

## **REGENERATION OF FERTILE PLANTS FROM SUNFLOWER (*Helianthus annuus L.*) - IMMATURE EMBRYO**

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*Received: March 18, 2010*

*Accepted: May 28, 2010*

### SUMMARY

Immature embryos from 15 sunflower genotypes (five restorers, five cytoplasmic male steriles, and five maintainers) were studied with the aim to shorten the seed to seed cycle, because seed maturation in sunflower takes 50-60% of the life cycle duration (120-150 days). This technique allows the production of fertile plants from immature embryos by reducing the breeding cycle. Ten days after pollination immature embryos were dissected from seeds grown in field plants (SGFP) and were transferred to MS medium allowing shoot and root development for 5-10 days. Young plantlets were transferred to soil, developed to maturity, and were then self pollinated and set seed. The first cycle of immature embryo-raised plants (ERP) was obtained. When these plants were at the flowering stage, 10-day-old embryos were dissected and the 2<sup>nd</sup> cycle of ERP was obtained. The plants at the flowering stage for obtaining the 3<sup>rd</sup> cycle are in the growth chamber at the moment.

The majority of cultured embryos developed into vigorous plantlets with 3-6 leaves. Out of the 1710 immature embryos, the average response of the explants was 93.1% (1591) showing morphogenesis with a minimum of 42.5 [N Record 109/Sanay 1-2(N)] and a maximum of 100% [(PR6404 (cms), Narmo Sanay 6-1 (cms), RIM 1-5 (cms), BGC0565 (N), N Record 109/Iskra (N), RHA 04, RHA 06, RHA 10, RHA 14, RHA 15]. Seventy percent of the developed plantlets had vigorous roots. They were transplanted into containers with a 1:1:2 peat: perlite: soil mixture (v/v) at 24 ± 2°C in 16 h/8 h (light/dark) in a growth chamber. Only 67.3% of them were grown to maturity, either self-pollinated or pollinated with maintainers and set seeds. The overall result was an average of 40-50 regenerated and matured plants per 100 immature zygotic embryos.

**Key words:** sunflower, immature embryo, fertile plant regeneration

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## INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops in both Turkey and the world. The development of hybrids with good agronomic performances is an important target in sunflower breeding programs. Sunflower is a highly cross-pollinated crop and any breeding program on this crop involves several breeding cycles. Obtaining homozygous lines for hybrid seed production in a short period of time is of great importance for commercial hybrid production. In the field, sunflower plants are grown from seeds during the growing season. Therefore, only one generation a year is used in the breeding program. In the Marmara Region in Turkey, sunflower seeds are sown from April to the beginning of May and the plants are generally harvested in early September. The seed to seed cycle takes about 120-150 days to complete to maturity phase for reseeded for the next generation. For that reason, a second year is necessary to obtain seeds under natural field conditions in the region. The length of one cycle is quite often a limiting factor in achieving a breeding aim in a reasonable amount of time. Sunflower breeders require a minimum of six generations with conventional methods for obtaining homozygous lines if the population is derived from a cross between two inbred lines (Hu *et al.*, 2008). It is also known that sunflower seeds keep their germination capacity for about six days after pollination and become dormant 16 days after pollination (Maiti *et al.*, 2006) and that seed maturation in sunflower takes 50-60% of the life cycle duration (Serieys, 1992). Consequently, reducing the duration of one cycle should be of great importance for a breeder aiming to obtain seed propagated cultivars or lines and new sources of maintainers (N), restorers (R) and cytoplasmically male sterile (*cms*) lines.

The application of new technologies such as *in vitro* tissue culture methods (anther culture, microspore culture, ovule culture, immature embryo culture from interspecific hybridization, *etc.*) accelerates the breeding process in sunflower. The sunflower embryo culture system was first developed for interspecific hybridization by Chandler and Beard (1983). Embryo culture has been used successfully by plant breeders in solving the problems of seed set, seed dormancy, slow seed germination, inducing embryo growth in the absence of symbiotic partner, shortening the breeding cycle, rapid seed viability test, obtaining rare hybrids and homozygous lines, and haploid production (Bhojwani and Razdan, 1996; Gürel *et al.*, 1991; Raghavan, 2003; Saji and Sujatha, 1998; Torresàn *et al.*, 1996; Yeung *et al.*, 1998; Zhong *et al.*, 1995). By using embryo culture, the breeding cycles of sunflower (Jambhulkar, 1995; Torresàn *et al.*, 1996), artichoke (Cravero and Cointry, 2007), wheat (Sharma and Gill, 1982), soybean (Roumet and Morin, 1997), orchids, roses and bananas (Yeung *et al.*, 1981), iris (Randolph, 1945), and azalea (Michishita *et al.*, 2001) have been shortened from one to three years to a few months depending on the plant. Jambhulkar (1995) has developed a rapid embryo-raised plant system for sunflower production from immature embryos, which allows five cycles in

316 days. The purpose of the present study was to develop plants under sterile conditions from immature embryos grown in *in vitro* culture. A technique has been developed that can be applied to propagate N, R and *cms* lines by speeding up the breeding process in hybrid seed production.

## MATERIALS AND METHODS

Five sunflower (*Helianthus annuus* L.) maintainers (N Record 109/Sanay 1-2, BGC0565, N Record 109/Iskra, RIM 1-3, Malkara Çerez/P4313), five restorers (RHA 04, RHA 06, RHA 10, RHA 14, RHA 15), and five *cms* lines (PR6404, Narmo Sanay 6-1, N Record 109/Sanay 1-2, RIM 1-5, Malkara Çerez/P4313) were grown under field conditions at Uludag University, Agricultural Research and Experiment Station in 2009. These genotypes were selected from the collection of Uludag University, Field Crops Department because of their early flowering and maturity. The plants were hand-pollinated and maintained until the seeds were mature. Ten to 12 days after pollination the heads were brought into the laboratory. The achenes from the outer 3-4 rows of the inflorescence were separated. To homogenize the embryos, achenes with dark color had been selected. The embryos with pericarps were sterilized with 20% commercial bleach with a few drops of detergent for 10 minutes. They were then rinsed three times with sterile distilled water. The immature embryos were excised from the pericarps, endosperm and embryo sac in order to produce the first cycle of plants. They were then placed onto the Murashige and Skoog (1962) medium with 2% sucrose and 0.8% agar at pH 5.6-5.7 as described by Jambhulkar (1995). All studies were carried out under sterile conditions. The Petri dishes, sealed with Parafilm to prevent moisture loss, were placed in the light (16h / 8h day / night) at  $25 \pm 2^\circ\text{C}$  and cultured for 4 weeks to form plantlets. Within two weeks of culture on MS, the developing embryos grew plantlets with strong roots (2-6 cm and branching) and a stem (3-5 cm) with 6-8 leaves (1 cm). Plantlets washed agar debris were then placed in 31 by 51 cm plastic multipot trays of 48 pots per tray containing a sterile 1:1:2 peat:perlite:soil mixture (v:v). The trays were covered with a plastic bag and incubated for a period of 7-10 days in the growth chamber at  $25 \pm 2^\circ\text{C}$  in the light (16h / 8h day / night), after which the plantlets were transferred to  $320 \times 2.70$  mm (16 lt) pots with regular soil, keeping 2-3 plants per pot in the same growth chamber. The plantlets were irrigated with sterile water three times a week in order to maintain a high humidity environment and fertilized every 10 days. Plantlets had flowers eight weeks after sowing and set seed. The number of plantlets regenerated and mature plants obtained was recorded. Developed seeds and empty seeds per head were counted. Some seeds from some of the regenerants were planted and germinated to produce sunflower plants. Thus, the 2<sup>nd</sup> cycle of ERP was obtained. Each genotype was represented with 10-15 Petri dishes (9 cm) with 10 embryos in each. Observations were made of the efficiency of immature embryos (EIE) (%), embryos developing plantlets, growth of shoots and

roots as score and intensity (%), and mean leaf number per explant (number) at the Petri dish stage. EIE was calculated through the rate number of plants obtained/ number of immature embryo excised. Shoot and root development on the plantlet was scored as described in Table 1. Rooting and shooting score data were rated from 0 to 4. A number of growth traits (head production, 50% of flowering dates, pollination dates, embryo emasculatation dates, transferred to soil dates) and agronomic traits (plant height, head diameter, plant width, leaf number, number of branches, number of filled seeds per head) were determined for each of the individual plants in the pots.

Table 1: Rooting and shooting score rating scale

Key	Description
0	No roots or shoots
1	Roots with up to 1 cm average length and shoots with up to 3 cm average length
2	Roots and shoots with 3-5 cm average length
3	Roots and shoots bigger than 5 cm average length

## RESULTS AND DISCUSSIONS

The first cycle of regeneration procedure was developed from immature embryos from field grown plants. Immature seeds removed as early as 10 days after pollination were successfully cultured *in vitro*. If the excised embryos were able to grow, most had a shoot and a root after a week. Out of the 1,710 immature embryos, the average response of the explants was 93.1% (1591), showing morphogenesis with a minimum of 42.5 [N Record 109/Sanay 1-2(N)] and a maximum of 100% [(PR6404 (*cms*), Narmo Sanay 6-1 (*cms*), RIM 1-5 (*cms*), BGC0565 (N), N Record 109/Iskra (N), RHA 04, RHA 06, RHA 10, RHA 14, RHA 15]. The regeneration response of immature embryos was scored after 3 weeks, evaluating the growth of the shoots and roots and its intensity. Within four weeks of culture on MS (Jambhulkar, 1995), the developing embryos mainly grew plantlets with strong roots (2-6 cm and branching) and a stem with 6-8 leaves (1 cm). The majority of the cultured embryos developed into plantlets in accordance with Jeannin and Hahne (1991). Seventy percent of the developed plantlets had vigorous roots. Plantlets were then transferred aseptically and without injury to plastic multipot trays for two weeks in a growth chamber. Although all of the cultured genotypes were grown in the MS medium during the first stage and were then transferred into the peat:perlite:soil mixture, 33% of them acclimatized (data not shown). RHA 10, RIM 1-3, N Record 109/Sanay 1-2, RIM 1-5, and PR6404 (33%) managed to produce mature plants in the growth chamber. Of the embryos that developed, 14.2% were in the peat:perlite:soil mixture and only 67.5% of them were grown to maturity, either self-pollinated or pollinated with maintainers and set seeds (Table 2). Plantlets bloomed 4-6 weeks later and set seed. Then, after two-three weeks of *in vitro* culture and about 15 days of acclimatization, the plantlets were transferred to the

pots at least four-five months earlier than would normally be possible, thus reducing seed-to-seed cycle.

Approximately 80 (14.2% of the immature embryos plated) primary plantlets were regenerated and moved to trays. Only about 67.5% of the transferred primary plantlets matured, self pollinated, and set seeds. This study reports the results of improvement in growing sunflower plants from *in vitro* immature embryo in the growth chamber. The plants were evaluated for their growth.

Table 3 shows a number of growth and agronomic performances of 1<sup>st</sup> generation plants obtained *in vitro*. There were no big morphological changes in the regenerated plants. In our study, rapid-cycling genotypes of sunflower were selected and completed their life cycles in about 106-112 days in field conditions, as compared to the 150-160 days of late flowering genotypes. In general, days to flowering of seed grown plants obtained *in vitro* ranged from 61 to 70 days and days to harvest of those plants ranged from 106 to 112 days, indicating the minimum need of 106 days for RIM 1-5 and 112 days for RHA 10 for completing regeneration in a year. However, the minimum requirement for completing the seed to seed cycle was 86 days when using the dwarf and early maturing genotype Morden. The 1<sup>st</sup> cycle of embryo raised plants was completed after 65 days by using the same genotype. Consequently, both immature embryo culture and selection of rapid-cycling lines of sunflower produced five generations per year (Jambhulkar, 1995).

Table 2: Estimation of efficiency of the regeneration procedure

Genotypes	Immature embryos plated	Plantlets transferred to peat:perlite:soil			Mature plants obtained		Seed number per head		
	No	No	%	EIE <sup>a</sup>	No	%	Empty	Developed	Total
RHA 10 (R)	127	25	19.7	14.2	18	72.0	487	283	770
RIM 1-3 (N)	121	5	4.1	1.7	2	40.0	40	120	160
N Record 109/ Sanay 1-2 ( <i>cms</i> )	114	22	19.3	16.7	19	86.4	275	231	506
RIM 1-5 ( <i>cms</i> )	80	27	33.8	17.5	14	51.9	513	183	696
PR6404	121	1	0.8	0.8	1	100	101	174	275
Total	563	80	14.2	9.6	54	67.5	1416	991	2407

EIE<sup>a</sup> = Efficiency of the immature embryo: the number of mature plants obtained/total number of immature embryos excised

The regenerated plantlets showed variations for plant height, head diameter, number of branches, plant width, number of leaves, and seed number per head. In general, plant height, plant width, seed number per head, and head diameter decreased when the *in vitro* technique was used. Of the genotypes used, PR6404 had the tallest plants (83 cm) and N Record 109/ Sanay the shortest (19.7 cm). All the plants transferred to soil produced flowers that contained both fertile and sterile seeds. When the plants were harvested, all the heads produced seeds ranging in number from 160 to 770 in total according to genotype. Similarly, Gopalkrishnan

*et al.* (1993) observed that the vegetative phase of embryo derived plants was very short, resulting in small plants (12-25 cm), flower buds and early flowering compared to plants grown in field conditions. Our results indicate that it is possible to produce viable seeds from plants grown *in vitro* and to obtain three generations a year.

The plant height of individual sunflower plants obtained from *in vitro* culture varied between 20 and 50 cm, as reported earlier by Hahne (2002). The size of the individual flowers ranged from a few ray flowers surrounding a small number of disk flowers to sunflower heads of reasonable size with more than 150 seeds in this study. The regenerated plants had a minimum of three and a maximum 16 small green leaves at harvesting. The development time from collecting immature embryos to harvesting seeds takes 4-5 months in our culture system.

Table 3: A number of growth and agronomic performances of 1<sup>st</sup> generation plants obtained from *in vitro*

Genotypes	Days to flowering	Days to harvest	Plant height (cm)		Head diameter (cm)		Number of branches		Plant width (mm)		Number of leaves	
			Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
RHA 10	70	112	26.1	11-50	2.9	1.5-6	1.7	0-6	4.3	2.5-6	9.6	6-13
NRecord109/Sanay	65	109	19.7	5-42	2.3	1-3.5	0.9	0-6	3.8	2-6.5	7.9	3-12
RIM1-5	61	106	33.4	6-62	3.2	1-5.5	1.4	0-6	5.1	4-6	11.1	5-16
PR6404	68	109	83	83	5.5	5.5	1.0	1.0	5.8	5.8	24	24
RIM1-3	65	109	22.3	10-35	2.8	2-3.5	4.5	2-7	3.5	2-3.5	9.5	8-11

The overall result was an average of 54 regenerated and matured plants per 563 excised immature embryos. Although the agronomic characters of *in vitro* regenerated plants decreased, more than 150 seeds were harvested for each genotype and at least 120 of them were viable and developed. This was sufficient for continuing on to the next generation. This method will be useful for breeders in the advancement of sunflower breeding. The three generations a year advanced the breeding of the genetic population. Therefore, homozygosity will be developed in two years if the population is derived from a cross between two inbred lines. In the future we are planning to improve this technique to make it possible to obtain at least 4-5 generations in a year.

It is also known that when the mature seeds of most sunflower lines are freshly harvested, they do not germinate due to dormancy. By using immature embryo culture, the dormancy problem can be solved easily. This technique will shorten the breeding cycle for sunflower, permitting the production of plantlets almost one third of a year earlier than when seeds grown in natural conditions in the field are used. In conclusion, embryo culture techniques avoid embryo abortion and seed dormancy. This technique can be useful in breeding schemes where small numbers of plants are needed per generation.

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