Effect of IAA and BAP Application in Varying Concentration on Zygotic Ovule Culture in *Nigella sativa* L.

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Abstract:. This study was carried out to test the effect of BAP (6-Benzylaminopurine) and IAA (Indole-3-acetic acid) hormones, both on embryo development in in vitro culture, and on obtaining hybrid plants of black cumin. Ovule culture was preferred in the study because the seeds were too small and embryo isolation was difficult. Ovule cultures were done by using populations obtained from producers in Ankara and Denizli in Türkiye and the Çameli variety. Hybridizations were done according to the semi-diallel hybridization method to gain zygotic embryos. MS (Duchefa Biochemie, Catalog No: M0222) mediums, with BAP or IAA (0.0, 0.5, 1.0, 2.0 mg/l) and BAP+IAA (1.0+1.0, 0.1+2.0, 2.0+0.5 mg/l) added, were used for ovule culture. As a result of the research, the highest success was gained for direct regeneration (1.7%) in the medium without hormone. The highest success gained for callus regeneration (2.3%) was in the medium having 2.0 mg/l BAP + 0.5 mg/l IAA added. The highest plant regeneration from callus was gained in the medium with an added 1.0 mg/l IAA as 7.7%.

Keywords: Auxin, black cumin, cytokinin, embryo culture, *Nigella sativa*

***Nigella sativa* L.'da Zigotik Ovül Kültürü Üzerine Farklı Konsantrasyonlarda IAA ve BAP Uygulamasının Etkisi**

Öz: *Bu çalışma BAP (6-Benzylaminopurine) ve IAA (Indole-3-acetic acid) hormonlarının Çörek otu bitkisinde in vitro kültürde embriyo gelişimi üzerine etkisini araştırmak ve melez bitkiler elde etmek amacıyla gerçekleştirilmiştir. Tohumların çok küçük olması ve embriyo izolasyonunun zor olması nedeniyle çalışmada ovül kültürü tercih edilmiştir. Ovül kültürleri, Türkiye'de Ankara ve Denizli'deki üreticilerden elde edilen popülasyonlar ve Çameli çeşidi kullanılarak yapılmıştır. Melezlemeler, zigotik embriyolar elde etmek için yarı diallel melezleme yöntemine göre yapılmıştır. Ovül kültürü için BAP veya IAA (0.0, 0.5, 1.0, 2.0 mg/l) ve BAP+IAA (1.0+1.0, 0.1+2.0, 2.0+0.5 mg/l) eklenmiş MS (Duchefa Biochemie, Katalog No: M0222) besiyerleri kullanılmıştır. Araştırma sonucunda en yüksek başarı doğrudan rejenerasyonda (%1,7) hormonsuz ortamda elde edilmiştir. Kallus rejenerasyonunda en yüksek başarı (%2,3) 2,0 mg/l BAP + 0,5 mg/l IAA eklenmiş ortamda elde edilmiştir. Kallustan en yüksek bitki rejenerasyonu %7,7 oranında 1,0 mg/l IAA eklenmiş ortamda elde edilmiştir.*

Anahtar Kelimeler: *Çörek otu, embriyo kültürü, Nigella sativa, oksin, sitokinin*

INTRODUCTION

Black cumin (*Nigella sativa* L.), a member of the Ranunculaceae family, originates from Eastern Mediterranean countries, Eastern and Southern Europe and Western Asia. It grows wild or is cultivated in the Balkans, Southern Europe, North Africa, the Middle East and India. It has a rich historical and religious background. The plant is widely grown for use as a spice in many countries, from Morocco to Northern India and Bangladesh, China, the Pacific, the Balkan countries, East Africa and Russia (Burdock, 2022). The genus Nigella has a total of 20 species, 14 of them are stated to be in Turkey's flora (Seçmen et al., 2000). Although there are many species of black cumin, only *N. sativa* and *N. damascene* species are grown commercially (Uysal, 2021).

The goal of biotechnological studies is to improve plant breeding: by shortening the process of plant breeding, providing the most suitable conditions for the growth of plants in preparing the ground; it is also to increase the capacity of traditional production systems so as to increase both yield and quality by growing healthy plants. In developing a new variety with the characteristics, we want; it is possible to obtain the desired results in a shorter time by using biotechnological methods. A longer time is needed when using classical breeding methods. With these techniques can be – gained new variations with somaclonal variation, haploid plant breeding with anther culture – breeding process can be shortened; inhibitor mechanisms can also be disabled, and it is possible to cross plant genera and species by embryo culture (Narayanaswami and Norstog, 1964; Kurt and Şavşatlı, 2005; Uysal and Sevindik, 2021).

Embryo culture technique was first used by Hanning in 1904. Mature embryos isolated from seed of Raphanus and Cochlearia were cultured in mediums containing mineral sugar and salt, and plantlets were obtained from them (Drew, 1997). Embryo culture is a technique that involves

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isolating primitive embryos formed after a period as maternal embryos or after fertilization, culturing them in the medium, and obtaining plants from them. Zygotic primitive embryo culture is used to obtain plants capable of survival after interspecific crosses. Ovule culture can be preferred instead of embryo culture in some plants have small seed. The aim of this study is to determine effect of IAA and BAP hormones on ovule culture and to obtain hybrid plants of black cumin.

MATERIALS AND METHODS

Ovule cultures were done by using populations obtained from producers in Ankara and Denizli in Türkiye and the Çameli variety. Hybridizations were done according to semi-diallel hybridization method to gain zygotic embryos. The black cumin plants were grown in greenhouse from November to May to make hybridizations. Seeds were sown every 15 days to save time during the hybridization phase. The plants were grown in 3.5-liter pots by using one part garden soil and one part barnyard manure. Five plants were grown in each pot. Hybridizations were done from 8.00 am to 10.00 am. Pollinations were done one day later after emasculations. After emasculation and hybridization, the flowers were isolated by using hybridization bags to avoid cross pollinations and labeled. The capsules including zygotic embryos were harvested 12 days following pollination. The harvested capsules were carried to laboratory in sterilized distilled water. The capsules were sterilized before ovule culture. For this purpose, the capsules were first treated with 70% ethanol for 10 sec., then treated with 10% commercial bleach (ACE brand containing 5%< chlorine-based bleach) for 5 min.; then washed with sterilized water 3 to 5 times. Ovule culture was preferred because the seeds in the black cumin plants were small, and the isolation of the embryo was difficult. For ovule culture, MS medium (Duchefa Biochemie, Catalog No: M0222) was used, with different added concentrations of Indole-3-acetic acid (IAA) or 6-Benzylaminopurine (BAP) (0.0, 0.5, 1.0, 2.0 mg/l) and BAP+IAA (1.0+1.0, 0.1+2.0, 2.0+0.5 mg/l).

IAA and BAP were first dissolved in 1 N NaOH (sodium hydroxide), then used. The MS medium was supplied as powder and added at 4.4 g per liter, the hormones were added if required, then sucrose was added; then the pH was adjusted to 5.8 and agar was added; then sterilization was done in autoclave at 121 °C for 15 minutes. Nine-cm diameter petri dishes were used for ovule cultures, 20 ovules being put in each petri dish. Five petri dishes were used for each medium, 100 ovules being cultured in each medium, totaling 300 ovules in 3 hybridization combinations. After sowing, the petri dishes were stored under 25°C temperature and 16 hours light photoperiod conditions.

The cultures were controlled every other day during the next 2 months and the regenerations were noted, if any. The plantlets, were regenerated *in vitro*, transferred to MS + 0.5 mg/l BAP and 0.1 mg/l IAA. When the plants had 2-3 cm root and 4-5 cm shoot, they were transferred to 1 litre pots containing one part garden soil and one part barnyard manure, one plant in each pot. The plants, were transferred to pots, were isolated by perforated bag for three days to adapt *in vivo*. The matured plants were harvested separately.

Microsoft Excel Package Program was used to calculate success rates and draw graphs from the data obtained as a result of the research. Analysis of variance method was applied by using SPSS V26 (PASW Inc., Chicago. IL. USA) statistical package program for the significance of the differences between the means. The significance of the difference between the analysis of variance and population means was tested using the significance levels obtained from the least significant difference test. It was also investigated as to which means were different using the Duncan multiple comparison test.

RESULTS AND DISCUSSION

The results of the cultured 100 ovules for each medium are given in Table 1.

As can be seen in Table 1, the highest success rate for direct germination was gained from cultured ovules in MS medium without hormone (MS0), 0.5 mg/l IAA and 2.0 mg/l BAP + 0.5 mg/l IAA from the combination of Denizli x Çameli and in MS0 from the combination of Ankara x Çameli as 2%.

When the research results are evaluated at the level of mediums, the highest overall success rate for direct germination was in MS0 as 1.7%. No direct regeneration was observed from the mediums 0.5 mg/l BAP, 1.0 mg/l BAP, 2.0 mg/l IAA, 1.0 mg/l BAP + 1.0 mg/l IAA and 0.1 mg/l BAP + 2.0 mg/l 1 IAA (Table 1). For direct germination, MS0 medium was statistically the best among the mediums studied in this research. The other mediums were statistically in the same group (Table 1).

Totally 12 hybrid plants were gained by direct germination, and all of them adapted to *in vivo* conditions. They matured and were harvested.

When the research results are analysed at the genotype level, the highest overall success for direct germination success rate was achieved in the Denizli x Çameli combination as 10.6%. In addition, Ankara x Çameli (0.5%) and Ankara x Denizli (0.1%) combinations had similar success rates on direct germination (Table 1).

The highest success rate was gained from cultured ovules for callus formation in MS+2.0 mg/l BAP + 0.5 mg/l IAA from the combination of Denizli x Çameli and in MS +1.0 mg/l BAP from the combination of Ankara x Çameli as 4% (Table 1).

Table 1. Numbers of callus and germination, and success rates (%) from cultured ovules

Medium* (mg/l)	Genotypes						General Success	
	Ankara x Çameli		Ankara x Denizli		Denizli x Çameli			
	Germ.	Callus	Germ.	Callus	Germ.	Callus	Germ.	Callus
MS0	2 (2.0%)	0	1 (1.0%)	0	2 (2.0%)	0	5 (1.7%) a	0
0.5 B	0	1 (1.0%)	0	2 (2.0%)	0	1 (1.0%)	0 b	4 (1.3%)
1.0 B	0	4 (4.0%)	0	2 (2.0%)	0	0	0 b	6 (2.0%)
2.0 B	1 (1.0%)	1 (1.0%)	0	0	0	3 (3.0%)	1 (0.3%) b	4 (1.3%)
0.5 I	1 (1.0%)	0	0	0	2 (2.0%)	0	3 (1.0%) b	0
1.0 I	1 (1.0%)	2 (2.0%)	0	0	0	1 (1.0%)	1 (0.3%) b	3 (1.0%)
2.0 I	0	0	0	0	0	0	0 b	0
1.0 B+1.0 I	0	0	0	2 (2.0%)	0	1 (1.0%)	0 b	3 (1.0%)
0.1 B+2.0 I	0	0	0	0	0	2 (2.0%)	0 b	2 (0.7%)
2.0 B+0.5 I	0	0	0	3 (3.0%)	2 (2.0%)	4 (4.0%)	2 (0.7%) b	7 (2.3%)
General Success	5 (0.5%)	8 (0.8%)	1 (0.1%)	9 (0.9%)	6 (0.6%)	12 (1.2%)	12 (0.4%)	29 (1.0%)

*B: BAP, I: IAA. Germ: Direct germination

When the research results are evaluated at the level of mediums, the highest overall success rate for callus formation was in MS+2.0 mg/l BAP + 0.5 mg/l IAA as 2.3%. No callus regeneration was observed from the mediums MS0, MS+0.5 mg/l IAA and MS+2.0 mg/l IAA (Table 1). There was no statistically significant difference between the mediums (please see Table 2).

When the research results are analysed at the genotype level, the highest overall success for callus formation was achieved in the Denizli x Çameli combination as 1.2%. In addition, Ankara x Çameli (0.8%) and Ankara x Denizli (0.9%) combinations had similar success rates on callus formation (Table 1).

The variance analysis table about callus formation and direct germination are given Table 2.

As shown for direct regeneration in Table 2, in terms of direct germination, there was a significant difference between the success averages according to the mediums ($p < 0.01$). There was no significant difference between the mean success rates according to genotypes ($p \not< 0.01$). Likewise, no significant difference was found between the mean success rates according to the medium and genotype interaction ($p \not< 0.01$). In terms of callus formations, no significant difference was found between the success averages of the medium ($p \not< 0.01$). No significant difference was found between the mean success rates according to genotypes ($p \not< 0.01$). Likewise, no significant difference was found between the mean success rates according to the medium and genotype interaction ($p \not< 0.01$).

Numbers of total plantlets obtained from callus and the average number of plantlets per callus are given in Table 3.

As can be seen in Table 3, the highest success rate was gained for plantlet formation from callus in MS+1.0 mg/l IAA as 23 numbers from the combination of Ankara x Çameli. Both of 2 calluses obtained in this medium regenerated into plantlets. In terms of overall success averages, the highest success rate was obtained from in MS+1.0 mg/l IAA medium (average 7.67 numbers) from the Ankara x Çameli combination (average 2.88 numbers). Another medium in which plants were formed from callus was the medium containing 0.5 mg/l BAP. In this medium, 7 plantlets were formed in the Ankara x Denizli combination and 2 plantlets in the Denizli x Çameli combination, and the overall average number of plantlets per callus was determined as 2.25 in this medium. Except for these two mediums, plantlets could not be formed in other mediums in which callus formation was detected. A total of 32 plantlets were obtained from a total of 29 calluses obtained during the research, and the average plantlet formation rate per callus was 1.10. The plantlets were transferred to the medium MS + 0.5 mg/l BAP and 0.1 mg/l IAA and 28 of them grew large enough to transfer to in vivo conditions. Of these, 24 matured and were harvested.

Table 2. Variance analysis table in terms of direct regeneration and callus formation

Variance source	Degrees of freedom	Sum of squares		Mean squares		F		P-value	
		Germ.	Callus	Germ.	Callus	Germ.	Callus	Germ.	Callus
Medium	9	2.960	3.307	0.329	0.367	**2.902***	1.361	0.004	0.214
Genotype	2	0.493	0.333	0.247	0.167	2.176	0.617	0.118	0.541
Interaction	18	1.640	7.133	0.091	0.396	0.804	1.468	0.692	0.114
Error	120	13.600	32.400	0.113	0.270				
Total	149	18.693	43.173						

*: $p < 0.01$

Table 3. Numbers of total plantlets obtained from callus and the average number of plantlets per callus

Medium*	**Genotypes**			Average Number of Plantlets
(mg/l)	Ankara x Çameli	Ankara x Denizli	Denizli x Çameli	
MS0	0	0	0	0
0.5 B	0	7 (%3.5)	2 (%2)	9 (%2.2)
1.0 B	0	0	0	0
2.0 B	0	0	0	
0.5 I	0	0	0	0
1.0 I	23 (%11.5)	0	0	23 (%7.7)
2.0 I	0	0	0	0
1.0 B+1.0 I	0	0	0	0
0.1 B+2.0 I	0	0	0	0
2.0 B+0.5 I	0	0	0	0
General success	23 (%2.9)	7 (%0.8)	2 (%0.2)	32 (%1.1)

*B: BAP, I: IAA.

In terms of germination status, the highest success rate (1.7%) was achieved in MS0 medium. According to the analysis of variance and Duncan multiple comparison results, it was concluded that medium has a significant and positive effect on germination. In contrast, Rezaei et al. (2018), Uysal (2021), and Uysal and Sevindik (2021) found higher success rates in mediums containing BAP and/or IAA.

The highest overall success in callus formation and germination was achieved in the Denizli x Çameli combination. Similarly, a high callus formation rate was obtained from the Denizli x Çameli combination in the study by Uysal and Sevindik (2021). Ankara x Çameli (0.8%) and Ankara x Denizli (0.9%) combinations have similar success rates in terms of callus formation. Ankara x Çameli combination also has a 0.5% success rate in terms of germination. A very low germination success rate was obtained in Ankara x Denizli combination (0.1%). According to the results of analysis of variance, no significant difference was found between the average of callus formation and germination success in terms of genotype.

DISCUSSION

Factors affecting success in embryo culture include genotype, age of embryos, composition of the medium, growing conditions of donor plants, light, temperature and humidity in the environments where the cultures develop (Hatipoğlu, 2012). In this study, all factors except for genotype and medium composition were applied equally for all crossing combinations. BAP (cytokinin) and IAA (auxin) hormones were added to the mediums at different concentrations. In *in vitro* regeneration, cytokinin hormones are used for shoot development and auxin hormones are used to promote root growth. Callus regeneration occurs when the concentrations of auxin and cytokinin used are high. The presence of these two hormones in equal amounts and at a balanced level in the medium also supports the formation of callus in *in vitro* culture (Hatipoğlu, 2012). In this study, the highest rate of direct regeneration was detected in the hormone free MS medium; and the absence of callus formation, especially the callus formation in the medium containing BAP or BAP+IAA, shows that the auxin and cytokinin hormones added to the medium promote callus formation in *in vitro* culture.

El-Mahrouk et al. (2018) made an ovule culture study in black cumin by using 6-benzyladenine (BA), kinetin (Kin), 2.4-dichlorophenoxyacetic acid (2.4-D), and α-naphthaleneacetic acid (NAA) for haploid plant production. As a result of that study, among different plant growth regulators (PGRs) tested, 2.4-D at 2 mg/l produced direct gynogenesis. The highest callogenesis percentage (100%) was obtained on MS medium containing 1 mg/l 2.4-D and 2 mg/l NAA.

Uysal and Sevindik (2021) used ovule culture in black cumin to improve hybrid plants. LS2.5 and MS mediums were used for this purpose. A total of 2904 ovules were cultured in the LS2.5 medium. 148 of them showed callus development. The callus formation rate was 5.10%, but plant regeneration could not be obtained from these calluses. A total of 3526 ovules were cultured in MS medium. Sixty plantlets were obtained from these ovules and plant formation rate was determined as 1.70%. 41 plants from these matured and were harvested.

Some of the *in vitro* studies using various plant hormones which gained high success are in *Nigella* spp.; Al-Ani (2008) cultured black cumin roots, hypocotyls and leaves on MS

medium supplemented with different concentrations of 2.4-D (0.0, 1.0, 2.0, 3.0, 4.0 mg/l) and Kinetin (0.0, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0 mg/l). The best callus production was obtained from leaf explants with 1 mg/l 2.4-D and 1.5 mg/l Kinetin. Chaudhry et al. (2014) had documented the callus response of different parts (leaf, epicotyl, hypocotyl and root) of in vitro grown seedlings of black cumin. This was determined to be on solid MS medium with different growth hormones, so as to generate inoculums for suspension cultures. The suspension culture was initiated in liquid MS medium supplemented with different hormones. The result of the study is that epicotyl region inoculated on MS medium supplemented with Kinetin (2 mg/l) + NAA (1 mg/l) produced faster growing creamy and friable callus compared to that of different parts of the seedlings and of different combinations used. Suspension cultures with Kinetin (2 mg/l) + NAA (1 mg/l) also showed the maximum growth rate and biomass accumulation followed by the medium including BAP (2 mg/l) + IAA (1 mg/l). Hypocotyls and cotyledon explants from black cumin seeds were cultured on MS media containing different types and various concentrations of growth regulators by ElNour et al. (2015). In this study black cumin explants were first cultured in the medium containing 1.0 mg/l and 5.0 mg/l Naphthalene acetic acid (NAA). As a result of the observation, it was reported that callus formation occurred rapidly in the second week. They reported that if the plant material is cotyledon, callus formation is slower when hypocotyls grown in 2.4-D medium in amounts of 5.0 mg/l and 0.5 mg/l are observed. Klimek-Chodacka et al. (2020) achieved high success in their study, using MS supplemented with mg/l BAP and 0.5 mg/l NAA for callus induction from hypocotyl and cotyledon explants. They had success rates of 83-100%. Somatic embryo formation and plant regeneration were successful on hormone-free media. Plant regeneration was observed at 76–95% of callus in *N. damescana*. Damanik et al. (2022) reported that MS medium modified with 2.4 D of 10 mg/l combination with GA_3 of 1 mg/l increased the percentage of callus growth. The combination of these two types of growth regulators also influenced the number of shoots, plant height and, number of roots. Dekami et al. (2023) made tissue cultures of *Nigella arvensis* using an MS medium supplemented with 2.4-D, NAA or IBA (0.0, 0.5, 1.0 and 1.5 mg/l) combined with kinetin or BAP (1.5 mg/l and 2.0 mg/l) to induce and grow callus in the presence and absence of light. In that study, the maximum callus induction (80.9%) was obtained with 1.0 mg/l 2.4-D and 1.5 mg/l kinetin in the presence of light. As observed; different parts on Nigella plants were used for regeneration in *in vitro* culture with mediums supplemented with various plant hormones, gaining various degrees of success.

CONCLUSIONS

In the present study, we have cultured black cumin's zygotic ovules in MS medium supplemented with BAP and IAA, gaining a success rate of 1.7% for direct regeneration in the medium without hormone, and 2.3% for callus regeneration in the medium having an added 2.0 mg/l + 0.5 mg/l IAA. The plants regenerations from calluses were 7.7% by using 1.0 mg/l IAA and 2.2% by using 0.5 mg/l BAP. The higher success rate can be achieved by using different hormones such as 2.4 D and Kinetin.

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