EFFECTS OF NAPHTHALENEACETIC ACID AND N6-BENZYLADENINE ON ANDROGENESIS IN *HELIANTHUS ANNUUS* L.

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ABSTRACT

The aim of this study was to investigate the effects of NAA and BA on androgenesis in sunflower anthers. Hybrid sunflower breeding line taken from Trakya Agricultural Research Institute was used as a material. 70 days old capitula were sterilized and then anthers obtained from different sized capitula and flowers were transferred to the MS medium including four different concentrations (0, 0.5, 1 and 2 mg/l) of NAA and/or BA. Anther cultures were incubated at photoperiod or continuous dark conditions. Observations showed that uninucleate microspores were available in flowers 3-4 mm in length. While maximum callus induction for photoperiod condition was 87% when MS medium including 2 mg/l NAA and 1 mg/l BA was used, it was 90% for continuous dark condition at the same medium. Both plant growth regulators had no effect on androgenesis when used alone. But the androgenetic stimulation was gradually rose when they used together with increasing concentrations. When only light effect on androgenesis taken into account, callusing was 26% and 12% for photoperiod and continuous dark conditions respectively. There was no regeneration when anthers were transferred to regeneration medium. Investigation on anther-derived callus showed that there were both haploid and diploid cells too in it. According to these results, it was proposed that callus were formed from microspores. Finally, it was determined that NAA, BA and light had significant effects on stimulating androgenesis in sunflower.

Keywords: *in vitro,* Sunflower, Anther Culture, Haploid Production

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important source of vegetable oil in the world and in Turkey. In Turkey, sunflower oil is often preferred as vegetable oil consumption. Therefore, the importance of sunflower is increasing in recent years. Hybrid varieties are generally used in sunflower agriculture. However, due to the use of the same gene source, it is approaching the upper limit of capacity of genetic productivity in varieties obtained using traditional breeding methods. It is only possible through the use of biotechnological methods to overcome this problem. Therefore, the studies in this context in recent years are getting important every day and new achievements are obtained every year.

In this study, anther culture which is one of the biotechnological methods for obtaining haploid plant has been studied. As known, while breeding process with traditional methods lasts 7-8 generation, it can be reduced to a single generation using double-haploid technology. In this context, with the aim of promoting androgenesis in the anthers of sunflower (Helianthus *annuus* L.), the effects of different hormone combinations have been investigated. And, cytological observations were performed to determine the ploidy level of obtained calli.

Choi (1991), argued that optimal levels of hormones are needed according to the physiological status of anther tissue for androgenesis to reach success. Several specific plant growth regulators are used in anther culture. Generally, auxins such as 2,4-D, IAA, IBA, NAA, or alternatively, cytokinins such as kinetin, zeatin, and riboside were used in initiation and regeneration medium (Luckett and Darvey 1992). Vijaya Priya et al. (2003) has argued that for callus induction in wild sunflower species the presence of 2,4-D with auxin and cytokinin at low concentrations is sufficient but additional auxin and cytokinin is not necessary to increase the amount of callus. Similarly, despite the best callusing rates are obtained with the use of 0,5 mg/L NAA and BA in the culture of anthers derived from interspecific hybrids of the sunflower, when the concentration was increased to 1 mg/L, there was no significant changes in the rate of callusing (Nurhidayah 1996). On the other hand, Gurel and Kazan (1998) reported that increasing BAP concentration for all NAA concentrations was constantly increased the rate of callusing from different genotype of sunflower explants taken from different somatic tissues. Based on these information, the effect of NAA and/or BA (0 - 0,5 - 1 and 2 mg/L) on sunflower anthers for callus induction were studied. In Turkey, haploid culture studies on sunflower are less common and this study has been made in terms of creating a basis for these studies.

MATERIALS AND METHODS

In this study, hybrid sunflower breeding lines which has resistance gene to the orobanche and downy mildew was used. Seeds were obtained from Trakya Agricultural Research Institute. Sunflower capitula (figure 1) were collected from the plants of 70 days old. Flower buds (figure 2) 3, 4, 5 and 6 mm in length were isolated from capitulum of 3, 4, 5 and 6 cm diameter. The anthers removed from these flowers were analyzed by the asetocarmine squash method under Olympus photomicroscope. For sterilization Anthers were shaken in 15% commercial bleach for 20 minutes and for 2 minutes in 70% alcohol solution then rinsed with sterile distilled water three times. The sterile anthers were transferred to culture medium at photoperiod (16/8 light/dark) or continuously dark conditions. MS basic medium was combined with four different concentrations of NAA and/or BA (0-0,5-1-2 mg/L). 0,1% and 0,5% PVP added to the medium to prevent browning observed in Anther. The calli were transferred to MS medium which includes 0,5 to 1 or 2 mg/L BA and/or 0,1 mg/L NAA for regeneration experiments. Some calli are reserved for cytological examination in order to determine ploidy level of the cell. Fresh calli were analyzed by the asetocarmine squash method under Olympus photomicroscope. The differences among the averages of all experimental groups were tested by one-way ANOVA. In this test, the differences were compared with Tukey test at 0,01 significance level.

RESULTS AND DISCUSSION

Microscopic examination on the anthers obtained from 3, 4, 5 and 6 mm length of flowers was made with the aim of determining the appropriate microspores for successful anther culture (figure 3). It was observed that uninuclear microspores were seen in flowers of 3 and 4 mm length. Meriç (2002) reported that 3, 4 and 4.5 mm in length sunflower flowers are in the consistent with our preliminary investigations. Therefore, in this study with the aim to obtain microspores in the uninucleate stage, anthers were provided from flower of 3 and 4 mm in length. Additionally, according to our study, it was observed that the flowers of 3-4 mm in length located in the uninucleate stage, but 5.5 cm and up in length are in the binucleate stage. The literature is capitulum of 3 to 4 cm in diameter.

Figure 1. **Sunflower capitula**

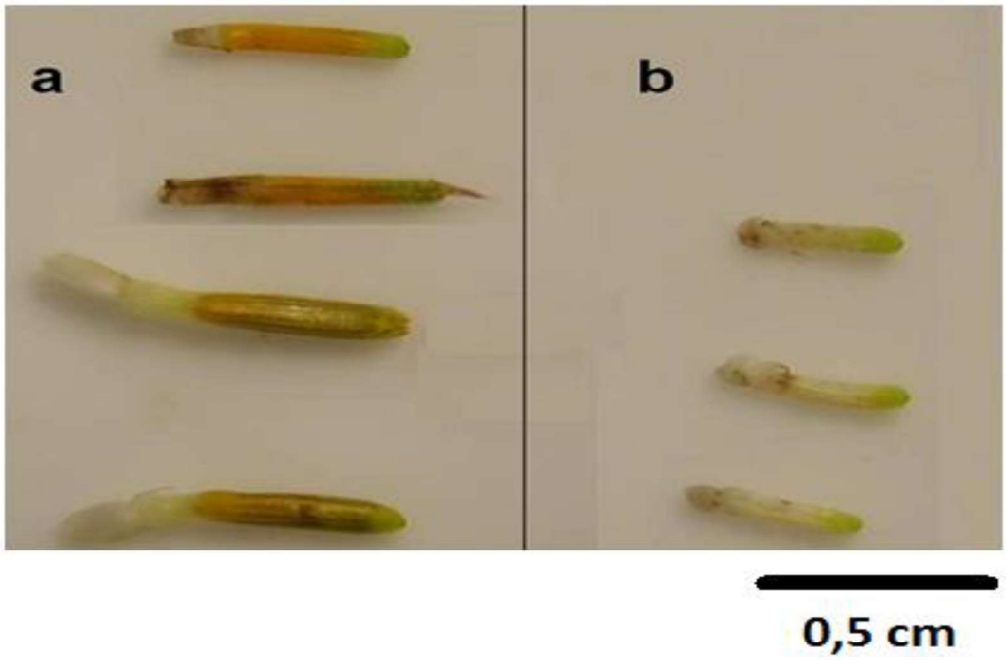


Figure 2. Sunflower tubular flowers. a) 5-6 mm in length, b) 3-4 mm in length

One of the most evident result of our study, any micspor response has not occurred in hormone-free basic MS medium (Table 1). All anthers in this medium browned and lost their vitality after 2 weeks even they showed some swelling at first. Vijaya Priya et al. (2003) studied on six different sunflower genotypes and reported that callus formation was not observed in hormone-free MS basic medium as similar to our findings. They explained this case by idea of the endogenous hormone levels in the anthers were not enough the promote callusing. We agree with this idea because of androgenic response does not occur in hormone - free MS medium in our study.

Table 1. Effect of NAA and BA on Callusing.

Light Regime	NAA mg/L	BA mg/L	Callusing %*
Photoperiod	0	0	0 [a]
"	0	0,5	0 [a]
"	0	1	0 [a]
"	0	2	0 [a]
"	0,5	0	0 [a]
"	0,5	0,5	7 [a]
"	0,5	1	27 [abcd]
"	0,5	2	47 [cde]
"	1	0	0 [a]
"	1	0,5	0 [a]
"	1	1	47 [cde]
"	1	2	50 [def]
"	2	0	0 [a]
"	2	0,5	73 [efg]
"	2	1	87 [g]
"	2	2	77 [fg]
Continuously Dark	0	0	0 [a]
"	0	0,5	0 [a]
"	0	1	0 [a]
"	0	2	0 [a]
"	0,5	0	0 [a]
"	0,5	0,5	0 [a]
"	0,5	1	20 [abc]
"	0,5	2	20 [abc]
"	1	0	0 [a]
"	1	0,5	0 [a]
"	1	1	0 [a]
"	1	2	7 [a]
"	2	0	0 [a]
"	2	0,5	40 [bcd]
"	2	1	90[g]
"	2	2	13 [ab]

*Values within each column followed by the same letters are not significantly different by the Tukey test at 0.01% probability level.

Another important result is that when NAA or BA used alone, there was no callus formation from anther (Table 1). On the other hand, when NAA and BA used together callus formation was observed in anther after two weeks (figure 4). Moreover, when NAA and BA concentration increased, the rate of callus formation is increased significantly. The best callus formation ratio (90%) was obtained on MS basic medium consisting of 2 mg/L NAA and 1 mg/L BA after 6 weeks (figure 5). Vijaya Priya et al. (2003), in their study, when 0.1 mg/L

NAA and 0.2 mg/L BA is added on the MS medium of, callus formation was observed depending on genotype ranged from 77% to 97%. When the rate of growth regulators are increased as 2.0 mg/L NAA and 1.0 mg/L BA, callus formation rates ranging still from 90% to 74% depending on the genotype was observed. They reported that the increasing concentrations of auxin and cytokinin (NAA and BAP) do not have a significant effect on the callusing rate. Similarly, Nurhidayah (1996) reported in his study that, the increasing concentrations of NAA and BA do not cause a significant increase in callus formation. These findings contradict with our findings. On the other hand, Gurel and Kazan (1998) reported that increasing BAP concentration for all NAA concentrations was constantly increased the rate of callusing from different genotype of sunflower explants taken from different somatic tissues. Although Vijaya Priya et al. (2003) has been reported that optimum doses of growth regulators are enough to callus induction and the increase of the dose does not influence the callusing rate, we claim that increasing the concentration of NAA and BA, increases callus formation rate based on the sunflower genotypes we used. As the different genotypes are used in each different study, this is believed to be the cause of these conflicts. Different genotypes and incubation conditions (constant light-dark-photoperiod) have various effects on callus formation. Considering the interaction between these factors, if the target is androgenetic stimulations in anthers of sunflower, our proposal is that concentration and combination of growth regulators must be optimized separately for each genotype and incubation condition.

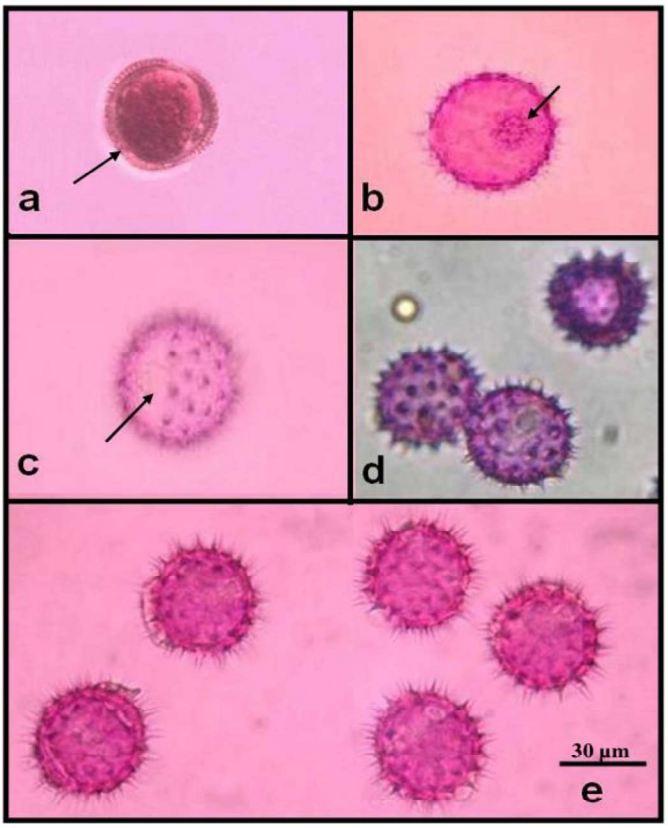


Figure 3. Sunflower microspores and pollens. a) early-uninucleate microspore stage b-c) late-uninucleate microspore stage d-e) binucleate pollens

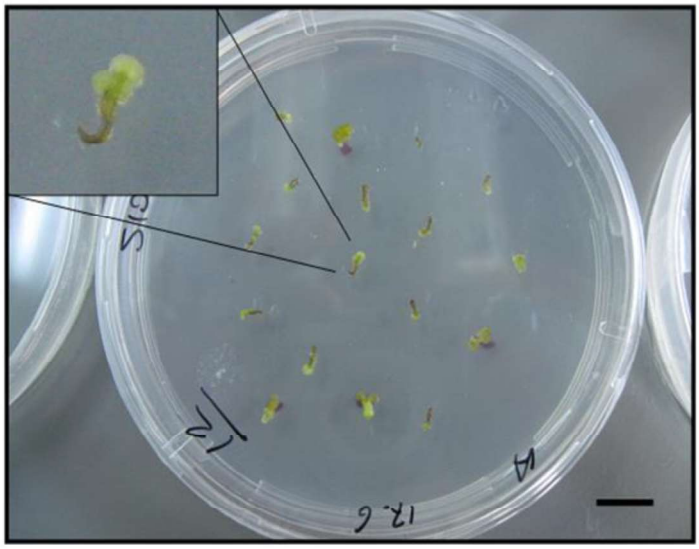


Figure 4. Second week of anther culture. MS medium includes 2 mg/L NAA and 1 mg/L BA. Bar=1 cm.

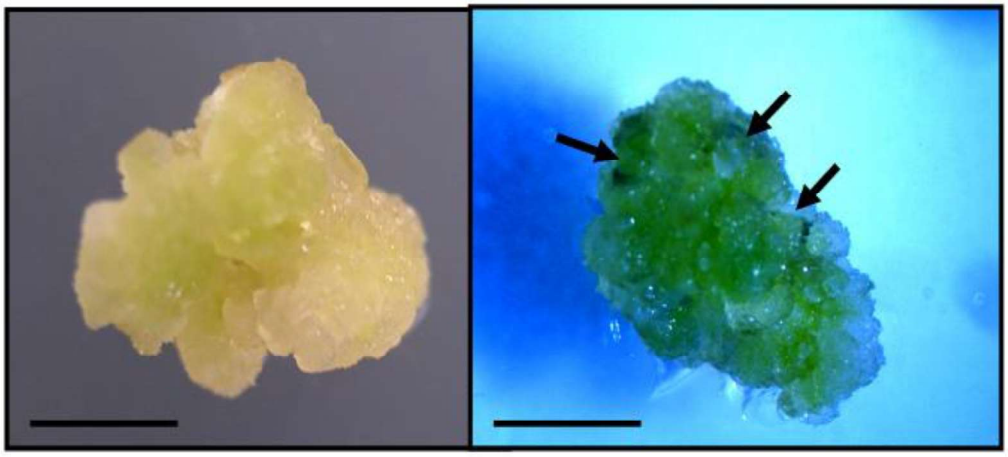
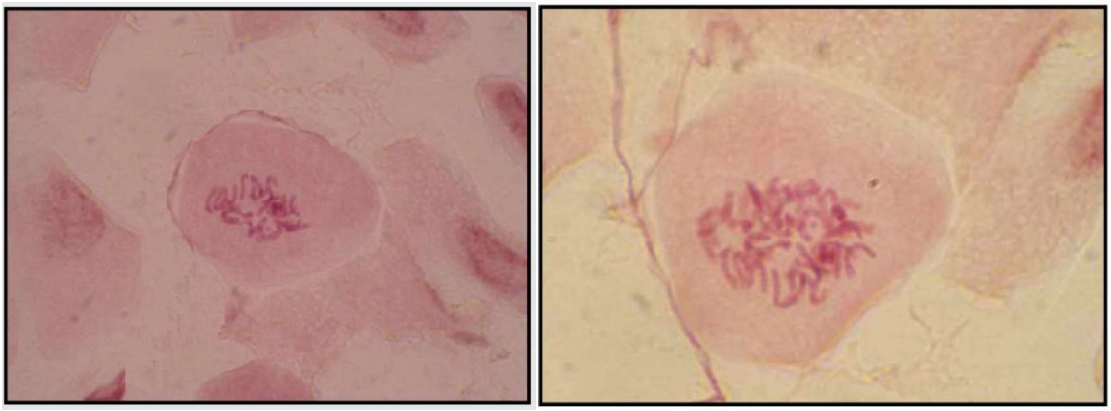


Figure 5. Sixth weeks old calli on MS medium includes 2 mg/L NAA and 1 mg/L BA. Yellowish callus occurred in dark condition on left, and green callus occurred in photoperiod condition on right. Bar=1 cm.

Previous studies have reported that by adding antioxidants such as PVP to the medium prevent browning (Roy and Sarkar 1991; Sudripta et al. 1999). In this study four different concentrations of PVP were tested with the reason of overcome the browning problem occurred in anther, but there was no statistically significant effect in terms of PVP. There was no shoot regeneration from callus in any experiment group. It is necessary to keep in mind that the low productivity is common in shoot regeneration from anther culture (Thengane et al. 1994). When the literature is studied it is outstanding that this case is common. Bohorova et al. (1985) has achieved anther callus around 70-100%, but failed to obtain shoot regeneration. Gürel et al. (1991a) reported low frequency direct embryo formation and shoot regeneration in the sunflower anther culture, but a whole plant could not be obtaioned. Gürel et al. (1991b) and Coumans and Zhong (1995) tested the isolated microspore culture and even they obtained continuous cell division and microcallus formation, they could not achieve shoot regeneration. A total of 200 callus have been examined, but chromosomes could be counted in small number of cells. However, both haploid and diploid cells were also observed in the callus (figure 6). The observation of haploid cells indicates that calli are microspore-derived. It is believed that diploid cells are spontaneous double-haploids as well.

Figure 6. Microscopy of sunflower callus. Hapliod (on left) and diploid (on Right) cells.



As a result, in this study it was found that the NAA should be used together with the BA to stimulate androgenesis from sunflower anther derived from flowers 3-4 mm in length. It is essential to product double-haploid plants originated from anther to contribute the sunflower breeding work in Turkey. Therefore, we hope the results of this study will be a guide for future studies.

This research which is the part of Sergun DAYAN's doctoral thesis was supported by Trakya University Academic Research Projects Unit (Project number: TÜBAP 2009-92).

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