trigiano and Gray, 2011). In the case of sunflower species, androgenesis can be induced on a basal medium such as Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), Nitsch and Nitsch (N6) media (Chu, 1978), Gamborg's media (B5) (Gamborg et al., 1968), or variations on these media supplemented with the same hormones concentration and sucrose. In the result, MS basal medium induced new shooting regeneration more than the other basal medium about 10-15% (Thengane et al., 1994; Nurhidayah et al., 1996; Todorova et al., 1997; Vijaya Priya et al., 2003). Several basal media consists of salt composition. The salt composition had moderate to dramatic effects on planting efficiency, embryogenesis and plant regeneration in barley (Mordhoest and Lorz, 1993). Complex organic compounds like coconut water, coconut milk, biotin and casein hydrolysate have been added to the media. MS medium supplemented with the casein hydrolysate was the best medium for regenerating shooting from sunflower anther (Vijaya Priya et al., 2003).

e) Plant growth regulators or hormones

For the plant growth regulators (PGRs), they are necessary to be added in anther culture medium because they control differentiation and growth in anther-derived callus until callus regenerates into a complete plant. Most sunflower genotypes require a low concentration of some PGRs in the media. For example in anther culture of sunflower genotypes, MS media supplemented with 0.5 mg/l benzylaminopurine (BAP) and 0.5 mg/l of α-naphthaleneacetic acid (NAA) could promoted callogenesis rate 86% and 92.7% embryo-like somatic (ELS) in sunflower (Nurhidayah et al., 1996). MS medium added with 2.0 mg/l of NAA and 1.0 mg/l of BAP induced embryonic callus rate about 30.4% in sunflower (Saji and Sujatha, 1998). MS medium supplemented with 250 mg/l of casein hydrolysate, 1.0 mg/l of

NAA, 2.0 mg/l of 2, 4- D and 0.5 mg/l of BAP produced more than 92.23% of callus induction in sunflower (Dodds and Roberts, 1995). MS basal medium added with 1.0 mg/l 2, 4-D and 0.5 mg/l BAP could be induced 10-15% of plantlets about in sunflower (Thengane et al., 1994).

The media containing PGRs, may be useful and benefit for callus or completely plantlet induction. The PGRs are also a main powerful reagent for chromosome variation (Karp, 1994). Both types and concentration of PGRs influence gametoclonal variation. However, unbalance of auxin and cytokinin may induce polyploids. The synthetic auxin, 2, 4-D is often associated polyploids and stimulate chromosome synthesis, while high cytokinin level did not affect to chromosome variation (Nehra et al., 1992). *In vitro* anther culture of sunflower, the gametoclonal variation through an intermediate callus phase could be induced and observed as phenotypic variation such as chlorophyll and carotenoid deficiencies, chimeric variegation, fasciated stem and capitulum, abnormal shoot development, and other morphological variations (Barotti et al., 1995).

2.8 Haploid in sunflower and chromosome doubling technique

Haploids are plants that contain a gametic chromosome number (n). The potential of haploidization in plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of *Datura* anthers (Guha and Maheshwari, 1966), which was followed by successful *in vitro* haploid production in tobacco (Nitsch and Nitsch, 1969). The successful use of the haploid technique to improve bread-marking quality holds true only when breeding aims are reconciled without compromising yield and quality. The numerous *in vitro* methods can be

divided into two categories (Figure 2.13). Androgenesis is the process by which haploid plants are developed from the male gametophyte. When anthers are cultures intact, the procedure is called anther culture (Figure 2.13A). For haploids derived via the female gametophyte are obtained through a process known as gynogenesis in which haploid cells are stimulated to develop into an embryo in an induced process similar to parthenogenesis (Trigiano and Gray, 2011). The embryo sac culture in sunflower consists of seven cells: egg cell, two synergids, and three antipodals. It can develop a new plant when it is cultured in synthetic medium supplemented with suitable hormone (Figure 2.13B).

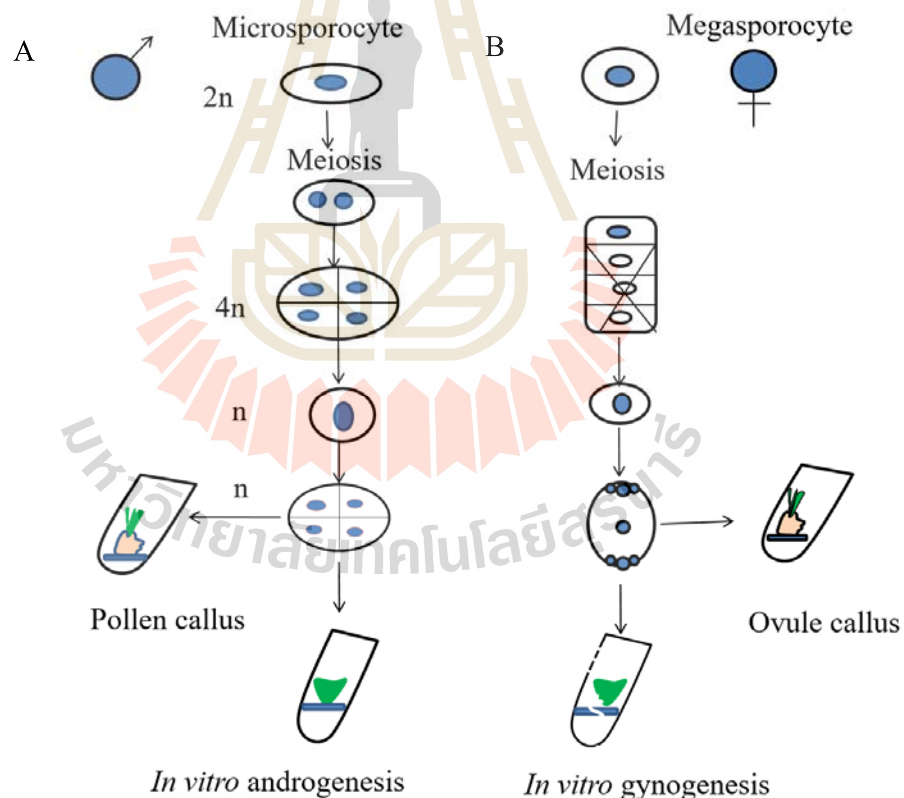

Figure 2.13 Schematic diagram of haploid production procedures. A) androgenesis, B) gynogenesis (Pierik, 1997).

In the development of inbred lines by investigation on the use of doubled haploid produced by androgenesis, chromosome doubling of haploid cells is a critical step in producing doubled haploid plants (Mohammadi et al., 2007). Colchicine agent is an important chromosome doubling agent which inhibits spindle formation resulting in doubling chromosome number during cell division process (Levan, 1938). The spontaneous chromosome doubling occurring during androgenesis in the nature of maize is an average of 10% (Buter, 1997), exceeding 50% in barley (Lyne et al., 1986). For artificial doubling chromosome, there were several investigations about the effect of colchicine on anther culture derived plants in wheat (Zammani et al., 2000), rice (Chen et al., 2001), sunflower (Downes and Marshell, 1983), and wild x cultivated sunflower interspecific hybrid (Jan, 1988). Colchicine has been the most successful agent when applied both *in situ* and *in vitro* conditions in sunflower (Todorova et al., 1997).

2.9 Polyploidy level determination

Polyploidy, the numbers of sets of the chromosome in the cell of a living thing, compose of more than three sets of chromosomes and has been significant occurrence in the evolution for genetic of cultivated plant species (Omezzine et al., 2012). Doubled haploid production through *in vitro* has long been associated with the phenomenon of gametoclonal variation. The gametoclonal variation may be a disadvantage or an advantage for plant breeding (Jain et al., 1996). The examples of gametoclonal variation in crop plants such as *Brassica napus* (2n=38) chromosome number occurred 2n, 3n, and 4n (Thomas and Wenzel, 1957), *Nicotiana sylvestris* (2n=24) chromosome number occurred n, 2n, 3n, 4n, mixoploid (McComb and

Mccomb, 1977). In addition, changes in polyploidy level during subculture as the influence of plant growth hormone conducted possible chromosome variation (Dolezal and Novek, 1984).

The polyploidy level of the anther derived callus is determined with several methods such as cytological markers or flow cytometry, chromosome staining method including acetocarmine and aceto-orcein, which counts on metaphase. But some species such as *Vitis vinifera* L. has very small size of chromosomes, the result cannot clearly elucidate its polyploidy. Consequently, polyploid level is evaluated by flow cytometry which measures DNA content in plant tissue. Plant species were determined for nuclear 2C DNA content with flow cytometry. It was reported in sunflower to be about 7.6 pg (Price, and Johnston, 1996), young leaf of sunflower about 6.21 ± 0.7 pg, embryos excised from achene of sunflower about 7.51 ± 0.06 pg, sunflower growing in shade under a canopy of trees about 6.99 ± 0.21 pg (Maiti et al., 2007), and *Lactuca sativa* about 5.74 pg (Arumuganathan and Earle, 1991). Pintos et al. (2007) reported that anther-derived embryos of cork oak treated with 0.1 mM oryzalin showed 50% diploid induction, while 1.3 mM colchicine induced a low rate of diploid. Martin and Widholm (1996) reported the ploidy of individual embryo-like structures (ELSs) of maize anther cultures induction of 27% mixoploid and 20% diploid.

CHAPTER III

MATERIALS AND METHODS

3.1 Correlation between morphological characteristics of floret bud, microspore features and microspore developmental stage

3.1.1 Plant materials and plant growth

This experiment used seeds of three sunflower varieties as shown in Table 3.1. Twenty-five seeds were grown in the field of Suranaree University of Technology (SUT) campus. The seeds were planted about 2.54 cm deep in the soil, 20-25 cm in row spacing. Young plants were watered every day around the root zone. Chemical fertilizer (N-P-K/16-16-16 ratio) was applied to root zone once every two weeks. Flower heads at the R5.1 reproductive stage were collected at 7.00 am for the experiment; some of the collected heads were immediately fixed in a solution of glutaraldehyde fixation for cytological and morphological studies, while the remaining of heads were used for anther culture study.

Table 3.1 Sunflower varieties and their origin for the experimental study.

Name	Sunflower variety	Origin
Suranaree 473 (S473)	Synthetic	SUT
Prado Red	Hybrid	USDA
Pacific 22	Hybrid	Pacific Seeds (Thai) Ltd.

SUT = Suranaree University of Technology, USDA = United States Department of Agriculture.

3.1.2 Measurement of disk floret bud and anther size

Fifty unopened disk florets were selected from whorl 1, 2 and 3 of the R5.1 reproductive stage (referred to Table 2.1) from three sunflower varieties; S473, Pacific22 and Prado Red. The floret characteristics included the length of disk floret (mm), anther diameter (mm), and anther width (mm) using stereomicroscope (Olympus SZX9, Germany). Images were captured with a software program (cellSens, The Netherlands) for a digital camera (Olympus DP22, Germany).

3.1.3 Determination of microspore viability and production

For microspore viability determination, fresh microspores were evaluated with iodine-potassium iodide (IKI) solution. The microspores were distributed on a glass slide and stained with a drop of 0.5% IKI and covered with a coverslip. The viable microspore was counted at 10 min after staining under a light microscope (Olympus BX51, German). The images were captured under software program (CellSens, The Netherlands) for windows using a digital camera (Olympus DP73, German). The percentage of microspore viability detected as a red color was calculated as below.

$$\text{Percentage of viability} = \frac{\text{No. of viable microspore X100}}{\text{Total number of microspore}}$$

For microspore counting, a hemacytometer was used (Godini, 1981). Ten anthers were randomly selected from each sunflower varieties and put into a 10 ml glass vial, up to 1 h after collection, where they were broken with the left in a tube at room temperature. Adding 8 ml of a volumetric solution composed of 2 ml of Tween 20 and one drop of safranine O solution (Bolate and Pirlak, 1999) into a vial and then shaken gently to allow the microspore grains releasing. The final volume of suspension was adjusted with to 10 ml of DI water. The chambers of hemacytometer were filled with a drop of microspore suspension and covered with a coverslip. The microspore was counted using a light microscope (Olympus BX51, Germany). The images were captured under software program (CellSens, The Netherlands) for windows using a digital camera (Olympus DP73, German).

3.1.4 Observation of anther structure using LM

The embedded anther sample blocks were cut with an ultramicrotome (Leica; EM UC7, Austria) at semi-thin section (1µm). The semi-thin sections were transferred onto a microscope slide and stained with 1% toluidine blue O (TBO) at 85 ^{o}C for 5 min (Chaffey et al., 2002). The sample was washed with tap water for 2-3 min and covered with a coverslip. The structural anther samples were observed under a light microscope (Zeiss Axiostar Plus, Carl Zeiss Pty Ltd; Germany) and captured with a software program (Zen blue 1012, Germany).

3.1.5 Determination of microspore features

Ten unopened disk florets were selected from whorl 1, 2 and 3 of the R5.1 reproductive stage from three sunflower varieties; S473, Pacific 22 and Prado Red (Figure 3.1). The anthers were dissected from tubular of the floret and then placed into a vial containing 5 ml DI water. The vial was gentle shake until microspore releasing. One drop of sample was placed on the microscope slide and covered with a coverslip. The sample was determined for microspore diameter, including a polar axis (P) and equatorial axis (E) under a light microscope (Olympus BX51, German). The images were captured under cellSen software (cellSens, The Netherlands) for windows using a digital camera (Olympus DP73, German). P/E ratio was also calculated.

3.1.6 Determination of microspore developmental stage

3.1.6.1 Sample preparation for microscope analysis (both light microscope and scanning electron microscope)

Fresh anthers from outermost three whorls of unopened floret buds at the R5.1 reproductive stage were separated. Twenty-five anthers were carefully dissected and grouped under the 10x magnifying lamp and immediately fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for overnight at 4 ^{o}C. The anthers were washed with 0.1 M phosphate buffer (pH 7.2) three times for 30 min, post-fixed with 2% osmium tetroxide in phosphate buffer (pH 7.2) for 2 h and washed with phosphate buffer three times for 30 min per each. The anthers were dehydrated with series of acetone in water: 30%, 50%, 70%, 90% and 100% (3 times) for 1 h at the same room temperature. The samples were infiltrated with a Spurr's resin: acetone mixtures (v:v) 1:3, 1:1, 3:1 for 3 h and in pure resin

overnight at 4 °C. The mixtures were embedded into blocks and were heated in dryer oven at 70 °C for 8 h (Spurr, 1969).

3.1.6.2 Determination of microspore developmental stage using light microscope (LM)

The sample block from 3.1.5.1 was sectioned at semi-thin sections (1 µm) with an ultramicrotome (Leica; EM UC7, Austria) and stained with 1% toluidine blue O (TBO) at 85 °C for 5 min (Chaffey et al., 2002). The microspores were observed using Zeiss Axiostar Plus (Carl Zeiss Pty Ltd; Germany) microscope and captured with Zen blue 1012 program. The percentage of microspore developmental stage including early and mid-late uninucleate microspore stages was recorded.

3.1.7 Ultrastructure of microspore and its surface morphology

3.1.7.1 Determination of ultrastructure of microspore using transmission electron microscope (TEM)

The sample block from 3.1.6.1 was sectioned at thin sections (60 nm) with an ultramicrotome (Leica; EM UC7, Austria), mounted on 200 mesh gold grids and were double-stained with 2% (w/v) uranyl acetate and 2.6% (w/v) lead citrate solution (Venable and Coggeshall, 1965) and then observed to reveal the nucleus and the microspore wall with transmission electron microscopy (TEM-Hitachi HT7700, Tokyo, Japan).

3.1.7.2 Determination of surface morphology of microspore using scanning electron microscope (SEM)

For sample preparation using scanning electron microscope (SEM) (Punt et al., 2007), fresh microspores were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.2) for 12 h at 4 °C and then washed

with 0.1 M phosphate buffer (pH 7.2) three times for 30 min, postfixed with 1% osmium tetroxide in distillate water (DI) for 1 h, washed with DI water three times for 30 min, dehydrated with an acetone series in water: 20%, 40%, 60%, 80%, and 100% (three times) for 1 h per each, microspores were dryed with critical point dryer (Samdri-PVT-3B, Ohio, USA). The microspores were fixed on a cover slide and then placed onto a stub and coated in the Sputter with gold-palladium. Surface morphology, sculpture element, aperture number and aperture characteristics of microspore were determined under SEM-Hitachi SU8020 (Hitachi, Japan) and operated at 5 kV.

3.1.8 Statistical analysis

The experimental data were analyzed by variance analysis. The mean square was analyzed by one-way analysis of variance (ANOVA). The means of data were analyzed with correlation analysis of variances at the 0.01 level. The means ± standard deviation (SD) were separated by Duncan's Multiple Range Tests (DMRT) at the 5% level of significance using IBM SPSS statistics 23.

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3.2 Effects of varieties, floret whorl, and culture medium on haploid plant production via anther culture

3.2.1 Callus and shoot induction on various medium through anther culture of three sunflower varieties

The flower heads at the R5.1 stage of three sunflower varieties were selected. These flower buds were transported in cooling boxes to the laboratory. The buds were thoroughly washed with running tap water for 30 min, followed by 70% (v/v) ethyl alcohol for 30 sec. The flower buds were surface sterilized with 15% (v/v)

Clorox solution containing a few drops of tween 20 for 10 min, 10% Clorox for 5 min, and then washed three times with sterile water. In the last step, anthers from 1-3 whorls of unopened disk florets were excised from the flower head. The filament was completely separated from the anthers with a blade. For callus induction, MS (Murashige and Skoog, 1962) media supplemented with various growth regulatory hormones and organic substances (Table 3.2) were applied. Media were composed of 30 g/l of sucrose and 8 g/l of solidified agar. The pH of media was adjusted to 5.7 using a 1 N HCl or 1 N NaOH before autoclaving. Twenty-five ml of the medium were poured into 100 mm in diameter of culture plates (3 plates each). Twenty-five anthers were cultured on each culture plate. The cultures were maintained at 25 ± 2 ºC in darkness condition for 7 days, after that, they were cultured with 3,000 lux of light and 16:8 (light: dark) photoperiod. Induction of callus was observed every 2 days until thirty days of cultures. Growth parameters including fresh weight, dry weight, callus size, the percentage of callus induction (PC) and percentage of embryogenic callus induction (PE) were calculated as below.

$$\text{Percentage of callus induction (PC; \%)} = \frac{\text{Number of callus X 100}}{\text{Total number of anthers}}$$

$$\text{Percentage of embryogenic callus induction (PE; \%)} = \frac{\text{Number of EC X 100}}{\text{Total number of anthers}}$$

Table 3.2 Callus induction.

Media	Basal	CH (mg/l)	CW (ml/l)	Auxin (mg/l)		Cytokinin (mg/l)	References
				NAA	2,4-D	BAP	
A1	MS	500	-	2	-	1	Vijaya Priya et al., 2003
A2	MS	-	100	2	-	1	This study
A3	MS	500	-	-	0.5	0.5	Phaosang et al., 2003
A4	MS	-	100	-	0.5	0.5	This study

Remarks: BAP = Benzylaminopurine, CH = casein hydrolysate, CW = coconut water MS = Murashige and Skoog medium, NAA = α-Naphthaleneacetic acid, 2, 4-D = 2,4 -Dichlorophenoxyacetic acid.

3.2.2 Shoot and root regeneration of embryonic callus derived from anther culture

Forty embryogenic calli (EC) of each variety, which are whitish, globular friable, dry, free of any differentiated structures such as root-like appearance, were selected and transferred to regeneration medium supplemented with various hormones for 30-40 days (Table 3.3). The percentage of shoot regeneration and root regeneration were calculated as below.

$$\text{Percentage of shoot regeneration (\%)} = \frac{\text{No. of regenerated shoot X 100}}{\text{Total embryogenic calli}}$$

$$\text{Percentage of root regeneration (\%)} = \frac{\text{No. of regenerated root X 100}}{\text{Total embryogenic calli}}$$

Table 3.3 Shoot induction medium.

Media	Basal	CH (mg/ml)	$AgNO_3$ (mg/ml)	Charcoal (%)	BAP (mg/l)
S1	MS	500	2	-	1
S2	MS	500	2	-	2
S3	MS	500	-	0.2	1
S4	MS	500	-	0.2	2

Remark: BAP = Benzylaminopurine, CH = casein hydrolysate, MS = Murashige and Skoog medium, $AgNO_3$ = silver nitrate.

3.2.3 Statistical analysis

The experimental data were analyzed by variance using IBM SPSS statistics 20. A completely randomized design was used in a 3x4x3 factorial arrangement (three genotypes x four medium x three whorls) in the experiment 3.3.1, while experiment 3.3.2 used a 3x4 factorial in completely random design (CRD). The experimental data were reported as the mean ± standard deviation (SD). The differences of mean values were separated using Duncan's Multiple Range Tests (DMRT) at the 5% level of significance.

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3.3 Effect of colchicine treatment on chromosome doubling of anther-derived embryos

3.3.1 Effect of concentration and timing condition of colchicine treatment on growth and survival rate of embryogenic calli

The embryogenic calli or active calli chartered such as whitish, globular, dry, and free of any differentiated structures were selected for chromosome duplication study. Colchicine (Sigma-Aldrich) was dissolved in distilled water and

then filtrated through millipore microfilter of 0.2 µm of porosity. Twenty-five active calli in each whorl groups were cut into 4-5 mm in size and soaked in sterilized aqueous colchicine solution at various concentrations 0 µM (control), 100 µM and 300 µM and incubated for 3 h and 6 h in dark condition (Soriano et al., 2007). After colchicine treatments, treated calli were washed with sterilized distilled water for three times then transferred to shoot induction media supplemented with 2 mg/l BAP, 500 mg/l CH, and 0.2% charcoal. The treated calli were evaluated for survival rate after 14 days of culture as below. Growth parameters including fresh weight, dry weight, and callus size were determined, as well as survival rate.

$$\text{Survival rate (\%)} = \frac{\text{Number of survival callus X 100}}{\text{Total number of treated calli}}$$

3.3.2 Determination of ploidy level using flow cytometry

To determine the ploidy levels of treated calli by flow cytometry, six active calli were removed and processed to release the nuclei (Galbraith et al., 1983). Preparation of nuclei suspension; approximately 50 mg of treated calli was placed in a glass Petri dish on ice. 100 µl of OTTO-I lysis buffer (pH 2-3) (Otto, 1992) was added for slicing and cropping with a sharp razor blade, followed by addition of 400 µl of the same buffer, and then incubated for 1 h. Then, the nuclei suspension was adjusted to 1 ml with the same buffer. The homogenate was filtered through 42 µm mesh nylon filter (Cell Strainer, Korea) into 2 ml microcentrifuge tube and centrifuged at 3,000 rpm for 5 min. The pellet was then incubated in 100 µl of OTTO-I lysis buffer for 10 min or was fixed into 70% ethyl alcohol at 4 ºC until use (kept the cells at -20 ºC for several months). The nuclei suspension was stained with 0.8 ml of 3:7 OTTO-I: OTTO II solution supplemented with 20 µl Propidium iodide

(Sigma) (stock solution 1mg/ml), and 20 µl Ribonuclease I (stock 1 mg/ml), gently mixed and then maintained in the dark condition for 15 min.

The suspension was analyzed by the Becton-Dickinson FACSlibur flow cytometry (Becton-Dickinson, USA). For DNA ploidy level, sunflower young leaves were used as the standard diploid suspension used (2C ploidy standard) to determine the G0/G1 peak. The histograms were obtained from the semi-logarithmic scale (log X-axis). For polyploidy confirmation, the positional peaks of calli were evaluated in relation to those of the parental plants.

3.3.3 Statistical analysis

A completely randomized design was used in a 3x3x3 factorial arrangement (three genotypes x three concentrations of colchicine x three duration time). The experimental data were reported as the mean ± standard deviation (SD). The differences of mean values were separated using Duncan's Multiple Range Tests (DMRT) at the 5% level of significance. All analysis were performed using IBM SPSS statistics v. 23 (IBM SPSS Inc., Chicago, IL).

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CHAPTER IV

RESULTS AND DISCUSSION

The goal of this study was to produce doubled haploid (DH) plants via anther culture in sunflower. Several factors that may affect DH plant production including varieties, floret whorl, culture medium, microspore developmental stage, and colchicine treatment were investigated to achieve the goal. Results are followed:

4.1 Correlation between morphological characters of floret bud, microspore features and microspore developmental stage

In this study, I conducted experiments to understand the relationship between floret bud features, microspore size and the microspore developmental stage. High resolution of light microscope (LM) and electron microscopes (both SEM and TEM) were used to document of the floret bud and microspore features.

4.1.1 Morphological characters of floret bud

Analysis of variance for disk floret length, anther length and anther width parameters showed significantly different among varieties, whorls and their interaction of varieties x whorls as presented in Table 4.1.

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Table 4.1 Analysis of variances for morphological characteristics in three sunflower varieties.

S.O.V	d.f.	Mean Square				
		DL	**AL**	**AW**	**MPF**	**PV**
Variety (V)	2	173.035*	5.5461*	0.2760*	441034427.08*	198.99*
Whorl (W)	2	9.175*	0.3803*	0.0595*	473663.20	3.08
V * W	4	2.523*	0.1144*	0.0024*	10411840.28*	2.28*
Error	18	0.056	0.0085	0.0024	1262413	26.78
% C.V		24.22	10.48	12.56	14.15	19.62

* = Significant at 0.05 probability level.

AL = anther length, AW = anther width, DL = disk floret length, MPF = microspore per flower, and PV = pollen viability.

Sunflower head is a small cluster of several florets at the top of stem or branch. It consists of ray and disk florets. The reproductive stage at R5 is the beginning of flowering, and this stage can be divided into substages of flowering (R5.1, R5.2,…, R5.9). In this study, the flower head at the R5.1 reproductive stage from three sunflower varieties; S473, Pacific 22, and Prado Red varieties (Figure 4.1A, C, E) and their floret buds were separated into individual whorls (Figure 4.1B, D, F). The disk florets are complete flower. Corolla, pappus and stamens are jointed on the top of ovary, purple corolla color is presented in Prado Red while yellowish corolla is presented in Pacific 22 and S473 (Figure 4.2).

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Figure 4.1 Sunflower heads at the R5.1 reproductive stage and dissected disk florets into whorls. A) Flower head of S473, B) Disk floret whorls of S473, C) Flower head of Prado Red, D) Disk floret whorls of Prado Red, E) Flower head of Pacific 22, and F) Disk floret whorls of Pacific 22. 1 = whorl 1, 2 = whorl 2, 3 = whorl 3.

Data of the different disk floret ages on the disk floret and anther sizes of three sunflower varieties (Table 4.2). The mean values of disk floret length of all varieties ranged from 7.86 to 15.53 mm. The longest average of disk floret length was 15.53 mm in S473, and the shortest 7.86 mm was in Prado Red. Among floret whorls of three varieties, the mean values of disk floret length of whorl 1 was the longest 13.92 mm, and decreased as the florets close to the center head. This result is similar to the finding of Isla et al. (2011) who reported that flower bud size of melon reduced with decrease in the age of flower bud.

Table 4.2 The mean values for disk floret features in three sunflower varieties.

Factors	**DL (mm)**	**AL (mm)**	**AW (mm)**	**MPF (grain/flower)**	**PV (%)**
Variety					
S473	15.53±0.9^{a}	4.85±0.39^{b}	1.24±0.1^{b}	24877±1106^{b}	93.01±0.1^{b}
Prado Red	7.86±0.07^{b}	3.56±0.07^{c}	1.22±0.0^{b}	14694±2003^{c}	90.81±5.9^{b}
Pacific 22	15.37±1.6^{a}	4.98±0.10^{a}	1.54±0.0^{a}	28106±1710^{a}	99.83±5.2^{a}
F-test	**	**	**	**	**
Whorl					
1	13.92±4.5^{c}	4.69±0.81^{a}	1.42±0.1^{a}	22384±7703	95.22±5.15
2	12.94±3.8^{b}	4.43±0.65^{b}	1.32±0.1^{b}	22819±5954	94.13±6.58
3	11.90±3.1^{a}	4.28±0.63^{c}	1.26±0.1^{c}	22475±4847	94.30±6.43
F-test	**	**	**	ns	Ns

Means within column followed by the same superscript letter are not significantly different at 1% level by DMRT.

** = Significant at 0.01 probability level. ns = not significant.

AL = anther length, AW = anther width, DL = disk floret length, MPF = microspore per flower, and PV = pollen viability.

Sunflower anther is a part of the stamen. The anther features of three sunflower varieties includes the fused of wall forming a cylinder and brown in color. Anther length and anther width parameters were determined. The longest anther length was 4.98 mm in Pacific 22 variety, and the shortest was in Prado Red variety with 3.56 mm. Among floret whorls, anther length and anther width in whorl 1 was larger than other whorls (Table 4.2). This result indicated that anther size varied among sunflower varieties, similar to the report of Mert and Soylu (2006) who reported that anther size (length and width) varied in chestnut cultivars.

4.1.2 Determination of microspore production per floret and pollen viability

Microspore per floret (MPF) is important for self-pollination inbred line production. Our result showed that the MPF was significant among varieties and the interaction between varieties x whorls, while whorl somehow was not significant as shown in Table 4.1.

The highest number of MPF was about 28,106 grains in Pacific 22 varieties followed by S473 varieties (24,877 grains) and Prado Red varieties (14,694 grains), respectively. No difference was detected among floret whorls (Table 4.2; Figure 4.2). The interaction between varieties and whorl was significant on MPF. The result showed that the highest MPF was noticed in whorl 1 of Pacific 22 varieties. Mert (2010) reported that the number of pollen grains per anther also varied significantly and ranged from 4,720-9,840 grains among walnut cultivars. The first study of pollen grain per flower was reported in two sunflower hybrids using a hemacytometer from disk floret located in external (21,924), middle (26,063) and internal (38,752) of DKOP 3845 cultivar, While DKOP 3945 cultivar had pollen per floret about 25,562 grains in external disk floret, 31,917 grains in middle disk and 36,938 grains in

internal disk floret (Astiz and Hermandez, 2013). Vear et al. (1990) observed and found that microspore number ranged from 25,218 grains to 40,788 grains in 14 inbred lines sunflower. Moreover, Dilkilic and Mestav (2011) reported the number of microspore ranged from 11,906 grains to 13,219 grains in quince, and Sumarmi et al. (2014) showed the result of 354.67±59.67 microspores grain in soybean.

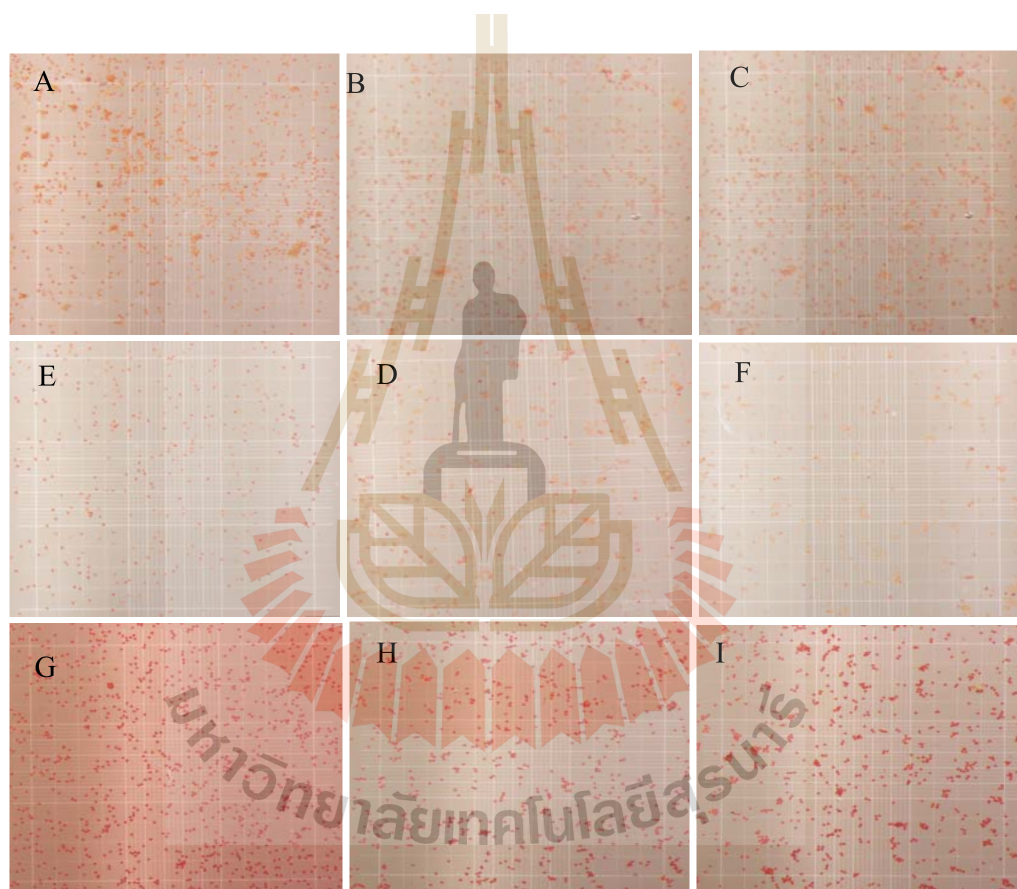

Figure 4.2 Images of microspore in three sunflower varieties. A) whorl 1 of S473, B) whorl 2 of S473, C) Whorl 3 of S473, D) Whorl 1 of Prado Red, E) whorl 2 of Prado Red, F) whorl 3 of Prado Red, G) Whorl 1 of Pacific 22, H) Whorl 2 of Pacific 22, and I) Whorl 3 of Pacific 22 (BAR = 100 μm).

The determination of microspore viability is very important in anther culture for plant breeding program. There are many factors affecting microspore quality such as heat and moisture conditions. Heat has been reported to be a great effect on microspore viability in *Triticum aestivum* (Saini et al., 1984), while the temperature highly affected on microspore viability in sunflower (Astiz and Hernandez, 2013).

Analysis of variance on pollen viability shows that varieties and their interaction are highly significant, while floret whorls had no significant effect for this trait ($p < 0.05$) as shown in Table 4.1.

Among three varieties, Pacific 22 has the highest pollen viability about 99.82%, followed by S473 (93.01%) and Prado Red (90.81%), respectively (Table 4.2, Figure 4.3). Different floret whorls exhibited differences in pollen viability. The highest pollen viability about 95.22% was observed in floret whorl 1, and the lowest about 94.30% in floret whorl 3.

In this study, the percentage of pollen viability in sunflower was similar to a study of Krudnak et al. (2013) who reported pollen viability at the level of 96.93% in S473, while Pacific 77 hybrid showed pollen viability about 98.33%. Astiz et al. (2013) reported pollen viability about 96.60% to 98.40% in two sunflower varieties. Elena (2013) reported pollen viability in F1 sunflower hybrids about 89.5% to 94.1% using acetic carmine test. Atlagic et al. (1993) reported pollen viability of hybrid *Helianthus tuberosus* x cultivated sunflower from 89.5% to 94.1%. Pollen viability of olive (*Olea europaea* L.) ranged from 35.5% to 84.3% (Mazzeo et al., 2014), and in cherry from 73.02% to 86.79% (Pirlak and Guleryuz, 2005).

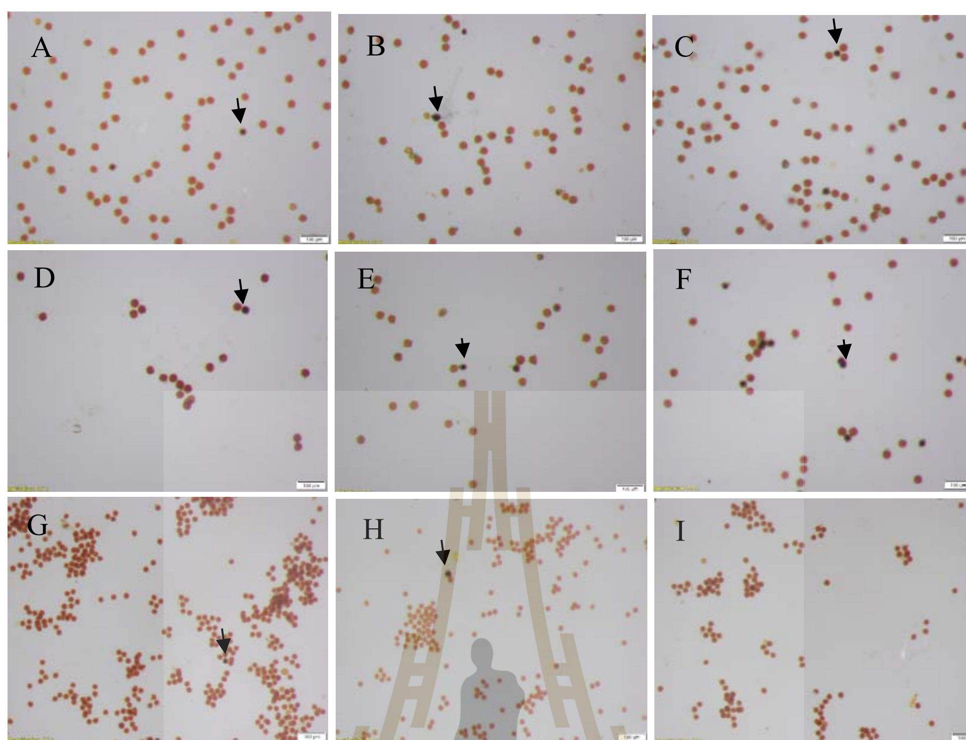

Figure 4.3 Images of IKI stained microspores viability in three sunflowers varieties. A) whorl 1 of S473, B) Whorl 2 of S473, C) Whorl 3 of S473, D) Whorl 1 of Prado Red, E) Whorl 2 of Prado Red, F) Whorl 3 of Prado Red, G) Whorl 1 of Pacific 22, H) Whorl 2 of Pacific 22, and I) Whorl 3 of Pacific 22. (arrow indicates microspore dead) (A-I, Bar = 100 μm).

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Correlation analysis is considered as an excellent tool to understand the interrelationship of different parameters. Expectedly, it was observed that the correlation of disk floret length (DL) had a strong positive correlation with anther length (r= 0.985**), anther width (r= 0.585**), microspore per flower (r= 0.929**), and pollen viability (r= 0.450) (Table 4.3). In addition, anther length had a positive relationship with anther width (r= 0.637**), microspore per flower (r= 0.886**), and pollen viability (r= 0.415). The microspore viability has a strong positive correlation

value (r= 0.568**) with microspore per floret. It also had a positive correlation with disk floret length (r= 0.450*) and anther length (r= 0.415*) (Table 4.3).

Table 4.3 Correlation of floret bud features of three sunflower varieties.

Variable	DL	AL	AW	MPF
DL	-			
AL	.985**	-		
AW	.585**	.637**	-	
MPF	.929**	.886**	0.315	-
PV	.450*	.415*	-0.059	.568**

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

AL = anther length, AW = anther width, DL = disk floret length, MPF = microspore per flower, and PV = pollen viability.

4.1.3 Anther structure and microspore features

Anther sections of unopened disk floret whorls namely 1, 2 and 3 of three sunflower varieties; S473, Pacific 22 and Prado Red were observed by light microscope. The results showed that anthers of all three varieties are similar in the structure and appear in butterfly-shape (Figure 4.4). Stomium is the point of separation between two locules in each lobe resulting in dehiscence of pollen release. The style is surrounded by anther cylinder. In anther tissues, there are distinct epidermal cells, which surround the reproductive cells and enlarge considerably just anthesis. Endothecium layer surrounds microspores. The anther contains five symmetrical pollen sacs called pentasporangiate in both S473 (Figure 4.4A-C) and Prado Red (Figure 4.4D-E) varieties. This finding was similarly recorded in

interspecific hybrid *Helianthus annuus* var. *macrocarpa* (DC) (Delio and Putt, 1980). While Pacific 22 hybrid has five-six pollen sacs in anthers (Figure 4.4G-I). The number of pollen sacs of this study was different to those of Smart et al. (1994), Baghali et al. (2011) and Lindstrom and Hernandez (2015) who reported four pollen sacs in the anther of CMS sunflower varieties and two hybrid sunflowers.

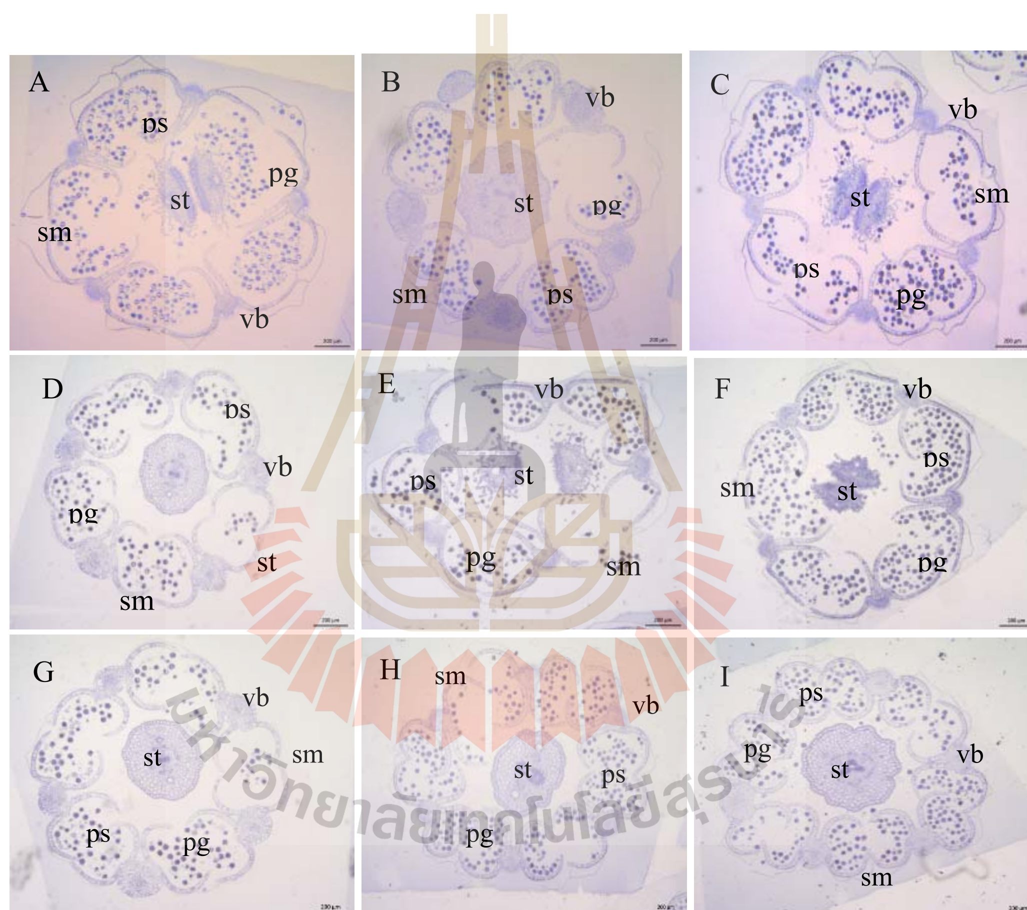

Figure 4.4 Thin anther sections from floret whorls in three sunflower varieties. A) Whorl 1 of S473, B) Whorl 2 of S473, C) Whorl 3 of S473, D) Whorl 1 of Prado Red, E) Whorl 2 of Prado Red, F) Whorl 3 of Prado Red, G) Whorl 1 of Pacific 22, H) Whorl 2 of Pacific 22, and I) Whorl 3 of Pacific 22. ps = pollen sac, pg = pollen grains, st = style, sm = stomium, vb = vascular bundle (A-I = 200 µm).

Significant differences were observed by analysis of variance (ANOVA) ($p \leq 0.05$). Two source of variance (varieties and whorls) had significant effects on three parameters (polar axis, equatorial axis, and ratio of P/E), while the interaction between varieties x whorls did not have significant effect as shown in Table 4.4.

Table 4.4 Analysis of variance for microspore features and microspore developmental stage in three sunflower varieties.

S.O.V	d.f.	P	E	P/E	EM	MM
Varieties (V)	2	236.5541*	240.0446*	0.0010*	300.0370*	506.2593*
Whorl (W)	2	18.8328*	19.8463*	0.0002	371.5926*	858.0370*
V * W	4	3.8786*	3.8714*	0.0002	83.8148	300.2592*
Error	18	0.3082	0.2511	0.0001	31.8148	37.8148
% C.V		23.22	16.06	28.12	24.22	26.70

*=Significant at 0.05 probability level.

E = equatorial view, EM = early uninucleate microspore developmental stage, P = polar view, P/E = polar view and equatorial ratio, MM = mid-to late uninucleate microspore developmental stage.

For microspore feature study, three main characteristics of microspore were identified including size, shape, and structure under the light microscope. Microspore grains has yellowish color. Microspore is isopolar, radial symmetry and covered with spines. The spine is conical from the base. The aperture number has three apertures (Figure 4.5).

Microspore size was determined following the method of Erdtman (1952). The length axis was measured in the polar axis (P), while width axis was measured in the equatorial axis (E). Of three varieties, the P and E axis of microspore were

significantly different. The means P ranged from 41.48 to 45.04 µm in S473, from 39.75 to 42.03 µm in Prado Red, and from 30.58 to 36.30 µm in Pacific 22, respectively. The mean E ranged from 39.64 to 42.59 µm in S473, from 38.10 to 40.84 µm in Prado Red, and from 28.71 to 34.09 µm in Pacific 22 as presented in Table 4.5. In different floret whorls there was significantly in statistical analysis in the P axis and E axis. The longest of the P and E axis was found in floret whorl 2, and the smallest the P and E axis was in floret whorl 1 (Table 4.5). Regarding the polar axis of all sunflower varieties, microspore is considered as ball-triangle, while the equatorial axis is slightly elliptical, similar to the report of Klimko et al. (2000).

The microspore shape may be referring to the ratio of the length of the polar axis (an imaginary straight line connecting the two poles) to the equatorial diameter (P/E). The means of P/E ranged from 1.02 to 1.08 among sunflower varieties, while it ranged 1.04-1.05 among whorls (Table 4.5). It is prolate-spheroidal in all three varieties.

Structure and aperture of microspore grain have three apertures, called tricoporate pollen. The aperture is composed of colpus with distinct pore and it is equally placed in the equatorial position of microspore (Figure 4.5A).

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Table 4.5 The mean values for microspore developmental stage and morphology of microspore in three sunflower varieties.

Factors	**P (µm)**	**E (µm)**	**P/E**	**EM (%)**	**MM (%)**
Variety					
S473	42.92±1.15^{a}	40.95±0.97^{a}	1.05±0.01^{a}	5.11±8.25^{b}	11.89±8.25^{b}
Prado Red	41.32±0.84^{b}	39.91±1.04^{b}	1.04±0.014^{b}	14.44±11.30^{a}	26.89±11.1^{a}
Pacific 22	33.35±2.31^{c}	31.53±2.33^{c}	1.06±0.01^{a}	15.67±7.86^{a}	19.33±16.05^{ab}
F-test	**	**	**	**	**
Whorl					
1	37.67±5.22^{c}	35.87±5.31^{c}	1.05±0.02	6.44±3.54^{b}	30.44±15.46^{a}
2	40.55±3.63^{a}	38.81±3.66^{a}	1.05±0.02	9.89±4.70^{b}	15.67±8.85^{b}
3	39.37±4.62^{b}	37.72±4.49^{b}	1.04±0.01	18.89±12.40^{a}	12.00±6.67^{b}
F-test	**	**	ns	**	**

Means within column followed by the same superscript letter are not significantly different at 1% level by DMRT.

* = Significant at 0.01 probability level. ns = not significant.

E = equatorial view, EM = early uninucleate microspore developmental stage, MM = mid-to late uninucleate microspore developmental stage, P = polar view, P/E = polar view and equatorial ratio.

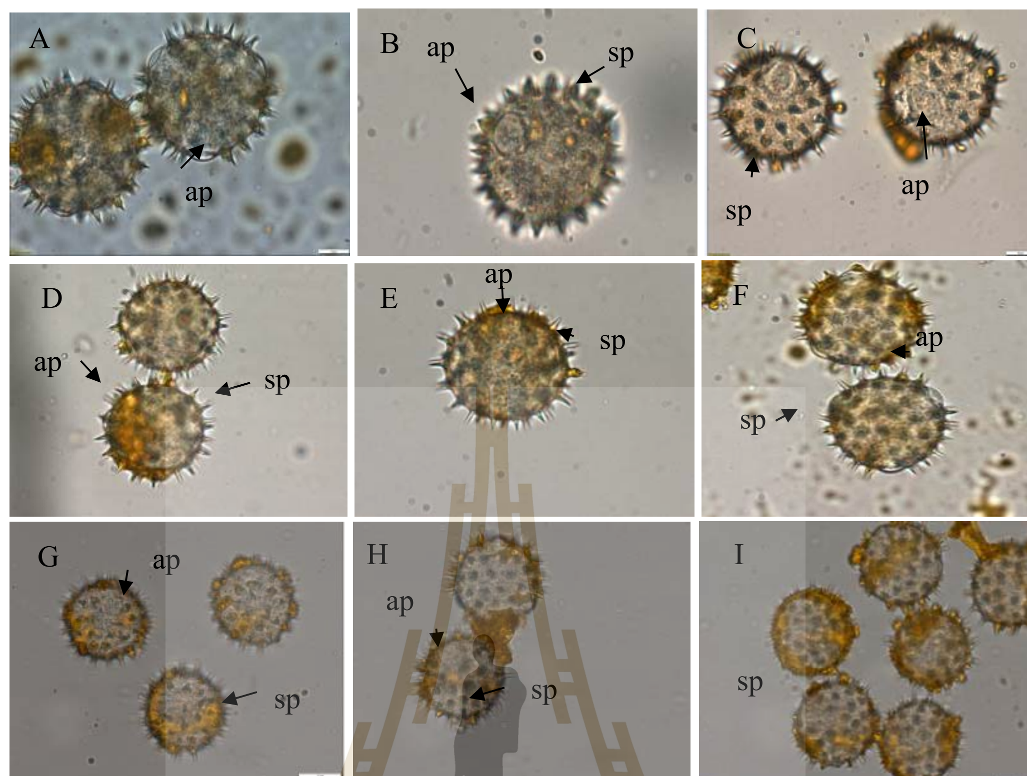


Figure 4.5 Light microscope images of microspore in three sunflower varieties. A) Whorl 1 of S473, B) Whorl 2 of S473, C) Whorl 3 of S473, D) Whorl 1 of Prado Red, E) Whorl 2 of Prado Red, F) Whorl 3 of Prado Red, G) Whorl 1 of Pacific 22,H) Whorl 2 of Pacific 22, and I) Whorl 3 of Pacific 22. ap = aperture, sn = sporopollenin, sp = spine (A-I, Bar = 10 µm).

Pollen grain and microspore are important characteristics for taxonomic study (Zafar et al., 2007). Polar, equatorial axis, P/E ratio and number of aperture characters were considered. In this study, microspore is quite homogeneous among studied varieties (isopolar, medium size, and tricolporate). Klimko et al. (2000) reported that microspore diameter (P) about 32.93 µm and its size depended on genotype and the P/E of pollen grain about 1.00 to 1.04 in sunflower. Rashid et al. (2013) reported that pollens of *Helianthus annuus* L. had the highest polar axis about 230 µm and

equatorial axis about 205.6 µm, while P/E ratio was found about 1.0. Walker and Doyle (1975) reported that the tricolporate pollen is the main and basic type found in most members of the Asteraceae family. However, *Graphistylis* spp. (Asteraceae) has P/E ratio about 0.97-1.02.

4.1.4 Determination of microspore developmental stages

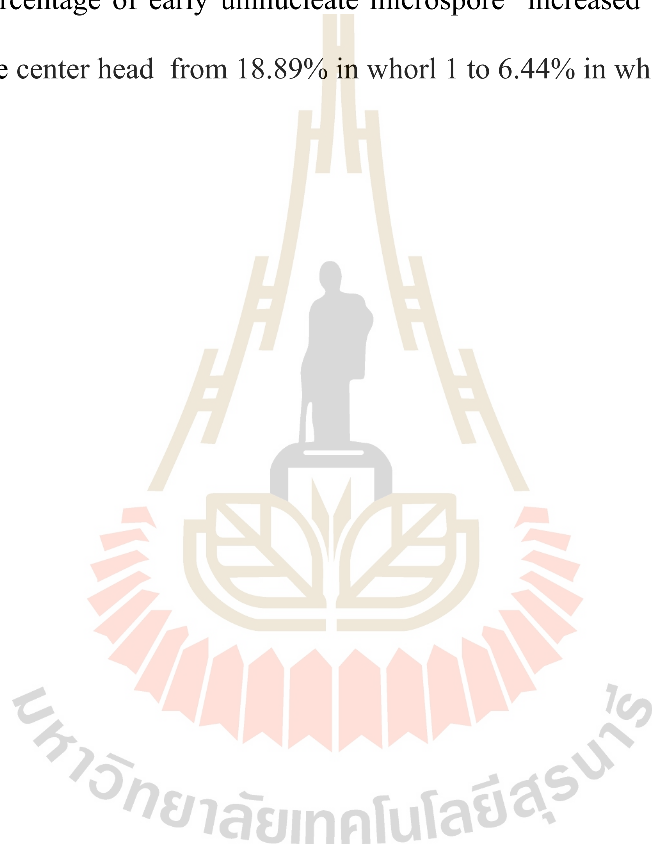
There are several methods for determination of uninucleate microspore developmental stages such as morphological markers and cytological markers. In the study, the cytological marker for determination of microspore developmental stage was applied.

Several researchers studied pattern of plant development and used the same R5.1 reproductive stage. Nucleus of microspore was stained with Toluidine Blue O (TBO). The microspore developmental stages were classified based on vacuole and nucleus location in cytoplasm. If microspore accumulated vacuoles, it was defined as early uninucleate microspore stage, while nucleus located at the central of cytoplasm of microspore, it was defined to mid-to late uninucleate microspore stage. The observation of microspore developmental stages in this work was presented in Figure 4.6.

The result of analysis of variance (ANOVA) ($p \leq 0.05$) showed that two sources of variance (varieties and whorls) had significant effect on early uninucleate microspore developmental stage (EM) and mid-to late microspore developmental stage (MM), while interaction between varieties x whorls did not have significant effect as shown in Table 4.4.

To compare among different varieties, the maximum percentage of mid-to late microspore was found in Prado Red variety about 26.89%, followed by S473 about 11.89% and Pacific 22 about 19.33%, respectively (Table 4.5). The percentage of

mid- to late uninucleate microspore was the highest about 30.44% in floret whorl 1, and decreased in floret whorl 2 about 15.67% and whorl 3 about 12.00% (Table 4.5). When compared among three disk floret whorls, the maximum mean value of the early uninucleate stage was found in Pacific 22 variety about 15.67%, while Prado Red and S473 variety had about 14.44%, and 5.11%, respectively as shown in Table 4.5. The percentage of early uninucleate microspore increased as the floret whorls closed to the center head from 18.89% in whorl 1 to 6.44% in whorl 3 (Table 4.5).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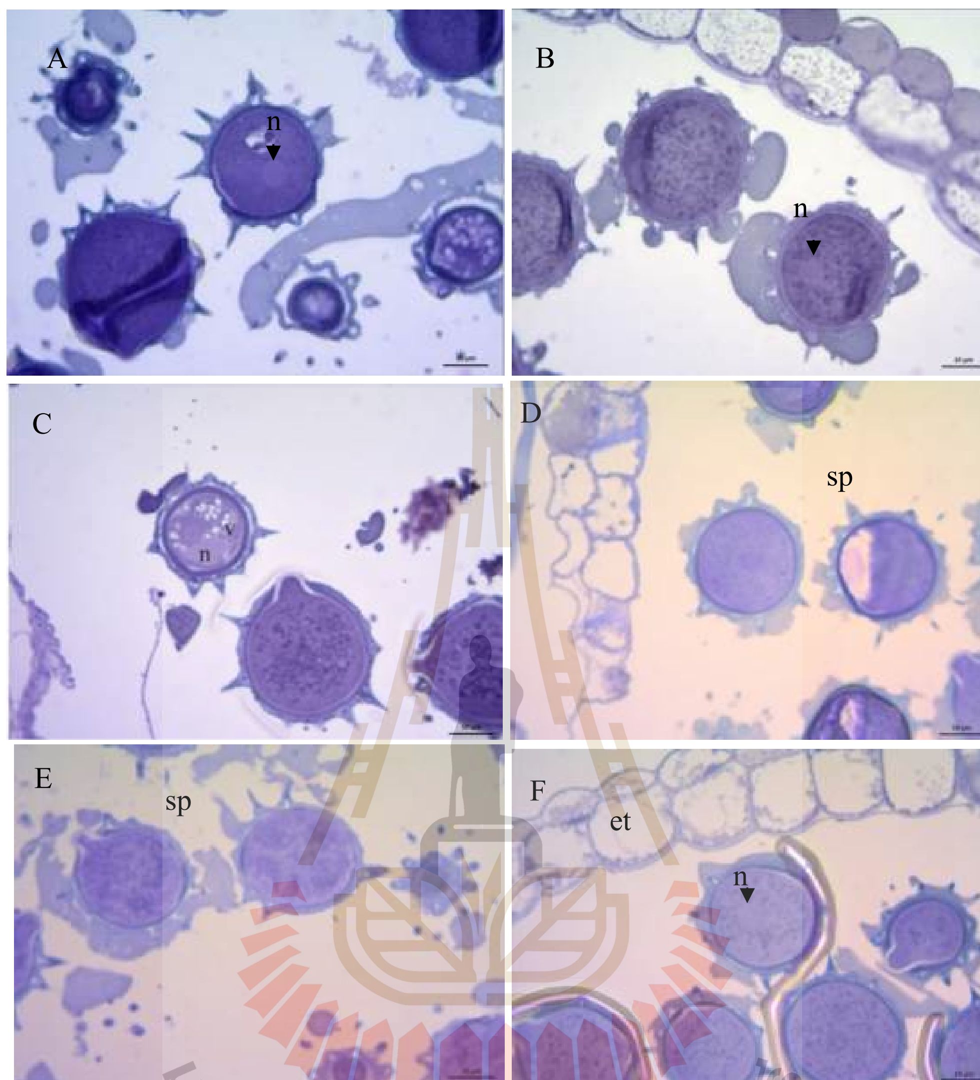

Figure 4.6 Microspores stained with Toluidine Blue O (TBO) under light microscope in three sunflower varieties. A) Floret whorl 1 of S473, B) Floret whorl 2 of S473, C) Floret whorl 3 of S473, D) Floret whorl 1 in Prado Red, E) Floret whorl 2 of Prado Red, F) Floret whorl 3 of Prado Red.

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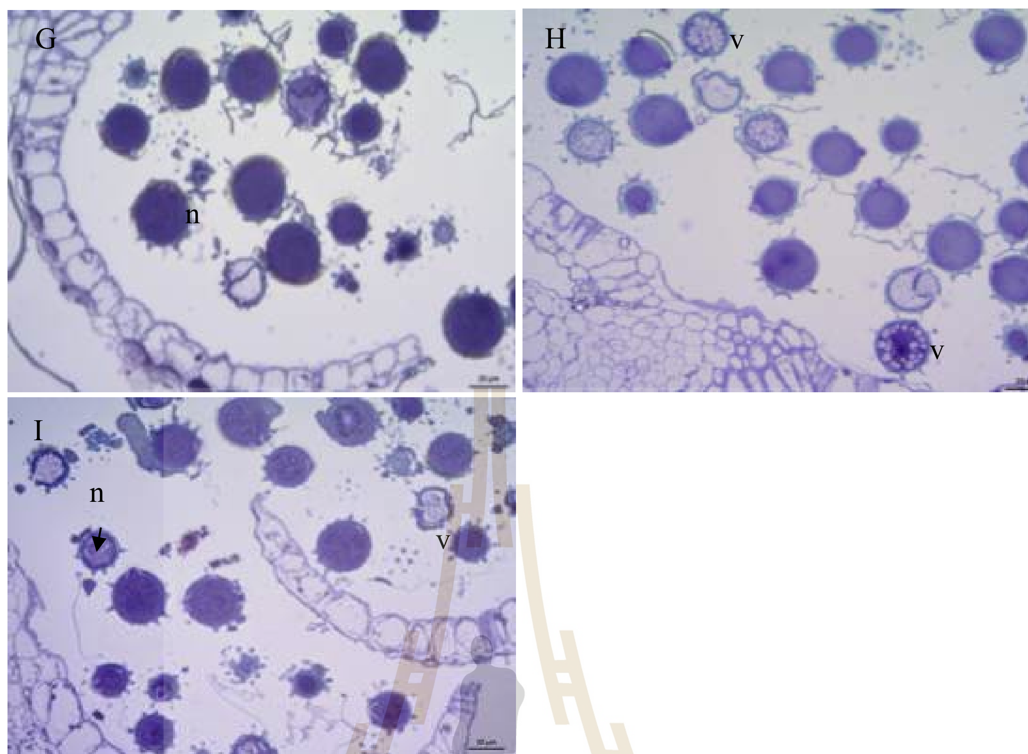

Figure 4.6 (Continued) G) Floret whorl 1 of Pacific 22, H) Floret whorl 2 of Pacific 22, I) Floret whorl 3 of Pacific 22, et = endothecium, n = nucleus, sp = spine, v = vacuole (A-F = Bar 20 μm).

For the relationship between microspore developmental stage and morphological characteristics as presented in Table 4.6 is observed that disk floret length (DL) was correlated with anther length (0.985**), anther width (0.579**), and P/E ratio (0.586**). For anther size, anther length was correlated with its anther width. Disk length of sunflower from different whorls was not correlated with uninucleate microspore developmental stage including early uninucleate microspore developmental stage and mid-to late uninucleate microspore developmental stage. While early uninucleate microspore developmental stage and microspore size including polar (P) (0.559**) and equatorial (E) (0.563**) axis were correlated.

Table 4.6 Correlation analysis of variances between related to morphological disk floret bud and microspore developmental stage of three disk floret whorls in sunflower varieties.

Variable	P	E	P/E	DL	AL	AW	EM	MM	MPF
E	.994**	-							
P/E	-.453*	-.543**	-						
DL	-0.346	-.397*	.586**	-					
AL	-0.311	-0.363	.579**	.985**	-				
AW	.402*	0.352	0.238	.585**	.637**	-			
EM	.559**	.563**	-0.335	-0.271	-0.228	0.041	-		
MM	0.227	0.265	-.408*	-0.308	-0.284	0.188	-0.249	-	
MPF	-.518**	-.555**	.552**	.929**	.886**	0.315	-0.275	-.464*	-
PV	-.586**	-.613**	.471*	.450*	.415*	-0.059	-0.259	-0.198	.568**

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

AL = anther length, AW = anther width, DL = disk floret length, E = equatorial view, EM = early uninucleate microspore developmental stage, P = polar view, P/E = polar view and equatorial ratio, MM = mid-to late uninucleate microspore developmental stage, MPF = microspore per flower, and PV = pollen viability.

These findings were similar to the study of Gonzalez-Melendi et al. (2008) who reported early uninucleate microspore stage in rapeseed, and Horner and Pearson (1978) who studied the accumulated vacuole in early uninucleate microspore in sunflower as well as the study of Hu et al. (1996) who reported early, mid-to late uninucleate microspore stages in the same flower bud of soybean.

Related study of Srivastava and Devaiah (1988) has also been reported about the correlations among 8 morpho-economic characters in 16 genotypes of jasmine. The weight of 100 flowers was positively associated with the flower diameter, petal width, petal length, anther length, floral bud length and width, and 100 bud weight.

Developmental stages of microspore have been early determined for plant breeding. Breeders used several techniques for discovering microspore stage. There are several methods to determine uninucleate microspore developmental stage such as using morphological markers including determination of flower bud size or anther size in tomato (Summers et al., 1992) and Mexican husk tomato (Escobar-Guzman et al., 2009), plant age and leave number in maize (de Moraes et al., 2008), bud length in soybean (Lauxen et al., 2003), flower head diameter in sunflower (Vijaya Priya et al., 2003), using R5.1 and R5.2 reproductive stage in sunflower (Phaosang et al., 2003; Krudnak et al., 2013), floret length in soybean (Summers, 1992), collora length in tobacco (Sunderland, 1984). Lauxen et al. (2003) reported that anthers with earlier microspore development were highly responsive for anther culture in soybean var. IAS5, while var. Brazilian showed best response for anther culture at early to late microspore developmental stage.

Few reports have been presented to correlate of anther length and microspore developmental stage such as Summers et al. (1992) reported that anther length 2.8-3.5 mm contained uninucleate microspore stage in tomato. In melon (*Cucumis melo*), the uninucleate stage of microspore appeared in anther length ranging from 1.9 to 2.0 mm (Islam et al., 2011). In maize, the early uninucleate microspore stage was observed in anther length about 7.16 mm, while late uninucleate stage was observed at 7.31 mm of anther length (de Moraes et al., 2008). During anther development, the development of the microspore is paralleled with changes in the anther. In addition,

the anther size and color have been correlated with changes of microspore stage (Parra-Vega et al., 2013).

4.1.5 Ultrastructure of microspore and its surface morphology

Table 4.6 shows the correlation between the sizes of microspore with the microspore developmental stage. Electron microscopes (TEM and SEM) were selected for the study of the ultrastructure and morphology of microspore.

Figure 4.7 describes a detailed wall of microspore grains in three sunflower varieties using TEM. The surface of microspore contains spines covered with sporopollenin. The exine is composed of ektexine which consists of tectum, columella and foot layer. Next layer is endexine with higher electron density and was very thick around the microspore cytoplasm. The tectum has ovale and round perforation. The structure of the tectal complex, inner of the spinular complex is composed of three distinct layers including an external micro-perforated, columellar layer, and internal micro-perforated. The microperforates under the spine was a large number of vestigial micro-foramina. The columellar layer is simple and branch (Figure 4.7A-I). The caveae is narrow, but it is the widest to occur in floret whorl 3 of Pacific 22 (Figure 4.7F). The foot layer is smooth with thickness. The endexine layer is thick and clear in all three varieties. The endexine layer of whorl 2 (Figure 4.7B), whorl 3 (Figure 4.7C) of S473 variety and whorl 3 (Figure 4.7F) of the Pacific 22 is thicker than other whorls. The inner layer is intine, thin layering which interacts between cytoplasm and pollen wall.

The type of columellae in ektexine or sexine of sunflower is called *Aster* (Moore et al., 2000). This study was similarly reported in pollen wall of the Asteraceae family under TEM by Coutinho and Dinis (2007) who found three main layers of exine that differ in thickness of pollen in *Pallensis maritime* (Asteraceae).

Similar observation was in *Senecio bergii* (Asteraceae) (Montes and Murray, 2015). However, Klimko et al. (2000) reported that the thickness of exine in sunflower pollen depended on varieties.

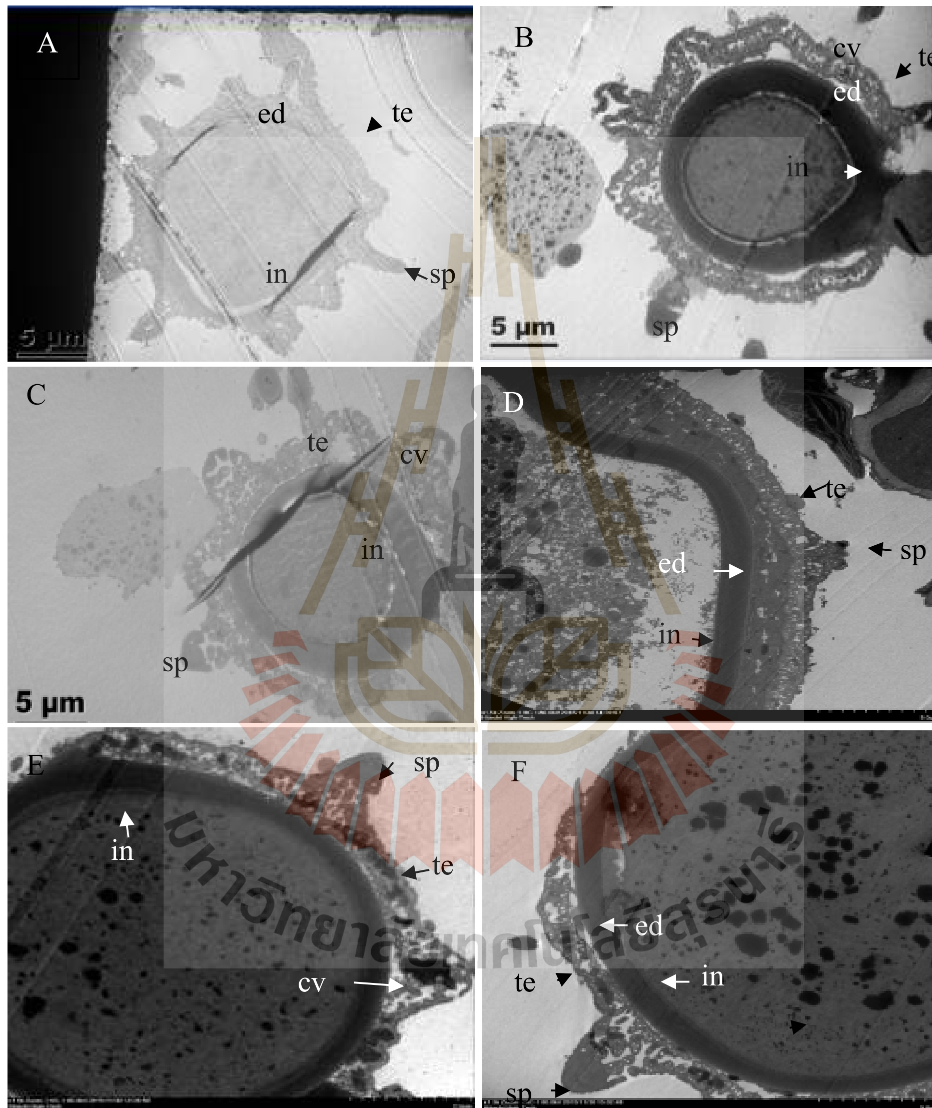

Figure 4.7 TEM micrographs of microspore wall in three sunflower varieties. A) Whorl 1 of S473, B) Whorl 2 of S473, C) Whorl 3 of S473, G) Whorl 1 of Prado Red, H) Whorl 2 of Prado Red, and I) Whorl 3 of Prado Red.

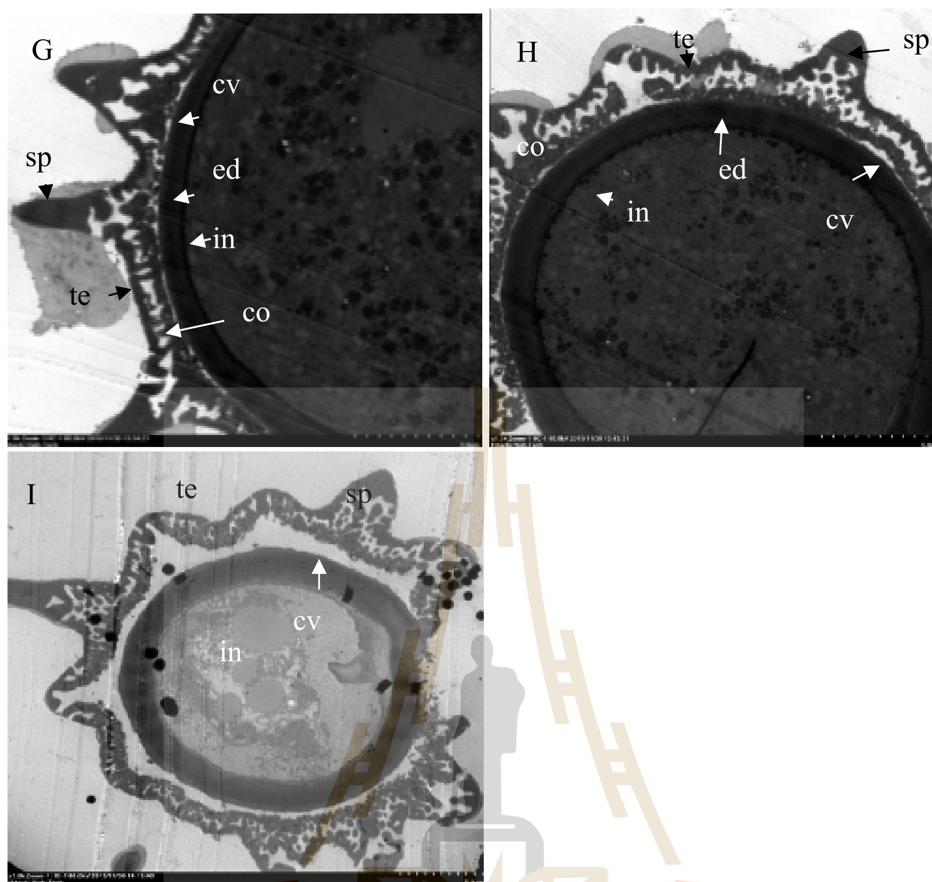

Figure 4.7 (Continued) D) Whorl 1 of Pacific 22, E) Whorl 2 of Pacific 22, and F) Whorl 3 of Pacific 22. ed = endexine, co = columellae, cv = caveae, in = intine, te = tectum, sp = spinulate (A-I = Bar 5.0 µm).

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The specificity of surface structure of microspore was observed by SEM. The external wall of pollen is very important for taxonomic classification and systematic in flowering plants (Ahmad et al., 2013). A majority of the palynological data has been derived from external structures and sculpturing element of pollen grains obtained from the SEM studies.

For SEM image of microspore sculpturing is referring the term of the surface of pollen wall. The results from this study showed that the pollen surface of three

varieties was commonly covered with spines. The spines are conical with covex and the base of spines is composed with microperforation. Each microspore apertureis composed of colpus with distinct pore and it is incongruence. Colpi is like-elongate aperture and crossed the equatorial axis of microspore. The colporate microspore grain, an aperture that includes a colpus type of ectoaperture with tapered extremities, a lalongate pore type of mesoaperture and the ectoaperture is sub terminal, broad in the equatorial area and acute at the ends. The endoaperture has granule colpus membrane. The pollen shape is prolate-spheroidal (Figure 4.8C).

The morphological characteristics (size, shape, aperture, sculpturing) of sunflower pollen agree with previous reports such as Gotelli et al. (2008) reported morphological pollen features of sunflower hybrid, Wortley et al. (2007) reported the morphological pollen feature of *Moquinia racemosa* DC (Asteraceae), and Coutinho et al. (2012) studied pollen grain morphology of *Praxelis* (Asteraceae) and tribe Dicomeae and funk (Asteraceae).

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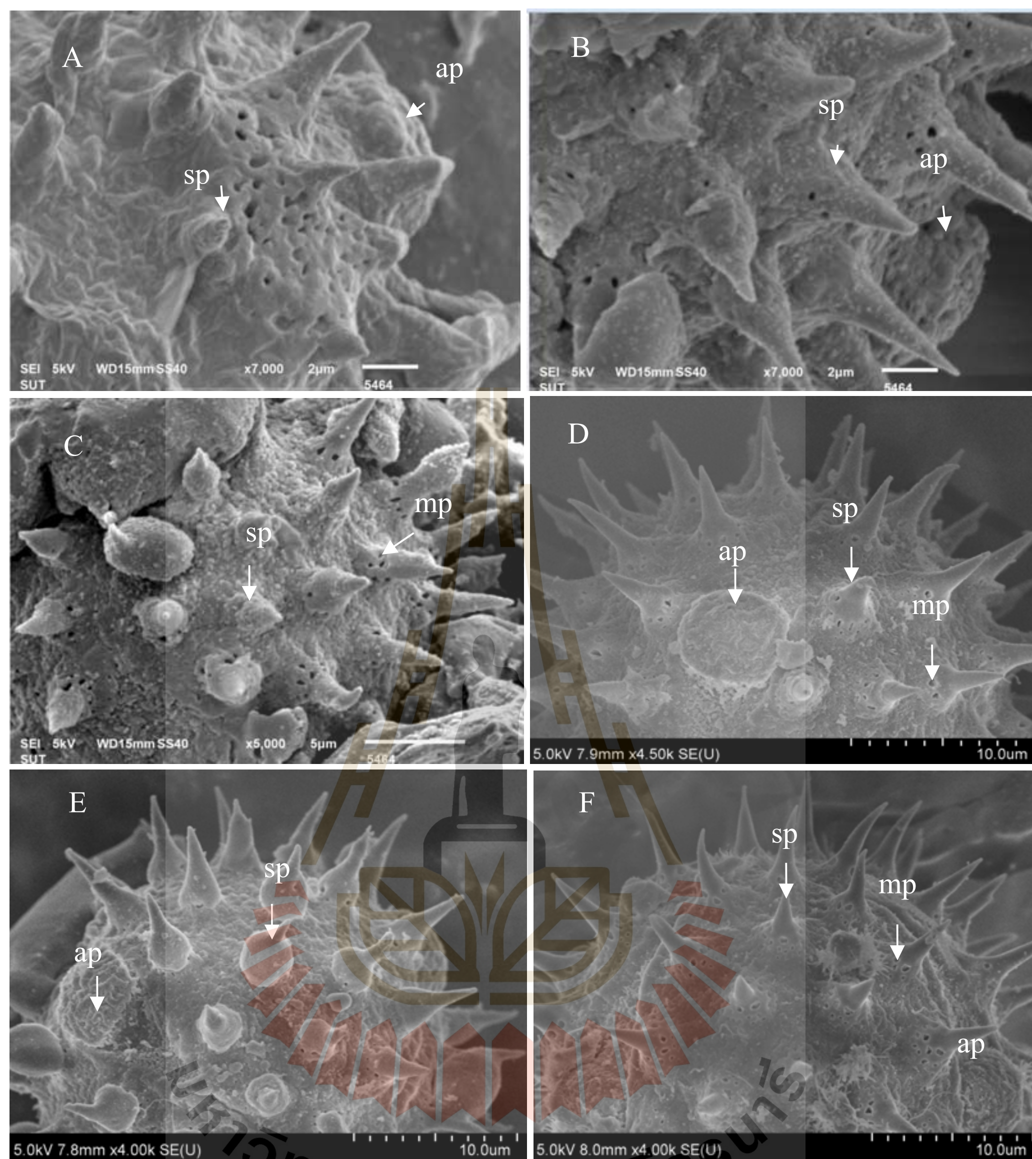

Figure 4.8 SEM images of microspore surface in three sunflowers varieties.

A) Whorl 1 of S473, B) Whorl 2 of S473, C) Whorl 3 of S473, D) Whorl 1 of Prado Red. E) Whorl 2 of Prado Red, F) Whorl 3 of Prado Red.

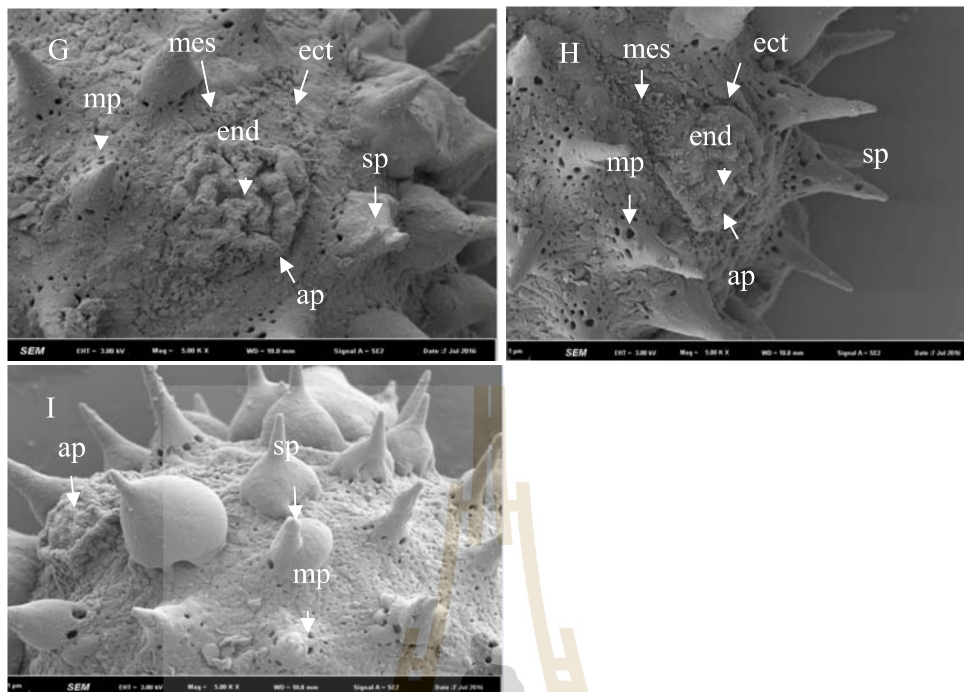

Figure 4.8 (Continued) G) Whorl 1 of Pacific 22, H) Whorl 2 Pacific 22, and I) Whorl 3 of Pacific 22. ap = aperture, ect = extoaperture, end = endoaperture, mes = mesoaperture, mp = microperforation, sp = spine (A-I = 10 µm).

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4.2 Effects of varieties, floret whorl, and culture medium on haploid plant production via anther culture

This study was conducted to determine the effects of varieties, floret whorl, and culture medium on haploid plant production via anther culture in order to determine a suitable condition for *in vitro* haploid plant production.

4.2.1 Callus induction on various medium through anther culture

Analysis of variance for different floret whorls was significant in the percentage of callus induction and the percentage of embryogenic callus parameters. The interaction of whorl x medium was significant in callus size, the percetage of callus induction and the percentage of embryogenic callus as presented shown in Table 4.7.

Analysis of variance for the medium on anther-derived callus were significant in all parameters including callus size, fresh weight, dry weight, the percentage of callus induction and the embryogenic callus. Different whorls were significant in the percentage of callus induction, and the percentage embryogenic callus. In different varieties there was were also significance in dry weight parameter as presented in Table 4.7.

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Table 4.7 Analysis of variances of callus growth parameters and the percentage of callus induction and the percentage of embryogenic callus in three sunflower varieties.

S.O.V	d.f.	CS	FW	DW	PC	PE
Variety (V)	2	0.184	0.002	0.0007*	14743.15	6.00
Whorl (W)	2	0.001	0.0004	0.0002	1133.26*	94.15*
Media (M)	3	30.739*	0.2279*	0.0266*	21782.04*	2455.97*
V *W	4	0.559	0.001	0.0002	356.37	30.65*
V * M	6	0.989*	0.0016	0.0003	675.79*	20.36
W * M	6	1.074*	0.001	0.0003	419.44*	33.28*
V * W * M	12	0.832*	0.0012	0.0002	80.86	15.32
Error	72	0.37	0.0014	0.0002	231.98	10.93
% C.V		28.55	22.6	10.02	15.88	12.06

* = Significant at 0.05 probability level

CS = callus size, DW = dry weight, FW = fresh weight, PC = the percentage of callus induction, and PE = the percentage of embryogenic callus.

Variety of donor plant, medium and microspore developmental stage are the important factors in success for anther culture. In this study, anthers of three sunflower varieties including S473, Prado Red and Pacific 22 were cultured on callus induction medium for 30 days. The results found that anthers of each variety produced friable calli to compact calli with similar color ranging from whitish, cream color, greenish and pale purple color. Anthers of S473 produced embryogenic calli in A1, A2 and A3 media, while A4 media failed to induce callus (Figure 4.9). The anthers of S473 synthetic variety produced callus at 42.28%. The anthers of Pacific 22 produced embryogenic callus and root in A1 media, and only callus and embryogenic calli produced in A2 and A3 medium (Figure 4.10). Prado Red variety produced pale

purple color, whitish and white calli at 36.42%, embryogenic calli with globular stage of embryogenesis and shoot in A2 medium, only embryogenic calli in A1 and A3 media, and no callus induction in A4 medium (Figure 4.11). The highest mean value of pro-embryo-like structures was recorded in Prado Red (10.37%), followed by Pacific 22 (9.70%) and S473 (9.63%), respectively (Table 4.7). For the analysis of morphology of anther-derived callus, the biggest size of callus was observed in Pacific 22 variety (2.025 mm), followed by S473 variety (2.019 mm) and Prado Red (1.897 mm), respectively. The maximum growth of callus in term of fresh weight parameter, the heaviest fresh weight of callus was (0.157 mg) was found in Pacific 22, and the lowest was detected in Prado Red (0.148 mg). The maximum of dry weight callus was 0.058 mg in Pacific 22, followed by S473 (0.050 mg) and Prado Red (0.051 mg), respectively (Table 4.7).

As shown in Table 4.7, significant differences in dry weight were determined among varieties. Among floret whorls, dry weight, the percentage of callus induction and the percentage of embryogenic calli were significantly different.

The success of anther culture in sunflower importantly depends on the use of microspore developmental stage at the time of culture. It is possible to choose disk floret with anther containing a suitable microspore developmental stage. In this study, the highest of the percentage of embryogenic calli (11.15%) was found in disk floret length about 12.94 mm and anther size 4.43 mm in length and 1.32 mm in width containing 9.82% of early uninucleate microspore developmental stage and 15.67% of mid-to late uninucleate microspore developmental stage in whorl 2. Moreover, and three directed shoot formation were obtained. While the lowest of the percentage of embryogenic calli was observed in whorl 3 (8.07%) in disk floret length about 11.90

mm and anther size 4.28 mm in length and 1.26 mm in width containing 18.89% of early uninucleate microspore developmental stage and 12.00% of mid-to late uninucleate microspore developmental stage. The correlation between disk floret and microspore developmental stage in this study was different from the report of Adhikari and Kang (2017), who reported a strong correlation between flower bud size (5.62-7.13 mm), anther size (2.60-4.38 mm) and microspore developmental stage which is imperative for practical anther culture in Campari tomato. Summers et al. (1992) also reported that anther length was a better predictive character in three different tomato cultivars for anther culture. Seguí-Simarro et al. (2011) reported that the selection of suitable flower bud length containing an optimal microspore developmental stage is important for androgenesis.

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Table 4.8 Comparison between the mean values (± SD) and callus induction characteristics in three sunflower varieties.

Factors	CS	FW	DW	PC	PE
Varieties					
S473	2.019±1.069	0.142±0.088	0.050±0.032[b]	42.28±33.88	9.63±8.77
Prado Red	1.897±1.131	0.148±0.089	0.051±0.032[ab]	36.42±30.08	10.37±9.27
Pacific 22	2.036±1.233	0.157±0.087	0.058±0.030[a]	38.74±27.03	9.70±9.67
F-test	ns	ns	**	ns	ns
Whorl					
1	1.974±1.228	0.148±0.091	0.055±0.035[a]	45.03±33.66[b]	10.48±9.6[a]
2	1.985±1.232	0.147±0.088	0.050±0.032[b]	37.69±61.02[a]	11.15±10.[b]
3	1.981±0.962	0.153±0.087	0.053±0.039[a]	31.72±24.75[a]	8.07±7.44[a]
F-test	ns	ns	**	**	**
Medium					
A1	2.584±0.325[a]	0.197±0.023[a]	0.067±0.010[b]	58.70±20.99[a]	12.69±20.99[b]
A2	2.828±0.517[a]	0.214±0.021[a]	0.079±0.012[a]	65.48±22.19[a]	21.88±22.19[a]
A3	2.053±0.791[b]	0.172±0.042[b]	0.058±0.016[b]	27.56±17.72[b]	5.04±17.72[c]
A4	0.00[c]	0.00[c]	0.00[c]	0.00[c]	0.00[d]
F-test	**	**	**	**	**

Means within column followed by the same superscript letter are not significantly different at p<0.01 level of probability using DMRT.

** = Significant at 0.01 probability level. ns = not Significant.

CS = callus size, DW = dry weight, FW = fresh weight, PC = the percentage of callus induction, and PE = the percentage of embryogenic callus.

The results showed that calli and embrygenic calli were produced through anther culture on A1, A2, A3 media excepted A4 medium. A2 medium containing 2 mg/l NAA, 1 mg/l BA and 10% CW gave a good response and regenerated shoot for both S473 and Prado Red. Direct shoot was observed in Prado Red (Figure 4.11) and direct root differentiation was observed in Pacific 22 (Figure 4.10). Embryogenesis, globular stage of the embryonic pathway was found in A1, A2 and A3 medium. A2

medium was the best synergist response for callus induction (65.48%), and the percentage of embrygenic calli (21.88%). The A1 medium gave callus induction about 58.70% and the percentage of embrygenic calli about 12.69%. The A3 medium gave 27.56% of gave callus induction, and 5.04% of embrygenic calli. The best callus growth was achieved in A2 medium with 2.2584 mm in size, 0.214 mg fresh weight, and 0.079 mg dry weight (Table 4.7).

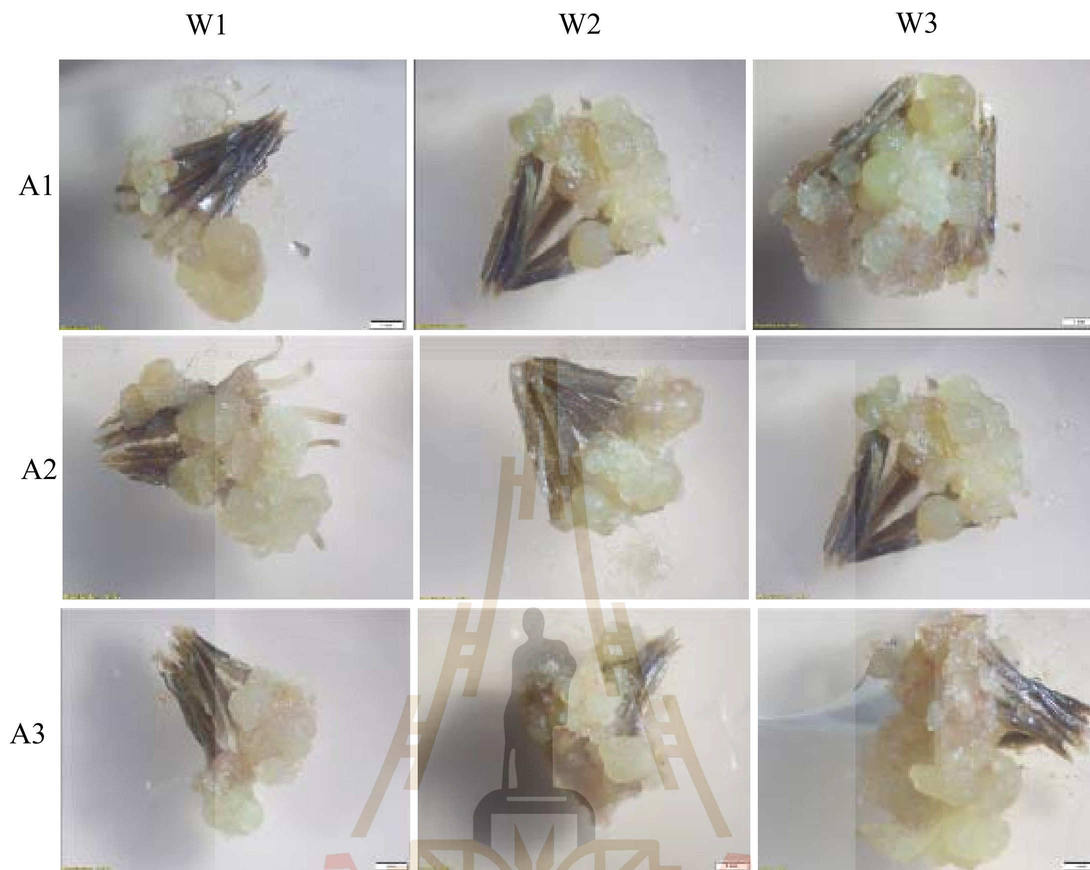

Figure 4.9 Anther- derived calli from different whorls of S473 variety on various callus induction medium. A = media, W = floret whorl.

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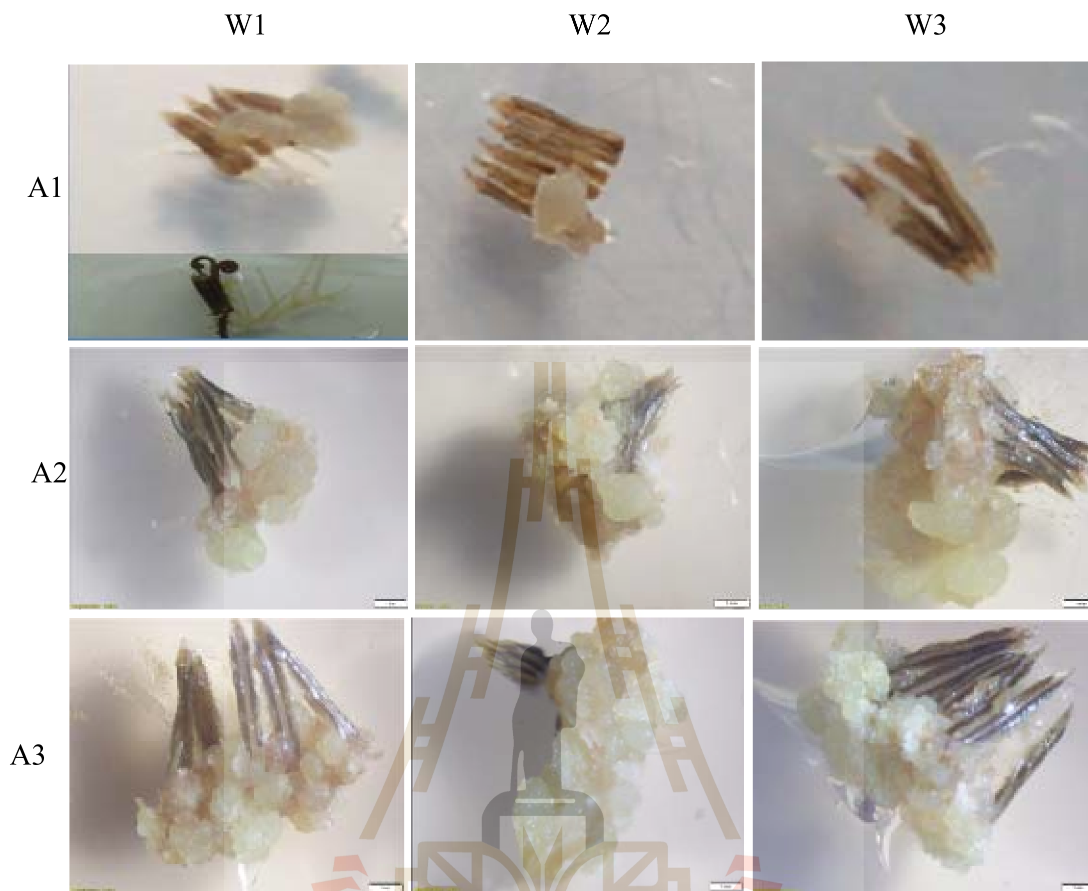

Figure 4.10 Anther-derived calli from different whorls of Pacific 22 variety on various callus induction medium. A = media, W = floret whorl.

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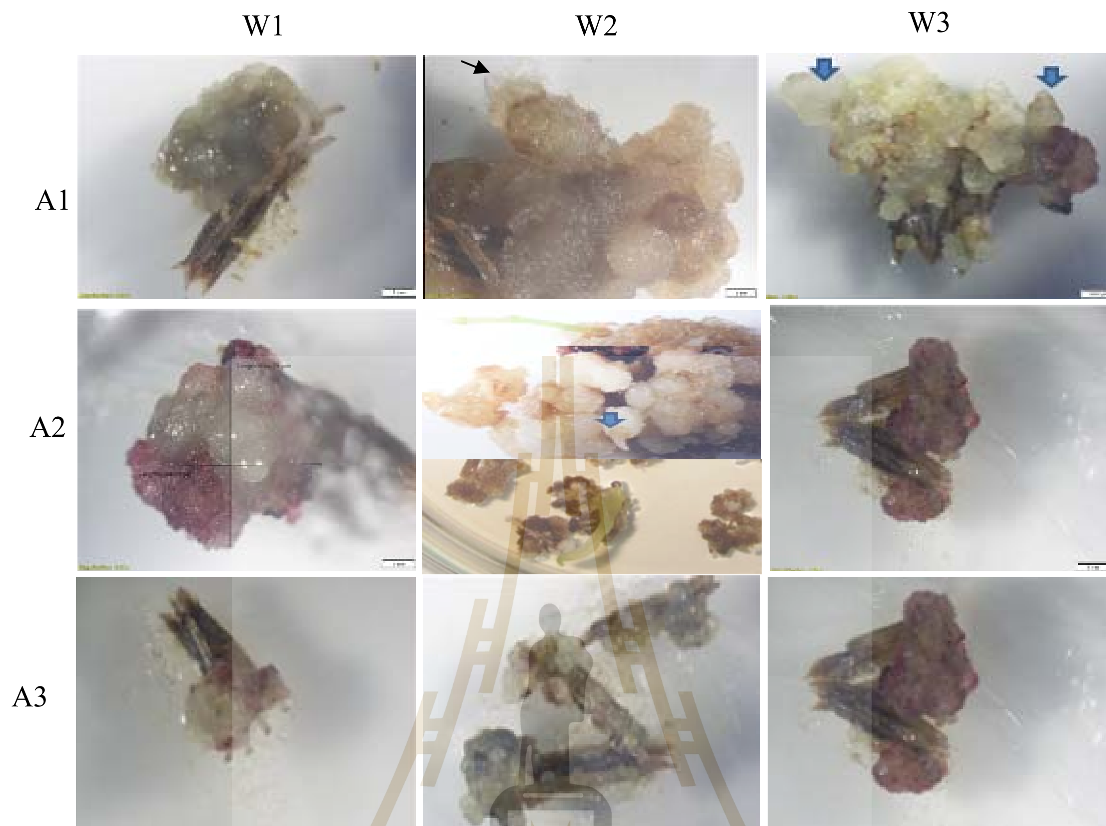

Figure 4.11 Anther-derived calli from different floret whorls of Prado Red variety on various callus induction medium. A = media, W = floret whorl, black arrow = organ, blue arrow = directed shoot formation.

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Analysis of variances for different varieties were significant in callus size parameter. The interaction between varieties x whorls was significant in the percentage of embrygenic calli parameter. The interaction between varieties x medium was significant in callus size and the percentage of callus induction parameters. The interaction between varieties x whorl x media was significant in callus size.

The percentage of callus induction has a strong positive correlation with callus size (r= 0.692**), fresh weight (0.713**), and dry weight (0.695**). The percentage of embrygenic calli was positively correlated with callus size (r= 0.666**), fresh weight (r= 0.669**), dry weight (r= 0.670**), and the percentage of callus induction (r=0.795**). Dry weight was correlated with callus size (r=0.809**) and fresh weight (r= 0.928**). Fresh weight was correlated only with callus size (r=0.814**) as shown in Table 4.9.

Table 4.9 Correlation analysis of variances of callus growth parameters in sunflower varieties.

Variations	CS	FW	DW	PC
FC	.814**	-		
DC	.809**	.928**	-	
PC	.692**	.713**	.695**	-
PE	.666**	.669**	.670**	.795**

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

CS = callus size, DW = dry weight, FW = fresh weight, PC = the percentage of callus induction, and PE = the percentage of embryogenic callus.

Exogenous hormones are important to produce haploid plant production through anther culture. Vijaya Priya et al. (2003) found that MS medium supplemented with 2 mg/l of NAA, 1 mg/l BA and 500 mg/l CH gave callus formation about 98.7% in cultivated sunflower, while MS medium supplemented with 1mg/l of NAA, 0.5 mg/l BA and 250 mg/l CH induced root formation in interspecific

hybrid *Helianthus annuus* L x *Helianthus decapetalus*. Nurhidayah et al. (1993) reported that MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BAP and 0.1 mg/l biotins could induce 187 shoots from interspecific sunflower. In strawberry (*Fragaria x ananassa* Duch) cv. Albion, anther-derived calli displayed 70.0% in MS medium supplemented with 0.4 mg/l BA, 0.1 mg/l IAA and 2.0 mg/l 2,4-D and shooting formation indicated at a level of 8.1% in MS medium supplemented with 2 mg/l IAA and 1 mg/l BA (Nguyen et al., 2012). In maize, the frequency of embryo-like structures (ELSs) was from 4.5 to 22% in Ypm medium (Mohammadi et al., 2007).

Sujatha and Prabakaran (2006) reported that interspecific *H. annuus* L. x *H. resinosus* showed good response and regenerated embryo-like structure through embryonic differentiation at a frequency of 98.7%, whereas interspecific *H. annuus* L. x *H. tuberosus* regenerated direct shoots through organogenesis.

The anther-derived callus had been optimized by Gurel et al. (1991). The various genotypes showed differential responsiveness towards callus initiation and play a major role in the success of haploid plant production under genetic control (Dayan, 2016). Furthermore, within species of *Hibiscus cannabinus* L. different genotypes exist in the ability to produce haploid plant (Ibrahim et al., 2015). Todorova et al. (1997) reported that doubled haploid production differed among sunflower genotypes, and found the maximum number of double haploid new plants in HB9203 hybrid about 20 haploid lines.

Nurhidayah et al. (1996) reported the frequency of callus induction from anther culture of interspecific *H. annuus* L. x *H. tuberosus* hybrid at 7.0% and shoot induction about 1.2%. Bohorova et al. (1985) showed that interspecific hybrid *H.*

annuus L. x *H. decapetalus* anther culture in MS medium gave direct shoot formation. Moreover, wild *Helianthus* species, e.g. *H. toberosus*, *H. laetiflorus*, *H. resinosus* as parents, seem to bear a higher shooting regeneration potential *in vitro* than the cultivar sunflower (Gurel et al., 1991; Friedt et al., 1997). Jeyamary and Jeyabalan (1997) reported synthetic medium supplemented with hormones thus impose a profound influence on the organogenesis but are dependent mostly on the genetic makeup of sunflower genotype. Miladinovic et al. (2012) reported that genetic variation in sunflower had significant effects on callogenesis, somatic embryogenesis, shooting and rooting formation through anther culture in sunflower species.

4.2.2 Shoot and root regeneration of embryogenic callus derived from anther

Analysis of variance for medium was significant in all parameters including fresh weight, dry weight, percentage of the shoot, percentage of the root and callus size parameters. Differences among varieties were not significant in all parameters. While the interaction between varieties x medium was significant in the percentage of the shoot and the percentage of the root as presented in Table 4.10.

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Table 4.10 Analysis of variance of anther-derived callus parameters on regeneration medium in three sunflowers varieties.

S.O.V	d.f.	Mean Square				
		CS	**FW**	**DW**	**PS**	**PR**
Variety (V)	2	1.75	0.009	5.88E-05	0.06	2.55
Medium (M)	3	37.80*	0.122*	0.001*	3.70*	3.52*
V * M	6	1.33	0.002	1.81E-05	0.06*	2.06*
Error	72	0.78	0.01	8.33E-05	0.93	1.25
% C.V		15.66	25.05	30.03	28.62	29.52

* = Significant at 0.05 probability level.

CS = callus size, DW = dry weight, FW = fresh weight, PS = percentage of shoot, and PR = percentage of root.

Four shoot induction media (S1-S4) were applied to shoot and root induction studies. The anther-derived callus was induced into shoot and root formation only on S4 shoot induction medium whereas no shoot or root was observed in other three tested medium (Table 4.11). Among various medium, S4 gave the maximum callus size (11.12 mm), callus fresh weight (0.33 mg), and dry weight (0.027 mg), whereas S1 and S3 gave exactly the same fresh weight (0.19 mg) and dry weight (0.015 mg). The S4 medium resulted in the highest the percentage of the shoot (1.85%) and root (1.94%) induction (Table 4.10).

Among different varieties, the best response for the percentage of shoot induction through anther-derived callus was Pacific 22 (1.04%) (Figure 4.12E, H), followed by S473 (0.26%) (Figure 4.12D), and Prado Red (0.13%) (Figure 4.12F), respectively. The maximum the percentage of root induction was observed in S473

(0.75 %), followed by Prado Red (0.54%) (Figure 4.13G) and Pacific 22 (0.17%) (Figure 4.12H). For callus growth parameters, S473 variety gave the best growth callus size (9.73mm), fresh weight (0.26 mg), and dry weight (0.021 mg), Where Prado Red showed the lowest callus size (9.33 mm), fresh weight (0.23 mg) and dry weight (0.018 mg) (Table 4.11).

Table 4.11 The mean values (± SD) of callus growth characteristics on various shoot induction medium in three sunflower varieties.

Factors	CS	FW	DW	PS (%)	PR (%)
Varieties					
S473	9.73±1.66^{b}	0.26±0.09^{a}	0.021±0.008	0.26±0.97^{b}	0.75±1.75
Prado Red	9.33±1.23^{a}	0.23±0.11^{b}	0.018±0.011	0.13±0.47^{b}	0.54±1.89
Pacific 22	9.38±1.25^{b}	0.24±0.11^{a}	0.20±0.011	1.04±2.47^{a}	0.17±0.70
F-test	**	**	ns	**	ns
Medium					
S1	8.75±0.89^{a}	0.19±0.57^{c}	0.015±0.011^{a}	0.00±0.00^{b}	0.00±0.00^{b}
S2	9.55±1.01^{b}	0.26±0.12^{b}	0.022±0.010^{b}	0.00±0.00^{b}	0.00±0.00^{b}
S3	8.50±0.78^{a}	0.19±0.05^{c}	0.015±0.001^{a}	0.00±0.00^{b}	0.00±0.00^{b}
S4	11.12±1.10^{c}	0.33±0.10^{a}	0.027±0.012^{c}	1.91±1.81^{a}	1.94±2.15^{a}
F-test	**	**	**	**	**

Means within column followed by the same superscript letter are not significantly different at 1% level by DMRT.

** = Significant at 0.01 probability level. ns = not significant.

CS = callus size, DW = dry weight, FW = fresh weight, PS = percentage of shoot, and PR = percentage of root.

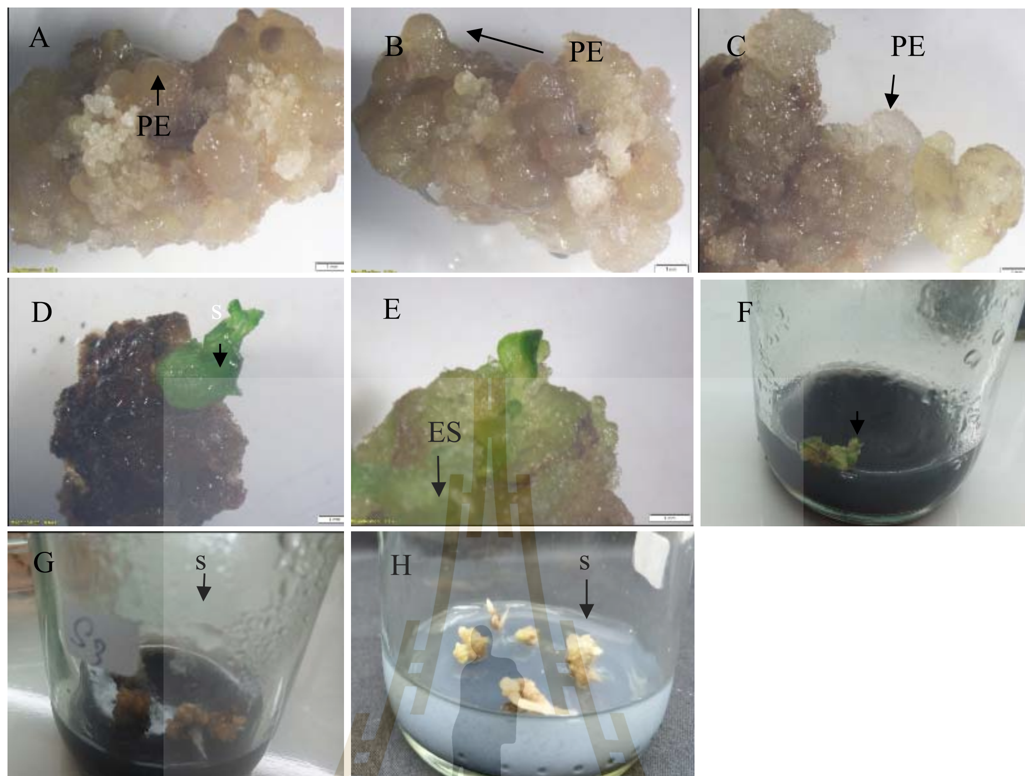

Figure 4.12 Embryogenic callus (EC) with pro-embryo (PE), shoot and root formation on shoot induction medium in three sunflower varieties. A) Embryogenic callus of Pacific 22, B) Embryogenic callus of S473, C) Embryogenic callus callus of Prado Red, D) Shoot of S473, E) Shoot of Pacific 22, F) Shoot of Prado Red, G) Root formation of Prado Red, and H) Shoot or root formation of Pacific 22. r = root formation, s = shoot formation.

4.3 Effect of colchicine treatment on chromosome doubling of anther-derived calli

The result of analysis of variances (ANOVA) for differences in colchicine concentrations and timing was significant in four parameters such as survival rate, fresh weight, dry weight and callus size. The interaction between concentrations x timing had a significant effect on the percentage of organogenesis. The analysis of variance for varieties, the interaction between varieties x time and varieties x concentration had a significant effect on the survival rate, while fresh weight, and dry weight parameters, but did not show significantly in callus size parameter (Table 4.12).

Table 4.12 Analysis of variance of morphological characteristics of anther-derived callus treated with colchicine in three sunflower varieties.

S.O.V	d.f.	Mean Square				
		SR	**CS**	**FW**	**DW**	**PS**
Varieties (V)	2	79.438*	5.158	0.007	0.001	29.498
Concentration (C)	2	3079.471*	281.898*	0.677*	0.004*	55.499
Timing (T)	2	5212.501*	1177.341*	1.429*	0.007*	54.208
V * T	4	94.861*	3.616	0.001	0	67.20
C * T	4	2.400*	3.426	0.001	0	175.26*
V * C	4	23.426*	1.734	0.004	0	46.27
V * C * T	12	33.939	2.655	0.002	0.00007	37.26
Error	120	34.859	3.556	0.005	0.001	48.04
% C.V		23.44	28.44	18.22	26.82	19.51

* = Significant at 0.05 probability level.

CS = callus size, FW = fresh weight, DW = dry weight, PS = percentage of organogenesis, SR = survival rate.

The embryogenic calli were treated with various concentrations and durations of colchicine and then these calli were cultured on the S4 regeneration medium from previous experiment. Shoots were successfully formed by sequential subculturing embryogenic calli on medium containing growth regulators 1 mg/l BAP, 500 mg/l CH and 0.2% charcoal every 2 weeks. After 14 days of culture on S4 medium, survival rate, callus growth (callus size, fresh weight and dry weight), percentage of organogenesis and chromosome content were determined. The results showed that the highest survival rate was identified for S473 at the level of 92.07%, followed by Pacific 22 (89.32%) and Prado Red (89.48%), respectively (Table 4.13). The maximum callus size was indicated in Prado Red (12.29 mm), while the minimum was in Pacific 22 (11.67 mm). The highest fresh weight parameter was presented in S473 (0.44 mg) and the lowest fresh weight was observed in Pacific 22 (0.40 mg). The maximum dry weight was observed in Pacific 22 (0.036 mg), both S473 and Prado Red had the same value of dry weight about 0.029 mg. The percentage of organogenesis from embryogenic callus of three sunflower varieties at different concentrations of colchicine was generally low, 0.56-7.32%, with an average of 7.32%, 1.99% and 0.56% at colchicine concentration of 0, 100 µM and 300 µM. The highest percentage of organogenesis was observed in S473, while the lowest was in Pacific 22. At different duration time for soaking, the highest percentage of organogenesis was observed in 0 h (7.31%), while lowest was observed at 3 h treatment.

Effects of colchicine concentration and duration time for induce chromosome set induction in sunflower callus were determined by ANOVA ($p \leq 0.05$) for two ploidy levels. Both 1N and 2N levels were significant as shown in Table 4.14.

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Polyploidy is important for the evolution of plant and constitutes genetic variation for new plant species as well as the creation of haploid line in breeding programs (Adams and Wendel, 2005). In this study, flow cytometer was applied to determine the ploidy level of the embryogenic calli treated with colchicine. The highest 1N level was found in Pacific 22 (44.62%), while the lowest 1N level was indicated in Prado Red (13.85%). At 2N level, ploidy level was highly indicated in Pacific 22 (24.61%), while the lowest 2N level was indicated in Prado Red (7.15%) (Table 4.12).

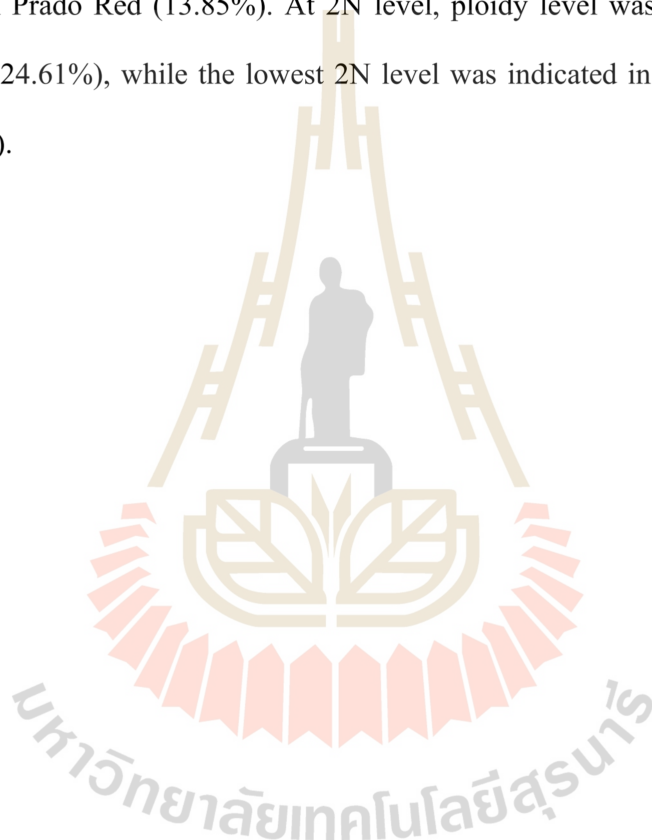

Table 4.13 Comparison of morphological characteristics and ploidy level of embryogenic calli in three sunflower varieties.

Factors	**CS (mm)**	**DW (mg)**	**FW (mg)**	**PO (%)**	**SR (%)**	**1N (%)**	**2N (%)**
Variety							
S473	11.67±0.03	0.029±0.003	0.44±0.01^{b}	1.39±5.78^{b}	92.07±0.83^{b}	16.39±4.08^{b}	12.37±2.60^{b}
Prado Red	12.29±0.03	0.029±0.003	0.41±0.01^{a}	1.05±4.02^{b}	89.48±0.83^{a}	13.85±5.04^{a}	7.15±2.53^{a}
Pacific 22	12.01±0.03	0.036±0.003	0.40±0.01^{a}	0.67±3.13^{a}	89.32±0.83^{a}	44.62±3.39^{c}	24.61±2.97^{c}
F-test	ns	ns	**	**	**	**	**
Colchicine concentrations (μM)							
0	18.95±1.76c	0.07±0.05^{c}	0.81±0.10^{c}	7.32±12.21^{b}	100.00±0.00^{c}	61.71±1.93^{c}	37.64±1.91^{c}
100	11.86±4.20^{b}	0.03±0.02^{b}	0.40±0.14^{b}	1.99±4.40^{a}	93.20±7.52^{b}	16.48±3.84^{b}	10.65±2.48^{b}
300	8.63±3.36^{a}	0.02±0.01^{a}	0.24±0.12^{a}	0.56±2.86^{a}	82.52±11.04^{a}	15.06±3.33^{a}	7.28±1.57^{a}
F-test	**	**	**	**	**	**	**
Duration (h)							
0	6.95±1.53^{a}	0.08±0.05^{a}	0.81±0.01^{c}	7.31±6.8^{a}	100.00±1.04c	61.71±1.93^{c}	37.64±1.90^{c}
3	13.55±3.16^{b}	0.03±0.01^{b}	0.43±0.10^{b}	0.58±2.86^{ab}	94.81±0.77^{b}	15.43±3.50^{a}	11.99±2.68^{b}
6	18.95±1.76^{c}	0.02±0.00^{c}	0.20±0.10^{a}	1.99±6.25^{b}	80.91±0.77^{a}	16.10±3.69^{b}	5.94±1.00^{a}
F-test	**	**	**	**	**	**	**

Means within column followed by the same superscript letter are not significantly different at 5% level by DMRT.

** = Significant at 0.01 probability level. ns = not significant.

DW = dry weight, CS = callus size, FW = fresh weight, PO = percentage of organogenesis, SR = survival rate, 1N = percentage of haploid level, and 2N = percentage of diploid level.

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The treated embryogenic callus cultured on S4 shooting medium for two weeks turned brown at the edge in some calli. Embryogenic callus without colchicine treatment gave the maximum survival rate about 100%. While 100 µM of colchicine gave the survival rate about 93.20% and 300 µM of colchicine treatment about 82.52%. In addition, callus size, fresh weight, and dry weight of treated calli were decreased in colchicine treatment compared with control treatment (Table 4.12). Ploidy level was resulted from colchicine treatment. Without colchicine treatment (control), the maximum survival rate was 100.0%, whereas 3 h and 6 h of exposure time showed 94.81% and 80.91% of survival rate, respectively. With colchicine treatment, the maximum 2N ploidy level (11.99%) was obtained from calli treated with colchicine for 3 h and the maximum (5.94%) was from calli treated with colchicine for 6 h (Table 4.12).

Hoveida et al. (2015) reported the polyploidy level determination using FMC in doubled haploid plants from anther culture of borage after colchicine treatment, and they found the maximum diploid level about 40%, while haploid level was found the maximum about 60%. Yu et al. (2009) reported 3N/6N ploidy level ratio 66.7/22.2 after rice callus treated with 6.26 µM colchicine.

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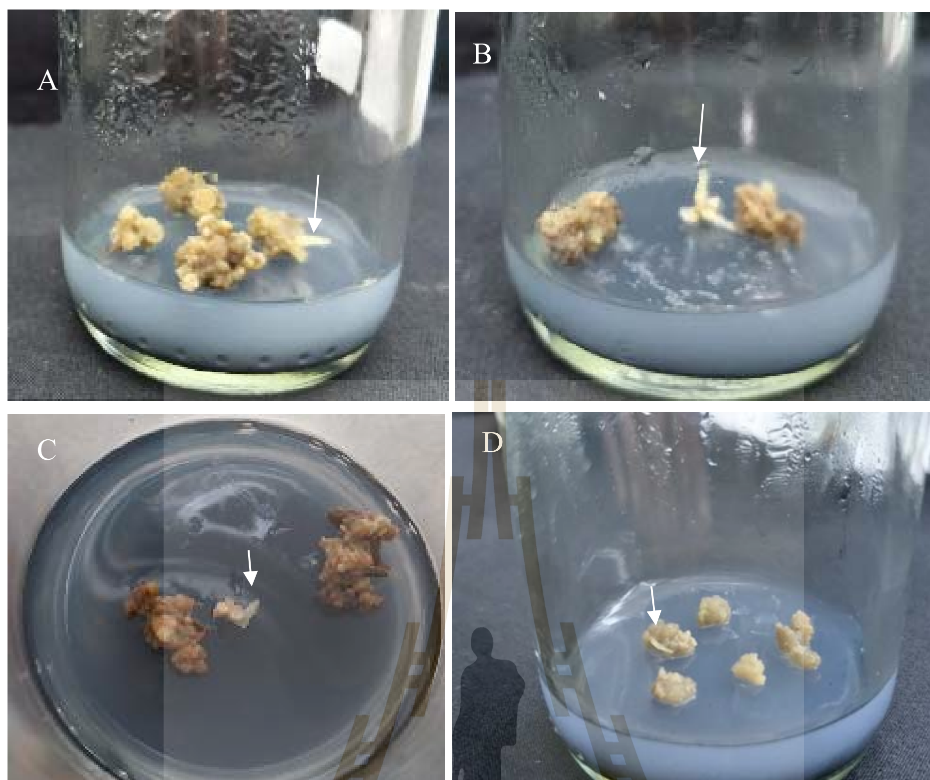

Figure 4.13 Images of organogenesis in the treated calli with colchicine. A) embryogenic callus treated 300 µM colchicine for 3 h of Pacific 22, B) embryogenic callus treated 100 µM colchicine for 6 h of Prado Red, C) embryogenic callus treated with 100 µM colchicine for 6 h, and D) embryogenic callus treated with 100 µM colchicine for 6 h of S473.

มหาวิทยาลัยเทคโนโลยีสุรนารี

Junpugdee and Te-chato (2010) reported effect of colchicine on callus growth in *Anthurium andraeanum* cv. Micky Mouse and they found the highest survival rate of the nodular calli in 0.2% colchicine for 72 h and the highest average of callus size at 1.92 µm in width and 1.91 µm in length. Kerdsuwan (2010) reported that *Rhynchostylis gigantea* var. *rubrum* Sagarik calli treated 0.2% colchicine for 72 h, 48 h and 24 h had callus survived at a level of 25%, 55% and 84%, respectively. Promma

(2010) reported that oil palm treated with 7.5 mM colchicine concentration affected survival rate and indicated at the level of 17.78%, while 2.5 mM colchicine concentration had the survived at the level of 71.11%.

Table 4.14 The ploidy level of the treated embryogenic calli with colchicine in three sunflower varieties.

S.O.V	d.f.	Mean Square	
		1N	2N
Variety (V)	2	5425.608*	1871.564*
Concentration (C)	1	36.380*	204.626*
Timing (D)	1	8.215*	658.966*
V * C	2	11.992*	259.635*
V * D	2	40.485*	874.879*
C * D	1	130.573*	16.226*
V * C * D	4	334.219*	105.301*
Error	45	0.731	0.4269
C.V%		17.6	20.42

* = Significant at 0.05 probability level.

1N = percentage of haploid level, and 2N = percentage of diploid level.

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CHAPTER V

CONCLUSION

Anther culture is one of the biotechnological tools of sunflower breeding program for increasing production yield and stability especially in the climate change world wide. In this thesis study, flower bud at the R5.1 reproductive stage of three sunflower varieties were employed for anther culture studies. Morphological and cytological parameters of floret, anther, and microspore were examined and analyzed under microscopic laboratory conditions. In addition, anther-derived calli were induced through callus and shoot induction medium, and their calli were treated with colchicine to induce chromosome doubling and evaluated for ploidy level through flow cytometry equipment. Summary of the study is as follows:

1. The morphology of three sunflower varieties with respect to floret whorls were investigated, and the results indicated the significant variation among the sunflower varieties and floret whorls. For morphological characteristics, disk length was higher in S473 (15.53 mm) and Pacific (15.37 mm) and lower in Prado Red (7.86 mm). The length and width of anthers were highest in Pacific 22, followed by S473, and Prado Red respectively. The same trends were recorded for disk length, anther length and anther width with respect to floret whorls, where higher values were shown in whorl 1 and lower in whorl 3.

2. The maximum number of microspore per flower was found in Pacific 22

(28,106 grains), followed by S473 (24,877 grains) and Prado Red (14,694 grains), respectively. Also, Pacific 22 variety has the maximum percentage of microspore viability with 99.83%. Furthermore, disk length and anther length parameters of floret characteristics maintain positive correlation with the percentage of microspore per flower and pollen viability, except anther width. The recorded results clearly indicate that Pacific 22 variety has the best morphological characters of floret which may be used for anther culture with high efficiency.

3. For histological study of sunflower disk floret, each disk floret contains a single anther that appears in butterfly-shape with five to six pollen sacs. Anther of S473 and Prado Red has five pollen sacs whereas Pacific 22 has six pollen sacs.

4. For cytological study of sunflower microspore, its polar view (P) and equatorial view (E) varied among varieties and whorls, but its ratio (P/E) was similarly observed in all three varieties. Furthermore, a negative relationship has been shown to exist between polar view (P) and equatorial view (E) of microspore and the percentage of microspore per flower and pollen viability.

5. In the flower bud at the R5.1 stage, individual floret whorls contained both early and mid-to late uninucleate microspore stage at different percentages. The microspore stages were significantly different in frequency of each stage of development among varieties and whorls. The outer most unopened disk floret had the highest percentage of mid-to-late uninucleate stage with 30.44% compared with the innermost unopened florets (whorl 3) with 12.00%. Among three varieties, Prado Red variety had the maximum percentage of mid-late uninucleate microspore stage about 26.89%, followed by Pacific 22 and S473 which are 19.33% and 11.89%, respectively.

6. Ultrastructure and surface morphology study of microspore with SEM and TEM revealed sculpturing element, aperture and microspore wall. Microspore surface of three sunflower varieties was similar in morphology where it is covered with spines and sporopollening substance. Microspore is prolate-spheroidal and has tricolporate aperture. Its wall is identified as an echinate type. The thickness of microspore exine seems to be varied among varieties and floret whorls.

7. Medium culture played an important role on callus and embryo induction of sunflower through anther culture. MS medium supplemented with 2 mg/l NAA, 1 mg/l BAP and 10% (v/v) CW induced the highest percentage of friable calli for all three sunflower varieties with 65.48%, but no response was observed in MS medium containing 0.5 mg/l 2, 4-D, 0.5 mg/l BAP and 100 mg/l coconut water. Some calli of S473 and Prado Red were directly induced into shoot, while some calli of Pacific 22 was directly induced into root formation. Analysis of variances confirmed that callus growth parameters (callus size, fresh weight, and dry weight), percentage of embryogenic callus and percentage of callus were significantly affected by medium.

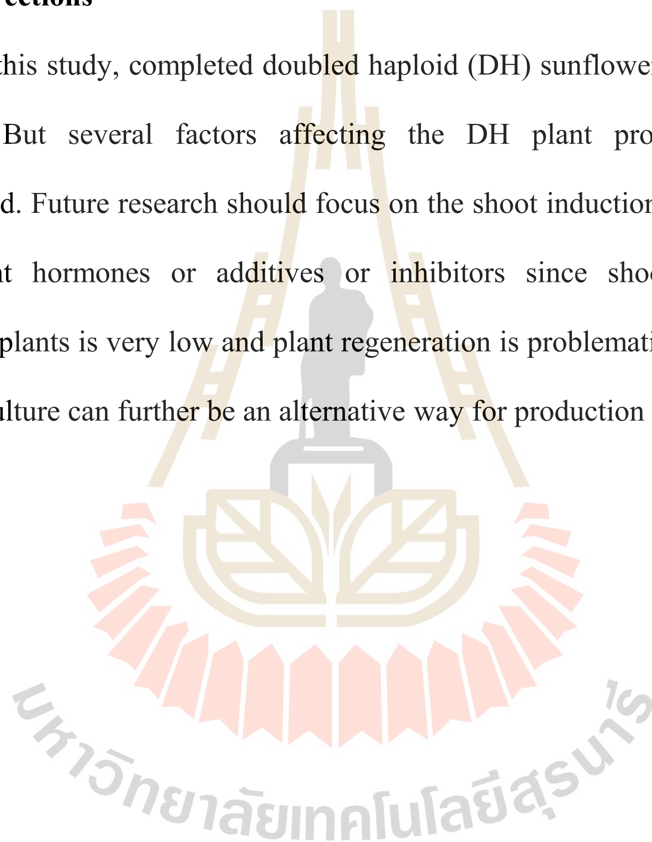
8. For shoot induction study of embryogenic calli-derived from anthers, MS medium supplemented with 2 mg/l BAP and 0.2% activated charcoal was found to be most suitable for shoot regeneration (1.85%) and root (1.94%) formation than other media which only embryogenic calli were produced. The addition of activated charcoal may express synergistic effect for anther culture, while silver nitrate may contribute antagonistic effect for organ induction from anther culture.

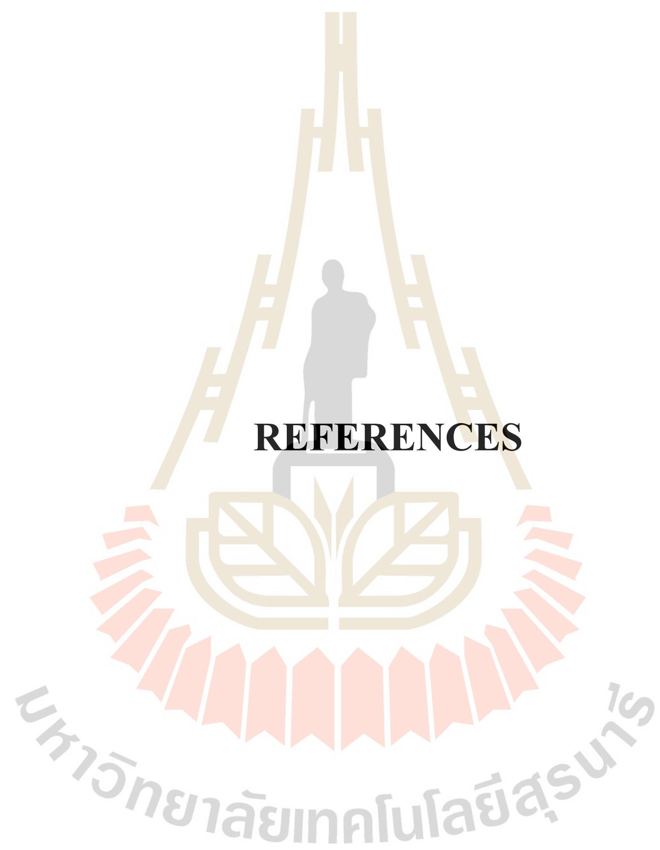
9. In chromosome doubling study, survival rate, shoot and root production of regenerating calli following in vitro colchicine treatment decreased with increasing colchicine concentration and treatment time. Cytological analyses of regenerated calli showed that chromosomes of control calli (not treated with colchicine) had

doubled that could be due to somatic cells of anther tissue. We found that 1N level about and 2N level decreased in colchicine treatment. It could be explained that in vitro colchicine treatment of regenerating calli of sunflower varieties was not effective in recovering diploid.

Future directions

In this study, completed doubled haploid (DH) sunflower plants could not be obtained. But several factors affecting the DH plant production have been investigated. Future research should focus on the shoot induction through addition of other plant hormones or additives or inhibitors since shoot production from sunflower plants is very low and plant regeneration is problematic. In addition, ovary or ovule culture can further be an alternative way for production of haploids.





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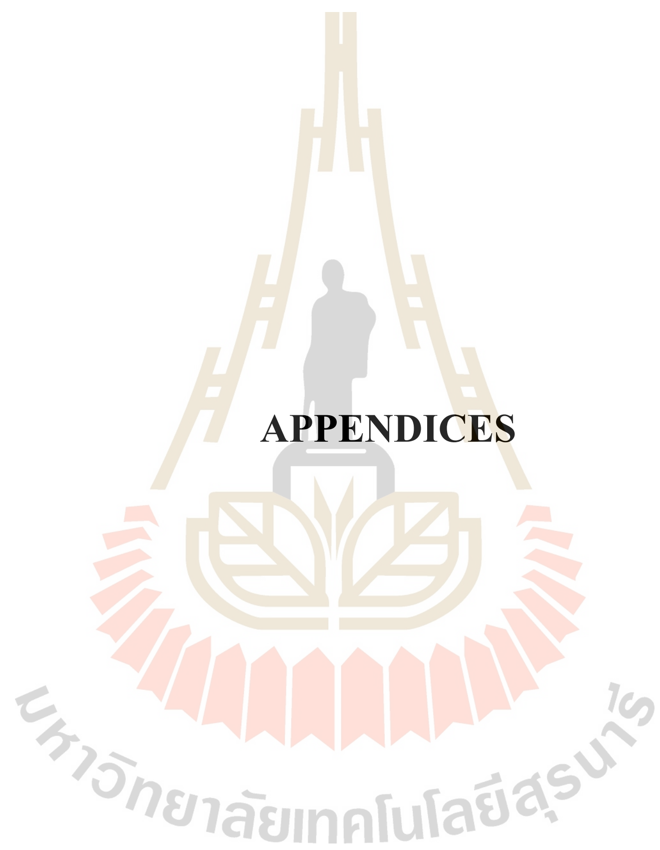
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APPENDICES

APPENDIX A

MURASHIGE AND SKOOG MEDIA

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

MSI (Major salts)

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

Preparation of stock MSI: each chemical component is dissolved in water then pour into a tank, to make a volume 1 liter. The solution is kept in a plastic container in the dark and store at 4-7 ºC.

MSII (Minor salts)

H_3BO_3	6.2 mg/l
$CoCl_2\ 6H_2O$	0.025 mg/l
$CuSO_4\ 5H_2O$	0.025 mg/l
$FeSO_4\ 7H_2O$	27.8 mg/l
$MnSO_4\ 4H_2O$	22.3 mg/l
KI	0.83 mg/l
$Na_2MoO_4\ 2H_2O$	0.25 mg/l

$ZnSO_4 \cdot 7H_2O$	8.6 mg/l

Preparation of stock MSII: each chemical component is dissolved in water then pour to a tank, to make a volume 1 liter. The solution is kept in a plastic container in the dark and store at 4-7 ^oC.

MSIII (iron chelated)

$FeSO_4.7H_2O$	5.57 g
Na_2-EDTA	7.45 g

Preparation of stock MSIII: each chemical component was dissolved in water then pour to a tank, to make a volume 1 liter. The solution is kept in a plastic container in the dark and store at 4-7 ^oC.

MSIV (Vitamins and organics)

Myo-Inositol	100 mg/l
Nicotinic Acid	0.5 mg/l
Pyridoxine-HCl	0.5 mg/l
Thiamine-HCl	0.1 mg/l

Preparation of stock MSIV: each chemical component is dissolved in water then pour into a tank, to make a volume 1 liter. The solution is kept in a plastic container in the dark and store at 4-7 ^oC.

A.2 Plant hormones

100 mg/l of NAA

Preparation of stock: the NAA is weighted at 100 mg and then dissolved in 1-2 ml of absolute alcohol, mixed thoroughly until dissolved and

brought the volume up to 100 ml with DI water. The solution is kept in a plastic container in the dark and store at 4-7 °C.

100 mg/l of 2,4-D

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

100 mg/l of BAP

Preparation of stock: the BAP is weighted at 100 mg and then dissolved in 1-2 ml of ethanol, mixed thoroughly until dissolved and brought volume up to 100 ml. The solution is kept in a plastic container in the dark and stored at room temperature.

A.3 Additive substances

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

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

Coconut water stock

Preparation of stock: the young coconut is washed. The coconut water drained out through micropyle and filtered through doubled cloth. The water was boiled at 80 °C at 1 h. The water is filtered by 0.20 μm paper filter and kept 4 °C.

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APPENDIX B

CHEMICAL REAGENTS

B.1 Chemical reagent solution for electron microscope

0.1 M Sodium phosphate buffer (pH 7.5)

Na_2HPO_4	17.79 g
NaH_2PO_4	15.60 g
EDTA	0.19 g

Preparation: all components are weighted and then added to sterile distilled water, mixed thoroughly until dissolved and adjusted pH 7.5 and brought volume up to 1,000 ml. Solution is sterilized by autoclaving at 121 ºC for 15 minutes.

B.2 Chemical reagent solution for pollen viability stain

1% Iodine-potassium iodide stains

Potassium iodide (KI)	1 g
Iodine	0.5 g

Preparation: all components are weighted and then added to distilled water, mixed thoroughly until dissolved and brought the volume up to 100 ml. The solution is filtered by 0.20 µm paper filter. After that, it is kept in dark bottle.

B.3 Chemical reagent for pollen quantity stains

1% Safranin

Preparation: dissolve 1g of safranine in 95% ethyl alcohol about 40 ml; made up to a volume 100 ml with distilled water. The solution is filtered by 0.20 µm paper filter. After that, it is kept in dark bottle.

B.4 Chemical reagent for thin section

0.5% mM Toluidine blue O

Toluidine blue O	0.5 g
Sodium borate	1.0 g

Preparation: dissolve both Toluidine blue O and Sodium borate in distilled water; made up to a volume 100 ml. Solution is filtered by 0.20 µm paper filter. After that it is kept in dark bottle.

B.5 Chemical solution for electron microscope

5% Glutaraldehyde (stock)

25% glutaraldehyde	10 ml

Preparation: 10 ml of 25% glutaraldehyde is diluted in DI water and pH 7.5 (adjusted with KOH) and then made up a volume to 100 ml. The solution is kept in brown glass bottles.

0.2 M Phosphate buffer (pH7.4)

$NaH_2PO_4.2H_2O$ (mol wt 156.01)	3.12 g
Na_2HPO_4 $12H_2O$ (141.96)	2.84 g

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Preparation: The chemicals substances are dissolved in 1,000 ml H_2O. and then is adjusted pH at 7.4

2.5% glutaraldehyde (must be EM grade) in 0.1M buffer.

0.2 M Phosphate buffer	1 part
5% Glutaldehyde	1 part

Preparation: 0.2 M Phosphate buffer 1 part is mixed in 5% glutaldehyde 1 part. After that it is adjusted pH to 7.4. The solution is kept in freeze.

1% Osmium tetroxide

OsO_4	1 g

Preparation: dissolve 1 g of OsO_4 in distilled water then make a volume to 100 ml. Before used should filter through Whatman filter paper.

B.6 Chemical reagents for flow cytometer

Otto Buffer I (Otto 1990)

0.1 M citric acid monohydrate	4.2 g
0.5 % (v/v) Tween 20	1 ml

Preparation: the chemicals are dissolved in sterilized distilled water then make a volume to 200 ml and filter through a 0.22 µm filter; store at 4 ^{o}C.

Otto II

0.4M $Na_2HPO_4 .12H_2O$	28.65 g

Preparation: the chemicals are dissolved in sterilized distilled water then make a volume to 200 ml and filter through a 0.22 µm filter; store at room temperature and re-filtered before each use.

Propidium iodide stock solution (1 mg/ml)

Propidium iodide 100 mg

Preparation: the chemical is dissolved in 100 ml H_2O and filter through a 0.22 µm filter, store at -20 °C in 1 ml aliquots.

RNase stock solution (1 mg/ml)

RNase (IIA Sigma) 100 mg

Preparation: the chemical is dissolved in 1 ml H_2O filter through a 0.22 µm filter heat to 90 °C for 15 min to inactivate DNases store at -20 °C in 1 ml aliquots.

B.7 Chemical for doubling chromosomes

5 mM colchicine stock

Colchicine 0.02 g

Preparation: the chemicals are dissolved in sterilized distilled water then made a volume to 10 ml and filter through a 0.22 µm filter; store at 4 °C and re-filter before each use.

100 µM colchicine

Preparation: 1 ml of 5 mM colchicine is diluted in 49 ml sterilized distilled water then made a volume to 50 ml.

300 µM colchicine

Preparation: 3 ml of 5 mM colchicine is diluted in 47 ml sterilized distilled water then made a volume to 50 ml.

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Publications

Khamsee Saensee, Thitiporn Machikowa, and Nooduan Muangsan. *In vitro* Anther Culture of Some Sunflower Varieties (*Helianthus annuus* L.). **The 8th Botanical conference of Thailand – BCT-8. 2-4 April 2014, Thailand**.

Awards -

Grants and Fellowships Scholarships for Potential Graduate Students at Suranaree University of Technology

Position and Place of Work -