ORGANOGENESIS OF SUNFLOWER MUTANT LINES

C. Carter
Plant Science Department
South Dakota State University
Brookings SD 57007

A. Nabipour
Department of Agronomy
University of Tehran
Karaj Iran

A. Sarrafi
Department of Biotechnology and Plant Breeding
BAP, Institut National Polytechnique-Ecole Nationale
Superieure Agronomique de Toulouse
Toulouse France

ABSTRACT

Genotypic variation in ability to regenerate via organogenesis was investigated in 104 F_5 sunflower (*Helianthus annuus*) mutant lines derived from AS-613, following gamma irradiation at 7500 rads and selection in M_2, M_3 and M_4. Cotyledonary explants were placed on regeneration media for 5 days, then cultured on development media for 3 weeks. Percent regeneration (PR), number of shoots per regenerating explant (SER), and other characteristics of regeneration were analyzed by general linear model (GLM) analysis of variance. GLM models were highly significant ($p<0.0001$), with R^2s of 0.50 and 0.57 for PR and SER, respectively. PR and SER differed among replicates and among lines. Mean PR ranged from 9% to 84%. Mean SER ranged from1.2 to 6.1 shoots. Most shoots arose from the proximal end of the explants. Genotypic variation was also evident in the ability to regenerate shoots from the central, marginal, or distal areas of the explants.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) can be regenerated by organogenesis or somatic embryogenesis from many tissues (Alibert et al., 1994; Robinson and Everett, 1990). Organogenesis has often been obtained from cotyledons of mature seed (Abdoli et al., 2003; Baker et al., 1999; Ceriani et al., 1992; Chraibi et al., 1991, 1992a, 1992b; Greco et al., 1984; Knittel et al., 1991; Nestares et al., 1996). Sunflower displays considerable genotypic variation for regeneration from cotyledons (Deglene et al., 1997; Sarrafi et al., 1996a, 1996b, 2000; Nestares et

al., 2002). Al-Chaarani et al. (2005) found that gamma irradiation improved genetic variability for organogenesis and other traits in sunflower.
The objective of the work described here was to characterize the organogenesis response of mutant lines derived following gamma irradiation of AS-613, an inbred line with high-organogenesis response developed at INP-ENSAT (Sarrafi et al., 2000).

MATERIALS AND METHODS

Seeds of AS-613 and 103 M_{4-5} mutant lines derived from AS-613 following gamma irradiation at 7500 rads and selection in M_2, M_3 and M_4 were provided by A. Sarrafi (INP-ENSAT). Achenes were washed 3 minutes in 70% ethanol, rinsed briefly in 1.2% sodium hypochlorite plus 0.08% Tween 20, and placed in distilled water while the pericarps were removed. Seeds were washed for 10 minutes in 1.2% sodium hypochlorite plus 0.08% Tween 20, with stirring, then rinsed 3 times in distilled water and twice in sterile distilled water. Seeds were placed on germination media consisting of 1/2 strength MS media (Murashige and Skoog, 1962) containing 15 g l^{-1} sucrose and 6 g l^{-1} Sigma Type A agar, pH 5.7, autoclaved 20 min at 120 C and 15 psi. Seeds were geminated for 14 to 19 hours at 21 to 27C and 16 hr light (60-115 umol m^{-2} s^{-1})/8 hr dark. There were 16 seeds per replicate and 3 replicates per line.

Seed coats were removed and the hypocotyl and primary shoot were excised from each seed. Each cotyledon was cut in half across the longitudinal axis, and the distal section was discarded. The proximal sections were placed abaxial side down onto regeneration (R) media consisting of full strength MS (Murashige and Skoog, 1962) with 50 mM KNO_3, 1 mM myo-inositol, 0.5 gl^{-1} casein hydrolysate, 4.4 uM BAP, 2.7 uM NAA, 30 gl^{-1} sucrose, 6 gl^{-1} Sigma Type A agar, pH 5.7, autoclaved 20 min at 120 C and 15 psi, and poured into 55X10 mm petri plates. Each plate contained 4 explants. Plates were sealed with Parafilm, labeled and placed in the culture room at ~21 to 27 C and 16 hr light/8 hr dark for 4 days in the first replicate and for 5 days in the second and third replicates. Explants were then transferred to development (D) media consisting of full strength MS (Murashige and Skoog, 1962) with 50 mM KNO_3, 1 mM myo-inositol, 0.5 gl^{-1} casein hydrolysate, 2.2 uM BAP, 0.05 uM NAA, 30 gl^{-1} sucrose, 6 gl^{-1} Sigma Type E agar, pH 5.7, autoclaved 20 min at 120 C and 15 psi, and poured into 100X20 mm petri plates. Plates were sealed with Parafilm and placed in the culture room at ~25 C and 16 hr light (60-115 umol m^{-2} s^{-1})/8 hr dark.

Data on the number of explants regenerating, number of shoots per explant, location of shoots (proximal, central, marginal and distal regions of the explant), amount of callusing (on a 0 to 5 scale), and explant weight were collected 2 weeks after explants were placed on development media. Where a primary shoot was inadvertantly left on an explant, this was noted but the primary shoot was not included in the calculation of percent regeneration or shoot numbers. Percent regeneration (PR) was calculated as PR=(r/e)*100, where r=the number of explants regenerating one or more shoots excluding the primary, per plate, and

e=the number of explants plated. Shoots per explant regenerating (SER) was calculated as SER=(s-sp)/e, where s= the total number of shoots per plate, sp=the number of primary shoots, and e=the number of explants plated. A cluster of 4 or 5 emerging shoot or leaf primordia was counted as a single shoot. The total number of shoots arising from regions other than the proximal, *i.e.*, from the central, marginal, or distal regions of each regenerating explant ($S_{CMD}ER$), was calculated as $S_{CMD}ER = (S_{CMD})/e$, where S_{CMD}=the number of central, marginal and distal shoots. Shoots were also analyzed separately for each location of origin, *i.e.*, proximal (S_PER), central (S_CER), marginal (S_MER) or distal (S_DER). Mean seed weight of each line was determined by weighing 8 individual seeds per line prior to preparation.

Data were analyzed by General Linear Model analysis of variance (Statistical Analysis Systems, Inc).

RESULTS

The analysis of variance models were highly significant ($p<0.0001$), with R^2s of 0.50, 0.57, and 0.55 for PR, SER, and $S_{CMD}ER$, respectively. Analyses of shoot numbers by location were also highly significant ($p<0.001$), with R^2s of 0.45 or higher.

PR and SER differed among replicates and among lines. Mean PR averaged over all lines was 50% in the first replicate, 53% in the second, and 67% in the third replicate. Mean PR was normally distributed (Figure 1) and ranged from 9% to 84%, with a mean of 56% when averaged over all lines and replicates.

Mean SER averaged over all lines was 3.2 in the first replicate, 2.1 in the second, and 2.0 in the third replicate. Mean SER ranged from 1.2 to 6.1 but

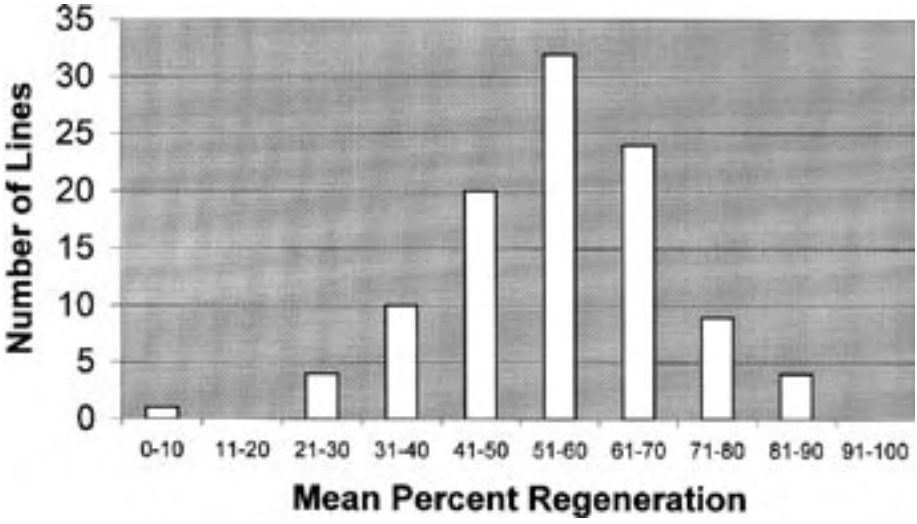

Figure 1. Distribution of mean percent regeneration (PR) among sunflower mutant lines.

was skewed to the lower end of the range (Figure 2), with a mean (averaged over all lines and replicates) of 2.4. The mean PR and SER for each line are given in Tables 1-2.

Most shoots arose from the proximal region of the explant. However, in 29 lines, at least 1 shoot per explant, on average, originated from central, marginal, or distal regions (Figure 3). The combined mean number of shoots at central, marginal and distal regions ranged from 0 to 3.9 per explant. Line M5-54-1 (Figure 4) had the most non-proximal shoots per explant with means of 2.4, 0.3, and 1.3 at central, marginal, and distal regions, respectively. Means and ranges of shoots at proximal, central, marginal and distal regions are given in Table 3.

Correlations among variables are given in Table 4. PR was positively correlated with SER and negatively correlated with callusing. There was no significant association of PR with explant weight. Proximal shoots (S_PER) were positively correlated with primary shoots inadvertantly left on explants, but all non-proximal shoots were negatively correlated with the presence of primary shoots. S_PER was negatively correlated with S_DER and not correlated with S_CER or S_MER.

Mean seed weight per line was not significantly correlated with mean PR or mean callus rating, but was positively correlated with mean explant weight ($r=0.35$, $p<0.001$) and mean S_PER ($r=0.21$, $p<0.05$), and negatively correlated with mean S_{CMD}ER per line ($r=-0.39$, $p<0.0001$).

Some lines regenerated multibranched shoots. M5-826-2 also produced what appeared to be ectopic shoot primordia on leaves of multibranched shoots (Figure 5).

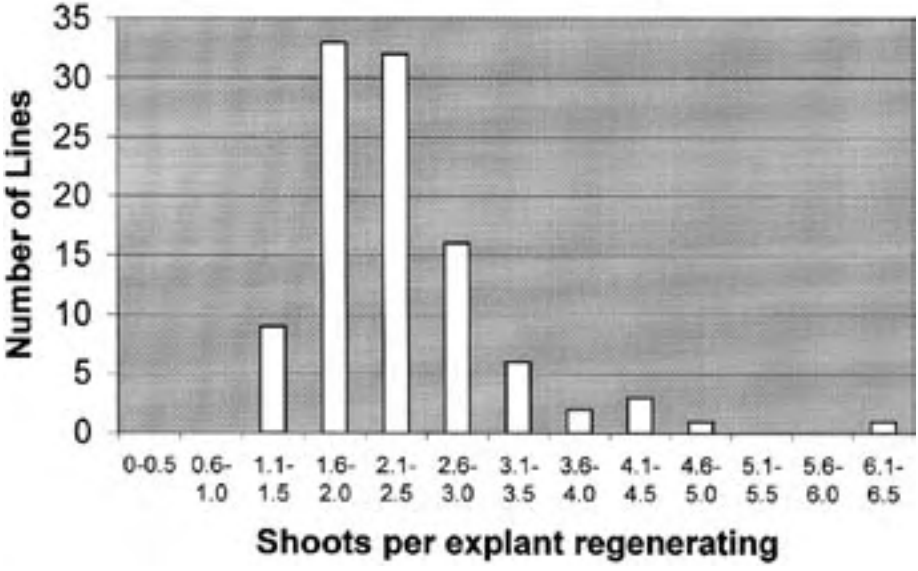

Figure 2. Distribution of mean number of shoots per explant regenerating (SER) among sunflower lines.

Table 1. Percent regeneration (PR) of sunflower mutant lines.

Line	PR[1]		Line	PR[1]		Line	PR[1]	
M5-378-1	84	*a*	M5-186-1	61	*abcdefghijklm*	M5-483-2	50	*cdefghijklmnop*
M5-495-1	82	*ab*	M5-368-2	61	*abcdefghijklmn*	M5-286-1	50	*cdefghijklmnop*
M5-826-2	81	*abc*	M5-214-1	60	*abcdefghijklmn*	M5-247-1	49	*cdefghijklmnop*
M4-904-1-1	81	*abc*	M5-796-2	60	*abcdefghijklmn*	M5-375-1	49	*cdefghijklmnop*
M5-85-2	78	*abcd*	M5-582-2	60	*abcdefghijklmn*	M5-785-1	49	*defghijklmnop*
M5-99-1	77	*abcde*	M4-901-2	59	*abcdefghijklmn*	M5-663-1	49	*defghijklmnop*
M5-44-1	77	*abcde*	M4-931-2	59	*abcdefghijklmn*	M5-696-1	49	*defghijklmnop*
M5-99-2	76	*abcde*	M5-53-2	59	*abcdefghijklmn*	M5-858-2	48	*defghijklmnop*
M5-135-2	74	*abcdef*	M5-435-2	58	*abcdefghijklmn*	M5-325-1	48	*defghijklmnop*
M5-54-1	73	*abcdef*	M5-96-1	58	*abcdefghijklmn*	M4-886-3	48	*defghijklmnop*
M5-129-2	72	*abcdefg*	M5-357-1	58	*abcdefghijklmn*	M5-46-1	47	*defghijklmnop*
M5-374-1	71	*abcdefgh*	M5-66-1	58	*abcdefghijklmn*	M5-472-1	47	*defghijklmnop*
M5-133-1	71	*abcdefgh*	M5-427-1	58	*abcdefghijklmn*	M5-502-2	45	*efghijklmnop*
M4-873-1	68	*abcdefghi*	M5-216-2	57	*abcdefghijklmn*	M5-281-1	43	*fghijklmnop*
M5-386-1	68	*abcdefghi*	M5-33-2	57	*abcdefghijklmn*	M5-179-1	43	*fghijklmnop*
M5-143-2	68	*abcdefghi*	M5-322-2	57	*abcdefghijklmn*	M5-676-1	42	*fghijklmnop*
M4-936-1	68	*abcdefghi*	M5-333-2	57	*abcdefghijklmn*	M5-854-1	42	*fghijklmnop*
M5-147-2	68	*abcdefghi*	M5-862-2	57	*abcdefghijklmn*	M5-522-1	42	*fghijklmnop*
M5-691-1	67	*abcdefghi*	M5-116-2	57	*abcdefghijklmn*	M5-568-2	42	*fghijklmnop*
M5-85-3	67	*abcdefghi*	M5-652-1	57	*abcdefghijklmn*	M5-417-1	40	*ghijklmnop*
M5-36-2	67	*abcdefghi*	M5-63-1	57	*abcdefghijklmn*	M5-842-2	39	*hijklmnop*
M5-42-1	66	*abcdefghi*	M4-886-2	55	*abcdefghijklmno*	M5-775-2	37	*ijklmnop*
M5-447-1	66	*abcdefghi*	M5-853-2	55	*abcdefghijklmno*	M5-106-1	36	*ijklmnop*
M4-917-1	66	*abcdefghi*	M4-894-2	54	*abcdefghijklmnop*	M5-865-1	33	*jklmnopq*
M5-78-1	66	*abcdefghi*	M5-381-1-1	54	*abcdefghijklmnop*	M5-531-2	32	*klmnopq*
M5-88-3	66	*abcdefghi*	M5-792-2	54	*abcdefghijklmnop*	M4-873-2	31	*klmnopq*
M5-477-2	65	*abcdefghi*	M5-485-1	53	*abcdefghijklmnop*	M5-795-2	31	*klmnopq*
M5-89-1	65	*abcdefghi*	M5-609-2	53	*abcdefghijklmnop*	M5-791-1	31	*lmnopq*
M5-118-3	65	*abcdefghi*	M5-213-1	53	*abcdefghijklmnop*	M5-548-1-1	30	*mnopq*
M4-900-2	65	*abcdefghi*	M5-225-2	53	*abcdefghijklmnop*	M5-331-2	30	*mnopq*
M5-38-1	64	*abcdefghij*	M5-485-2	52	*abcdefghijklmnop*	M5-39-2-2	30	*nopq*
M5-263-2	64	*abcdefghij*	M4-871-1	52	*abcdefghijklmnop*	M5-796-1	25	*opq*
M5-338-2-2	62	*abcdefghijk*	AS-613	51	*bcdefghijklmnop*	M5-862-1	24	*pq*
M5-133-2	62	*abcdefghijkl*	M5-771-1	51	*bcdefghijklmnop*	M5-743-3	9	*q*
M5-52-1	61	*abcdefghijklm*	M5-509-1	50	*cdefghijklmnop*			

[1] Means followed by the same symbol do not differ by Student-Newman-Keuls' test.

Table 2. Number of shoots per explant regenerating (SER) of sunflower mutant lines.

Line	SER[1]		Line	SER[1]		Line	SER[1]	
M5-54-1	6.1	*a*	M5-63-1	2.4	*cdefgh*	M5-417-1	1.9	*efgh*
M5-378-1	4.7	*b*	M5-338-2-2	2.4	*cdefgh*	M5-213-1	1.8	*efgh*
M5-36-2	4.1	*bc*	M5-263-2	2.4	*cdefgh*	M5-88-3	1.8	*efgh*
M4-904-1-1	4.1	*bcd*	M5-46-1	2.4	*cdefgh*	M6-609-2	1.8	*efgh*
M5-495-1	3.7	*bcde*	M5-116-2	2.3	*cdefgh*	M4-873-2	1.8	*efgh*
M5-33-2	3.6	*bcdef*	M5-835-2	2.3	*cdefgh*	M5-39-2-2	1.8	*efgh*
M5-52-1	3.5	*bcdefg*	M5-865-1	2.3	*cdefgh*	M5-216-2	1.8	*efgh*
M5-509-1	3.5	*bcdefg*	M5-836-1	2.3	*cdefgh*	M4-894-2	1.8	*efgh*
M5-186-1	3.2	*bcdefgh*	M4-931-2	2.3	*cdefgh*	M4-917-1	1.8	*efgh*
M5-179-1	3.2	*bcdefgh*	M5-129-2	2.3	*cdefgh*	M5-842-2	1.8	*efgh*
M5-133-2	3.1	*bcdefgh*	M5-743-3	2.2	*cdefgh*	M5-568-2	1.8	*efgh*
M5-826-2	3.1	*bcdefgh*	M5-477-2	2.2	*cdefgh*	M5-771-1	1.7	*efgh*
M5-375-1	3.0	*bcdefgh*	M5-435-2	2.2	*cdefgh*	M5-78-1	1.7	*efgh*
M5-38-1	3.0	*bcdefgh*	M5-582-2	2.2	*cdefgh*	M5-247-1	1.7	*efgh*
M4-871-1	2.9	*bcdefgh*	M5-775-2	2.2	*cdefgh*	M5-785-1	1.7	*efgh*
M5-485-2	2.9	*bcdefgh*	M4-886-2	2.2	*cdefgh*	M5-99-2	1.7	*efgh*
M5-374-1	2.9	*bcdefgh*	M4-936-1	2.2	*cdefgh*	M5-548-1-1	1.7	*efgh*
M5-99-1	2.9	*bcdefgh*	M5-143-2	2.2	*cdefgh*	M5-858-2	1.7	*efgh*
M5-214-1	2.9	*bcdefgh*	M4-901-2	2.2	*cdefgh*	M5-96-1	1.7	*efgh*
M5-42-1	2.8	*cdefgh*	M5-66-1	2.2	*cdefgh*	M5-652-1	1.6	*efgh*
M5-357-1	2.8	*cdefgh*	M5-85-3	2.2	*cdefgh*	M5-325-1	1.6	*efgh*
M5-531-2	2.8	*cdefgh*	M5-118-3	2.1	*cdefgh*	M5-792-2	1.6	*efgh*
M5-85-2	2.8	*cdefgh*	M5-368-2	2.1	*cdefgh*	M5-472-1	1.6	*efgh*
M4-886-3	2.7	*cdefgh*	M5-795-2	2.1	*cdefgh*	M5-796-1	1.5	*efgh*
M5-135-2	2.6	*cdefgh*	M5-427-1	2.1	*cdefgh*	M5-676-1	1.5	*efgh*
M5-485-1	2.6	*cdefgh*	M5-854-1	2.0	*cdefgh*	M5-381-1-1	1.4	*fgh*
M5-322-2	2.6	*cdefgh*	AS-613	2.0	*cdefgh*	M5-663-1	1.4	*fgh*
M5-281-1	2.6	*cdefgh*	M5-791-1	2.0	*cdefgh*	M5-286-1	1.4	*fgh*
M5-53-2	2.5	*cdefgh*	M5-447-1	2.0	*defgh*	M5-106-1	1.4	*fgh*
M5-44-1	2.5	*cdefgh*	M4-900-2	2.0	*defgh*	M5-696-1	1.4	*gh*
M4-873-1	2.5	*cdefgh*	M5-147-2	2.0	*efgh*	M5-862-1	1.3	*gh*
M5-483-2	2.4	*cdefgh*	M5-225-2	1.9	*efgh*	M5-331-2	1.3	*gh*
M5-89-1	2.4	*cdefgh*	M5-691-1	1.9	*efgh*	M5-522-1	1.3	*gh*
M5-796-2	2.4	*cdefgh*	M5-133-1	1.9	*efgh*	M5-333-2	1.2	*h*
M5-502-2	2.4	*cdefgh*	M5-962-2	1.9	*efgh*			

[1] Means followed by the same symbol do not differ by Student-Newman-Keuls' test.

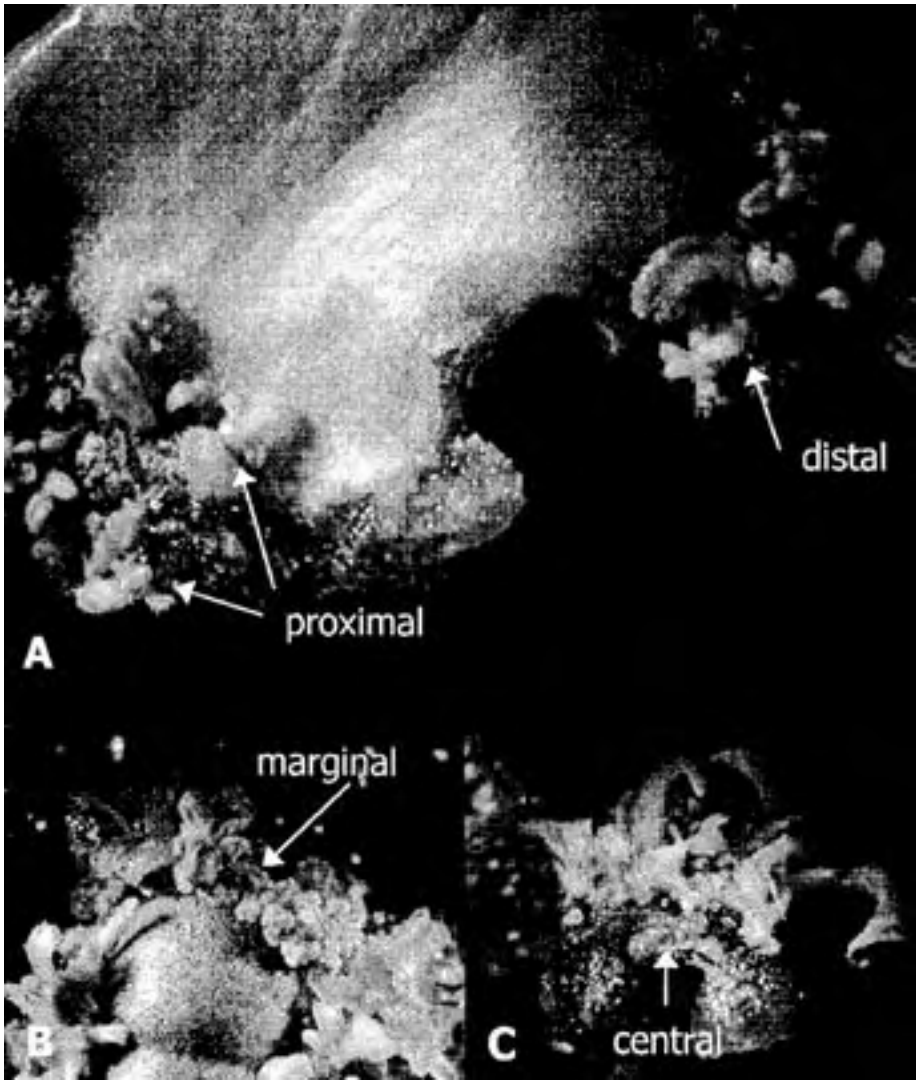

Figure 3. Shoots originating at A. proximal and distal; B. marginal; and C. central regions of explants.

DISCUSSION

The mutant lines displayed extensive variation for organogenesis parameters, and means differed significantly among genotypes. Although most shoots originated at the proximal end of explants, 28% of the lines also regenerated shoots at other explant regions, and proximal shoots were negatively correlated or not correlated with central, marginal or distal shoots, suggesting the possibility of independent genetic effects in regeneration potential from different explant regions. Shoot production was negatively correlated with callusing, which has been found to inhibit shoot development (Ceriani et al., 1992; Knittel et al., 1991).

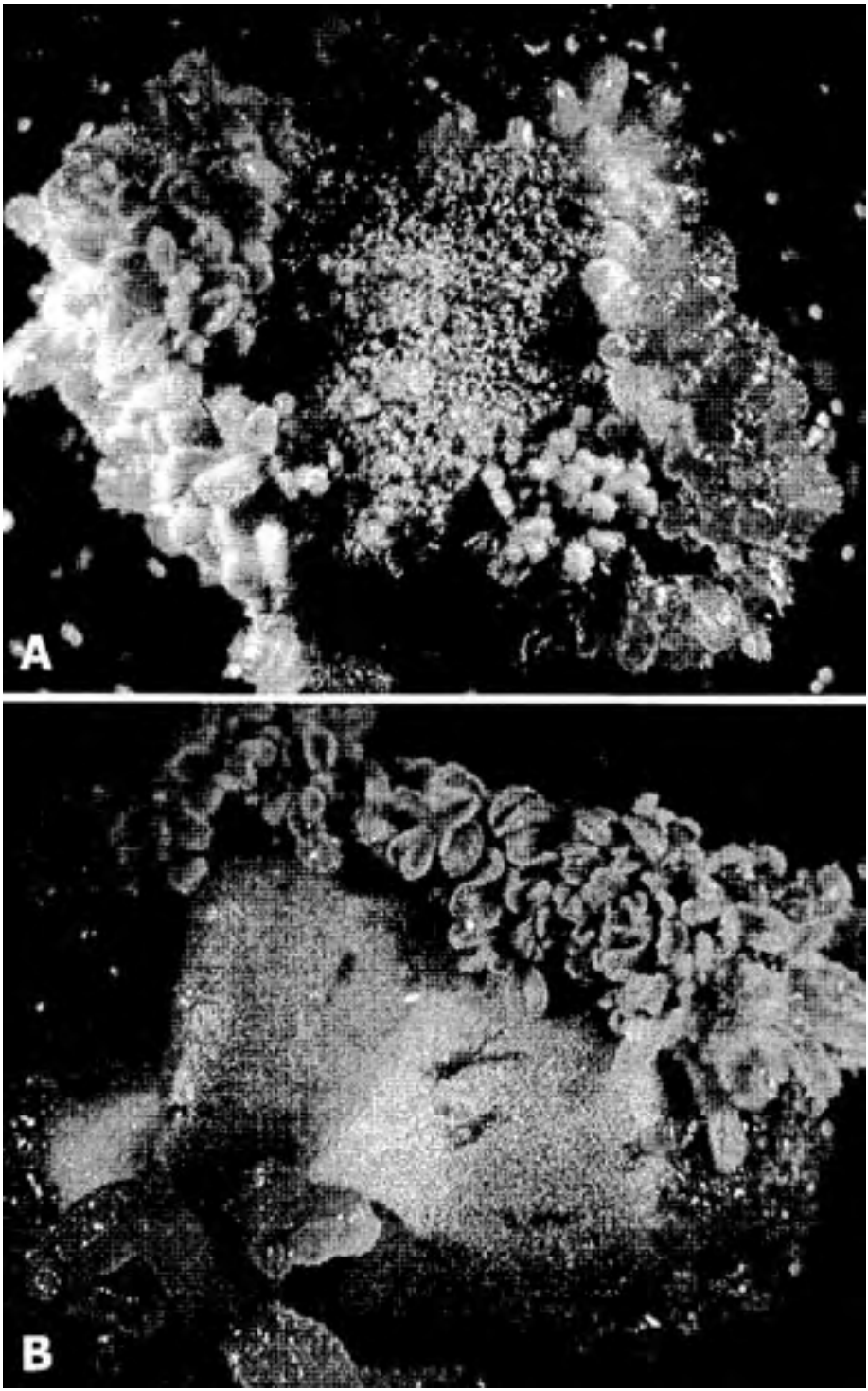

Figure 4. Explants of M5-54-1. A. Extensive shoot proliferation at proximal, distal and marginal regions; B. One proximal and 4 distal shoots.

Table 3. Mean and range of shoots originating at 4 explant regions.

Region of shoot origin	Mean	Minimum	Maximum
Proximal	2.0	0	17.5
Central	0.2	0	20.0
Marginal	0.1	0	9.5
Distal	0.4	0	7.2

Table 4. Correlations among percent regeneration (PR), shoots per explant regenerating (SER), proximal shoots per explant regenerating (S_PER), central shoots per explant regenerating (S_CER), marginal shoots per explant regenerating (S_MER), distal shoots per explant regenerating (S_DER), primary shoots, callus, and explant weight (ExplWt).

Variable	PR	SER	S_PER	S_CER	S_MER	S_DER	Primary	Callus	ExplWt
PR		0.2****	-0.08***	0.09****	0.06**	0.21****	0.06**	-0.09***	0.001 ns
SER			0.40****	0.65****	0.51****	0.52****	-0.07**	0.02 ns	0.06*
S_PER				-0.05 ns	0.01 ns	-0.05 *	0.50****	-0.19****	0.03 ns
S_CER					0.18****	0.13****	-0.06**	0.02 ns	0.004 ns
S_MER						0.16****	-0.08***	0.05*	0.02 ns
S_DER							-0.13****	0.09****	0.06 ns
Primary								-0.27****	-0.01 ns
Callus									0.53****
ExplWt									

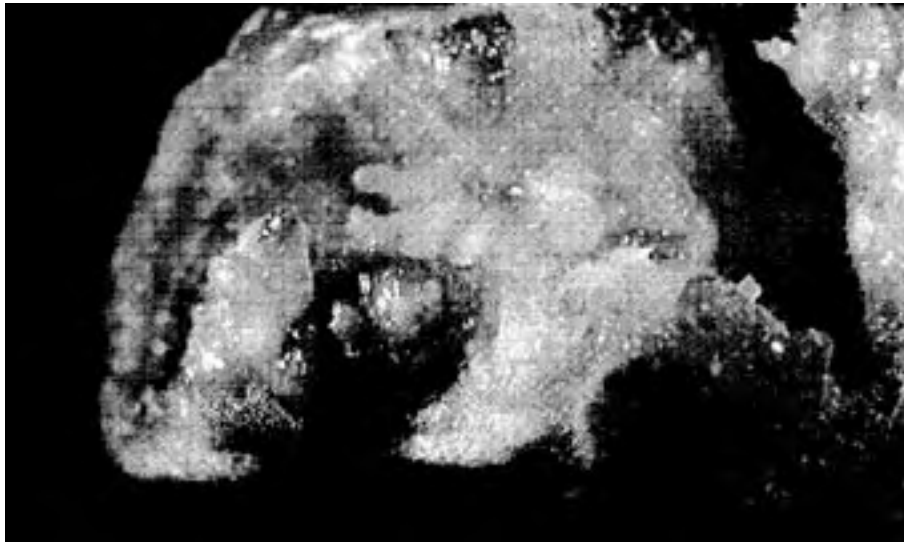

Figure 5. Possible ectopic shoots arising along the margin of a leaf of a multibranched organogenic shoot of M5-826-2.

LITERATURE CITED

Abdoli M, Moieni A, Dehghani H. 2003. Effects of genotype and cotyledon section on organogenesis in sunflower. Iran J Biotechnol 1: 234-238.

Al-Chaarani GR, Gentzbittel L, Barreault G, Lonoble S, Sarrafi A. 2005. The effects of gamma rays and genotypes on sunflower organogenesis traits. Plant Cell Tissue Organ Culture (in press).

Alibert G, Aslane-ChanabJ C, and Burrus M (1994) Sunflower tissue and cell cultures and their use in biotechnology. Plant Physiol Biochem 32: 31-44.

Baker CM, Muñoz-Fernandez N, Carter CD. 1999. Improved shoot development and rooting from mature cotyledons of sunflower. Plant Cell, Tissue and Organ Culture 58: 39-49.

Ceriani MF, Hopp HE, Hahne G, Escandon AS (1992) Cotyledons: an explant for routine regeneration of sunflower plants. Plant Cell Physiol 33:157-164.

Chraibi KMB, Castelle J-C, Latche A, Roustan J-P, Fallot J (1992a) A genotype-independent system of regeneration from cotyledons of sunflower (*Helianthus annuus* L.). The role of ethylene. Plant Sci 86: 215-221.

Chraibi KMB, Castelle J-C, Latche A, Roustan J-P, Fallot J (1992b) Enhancement of shoot regeneration potential by liquid medium culture from mature cotyledons of sunflower (*Helianthus annuus* L.). Plant Cell Rep 10: 617-620.

Chraibi KMB, Latche A, Roustan J-P, Fallot J (1991) Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. Plant Cell Rep 10: 204-207.

Deglene L, Lesignes P, Alibert G, Sarrafi A. 1997. Genetic control of organogenesis in cotyledons of sunflower (*Helianthus annuus*). Plant Cell Tissue Organ Culture 48: 127-130.

Greco B, Tanzarella OA, Carozzo G, Blanco A (1984) Callus induction and shoot regeneration in sunflower (*Helianthus annuus* L.). Plant Sci Lett 36: 73-77.

Knittel N, Escandon AS, Hahne G (1991) Plant regeneration at high frequency from mature sunflower cotyledons. Plant Sci 73: 219-226

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Nestares G, Zorzoli R, Mroginski L, Picardi L (1996) Plant regeneration from cotyledons derived from mature sunflower seeds. Helia 19: 107-112.

Nestares G, Zorzoli R, Mroginski L, Picardi L. 2002. Heritability of *in vitro* plant regeneration capacity in sunflower. Plant Breeding 121: 366-368.

Robinson KEP, Everett NP (1990) Sunflower (*Helianthus annuus* L.): Establishment of cultures, transformation, and the regeneration of plants. In: Bajaj YPS (Ed), Biotechnology in Agriculture and Forestry Vol 10, Legumes and Oilseed Crops, pp 434-452.

Sarrafi A, Bolandi AR, Berville A, Alibert G. 1996a. Analysis of cotyledon culture to measure genetic variability for organogenesis parameters in sunflower (*Helianthus annuus* L.). Plant Sci 121: 213-219.

Sarrafi A, Roustan JP, Fallot J, Alibert G. 1996b. Genetic analysis of organogenesis in the cotyledons of zygotic embryos of sunflower. Theor Appl Genet 92:225-229.

Sarrafi A, Kayyal H, Al-Chaarani GR, Cantin F, Châline AS, Durielle E. 2000. Inheritance of organogenesis parameters in cotyledons of sunflower (*Helianthus annuus* L.). J Genet & Breed 54: 227-231.