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Obtaining haploid plants by irradiated pollen culture in oil seed crops

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Abstract

Haploid plant production is of great importance to shorten the breeding period in plant breeding programs. Obtaining pure lines in plant growing programs require an intensive work with huge labor and time. Obtaining one hundred percent homozygous pure lines is a key point for the improvement and development of new cultivars. Haploid plants with a single set of homozygous chromosomes have become a valuable tool in plant breeding. Dihaploid plants that are homozygous at all loci with doubling of their chromosomes can be propagated by seed and reach full homozygosity in a single generation. Traditional methods take seven years to reach homozygosity. Dihaploidization methods provide significant advantages in terms of gaining homozygosity in a short period of one year and bringing pure lines into agriculture. Anther culture and irradiated pollen technique are among the most widely used techniques in this respect; where physical or chemical agents are used to induce mutated pollen grains and anthers that are subsequently employed to develop dihaploids through *in vitro* cultures. These techniques are a good source to facilitate gene mapping, cytogenetic research, and evolutionary studies. Irradiated pollen culture techniques have been applied to many oilseed crops to obtain pure lines. This study highlights some salient features of producing dihaploids using irradiated pollen grains and their maintenance.

Introduction

There is a deficit in production and demand of vegetable oil, therefore, Türkiye spends a large amount of budget to import vegetable oil to meet the demand for increased vegetable oil and products (Arıoğlu et al., 2020). In addition to increasing the current cultivation areas, improving cultural techniques, and preferring high-yielding varieties can be good solutions to increase yield (Şanver & Göksoy, 2019).

It is essential to breed varieties suitable for changing climatic conditions especially to cold, heat, and drought stresses, which are the main constraints that limit the yield of many crops. Haploid plant studies were reported first by Blakeslee et al. (1922) and have since become an important topic for researchers, especially in plant breeding. Haploid plants can be obtained by *in vitro* culture of anthers or isolated microspores (androgenesis), ovules (gynogenesis) or *in vitro* rescue of parthenogenetic embryos induced by *in situ* pollination with irradiated pollen (parthenogenesis) (Gonzalo et al., 2011). It can occur spontaneously in nature and can also be induced in different ways. Irradiation of pollen grains and anthers is one method among them.

Initial research aimed to evaluate the effects of radiation on pollen germination and pollen tube growth. Sestili and Ficcadenti (1996) has reported that radiation could also be used to render pollen generatively ineffective without affecting the ability to stimulate egg cells, thus allowing formation of parthenogenic embryos.

The leading plants from which vegetable oil is obtained include soybean, sunflower, cottonseed (cotton), rapeseed, peanut, sesame, safflower, castor oil, poppy, flax, hemp, jojoba, corn (from corn germ), olive, date palm, and coconut. Most of them except jojoba,

date palm, and coconut can be successfully cultivated in Türkiye with their high adaptation capabilities. The development of fast and effective methods and their adaptation to the agronomic process will make a great economic contribution to Turkish vegetable seed oil industry (Arıoğlu et al., 2010).

Haploid methods used in oilseed plants

Haploid and double haploid methods is desired by plant breeders in order to shorten the process of obtaining and developing new varieties. Double haploid plants are homozygous at each locus. They can be used as parents to grow F1 hybrids. Many studies have successfully obtained haploid plants in different plant species. The induction of maternal haploid embryos by pollination with irradiated pollen has been successfully used in many species (Grouh et al., 2017; Kurtar et al., 2020; Shahhosseini, 2022; Zhao et al., 2022).

Due to the successful use of haploid and doubled haploid technology in maize, the technique has also been investigated and developed for various oilseed crops with varying success rates. There has been limited success in soybeans. Several successful *in vitro* anther culture experiments have been conducted to produce haploid plants in soybean but are not commercially sufficient and viable (Lulsdorf et al., 2011).

Two methods, 'maternal haploid' and 'paternal haploid', are used to obtain haploid plants by *in vivo* technique. The method of using the reducing line as the pollen donor, i.e., the father, is called paternal haploid, and the method of using the reducing line as the pollen receiver, i.e., the mother, is called maternal haploid (Yorgancılar et al., 2019). Maternal induction is the most widely used and preferred method in modern maize breeding programs due to its higher efficiency and more reliable source of stable inducer lines than paternal induction. In a program to develop soybean doubled haploid lines using *in vivo* haploid induction, maternal induction would be the preferred method due to the possibility of using male sterile lines (Friederich, 2020).

Aktaş (2018) aimed to develop a haploidization procedure in his study with sunflower (*Helianthus annuus* L.). Therefore, the researcher used irradiated pollen technique as an alternative to the previously studied anther and microspore cultures. 16 dissimilar sunflower breeding lines were used as experimental material. Results showed that the effectiveness of the method is highly dependent on the genotype of the recipient lines.

Studies have shown that it is possible to use irradiated pollen to produce double haploids through parthenogenesis (Aktaş et al., 2018; Bidney & Scelonge, 1997; Kaya, 2004; Todorova et al., 1997).

Sesame (*Sesamum indicum* L.) is one of the most important oil crops. Gamma irradiation is a reasonable tool to induce variability in sesame and is advantageous in increasing pollen viability (Audu et al., 2021; Ryu, Doo & Kim, 1992).

Haploid studies for the variety development in cotton breeding studies are helpful to shorten breeding period and providing economic gains (Bajaj & Gill, 1997; Korkunç et al., 2017).

Brassica napus L. is one of the most important oil crops in the world. It is more prone to tissue culture studies than other Brassicaceae. Mutants with high oil content and high oleic acid, low linolenic and erucic acid contents were obtained through mutations and microspore culture (Ahmad, Macdonald & Ingram, 1991; Fletcher et al., 1998; Kučera et al., 2002; Seyis et al., 2014).

Obtaining haploid with irradiated pollen

Obtaining a sufficient number of haploids in breeding programs has been among the objectives of plant breeders. Interspecific crosses, delayed pollination, temperature shocks, use of irradiated pollen, use of chemicals, and plant growth regulators have been tried (Dwivedi et al., 2015; Puolimatka & Pauk 2000; Zheng et al., 2001).

Errors that occur during DNA replication cause mutation and then natural radiation in the environment triggers this mutation that causes a change in the hereditary material. The individuals modified as a result of this natural mutation is known as a spontaneous mutant. With the discovery of ionizing (X and γ-rays) and non-ionizing (UV) radiation, the mutations can also be realized artificially (Spencer-Lopes et al., 2018).

Various types of radiations are available for plant mutation. Gamma rays, which induce disruption of the unstable nucleus of an atom, are generally preferred and widely used. They have a shorter wavelength and therefore have more energy compared to x-rays. They can also be placed in a climate chamber, greenhouse, or field with the advantage of using them at dissimilar stages of plant development (Spencer-Lopes et al., 2018). Cobalt-60 (^{60}Co) and Cesium-137 (^{137}Cs) isotopes are the main sources of gamma rays (Lerouge & Simmons, 2012).

Pollen irradiation can give plants new positive or negative traits by making some hereditary changes in the structure and number of chromosomes or the physical and chemical structures of their genes (Yaman, 2014). The success of the irradiated pollen technique depends on the genotype of the plant, the physiological stage of the female plant, culture conditions, ambient compositions, and radiation dose (German, 2011).

The stages of obtaining haploid plants with irradiated pollen are as follows;

i. Cultivation of the donor plant: The oil crops are grown in fields or greenhouses.
ii. Emasculations: Removal of anthers before anthesis to prevent self-pollination. Then they are covered.
iii. Pollen collection and irradiation: Pollen at the appropriate stage is collected and irradiated at different doses.

iv. Emasculated irradiated pollen given to the plant: Pollens irradiated at different doses are given to the emasculated plant and covered.
v. Embryo rescue: 12-20 days after pollination, the trays are collected and brought to the laboratory environment and the immature embryos are regenerated to obtain plants.
vi. Haploid plant regeneration and ploidy: Haploid plants regenerated from embryos are grown under climatic chamber conditions.
vii. Ploidy level determination: Ploidy analyzer or flow cytometry devices are used to determine ploidy levels.
viii. Colchicine treatment: Different doses of colchicine are applied to plantlets that have completed root formation.
ix. Doubled haploid plants: Colchicine applied plantlets are evaluated morphologically and cytologically. Necessary maintenance procedures are carried out in the greenhouse for those which are found to be double haploid.

Gamma radiation in plant breeding

The induction of mutations through physical agents allows the acquisition of genetic variations of agricultural importance that are not found in plants existing in nature (Pérez-Jiménez et al., 2020). The formation of free radicals, induced by the exchange promotes structural and metabolic changes in the plant. For example, 50 Gy gamma radiation produced a sensitizing effect on chloroplasts. Moreover, high Gy doses affect protein synthesis, hormonal balance, enzymatic activity, gas, and water exchange (Riviello-Flores et al., 2022). Nowadays, new variations are needed to develop more nutritious, resistant, and productive varieties (Amri-Tiliouine et al., 2018; Shuryak et al., 2019). Obtaining these variations in a controlled manner in a short time has enabled the rapid development of breeding studies.

Gamma-ray is the most efficient source for irradiation. Its simple application, good penetration, reproducibility, high mutation frequency, and low lethality problems are of great advantage (Chahal & Gosal, 2002). The most widely used gamma-ray sources are Cobalt-60 (^{60}Co) and Cesium137 (^{137}Cs). Gamma-irradiated pollen can germinate on the stigma, grow, and reach the embryo sac. Although it cannot fertilize the egg cell and polar nuclei, it stimulates the development of haploid embryos (Blasco et al., 2016). This method requires immature embryo rescue under *in vitro* conditions.

Radiation dose and source

Radiation causes changes in the generative nucleus of sperm cells that induce parthenogenetic development of the egg to form haploid embryos (Dal et al., 2016). Beneficial mutations that can be induced by physical mutagenic agents such as ionizing radiation (X-rays and gamma rays), non-ionizing radiation (ultraviolet) and corpuscular radiation (protons, neutrons, alpha and beta particles) are the changes in genotypic structure that increase the variability of species and facilitate their adaptation to various selection pressures. These sources have a 94% effect on the generation of mutant types (Al-Safadi & Simon, 1990; Ludovici et al., 2020; Spencer-Lopes et al., 2018).

Although seeds or whole plants can be irradiated, pollen irradiation has advantages such as the rare formation of chimeras and homozygous plants. The disadvantages are the difficulty of obtaining material and the duration of viability. Efficient radiation doses depend on pollen type. In many practical cases, the absorbed dose is not measured directly but is calculated from the measured number of ions produced in the air by ionizing radiation (Spencer-Lopes et al., 2018).

Plants are affected by physical and chemical mutagens to varying degrees. One of the most important factors for successful mutation breeding and achieving the goal of the study is the determination of the optimum mutagen dose. The dose required for a given experiment depends on the desired return, but mutagenic treatment can also have undesirable consequences, such as death and infertility. The genotype of the selected material can also alter the susceptibility to mutagenic treatments (Kundu et al., 2014, Kundu et al., 2016). Lower doses are generally preferred, as dose increases can lead to undesirable severe mutations. Therefore, preliminary analyses are required to determine the appropriate doses for each plant material.

Pollen irradiation

Artificial mutation of plant pollen using ionizing radiation (protons, neutrons, alpha, beta, gamma, and x-rays) causes chemical changes in the plant. It acts together with molecules and atoms on the production of free radicals in the cell. These radicals cause changes in the physiology, biochemistry, anatomy, and morphology of plants depending on the radiation levels. The amount of moisture in the material is also important for the effect of radiation on DNA. Radiation causes random ionization and excitation events in the environment it passes through.

In practice, male flowers are collected one day before flowering in plants grown under controlled conditions in the greenhouse or the open field, hermaphrodite flowers (emasculated) are castrated and closed to prevent uncontrolled pollination. Afterward, the petals of the male flower are removed, the pollen grains are collected and irradiated with gamma-ray doses (250, 350, 450, and 550 Gy) determined according to the plant using a radiation source (e.g.,^{60}Co) and then stored at room temperature overnight and the next day the female flowers are pollinated using gamma-irradiated pollen.

Irradiated pollen surviving test

Sensitivity tests must be performed to determine the mutagen dose. Determining the most effective and

efficient mutagen dose (RD50, EMD) is a prerequisite for success. Irradiation dose, irradiation duration, pollen age, and genotypes can affect pollen viability. The viability, which indicates the quality characteristics of irradiated pollen, varies according to genotype, and is highly affected by the gamma-ray dose applied (Hayati et al., 2022).

The viability of pollen kept at room temperature and 50% humidity decreases rapidly (Giovannini et al., 2017). Therefore, irradiated pollen should be stored under cold conditions. In order to measure the response of irradiated pollen to irradiation and to determine the effect of different doses, it is important to determine the appropriate dose by pollen viability tests (Kurtar et al., 2020). Different pollen viability tests are performed to determine viability percentages.

2,3,5, triphenyl tetrazolium chloride (TTC) (1%) solution is preferred to determine pollen viability. A viability test should be performed before aceto-orcein staining, since this may stain all pollen. Pollen viability is separated according to pollen character (viable, semi-viable, non-viable). After dropping 1 drop of the prepared solutions on the slide, the pollens are sprinkled on this drop covered with a coverslip, holding it at room temperature for 4-6 hours or more to before observing them under a light microscope. In the preparations examined under the microscope, the dark-stained, light and non-stained pollens on slides could be identified as living, semi-living and non-living in the same order (Özer, 2016; Stanley & Linskens, 1974).

In the iodized potassium iodide (IKI) method, 1 g of potassium iodide and 0.5 g of iodine are dissolved in 100 mL of distilled water for the IKI solution. Pollen viability counts are made five minutes after pollen is placed in a solution of IKI. Pollen grains with dark spots (dark red or brown color) are considered alive (Sulusoglu & Cavusoglu, 2014).

In vivo pollination

Pollen and floral biology of the plants to be used in the studies should be known for inducing better and improved conditions for their use in breeding programs. Emasculated flowers are covered with a cloth bag to eliminate the risk of contamination before pollination with irradiated pollen. Emasculation is carried out one day before the separation of anthers. After irradiation, pollen kept at +4℃ is released early the next morning to ensure pollination in field conditions. The female flowers are then isolated again with cloth bags to prevent unwanted pollen contamination.

To promote fruit or seed development after pollination with irradiated pollen, it has been proposed to apply growth regulators to the calyx of pollinated flowers; however, this may induce parthenocarpy rather than haploidy (Sestili & Ficcadenti, 1996). *In vitro* culture is necessary to rescue haploid plants in many cases. Embryos formed after pollination are isolated and regenerated under *in vitro* conditions on dissimilar nutrient media pre-optimized according to the genotypes.

Embryo rescue and chromosome doublin

Gamma-irradiated pollen can germinate on the stigma, grow along the style, and reach the embryo sac. Although it cannot fertilize the egg cell and polar nuclei, it stimulates the development of haploid embryos (Musial & Pzrywara, 1998). This method requires immature embryo rescue under *in vitro* conditions (Blasco et al., 2016).

To preserve the immature embryos formed in the flowers of plants pollinated with irradiated pollen, field controls must be carried out. The application of this method starts with the castration of the donor plant. Pollen is collected from the donor plant and exposed to the appropriate dose of irradiation. Embryo maturation time varies according to species (Shu et al., 2011). This timing occurs 10-15 days after pollination depending on the climate.

During this process, the embryos are removed, placed in the most suitable nutrient medium for regeneration under *in vitro* conditions and allowed to develop. The most important issue in plant development and rooting is determining the ploidy level. Some plants may exhibit spontaneous folding.

Knowing the ploidy level of living plants is very important for the effective use of genetic resources. Ploidy level in a plant greatly affects the performance of the plant (Sakiroglu & Kaya, 2012). Two methods are generally used to determine the ploidy level in plants. The first of these methods is to determine the number of chromosomes with the help of a microscope and the other is the flow cytometry method. However, it is a significant disadvantage that the processes are long and laborious. Moreover, it is known that it is not a very efficient method as it will increase the possibility of error for plants with small chromosomes (Wanner et.al., 1991). Flow cytometry (FCM) has become a convenient and useful tool for determining ploidy levels in plant breeding. It is a device and technique for quick and reliable measurement of cells or other biological particles for physical or chemical properties in a liquid stream. By using fluorescent dyes that bind to the structure of DNA in this technique, usable information is created by transmitting different wavelengths of the device to the system by laser radiation. Propidium iodide, ethidium bromide and acridine orange are fluorescent dyes commonly used in flow cytometry (Bohanec, 2003; Demirel, 1995; Demirel et.al. 2019).

Aktaş (2018) used the irradiated pollen technique in this study and obtained haploid plants with embryo recovery. He reported spontaneously formed six haploids in his study.

In order for haploid plants to attach seeds and continue their generation, chromosome numbers must be multiplied with the help of chemicals such as hexachlorocyclohexane, acenaphthene, chloral hydrate, ethyl mercuricloride, nitrogen perotoxide, colchicine, caffeine, sulfinilamide. After the chromosomes of the plants are folded, they are transferred to the greenhouses for acclimatization.

Conclusion

Developing tolerance to increased stress in plants is crucial for the development of the future agricultural sector and to reduce the risk. As in other plants, a number of physiological, biochemical, and molecular mechanisms are involved in the development of tolerance to various stresses in oil crops.

With the use of effective scientific and analytical approaches in agriculture, the number of faster and more effective breeding studies will increase in the future. Climate change, pollution, changing natural areas, population growth, and intensive agricultural practices increase the importance of agricultural production and biodiversity. The decrease in biodiversity brings along many environmental and economic problems. With the development of biotechnology, efforts to increase our agricultural resources and quality should continue rapidly. Biotechnological approaches that can be used to increase the effectiveness of breeding programs increase the effectiveness of the studies and ensure the growth and sustainability of the agricultural economy.

To meet the increasing population demand, mutation breeding can be considered a good option to modify existing superior varieties.

Gamma irradiation, an irradiation method used in combination with haploid techniques as a tool for plant breeding, offers several opportunities in agricultural and food applications. If irradiation technology is used in conjunction with targeted biotechnological methods, it has a potential to play an important role in the accelerated breeding.

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