Cilt 12, Sayı 2, Sayfa 145-150, 2023 Volume 12, Issue 2, Page 145-150, 2023
Derleme https://doi.org/10.46810/tdfd.1132699 Review
BİNGÖL ÜNİVERSİTESİ
Türk Doğa ve Fen Dergisi
Turkish Journal of Nature and Science
www.dergipark.gov.tr/tdfd

A New Method for Obtaining Haploid Plant Shed-Microspore Culture

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(Received: 22.06.2022, Accepted: 12.06.2023, Online Publication: 22.06.2023)

Keywords
Pepper, Shed-microspore Culture, Androgenesis, Dihaploidisation

Abstract: In plant breeding studies, dihaploidisation method is used to obtain homozygous pure lines used to develop varieties in a shorter time. Dihaploidisation method is applied in most types of vegetables such as wheat and corn, as well as in vegetable species. Androgenesis methods (anther and microspore culture) and gynogenesis and parthenogenesis methods (ovula and ovary culture) are used to obtain haploid plants. One of the androgenetic methods, shed-microspore culture has been a new alternative technique to anther and microspore cultures, and successful results have been obtained. The most important feature of this technique is the presence of a double-layered nutrient medium in a single petri dish, and the culture of microspores in the anthers in solid and liquid nutrient mediums. In this study, the definition and application of microspore culture and shed-microspore cultures, studies conducted in the world and in our country and their developments in recent years were discussed.

Haploid Bitki Elde Etmede Yeni Bir Yöntem Shed-Mikrospor Kültürü

Anahtar Kelimeler
Biber, Shed-Mikrospor Kültürü, Androgenesis, Dihaploidizasyon

Öz: Bitki ıslah çalışmalarında çeşit geliştirmek için kullanılan homozigot saf hatların daha kısa zamanda elde etmede dihaploidizasyon yönteminden yararlanılmaktadır. Dihaploidizasyon yöntemiyle sebze türlerinde uygulandığı gibi buğday, mısır gibi çoğu türde de uygulanmaktadır. Haploid bitki eldesi için androgenesis yöntemleri (anter ve mikrospor kültürü) ile ginogenesis ve partenogenesis yöntemleri kullanılmaktadır. Androgenetik yöntemlerden shed-mikrospor kültürü ise anter ve mikrospor kültürlerine alternatif yeni bir teknik olmuş başarılı sonuçlar elde edilmiştir. Bu teknik en önemli özelliği tek bir petride çift katlı besin ortamının olması, anterlerın içindeki mikrosporların katı ve sıvı besin ortamına kültüre alınmasıdır. Bu çalışmada mikrospor kültürü ve shed-mikrospor kültürlerinin tanımı, uygulanışı ile dünyada ve ülkemizde yapılan çalışmalar ile son yıllardaki gelişmeleri incelenmiştir.

1. INTRODUCTION

With the increase in the world population, the demand for food has increased due to the rapid development of urbanization, the decrease in agricultural lands and the changes in climatic conditions. They are among the leading important vegetables in our country and world production, especially in tomato, pepper, eggplant and potato species from the *Solanaceae* family. Increasing food production needs to be done in a reliable and healthy way. This is not sufficient with traditional methods. Plant biotechnology offers alternative solutions to many problems that are difficult or unsolvable with classical breeding methods; It offers easier, faster, economical, efficient and quality methods for plant breeding.

All of the techniques that enable the plant's organs, tissues and cells to be isolated and cultured using artificial food sources in sterile conditions and to improve their genetic characteristics are all within plant biotechnology. Today,

rapid and technological developments in the fields of plant biochemistry, plant physiology and molecular biology help researchers to prevent some problems in plant breeding. Accordingly, with the development of new techniques and protocols, there are important developments in the field of breeding and development of the plants grown. Since researchers shorten the concept of time in breeding, the haploid method has an important place in its use in the field of vegetable breeding. Searchers shorten the concept of time in breeding, the haploid method has an important place in its use in the field of vegetable breeding. Anther culture and microspore culture, which are more preferred in obtaining haploid plants, are among the methods that have become common.

While much faster and shorter-term studies are needed in breeding studies due to reasons such as the increase in population, rapid development of urbanization and changes in climatic conditions with the decrease of agricultural lands, androgenesis methods (anther and microspore culture) and gynogenesis and parthenogenesis methods to obtain haploid plants. culture) is applied [1-8]. From methods of obtaining haploid plants to microspore culture and anther culture shed-microspore culture, which is an alternative new method, is different from anther and microspore culture. It is a method of culturing in a suitable liquid nutrient medium prepared as a whole, by removing the filaments of the appropriate anthers and without damaging the anthers. In microspore culture, embryogenesis is the method of creating embryos from gametophytic development by using anther culture or microspore culture methods of immature male gametophytes during in vitro culture. In microspore culture, haploid plants can be obtained directly from embryos obtained from microspores or by organogenesis from calli obtained from microspores [22]. Haploid embryos can be transformed into homozygous DH plants after being treated either by themselves or with chromosome folding agents and can be used in breeding programs. Haploid embryos can be transformed into homozygous double haploid plants either spontaneously or after treatment with chromosome folding agents and breeding used in their work.

The first study in microspore culture was performed by Kameya and Hinata in 1970 [9]. Researchers have done many studies on microspore culture [9-27] and have achieved successful results. It is reported that one of the most important factors affecting the success in microspore culture is the isolation of microspores. For isolation, buds or anthers are left in different environments according to the characteristics of the species, and it has been reported that the anthers are crushed in these environments, allowing microspores to be released [28-35]. In recent studies, the shed-microspore culture, which is a new method and continues to be developed, is being studied on hot pepper, and its studies are continuing in the sweet pepper group. It is thought that this technique will be further developed and made widespread, and it will shed light on the field of plant breeding and plant breeders. Shed-microspore culture, one of the androgenetic methods, is a new alternative technique to anther and microspore cultures and successful results have been obtained. It was first applied and successful in Indonesia's local hot pepper. This method is called the shed microspore method. In the method, microspores in the anthers are cultured in solid and liquid nutrient medium in a double-layer nutrient medium.

The most important difference of the Shed-Microspore culture method is that it is completely separated from the filaments of the anthers isolated from the flower buds, by crushing or separating them, without isolating the microspores inside and without damaging the anthers, and they are cultured in a double-layered nutrient medium. Microspores within the anthers are cultured freely floating into the liquid layer of the bilayer nutrient medium. The microspores that develop inside the anthers benefit from the nutrient medium and are allowed to develop freely in the liquid medium. In the Shed-Microspore culture technique, embryoids and callus tissues do not form on the cultured anthers as in anther culture. All of the embryos are embryos consisting of haploid microspores by completing their formation and development in the liquid medium. Observing and examining embryos in liquid medium is much more functional than other methods.

When the shed-microspore culture is compared with other (anther and microspore culture) methods [36]; due to the double-layered nutrient medium, additional substances can be added to the nutrient medium in the next steps, and the liquid layer can be renewed when necessary. If a large number of embryos are formed in a single petri dish, the liquid nutrient medium is renewed without any intervention to the embryos, and their development continues. With the liquid layer, antibiotics can be added to the nutrient medium quickly and effectively, which will protect them from possible infections. Since the anthers are isolated as a whole and microspores develop easily during the developmental stage, the highest rate of embryo is formed. The researchers reported that the haploid plant percentage was higher when colchicine applications to plantlets obtained from the Shed-microspore culture technique had better results compared to other methods. The determination of ploidy levels of the plantlets obtained by this method is facilitated by flow cytometry and chloroplast counting technique.

In this study, the definition and application of microspore and shed-microspore cultures, their embryo production potential, studies conducted in the world and in our country and their recent developments were examined.

1.1. Microspore Culture Studies

Obtaining the first haploid plant by microspore culture method it was reported that Nitsch succeeded in 1974 and the first microspore culture studies were applied in 1989 by Pesticelli et al. in tobacco and corn plants [5]. One of the most important factors affecting androgenesis is the developmental stage of microspores, and researchers report that unless microspores at the appropriate stage are cultured, there will be no development in pollen embryogenesis even if other culture conditions are met

[37]. It is known that there are many factors affecting the number of androgenic microspores and the number of embryos obtained per microspore. Some of the factors that will affect the success of microspore culture are genetic and depend on the genotype of the donor plants from which the microspores are taken. Another part is the microspore culture technique. It is related to the environmental conditions during its implementation [38]. Yin and his colleaguees reported that conducted a study of microspores isolated from pepper anther have developed a system for rapid progression by initiating embryoids in culture medium [39].

As a result of the study, it was determined that low temperature pretreatment, combinations of plant growth regulators, activated carbon concentrations and temperature pretreatments were the most important factors affecting the embryoid formation of pepper microspores isolated in vitro. In another study, it was reported that it was successful in increasing embryo formation with temperature applications [40]. Studies such as different nutrient media and pre-applications have been carried out to obtain haploid plants by anther culture method in local peppers originating from Turkey and successful results have been reported [41]. Examined all the factors affecting the androgenesis developmental stage in the culture medium of microspores isolated from Hungarian and Spanish local pepper genotypes [42]. As a result of the study, it was reported that the anthers with 80% mononuclear and 20% double-core microspores were successful and high yield was obtained. They stated the high frequency of embryo production and plant regeneration in the culture of microspores isolated from pepper [43].

1.2. Shed-Microspor Culture Studies

In Indonesia, six hot and four rolled local pepper genotypes and two bell pepper genotypes for comparison purposes, anthers at single seed stage were transferred to the nutrient media. The researcher, who opened the anthers one to two weeks after the medium and dispersed the microspores on the nutrient medium, named this method as the "Shed microspore" technique. It has been reported that all hot and curly pepper genotypes used were successful, and curly peppers were less successful than other hot peppers [44].

Aimed to create a new method and protocol to increase the DH (double haploid) rate by applying different additions by using anther and microspore culture methods in the local hot pepper genotypes of Indonesia [36]. In the experiment, anthers containing 50% single-core microspores were used. As preliminary applications before media transfer; After keeping the flower buds at 4°C for 24 hours, they were incubated at 9°C for a week and grown at 28°C in complete darkness. Unlike the nutrient media applied in the microspore and shed microspore culture method, NN media with solid first layer and liquid second layer were applied. In the study, it was reported that 0-2% activated charcoal added to the solid medium increased the number of embryos and decreased the number of embryos by 2%. It has been reported that one hundred eight plants, one hundred four haploids, sixty-one diploids, two triploids and one tetraploid were obtained from 6 hot pepper genotypes.

The same researchers [45] used the material they used in their anther and microspore culture studies in another study they conducted. Success rates were investigated by using different antibiotic combinations with the shed microspore culture method in order to prevent infections assumed to originate from these donor plants, which used Indonesian local hot pepper genotypes, and to increase the phytotoxic efficiency of different antibiotics. In the study, it was reported that chloroplast count and ploidy levels were determined exactly and accurately in flow-cytometry and leaf stomata of plantlets obtained by applying shed microspore culture method and two-layer solid and liquid media. In addition, as a result of four different antibiotic combinations, it has been reported that the most appropriate antibiotic component against infections originating from donor plants is 20 mg/l Rifampicin + 100 mg/l Timentin. The following picture shows the stages of the shed microspore (Fig1).

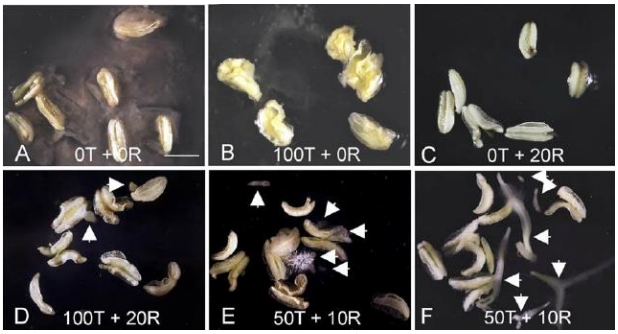

Figure. 1. Shed-microspore culture of Indonesian hot pepper (C. annuum L.) 'Gada' after single and combination treatments of antibiotics. (A) Control without antibiotic treatment (0T+0R) showing contaminated culture; (B) culture treated with 100 mg/l timentin (100T+0R) showing contamination; (C) culture treated with 20 mg/l rifampicin (0T + 20R) showing no contamination, but without undergoing embryogenesis; (D) culture treated with 100 mg/l timentin + 20 mg/l rifampicin (100 + 20R) showing no contamination, where some embryos can be seen (arrows); (E and F) culture treated with 50 mg/l timentin + 10 mg/l rifampicin (50T + 10R) showing no contamination, where more embryos can be seen (arrows). The culture was with 0.5% activated charcoal in solid medium. Donor-plants for the anthers were grown in Indonesia. (A–E) After 5 weeks of culture; (F) after 6 weeks of culture. Bar = 4 mm.[36,45].

Aimed to achieve success by using the shed microspore culture method in order to increase the embryo number and embryo quality in hot pepper (*Capsicum annuum* L.) genotypes [46,47]. The protocol applied in Shed microspore culture; 1% activated charcoal was kept at 4°C for 1 day and exposed to 9°C for 1 week, then it was subjected to growth conditions at 4 different temperatures by applying 5 different growth nutrients in addition to 2.5 µM zeatin and 5 µM IAA to 28°C completely dark and liquid environments. As a result of the study, the number of embryos and quality of the petri dishes to which 50 and 100 µM were added in the 3rd week in liquid medium decreased, but in the petri dishes to which 2.5 µM zeatin + 5 µM IAA was added, 35.3 in 6 anthers, 24.7 in the medium with growth regulator, 27.3 in which 2.5 µM zeatin was added. There were 18.4 normal embryos with 5 µM.Within the scope of the study, in 6 anthers, 20.6

embryos were formed at 28℃, 20.2 at 25℃, 19.5 at 23℃, 17.1 at 21℃, and 7.5 at 18℃. According to the obtained data, it has been reported that the medium with 2.5 µM zeatin + 5 µM IAA addition and the environment with 28°C temperature are more successful than the other applied conditions for normal embryo formation.

In a study by [48], it was aimed to determine the response of commercial varieties of 3 different pepper genotypes by applying the shed microspore method. In the experiment, anther sizes, anthocyanin levels and developmental stages of microspores in anthers, embryo formation and plant transformation rate, embryo formation potential of liquid nutrient medium in shed microsporda method were investigated. [49], anther and shed microspore culture techniques were applied to 2 commercial pepper spikes (Lumbard RZ F1) and Cubanelle (Üçbuun F1) cultivars to observe haploid and dihaploid plant production and anther development stages. Anther culture was made in MS [50] medium, after collecting the pepper buds, which came to the stage of ingestion in the shed microspore technique, after 24 hours of pre-treatment at +4℃, the anthers were transferred to NN medium and covered with a liquid layer by adding +2% maltose to NN medium. The pH of the nutrient medium was 5.7-5.8 as semi-solid - semi-liquid. The findings obtained in the research; In anther culture, the rate of embryo development from Lumbard RZ F1 variety is 8.94% and the rate of embryos turning into plants is 23.63%, while the rate of embryo formation in Üçburun F1 is 22.18% and the rate of embryos turning into plants is 22.22%, while the rate of embryos turning into plants is 22.22% in shed microspore method. It has been reported that embryo development was detected only in Lumbard RZ F1 variety. In the study conducted by [51], the effect of the use of agitator on embryo yield in 29 ornamental pepper genotypes (commercial and local) shed microspore method was examined, while the bud morphologies and bud sizes of the genotypes at the culture stage of microspores were also examined. Before removing the anthers of the flower buds, pre-chilling was done at +4 ℃ and 24 hours in the dark, and the anthers shed microspore (semi-solid-semi-liquid medium) method was applied. It was determined that the success rate of the medium on the shaker was higher in terms of embryo formation, and the embryo formation performance was more successful in the medium on the shaker than the control group.

In this study, it was aimed to determine the morphological and molecular characterization of Gaziantep local pepper (GB) genotypes and the possibilities of obtaining double haploid (DH) lines by anther culture and shedmicrospore culture method. In the study, 96 pepper genotypes, including 81 different GB genotypes and 15 standard varieties, collected from the region were used. For the first time, the shed microspore method was applied to local pepper genotypes. As a result of the study, in shed microspore culture in GB local pepper genotype; While the total number of anthers was 920 and the number of developing anthers was 140 in total, the highest embryo formation rate; ıt has been reported that 5% was achieved [52].

2. CONCLUSION

In this study, the effectiveness of dihaploidization methods in obtaining homozygous pure lines used to develop varieties in breeding studies in a shorter time and the results obtained are summarized. Anther and microspore cultures, which provide haploid plant production among tissue culture techniques, have an important place in plant breeding studies. However, shed microspore, which is one of the new methods in tissue culture, is reported to be successful in literature studies. Studying new methods will increase the success rate in plant species that are difficult to study in breeding. The use of these new methods is limited in economically important plants, especially in our country, and there are few studies. However, it is very few and insufficient and it is very important to increase the studies on this subject. Studies with shed-microspore culture, which is an alternative method in anther and microspore culture methods, have shown in studies that the success rate has increased and it has become increasingly widespread in the world recently. At the same time, it has been shown in literature studies that it will be beneficial to many subjects that have not been studied yet in tissue culture. For this reason, it is necessary to carry out studies in this direction and to develop protocols for future studies or to create new protocols. Thus, when shed-microspore culture, which is an efficient and promising technique, is applied, an important gap in breeding studies will be filled. The improvement of this method will be able to offer more successful solutions in breeding studies.

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