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Full Length Research Paper

Genotype dependent callus induction and shoot regeneration in sunflower (*Helianthus annuus* L.)

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This study aims to observe the effect of genotype, hormone and culture conditions on sunflower (*Helianthus annuus* L.) callus induction and indirect plant regeneration. Calli were obtained from hypocotyl and cotyledon explants of five different sunflower genotypes; Trakya 80, Trakya 129, Trakya 259, Trakya 2098 and Viniimk 8931, which are commercially important for Turkey. Seeds germinated on Murashige and Skoog (MS) media contained no hormones. Hypocotyl and cotyledon explants were cultured on MS media supplemented with 1 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) and different percentage of callus inductions were obtained. Calli were cultured on MS + 1 mg/l BA (6-benzylaminopurine) and 0.5 mg/l NAA (α-naphthalene acetic acid). Some genotypes showed high regeneration response while others showed lower on the same media with hypocotyl and cotyledon derived calli. This study showed that genotypic differences affect callus induction and plant regeneration in sunflower tissue culture studies.

Key words: Sunflower, tissue culture, callus, hypocotyl, cotyledon, organogenesis.

INTRODUCTION

Compositae family member sunflower (*Helianthus annuus* L.) has a great importance in industrial crops with its high oil content. The technology for processing sunflower-oil into biodiesel-oil has been developed lately, and as a result; the necessity of good quality sunflower oil is increasing rapidly (Ikeda et al., 2005). Thus in breeding programs, increased disease resistance and high oil content have been the main aim for sunflower (Weber et al., 2000). In recent years, biotechnological techniques such as tissue culture and gene transfer systems have been used for improvement of sunflower, but these techniques are mainly limited by the tissue culture response of commercial varieties (Nestares et al., 2002).

In sunflower, reports on shoot regeneration from hypocotyls, cotyledons, leaves and meristematic tissues of young plantlets are available, but regeneration ability and precocious flowering, vitrification or poor rooting (Sujatha and Prabakaran, 2001). Furthermore, regeneration quality were either low with abnormal morphogenesis, frequency by organogenesis essentially depends on genotype and its interaction with culture conditions (Ozyigit et al., 2006). Sarrafi et al. (1996) studied genetic analysis of organogenesis in sunflower and observed that both shoot and root regeneration were under genetic control (narrow-sense heritability = 68.6%) and evidence of a cytoplasmic effect and nucleo-cytoplasm interaction was also described by Deglene et al. (Sarrafi et al., 1996; Deglene et al., 1997). Recently, Petitprez et al. (2005) reported genetic control of somatic embryogenesis using hypocotyl epidermis layers of sunflower in liquid media (Petitprez et al., 2005).

Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium (auxins and cytokinins) (Shah et al., 2003). The specific concentrations of plant regulators, needed to induce callus formation, varies from species to species and can even depend on the source of explant (Charriere et al., 1999). Furthermore, genotype is one of the most important factors for callus induction like shoot regeneration in tissue culture studies (Sarrafi et al., 1996;

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Punia and Bohorova, 1992). In different explants of *Helianthus*, cotyledons and young hypocotyls are advantageous since they are easily and quickly available and posses a high potential for direct and indirect ways of regeneration (Ozyigit et al., 2002). Many reports showed that for *Helianthus* genus, 1 mg/l 2,4-D is convenient for callus formation in both hypocotyl and cotyledon explants and 1 mg/l BA and 0.5 mg/l NAA together are also convenient for shoot regeneration (Ozyigit et al., 2002, 2006).

In this study, genotype dependent callus induction and indirect shoot regeneration of five commercially important cultivars of sunflower in Turkey was reported. Four of them were originated and certificated in Turkey while Viniimk 8931 originated in Russia. In this study, the same media, hormones and culture conditions were used and it was seen that genotype is an important factor for callus induction and indirect organogenesis.

MATERIALS AND METHODS

Sunflower (*H. annuus* L.) seeds; Trakya 80, Trakya 129, Trakya 259, Trakya 2098, Viniimk 8931 were obtained from Trakya Agricultural Research Institute, Edirne-Turkey. Seed coats were removed with sterile sculpture and pliers prior to surface sterilization and they were surface sterilized by immersion in 70% ethanol (Sigma Chemical Co.) for 3 min, followed by stirring in 20% commercial bleach (ACE Lever Co.) for 20 min. The surface sterilized seeds were rinsed 3 times with sterile distilled water for 5 min and they were dried onto sterile filter papers. Seeds were germinated on hormone free MS (Murashige and Skoog) medium. The medium contained 1 mL MS vitamin solution, 4.3 g basal salt mixture, 30 g sucrose and 9 g agar (Sigma Chemical Co.). The pH of the media was adjusted to 5.7 with 1 M NaOH (Merck) before autoclaving. Seeds were kept at growth chamber with photoperiod of 16 h light (7500 lx) and 8 h dark, at 25°C and 70% humidity.

After 10 days, hypocotyls and cotyledons were dissected out from seedlings. Hypocotyls were cut into 0.5 cm long pieces and cotyledons were cut transversally into two parts and then cultured on Murashige and Skoog (MS) media supplemented with 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) for callus induction. 40 explants were used from 5 genotypes for each treatment. Both hypocotyl and cotyledon explants became larger in 7 days and then, approximately 15 - 21 days after culturing period calli were formed. Calli of 5 different genotypes were separated in 2 groups. One group cultured on MS supplemented with 1 mg/l BA and 0.5 mg/l NAA and the other group cultured on hormone free MS media. All genotypes showed different regeneration responses on MS with 1 mg/l BA and 0.5 mg/l NAA but none of them gave any regeneration responses on MS without any hormones. While some of the hypocotyl derived calli produced air roots, most of the cotyledon derived calli transformed to brownish-black. Regenerated plantlets were rooted in hormone free MS and MS supplemented with 1 mg/l Indole-3-butyric acid (IBA). Rooted plants transferred to the soil 3 - 4 weeks later. Callus induction and regeneration ratios of different genotypes and explants were calculated.

RESULTS

The germination frequency was around 90% in 5-tested cultivar's seeds. After germination, 10-day-old hypocotyl and cotyledon explants were cultured on MS media supplemented with 1 mg/l 2,4-D. Hypocotyl and cotyledon explants became larger after 1 week of culturing, and then callus were formed in 2 - 3th weeks (Figure 1a-b). For all genotypes, callus inductions from hypocotyls were faster than cotyledons. Callus induction of hypocotyl explants on MS media supplemented with 1 mg/l 2-4 D was between 89 - 100% as seen in Table 1. Trakya 129, Trakya 2098 and Viniimk 8931 genotypes gave the best values (100%). Trakya 159 showed 95% and Trakya 80 showed 89%. Callus induction was slower in cotyledon explants in the same culture conditions and they gave also lower values. Callus induction was between 57-90% and Trakya 259 gave the best value with 90%. Other genotypes showed values as followed; Trakya 80 (81%), Trakya 129 (89%), Trakya 2098 (57%) and Viniimk 8931 (67%). Callus induction for hypocotyls was observed in 2 weeks in all genotypes but for cotyledons, in 3 weeks. On the other hand, Trakya 259's cotyledons formed calli in 2th week like hypocotyl explants as seen in Figure 1b.

Calli were separated in two groups. One group transferred and cultured on MS supplemented with 1 mg/l BA and 0.5 mg/l NAA and the other group cultured on hormone free MS media. Shoot regeneration was started in two or three weeks (Figure 1c-d) on MS supplemented with 1 mg/l BA and 0.5 mg/l NAA. Regeneration efficiency was between 11 and 31% in hypocotyl explants and Trakya 259 genotype gave the best regeneration response (31%). Other genotypes showed values as followed (Table 1); Trakya 80 (29%), Trakya 129 (25%), Trakya 2098 (11%) and Viniimk 8931 (24%). In cotyledon explants, regeneration efficiency was between 7 - 18% and Trakya 259 gave the best response like the hypocotyl derived calli. Other responses were (Table 1); Trakya 80 (11%), Trakya 129 (10%), Trakya 2098 (7%) and Viniimk 8931 (13%). On the other hand none of the calli in both explant types showed any regeneration responses on MS without any hormones despite frequent subculturing. Some of hypocotyl derived calli formed thin and long roots (Figure 1f) and most of the cotyledon derived calli transformed to brownish-black (Figure 1e).

For rooting, regenerated shoots, which reached from 2 to 3 cm were transferred on MS media supplemented with 1 mg/l IBA (Figure 1h) or hormone free MS medium (Figure 1g). All genotypes were rooted well in three or four weeks and became ready to be transferred to soil. In both media, rooting efficiency was between 95 and 100%, but roots were thicker and denser on MS medium supplemented with 1 mg/l IBA.

DISCUSSION

As many tissue culture studies like sunflower, callus can be obtained from different explants with various plant hormones and hormone combinations (Ozyigit et al., 2006; Shah et al., 2003; Punia and Bohorova, 1992). In our study, the results have indicated that MS medium supplemented with 1 mg/l 2,4-D is quite suitable for callus

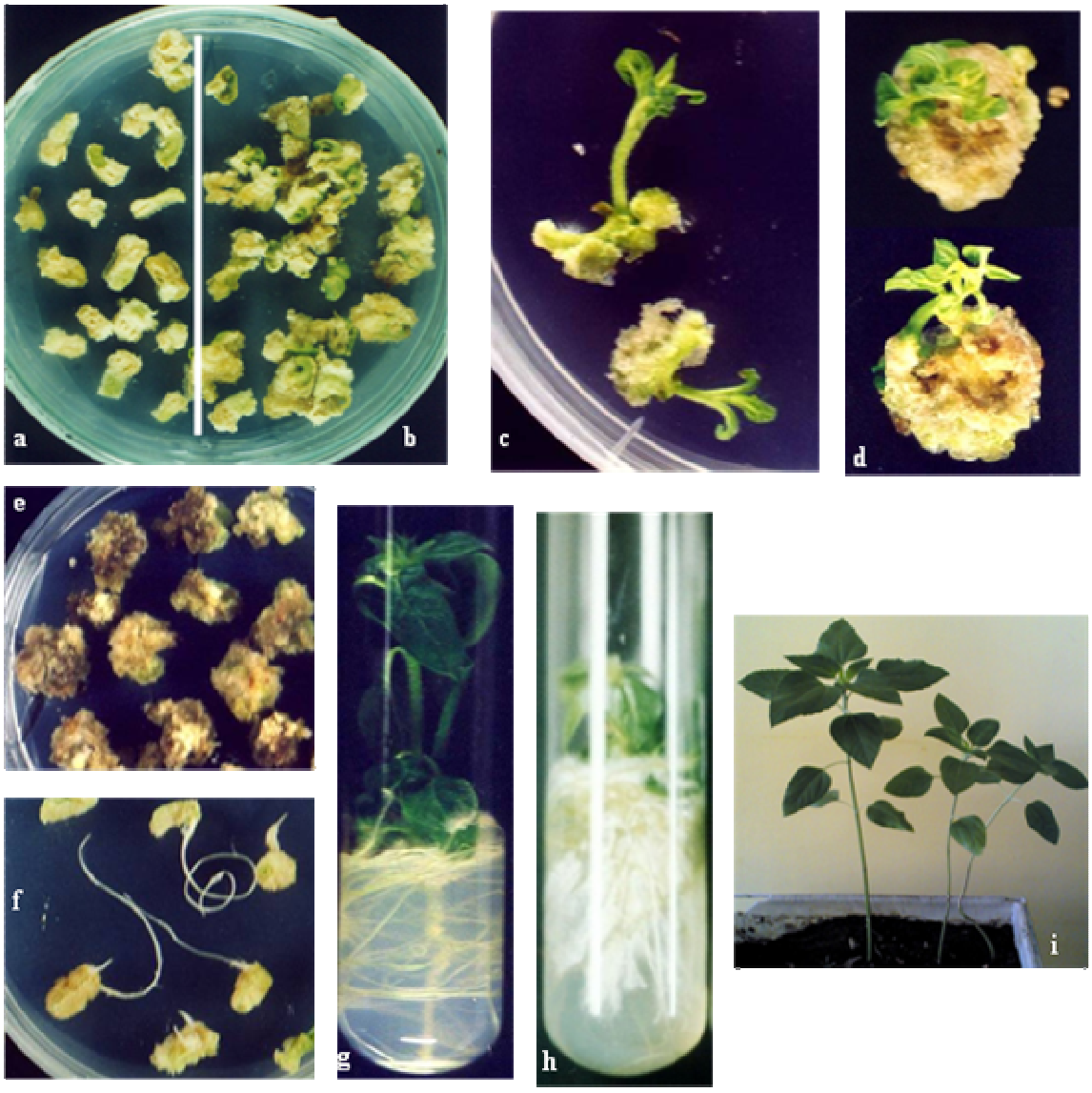

Figure 1. Callus induction from 10 day old hypocotyl **(a)** and cotyledon **(b)** explants of five different sunflower genotypes on MS media supplemented with 1 mg/l 2-4 D in 2-3 weeks. Indirect shoot regeneration from hypocotyl **(c)** and cotyledon **(d)** derived calli on MS media supplemented with 1 mg/l BA and 0.5 mg/l NAA. Browning and blackening of cotyledon derived calli on MS with no hormones **(e)**. Unwilling early root induction on MS with no hormones **(f)**. Root induction on hormone-free MS **(g)** and on MS + 1 mg/l IBA **(h)**. Plant development in climate room conditions **(i)**.

induction from hypocotyl and cotyledon explants of 5 different sunflower genotypes. In a similar study, explants were cultured on MS supplemented with 1.0 mg/l BA, 0.1 mg/l GA_3 (Gibberellic acid), 500 mg/l CA (Casamino acid) and 40 mg/l AS (Adenine sulphate) and callus was obtained from stem (79 - 98%), leaf (84 - 100%), bud (94.2 - 100%), and cotyledons (27 - 62%) of 6 different *Helianthus* genotypes (Punia and Bohorova, 1992). In one of the other reports, hypocotyl and cotyledon explants of 5 different genotypes were cultured on 7 different media and callus formation was 100% on MS1 and MS2 in all genotypes. MS1 was not supplemented with any hormones, MS2 was supplemented with 30 mg/l maltose, 400 mg/l Glutamine, 4 mg/l KIN (Kinetin), 2 mg/l NAA but no sucrose (Gürel, 1994). In our previous study, we have also obtained callus (80 - 92%) using the mature embryos of the same genotypes in the same culture conditions (Ozyigit et al., 2006).

The results above showed that, genotype and explant type have a considerable role for callus induction. In general, 7-day-old cotyledon explants and combinations of BA, NAA and GA_3 (Gibberellic acid) are used in other

Table 1. Callus induction (on MS medium supplemented with 1 mg/l 2,4-D) and shoot regeneration (on MS medium supplemented with 1 mg/l BA and 0.5 mg/l NAA) from hypocotyl and cotyledon explants of five different sunflower genotypes.

Genotypes	Callus Induction (%)		Shoot Regeneration (%)	
	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon
Trakya 80	89 ± 1.52	81 ± 1.92	29 ± 1.64	11 ± 3.49
Trakya 129	100	89 ± 2.30	25 ± 5,22	10 ± 2.75
Trakya 259	95 ± 2.15	90 ± 2.03	31 ± 3.62	18 ± 3.11
Trakya 2098	100	57 ± 5.35	11 ± 4.16	7 ± 2.42
Viniimk 8931	100	67 ± 4.00	24 ± 2.34	13 ± 1.92

The mean values of five independent experiments and their standard errors are presented.

tissue culture studies (Punia and Bohorova, 1992). However, in this study only auxin (2,4-D) was used and high percentage of calli obtained from 10-day-of hypocotyl explants, but callus induction was slower and frequency was lower with 10-day-old cotyledon explants in five tested genotypes. In addition, Trakya 2098 genotype gave one of the highest callus inductions with hypocotyl explants (100%), but the lowest callus induction (57%) with cotyledon explants. This result showed that source of the explant together with genotype has a respected role in tissue culture studies.

Calli of five different genotypes were separated in 2 groups. One group cultured on hormone free MS media and the other group cultured on MS media supplemented with 1 mg/l BA and 0.5 mg/l NAA. It was observed that all cotyledon derived calli became brownish black and dead (Figure e) and some of the hypocotyl derived calli developed thin and long roots on MS without any hormones. In addition, different changes occurred on MS media supplemented with 1 mg/l BA and 0.5 mg/l NAA, both hypocotyl and cotyledon derived calli. Some of them became brownish black and die, some formed only roots like on MS + no hormones and some developed shoots (Figure c-d). Shoot regeneration was better with hypocotyl derived calli than cotyledon derived calli and in both types of explants, Trakya 259 showed the best regeneration efficiency.

In our study, the best condition for shoot induction of sunflower was found to occur on MS media with 0.5 mg/l NAA combined with 1 mg/l BA. Using hypocotyl explants, 5 different genotypes and 7 different media have been tested and shoot regeneration and callus formation was observed. MS media with 1 mg/l GA_3, 3 mg/l kinetin and 0.5 mg/l NAA were used and regeneration was 4% for 7 day old cotyledons of Semu FV/89 Festive genotype (Gürel, 1994). In addition Paterson and Everett observed good regeneration and somatic embryogenesis for 12 day old hypocotyls on MS supplemented with 6.9 g/l KNO_3, 40 mg/l adenine sulphate, 500 mg/l casamino acids, 1 mg/l BAP, 1 mg/l NAA and 0.1 mg/l GA_3 (Paterson and Everett, 1985).

Using cotyledon explants (10 day old) on same medium, Knittel et al. (1991) observed the best regeneration efficiency with 4 -10 day old explants of 8 different genotypes on 17 different combinations of BA and NAA. They found that using 1 mg/l BA and 0.5 mg/l NAA gave the best result for HA300B genotype (80%) (Knittel et al.). Khalid et al. (1992) used 1 - 4 day old cotyledons of 3 different genotypes in a liquid medium with 4.4 µM BA and 5.4 µM NAA and found that the best regeneration (59 %) belongs to 2 day old cotyledons of R897 genotypes after 4 weeks (Khalid et al., 1992). Ceriani et al. (1992) used 20 different genotypes and did not get any regeneration for 9 of them, but the best regeneration was obtained on MS medium, which contains 1 mg/l BA and 0.75 mg/l NAA (90 %) (Ceriani et al., 1992). As it can be seen from the studies above, especially in sunflower tissue cultures, hypocotyl and cotyledon explants are good regeneration materials that show different regenerative behavior when kept in a culture, depending on their genotype.

In our other 2 researches we studied direct regenerations with hypocotyl and cotyledon explants and indirect regenerations with mature embryos of same genotypes and same culture conditions. We observed different regeneration responses with these genotypes. For example, although we observed the highest regeneration responses with hypocotyls (direct), cotyledons and embryos (indirect) we could not obtain direct regeneration responses with cotyledon explants of Trakya 259. While Trakya 129 was the best with cotyledon explants and direct regeneration, it was not good with mature embryos and indirect regeneration in the same hormone and culture conditions (Ozyigit et al., 2002, 2006). In this case, when we decide to study with a genotype, we have to comparatively examine that genotype with the others. Because one part of a genotype can be more adaptive than other parts of other genotypes. This condition shows that, explant source is also an important factor like genotype.

Efficient regeneration is a very important in the biotechnological improvement of sunflower. Two strategies may be adapted to ensure facile regeneration of shoots from genetically manipulated cells. One is to select the most adaptive genotype and explant source, the other is to optimize environmental and/or culture conditions to maximize the genetic potential.

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