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# SOME FACTORS AFFECTING THE PLANT REGENERATION IN SUNFLOWER (*Helianthus annuus* L.)

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## ABSTRACT

*High frequency of plant regeneration in sunflower was developed in order to use in modern sunflower breeding. Hypocotyl and cotyledon explants grown either in the light or in the dark of sunflower line and hybrids were investigated for their morphogenic potential on media containing a range of hormonal combinations including 1-naphthaleneacetic acid and 6-benzylaminopurine (0.5-1.5 mg/l). The amount of callus production in each explant was influenced by the age of seedlings from which the explants were taken, the type of explant, cultivars and the interaction between cultivars and light. Regeneration of embryo and shoot varied from 0% to 27% depends on the experiment. Although cotyledon explants taken from older seedlings produced statistically the lower amount of callus per explant, the morphogenic capacity of younger seedlings (4-day-old) was generally much higher than older seedlings.*

## Introduction

The sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops in the world. The lack of suitable genetic resources in modern varieties affects negatively the obtainable of new sunflower hybrids possessing high disease resistance and new oil and protein qualities. New technologies are necessary for broaden the genetic variation of cultivated sunflower. Biotechnology involving tissue culture and genetic engineering might be useful tool to exploit genetic variation (10). Transgenic sunflower plants created by genetic manipulations will contribute to the germplasm development. Successful transformation system requires cell cultures competent for efficient plant regeneration as well as effective methods for gene transformation. Although biotechnology offers great opportunity of regeneration new varieties, the application of this technique largely depends on the plant regeneration from organs, tissues or protoplasts (7). The first sunflower plantlet regeneration was reported by Sadhu (17). Since then many successful attempts have been done by various scientists (4, 6, 13). Plant regeneration via tissue culture have been achieved in earlier studies with many sunflower inbred and hybrid lines by applying either organogenesis (3, 4, 16), somatic embryogenesis (5, 6, 8, 13), androgenesis (12), embryo rescue (5) and protoplast (1, 2).

The high frequency of plant regeneration, the normal morphology of regenerated plants and seed production are necessary for the use of these techniques in sunflower breeding. The studies showed that sunflower is a recalcitrant plant (15). The process of sunflower regeneration is affected by many factors either singly or in combination. These include genotype (14, 15), composition of culture medium (14), the age of explant (4, 9, 13), explant type (3), and environmental factors (6).

In order to exploit the potential use of sunflower tissue culture in plant breeding,

it was necessary to develop the optimum techniques for the initiation of callus cultures followed by regeneration of plantlets. The effects of genotype, the age of seedlings from which the explants were taken, the type of explant and the concentrations of hormone in the callus induction medium were investigated in this research.

## Materials and Methods

### Plant Material and Culture Conditions

The experiments were conducted at Uludağ University, Agricultural Faculty, Field Crops Department, Plant Tissue Culture Laboratory in 1998-1999. The first experiment was performed using the two different explants (hypocotyl and cotyledon) excised from 10 day-old seedlings of 3 hybrid sunflower cultivars (Sunbred 281, Sunbro and Pioner). The cotyledon explants of two hybrid cultivars (Sunbred 281 and Pioner) and one restorer line developed by our department (res 89/2) were taken from the seedlings of different ages (4, 9 and 15 days) grown in the dark in the second experiment. In the third experiment, cotyledon explants excised from 3-day-old seedlings, grown either in the dark or in the light were cultured on MS callus induction medium as described by Fiore *et al.* (6), supplemented with 3 different range of hormonal combinations, including 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) (0.5, 1.0 and 1.5 mg/l). Before surface sterilization, the seeds were rinsed 5-6 times in sterile distilled water. The seeds were sterilised by agitation with 70 % ethanol (2 min) followed by 60 % (v/v) NaOCl for 30 min, after whitch NaOCl was removed by rinsing 7-8 times with sterile water and were placed onto petri dishes (20 seeds/dish) which contained Murashige and Skoog (MS, 11) basal salts supplemented with 1% sucrose, 0.1 g/l myo inositol, 0.8% agar for germination. Explants were placed onto callus induction containing 1 mg/l NAA, 1 mg/l BAP, 0.1 mg/l gibberallic acid, 100 mg/l myo-inositol, 6.9 mg/l potasium nitrate, 3 % sucrose and 0.8 % agar at the 2 experiments. Cultures were incubated at 26 °C ± 1 in dark and light (16 h/8 h day/night). After 2 weeks the cultures were subcultured into the same media. Regenerated shoots were excised from callus and placed onto a VM and root medium described by Fiore *et al.* (1997). After 4-5 weeks plantlets reached sufficient size (6-8 cm in length) and then were transferred into sterile soil for acclimatisation. They were watered with sterile water and the pots were covered with plastic bags to maintain high humidity conditions. Plantlets were incubated in room temperature and plastic bags were completely after 2-3 weeks.

### Observations and Statistical Analysis

Callus response (%), callus production per cotyledon explant (score), root production (%) and production of embryo like structure and shoot regeneration (%) data were taken from explants subcultured into the same media after 2 weeks. The percentage of explant pieces producing callus was scored with an estimate of callus production in each cotyledon explant according to the following scale: **0:** explant did not produce callus, **1:** up to 25% of the area of the explant piece produced callus, **2:** 26-50% of the area of the explant piece produced callus, **3:** 51-75% of the area of the explant piece produced callus, **4:** 76-100% of the area of the explant piece produced callus.

Data obtained from an average of callus production per explant as score were analysed using the completely randomised design. The means were separated by LSD test at 5% level. The embryo like structure and shoot regeneration per responding callus and root production per responding callus are showed as percentage with standard errors.

## Results and Discussion

The F values from analysis of variance summarized in **Table 1** present significant

TABLE 1

**F values for callus production per explant (score) of sunflower genotypes belong to 1, 2 and 3 experiments**

| EXPERIMENT 1 | Callus production/ explant (Score) | EXPERIMENT 2 | Callus production/ explant (Score) | EXPERIMENT 3 | Callus production/ explant (Score) |
|---|---|---|---|---|---|
| A | 0.16 | A | 19.85** | A | 20.05** |
| B | 29.3** | C | 7.71** | D | 1.83 |
| AxB | 0.60 | AxC | 1.9 | E | 2.91 |
| | | | | AxD | 5.94** |
| | | | | AxE | 0.49 |
| | | | | DxE | 1.35 |
| | | | | AxDxE | 1.65 |

A: Genotypes, B: Explant, C: Seedling age, D: Light, E: Hormone concentration
scoring system (see Materials and Methods). ** significant at $\alpha = 0.01$

TABLE 2

**Influence of cultivar and explant on the callus production of 10-day-old seedlings of 3 sunflower genotypes**

| Genotypes | Mean responding callus per treatment (score)* | | |
|---|---|---|---|
| | Ekxplant | | |
| | Hypocotyl | Cotyledon | Genotypes means |
| Sunbro | 2.78 | 1.85 | 2.32 |
| Pioner | 2.84 | 2.04 | 2.44 |
| Sunbred 281 | 3.00 | 1.72 | 2.36 |
| Explant means | 2.87A | 1.87B | |

scoring system (see Materials and Methods). Means were calculated from 60 replicated samples for each treatment.

TABLE 3

**Influence of cultivar and seedling age on the callus production of 3 sunflower genotypes**

| Genotypes | Mean responding callus per treatment (score)* | | | |
|---|---|---|---|---|
| | Seedling age (day) | | | |
| | 4 | 9 | 15 | Genotypes means |
| res 89/2 | 2.16 | 2.0 | 2.19 | 2.12 B |
| Sunbred 281 | 2.39 | 2.67 | 2.91 | 2.66 A |
| Pioner | 2.5 | 2.41 | 2.94 | 2.62 A |
| Seedling age means | 2.35 B | 2.36 B | 2.68 A | |

scoring system (see Materials and Methods).Means were calculated from 60 replicated samples for each treatment.

values in terms of different treatments used for all 3 experiments. Data on callus formation of explants, genotypes, age of seedling and interaction between genotypes and light on callus induction medium influenced callus production and the regeneration of sunflower differently.

Almost all of the treatments induced callus formation on both explants. The effect of genotypes and explant on the callus production of 10-day-old sunflower seedlings showed that hypocotyl explants had more callus formation per explant than cotyledon explants (**Table 2**). However, the morphogenic capacity of hypocotyls much lower than cotyledons. Hypocotyl explants produced only root structure on callus while cotyledon explants had embryo like structure and shoot formation at all genotypes (**Table 5**).

The amount of callus production in each genotypes was influenced by the age of seedlings from which explants were taken (**Table 3**). Genotypes Sunbred 281 and Pioner resulted in higher proportion of callus formation than Sanbro in the second experiment.

Significant genotypes effect was also found in the third experiment. Cotyledon explants of sunflower genotypes, grown either in the light or in the dark, investigated

TABLE 4

**Influence of cultivars, light and hormonal composition of the medium on the callus production of 2 sunflower genotypes**

| Genotypes | Light | Mean responding callus per treatment (score)* Hormonal composition of the medium | | | Genotypes means | Genotypes x Light means |
|---|---|---|---|---|---|---|
| | | 0.5 mg/l NAA 0.5 mg/l BAP | 1.0 mg/l NAA 1.0 mg/l BAP | 1.5 mg/l NAA 1.5 mg/l BAP | | |
| | Light | 1.94 | 2.28 | 2.44 | 2.07 B | 2.22 B |
| Sunbred 281 | Dark | 1.84 | 2.07 | 1.88 | | 1.93 C |
| | Light | 2.22 | 2.54 | 2.37 | 2.41 A | 2.38 AB |
| Sunbro | Dark | 2.47 | 2.38 | 2.54 | | 2.46 A |
| Hormonal composition means | | 2.11 | 2.32 | 2.30 | | |

scoring system (see Materials and Methods).Means were calculated from 60 replicated samples for each treatment.

TABLE 5

**Influence of cultivar and explant on the regeneration of sunflower**

| Genotypes | Eksplant | Callus response (%) | Morphogenic callus | |
|---|---|---|---|---|
| | | | Embryo like structure and shoot regeneration per responding callus (% ± SE) | Root production per responding callus (% ± SE) |
| Sunbro | Hypocotyl | 98.9 | 0 | 0 |
| | Cotyledon | 98.0 | 8.0 ± 2.0 | 0 |
| Pioner | Hypocotyl | 86.0 | 0 | 0 |
| | Cotyledon | 96.0 | 4.0 ± 0.8 | 30.0 ± 4.2 |
| Sunbred 281 | Hypocotyl | 100 | 0 | 0 |
| | Cotyledon | 100 | 8.0 ± 1.0 | 0 |

scoring system (see Materials and Methods).Means were calculated from 60 replicated samples for each treatment.

for their morphogenic potential on media containing a range of hormonal combinations including NAA and BAP illustrated that callus production per cotyledon explant depended on the genotypes (**Tables 1** and 4). Cotyledon explants taken from 4-day-old seedlings of Sanbro produced significantly higher amount of callus per explant than Sunbred 281 (**Table 4**). In spite of the fact that, cotyledon explants taken from older seedlings produced more callus, the younger seedlings (4-day-old) induced higher adventitious shoot formation (**Table 3** and **6**). Generally, the high percentage of root formation occurred on the callus when the age of the seedling increased.

Embryo like structure and shoot regeneration varied from 0 % to 27 % while rooting efficiency was between 6.8 % and 68.5 % depended on the experiments (**Table 5, 6, 7**). These results also demonstrated that the genetic structure of the plant controls in vitro sunflower regeneration. The percentage mean of callus induced embryo like structure and shoot formation was 0-27.2% for all ages. On the other hand, root production per responding callus varied between 19.0% and 68.5% (**Table 6**). There was also significant interaction between light and genotypes in terms of callus production (Table 1). Sunbred 281 showed the highest regeneration response in light while Sanbro had the highest regeneration in dark.

The results have indicated that selection of

TABLE 6

**Influence of cultivar and seedling age on the regeneration of sunflower**

| Genotypes | Eksplant | Callus response (%) | Morphogenic callus | |
|---|---|---|---|---|
| | | | Embryo like structure and shoot regeneration per responding callus (% ± SE) | Root production per responding callus (% ± SE) |
| Sunbred 281 | 4 | 100 | 27.2 ± 8.9 | 30.8 ± 12.4 |
| | 9 | 100 | 10.52 ± 3.6 | 68.0 ± 6.6 |
| | 15 | 100 | 6.2 ± 3.8 | 68.5 ± 10.3 |
| Pioner | 4 | 100 | 8.0 ± 3.7 | 19.0 ± 8.7 |
| | 9 | 100 | 4.2 ± 1.8 | 51.5 ± 4.2 |
| | 15 | 100 | 0.8 ± 0.3 | 49.6 ± 5.9 |
| Sunbro | 4 | 100 | 20.4 ± 5.0 | 19.2 ± 5.8 |
| | 9 | 100 | 0 | 24.9 ± 5.5 |
| | 15 | 100 | 0 | 55.7 ± 5.8 |

scoring system (see Materials and Methods).Means were calculated from 60 replicated samples for each treatment.

TABLE 7

**Influence of cultivar, hormone combinations and light conditions on the regeneration of sunflower**

| Genotypes | Light conditions | Hormonal composition of the medium | Callus response (%) | Morphogenic callus | |
|---|---|---|---|---|---|
| | | | | Embryo like structure and shoot regeneration responding callus (% ± SE) | Root production per responding callus (% ± SE) |
| Sunbred 281 | Light | 0.5 mg/l NAA 0.5 mg/l BAP | 100.0 | 17.0 ± 4.2 | 10.0 ± 3.7 |
| | | 1.0 mg/l NAA 1.0 mg/l BAP | 100.0 | 12.3 ± 6.9 | 16.8 ± 2.4 |
| | | 1.5 mg/l NAA 1.5 mg/l BAP | 100.0 | 8.0 ± 1.6 | 18.4 ± 4.6 |
| | Dark | 0.5 mg/l NAA 0.5 mg/l BAP | 92.4 | 0 | 14.2 ±3.9 |
| | | 1.0 mg/l NAA 1.0 mg/l BAP | 100.0 | 12.52 ± 5.64 | 25.2 ± 6.8 |
| | | 1.5 mg/l NAA 1.5 mg/l BAP | 99.0 | 1.7 ± 1.6 | 27.5 ±10.5 |
| Sunbro | Light | 0.5 mg/l NAA 0.5 mg/l BAP | 99.1 | 7.0 ± 2.8 | 29.5 ± 6.3 |
| | | 1.0 mg/l NAA 1.0 mg/l BAP | 99.0 | 6.0 ± 2.4 | 24.2 ± 3.3 |
| | | 1.5 mg/l NAA 1.5 mg/l BAP | 100.0 | 7.0 ± 2.5 | 24.0 ± 4.7 |
| | Dark | 0.5 mg/l NAA 0.5 mg/l BAP | 100.0 | 14.2 ± 3.5 | 17.5 ±2.5 |
| | | 1.0 mg/l NAA 1.0 mg/l BAP | 100.0 | 3.4 ± 1.1 | 8.3 ± 3.8 |
| | | 1.5 mg/l NAA 1.5 mg/l BAP | 99.2 | 7.5 ± 2.8 | 6.8 ± 1.3 |

*scoring system (see Materials and Methods).Means were calculated from 60 replicated samples for each treatment.

genotypes had a certain role in callus production per cotyledon explant. Punia and Bohorova (16) demonstrated similar results with six wild species of sunflower. They showed that the effect of genotype, explant and medium were very important on callus induction and plant development. The effect of genotypes on sunflower regeneration was also investigated by Paterson and Everett (13) who revealed that regeneration potential in sunflower was under multigene control with incomplete dominance of genes.

In all tested hormonal combinations, Sunbred 281 grow in MS medium containing BAP (0.5 mg/l) in dark did not produce any embryo like structure and shoot regeneration but callus and root production were occurred. Ceriani et al. (3) were also found similar results. They reported that shoot differentiation was replaced by callus proliferation in media supplemented with auxin. MS medium with 0.5 mg/l NAA, 0.5 mg/l BAP and 1.0 mg/l NAA, 1.0 mg/l BAP induced nodular and friable callus respectively.

Optimized procedures for the callus induction and rapid production of regenerated plantlets of sunflower have been established in order to facilitate studies of the transformation and clonal propagation. The efficiency of regeneration system can be improved with the examination of other factors such as explant manipulation and physical culture conditions.

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