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In Vitro Tissue Culture Studies in Sunflower (*Helianthus* spp.)

Nazan DAGUSTU[1*]

[1] Uludağ Üniversitesi, Ziraat Fakültesi, Tarla Bitkileri Bölümü, Bursa.

[*] Corresponding author e-mail: ndagustu@uludag.edu.tr

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ABSTRACT

Sunflower (*Helianthus annuus* L.) is the fourth most important oilseed crop in the world in terms of total yearly production, after soybean, rapeseed, and groundnut. The classical breeding studies made from 1880 until today have been focused on major characters, such as high fat, pests and diseases resistance. The narrow genetic base in sunflower causes a major problem at breeding and selection of desired sunflower lines eg. cytoplasmic male sterility, disease and insect pest resistance, fertility-restoration, agronomic and seed-oil characteristics, drought tolerance, protein content, imidazolinone resistance and fatty acid composition. The growth or maintenance of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro* described as plant tissue culture, is an important tool in both basic and applied researches as well as in commercial application. The first and important step of tissue culture is development of *in vitro* regeneration system. Researchers have successful attempts by various explants, such as immature embryo mature embryo, meristem, anther, hypocotyl, protoplast, cotyledonand embryo sac. Although the specific techniques like, embryo culture, organogenesis, somatic embryogenesis have been succesfully used, anther culture, microspore culture ovule cultureneeds to be improved.

Keywords: sunflower, plant tissue culture, *in vitro* regeneration

Introduction

Sunflower (*Helianthus annuus* L.) is the fourth most important oilseed crop in the world in terms of total yearly production, after soybean, rapeseed, and groundnut. The classical breeding studies started from 1880 until today have been focused on major characters, such as high fat, pests and diseases resistance (Faure *et al*., 2002; Seiler and Gulya, 2004; Vassilevska-Ivanova *et al*., 2014). The conventional breeding studies have resulted in cultivars with improved agronomic characteristics. However, the lack of suitable genetic resources in modern sunflower varieties affects negatively development of new sunflower hybrids possessing high disease resistance and new oil and protein qualities, highy tolerence to stress conditions (drought, salt etc.). New technologies are necessary to broaden the genetic variation of cultivated sunflower. Biotechnology involving tissue culture and genetic engineering might be useful tool to exploit genetic variation (Larkin and Scowcroft, 1981). The main target of tissue culture and molecular techniques is crop improvement.Plant tissue culture is powerful tool for studying basic and applied problems in plant breeding. The growth or maintenance of cells, tissues, organs, and their components under defined physical and chemical conditions *in vitro* described as plant tissue culture, is an important tool in both basic and applied researches as well as in commercial application (Thorpe, 1990). Plant cell tissue culture technologies also have considerable potential for genetic improvement of sunflowers, with the generation of somaclonal variant, transgenic and somatic hybrid plants illustrating the potential of these approaches to promote conventional breeding. Molecular techniques facilitate the selection of germplasm for incorporation into sunflower breeding programs.

Application of various tissue culture methods to sunflower crop improvement, throught regeneration via both organogenesis and somatic embryogenesis, interspecific hybridization and embryo culture, haploid production, somaclonal variation, protoplast culture are subjects of the review.

Tissue culture and plant regeneration

The application of biotechnological methods (whole plant regeneration *via* tissue culture and molecular studies) for most sunflower breeding studies to improve the characteristics of sunflower (salinity tolerence, drought and disease resistant, *in vitro* mutagenesis, and somatic embryogenesis eg.) are limited mainly by the difficulty of regenerating plants in a reproducible and efficient way (Flick *et al*., 1983, Phillips, 2004). Sunflower species are highly recalcitrant nature and difficult to regenerate plants especially when they are subjected to *in vitro* conditions (Moghaddasi, 2011; Davey and Jan, 2010; Nichterlein and Horn, 2005; Mezzarobba and Jonard, 1986). Thus, the first and important step of tissue culture is development of *in vitro* regeneration system. Development of a highly regenerable tissue culture system for many *Helianthus* species for crop improvement would be possible with techniques such as embryo rescue, somatic hybridization, somaclonal variation and morphogenesis. Sunflower tissue culture study was started with sunflower tumor cells in 1940's (Hildebrandt *et al*., 1946). It is reported that the sunflower tumor cells continued to proliferate in the absence of *Agrobacterium tumefaciens* (White and Braun, 1941), only root regeneration from tumor cells was obtained but there was no report of shoot regeneration from those tumors. In early, most studies of *Helianthus annuus* L. (*H. annuus* L.) tissue culture were concerned about crown gall cells. In 1954, *H. annuus* with non tumorous callus was obtained from growing stem or hypocotyl segments on simple media with auxin (Henderson, 1954; Kandler, 1952). In the same year, Henrickson (1954) cultured shoot tips of 5 day old *H. annuus* cv. 'Mammoth Russion' on a modified White's medium to obtain whole plant. It is shown that a whole plant without callus formation can grow from a single shoot tip but flowered in culture within 3 months. Rogers *et al*., (1974) successfully established callus from a CMS sunflower line, but this callus induced only roots. The first whole plant regeneration was obtained from callus tissue isolated from the stem pith tip of a 2 month old sunflower plant in medium with 1 mg/l IAA by Sadhu in 1974. Since then many successful attempts on sunflower regeneration protocols have been done by various explants, such as immature embryos (Finer, 1987; Jeannin *et al*., 1995; Dağüstü *et al*., 2010; Encheva *et al*., 2004), mature embryo (Ozyigit *et al*., 2007), meristems (Paterson, 1984), shoot tips and 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 and Dahanayake, 2015), roots and stems (Inoka and Dahanayake, 2015), anthers, ovaries (Badea *et al*., 1989; Mohmand and Quraishi, 1994; Thengane *et al*., 1994; Nurhidayah *et al*., 1996), hypocotyls (Lupi *et al*.,1987; Mohmand and Quraishi, 1994; Müller *et al*.,2001; Sujatha *et al*., 2012; Greco *et al*., 1984), protoplasts (Guilley and Hahne, 1989; Fischer *et al*., 1992; Henn *et al*., 1998; Rákosy-Tican *et al*., 2007), and cotyledons (Fiore *et al*., 1997; Greco *et al*., 1984; Sujatha *et al*., 2012; Ceriani *et al*., 1992), embryo sacs (Popielarska and Przywara, 2003; Popielarska, 2005). Greco *et al*., (1984) also pointed that every part of the seedlings except roots was cabable of regeneration in sunflower. Punia and Bohorova (1992) studied with six wild species of sunflower showed that the effect of genotype, explant and medium were very important on callus induction and plant development. The parallel results were obtained by Dağüstü (2002) that the effect of genotype, age of seedlings and interactions between genotype and light in sunflower genotypes were very important on callus induction and plant regeneration of sunflower genotypes. Plant regeneration parameters have been shown to be under quantitative genetic control in sunflower (Sarrafi *et al*., 1996ab; Delgene *et al*., 1997; Berrios *et al*., 1999a).

Application of tissue culture methods in sunflower breeding

Organogenesis and Somatic Embryogenesis

A variety of techniques for regeneration by organogenesis (Pugliesi *et al*., 1993; Witrzens*et al*., 1988; Power, 1987; Espinasse and Lay, 1989; Chraibi *et al*., 1992; Ceriani *et al*., 1992; Berrios *et al*., 1999b) or somatic embryogenesis (Finer, 1987; Freyssinet and Freyssinet, 1988; Espinasse and Lay, 1989; Pelissier *et al*., 1990; Prado and Berville, 1990; Jeannin and Hahne, 1991; Nestares *et al*., 1996) have been described in sunflower. Sunflower regeneration capacity by organogenesis is highly variable and depends upon genotype, specific media components, the explant type, age of seedling, concentrations of hormones in callus induction medium, lighting conditions and tissue culture methods (Moghaddasi, 2011; Dagüstü, 2002).

Interspecific Hybridization and Embryo Culture

Embryo rescue culture is the growth of an immature embryo under sterile conditions *in vitro* for obtaining a viable plant (Bürün and Gürel, 2001). Embryo culture has been used successfully by plant breeders in solving the problems of seed set, seed dormancy, slow seed germination, inducing embryo growth in the absence of symbiotic partner, shortening the breeding cycle, rapid seed viability test, obtaining rare hybrids and homozygous lines, and haploid production (Bhojwani and Razdan, 1996; Chandler and Beard, 1983; Dağüstü *et al*., 2012; Gürel *et al*., 1991ab; Raghavan, 2003; Torresàn *et al*., 1996). Dagustu *et al*., (2010) managed to regenerate fertile plants from sunflower (*H. annuus* L.) *via* immature embryo culture. Embryo culture proved to be an useful tool to overcome post-zygotic hybrid incompatibility in different *Helianthus* spp. and significantly increases the efficiency of distant hybridization (Nenova *et al*., 2014).

The wild *Helianthus* species are of considerable interest as a source of genetic variation for economically important characters such as fatty acid composition, male sterility, fertility restoration, protein content, disease resistance, drought tolerance, salt tolerance, herbicide tolerance, chemical constituents, garden and ornamental sunflower types with novel plant and flower characteristics (Breccia *et al*., 2009; Chandler and Jan, 1985; Christov, 2012; Christov, 2013; Fernandez-Martinez *et al*., 2010; Jan and Chandler, 1985; Kaya, 2015; Mandel *et al*., 2011; Miller and Al-Khatib, 2004; Petcu and Pacureanu, 2011; Sauca and Lazar, 2011; Seiler, 2012; Seiler and Rieseberg, 1997; Seiler *et al*., 2017; Škorić, 2009; Serieys and Christov, 2005; Sukno *et al*., 1999; Tan *et al*., 1992; Tosun and Ozkal, 2000). The use of interspecific hybridization in sunflower has started in 1916, when the Russian scientist Sazyperow produced an interspecific hybrid between *H. annuus* and *H. argophyllus* T. and G. in an attempt to develop sunflower with resistance to rust (Cockerell, 1929). Interspecific hybridization in Russia continued with Galina Pustovoit's research on perennial *Helianthus tuberosus* L. (Škorić, 1988). The first regeneration from interspecific sunflower species was obtained from partial compatible *H. annuus* and perennial tetraploid *H. decapetalusvia* embryo rescue culture by Georgeiva - Todorova *et al*., (1980). With improvement of embryo rescue, 33 interspecific hybrids with an overall success rate of 41%, producing many new hybrid combinations was developed Kräuter *et al*., (1991).In recent years many successful studies have been carried on with embryos arising from interspecific and intergeneric hybrids in sunflower by embryo rescue (Sukno *et al*., 1999; Christov, 2008; Dağüstü *et al*., 2010; Faure *et al*., 2002).At first time, Chandler and Beard (1983) developed a two-step embryo culture procedure that produced 53 interspecific cross combinations without multiple pollinations. Out of 53 interspecific crosses, 21 combinations developed regeneration that had not been accomplished using conventional procedure. Development of chromosome doubling with colchicine greatly accelerated interspecific hybridization in sunflower(Jan, 1988). Today, lines with the high and middle oleic acid content have been successfully transferred to cultivated sunflower genotypes by interspecific hybridization, mutation breeding and gene transfer methods (Sukno *et al*., 1999; Abbas Mohamed 2005). Jambhulkar (1995) developed a rapid embryo-raised plant system for sunflower production from immature embryos, which allows five cycles in 316 days. In the recent study the breeding cycle of sunflower using immature embryo culture was shortened by taking 4 generations in a year (Dagustu *et al*., 2012). Nenova *et al*., (2014) showed succesfull results using embryo culture on interspecific hybridization between cultivated sunflower (*Helianthus annuus* L.) and the perennial species *Helianthus ciliaris.* Four lines 1131/н, 1135/н, 1145/н, 1171/p, 1161/p and 1151/p among the selected possessed complete resistance to the pathogens of downy mildew and broomrape and some lines had higher seed oil content.

Haploid Production

Haploid plants are plants with a gametophytic chromosome number and doubled haploid plants are haploids that have undergone chromosome duplication. Anther and microspore culture for haploid production greatly reduce the time required for development of improved cultivars by providing homozygous doubled haploids within a comparatively short time (Bürün and Gürel, 2001). Several methods have been applied for the production of haploid sunflower plant through gynogenesis (Yang *et al*., 1985; Gelebart and San, 1987), anther culture (Bohorova *et al*., 1985; Gürel *et al*., 1991a; Pugliesi *et al*., 1993; Saji and Sujatha, 1998; Thengane *et al*., 1994; Yang *et al*., 1990; Zhong *et al*., 1995) microspore culture (Gürel *et al*., 1991b; Coumans and Zhong, 1995) and induced parthenogenesis (Todorova *et al*., 1997). Thengane *et al*. (1994) cultured anthers of 4 genotypes and the only one genotype (eg. interspecific hybrids) regenerated plantlets from embryos. Nenova *et al*. (2000) had successes with anthers of some wild species by direct organegenesis. Several publications have described extensive callusing induced from anthers of various interspecific sunflower hybrids

cultured *in vitro* (Bohorova *et al*., 1985; Mezzarobba and Jonard, 1986). Optimization of anther culture with regard to the induction of callus formation and direct embryogenesis was studied with interspecific hybrids of *H. annuus* with *H.tuberosus, H. laetiflorus* and *H.resinosus* by investigating six different induction media and four regeneration media by Nurhidayah *et al*., (1996). Sunflower proved to be very recalcitrant in anther culture and culture response is strongly affected by physical, nutritional, physiological and genetical factors (Gürel *et al*., 1991b; Mezzarobba and Jonard, 1986) and the regeneration rates are very low. Although the results of anther culture of cultivated sunflower has been unsatisafactory so far, whereas the interspecific hybrids have been successfully used in anther culture of sunflower (Alissa *et al*., 1985; Bohorova *et al*., 1985; Mix, 1985; Jonard and Mezzarobba, 1990; Nurhidayah *et al*., 1996).

Isolated microspore culture of cultivated sunflower was assayed by Coumans and Zhong (1995) in order to avoid development of the anther wall and other somatic tissues. It is reported that viability and initial division rate of microspore response was increased and sustained division and microcallus formation were achieved after addition of aminocyclopropane carboxylic acid, an ethylene precursor. The only groups of cells had hairy types of structures developed into calluses.

An alternative method for haploid production in *Helianthus* spp. are induced parthenogenesis. It is an applicable approach for rapid production of doubled haploid lines in sunflower. Todorova *et al*., (1997) used irradiated pollen-induced parthenogenesis obtained the number of agronomically useful DH lines that were fertile and resistant to downy mildew. But the efficiency of the method was depending on the female genotype and pollen donors.

Somaclonal Variation

Genetic variation is revealed in crops and their progenies raised through cell and tissue culture techniques. This is defined as somaclonal variation (Larkin and Scowcroft, 1981). Many types of genetic changes occur in somaclonal variation including alterations in DNA sequence e.g. single gene mutation, transposition, amplification; in gross chromosome structure e.g. duplications, translocations, deletions; in chromosome number e.g. polyploidy or aneuploidy; and in chloroplast or mitochondrial genomes. This types of changes are stable through succeeding generations. However, the variation exposed as a result of a tissue culture cycle can be non-heritable (epigenetic) which would not be transmitted through meiosis and it may be reversible during the life of a plant. Hence it is worthless for sexually propagated plant production. Changes have also been identified that are both heritable and unstable (Karp, 1990). It is believed that somaclonal variants can be enhanced for some characters during culture *in vitro*, including resistance to disease pathotoxins and herbicides and tolerance to environmental or chemical stress. However, at present few cultivars of any agronomically important crops have been produced through the exploitation of somaclonal variation as in sunflower. Pugliesi *et al*., (1991) showed plant regeneration and genetic variability in tissue cultures of sunflower cotyledons. Somaclonal variation and *in vitro* selection can be applied in sunflower in many aspects. Examples of beneficial changes have included higher oil content in seed, higher 1000 seed weight, good combining ability, full resistance to *Phomopsis helianthi*, shorter vegetation period and reduced height (Encheva *et al*., 2003; 2004).

Protoplast Culture

Sunflower protoplasts from various sources (mesophyll, stems, cotyledons anhypocotyls) have been tested for their capacity to divide in culture (Lenee and Chupeau, 1986), only 6% of the initially plated protoplasts reached the stage of calli in a medium containing glutamine or ammonium succinate as sole sources of nitrogen and a reduced amount of naphthalene acetic acid (NAA) (0.1 mg/l).Bohorova *et al*., (1986) isolated and cultured protoplasts of wild and cultivated sunflower and obtained roots and meristematic regions from protoplast derived callus. The scientists managed regeneration of fertile plants from *H. petiolaris* and *H. annuus* protoplasts in 1991 (Burrus *et al*., 1991; Chanabé *et al*., 1991). Simple and efficient method for routine callus formastion from calli protoplasts and for plant regeneration from hypocotyl protoplasts of a sunflower commercial cultivar Girapac SH222 was described by Santos and Caldeira (1998). Plant regeneration from hypocotyl protoplasts of sunflower was also achieved for two cvs.'Florom 328' and 'Turbo' by Rákosy-Tican *et al*., (2007).

In conclusion, sunflower regeneration capacity by organogenesis and somatic embryogenesis are highly variable and depend upon genotype, specific media components, the explant type, age of seedling, concentrations of hormones in callus induction medium, lighting conditions and tissue culture methods. Although much work focused on establishment double haploid production, protoplast culture in sunflower, none of the improvements was efficient and applicabale to a wide range of genotypes in plant breeding programmes. The further researches are necessary to optimize tissue culture protocols for use in sunflower breeding programmes.

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