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Original scientific article

DEVELOPMENT OF BREEDING LINES BY ZIGOTIC OVULE CULTURE IN *Nigella sativa* L.

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This study was carried out in order to develop an embryo culture technique and to establish breeding lines with this technique. Ovule culture was preferred in the study because the seeds were too small and embryo isolation was difficult. Embryo cultures were done by using populations obtained from producers in Samsun, Denizli, Isparta, Mersin in Turkey and Çameli black cumin variety. Hybridizations were done according to the semi-diallel hybridization method to gain zygotic embryos. LS2.5 and MS (Sigma Aldrich Catalog number: M5519) mediums were used for ovule culture and MSD4 medium was used growing the plants obtained from the ovules. As a result of the research, a total of 2904 ovules were cultured in LS2.5 medium. 148 of them showed callus development; callus formation rate was 5.10%. The highest callus formation rate in the investigated combinations was obtained from Çameli x Denizli combination at 7.26%. 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.

Keyword: Black cumin, embryo culture, in vitro, *Nigella sativa*, ovule culture

INTRODUCTION

Ranunculaceae is a large family of plants, represented by about 2,500 species belonging to about 60 genera in the world and spreading almost all over the world. In addition to favoring

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temperate and cool zones in both hemispheres, it shows a high diversity, especially in the temperate zones of the Northern hemisphere (YILDIRIM and GÜL, 2018). Black cumin (*Nigella sativa* L.) is a single-year plant from the family Ranunculaceae. It is cultivated in many countries especially Eastern Mediterranean countries (ILISULU, 1992). Black cumin has a total of 20 species, 14 of them are stated to be in Turkey's flora (SECMEN *et al.,* 2000). Although there are many species of black cumin, only *N. sativa* and *N. damascena* species are grown commercially. Black cumin is known to have a rich historical background in herbs used in medicine and has been used for many years to preserve food and to enhance flavor. The seeds of black cumin were found in the tomb of the 18th dynasty Pharaoh of Tutankhamen, in the Valley of the Kings. It is reported that the oil obtained from this plant was used by the Egyptian queen Cleopatra to provide health and beauty. Dioskorides used nigella oil to reduce nasal obstructions, to relieve nasal congestion, headache and toothache (RAGAA, 2010). In recent years, it has been reported that the fixed and essential oil of black cumin is beneficial with antibacterial, antitumor, sedative, analgesic and blood sugar lowering effects (NICKAVAR *et al.,* 2003).

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; and to increase the capacity of traditional production systems in order to increase yield and quality by growing healthy plants. In order to develop a new variety with the characteristics we want, it is possible to obtain the same results in a shorter time by using biotechnological methods, while a long time is needed when using classical breeding methods. With these techniques -clonal reproduction with somaclonal variation and rapid reproduction, 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 SAVSATLI, 2005).

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 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. The aim of this study is to develop an embryo culture technique which can be applied in black cumin after hybridization.

MATERIALS AND METHODS

Embryo cultures were made by using populations obtained from producers in Samsun, Denizli, Isparta, Mersin in Turkey and Çameli black cumin variety (Figure 1). Hybridizations were done according to semi-diallel hybridization method to gain zygotic embryos. The black cumin plants were grown in greenhouse from October to June to make hybridizations. The plants were grown in 3 liters pots by using one part garden soil and one part commercial plant soil (Mixflor). Three plants were grown in each pot. Hybridizations were done from 9.00 am to 11.00 am. Pollinations were done after emasculations one or two days later depending on weather conditions. After emasculation and hybridization the flowers were isolated by using hybridization bags to avoid cross pollinations and marked. The capsules including zygotic

embryos were harvested 10 or 12 days following pollination. The harvested capsules were carried to laboratory in sterilized distilled water. The capsules were sterilized before embryo culture. For this purpose the capsules were first treated with 70% ethanol for 30 sec., then treated with 10% bleach (ACE) for 10 min. and 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. LS2.5 and MS (Sigma Aldrich Catalog number: M5519) mediums were used for ovule culture. The plants gained from the ovules were grown in MSD4 medium. The mediums used in this study are shown Table 1. The MS medium was supplied as powder and added at 4.4 g per liter, then sucrose was added; then the pH was adjusted to 5.8 and agar was added and sterilized in autoclave at 121°C for 15 minutes. While preparing MSD4 and LS2.5 mediums, stock solutions were prepared and then the mediums were prepared from these stock solutions. When preparing stock solutions, the macro elements and micro elements were prepared separately, Na_2-EDTA and $FeSO_4.7H_2O$ were prepared as a stock solution, potassium iodide (KI) were prepared as a stock solution, and each vitamin and hormone was prepared separately as a stock solution. Myo-Inostol and sucrose were added to the medium each time by weight while preparing the medium. After the medium was prepared, pH was adjusted to 5.8; then agar was added and sterilization was carried out in an autoclave at 121° C for 15 minutes. Microsoft Excel Package Program was used to calculate success rates and draw graphs from the data obtained as a result of the research.

Figure 1. Location of Samsun, Denizli, Isparta and Mersin (ANONYMOUS, 2020)

Table 1. Content the nutrient mediums used the study

Chemical Matter	LS2.5	M5519	MSD4
KNO_3	1900	1900	1900
$CaCl_2.H_2O$	440	332.2	440
NH_4NO_3	1650	1650	1650
$MgSO_4.7H_2O$	370	180.7	370
KH_2PO_4	170	170	170
Na_2-EDTA	37.25	37.25	37.25
$FeSO_4.7H_2O$	27.85	27.85	27.85
H_3BO_3	6.2	6.2	6.2
$MnSO_4.H_2O$	22.3	16.9	22.3
$ZnSO_4.7H_2O$	8.6	8.6	8.6
KI	0.83	0.83	0.83
$Na_2MoO_4.H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025
Glycine	-	2.0	-
Myo-Inostol	100	100	100
Nicotinic acid	-	0.5	0.5
Pyrodoxine-HCl	-	0.5	0.5
Thiamine HCI	1.0	0.1	0.1
2.4-D (2,4-Dichlorophenoxyacetic acid)	2.5	-	-
BAP (6 benzylaminopurine)	-	-	0.5
Sucrose	10 000	10 000	30 000
Plant Agar (Ducefa)	7 000	7 000	7 000
PH	5.8	5.8	5.8

RESULTS AND DISCUSSION

In this study, the number of flower buds emasculated, the number of pollinated flower buds, the number of hybrid capsules harvested, and the success rates in hybridization data are given in Table 2 and Fig. 2. A total of 149 flower buds were emasculated during the study and 140 of them were pollinated. A total of 123 hybrid capsules were obtained from these buds which were pollinated, and the overall success rate of hybridization was 87.86%. When the success rates were examined according to the combinations, the highest success rate was obtained from Çameli x Mersin and Denizli x Mersin combinations with 92.31%, followed by Samsun x Mersin combination with 91.67%. The lowest success rate in hybridization was obtained from the combination of Samsun x Denizli with 84.62% (Table 2 and Fig 2). The success rate of hybridization was so high that black cumin flowers were large enough for hybridization and the pollination was carried out 1-2 days after emasculation. The results of the hybridization breeding program made on black cumin were not found in the literature reviewed. During this study, a total of 6430 zygotic embryos were cultivated in LS2.5 and MS medium.

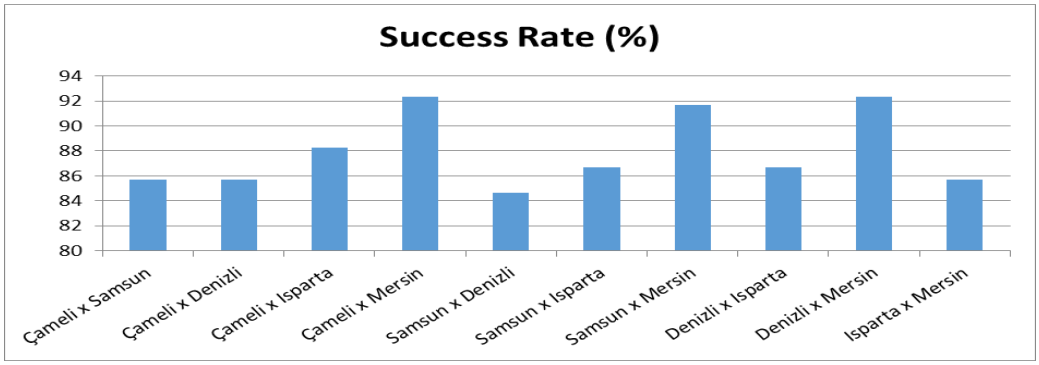

Figure 2. Success rate in hybridizations

Table 2. Data on hybridization studies

Combination	The number of flower buds emasculated	The number of pollinated flower buds	The number of hybrid capsules harvested	The success rates in hybridization (%)	The number of ovules cultured
Çameli x Samsun	16	14	12	85.71	636
Çameli x Denizli	15	14	12	85.71	674
Çameli x Isparta	17	17	15	88.24	737
Çameli x Mersin	13	13	12	92.31	632
Samsun x Denizli	14	13	11	84.62	576
Samsun x Isparta	15	15	13	86.67	674
Samsun x Mersin	14	12	11	91.67	563
Denizli x Isparta	16	15	13	86.67	661
Denizli x Mersin	15	13	12	92.31	632
Isparta x Mersin	14	14	12	85.71	645
Total	149	140	123	87.86	6430

The maximum number of ovules was cultured from Çameli x Isparta combination with 737 ovules, and the lowest number of ovules from Samsun x Mersin combination was 563. During the research, the number of ovules cultured in LS2.5 medium, the number of calluses obtained from the cultured ovules, the number of plants, and the success rates, are given in Table 3. A total of 2904 ovules were cultured in LS2.5 medium; 148 of these showed callus development, and the callus formation rate was realized as 5.10%. Among the investigated combinations, the highest callus formation rate was obtained from Çameli x Denizli combinations with 7.26%, followed by Çameli x Isparta combinations with 6.43%. The lowest

success rate was obtained from Isparta x Mersin combinations with 3.74%. No plant growth occurred in the LS2.5 medium.

Table 3. Numerical data on the development of ovules cultured in LS2.5 medium during the research

Combinations	The number of cultured ovules	The number of callus obtained	Callus creation rate (%)	The number of plants obtained from callus
Çameli x Samsun	286	18	6.29	-
Çameli x Denizli	303	22	7.26	-
Çameli x Isparta	342	22	6.43	-
Çameli x Mersin	275	11	4.00	-
Samsun x Denizli	274	13	4.74	-
Samsun x Isparta	298	15	5.03	-
Samsun x Mersin	254	10	3.94	-
Denizli x Isparta	292	14	4.79	-
Denizli x Mersin	286	12	4.20	-
Isparta x Mersin	294	11	3.74	-
Total	2904	148	5.10	-

The number of ovules cultured in MS medium, the number of plants obtained from cultured ovules and the number of plants matured by transferring to pots and their success rates are given in Table 4. A total of 3526 ovules were cultured in MS medium and 60 plants were obtained, and the plant formation rate was determined as 1.70%. 54 of these plants reached sufficient size to be transferred to pots. 54 of these plants reached sufficient size to be transferred to pots (Table 4). The highest numbers of ovules were cultured in Çameli x Isparta combination, totalling 395. The lowest numbers of ovules were cultured in Samsun x Denizli combination, with a total of 302. When the success rates obtained from the cultured ovules were evaluated at the combinations level, the highest success rate was obtained from Çameli x Isparta combination with 2.53% followed by Çameli x Denizli combination with 2.16%. The lowest success rate was obtained from Denizli x Mersin combination with 0.87%. The highest numbers of plants were those transferred to the pots in the Çameli x Isparta combination a total of 9, 7 plants of which adapted to the external environment and matured. The other 2 plants could not adapt to the external environment. The least number of plants transferred to the pots were those in the combination Denizli x Mersin totaling 3; all of these plants adapted to the external environment and matured. When the nutrient media used for embryo culture are examined, LS2.5 medium is found to contain a high level of auxin hormone. In plant tissue culture, auxin hormones used in low amounts promote root formation; auxin hormones in high amounts promote callus formation on the explants (ELHAN *et al.*, 2004; HATIPOĞLU, 2012). The results of this study support this situation. MS medium did not contain hormones. For this reason, direct plant regeneration has been realized from the cultured embryos and no callus formation has been observed. However, in general, the rate of plant formation of embryos was low. Success in embryo culture is affected by many factors. The most important factors at these points are the composition of the nutrient medium, the genetic structure of the plant, age of the cultured embryos and culture conditions

(BRIDGEN, 1994; UYSAL, 2007; HATIPOGLU, 2012). In terms of the composition of the nutrient medium, some hormones can promote embryo development. In this study, all of the mentioned factors may have had an effect in lowering the success rate of cultured embryos. It is also possible that black cumin has a poor ability to adapt in vitro. We saw that embryo culture studies were not studied in black cumin when the literature reviewed. Therefore, the results obtained from this study could not be compared with previous studies. One of the most important problems of tissue culture studies is that the adaptation of plants obtained after tissue culture to the external environment is difficult (KURT, 2004). Since the plants are adapted to a controlled environment where all conditions are controlled, when they are taken out, serious stress factors are formed on the plants and the plants suffer from adaptation problems. Therefore, the frequency of adaptation to the external environment may be very low depending on the plant genotype. Based on this study, we can say that the adaptability of black seed is relatively good.

Table 4. Numerical data on the development of ovules cultured in MS medium during the research

Combinations	The number of cultured ovules	The number of plants obtained	Success rate (%)	Number of Plants Transferred to Pots	The number of matured plants
Çameli x Samsun	350	6	1.71	6	4
Çameli x Denizli	371	8	2.16	6	4
Çameli x Isparta	395	10	2.53	9	7
Çameli x Mersin	357	6	1.68	6	5
Samsun x Denizli	302	5	1.66	4	3
Samsun x Isparta	376	6	1.60	6	5
Samsun x Mersin	309	5	1.62	5	3
Denizli x Isparta	369	5	1.36	4	3
Denizli x Mersin	346	3	0.87	3	3
Isparta x Mersin	351	6	1.71	5	4
Total	3526	60	1.70	54	41

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REFERENCES

ANONYMOUS (2020): http://cografya.sitesi.web.tr/haritalar/bos-haritalar/bos-turkiye-haritasi-5/ (03.04.2020)

BRIDGEN, M.P. (1994): A Review of Plant Embryo Culture. Hort Sci., *29(11):* 1243-1246.

DREW, R.A. (1997): The Application of Biotechnology to the Conservation and Improvement of Tropical and Subtropical Fruit Species. Seed and Plant Genetic Resources Service Food and Agriculture Organization of the United Nations Rome.

ELHAN, H., M.M., EL-OLEMY, M.S., AL-SAID (2004): Enhancement of Somatic Embryogenesis and Production of Developmentally Arrested Embryos in *Nigella sativa* L. Hort Science., *39(2):*321-323.

HATIPOGLU, R. (2012): Bitki Biyoteknolojisi. Textbook, Çukurova University, Agricultural Faculty, Field Crop Department, Number: 176, Adana, Turkey.

ILISULU, K. (1992): İlaç ve Baharat Bitkileri. Ankara University, Agricultural Faculty, Number: 1256 Ankara, Turkey.

KURT, O. (2004): Bitki islahı. Textbook. J. of Fac. of Agric. OMU. Number: *43.*

KURT, O., Y., ŞAVŞATLI (2005): A general view to plant biyotechnology. J. of Fac. of Agric. OMU, *20 (3):*126-133.

NARAYANASWAMI, S., K., NORSTOG (1964): Plant embryo culture. The Botanical Review, *30* (4): 587-628.

NICKAVAR, B., F., MOJAB, K., JAVIDNI, M.A.R., AMOLI (2003): Chemical Composition of the Fixed and Volatile Oils of *Nigella Sativa L.* from Iran. Zeitschriftfür Naturforschung, *58* (9): 629-631.

RAGAA, H.M.S. (2010): Clinical and therapeutic trials of *Nigella sativa*. TAF Prev. Med. Bull., *9*(5): 513-522

SEÇMEN, Ö., Y., GEMICI, G., GÖRK, L. BEKAT, E., LEBLEBICI (2000): Tohumlu bitkiler sistematiği. textbook, Ege University, Science Faculty Number: 116. İzmir, Turkey.

UYSAL, H., F., SEYIS, O., KURT (2007): Tarla bitkilerinde melezleme bariyerlerinin aşılmasında alternatif bir yöntem embriyo kültürü. J. Fac. Agric. OMU, *22*(1): 116-122.

YILDIRIM, H., R., GÜL (2018): *Ranunculus bullatus* L.'un (Ranunculaceae) Türkiye'de varlığı üzerine. Bağbahçe Bilim Dergisi., *5*(1):10-14.

RAZVOJ OPLEMENJIVAČKIH LINIJA KULTUROM EMBRIONA KOD *Nigella sativa* L.

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Izvod

Ova studija je sprovedena u cilju razvoja tehnike kulture embriona i dobijanje oplemenjivačkih linija za uzgoj ovom tehnikom. U studiji je bila poželjnija kultura ovula, jer je seme bilo premalo i izolacija embriona je bila teška. Kultura embriona rađena je korišćenjem populacija dobijenih od proizvođača iz Samsuna, Denizlija, Isparte, Mersina u Turskoj i sorte crnog kima Cameli. Hibridizacije su rađene prema metodi poludialelne hibridizacije da bi se dobili zigotični embrioni. Za kulturu jajnih ćelija korišćeni su medijumi LS2.5 i MS (Sigma Aldrich Kataloški broj: M5519), a za gajenje biljaka dobijenih iz ovula korišćen je medijum MSD4. Kao rezultat istraživanja, ukupno 2904 ovule su gajene u medijumu LS2,5. 148 ih je pokazalo razvoj kalusa; stopa formiranja kalusa bila je 5,10%. Najveća stopa formiranja kalusa u ispitivanim kombinacijama dobijena je iz kombinacije Cameli x Denizli od 7,26%. Regeneracija biljaka nije se mogla dobiti iz ovih kalusa. Ukupno 3526 ovula je kultivisano u medijumu sa MS. Iz ovih jajnih ćelija dobijeno je šezdeset biljaka, a stopa formiranja biljaka je utvrđena kao 1,70%. 41 biljka je sazrela i požnjevena.

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