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Review/Derleme

HISTORY OF *in vitro* CULTURE STUDIES ON *Helianthus annuus* L. IN TURKEY

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Abstract: Tissue culture techniques offer important approaches about sunflower breeding and germplasm conservation. The available data on the subject in Turkey was reviewed in order to encourage the researchers to study on tissue culture of sunflower. *In vitro* studies on sunflower in Turkey started in the first half of the 90s. A large number of *in vitro* culture studies on sunflower using anthers, hypocotyls, cotyledons, petioles of cotyledon, shoot-tips, mature embryos, immature embryos, leaves, petioles, immature cotyledons and microspores as different explants have been published. Microspore culture, anther culture, embryo culture, slow growth storage, micropropagation and gene transfer with *Agrobacterium* were also used in these culture studies. Although these studies formed an important scientific knowledge about sunflower tissue culture in the country, it is still not sufficient. Therefore, there is an urgent need to make more *in vitro* studies on sunflower which is an important agricultural plant for Turkey. The transfer of the results of these studies to agricultural applications is also essential from a sectoral standpoint.

Key words: Tissue culture, sunflower, breeding, germplasm conservation.

Türkiye'de *Helianthus annuus* L. Üzerine Yapılan *in vitro* Kültür Çalışmalarının Tarihi

Özet: Doku kültürü teknikleri, ayçiçeği ıslağı ve germplasm koruması konusunda önemli yaklaşımlar sunmaktadır. Bu derleme, ülkemizde bu alandaki bilimsel birikimi göz önüne sermek ve araştırmacıları ayçiçeği doku kültürü üzerine çalışmaya teşvik etmek için yazılmıştır. Türkiye'deki *in vitro* ayçiçeği çalışmaları 90'ların ilk yarısında başlamıştır. Günümüzde anter, hipokotil, kotiledon, kotiledon petiyolü, sürgün ucu, olgun ve olgunlaşmamış embriyo, yaprak, petiyol, olgunlaşmamış kotiledon, ve mikrospor gibi farklı eksplantların kullanıldığı birçok ayçiçeği *in vitro* kültür çalışması yayınlanmıştır. Buna ek olarak, Türkiye'de yapılmış bu çalışmalarda mikrospor kültürü, anter kültürü, embriyo kültürü, büyümenin yavaşlatılması ile depolama, mikroçoğaltım ve *Agrobacterium* ile gen transferi gibi teknikler kullanılmıştır. Bu araştırmalar Türkiye'deki ayçiçeği üzerine yapılan doku kültürü çalışmaları ile ilgili önemli bir bilgi birikimi oluştursa da henüz istenilen yeterlilikte değildir. Bundan dolayı acil olarak Türkiye için önemli bir tarımsal bitki olan ayçiçeği üzerinde daha fazla *in vitro* araştırma yapmaya gerek duyulmaktadır. Ayrıca bu çalışmalardan elde edilen bilgilerin tarımsal uygulamalara aktarılması sektörel açıdan gereklidir.

Anahtar kelimeler: Doku kültürü, ayçiçeği, ıslah, germplasm koruması

Introduction

Sunflower is an important agricultural plant as a source of vegetable oil and is the second most planted crop after soybean in world vegetable oil production (Weber *et al.* 2003). Sunflower oil is often preferred for vegetable oil consumption in Turkey. Classical breeding techniques as population breeding and selection, hybridization, backcross breeding, inbreeding, mutation breeding, genus and species hybrids, polyploidy breeding are being used in sunflower agriculture (Demir & Turgut 1999). However, due to the use of the same gene source, the varieties obtained using traditional breeding methods are about to reach their upper limits of capacity of genetic productivity. Therefore, the establishment of tissue culture systems has a great importance for genetic manipulation of sunflower in order to obtain agronomically important varieties (Nestares *et al.* 2002).

Tissue culture techniques offer several advantages. For example, protoplast culture is an advantageous technique in terms of gene transfer and interspecific hybridization (Kaya 2004). Double-haploid plants obtained from haploid tissues (i.e. anther, microspore and ovary) are useful for obtaining pure lines. The duration of a breeding process which lasts for 7-8 years using conventional techniques can be reduced by biotechnological methods.

A large number of studies have been published during the last few decades about sunflower regeneration protocols using different explants types such as shoot tips, embryonic axes (Paterson 1984, Malone-Schoneberg *et al.* 1994, Elavazhagan *et al.* 2009), leaves (Greco *et al.* 1984, Lupi *et al.* 1987, Paterson 1984, Inoka & Dahanayake 2015), immature embryos (Finer 1987, Prado

& Berville 1990, Jeannin *et al.* 1995, Lucas *et al.* 2000, Dağüstü *et al.* 2010), hypocotyls (Lupi *et al.* 1987, Mohmand & Quraishi 1994, Müller *et al.* 2001, Sujatha *et al.* 2012), protoplasts (Guilley & Hahne 1989, Fischer *et al.* 1992, Henn *et al.* 1998), mature cotyledons (Greco *et al.* 1984, Brar & Roberts 2006, Sujatha *et al.* 2012), anthers, ovaries (Badea *et al.* 1989, Mohmand & Quraishi 1994, Thengane *et al.* 1994) roots and stems (Inoka & Dahanayake 2015). More detailed information on *in vitro* sunflower studies can be found within the review studies of Moghaddasi (2011) and Davey & Jan (2010).

Despite these developments in tissue culture of sunflowers, *in vitro* sunflower studies in Turkey are still not at the desired level when compared with similar studies throughout the world. The available data on the subject in Turkey was reviewed in order to encourage the researchers to study on tissue culture of sunflower.

History of Tissue Culture Studies on Sunflower in Turkey

The first sunflower tissue culture studies carried out in Turkey date back to early 90s. In one of these studies, Emiroğlu *et al.* (1993) examined the effects of culture medium components and genotype on the shoot and embryoid formation from anther. They used 29 different sunflower genotypes including hybrids and commercial varieties and about 21000 anthers were isolated and cultured on different nutrient media. Although meristemoid structures on callus masses, direct shoot formation or shoot like macroscopic structures and embryoids were observed, the researchers reported that none of these structures developed into plantlets and that subcultures failed.

In a subsequent study, cotyledon and hypocotyl explants of 5 sunflower genotypes were cultured on 7 different culture media one of which was MS (Gürel 1994). Gürel (1994) reported that callusing was 100% on MS medium without hormones and MS medium including 4mg/l kinetin + 2mg/l NAA (Naphthalene acetic acid). In this study, 30mg/l maltose was used instead of sucrose.

In the second half of the 90s, Gürel & Kazan (1998) studied different regeneration protocols using cotyledon, petiole of cotyledon, hypocotyl and shoot-tip as explants and plant growth regulator combinations to develop a plant regeneration system from *H. annuus*. The results showed, considering the shooting capability of these four different explant types, that shoot-tip explants were the most responsive. Gürel & Kazan (1998) also reported that genotypic variation was the most critical factor for shooting from shoot-tips.

Gürel & Kazan (1999) performed another study on gene transfer to sunflower genotypes via *Agrobacterium tumefaciens* Smith & Townsend, 1907. They examined several factors and observed that genotypes had significant variations in their transformation efficiencies (from 0.0 to 82.7% GUS positive), and hybrids were more responsive to *A. tumefaciens* infection than inbred lines. They also suggested that particle bombardment of explants before the inoculations with *A. tumefaciens* did not affect the transformation positively.

Gene transfer to the sunflower is a very important point for breeding new lines and/or varieties and for a successful transfer procedure, establishment of tissue culture and regeneration systems are necessary. Considering this necessity, Özyiğit *et al.* (2002) performed a study to establish tissue culture and regeneration systems of five commercially important sunflower genotypes named as Trakya 259, Trakya 80, Trakya 129, Trakya 2098 and Viniimk 8931. They cultured hypocotyl and cotyledon explants on MS medium including various plant hormones and reported that the maximum shoot regeneration rate was 40% (hypocotyl explants of Trakya 259 cultured MS medium including 1mg/1 BAP + 0.5mg/l NAA). On the other hand, the shoot regeneration rate of cotyledon explants was lower in comparison with hypocotyl explants. Additionally, Trakya 259 and Trakya 80 genotypes were not responsive on the same medium with cotyledon explants. Obtaining whole plant is critical point for regeneration and micropropagation systems. Özyiğit *et al.* (2002) achieved rooting of all regenerated shoots on MS medium including 1mg/1 IBA and they suggested that their results could be used for improvement of gene transfer protocols to these sunflower genotypes which are commercially important.

Arda (2004) studied on regeneration protocols using cotyledon and hypocotyl explants of 15 sunflower hybrids and one native sunflower variety as the control group and found that regeneration and callus formation differed between the native and hybrid varieties.

In addition to the explants listed above, mature embryos of different sunflower genotypes (Trakya 80, Trakya 129, Trakya 259, Trakya 2098, and Viniimk 8931) were also used (see Özyiğit *et al.* 2006). Özyiğit *et al.* (2006), reported that callus development and efficient shoot and root organogenesis are obtained from five different sunflower varieties. The Trakya 259 genotype showed the best shoot regeneration (44%) and all regenerated shoots were rooted on MS medium including 1mg/l IBA (Indole-3-butyric acid) and on MS medium without any hormones. The researchers suggested that mature embryos could be an alternative source for indirect plant regeneration and gene transfer systems for different sunflower genotypes. In another study, cotyledon and immature embryo explant of ten different lines of sunflower were cultured on MS medium supplemented with different concentrations of Kinetin, NAA and 20mg/l sucrose (Binboğa Meral 2007a). The results of this study showed that shoot induction was observed on both explant types cultured on MS medium including any concentration of kinetin and NAA. Binboğa Meral (2007b) studied on *H. annuus* for tumor formation using *A. tumefaciens*. Immature cotyledons, leaves and petioles from one week old *in vitro* plantlets of sunflower were treated with oncogenic A281 strain of *A. tumefaciens*. The

results suggested that induction of tumors started on all explant types within 6-7 days of culture period and after 4 weeks of culture and tumor formation was 100% on all explants types.

Özyiğit *et al.* (2007) studied on the effects of genotype, plant growth regulator and culture requirement for callusing and indirect plant regeneration from sunflower. They reported that callus tissues were obtained from hypocotyls and cotyledons of 5 different sunflower genotypes. Seed germination occurred on hormone free MS medium and various percentage of callus inductions were observed on hypocotyl and cotyledon explants which were cultured on MS medium containing 1mg/l 2,4-D (2,4-Dichlorophenoxyacetic acid). Some genotypes showed high regeneration response while others showed lower on the same media with hypocotyl and cotyledon derived calli. In conclusion, Özyiğit *et al.* (2007) suggested that genotype effected callusing and regeneration in sunflower tissue culture.

Seed maturation of sunflower lasts 120-150 days (it takes 50-60% of the life cycle) therefore, to shorten the seed to seed cycle is very important for reducing the breeding cycle of sunflower. For this reason, Dağüstü *et al.* (2010) studied with immature embryo explants of fifteen genotypes (five restorers, five cytoplasmic male steriles and five maintainers). In their procedure, they dissected immature embryos from seeds of field-grown plants at the 10^{th} day of pollination and cultured them on MS media allowing shoot and root development for 5 to 10 days. Then the plantlets were acclimatized and allowed to growth. These *in vitro* derived plants were self-pollinated and set to seed. By doing so, the researchers obtained the first cycle of immature embryo-derived plants. At the end, the third cycle was also obtained by the same method in the growth chamber. The results showed that most of the cultured embryos developed into healthy shoots with 3-6 leaves and 70% of the developed shoots had vigorous roots. When the researchers acclimatized the plants, only 67.3% of them were maturated and set seeds. Finally, Dağüstü *et al.* (2010) reported that they obtained approximately 40-50 mature regenerants per hundred immature zygotic embryos.

As described above, double-haploid plants obtained from haploid tissues are very important for obtaining plants which is homozygous for all characters of nuclear genome. Therefore, Dayan (2011) studied the effects of genotype, light, NAA and BA on the induction of anther androgenesis in two sunflowers cultivars and found that androgentic responses of sunflower's anthers were guided based on the effects of these different parameters.

Conservation of plant breeding lines is as much important as breeding them. Arda *et al.* (2012) studied on *in vitro* sunflower plants preserved by slow growth storage. They stored the mature embryo derived plants at low temperature and determined the anatomical and karyological changes. In the anatomical evaluation, structures such as epidermis, cortex parenchyma, parenchyma and vascular bundles of experimental group plants showed some major differences compared to the control group plants. All of these differences disappeared at normal culture conditions and plants showed healthy development. In conclusion, they recommended that slow growth storage of sunflower is a practicable method for germplasm conservation of sunflower.

Dağüstü *et al.* (2012) studied on immature embryo culture of sunflower to decrease the generation period in breeding programs. They obtained the four cycles of immature embryo-raised plants using the technique of immature embryo culture. The regenerated plants showed no morphological changes in their study. They suggested that all the agronomic characters examined on *in vitro* regenerated plants decreased compared to field grown plants.

Kılıç *et al.* (2016) used the Comet assay method to understand the genotoxic effects of plant growth regulators and some other parameters on sunflower callus tissues. Anthers with uninucleate-microspores obtained from capitula of Hybrid *H. annuus* genotypes which were grown at field were used for culture. Cultured anthers were pretreated with cold (24, 48 or 72 hours) at 4°C and heat (0, 2, 4, 8 or 12 days) at 35ºC in the dark. Different plant growth regulators (0.5mg/l IAA+0.5mg/l BAP, 0.5mg/l NAA+0.5mg/l BAP, 0.5mg/l 2,4-D+0.5mg/l BAP) were added to media. Obtained calli from all experiments were used as a material to determine the DNA damage levels by the Comet assay. Researchers reported high frequency callus induction (95%) at 35ºC for 2 days on the MS medium including 0.5mg/l 2,4-D and 0.5mg/l BAP. Additionally, they evaluated different levels of DNA damage in examples of callus nuclei.

Another study on anther culture of four hybrid sunflower genotypes aimed to determine the effects of different growth conditions (field and growth chamber), two pretreatments (cold and heat) and different media compositions on androgenetic ability of the hybrids (Akgül *et al.* 2016). Anthers with late uninucleate-microspores obtained from capitulums were used for culture. Maximum callus induction rates were found to be 34.4% (when anthers cold pretreated for 24 hours) and 41% (when anthers heat pretreated at 35ºC for 2 days) on the medium including 0.5mg/l NAA. These results suggested that factors such as genotype, growing conditions of donor plant, pollen phase, pretreatments and culture media compositions effected anther culture.

Doğan *et al.* (2016) who studied on microspore culture of different hybrid sunflower cultivars claimed that androgenic methods are used to obtain haploids such as anther and microspore culture. In these methods, genetic potential of cultivars is very important for a successful culturing. Doğan *et al.* (2016) selected different hybrid sunflower cultivars and evaluated their responses to isolated microspore culture. The effects of different plant growth regulators and media composition on androgenic

microspore culture were studied in their study. As a result, the authors claimed that further studies with isolation method, different media compositions and culture conditions will be necessary in order to develop an efficient microspore isolation and culture technique in sunflower.

Conclusion

In conclusion, when the above studies are examined, it appears that several studies about *in vitro* sunflower culture using different explants types in Turkey have been published in last few decades. Anthers (Emiroğlu *et al.* 1993, Dayan 2011, Kılıç *et al.* 2016, Akgül *et al.* 2016), hypocotyls and cotyledons (Gürel 1994, Gürel & Kazan 1998, Özyiğit *et al.* 2002, Arda 2004, Özyiğit *et al.* 2007), petioles of cotyledon and shoot-tips (Gürel & Kazan 1998), mature embryos (Özyiğit *et al.* 2006, Arda *et al.* 2012), immature embryos (Binboğa Meral 2007a, Dağüstü *et al.* 2010, Dağüstü *et al.* 2012), leaves, petioles and immature cotyledons (Binboğa Meral 2007b) and microspores (Doğan *et al.* 2016) were used as an explant in these studies.

Microspore culture (Doğan *et al.* 2016), anther culture (Emiroğlu *et al.* 1993, Dayan 2011, Kılıç *et al.* 2016, Akgül *et al.* 2016), embryo culture (Özyiğit *et al.* 2006, Arda *et al.* 2012, Binboğa Meral 2007a, Dağüstü *et al.* 2010, Dağüstü *et al.* 2012), slow growth storage (Arda *et al.* 2012), micropropagation (Özyiğit *et al.* 2002) and gene transfer with *Agrobacterium tumefaciens* (Gürel & Kazan 1999, Binboğa Meral 2007b) are the techniques which were used in sunflower culture studies in Turkey.

Consequently, all of these available studies are evaluated as promising in tissue culture of sunflower. Nevertheless, more studies are still needed for the development of new breeding lines and/or varieties of sunflower, which is commercially registered. Although some studies are ongoing in different laboratories in Turkey, there is still a major need to devote more resources to this research field. Planning of master and doctoral studies on sunflower tissue culture techniques in universities may be an important step for improvement. I hope that this review will positively contribute to sunflower tissue culture studies in Turkey.

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