Genetics & Applications

Institute for Genetic Engineering and Biotechnology University of Sarajevo

Research Article Open access

Induction of haploid plants for speed-up breedıng in sunflower (*Helianthus annuus* L.) by pollen irradiation

Yunus Emre Aktaş[1], Yıldız Aydin[2], Ahu Altinkut Uncuoglu[1*]

[1]Marmara University, Faculty of Engineering, Department of Bioengineering, Istanbul, Turkey

[2]Marmara University, Faculty of Sciences, Department of Biology, Istanbul, Turkey

DOI: 10.31383/ga.vol7iss1ga05

***Correspondence**

E-mail: *ahu.uncuoglu@marmara.edu.tr*

Received

March, 2023

Accepted

June, 2023

Published

June, 2023

Keywords

Sunflower, Haploid induction, Irradiated pollen, Embryo rescue

Abstract

The creation of homozygous plants is essential for both genetic studies and the production of hybrid seeds, particularly in plants that were pollinated by an alien species. Dihaploid (DH) technology is an important application in obtaining homozygous lines used to plant speed breeding programs. The generation of haploid embryos by pollen irradiation and pollination of female flowers with irradiated pollen has been successfully applied. The influence of irradiated pollen on the induction of haploids in sunflower (*Helianthus annuus* L.) has been evaluated on 16 different sunflower lines from Turkey's National Sunflower Breeding Program. In this study, pollen grains were irradiated with varying dosages of Gamma rays (500-1000 Gy) to generate parthenogenetic haploid embryos in oilseed sunflowers. At a period of 12–20 days following the process of pollination, embryos were plated into modified MS culture media under a 16/8 h photoperiod and a temperature of 25°C. Parthenogenetic 18457 embryos at different developmental stages were rescued in vitro and 650 were germinated. The ploidy analysis of regenerants was made with chromosome count and flow cytometry. Except for 500 Gy, all dosages were effective for inducing embryos and haploid plants. Obtained results indicate that parthenogenetic haploid embryos can be produced in K3AD SN:8, IMI 069, and IMI 044 sunflower genotypes by selecting the appropriate Gamma ray dose and embryonic developmental stage. This study was focused to recover sunflower doubled haploids to accelerate the production of non-segregating lines for breeding.

Introduction

Common sunflower (*Helianthus annuus* L., 2n = 2x = 34, haploid genome size ~3.5 Gbp) is widely cultivated as oilseed and confection crop types (Pegadaraju et al., 2013), which belongs to the genus *Helianthus*, which has 52 species and 19 subspecies (Blinkov et al., 2022). In order to meet the world's food demand, crop development is essential for enhancing agricultural production. The development of varieties with desirable traits is necessary for breeders to carry out economic operations and achieve sustained agricultural development. The generation of homozygous plants is crucial for both genetic studies and the production of hybrid seeds in cross-pollinated plants, such as sunflower, which are encountered at greater rates. It is necessary to generate varieties with desirable traits as well as sustainable agricultural growth and economic activities for breeders. Doubled haploid (DH) technology, as opposed to conventional backcrossing and selection methods, can speed up and improve the efficiency of the development of true breeding lines (Mabuza et al., 2023). Obtaining haploids has several advantages for the advancement of genetic studies, particularly in foreign pollination plants where there is no available DH induction procedure/technique that can be efficiently used in breeding programs.

Sunflower is an important raw material that accounts for a significant portion of global vegetable oil production (Kaya, 2004). Owing to their superior production performance, quality, and uniformity, farmers choose hybrid sunflower lines. The conventional method for breeding a new crop variety is selecting parental genotypes with the required qualities, crossing them, and then selecting and advancing successive generations of superior offspring to develop candidate cultivars.

In our country and worldwide, sunflower breeding programs are generally based on hybrid breeding. In these breeding programs, the target traits are higher seed yield and oil content, earliness, resistance to diseases and pests, etc. Developing inbred lines with traits and newly developed hybrids should have much higher yield potential in the future (Kaya et al., 2009).

Conventional breeding methods can take up to ten years. With current methods such as doubled haploid breeding (Dwivedi et al., 2015) and speed breeding (Hickey et al., 2019), the length of each crop breeding cycle can be decreased. For plant breeders and geneticists, the haploidization processes are extremely useful since they facilitate the rapid development of inbred lines. Haploid plants derive from gametes that include one set of chromosomes containing only half of the genetic information of a somatic cell and they are incapable of fertilization and producing healthy offspring (Gilles et al., 2017).

Breeders place the greatest value on the ability of haploids to achieve homozygosity in a single generation. As a result, it is possible to create doubled haploid lines and acquire complete homozygosity without the need for numerous generations of back-crossing.

The two groups that make up the category of haploid induction techniques are in vitro and in situ techniques. This may be accomplished with anther/microspore or ovary/ovule explants. The regeneration response of cells, in a particular species, yields varying results, and donor genotype has a significant impact on cell responsiveness. Irradiated pollen, inter-specific crosses, and haploid inducer lines are examples of the types of pollination strategies that are utilized in situ research procedures (Gilles et al., 2017). Even though immature embryo rescue procedures may be able to cut the amount of time needed in half, the generation of inbred lines via self-pollination still takes a lot of time (Zhong et al., 1995). Sunflower haploids have already been created using anther culture (Zhong et al., 1995; Saji and Sujatha, 1998), gynogenesis (Yang et al., 1985), and microspore culture (Gürel et al., 1991), but the techniques are difficult to replicate. Sunflower has been resistant to all in vitro growing procedures, making haploid approaches difficult to create (Hahne, 2001). This was also demonstrated in our prior research on sunflower microspores and another culture (Çakmak et al., 2017; Akgül et al., 2020). When neither androgenogenesis nor gynogenesis is successful, parthenogenetic haploid induction may be a viable option. For a number of haploid induction experiments where the genotype of the germinated plant is maternal, in situ, parthenogenetic induction is commonly used such as in loquat (Blasco et al., 2016) and carrot (Adamus and Michalic, 2003). This approach comprises the pollination of female florets with pollen that has been exposed to radiation, followed by the isolation of embryos generated from the ovules by parthenogenesis and their subsequent cultivation in vitro. Using an adequate amount of γ -irradiation is one of the most crucial aspects in the formation of parthenogenetic haploid and doubling haploid plants, as well as embryo cultivation. Irradiation does not stop pollen from germinating on the stigma at quantities that are considered acceptable for viability; however, it does stop pollen from fertilizing the ovules. Still, it can speed up the rate of egg cell division and embryo development. Both the genotype of the plant and the amount of radiation it receives influence how the pollen reacts to being irradiated. Irradiation pollen induction relies on the genotypes of both donor and recipient plants as their primary sources of variation. The most frequently used radiation sources are 137Cs and 60Co. In several species, such as winter squash (Kurtar and Balkaya, 2010) and loquat (Blasco et al., 2016), gamma-induced pollination with irradiated pollen has been successfully exploited to create maternal haploid embryos. The first effort at producing haploid sunflower plants by gamma-induced parthenogenesis was made in 1993 at the Dobrudzha Agricultural Institute in Bulgaria (Encheva et al., 2017). Bulgarian researchers presented the first report on the successful cultivation of *H. annuus* L. using this technique in 1997 (Todorova et al.,1997). Todorova et al. (1997) utilized irradiation pollen-induced parthenogenesis to create agronomically valuable viable DH sunflower lines. Unfortunately, not all genotypes were adequately responsive to haploidization procedures. They used four hybrids Albena, Viki, Euroflor, and HB 9203 as female recipient parents, but doubled haploids were obtained only from the sunflower hybrids Albena and Viki. Todorova (1999) demonstrated that the genotype of the pollen donor influences the induction of parthenogenesis. Similarly, various recipient lines react differentially to pollen irradiation of the same genotype. The objective of the present study was to evaluate the response of sunflower genotypes from the National Sunflower Breeding Program of Turkey to in situ-induced parthenogenesis by pollination with gamma-irradiated pollen followed by in vitro embryo rescue. The purpose of identifying DH plants from sunflower crops is to speed the development of the disease- and stress-resistant breeding lines, among other desired features.

Materials and methods

Plant material and Pseudofertilization

The research was conducted using 16 distinct sunflower lines, 9 of which were Cytoplasmic Male Sterile (CMS) and 7 of which were emasculated. A total of 80 individuals, 50 of which were CMS and 30 of which were emasculated, were supplied by the Thrace Agricultural Research Institute (TARI), and all plants were grown in fields maintained by TARI. The Pollen grains were collected from TARI fields, and the collected pollen grains were subjected to gamma irradiation (using a Cobalt 60 source) at a dose of between 500 and 1000 Gy. The staining method was utilized in order to determine the pollen's vitality. Aniline blue, also known as cotton blue, was prepared in lactophenol and used to stain both the control and irradiated pollens (Mclean and Cook, 1941). Following the placement of the pollen in

tubes with a volume of 2 milliliters, one or, at most, two drops of cotton blue were added to each tube before the tubes were gently shaken. Following a five-minute incubation time, a few drops of a cotton blue and pollen combination were poured on the slide, and then the slide was covered with a coverslip. The slide was then examined under a microscope. The KAMERAM app was used in conjunction with the KAMERAM digital camera in order to take photographs of the preparations before they were analyzed using the Olympus BX-51 light microscope.
Before anthesis, immature flower buds of ovule donors, excluding CMS lines, were manually emasculated and bagged daily to prevent unintended outcrossing. The flower buds of the ovule donor were fertilized with irradiated pollen. Flowers that had been pollinated were bagged to prevent pollen contamination (Figure 1). After 12 to 20 days following pollination, the paper bags were removed and flower heads were taken for laboratory analysis. Before cultivation, the embryonic stages were established. Experiments were conducted at the Biology Department's laboratory and greenhouse complex at Marmara University in Istanbul, Turkey.

Figure 1. a) Collected pollen bulk before irradiation, **b)** Emasculation **c)** Pollination with irradiated pollen, **d)** Bagged flower-heads.

In vitro embryo culture

The seeds were extracted using aseptic procedures, and the surface of the seeds was disinfected using a 20% commercial bleaching solution mixed with 2-3 drops of Tween 20 and stored in sterile bottles. At last, the seeds were washed 5-6 times with deionized and sterile water to eliminate all of the detergent foams (Dagustu, 2010). Individually sterilized seeds were opened in a laminar airflow cabinet. The embryos were originally cultivated in Petri dishes (9 cm in diameter and 1.5 cm in height) using approximately 20 ml of modified MS (Murashige and Skoog, 1962) culture medium (Aspiroz, 1987; Freyssinet, 1988; Dagustu, 2010) (Table 1) and placed in a growth chamber with a 16/8-hour light/dark cycle and a temperature of 25 +/-1 °C. The embryos that had grown roots and shoots in the lab were moved to vials. The care was taken to wash the solid medium off the roots. The potted plants were covered with a beaker, and the cover was slowly taken off so that the plants could acclimatize to lower humidity (Figure 2). Once the plants, they were transferred into a greenhouse.

Table 1. Culture medium utilized for embryo rescue investigations

Media	Salts	Vitamins	Sucrose	Plant Growth Regulators	Literature
A	1/2 MS	B5	20 g/L	—	Aspiroz, 1988
D	MS	MS	20 g/L	—	Dagüstü, 2010
FM1	MS	MS	90 g/L	0.05 mg/L BAP*	Freyssinet, 1988
FM2	MS	MS	30 g/L	0.01 mg/L BAP	
FM3	B5	B5	10 g/L	0.02 mg/L IAA**	

*BAP: Benzylaminopurine, **IAA: Indole-3-acetic Acid

Figure 2: Acclimatization stages of regenerated plantlets. a) *In vitro* regenerated plant on solid medium. b) Cleaning MS medium from the roots of regenerated plant. c) Potting the regenerated plants. d) Covering the potted plantlets with transparent plastic or beaker.

Determination of ploidy level

Fresh leaf samples from in vitro-grown haploid and diploid (control) plants were used for flow cytometry. After chopping 0.5 cm2 of leaf samples in 1.0 ml of nuclei isolation buffer (Partec), the mixture was filtered through a 50 µm nylon filter and stained with 3.0 ml of coloring solution containing 1 mgL-1 of DAPI (4′-6-diamidino-2-phenylindole HCl). Sample ploidy was determined using a flow cytometer (Partec, Munster, Germany). Ploidy of the H. annuus seedlings was determined based on the relative peak positions of the samples to standard (Rye).

In vitro-grown embryo-cultured plantlets were harvested at around 1 cm in length, with the active root tips harvested shortly thereafter. When root tips were collected, they were placed in ice water and stored in a refrigerator at 4 °C for 24 hours. Thereafter, they were maintained at 4 °C until needed, after which they were fixed in a 3:1 v/v solution of ethanol and acetic acid. Once the root tips were rinsed twice in distilled water, they were hydrolyzed in 1 N HCl for 18 minutes at 60 °C to prepare them for microscope preparations. After hydrolysis, the root tips were stained in Feulgen stain for 1 hour and then rinsed in distilled water. The crushed, acetocarmine-stained root tips were examined using an optical microscope, and the images were captured at 100x magnification.

Results

Embryo induction and growing of haploid plants

In order to determine the most effective haploidy technique in sunflower, this study examined three doses of gamma irradiation on a variety of flower heads from 16 distinct sunflower genotypes. A total of 18457 embryos were successfully grown in vitro and haploids were obtained from the sunflower line K3AD SN:8, IMI 069, and IMI 044. Both the pollen's Gamma irradiation dose and the media's composition had major effects on the rate of embryo induction (Table 2).

Table 2 displays the effects of different gamma-ray dosages on the number of embryos. The most efficient irradiation dose was determined to be 1000 Gy with a germination rate of 5.35 %. The dose-response curve for 750 Gy showed that it was superior to 500 Gy for certain genotypes, with a germination rate of %4.41. Comparing the impacts of various media compositions on their germination efficiency, the D medium yielded the highest germination rate at 4.2%, followed by A and FM culture media at 4.1% and 1.3%, respectively, for sunflower lines collected 12 to 16 days after pollination. In order to assess the most convenient stage of embryo evolution for successful embryo rescue, immature seeds were harvested at 17-21 days after pollination, which represented an interval between seed development and fruit enlargement stages. Significantly more seeds germinated in fruits collected 17-21 days after pollination with irradiated pollen than in fruits taken 12-16 days after pollination, and germination rates increased to 78%, 64%, and 48% for A, D, and FM medium compositions, respectively.

The embryos with substantially more endosperm were regenerated faster and resulted in the growth of healthy plants. This option reduced our time and consumption of resources.

Table 2: The effects of different gamma-ray doses and media composition on *in vitro* germination percentages of embryo used for *in situ*-induced parthenogenesis.

Effect of different gamma-ray doses				**Effect of different media composition**			
Dose (Gy)	Number of induced embryos	Number of plants regeneration	Rate of plants regeneration	Media composition	Number of induced embryos	Number of plants regeneration	Rate of plants regeneration
500	5495	27	0,49	Aspiroz, 1988	7639 (5916 CMS/ 1723emasculated	317	4,15
750	6106	269	4.41	Dagüstü, 2010	7024	295	**4,20**
1000	6856	367	**5.35**	Freyssinet, 1988	3798	48	1.3

Ploidy level

Flow cytometry and chromosome counting validated the ploidy level of the plantlets grown in vitro. In addition to the DNA content, chromosome counting proved that a haploid plant was obtained through irradiation pollen cultivation. Through flow cytometry analysis it was confirmed that 18,75% of genotypes (3 of the 16) (K3AD SN:8, IMI 069 and IMI 044) were haploid. The counting of chromosomes from the actively growing root tips of all of the in vitro-cultured plantlets revealed that parthenogenetic haploid plants with 17 chromosomes were acquired, and that diploids with 34 chromosomes were found. Five more haploid plantlets were identified (Figure 3).

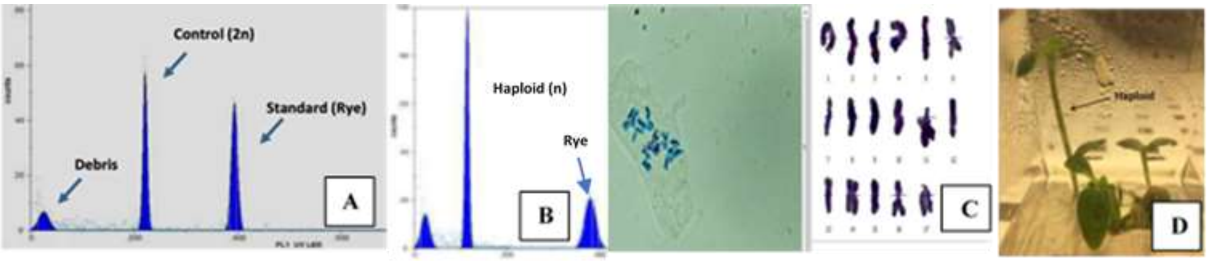

Figure 3: (A) Flow cytometry histogram of a mixture of tissues from diploid control sunflower plant (2n) and the haploid (B) obtained by induced parthenogenesis (n). (C) Chromosomes count on sunflower root piece of the haploid cell (n = 17). (D) *In vitro* cultured haploid sunflower plant.

The objective of this study is to establish a way of haploidization for sunflower (*Helianthus annuus* L.), and the approach based on the irradiation of pollen was selected as an alternative to the previously explored anther and microspore culture. When using alternate methods, finding haploid regeneration in sunflowers can be challenging. The anther culture was one of the methods that we used in the past with the intention of inducing haploidy. However, only diploid plants could be regenerated through embryogenesis, and no haploids were found, despite extensive study of the impact of various factors (Çakmak et al., 2019). Therefore, in our current study, we focused on obtaining haploid sunflowers using the approach of pollen irradiation.

Pollen irradiation can be used to induce gynogenic development of the embryo sac, which could lead to the production of doubled haploid lines in sunflower. The radiation exposure, genotype, and timing of harvesting sunflower flower heads all have a role in determining how effective embryo induction is in this species. Under controlled conditions, we were able to obtain haploid plants by induced parthenogenesis. The optimal pollen irradiation dose for initiating parthenogenesis varies from species to species. For example, the best dose for haploid production is 300 or 400 Gy in Iris pseudacorus (Grouh et al., 2015), 300 Gy in loquat (Blasco et al., 2016), 250 Gy in melon (Hooghvorst et al., 2020). The genotypes' responses ranged from 0% to 70% in the current investigation and the breeding line “SURES K2AD SN-2/15” had the most successful regeneration ratio of 70.85% according to overall germination ratios. Irradiation doses of 750 Gy and 1000 Gy were shown to be effective in sunflower. We observed that 750 Gy was the best dose for recovering haploid embryos from sunflower; when lower than 750 Gy dose was utilized, no haploids were identified.

In this investigation, flower heads were retrieved 12 to 16 days following the pollination with irradiated pollen. Observations under a microscope revealed that the majority of seeds contained embryos, however, the rate of regeneration was low for relatively weak seeds. To increase the regeneration rate, the interval between harvesting and culturing embryos was extended to 17-21 days, and only the strongest embryos were cultured, while germination rates increased. We determined the embryo germination rates as 0,4%, 0,6%, and 0,3% in A, D, and FM media respectively for 12-16 days after pollination. This ratio was conducted as 78%, 64%, and 48% in A, D, and FM media respectively for 17-21 days. This modification made our results comparable to those of Todorova. Todorova (1997) obtained 1107 plants from 2279 embryos after 12-16 days after pollination with a germination rate of 48.5%. These robust embryos could also germinate in soil without the need for an embryo rescue phase. At the two- to three-leaf stage, the ploidy level of 912 plantlets was evaluated by flow cytometry, and the results were published by Todorova (1997). These numbers indicated that 296 of the plants were haploid, while the remaining plants were diploid. This group of 296 plants had their haploid status rechecked after 20 days. Out of the total number of plants, 239 were diploid, 25 were still haploid, and 32 were mixoploid (containing diploid and haploid cells). This finding demonstrates that spontaneous diploidization occurs at a very high rate. In order to confirm this possibility for the sake of this research, self-pollinating diploid seedlings were bagged and seeds were collected. When we rechecked self-pollinated seedlings, we confirmed that they were diploid. When compared with anther culture, the results demonstrated that parthenogenesis, which is triggered by pollen irradiation, is a more effective method for producing haploid sunflower plants.

Conclusion

Because of the effects of climate change on crop production, particularly sunflower, plant breeding techniques need to be quick and effective in order to produce new cultivars with an adequate level of tolerance to both biotic and abiotic stress. Gynogenesis techniques, and more specifically irradiated pollen, may be an effective means of accomplishing this goal in sunflower. In this study, the generation of haploid embryos by pollen irradiation and pollination of female flowers with irradiated pollen has been successfully applied. Pollen grains from 16 different sunflower lines were irradiated with varying dosages of Gamma rays (500-1000 Gy) to generate parthenogenetic haploid embryos in oilseed sunflowers. Parthenogenetic 18457 embryos at different developmental stages were rescued in vitro and 650 were germinated. Except for 500 Gy, all dosages were effective for inducing embryos and haploid plants. By choosing the proper Gamma ray dose and embryonic developmental stage, it was possible to produce parthenogenetic haploid embryos. Flow cytometry analysis confirmed 3 out of 16 genotypes (K3AD SN:8, IMI 069 and IMI 044) (18,75%) to be haploid. This is the first study that in situ parthenogenesis has been shown to be effective in inducing haploid embryos by use of irradiated pollen in sunflower.

Acknowledgments

We gratefully acknowledge the financial support of the Marmara University Research Foundation BAPKO (Project No: FEN-C-YLP-081117-0627).

Conflict of Interest

The authors declare no conflict of interest.

References

Adamus A, Michalik B (2003) Anther cultures of carrot (*Daucus carota* L.). Folia Horticulturae 15:49–58.

Akgül N, Dogan, E, Altinkut Uncuoglu A, Aydin Y (2020) In vitro androgenic response of sunflower. In: Recent Advances in Plant Science, Nova Science Publishers, Inc. ISBN: 978-1-53617-265-2. Editors: Filiz Vardar, Yildiz Aydin, Ahu Altinkut Uncuoglu, pp: 363-378.

Aspiroz HS, Vincourt P, Serieys H, Galais A (1987) La culture in vitro des embryos inmatures dans l′ accelération du cycle de sélection des dignées de tournesol et ses effects morphovégétalifs. Helia 10, 35-38.

Blasco M, Badenes ML, del Mar Naval M (2016) Induced parthenogenesis by gamma-irradiated pollen in loquat for haploid production. Breeding Science 66: 606–612.

Blinkov AO, Varlamova NV, Kurenina LV, Khaliluev MR (2022) The Production of *Helianthus* Haploids: A Review of Its Current Status and Future Prospects. Plants 11: 2919.

Çakmak E, Altınkut Uncuoğlu A, Aydin Y (2019) Evaluation of in vitro genotoxic effects induced by in vitro anther culture conditions in sunflower. Plant Signaling and Behavior 14:9.

Dagustu N, M. Sincik G. Bayram M. Bayraktaroglu (2010) Regeneration of fertile plants from sunflower (*Helianthus annuus* L.) Immature embryo. Helia 33(52): 95-102.

Dwivedi SL, Brittt AB, Tripathi L, Sharma S, Upadhyaya HD, Ortiz R (2015) Haploids: Constraints and opportunities in plant breeding. Biotechnology Advances 33, 812-829.

Encheva J, Ivanov P (2017) Sunflower commercial hybrids, developed by biotechnologically obtained lines. In Proceedings of the Congress Book of The 2nd International Balkan Agriculture Congress, Tekirdağ, Turkey, 16–18 May 2017, pp. 717–726.

Freyssinet M, Freyssinet G (1988). Fertile plant regeneration from sunflower (*Helianthus annuus* L.) immature embryos. Plant Science 56(2):177-181.

Gilles L, Martinant J, Rogowsky P, Widiez T (2017) Haploid induction in plants. Current Biology 27: R1089–R1107.

Grouh MSH, Sousaraei N, Akbari M, Rahimi, Bayat HR (2015) Induction of haploid plants in iris (Iris pseudocorus) by pollen irradiation. Turkish Journal of Agriculture and Forestry 39:596-600.

Gürel A, Kontowski S, Nichterlein K and Friedt W (1991). Embryogenesis in microspore culture of sunflower (*Helianthus annuus* L.). Helia 14: 123-128.

Hahne G (2001) Sunflower. In: Hui YH, Khatchtourians GG, McHughen A, Nip WK, Scorza R (eds) Handbook of transgenic plants. Dekker, New York, pp 813–833.

Hickey LT, Hafeez A, Robinson H, Jackson SA, Leal-Bertioli S (2019) Breeding crops to feed 10 billion. Nature Biotechnology 37:744-754.

Hooghvorst I, Torrico O, Hooghvorst S, Nogues (2020). In situ parthenogenetic doubled haploid production in melon “Piel de Sapo” for breeding purposes. Frontiers in Plant Science 11: 378.

Kaya Y (2004). Confectionery sunflower production in Turkey. In Proceedings of the 16th International Sunflower Conference. Fargo, ND, USA. Ed. GJ Seiler, pp. 817-822.

Kaya Y, G. Evci, Durak S, Pekcan V, Gücer T (2009) Yield components affecting seed yield and their relationships in sunflower (*Helianthus annuus* L.). Pakistan Journal of Botany 41(5): 2261-2269.

Kurtar ES, Balkaya A (2010) Production of in vitro haploid plants from in situ induced haploid embryos in winter squash (Cucurbita maxima Duchesne ex Lam.) via irradiated pollen. Plant Cell, Tissue, and Organ Culture 102: 267–277.

Mabuza LM, Mchunu NP, Crampton BG, Swanevelder DZH (2023) Accelerated Breeding for *Helianthus annuus* (Sunflower) through Doubled Haploidy: An Insight on Past and Future Prospects in the Era of Genome Editing. Plants 12: 485.

McLean RC, Ivimey-Cook WR (1941) Plant science formulae. McMillan & Co. Ltd., London.

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

Pegadaraju V, Nipper R, Hulke B, Qi L, Schultz Q (2013) De novo sequencing of sunflower genome for SNP discovery using RAD (Restriction site Associated DNA) approach. BMC Genomics 14:556.

Saji KV, Sujatha M (1998) Embryogenesis and plant regeneration in anther culture of sunflower (*Helianthus annuus* L.). Euphytica 103(1): 1-7.

Todorova M, Ivanov P, Shindrova P, Christov M, Ivanova I (1997) Doubled haploid production of sunflower (*Helianthus annuus* L.) through irradiated pollen-induced parthenogenesis. Euphytica 97(3): 249-254.

Todorova M, Ivanov P (1999) Induced Parthenogenesis in Sunflower: Effect of Pollen Donor. Helia 22:49–56.

Yang HY, Zhou C, Cai D, Yan H, Wu Y and Chen YM (1985) In vitro culture of unfertilized ovules in *Helianthus annuus* L. In: Haploids of Higher Plants In vitro Hu Hand Yang HY (eds), China Academic Publishers, Beijing, Springer Verlag, Berlin, pp. 182-191.

Zhong D, Michaux-Ferriére N, Coumans M (1995) Assay for doubled haploid sunflower (*Helianthus annuus* L.) plant production by androgenesis: fact or artifact? Part 1. In vitro anther culture. Plant Cell, Tissue and Organ Culture 41(2): 91-97.