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THE SHORT BREEDING CYCLE PROTOCOL EFFECTIVE ON DIVERSE GENOTYPES OF SUNFLOWER (*Helianthus annuus* L.)

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ABSTRACT

Immature embryo culture of sunflower (*Helianthus annuus* L.) was studied for shortening the generation time in breeding programs. The seed development from pollination to maturity in sunflower takes 50-60% (60 days) of the life cycle duration (120-150 days). This technique allows the production of fertile plants from immature embryos of 11 sunflower genotypes. Immature embryos of 10-12 days after pollination were dissected from seed grown plants (SGP), were transferred into 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 (IERP) was obtained. The four cycles of IERP were obtained from immature embryo culture technique in contrast to one generation per year with conventional breeding. The majority of cultured embryos developed into vigorous plantlets with 3-6 leaves. Out of 1320 immature embryos, the average response of the explants were 92.1% (1216). The 75% of the developed plantlets had vigorous roots and were transplanted into viol containing 1:1:2 peat: perlite: soil mixture (v/v) at 24 ± 2°C in 16h/8 h (light/dark) in the growth chamber. The only 70.3% of them was grown to maturity, self-pollinated and set seed. The overall result was average 40-45 regenerated and matured plants per 100 immature zygotic embryos. The regenerated plants also showed no morphological changes. The analysis of variance for all agronomic characters (plant height, head diameter, number of leaves, stem diameter, number of branches and seed number per head) taken from the mean of four generations *in vitro* grown plants resulted in significant differences among genotypes at 5% level. All the agronomic characters examined at *in vitro* regenerated plants decreased compared to field grown plants.

Key words: fertile plant regeneration, immature embryo, shortening the breeding cycle, sunflower.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops in both Turkey and the world. Development of hybrids which have valuable agronomic performances is an important target in sunflower breeding program. Sunflower is a highly cross-pollinated crop and a breeding program of these crops involves several breeding cycles. Obtaining homozygous parental lines for hybrid seed production in short period of time are of great importance for commercial hybrid production. In general sunflower plants were grown from seeds once a growing season in the field conditions in many countries. Therefore, only one generation a year is taken during classical breeding program. The homozygous lines and conversion of an inbred to a cytoplasmic male sterile (CMS) line can be obtained within minimum 6 generations by conventional methods (Jambhulkar, 1995; Hu et al., 2008). On the other hand, it is also known that sunflower seeds keep germination capacity about 6 days after pollination, becomes dormant 16 days after pollination (Maiti et al., 2006), remain dormant for 45-60 days (Jambhulkar, 1995) and seed maturation in sunflower takes 50-60% of the life cycle duration (Serieys, 1992).

A field grown sunflower crop requires at least 3-4 months from sowing to maturity for reseeding the next generation. Of this time, seed development from pollination to maturity takes 2 months of the life cycle duration (Hahne, 2002). *In vitro* immature embryo culture reduces the flowering and seed maturation, shortening the entire lifecycle, thus increasing the number of generation that can be produced annually. The length of one cycle is quite often limiting factor for performing a breeding aim in a reasonable period of time. This system can also reduces the cycle of backcross and selfing of lines, thus breeding process speed up and overcome seed dormancy problem. Consequently, reducing the duration of one cycle should have a great importance for the breeder to obtain seed propagated cultivars, lines and new source of maintainers (B), restorers (R) and cytoplasmic male sterile (CMS) lines.

Application of new technologies such as *in vitro* tissue culture methods in sunflower (anther culture, microspore culture, ovule culture, immature embryo culture etc.) accelerates the breeding process. Embryo culture has been used successfully by plant breeders to solve 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, haploid production (Yeung et al., 1981; Chandler and Beard, 1983; Gürel et al., 1991; Zhong et al., 1995; Bhojwani and Razdan, 1996; Torresàn et al., 1996; Saji and Sujatha, 1998; Raghavan, 2003). By using embryo culture, breeding cycle 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 banana (Yeung et al., 1981), iris (Randolph, 1945) azalea (Michishita et al., 2001) were shortened from 1 to 3 years to a few months depended on the plants. The sunflower embryo culture system was first developed for interspecific hybridization by Chandler and Beard (1983). Jambhulkar (1995) developed a rapid embryo-raised plant system for sunflower production from immature embryos, which allow five cycles in 316 days. The purpose of the present study is to increase the generation time *via* immature embryo culture. This technique was developed for rapid propagation of parental lines (B, R and CMS) and hybrids by speeding up the breeding process in sunflower industry.

MATERIALS AND METHODS

The 11 sunflower genotypes (Record 109/Sanay 3-5 B, Record 109/Sera B, BGC0565, IMIN3, RHA 04, RHA 07, RHA 14, RHA 16, IMICMS6 X IMIN6, T0910131-2, T091241-3) were obtained from 3 different sources (sunflower collections of Field Crops Department, Uludağ University, Bursa; Trakya Agricultural Research Institute, Edirne; TMT Ltd. Şti., Tekirdağ, Turkey). These genotypes were chosen for their early flowering and maturing capacity and the shortest plant height within population. Sunflower collections were planted into 5 m lines with 0.65 m row spacing and 0.3 m plant-plant spacing at Uludağ University, Agricultural Research and Experimental Station in 2010.

The plants were hand-pollinated, and maintained until the seeds were set. The first regeneration cycle has started from seed grown plants (SGP). Ten to 12 days after pollination the heads were brought into the laboratory. The achenes placed at the outer 3-4 rows of the inflorescence were separated. For homogenize the embryos, achenes with dark color had been selected. The embryos with pericarps were surface sterilized in 20% commercial bleach with a few drops of detergent for 15 minutes. They were then rinsed three times in sterile distilled water. The immature embryos were excised from the pericarps, endosperm and embryo sac for producing the first cycle of plants and placed into Murashige and Skoog (MS, 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 were sealed with parafilm to prevent moisture loss and placed in the light (16h/8h day/night) at 25 ± 2 ^{o}C and cultured until plantlets production. Within 1-2 weeks of culture in MS, developing embryos grew plantlets with strong roots (2-6 cm and branching) and 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 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 ± 2 ^{o}C in the light (16 h/ 8 h day/night), after which the plantlets were transferred to 320 X 270 mm (16 lt) pots with regular soil, keeping 5 plants per pot at the same growth chamber. Three pots were set up for each genotype. The plantlets were irrigated with sterile water 3 times weekly to maintain a high humidity environment and fertilized every 10 days. Plantlets had flowers approximately 7-8 weeks after sowing and set seed.

The regeneration response of immature embryos was scored after 2 weeks, evaluating the growth of the shoot, root and their intensity. The number of plantlets regenerated and mature plants obtained were recorded. Each genotype consisted of 10-15 petri dishes (9 cm) with 10 embryos in each. Observations on efficiency of the immature embryos (%), embryos developing plantlets (number) were evaluated.

A number of growth parameters (head production, 50% of flowering dates, pollination dates, embryo emasculation dates, transfer to soil dates) and agronomic traits (plant height, head diameter, stem diameter, number of leaves per plant, number of branches, number of seeds per head) were determined for each of the individual plants in the field and plants grown in pots. Five plants were randomly selected from each genotype grown from original seeds in the field conditions. Mean data were presented with standard deviations. For *in vitro* grown plants, 3 pots with 5 plants each were subjected to variance analysis in the completely randomized design with JUMP statistical program. The agronomic performances of *in vitro* grown plants were determined by the mean of four generations. The significance of genotypes was calculated at the 0.05 probability level by the Student's t-test. The experiments were carried out for 2 years to obtain reliable results.

RESULTS AND DISCUSSION

The first cycle of immature embryo raised plants (IERP) had taken 85-90 days after sowing. The 2nd, 3rd and then 4th cycle of IERP were obtained from *in vitro* grown plants. Immature seeds removed as early as 10-12 days after pollination were successfully cultured *in vitro*. All of the cultured genotypes developed and produced plantlets at the first stage in MS medium. Within two weeks, developing embryos mainly grew plantlets with strong roots (2-6 cm and branching) and stem with 6-8 leaves (~1 cm) as described by Freyssinet and Freyssinet (1988).

The well developed plantlets were selected to transfer into viol containing peat:perlite:soil mixture for acclimatization. The plants were kept for two weeks in growth chamber. Almost all of the plantlets grew healthy in the viols in green house conditions and were phenotypically similar to original seeds. They were generally short, often branched with a flower on each branch. The only 15 of them placed into pots (5 plantlets /

pot). They survived to flower and set seed after 8 weeks. The premature flowering did not occur *in vitro*. Mature plants exhibited normal phenotype and were fertile. When the unshaped and small plantlets were transplanted to soil, they did not survive. Days from culturing to harvesting of mature seeds were almost 80-95 days depended on the genotype.

Out of 1320 immature embryos, the average response of the explants was 92.1% (1216) showing morphogenesis with a range of 90-100%. The majority of cultured embryos developed into plantlets like Jeannin and Hahne (1991). Of the embryos that placed into embryo growth medium, 85% was in peat:perlite:soil mixture and the only 77.5% of them was grown to maturity, self-pollinated and set seeds. This study reports the results of improvement of the growing sunflower plants from *in vitro* immature embryo culture in the growth chamber.

The mean values for agronomic performances of SGP and IERP sunflower genotypes are given in Table 1 and Table 2. Although there were no big morphological changes in *in vitro* regenerated plants, they showed variations for plant height, head diameter, stem diameter, number of branches, number of leaves per plant and number of seed per head.

Table 1. The agronomic performances (plant height, head diameter and number of leaves) of seed grown and *in vitro* grown plants in sunflower genotypes.

Genotypes	Plant height (cm)		Head diameter (cm)		Number of leaves (no)	
	SGP	IERP*	SGP	IERP*	SGP	IERP*
RHA04	93.0 ± 2.6	40.8ef	7.5 ± 0.2	2.06de	21.0 ± 1.1	16.4bc
RHA 07	97.4 ± 3.8	41.2ef	9.0 ± 0.3	2.14de	22.4 ± 0.5	17.0bc
RHA 14	84.8 ± 3.4	60.6b	6.1 ± 0.3	4.50a	21.2 ± 0.4	17.3bc
RHA 16	58.8 ± 6.0	51.04c	5.2 ± 1.0	2.06de	18.6 ± 1.9	18.3ab
T0910131-2	70.4 ± 1.9	41.8ef	11.0 ± 0.6	2.32cd	24.6 ± 2.4	20.2a
T091241-3	120.0 ± 8.4	61.2b	10.6 ± 1.4	2.82bc	18.4 ± 1.6	18.3ab
IMIN3	75.8 ± 5.8	41.9ef	7.5 ± 1.1	1.54f	22.4 ± 1.4	16.2c
IMICMS6 X IMIN6	78.8 ± 4.1	37.9f	7.5 ± 0.8	1.77ef	22.8 ± 0.9	15.6c
Record 109/Isera	70.2 ± 3.1	45.2de	5.3 ± 0.1	2.37cd	19.0 ± 0.5	17.0bc
Record 109/Sanay 3-5(B)	81.4 ± 7.9	69.1a	7.8 ±0.9	2.67bc	20.8 ± 0.8	16.8bc
BGC0505	68.0 ± 2.4	48.4cd	4.5 ± 0.2	3.08b	18.6 ± 0.9	16.6bc

*the mean of 4 generations were taken from *in vitro* grown plants

Table 2. The agronomic performances (stem diameter, number of branches and number of seed per head) of seed grown and *in vitro* grown plants in sunflower genotypes.

Genotypes	Stem diameter (cm)		Number of branches (no)		Number of seed per head (no)	
	SGP	IERP*	SGP	IERP*	SGP	IERP*
RHA04	0.46 ± 0.0	0.38c-e	14.6 ± 0.7	3.12bc	558.3 ±33.5	16.3e
RHA 07	0.54 ± 0.0	0.47bc	8.6 ± 0.5	3.31b	579.3 ±148.9	41.5ab
RHA 14	1.86± 0.0	1.28a	7.4 ± 0.4	4.30a	646.3 ±167.8	72.7a
RHA 16	0.86± 0.0	0.36de	8.0 ± 0.7	1.79ef	172.7 ±58.0	22.1c-e
T0910131-2	2.04 ± 0.2	0.43b-d	2.8 ± 1.4	1.04g	841.7 ±160.9	17.7de
T091241-3	1.64 ± 0.2	0.39b-e	3.6 ± 0.4	1.50fg	787.0 ±133.4	30.1b-e
IMIN3	0.56± 0.2	0.47bc	10.4 ± 0.5	2.56cd	382.0 ±58.9	32.2a-c
IMICMS6 X IMIN6	0.88± 0.0	0.41b-e	10.6 ± 0.9	2.44c-e	535.3±21.5	18.8de
Record 109/Isera	0.60± 0.0	0.49b	7.6 ± 0.7	3.11bc	415.7±84.0	39.3a-c
Record 109/Sanay 3-5(B)	0.40± 0.0	0.31e	10.4 ± 1.2	2.13d-f	494.7±27.1	35.8a-c
BGC0505	0.72± 0.0	0.43b-d	7.0 ± 0.3	3.80ab	508.7±81.1	38.9a-c

*the mean of 4 generations were taken from *in vitro* grown plants

The seed grown plants had generally higher agronomic performances compared to *in vitro* grown plants as reported by Jeannin and Hahne (1991). In general, the plant height, stem diameter, number of seed per head, head diameter decreased when *in vitro* technique was used as indicated by Encheva et al., (2003) and Gopalkrishnan (1993). The possible reason for decreasing of the agronomic performances is growing conditions of the plants. The plant height of the individual sunflower plants obtained from *in vitro* culture varied between 37.9 and 69.1 cm as it was revealed earlier by Hahne (2002). Record 109/Sanay A 3-5 had the highest plant height (69.1 cm) while IMICMS6 X IMIN6 had the shortest plant height (37.9 cm). The plant height of seed grown plants varied between 58.8 and 120.0 cm. It was lower than 100 cm except T091241-3 (120.0 cm). The regenerated plants had minimum 1.54 and maximum 4.5 cm head diameter at harvest stage. The similar results with reduced head diameter of sunflower after *in vitro* grown conditions were published by Encheva et al., (2003). The size of the individual flowers chanced from a few ray flowers surrounding a small number of disk flowers to sunflower heads of reasonable size with more than 70 seeds in this study. The seed production of SGP varied from 173 to 842 while *in vitro* grown plants had 16-73 seeds (average of 24 seeds) under our growth conditions.

All the plants transferred to soil produced flower, contained both fertile and sterile seeds. When the plants were harvested, all the heads produced seeds changing from 16.3 to 72.7 in total depended on the 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 field-grown plants. The possible reason for decreasing of the agronomic performances is growing conditions of the plants.

One of our study conducted at field conditions in Marmara region showed that the agronomic features of sunflower in the field conditions was very high (Göksoy et al., 2003). Our results indicated that it is possible to produce viable seeds from *in vitro* grown plants and to obtain four generations a year.

The overall result was average 54 regenerated and matured plants per 563 excised immature embryos. Although agronomic characters of *in vitro* regenerated plants decreased, more than 16 seeds were harvested for each genotype and majority of them was viable. Therefore, it was sufficient for continuing to the next generation. This method will be useful for breeders to advance sunflower breeding. The four generations in a year advanced the breeding of genetic population. Therefore, homozygosity may be developed in 2 years if the population is derived from a cross between two inbred lines.

It is also known that when the mature seeds of most sunflower lines are freshly harvested, they do not germinate due to dormancy (Gopalkrishnan et al., 1993; Hahne, 2002). By using immature embryo culture 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 (waiting for mature seed collection, sowing and germination) seed grown in field. 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.

The rapid cycling genotypes of sunflower selected in our conditions were completed their life cycles in about 106-112 days in the field conditions compared to the 150- 160 days of late flowered genotypes (Data were not shown). In general, the genotypes selected above needed 85-95 days for completing one generation by *in vitro* immature embryo culture.

Consequently, both immature embryo culture and selection of possible rapid cycling lines of sunflower produced 4 generations per year. However, the minimum requirement for completing the seed to seed cycle was 86 days by using dwarf and early maturing genotype Morden and, the embryo raised plants completed their 1st cycle at 65 days. Thus, the researcher managed to obtain 5 generations in a year (Jambhulkar, 1995).

Based on the results from the present study, the time required to recover immature embryos averaged 3 months. The length of time includes the following steps: 1) 2 weeks in *in vitro* conditions for production of plantlets 2) 2 weeks acclimatization period from *in vitro* to growth room chamber conditions, 3) 4-5 weeks of plant growth in the green house to flowering and pollen shed, 4) 2 weeks to allow the seeds to fill and discarded immature embryo.

This study reports the results of improvement growing sunflower plants from *in vitro* immature embryo culture in the growth chamber and green house conditions. This system is available for use by sunflower breeders and researches to create hybrids, parental lines in a very short time at breeding studies.

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