Double Haploid Technique: In Soybean and Other Species

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Abstract

Recent work however, has been directed towards development of new plant varieties by using double haploid technology. Research has resulted in successful development of haploid and doubled haploid plants from several crop species. Double haploid technology refers to the use of the microspore or anther culture (ovary culture in few species) to obtain haploid embryos from microspores. The ploidy level could be doubled autonomously or by chemical treatments to obtain plants with 100% homozygosity. This technology is available in some crops including soybean to speed up the breeding procedure. The anther culture technique used in *Glycine max*, (2n = 40) results in haploid plants. The chromosome number could be doubled by chemical treatment i.e. Colchicine treatment results in double haploid plants with 100% homozygosity.

Keywords: Doubled haploid, soybean, anther culture.

Introduction

Oil seeds are important source of edible oil production and are cultivated on a large scale in the world. It plays important role in the country’s economy. However, in Pakistan its production is very low and hence Pakistan is the third largest importer of edible oil in the world.

Soybean (*Glycine max. L.*) is the most important crop among the oil seed crops. Soybean originated in north eastern china and was domesticated about 1100 B.C. (Hymowitz, 1970). The major producing countries of soybean are United States of America, China, Brazil, and Argentina. Soybean seed contain about 40% protein and 20% oil on dry weight basis. Although soybean is a highly self pollinated crop, however, insects may carry the pollen but the frequency of cross pollination is less
Soybean is a member of the genus Glycine and family leguminosea and has a chromosome number of 2n=40 (Hadley and Hymowitz., 1973). The cultivated soybean is erect, bushy and leafy, varies in height (from 30 cm to 1 m). It is an annual crop having different varieties. Every locality has its own indigenous germplasm.

Different breeding methods are used by the soybean breeders to develop soybean varieties. Introduction, selection and hybridization are the principal breeding methods. The conventional breeding of soybean takes several years for variety development. For this purpose, the soybean breeders have turned to the non conventional methods to develop 100% homozygous varieties in shortest possible time.

Double haploid technology is one of the non conventional methods which refer to the use of microspore or anther culture to obtain haploid embryo. Guha and Maheshwari (1964) first reported the direct development of haploid embryo from microspores of *Datura innoxia* by anther culture in vitro.

Anther culture technique has been refined and extended to 200 species belonging to 60 genera and 26 families of Angiosperm. The first pollen derived plants obtained from cultured anthers in China were rice and wheat. The anthers of a floret contain microspores, the male sexual cells. In contrast to vegetative cells, in microspores the number of chromosomes is reduced by half. On specific media microspores within the anther, regenerate into so called haploid plants having single set of unpaired chromosome which are sterile, using colchicines for chromosome doubling i.e. produce fertile homozygous doubled haploids.

Advantages of double haploid plants in plant breeding are:
1. Saving time in producing homozygous plants
2. Easy selection of characteristics i.e. genotype or phenotype
3. Reduced time to develop new variety
4. Use of double haploid as basic germ plasm for breeding new variety.

The objective of this review paper is to collect the relevant literature, on the title of double haploid technique in soybean, and to get the latest information in the discipline of plant breeding and genetics, which will be helpful for understanding of the researcher and onward access of the researcher working in this particular area of the discipline of plant breeding.

**Fundamental Informations**

Haploids may occur spontaneously in nature, or they may be induced artificially. Induced haploids can generally be obtained by the stimulation of the egg, synergids, or sperm by a number of methods, including ionizing irradiation and radioisotopes, thermal shocks, distant hybridization, delayed pollination, application of abortive pollen, spraying with various chemicals.

**Protocol for anther culture**

The immature anthers containing uninucleate pollen at the time of first mitosis are the most suitable material for the induction of androgenesis. The flower buds are
 sterilized with 1% (w/v) solution of sodium hypochlorite, or 5% (v/v) solution of a commercially available disinfectant such as Clorox. They are washed a couple of times with sterile distilled water, and the anthers are dissected out and cultured on agar solidified medium. The flower buds obtained from plants grown in greenhouses. An excision is made on one side of the flower bud, the stamens are gently squeezed out and collected in a sterile Petri dish.

Basal medium of MS with various additives have been employed. The usual level of sucrose is 2-4%; however, higher concentrations (8-12%) favor androgenesis in cereals. The cultures are incubated at 24-28 °C in a 14-hr daylight regime at about 2000 lux.

The anthers normally start to undergo pollen embryogenesis within 2 weeks, and either directly develop into haploid plants in about 6 weeks (in tobacco) and are capable of transferring to the soil, or undergo proliferation to form callus which can be induced to differentiate plants.

**Induction of androgenesis**

Haploid plantlets are formed in two distinct ways, by direct androgenesis or by organogenesis from haploid callus tissue.

The anthers containing uninucleate pollen cultured on basal medium turn brownish within 2 weeks without any visible signs of growth. After 3-4 weeks in culture, small white protuberances, the embryos, appear which eventually develop into plantlets.

Microscopic observations reveal that within the excised anthers the microspores exhibit various modes of development. The microspore nucleus either undergoes a normal mitosis and forms a vegetative and a generative nucleus or divides to form two “similar looking” nuclei. In some pollen the vegetative nucleus, while in others the two “similar looking” nuclei, undergo further repeated divisions to form multinucleate pollen. The generative nucleus remains quiescent or divides a couple of times and aborts. As a result of repeated division pollen with up to 30 nuclei may be formed with no walls separating the nuclei. Such pollen do not take part in androgenesis and generally abort.

At the early stages of development it is not possible to differentiate between the multinucleate type of development and that which leads to the formation of callus. However, after about 2 weeks, pollen are observed to be larger than the multinucleate and the embryonal type and contain only a few cells. These cells increase in size, exerting pressure on the exine which bursts open and the contents are released in the form of callus. At the same time, in some cases, shoots differentiate from the callus. The plantlets originating from the callus are generally undesirable, as they exhibit various levels of ploidy.

**Culture of Isolated Pollen**

A hanging drop method were used to culture isolated pollen of *Brassica oleracea* and *Brassica oleracea* x *Brassica alboglabra*. The method involved placing a drop of medium containing 50-80 grains on the cover glass, which is then inverted over a
cavity slide and sealed with paraffin. Before inverting the cover glass, a column of paraffin is raised in the center, so that when inverted, it touches the bottom of the cavity slide. This facilitates the aeration as well as the movement of the pollen when the slide is rotted. Cell clusters were formed from isolated pollen after 4 weeks in a medium containing coconut water. Making use of the observations that thermal shocks given to plants can change the mode of division of the pollen nuclei. It was reported that trauma given to pollen at the time of the first mitosis considerably enhanced the number of pollen cells undergoing androgenesis.

Partial success along the same lines was also reported in tomato. Later a fully synthetic medium was substituted for the anther extract and obtained plantlets from *Nicotiana tabacum* cv. Red Flowered and Coulo. This work was then extended to another commercially important tobacco cultivar.

The isolated pollen can be cultured by the methods which follow:

**Nurse culture technique**
The isolated pollen of *Lycopersicon esculentum* was induced to form haploid callus. In this method the anthers are placed horizontally on top of the basal medium within a French square container. A filter paper disk is placed over the intact anther, and about 10 pollen grains (in suspension) are then placed on the filter paper disk.

The controls are prepared in exactly the same way, except that the pollen are placed on a filter paper disk kept directly on the agar. With this method the control did not grow at all, while the pollen kept on the nurse cultures had a planting efficiency of up to 60%, and clusters of green parenchymatous cells were formed on the filter paper disk in 2 weeks. These clones were observed to be haploid. Androgenesis was successfully induced in *Nicotiana tabacum* microspores using petunia callus as a nurse tissue.

**Preculture Method**
The technique for the culture of isolated pollen involves the following steps. The anthers are aseptically removed and cultured after 4 days of incubation at 27 °C, the pollen are squeezed out of the anther into the liquid medium. One liter of the medium contains KNO₃ (8.9 mM), NH₄NO₃ (8.9 mM), MgSO₄ 7H₂O (0.75 mM), CaCl₂ (1.42 mM), KH₂PO₄ (0.50 mM), FeEDTA (100 mM), glutamine (5.5 mM), serine (0.95 mM), Myoinositol (0.03 M), Zeatin (0.046 mM), indoleacetic acid (0.57 uM), and sucrose (0.06-0.23 M). After filtration the pollen are centrifuged to form a pellet. The pollen are washed twice, centrifuged again, and suspended in fresh medium with a density of about 5 x 10⁴ pollen per ml. Aliquots of 2 ml are then dispensed in thin layers in small Petri dishes or 25 ml Erlenmeyer flasks. However, if the material is available in microquantities, it can be grown in drop cultures. To prepare such cultures, a drop of silicon is placed in the center of a small (5 cm) sterile plastic Petri dish, and a cover slip (22 x 22 mm) is gently lowered onto the drop. Then a drop of 250-500 μl of the pollen suspension is pipetted onto the cover slip. To prevent dessication, the Petri dishes are sealed with “Parafilm M” and the cultures incubated for the first 4-6 days under low light (500 lux), and then maintained in 14-hr daylight regime of 2000 lux at 28 °C. The induced pollen undergo normal androgenesis and
eventually produce haploid plants. Recently these techniques have been considerably modified and refined to obtain high percentages of pollen embryogenesis. For instance, rye pollen suspension may be purified to separate the viable microspores. Likewise, androgenesis can be induced in pollen cultures of tobacco. The serial culture of anthers in the liquid medium enables the embryogenic pollen to discharge in the medium in good quantities. Such pollen can then be filtered and cultured.

Following are some of the important points which have emerged from the work on the culture of isolated pollen of *Nicotiana* and *Datura*.

Growth and morphogenesis are adversely affected if the isolated pollen are not washed before culturing. Unwashed pollen either fail to grow or have a tendency toward callus formation.

Cold treatment has been used to increase the frequency of androgenesis. Close examination reveals that even in cold-treated pollen, normal mitosis usually takes place to form a vegetative and a generative nucleus. The cold treatment does not induce androgenesis, but it enhances the viability of cultured pollen, and causes repression of the gametophytic differentiation which results in higher frequency of androgenesis.

Androgenesis in isolated tobacco pollen can be induced at various stages of development (ranging from uninucleate microspore, during and after first mitosis to late binucleate), but frequency of success is most frequently achieved using anthers taken at late uninucleate to binucleate pollen stage.

The frequency of androgenesis can be enhanced considerably by raising the sucrose and myo-inositol concentrations in the medium.

**Homozygous plants**

Homozygous plants can be obtained in a relatively short time by the production of haploids and by doubling their chromosomes. This technique has been successfully used in China where several varieties of wheat and rice have been developed. The duplication of the chromosomes can be achieved by a number of methods.

**Endomitosis**

Haploid cells are, in general unstable in culture and have a tendency to undergo endomitosis to form diploid cells. This property of cell cultures has been exploited for obtaining homozygous plants. A small segment of stem from a haploid plant is grown on an auxin cytokinin medium to induce callus formation. During callus growth and differentiation there is a doubling of the chromosomes by endomitosis to form diploid homozygous cells and ultimately plants.

**Colchicine Treatment**

Colchicine has been used extensively as a spindle inhibitor to induce chromosome duplication and to produce polyplloid plants. This has been employed for obtaining homozygous diploid plants from haploid cultures. While still enclosed by the anther, the young plantlets are treated with 0.5% colchicine solution for 24-48h washed thoroughly, and replanted. The fertile homozygous plants thus obtained can be used for producing inbred lines used to produce hybrids.
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References


656

Saleem Khan et al