

CYTOLOGICAL AND FOOD RESERVE CHANGES IN SUNFLOWER COTYLEDONS *In Vitro*

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Abstract

Cotyledons, food reserve organs, have been routinely used as explants in sunflower (*Helianthus annuus* L.) tissue culture, but the underlying mechanism is not well understood. The objective of this study was to investigate the changes of the cytological characteristics and chemical content of cotyledon explants under *in vitro* culture and their relationship with shoot regeneration ability. Cotyledons of 0-, 1-, and 7-day old germinating seeds of the F1 hybrid (Pacific 22 × Prado Red) and Prado Red varieties were placed with the adaxial side on a modified MS medium for 21 days, then subjected to cytological and food reserve composition analysis. The results showed that there was a statistically significant difference among cotyledon ages and conditions in terms of cytological features and food reserve composition. The cell numbers per area were lower in cotyledons cultured *in vitro* compared to those in germinating seeds, whereas the cell area and cotyledon thickness exhibited opposite results. The *in vitro* cotyledons showed lower contents of total fats, total sugar, and sucrose, but a higher content of protein. Correlation analysis demonstrated a positive relationship between shoot regeneration with the protein and cell area, but a negative relationship with cell numbers, indicating that high protein content and cell expansion may contribute to shoot regeneration ability in sunflowers.

Keywords: Cotyledon, protein, regeneration, sunflower, total fat

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Introduction

Sunflower (*Helianthus annuus* L., Asteraceae) has become one of the seeds in high global demand for oilseed crops after soybean, rapeseed, and groundnut and it has a flower value worldwide due to the beauty of its inflorescence both as a cut flower for decoration and as a plant in gardens and parks (Miklic *et al.*, 2008; Mayor *et al.*, 2010; Eurostat Statistics Explained, 2015). The application of efficient and reliable regeneration protocols is one of the main requirements for genetic improvement of this oilseed crop. *In vitro* regeneration through organogenesis or somatic embryogenesis using a variety of explants including hypocotyls, leaves, stems, cotyledons, shoot apices, and immature zygotic embryos has been reported (Knittel *et al.*, 1991; Hewezi *et al.*, 2003; Ozyigit *et al.*, 2006; Elavazhagan *et al.*, 2009; Sujatha *et al.*, 2012; Inoka and Dahayaka, 2015). Among various explants, the use of cotyledons gives highly efficient plant regeneration (Ozyigit *et al.*, 2006; Sujatha *et al.*, 2012).

The cotyledon is a relatively leaf-like similar to a seedling with a distinct palisade and spongy parenchyma, epidermis layers, and vascular cambium. Both the palisade and spongy parenchyma are storage reserves for seedlings. The main storage reserves of the sunflower seed are the lipid followed by protein, total sugar, and sucrose (Rosa *et al.*, 2009). Shoot organogenesis from sunflower cotyledons has been obtained from seeds and seedlings. The age and orientation of cotyledons have been previously reported to have effects on plant regeneration (Knittel *et al.*, 1991; Pugliesi *et al.*, 1991; Dağüstü, 2002), but the underlying mechanism is not well known. Thus, it is quite probable that variations in shoot regeneration ability due to the food reserve composition and cell changes in the cotyledons can affect the ability to regenerate. In this study, we therefore investigated cytological and chemical changes in cotyledons cultured *in vitro*, and the relationship between these changes and the shoot regeneration ability was also determined. The information from this study will assist the

improvement and higher efficiency of shoot regeneration of the sunflower.

Materials and Methods

Plant Preparation

Seeds of 2 sunflower varieties (*Helianthus annuus* L.), the F1 hybrid (Prado Red x Pacific 22) and Prado Red, were surface sterilized under aseptic conditions by soaking with 20% Clorox® (commercial bleach) and with 2 drops of Tween 20 for 4 h. The seeds were washed 5 times with sterile distilled water for 5 min and then rinsed in 70%(v/v) ethyl alcohol for 1 min. The surface sterilized seeds were germinated in a bottle with sterile paper and incubated in a culture room at a temperature of 25±2°C for 7 days. Three stages of germinating seeds at days 0, 1, and 7 were used as explants for this study.

In vitro Culture

Cotyledons of 0-, 1-, and 7-day old germinating seeds from 2 sunflower varieties were excised and cut transversely into 2 pieces. Only the adaxial sides of the cotyledons were cultured on MS basal salt medium (Murashige and Skoog, 1962) supplemented with 1 mg/l of benzyladenine (BA), 30 g/l of sucrose, and 2 g/l of gellan gum (Kelcogel, CP Kelco U.S., Inc., Atlanta, GA, USA). The pH was adjusted to 5.7-5.8 before autoclaving at 121°C for 20 min. All cultures were maintained at 25±2°C under a photoperiod of 16/8 h (day/night). Regeneration parameters including callus induction, shoot induction, the number of shoots per explant, and root induction were observed at 21 days after culture. The following parameters were determined as follows (shoot >4 mm was counted) (Azadi *et al.*, 2002):

$$\text{Callus induction (\%)} = \frac{PC}{E}$$

$$\text{Shoot induction (\%)} = \frac{PS}{E}$$

$$\text{Number of shoots per explant} = \frac{S}{E}$$

$$\text{Root induction (\%)} = \frac{PR}{E}$$

where PC = the number of explants producing calluses; E = the total of explants; PS = the number of explants producing shoots; S = the number of shoots; PR = the number of explants producing roots.

Cytological Study

The cotyledon sections of 2 μm thickness were obtained on a microtome (Automatic MT-3, NK Systems Ltd., Tokyo, Japan). The sections were dehydrated by ethanol series, fixed on microscope slides, and stained with 0.05% (v/v) safranin O solution for cell wall indication. The images of the sections were observed under an Olympus BX51 microscope (Olympus, Hamburg, Germany) and captured using a digital microscope camera DP73 (Olympus, Germany). Cell numbers, cell area, and cotyledon thickness were counted and measured under a light microscope.

Total Fat Analysis

The Soxhlet method of Manirakiza *et al.* (2001) was used for total fat analysis. The powder sample at 0.5 g was placed into an extraction thimble and transferred into a Soxtec 2050 (Foss, Hilleroed, Denmark). Ten ml of petroleum ether were filled into the bottle and put into the heating mantle for 1 h. The solvent was evaporated by using the vacuum condenser. The sample was incubated at 105°C for 30 min until the sample was completely dry. The bottle was reweighed and the dried content was determined. The total fat was calculated as follows:

$$\text{Total fat (\%)} = (W2 - W1) \times \frac{100}{S} \quad (1)$$

where W1 = the weight of the empty flask (g); W2 = the weight of the flask and extracted fat (g); and S = the weight of the sample (g).

Total Protein Analysis

The total protein analysis was done according to the Kjeldahl method of Helrich (1990). One g of the powdered sample was digested in the digestion flask by boiling with

14 ml of concentrated H_2SO_4 and 2 tablets of the catalyst (3.5g K_2SO_4 and 0.4g CuSO_4) at 420°C for 1 h. The samples were cooled at room temperature and 70 ml of distilled water was added with a mixed indicator (0.1 g of bromocresol green: 0.1 g of methyl red in 100 ml ethanol). Ammonia was steam distilled from the digest and 50 ml of 40 NaOH was added into the receiving flask and was titrated in excess 0.1 N HCl until the solution changed color from purple to pink. The determination of the blank was made with reagent alone. The total protein was calculated as follows:

$$\% \text{ Kjeldahl nitrogen} = \frac{(V_s - V_B) \times M \times 14.007}{W \times 10}$$

$$\text{Total protein (\%)} = \% \text{ Kjeldahl nitrogen} \times F$$

where V_s = the volume (ml) of standard acid used to titrate a test; V_B = the volume (ml) of standard acid used to titrate the reagent blank; M = the molarity of the standard HCl; W = the weight (g) of the test portion or standard; 14.007 = the atomic weight of nitrogen; 10 = the factor to convert mg/g to percent; F = the protein-nitrogen conversion factor for the plant (5.30 for sunflower seed).

Total Sugar and Sucrose Analysis

Soluble total sugar was extracted with the phenol-sulfuric method (Dubois *et al.*, 1956) and sucrose was extracted with the resorcinol-HCl method (Robbins and Pharr, 1987). For total sugar extraction, 100 mg of fresh samples were placed into a test tube, filled with 6 ml of 80% ethanol, boiled in water for 2 min, and then placed in a water bath at 65°C for 2 h. One hundred μl of supernatant was taken into a test tube. Then 500 μl of 5% phenol and 1 ml of sulfuric acid were added into the samples. After 30 min, the samples were measured in absorbance at 490 nm with a T80+ UV/VIS spectrophotometer (PG Instruments. Ltd., Lutterworth, UK). The total sugar content was calculated using glucose as the standard curve. For sucrose analysis, 500 μl of supernatant was filled into a test tube. The sample had 100 μl of 0.2 N NaOH added, was boiled in a water bath at 100°C for 10 min, had 250 μl of 1%

resorcinol and 750 μ l of 30% HCl added, and was incubated at 80°C for 10 min. The solution sample was analyzed with the UV spectrophotometer at 520 nm. The sucrose concentration was calculated using sucrose as the standard curve.

Statistical Analysis

Data analysis was conducted through analysis of variance (ANOVA) for a completely randomized design. Significant mean differences were determined with Duncan's multiple range tests (DMRT) at 0.05%. Correlation analysis among *in vitro* regeneration parameters, cytological characters (cell numbers, cell area, and cotyledon thickness), and the food reserve composition (total fat, total protein, total sugar, and sucrose) was conducted using the Pearson product-moment test. All data were analyzed using the SPSS package version 16.

Results and Discussion

Effect of Cotyledon Age on Shoot Regeneration

Cotyledon explants grown in MS medium turned green 3-4 days after culture. Their size increased about 1- to 3-fold and showed the first sign of a callus approximately 7 days after culture. For the F1 hybrid variety, all parameters consisting of shoot induction, callus induction, root induction, and the number of shoots per explant were significantly different among the ages ($P \leq 0.01$) (Table 1). On the other hand, only shoot induction was significant among the ages in the Prado Red

variety. Cotyledons of 1-day old germinating seeds cultured *in vitro* gave the highest percentage of shoot induction at 18.06% and 26.67% in the F1 hybrid and Prado Red, respectively. The number of shoots per explant in the F1 hybrid and Prado Red were 0.94 and 1.10 shoots per explant, respectively, as shown in Table 1. In this experiment, it was demonstrated that shoot induction of both varieties was significantly affected by the age of the cotyledons.

The results of this study were in agreement with most of the results of Ozyigit *et al.* (2006) who reported that cotyledon explants of 5 different sunflower genotypes gave shoot primordia of about 18.00% and had the highest callus induction of about 90.00%. The difference in shoot induction percentage might be due to the genotype, plant hormone, and age differences. The genotype is one of the most important factors for callus and shoot induction in tissue culture studies (Punia and Bohorava, 1992). Dağüstü (2002) reported that the increase of the age of cotyledon explants highly affected the capacity and developmental stage in sunflower organogenesis. Knittel *et al.* (1991) reported that the best regeneration was obtained from 4- to 10-day old seedlings in different combinations of BA and naphthalene-acetic acid. They found that using 4-day old cotyledons gave the best result, while Pugliesi *et al.* (1991) found that cotyledons of 1- and 2-day old seedlings on medium with 18.6 μ M of kinetin and 5.71 μ M of indole-3-acetic acid obtained adventitious shoots that originated with 4-10 buds per explant.

Table 1. The mean values ($\pm$ SD) of regeneration parameters for *in vitro* culture of cotyledon explants for 21 days

Variety	Age (day)	<i>In vitro</i> culture data			
		Callus induction (%)	Shoot induction (%)	No. of shoots per explant	Root induction (%)
F1 hybrid	0	34.72 $\pm$ 4.95 ^b	3.89 $\pm$ 2.49 ^b	0.46 $\pm$ 0.21 ^b	2.22 $\pm$ 1.71 ^b
	1	51.11 $\pm$ 6.39 ^a	18.06 $\pm$ 5.32 ^a	0.94 $\pm$ 0.13 ^a	17.78 $\pm$ 4.81 ^a
	7	48.05 $\pm$ 3.02 ^{ab}	3.61 $\pm$ 2.82 ^b	0.17 $\pm$ 0.11 ^b	0 ^b
<i>F-test</i>		**	**	**	**
Prado Red	0	37.25 $\pm$ 3.54	6.67 $\pm$ 2.84 ^b	0.58 $\pm$ 0.21	3.33 $\pm$ 2.78
	1	37.50 $\pm$ 4.38	26.67 $\pm$ 6.65 ^a	1.10 $\pm$ 0.17	3.61 $\pm$ 1.66
	7	35.56 $\pm$ 4.66	12.78 $\pm$ 3.66 ^b	0.73 $\pm$ 0.16	0.56 $\pm$ 0.56
<i>F-test</i>		ns	**	ns	ns

Means in the same column followed by different letters are significantly different according to the Duncan's multiple range test (DMRT) at $P \leq 0.05$. (** indicates significant *P*-value at 0.01 level; ns = not significant)

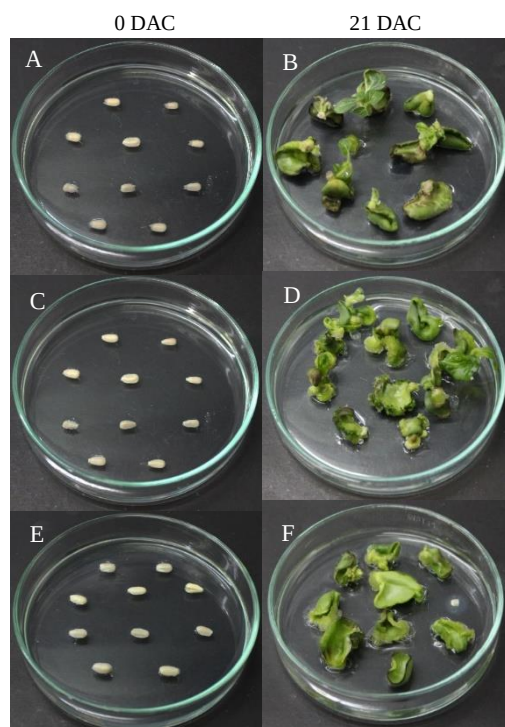


Figure 1. Changes of cotyledon explants at 0 day and 21 days after culture (DAC) of F1 hybrid variety on MS medium with 1 mg/l of BA: (A-B) 0-day old cotyledons, (C-D) 1-day old cotyledons, (E-F) 7-day old cotyledons (bar = 1 cm)

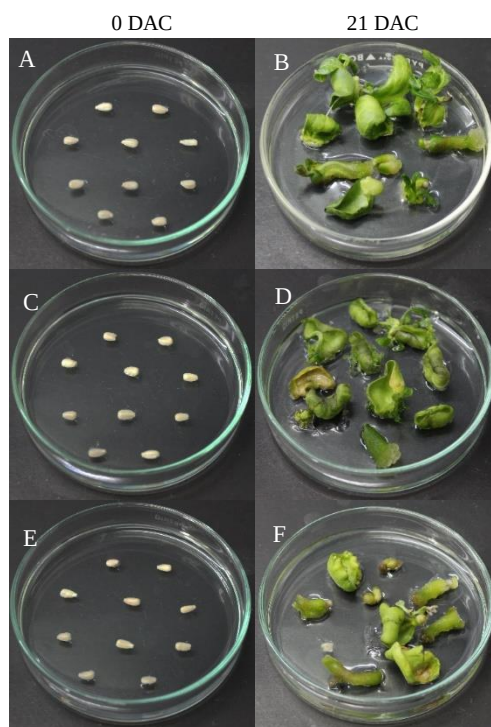


Figure 2. Changes of cotyledon explants at 0 day and 21 days after culture (DAC) of Prado Red variety on MS medium with 1 mg/l of BA: (A-B) 0-day old cotyledons, (C-D) 1-day old cotyledons, (E-F) 7-day old cotyledons (bar = 1 cm)

Cytological Change of Sunflower Cotyledons

The anatomical study showed that the cotyledons of mature sunflower seeds were comprised of several distinct tissue regions: the food reserve parenchyma, procambium, and epidermis. The storage parenchyma includes 2 types: adaxial parenchyma (rectangular shape) and abaxial parenchyma (spherical shape). The small vascular tissues of the cotyledon are scattered in the center of the cotyledon. The outermost layer is covered by a layer of epidermal cells (data not shown). Within 3-4 days after seed imbibition, the radicle, plumule, and expanded cotyledons appeared. At 7 days, the adaxial parenchyma was differentiated into the palisade parenchyma and the abaxial parenchyma was differentiated into the spongy parenchyma. After culture on MS medium

containing 1 mg/l BA for 21 days, the cotyledon explants had expanded in size and turned green, and some produced a callus and/or adventitious shoots (Figures 1 and 2).

Analysis of variance for cell numbers showed a significant difference in the condition and age, and the interaction between condition and age. The cell area was significant in all parameters except the interaction between the variety and age. The cotyledon thickness parameter was significant in the condition and age of the cotyledon but not the interaction between them, as presented in Table 2 ($p \leq 0.05$).

For the *in vitro* condition, cotyledon explants had lower cell numbers but larger cell areas and thicknesses than those in germinating seeds (Table 3). Similar results were obtained in older cotyledons compared with younger

Table 2. Analysis of variance for the effect of condition, variety and age on cytological characteristics of cotyledon explants

Source of variation	df	Mean square		
		Cell number (cell/ μm^2)	Cell area (μm^2)	Cotyledons thickness (mm)
Condition (C)	1	13417.361*	131017058.758*	0.065*
Variety (V)	1	17.361	1124501.181*	0.007
Age (A)	2	1694.694*	5455338.713*	0.180*
C * V	1	103.361	472122.442*	0.000
C * A	2	417.527*	688865.834*	0.001
V * A	2	89.194	25256.175	0.003
C * V * A	4	94.694	23096.105*	0.001
Error	24	29.306	25242.868	0.001
C.V. (%)		27.55	23.85	11.76

(* indicates significant *P*-value at 0.05 level)**Table 3. The mean values ($\pm$ SD) for cell number, cell area and cotyledon thickness at different ages of sunflower cotyledons**

Condition	Variety	Age (day)	Cytological data		
			Cell number (cell/ μm^2)	Cell area (μm^2)	Cotyledon thickness (mm)
In germinating seed	F1 hybrid	0	77.67 $\pm$ 5.51 ^a	1610.43 $\pm$ 69.89 ^c	0.89 $\pm$ 0.06 ^b
		1	52.67 $\pm$ 5.69 ^b	1792.49 $\pm$ 22.67 ^b	0.93 $\pm$ 0.05 ^b
		7	55.67 $\pm$ 2.51 ^b	2422.96 $\pm$ 129.37 ^a	1.08 $\pm$ 0.02 ^a
	F-test		**	**	**
	Prado Red	0	81.67 $\pm$ 12.89 ^a	1430.87 $\pm$ 68.62 ^c	0.94 $\pm$ 0.04 ^c
		1	51.33 $\pm$ 3.78 ^b	1718.02 $\pm$ 171.80 ^b	1.04 $\pm$ 0.04 ^b
		7	38.67 $\pm$ 7.02 ^b	2303.68 $\pm$ 111.71 ^a	1.17 $\pm$ 0.05 ^a
F-test		**	**	**	
In vitro	F1 hybrid	0	27.33 $\pm$ 4.16 ^a	5259.16 $\pm$ 69.88 ^c	0.87 $\pm$ 0.03 ^c
		1	19.33 $\pm$ 4.16 ^b	5805.86 $\pm$ 195.37 ^b	0.95 $\pm$ 0.03 ^b
		7	13.33 $\pm$ 2.08 ^b	6894.25 $\pm$ 238.83 ^a	1.16 $\pm$ 0.06 ^a
	F-test		**	**	**
	Prado Red	0	27.67 $\pm$ 1.53 ^a	4578.44 $\pm$ 261.29 ^c	0.96 $\pm$ 0.04 ^c
		1	23.33 $\pm$ 2.08 ^b	5123.38 $\pm$ 126.91 ^b	1.07 $\pm$ 0.05 ^b
		7	15.00 $\pm$ 2.65 ^c	6509.91 $\pm$ 222.72 ^a	1.22 $\pm$ 0.03 ^a
F-test		**	**	**	

Means in the same column followed by different letters are significantly different according to the Duncan's multiple range test (DMRT) at $P \leq 0.05$. (** indicates significant *P*-value at 0.01 level)

ones (Table 3; Figures 3 and 4). This result means that cells expand during germination and under the *in vitro* condition leading to the increase in cotyledon thickness. The results of this study demonstrated that great expansion of the cotyledon was obtained through *in vitro* culture, which is similar to the report of Munshi *et al.* (2007) and Sinha (2014) who reported that *in vitro* cotyledons had more cell expansion than the seedling condition which may be caused

by the cytokinin hormone added into the culture medium. Cotyledon organogenesis through *in vitro* culture was affected by several factors including gene hierarchies, gene expression, and the hormone function (Chandler, 2008).

Food Reserve Change of Sunflower Cotyledons

The analytical results of the food reserve are summarized in Table 4 and showed

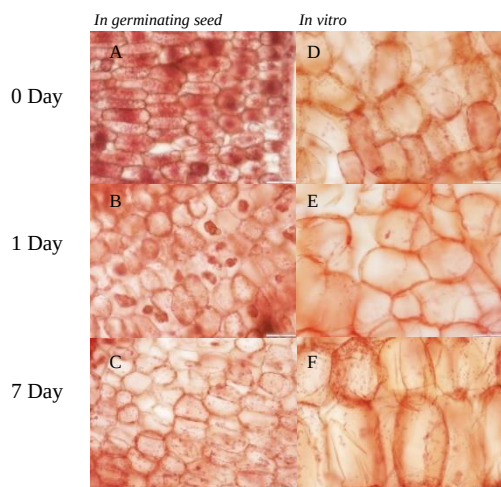


Figure 3. Cytological images of cotyledon explants in F1 hybrid variety under a light microscope: (A-C) cotyledons of 0-, 1-, and 7- day old germinating seeds, respectively; (D-F) 21 days *in vitro* cotyledons of 0-, 1-, and 7 -day old germinating seeds, respectively (bar = 50 μ m)

significance for all sources in the total protein and total sugar parameters. Total fat and sucrose were significant in condition, variety, and age at the $p \leq 0.05$ level (Table 4). Total fats were the most abundant reserve compounds in the cotyledons of germinating seeds in both varieties, accounting for 52-54% (Table 5). Total fats decreased as the age of the germinating seeds increased and were lower in cotyledon explants cultured *in vitro*, which may be caused by degradation of the lipid body as a nutrient source for development. Proteins were the second most represented compounds in sunflower cotyledons accounting for 24-26%. The total protein content increased during seed germination and there were higher levels in the cotyledon culture *in vitro* (Table 5). The total sugars or soluble sugars were the third sources represented in the sunflower seeds. Changes in total sugar and sucrose contents in the cotyledons during germination and the *in vitro* culture were similarly observed in both sunflower varieties. The highest value of total sugar and sucrose was in 0-day old germinating seeds while the lowest content was in 7-day old

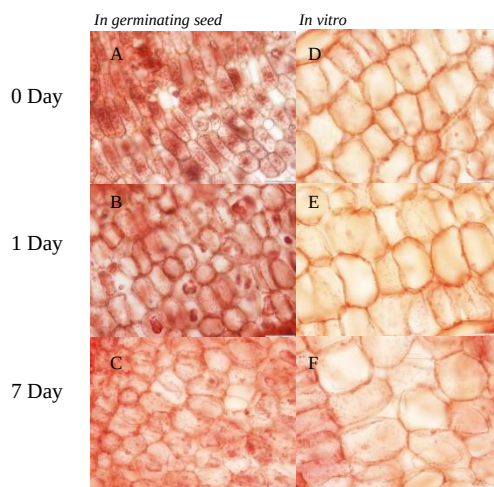


Figure 4. Cytological images of cotyledon explants in Prado Red variety under a light microscope: (A-C) cotyledons of 0-, 1-, and 7- day old germinating seeds, respectively; (D-F) 21 days *in vitro* cotyledons of 0-, 1-, and 7 -day old germinating seeds, respectively (bar = 50 μ m)

germinating seeds. In addition, *in vitro* cotyledons had total sugar and sucrose lower than the germinating seeds (Table 5).

The results of the cytological and chemical analysis agreed with the findings of Munshi *et al.* (2007) and Radic *et al.* (2009) who reported that, in the sunflower, seed oil had the highest content (36.82%), followed by protein (23.61%). Balasaraswathi and Sadasivam (1997) reported that total fat decreased significantly after 72 h of sunflower seed germination. In the sunflower oilseed species, loss of oil reserve during germinating occurred far more quickly (Lima *et al.*, 2014). A similar finding in cactus was also reported (Alencar *et al.*, 2012). *In vitro* cotyledons of sunflower produced more protein than cotyledons of germinating seeds which may be manipulated by changing the nutrient composition due to photosynthesis, similar to the finding of Morard and Henry (1998).

We also reported about the correlation analysis of the cytological, chemical change, and shoot induction parameters. The results showed that the shoot induction was positively correlated with cell area ($r = 0.569^{**}$) and total

Table 4. Analysis of variances for the effects of condition, variety and age on chemical parameters of sunflower cotyledons

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

(* indicates significant *P*-value at 0.05 level).**Table 5.** The mean values ($\pm$ SD) for chemical analysis at different ages of sunflower cotyledons

Condition	Variety	Age (day)	Chemical analysis			
			Total protein (%)	Total fat (%)	Total sugar (%)	Sucrose (%)
In germinating seed	F1 hybrid	0	24.09±0.08 ^c	54.00±0.62	10.41±0.50 ^a	3.88±1.47 ^a
		1	25.17±0.11 ^b	53.19±0.50	9.73±0.07 ^b	3.81±1.04 ^a
		7	26.28±0.17 ^a	52.66±0.86	7.03±0.09 ^c	2.65±0.63 ^b
	F-test		**	ns	**	**
	Prado Red	0	26.21±0.05 ^a	53.96±0.06 ^a	8.77±0.65 ^a	4.37±3.18 ^a
		1	25.93±0.03 ^b	52.02±0.19 ^b	5.09±0.25 ^b	3.86±0.80 ^a
		7	25.26±0.05 ^c	51.84±0.21 ^b	4.39±0.07 ^b	2.54±0.27 ^b
	F-test		**	**	**	**
	In vitro	F1 hybrid	0	30.99±0.06 ^c	4.98±0.06 ^a	5.29±0.78 ^a
1			31.96±0.08 ^b	4.03±0.59 ^b	3.59±0.16 ^b	1.25±0.29 ^{ab}
7			33.11±0.07 ^a	3.17±0.02 ^c	3.30±0.03 ^b	1.10±0.38 ^b
F-test			**	**	**	**
Prado Red		0	36.97±0.06 ^c	8.93±0.05 ^a	6.63±0.08 ^a	1.91±0.74 ^a
		1	38.81±0.01 ^b	7.95±0.10 ^b	5.86±0.19 ^b	1.49±0.04 ^b
		7	39.84±0.04 ^a	6.88±0.01 ^c	5.10±0.02 ^c	1.44±0.12 ^b
F-test			**	**	**	**

Means in the same column followed by different letters are significantly different according to the Duncan's multiple range test (DMRT) at $P \leq 0.05$. (** indicates significant *P*-value at 0.01 level; ns = not significant)

protein (0.357*), while it had a negative correlation with cell numbers ($r = -0.506^{**}$), as shown in Table 6. This result indicated that increased protein content and cell size may play a role in shoot induction of the sunflower.

Conclusions

This study demonstrated that some cytological and chemical parameters of sunflower cotyledons varied among the ages of the germinating seeds

and the conditions. Cell numbers and total fats decreased as the age of the germinating seeds increased and were lower when cultured *in vitro*, whereas proteins and cell area increased as the age increased and had higher values in cotyledon explants cultured *in vitro*. The changes in total fat and protein content were related to shoot regeneration ability. Therefore, shoot regeneration efficiency may be influenced by these cytological and chemical changes.

Table 6. Correlation analysis between cytological features, chemical content and shoot induction parameters of sunflower cotyledon *in vitro*

Variable	Cell area	Cell number	Cotyledon thickness	Total protein	Total fat	Total sugar	Sucrose	Callus induction	Shoot induction
Cell number	-0.889**								
Cotyledon thickness	0.571**	-0.641**							
Total protein	0.254	-0.344*	0.359*						
Total fat	0.108	0.018	-0.134	-0.871**					
Total sugar	-0.239	0.377*	-0.513**	-0.501**	0.629**				
Sucrose	0.082	0.035	-0.337*	-0.782**	0.897**	0.755**			
Callus induction	0.856**	-0.753**	0.305	0.063	0.194	-0.208	0.169		
Shoot induction	0.569**	-0.506**	0.312	0.357*	-0.095	-0.110	-0.013	0.498**	
Root induction	0.301	-0.254	0.043	0.014	0.069	-0.094	0.174	0.472**	0.483**

(** indicates significant *P*-value at 0.01 level; * indicates significant *P*-value at 0.05 level)

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