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Relationship between floret size and anther culture response in an ornamental sunflower

Khamsee Saensee[1], Thitiporn Machikowa[2], Yalcin Kaya[3], Nooduan Muangsan[1,] *

[1] School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand
[2] School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, 30000, Thailand
[3] Genetic and Bioengineering Department, Engineering Faculty, Trakya University, Edine, 22030, Turkey
*Corresponding author: nooduan@sut.ac.th

Abstract

A correlative approach using light microscope and electron microscope was carried out in this study to assess floret characteristics and microspore developmental stages in an ornamental sunflower cv. Prado Red. The relationship between microspore stage with anther culture response on various culture media was also investigated. The results revealed a decrease in the percentages of mid-to late- uninucleate microspores from 39.33% of the outer unopened disk floret whorl 1 to 15.33% of the inner floret whorl 3. Disk floret length and anther size were positively correlated with the microspore stage. The callus induction was significantly affected by culture medium but not disk floret whorls and the interaction between them, while the percentage of embryogenic calli was significantly affected by culture media and floret whorls but not the interaction between them. Among the four tested media, MS medium supplemented with 2 mg l^{-1} α-napthalene acetic acid (NAA), 1 mg l^{-1} N^6-benzylaminopurine (BAP) and 100 ml l^{-1} coconut water produced the highest callus induction (60.44%) and embryogenic callus production (20.45%). Hence larger disk florets with higher percentages of mid-to late-uninucleate microspores responded well and produced embryogenic callus in the optimum culture medium.

Keywords: Anther; Callus; *Helianthus annuus* L.; Microspore stage; Ornamental sunflower

1. Introduction

Ornamental sunflowers **(***Helianthus annuus* L**.-** Asteraceae**)** are mainly for their aesthetic value**.** The economic role of sunflowers as ornamental plants has increased worldwide **[**1**].** Recently, numerous ornamental sunflower cultivars have been developed and grown commercially as cut flowers with a wide range of available colors ranging from; orange, cream, red, to bi**-**or multi**-**colored**.** Most cultivars are pollen**-**free cultivars which are preferred by growers because they have longer vase life **[**2, 3**].** The new varieties of ornamental sunflowers have no concerns in yield and oil content, unlike oilseed varieties**.** The breeding targets desirable structures and aesthetic colors of the disk and ray florets to create a high quality inflorescence **[**4**].** Floral colors and types are controlled by one or two genes with different effects, and floral colors mainly depends on the presence or absence of anthocyanin pigmentation **[**5**].**

Androgenesis techniques play a significant role in shortening plant breeding programs as only a single generation is needed, while conventional breeding usually requires a minimum of six generations to obtain pure lines **[**6**].** As a result of these techniques, double haploid **(**DH**)** lines are generated and valued for use in breeding programs because multiple generations of back**-**crossing are not required to fix traits**.** Double haploid lines are also useful in hybrid breeding and in the development of mapping sunflower populations**.** The generated DH lines in sunflowers by anther culture have been presented in a number of studies **[**7, 8, 9, 10, 11, 12, 13**].** However, anther culture results have been rather unsatisfactory for application in sunflower breeding **[**9, 10, 11**].** The degree of anther culture response depends on many factors such as the stage of microspore development, condition of donor plants, genotypes, components of medium, and cultural conditions **[**8, 13, 14, 15, 16**].** In this study, we

aimed to determine disk floret morphological traits, the stage of microspore development using light microscope **(**LM**)** and transmission electron microscope **(**TEM**)**, and evaluated their relationships pertaining to the efficiency of anther**-**derived embryos on different media in an ornamental sunflower cv**.** Prado Red**.**

2. Materials and Methods

2.1 Plant materials and sample collection

The ornamental sunflower (*Helianthus annuus* L.) cv. Prado Red, multi-stemmed with up to twenty deep, mahogany-red flowers, was used to investigate the relationship between the floret features, microspore developmental stage and anther culture response. The plants were grown on Suranaree University of Technology campus fields from November to December 2015. We selected the flower buds at the R5.1 reproductive stage and collected them at 7.00 am in order to determine the microspore stage and to use in the anther culture study.

2.2 Histological analysis

For the histological studies of anthers and microspores using light microscope (LM) and transmission electron microscope (TEM), the three outermost whorls of unopened disk florets, named whorl 1, 2 and 3 (Figure 1a) of flower buds at the R5.1 reproductive stage were selected. Anthers were carefully excised and immediately fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4 ºC. On the next day, the anthers were washed in a 0.2 M sodium phosphate buffer (pH 7.2) three times for 10 min each, postfixed with 2% osmium tetroxide in phosphate buffer (pH 7.2) for 2 hrs, washed with phosphate buffer three times for 10 min each, and dehydrated in a series of acetone in water: 30%, 50%, 70%, 90% and 100% for 1 h each, at room temperature. Infiltration in spurr resin was carried out with mixtures of resin: acetone (v:v) of 1:3, 1:1, 3:1 for 3 h each, then in pure resin for 24 hrs at 4 ºC for three times. The specimens were then placed into blocks, filled with resin, and allowed to be polymerized for 8 hrs at 70 ºC as described by Spurr [17]. Semi-thin sections (1 µm) of anthers were cut from the polymerized blocks in an ultramicrotome (Leica EM UC7, Austria), stained with 1% toluidine blue at 85 ºC for 5 min [18], and observed on an optical microscope (Zeiss Axiostar Plus, Germany). Images were captured using a program Zen blue 1012 software. The percentages of early and mid- to late-uninucleate microspore stages were determined. For TEM analysis, thin sections (60 nm) were also cut, mounted on copper grids of 200 mesh and contrasted with uranyl acetate-lead citrate as described by Venable and Coggeshall [19] to reveal the microspore wall (TEM-Hitachi HT7700, Japan).

2.3 Measurements of disk floret, anther and microspore

The measurements of unopened disk floret length (mm), anther length (mm), and anther width (mm) were performed using a stereomicroscope (Olympus SZX 9, Germany) at 0.63X, while microspore diameter (µm), polar view (P) and equatorial view (E) were obtained using an optical microscope (Olympus BX5, Germany) at 400X. Images were captured using Olympus cellSens Standard software. For each parameter, mean values were calculated from 30 florets, 30 anthers and 30 microspores.

2.4 Pretreatment and sterilization of sunflower heads

Flower buds at the R5.1 stage were collected before anther dehiscence then the heads were kept in a cool block. In the laboratory, the edge of the head was trimmed and then washed with running tap water for 15 min. It was subsequently disinfected with 70% ethyl alcohol for 30 sec and surface-sterilized with 10% commercial clorox added with 2-3 drops cold Tween 20® for 10 min. The sunflower heads were washed four times with sterile DI water for 1 min and dried on sterile paper in the sterile Petri dish before use.

2.5 Anther culture

Twenty-five anthers were cultured per treatment on Murashige and Skoog medium [20] modified with various hormones and additives and 30 g l^{-1} sucrose [10, 21] (Table 1), incubated at 25 ± 2 ºC in dark conditions for 5 days and kept under 16 hrs illumination with 3000 lux light intensity. After 30 days of culture, callus size (mm), fresh weight (mg), dry weight (mg), percentage of callus induction, and percentage of embryogenic callus were recorded. The images were captured with Olympus cellSens Standard software.

Table 1 MS medium supplemented with hormones and additives for callus induction from anthers

Media	Basal	CH (mg l^{-1})	CW (ml l^{-1})	Auxin (mg l^{-1})		Cytokinin BAP
				NAA	2,4-D	
A1	MS	500	-	2.0		1.0
A2	MS	-	100	2.0		1.0
A3	MS	500	-	-	0.5	0.5
A4	MS	-	100	-	0.5	0.5

CH: casein hydrolysate; CW: coconut water; NAA: α-naphthalene acetic acid;
2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: N^6- benzylaminopurine.

2.6 Statistical analysis

The experiment was designed as a CRD (Completely Random Design). All statistical data were analysed using the IBM SPSS statistics v.20 software and are presented as mean ± SD with three replicates. Means were compared by DMRT (Duncan Multiple Range Test) at $\alpha \leq 0.05$. The data on callus size, fresh weight, dry weight, percentage of callus induction and percentage of embryogenic callus were recorded and analysed using one-way analysis of variance (ANOVA). Pearson's correlation was used to examine the relationship between floret morphological characters, percentages of microspore stage, and percentage of anther-derived embryo.

3. Results

3.1 Determination of microspore developmental stage

To determine the microspore developmental stage of an ornamental sunflower cv. Prado Red, the semi-thin sections of anthers were studied by LM and TEM. A similar structure of anthers from floret whorl 1, 2 and 3 were observed. Each floret was composed of five anthers united as a tube enclosing the style. The anther wall was composed of the epidermal layer and the elongated endothecium cells, and contained uninucleate microspores (Figure 2a, b, c). The uninucleate microspore had a thick exine wall and uninucleus located in the cytoplasm. If a microspore showed a vacuole within the cytoplasm and the nucleus came to lie at the periphery, then it was categorized as early uninucleate microspore, while the microspore contained uninucleus located at the central of cytoplasm and lacks of vacuole that was classified as mid-to late- uninucleate microspore (Figure 2d, e, f). Based on vacuole accumulation, we observed 39.33%, 26.00% and 15.33% of mid-to late- uninucleate microspore in floret whorl 1, 2, and 3, respectively (Table 2) indicating that the larger (outer) disk florets have more percentages of mid-to late- uninucleate microspores compared to the smaller (inner) florets.

Based on TEM observation, the microspore had a double exine, which was an outer layer consisting of thick collumellae inserted by cavus, and inner layer (endexine). In this study, we found a difference in thickness of exine structure among floret whorls. Microspores in floret whorl 1 had thicker exine (Figure 3a) compared to those in floret whorl 2 (Figure 3b) and whorl 3 (Figure 3c). Moreover, the uninucleate microspores from floret whorl 1 had less dense cytoplasm compared with those from whorl 2 and 3.

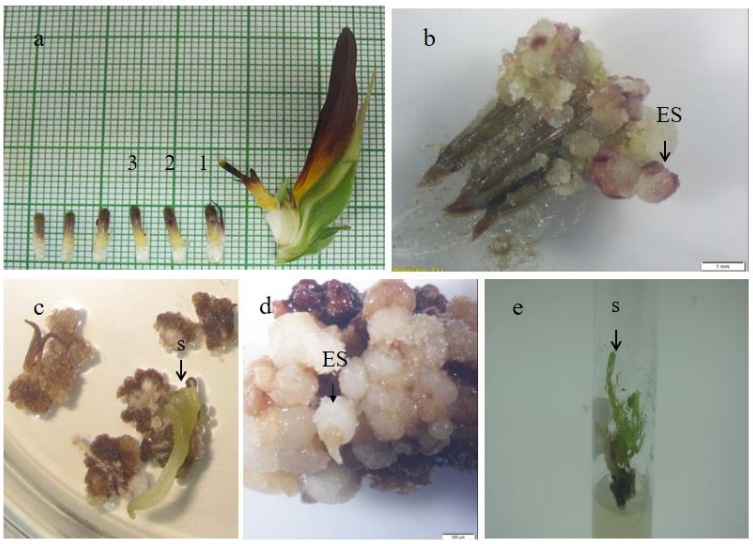

Figure 1 Ornamental sunflower florets at the R5.1 stage cv. Prado Red and embryogenic calli via anther culture. (a) Unopened disk florets under this study, whorl 1, 2, and 3, (b) Anther-derived calli and embryogenic-like structure (ES) after 14 days of culture, (c) Shoot regeneration after 14 days of culture, (d) ES after 30 days of culture, and (e) Shoot regeneration after 30 days of culture. ES: embryogenic-like structure, s: shoot.

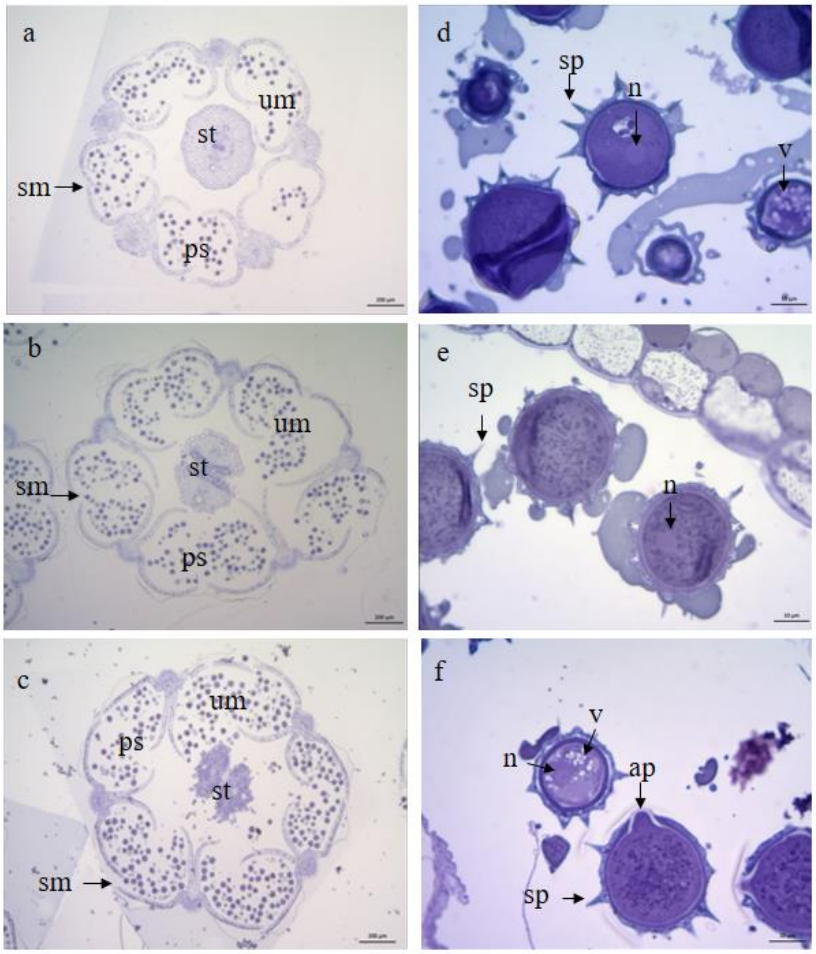

Figure 2 Structure of anthers and microspores with light microscope. (a) Anther of floret whorl 1, (b) Anthers of floret whorl 2, (c) Anther of floret whole 3, (d) Uninucleate microspores of floret whorl 1, (e) Uninucleate microspores of floret whorl 2, and (f) Uninucleate microspores of floret whorl 3. ap: aperture, n: nucleus, ps: pollen sac, sm: stomium, st: style, sp: spine, um: uninucleate microspore. Bar =200 µm (a, b, c), Bar= 10 µm (d, e, f).

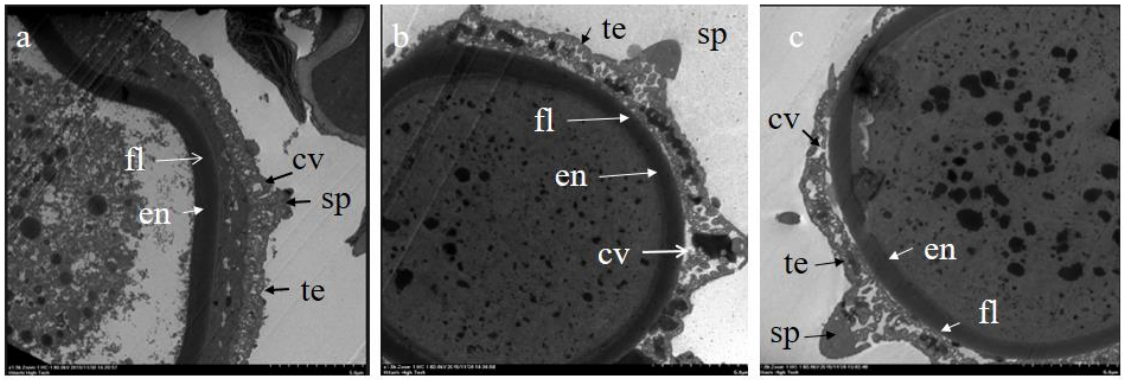

Figure 3 TEM images of microspores from different floret whorls. (a) Floret whorl 1, (b) floret whorl 2, and (c) floret whorl 3. cv: cavus, en: endexine, fl: foot layer, te: tectum, sp: spine. Bar =5 µm.

3.2 Relationship between floret characteristics and microspore stages

The morphological characters of the floret including disk floret length (DL), anther length (AL), and anther width (AW), and of the microspore including the polar view diameter (P), equatorial diameter (E), and polar per equatorial ratio (P/E ratio), were used to determine the relationship with the microspore stage. Our results showed that microspores from floret whorl 1, 2 and 3 were similar in morphology as isopolar in which the two poles were equal, medium size (40.43 to 41.97 µm), and had prolate shapes (P/E 1.03 to 1.05) (Table 2).

A strong correlation ($R = 0.882$) was found between disk floret length and microspore stage in which large disk florets (whorl 1) contained a higher percentage of mid-to late- uninucleate microspore (39.33%) compared to small florets (whorl 3) which contained a lower percentage of mid-to late- uninucleate microspore (15.33%). In other words, small florets had a high frequency of early uninucleate microspores (26.67%) compared to large disk florets (6.00%). The averages of disk floret length, anther length, anther width, microspore polar view, and equatorial view except P/E ratio all differed significantly among floret whorls based on DMRT's test ($P \leq 0.05$) (Table 2). To assess which variable, disk floret length or anther size, is a better predictor of microspore developmental stage, correlation coefficients were generated and found to be significant for both variables (Table 3). Significant positive correlations were found between disk floret length or anther size in terms of anther length and anther width with the percentage of mid-to late- uninucleate microspore ($P \leq 0.01$), whereas P and E were negatively correlated with it.

Table 2 Floret characteristics and microspore stages of three floret whorls in ornamental sunflower cv. Prado Red

Whorl no.	P (µm)	E (µm)	P/E	DL (mm)	AL (mm)	AW (mm)	EM (%)	MM (%)
1	40.43± 0.83^{b}	38.67± 0.57^{b}	1.05± 0.02	7.93± 0.02^{a}	3.63± 0.04^{a}	1.28± 0.02^{a}	6.00± 2.00^{b}	39.33± 5.03^{a}
2	41.49± 0.33^{a}	40.34± 0.58^{a}	1.03± 0.02	7.87± 0.01^{b}	3.56± 0.02^{a}	1.21± 0.01^{b}	10.67± 2.31^{b}	26.00± 3.46^{b}
3	41.97± 0.05^{a}	40.72± 0.26^{a}	1.03± 0.01	7.78± 0.02^{c}	3.48± 0.01^{b}	1.17± 0.01^{c}	26.67± 12.22^{a}	15.33± 5.03^{c}
Mean	41.29± 0.40	39.91± 0.47	1.04± 0.02	7.86± 0.02	3.56± 0.02	1.22± 0.01	14.44± 2.18	26.89± 4.51

The values are mean ± SD of variance for polar view (P), equatorial view (E), P/E ratio (P/E), disk floret length (DL), anther length (AL), anther width (AW), early uninucleate microspore (EM), and mid-to-late uninucleate microspore (MM). Means with the same letters within a column are not significantly different ($P \leq 0.05$).

Table 3 Correlation analysis of variances of three floret whorls in ornamental sunflower cv. Prado Red.

Variations	P	E	P/E	DL	AL	AW	EM
E	0.865**	-	-	-	-	-	-
P/E	-0.213	-0.660	-	-	-	-	-
DL	-0.707*	-0.787*	0.403	-	-	-	-
AL	-0.611	-0.705*	0.343	0.953**	-	-	-
AW	.-810**	-0.944**	0.618	0.906**	0.848**	-	-
EM	0.634	0.657	-0.349	-0.759*	-0.708*	-0.779*	-
MM	-0.816**	-0.761*	0.196	0.882**	0.871**	0.849**	-0.671*

** Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level.
Abbreviations: P: polar view, E: equatorial view, P/E: ratio of polar view per equatorial view, DL: disk floret length, AL: anther length, AW: anther width, EM: percentage of early uninucleate microspore, MM: percentage of mid-to late- uninucleate microspore

3.3 Effect of different culture media on anther culture response

Within 7-14 days of culture initiation on various medium, the anther walls turned brown and generated calli which were friable light yellow, or in some cases light purple. The MS medium (A2) supplemented with 2 mg l^{-1} α-napthalene acetic acid (NAA), 1.00 mg l^{-1} N^6-benzylaminopurine (BAP) and 100 ml l^{-1} coconut water produced the highest frequency of callus induction (60.44%), followed by A1 and A3 media which were about 58.22% and 27.00%, respectively, while A4 medium had no callus induction. Callus growth including callus size, fresh weight and dry weight, was the highest in A2 medium followed by A1 medium and A3 medium, respectively (Table 4). Anther-derived calli in A2 medium developed into 20.45% of embryo-like structures (ES) and some of these calli developed into shoot or root primordia (Figure 1b-e). A significant difference of percentage embryogenic callus induction was observed among various culture medium, A2 medium showing 20.45% of embryo-like structures (ES), A1 showing 14.07% of ES, A3 showing 6.69% of ES, and A4 showing no response ($p < 0.05$). It was noted that no complete plantlets were obtained from this study. In addition, the analysis of variance revealed that culture medium significantly affected all mean parameters as shown in Table 5 ($p < 0.05$).

3.4 Effect of floret whorls on anther culture response

The effects of various floret whorls on anther culture are shown in Table 6. The highest anther-derived calli was observed at 41.17% in floret whorl 1 followed by 39.92% and 28.50% in floret whorl 2 and 3, respectively. The ANOVA revealed that floret whorls did not significantly affect any mean parameters except embryogenic-like structure induction (PE) as shown in Table 5 ($p < 0.05$). Based on this result, it offers more flexibility that all three disk floret whorls from the R5.1 reproductive stage can be used for callus induction, but only floret whorl 1 and 2 produced embryogenic calli with high frequency.

Table 4 Effect of culture media on callus growth and anther-derived embryos in ornamental sunflower cv. Prado red.

Media	CS (mm)	FW (mg)	DW (mg)	PC (%)	PE (%)	Type of callus
A1	2.506±0.273^{ba}	0.198±0.016^{b}	0.065±0.010^{b}	58.22±22.73^{a}	14.07±5.97^{b}	Friable light yellow-purple
A2	2.683±0.199^{a}	0.219±0.019^{a}	0.080±0.008^{a}	60.44±19.32^{a}	20.45±8.22^{a}	Friable light yellow-purple
A3	2.390±0.172^{b}	0.176±0.026^{c}	0.058±0.012^{b}	27.00±17.12^{b}	6.96±3.04^{c}	Friable light yellow-purple
A4	0.000^{c}	0.000^{d}	0.000^{c}	0.00^{c}	0.00^{d}	No response
Mean	1.900±0.161	0.148±0.015	0.051±0.008	36.42±14.79	10.37 ±9.27	

The values are me ± SD of variance for anther-derived callus size (CS), fresh weight (FW), dry weight (DW), percentage of callus induction (PC) and percentage of embryogenic-like structure (PE). Means with the same letters within a column are not significantly different ($P \leq 0.05$).

Table 5 Analysis of variance for anther culture response in ornamental sunflower cv. Prado Red.

S.O.V	d.f.	CS	FC	DC	PC	PE
Whorl (W)	2	0.161	0.0004	0.0001	31394.97	130.466*
Media (M)	3	43.576*	0.2715*	0.0334*	21918.972*	703.121*
W * M	6	0.069	0.0009	0.0002	1337.278	36.865
Error	24	0.923	0.0091	0.0022	6969.333	417.357

*= Significant at 0.05 probability level. Abbreviation: callus size (CS), fresh weight (FC), dry weight (DC), percentage of callus induction (PC) and percentage of embryogenic-like structure induction (PE).

Table 6 Effect of floret whorls on anther culture response in ornamental sunflower cv. Prado Red.

Whorl no.	CS (mm)	FC (mg)	DC (mg)	PC (%)	PE (%)
1	1.958±1.209	0.150±0.095	0.053±0.033	41.17±33.78	10.78±8.98[b]
2	1.930±1.176	0.151±0.093	0.051±0.033	39.92±32.55	13.45±11.60[a]
3	1.804±1.10	0.143±0.089	0.048±0.033	28.50±23.80	6.88±5.88[b]
Mean	1.897±1.131	0.148±0.092	0.051±0.033	36.42±19.720	10.37±9.27

The values are mean ± SD of variance for anther-derived callus size(CS), fresh weight (FC), dry weight (DC), percentage of callus induction (PC) and percentage of embryogenic- like structure induction (PE). Means with the same letters within a column are not significantly different ($P \leq 0.05$).

4. Discussion

Haploid breeding is a very desirable way to select homozygous lines within a short time period. Investigating the precise stage of the microspore development in order to promote efficient embryo production through anther culture is critical. The present investigation discusses the scope of microscopy analysis of microspore stage in different disk floret whorls for anther culture response in an ornamental sunflower cv. Prado Red. According to previous studies, the anther culture developmental pathway depends on various factors such as genotypic species, medium and microspore developmental stages [8, 13, 14, 15, 22]. In this study, it was first reported that floret length and anther size in sunflower were positively correlated with the microspore developmental stages. The large florets contained higher percentages of mid-to late- uninucleate microspores compared with small florets. Other studies found similar results that bud length and the stage microspore stage were correlated in cultivated tomato [23], chickpea [24], and ornamental pepper [25]. However, differences in microspore developmental stages were observed among cultivars [26]. The variation of mid-to late- uninucleate microspore stages among whorls of the same flower bud could be accounted for the slightly temporal difference in the flower development. Our results confirm that microsporogenesis in anthers of the inner floret whorl is delayed when compared with anthers of the outer floret whorl, similar to previous observations in soybeans [26].

Sunflower anther culture success was achieved using an early uninucleate microspore stage [27], and mid-to late- uninucleate microspore stage [7, 15]. According to our study, anthers of all three floret whorls at the R5.1 stage contained both microspore stages and could be induced into callus and embryogenic-like structures. However, the composition of medium was one of the main factors affecting haploid plant induction from anther culture [8, 9, 10, 11]. For the anther-derived callus induction study, the anthers containing uninucleate stage showed a wide range of callus responses among four tested media. The MS medium supplemented with combination hormones of NAA, BAP and CW gave maximum callus induction at 60.44%, lower than previous report by Vijaya Priya et al. [10] who observed 98.27% anther callus induction in the same medium. Moreover, Sujatha and Prabakaran [11] reported that MS medium added with NAA and BAP were capable of inducing 100% callus regeneration from sunflower anthers. The difference between our result and previous observations could be due to the genotypic difference, physiology of donor plant, and/or culture conditions. Some sunflower cultivars require complex organic compounds such as coconut water and casein hydrolysate for anther-derived callus and shoot induction [10, 28]. Moreover, the anther-derived callus capacity was thought to be related to the

accumulation of substances and phenolic compounds [29]. Further refinement of the culture medium is necessary to improve the efficiency of plant regeneration of this ornamental plant.

5. Conclusions

In conclusion, disk floret length and anther size in sunflower were correlated with the microspore developmental stages, and hence might be used as indicators for microspore stage. The outer disk floret whorls contained a higher percentage of mid-to late- uninucleate microspores compared to inner whorls. Fewer calli were produced as floret length or anther size decreased, resulting in poor callus induction from younger florets which contained lower percentage of mid-to late- uninucleate microspores. Moreover, our results confirmed that the culture medium significantly affected callus regeneration from anthers. Further study, however, is necessary to increase the rate of shoot induction and then the conversion to plantlets for this cultivar.

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