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Induction of Microspore Embryogenesis in Ornamental Kale by Gamma Irradiation and High Temperature Stress

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ABSTRACT

The effect of gamma irradiation with high temperature treatments on the microspore embryogenesis were evaluated in ornamental kale (*B. oleracea* var. *acephala* cv. Chidori Red F1). Four different gamma doses (50, 75, 100 and 300 Gy) were applied to the buds that were at the late uninucleate stage microspore. Buds to which gamma applied were kept in 4°C under dry (in petri dish) and liquid (in NLN-13 medium) storage conditions. Microspores isolated after 29 h following the gamma treatment were taken and cultured in NLN-13 medium (40.000 microspore mL^{-1}). In order to induce embryo induction, microspores were kept in 32 and 35°C during the first 2 days of the culture. The effect of the gamma irradiation treatment on ornamental kale varied by the temperature. According to the 12 day embryo yields after planting 32C°+50 Gy (5.54 embryo/petri) and 35°C + 75 Gy (5.20 embryo/petri) in ornamental kale were found effective treatments. The effect of keeping the irradiated buds under liquid storage conditions (in NLN-13 medium) up to isolation time was seen on providing embryo induction depending on temperature. As a result at both temperature treatment (32 and 35°C) on condition that microspores are isolated immediately after irradiation treatment intermediate doses below 100 Gy is thought to be more effective in stimulating the formation of the embryo.

Key words: *Brassica oleracea*, microspore embryogenesis, gamma irradiation, heat shock

INTRODUCTION

Anther and microspore cultures can be utilized in order to shorten the breeding period in different species for obtaining haploid embryos. Anther culture studies were successfully performed on rice (Bagheri and Jelodar, 2008; Niroula and Bimb, 2009), mazie (Hassan *et al*., 2001), eggplant (Khatun *et al*., 2006), pepper (Koleva-Gudeva *et al*., 2007). On the other hand, on *Brassica* species it was noted that embryo formation rate was low. It is noted that microspore culture method on *Brassica* species can be successfully applied (Zhang *et al*., 2008; Prem *et al*., 2008). Through applying various stress factors such as high temperature, colchicine and gamma irradiation on the microspores isolated from anthers or buds in order to stimulate haploid embryo formation from microspores in *Brassicas*, gametophytic rings can get broken and microspores can develop in the direction of embryo formation (Touraev *et al*., 1997).

High temperature treatments become more effective when they are applied after microspores isolated are taken into culture. Microspores can be programmed towards the direction of embryo development through stimulating symmetric nuclear fission as a result of high temperature-shocks

applied in the first several days of the culture period (Custers *et al*., 1994). More successful results are noted to be obtained from treatments on 30°C for 2 days (Wan *et al*., 2011) and 30°C for 5 days (Iqbal and Moellers, 1999) in *B. napus*, 30°C for 2 days in broccoli (Duijs *et al*., 1992), 33°C for 1 day in Chinese cabbage (Cao *et al*., 1994), 32°C for 2 days in cauliflower, white head cabbage and *B.oleracea* var. *sabauda* in (Ferrie *et al*., 1999), 32.5°C for 1 day in *Brassica oleracea* var *costata* (Dias and Correia, 2002), 32°C for 2 days in ornamental kale (Zhang *et al*., 2008), 32°C for 2 days in *Eruca sativa* (Leskovsek *et al*., 2008), 32.5°C for 10-11 days in *B. juncea* (Prem *et al*., 2008).

Gamma irradiation is a stress factor more rarely used in comparison with other stress treatments (high temperature, colchicine etc) performed for stimulating embryo formation in *Brassica* species and can stimulate embryo formation from microspores (Shariatpanahi *et al*., 2006). Indeed, only two article of *Brassica* about the types of applications achieved with gamma. Pechan and Keller (1989) applied 0, 450 and 900 Gy gamma rays on the buds including at the late uninucleate stage microspores in *Brassica napus* L. After the irradiation process on the buds irradiated, microspores isolated 4-5, 6-7 and 8-10 days later were taken into culture in 25 and 32°C. It was stated that embryo formation ratio was higher than in pollens irradiated in comparison with the control in all treatments and average 2% embryo formed in 32°C temperature treatment whereas embryo formed less than 0.01% in 25°C temperature treatment (Macdonald *et al*., 1988). Buds from *B. napus* 0 Gy, 5 Gy, 10 Gy and 15 Gy gamma-ray applications, the highest formation of embryos of non-irradiated received (11.3 embryos/bud), followed by 5 Gy (7.3 embryo/bud) and 15 Gy (7.0 embryos/Petri dishes) doses reported. Gamma-ray applied to the isolated microspores, again the control group (25.0 embryos/bud) were found more successfully, this 5 Gy (19.4 embryos/bud) and 15 Gy (19.2 embryos/bud) doses, respectively.

The aim of this study the effect of gamma irradiation with high temperature treatments to ornamental kale (*B. oleracea* var. *acephala* cv. Chidori Red F1) on the microspore embryogenesis was investigated.

MATERIALS AND METHODS

Plant materials and growth conditions: The research was performed between 2008-2009 in the laboratory of Ankara University, Faculty of Agriculture, Department of Horticulture and under plantation conditions. Ornamental kale (cv. Chidori Red Fi) was used as plant material. Seeds were sown in August 2008 on peat-filled viols. Seedlings were planted when 3-4 leaf stages with intervals of 30×60 cm within September 2008. Plants were left in the field from winter to spring and then buds were collected after the flowering.

Gamma irradiation: Flower buds (2.4-4.4 mm length) containing microspores at the late uninucleate stage of development were taken to the Turkish Atomic Energy Authority so as to perform gamma irradiation. The source power of the device which will perform the gamma radiation was determined as 0,938 Kgy h^{-1}. Four different gamma doses (50 Gy, 75 Gy, 100 Gy and 300 Gy) were used for flower buds. Buds to which gamma applied were kept in 4°C under dry (directly in petri dishes) and liquid (NLN-13) conditions. The 29 h later than irradiation, microspores were isolated.

Microspore isolation: Buds to which gamma rays were applied were collected and sterilized in 10% sodium hypochlorite solution for 10 min, they were disinfected by after rinsing in bidistillated

water 3 times for 6 min each. 40 buds were used in each isolation. The buds were macerated with a glass rod in 3.5 mL^{-1} NLN-13 medium (Lichter, 1982) containing 13% sucrose (hormone-free, pH: 6.1) and microspores were made free. After that, microspore suspension was sifted through sieves with 40 μm holes, the residue on the sieve and beaker was washed again with 6.5 mL NLN-13 medium and transferred to glass beakers. Sieved suspension was taken in centrifuge tubes (Falcon polypropylene tubes), centrifuged in 900 rpm speed in 4°C 3 times for 3 min each and microspore residue was attained.

Temperature treatment and culture of microspores: Microspores were resuspended in fresh NLN-13 medium (40.000 microspore mL^{-1}). Five-mililiter aliquots of microspore suspension were dispensed into 60×15 mm sterile petri dishes (200.000 microspore/petri). Dishes were incubated in the dark at 32 and 35°C for 48 h to induce embryogenesis and then at 25°C in the dark conditions. When 2 or more embryos were seen with naked eye (approx. 11-12 days after the isolation) petri dishes were taken on the orbital shaker (45 rpm) (Gerhardt, Laboshake 500) and kept for 3 weeks under dark conditions in 25°C.

Performing embryo yield: Embryo counts per petri dish were made 2 (12th day) and 3 (19th day) weeks after the isolation under the binocular microscope.

Statistical analysis: Embryos were counted per petri dish (6 petri dishes for each treatment, 4 repetitions for treatment and totaly 24 petri dishes per treatment). The obtained data were analyzed using factorial variance analysis (gamma × temperature × storage condition treatments). Differences among gamma, temperature and storage condition treatments were tested using Duncan's multiple range test at $p = 0.05$ (Duncan, 1955). Statistical analysis was performed using SPSS statistical package program.

RESULTS

Embryo yields acquired at the end of 12th day are given in Table 1. The factors were not evaluated independent since "dose x temperature x storage conditions' interaction was found statistically significant. Higher embryo yields were reached in both temperature degree and under liquid (NLN-13) storage conditions in all doses. Accordingly, the most successive combinations were determined as 32°C+50 Gy (5.54 embryo/petri) ve 35°C+75 Gy (5.20 embryo/petri). It was determined that embryo yields decreased in doses above 100 Gy, in both temperature degree and under both storage conditions (Table 1).

Embryo yields acquired at the end of 19th day are given in Table 2. When analyzed the embryo yields, it draws attention that there emerged an evident decrease in embryo yields compared to 12th day. According to the embryo yields performed 19th day, it draws attention that results are similar to the results of 12th day. The highest embryo yield was still attained from 32°C+50 Gy (2.09 embryo/petri) and 35°C+75 Gy (3.16 embryo/petri) (Table 2).

In examinations made under the binocular microscope approximately 3 weeks after the isolation (19th day), differentiated structures (Fig. 1a-c), together with embryo formations (Fig. 1e, f) were observed.

Table 1: Effect of different storage conditions, high temperature and gamma irradiation treatments in ornamental kale on embryo yield (12th day)

		Storage conditions of buds	
		Mean yield (embryos/petri[1])	
Temperature (°C)	Gamma dose (Gy)	Dry	Liquid
32	Control	3.98±0.82aA[1]	4.32±0.83aAB[1]
	50	4.79±1.45aA[1]	5.54±0.36aA[1]
	75	1.53±0.32aB[1]	3.70±1.00aB[2]
	100	0.22±0.10aB[1]	0.23±0.11aC[1]
	300	0.49±0.21aB[1]	0.17±0.06aC[1]
35	Control	2.09±0.27aAB[1]	2.23±0.41aBC[2]
	50	3.16±0.86aA[1]	3.79±0.98aAB[2]
	75	1.45±0.59bAB[1]	5.20±1.06aA[1]
	100	0.37±0.15aB[1]	0.54±0.26aCD[1]
	300	0.30±0.15aB[1]	0.37±0.14aD[1]

[1]5 mL^{-1} NLN-13/60×15 mm petri dish (40.000 microspore mL^{-1} = 200.000 microspore/petri dish). Different capital letters in a same column show significant differences among the gamma doses ($p<0.05$). Different small letters in a same temperature show significant differences among the storage conditions ($p<0.05$). Different figures in a same gamma dose show significant differences among the temperatures ($p<0.05$)

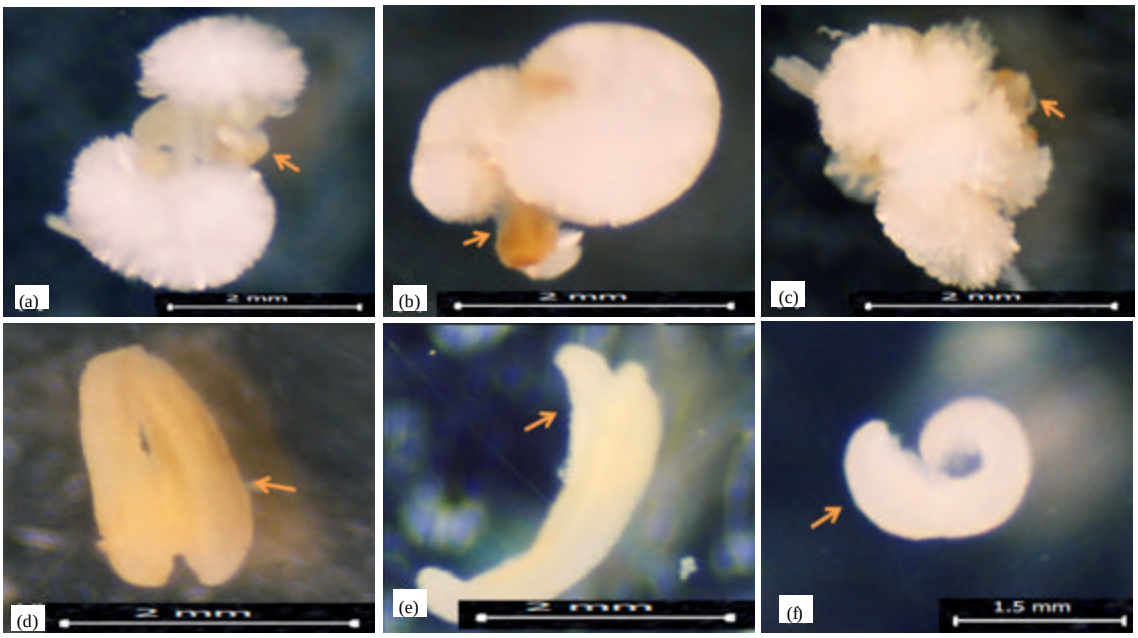

Fig. 1: *In vitro* microspore embryogenesis (a, b, c) differentiated structures (32°C+50 Gy-liquid), (d) uncrushed anther tissue, (e) 12 daily late torphedo embryo, (f) 19 daily developed embryo (32°C+50 Gy-liquid) in ornamental kale.

Table 2: Effect of different storage conditions, high temperature and gamma irradiation treatments in ornamental kale on embryo yield (19th day)

		Storage conditions of buds	
		Mean yield (embryos/petri[1])	
Temperature (°C)	Gamma dose (Gy)	Dry	Liquid
32	Control	1.51±0.97aA[1]	1.50±0.50aAB[1]
	50	1.28±0.72aA[1]	2.09±0.73aA[1]
	75	0.36±0.23aA[1]	1.92±0.58aA[1]
	100	0.16±0.08aA[1]	0.22±0.11aB[1]
	300	0.44±0.23aA[1]	0.21±0.08aB[1]
35	Control	0.79±0.47aA[1]	1.94±0.06aAB[1]
	50	0.41±0.24aA[1]	1.83±1.13aAB[1]
	75	0.20±0.20bA[1]	3.16±0.98aA[1]
	100	0.27±0.11aA[1]	0.81±0.33aB[1]
	300	0.23±0.14aA[1]	0.82±0.28aB[1]

[1]5 mL^{-1} NLN-130/60×15 mm petri dish (40.000 microspore mL^{-1} = 200.000 microspore/petri dish). Different capital letters in a same column show significant differences among the gamma doses ($p<0.05$). Different small letters in a same temperature show significant differences among the storage conditions ($p<0.05$). Different figures in a same gamma dose show significant differences among the temperatures ($p<0.05$)

DISCUSSION

Gamma irradiation can be utilized in order to stimulate embryo formation from microspores and anthers. However studies on this subject in Brassica species are limited in number (Macdonald *et al*., 1988; Pechan and Keller, 1989). *B. napus*, 450 Gy and 900 Gy doses were used and 900 Gy dose (5.98% embryo formation) was founded more effective in terms of embryo formation (Pechan and Keller, 1989). However, Macdonald *et al*. (1988), isolated microspore from *B. napus* applied low doses of gamma and non-irradiated (control group) treatment a higher embryo (25.0 embryo/bud) obtained. And then 5 Gy (19.4 embryos/bud) and 15 Gy (19.2 embryos/bud) doses, respectively.

Due to the distance of gamma rays source, it was not possible to perform isolation from buds irradiated soon after irradiation. Therefore, buds irradiated in ornamental kale were conveyed to the laboratory 29 h later, the buds were maintained in 4°C during that time. Buds irrigated before isolation were kept in 4°C under dry and liquid conditions. Higher embryo values were attained from 35°C+75 Gy (5.20 embryo/petri) ve 32°C+50 Gy (5.54 embryo/petri) applications to ornamental kale than control (Table 1). It is considered that performing isolation soon after the irradiation has a significant impact in getting these results. In a study conducted by Pechan and Keller (1989) on *Brassica napus*, even though the effectiveness of gamma rays treatment was mentioned, embryo varied at a quite lower rate. In our study, higher embryo count was achieved in ornamental kale than the count declared by Pechan and Keller (1989). It is thought that lower doses we selected and species variability had an impact on it. Indeed, *B. napus* ssp. *Metzg oleifera*. (Sins) microspores stimulating the formation of embryos used in lower doses of gamma (Macdonald *et al*., 1988). However, outside of *Brassica* species in different types of anther/or microspores stimulating the formation of embryos used in low doses of gamma. Gamma irradiation with the dosage of 10 Gy significantly increased induction of embryogenesis in cultured Nicotiana and Datura anthers

(Sangwan and Sangwan, 1986). Combination of a 4 Gy irradiation followed by incubation at 10°C for 9 days significantly increased anther culture response in tomato (Shtereva *et al.*, 1998). Barley is a study of doses of 5 Gy and 10 Gy dose is reported to be effective (Arabi *et al.*, 2005).

Performing the embryo counts at 2 different times including 12th and 19th days is significant for determining successive treatments and the changes that occurred in the structures and quality of embryos in 1 week. While embryo count is higher at the end of 12th day in almost all applications, it was determined that the structure and quality of embryos had been damaged due to the infection in the counts on the 19th day and that there was a considerable decrease compared to the 12th day. These results concordant with findings of the research which state that ventilation and medium renewal applications by shaking at intervals of 3 days in order to reduce the effect of toxic substances that may have accumulated in the medium in *Brassica* species reduced the embryo count on the following days which was higher on previous days (Lighter, 1989).

CONCLUSIONS

As for the future studies, it was determined that testing 100 Gy and lower doses can be more beneficial for elucidating the effect of gamma rays treatment only through isolating them soon after radiation. Selection of effective gamma dose and distance of the location where the irradiation is carried out affect the achievement in stimulating the embryo formation. Additionally, it was concluded that the high temperature treatment of 32 and 35°C for 2 days can be utilized in order to attain embryos from microspores in studies to be performed on ornamental kale times in the future.

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REFERENCES

Arabi, M.I.E., B. Al-Safadi, M. Jahwar and N. Mir-Ali, 2005. Enhancement of embryogenesis and plant regeneration from barley anther culture by low doses gamma irradiation. *In vitro* Cell. Dev. Biol. Plant, 41: 762-764.

Bagheri, N. and N.B. Jelodar, 2008. Combining ability and heritability of callus induction and green-plant regeneration in rice anther culture. Biotechnology, 7: 287-292.

Cao, M., Y. Li, F. Liu and C. Dore, 1994. Embryogenesis and plant regeneration of pakchoi (*B. rapa* L. ssp. *Chinensis*) via *in vitro* isolated microspore culture. Plant Cell Rep., 13: 447-450.

Custers, J.B.M., J.H.G. Cordowener, Y. Nollen, H.J.M. Dons and H.J.M. Van Lookeren Campagne, 1994. Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Rep., 13: 267-271.

Dias, J.S. and M.C. Correia, 2002. Effect of medium renovation and incubation temperature regimes on tronchuda cabbage microspore culture embryogenesis. Sci. Hortic., 93: 205-214.

Duijs, J.G., R.E. Voorrips, D.L. Visser and J.B.M. Custers, 1992. Microspore culture is successful in most crop types of *Brassica oleracea* L. Euphytica, 60: 45-55.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometrics, 11: 1-42.

Ferrie, A.M.R., D.C. Taylor, S.L. Mackenzie and W.A. Keller, 1999. Microspore embryogenesis of high sn-2 erucic acid *Brassica oleracea* germplasm. Plant Cell, Tissue Organ Cult., 57: 79-84.

Hassan, L., S.D. Ahmad and A. Okumus, 2001. The direct regeneration of maize haploids through anther culture. J. Biological Sci., 1: 900-901.

Iqbal, M.C.M. and C. Moellers, 1999. Double haploids in *Brassica napus* L. Breeding programs. Pak. J. Biol. Sci., 2: 1197-1202.

Khatun, F., M.B. Meah and K.M. Nasiruddin, 2006. Regeneration of eggplant through anther culture. Pak. J. Biol. Sci., 9: 48-53.

Koleva-Gudeva, L.R., M. Spasenoski and F. Trajkova, 2007. Somatic embryogenesis in pepper anther culture: The effect of incubation treatments and different media. Sci. Hortic., 111: 114-119.

Leskovsek, L., M. Jakse and B. Bohanec, 2008. Doubled haploid production in rocket (*Eruca sativa* Mill.) through isolated microspore culture. Plant Cell Tissue Organ Cult., 93: 181-189.

Lichter, B., 1982. Induction of haploid plants from isolated pollen of *B. napus*. Z. Pflanzenphysiol., 105: 427-434.

Lighter, R., 1989. Efficient yield of embryoids by culture of ýsolated microspores of different *Brassicaceae species*. Plant Breed., 103: 119-123.

Macdonald, M.V., M.A. Hadwiger, F.N. Aslam and D.S. Ingram, 1988. The enhancement of anther culture efficiency in *Brassica napus* ssp. oleifera Metzg. (Sinsk.) using low doses of gamma irradiation. New Phytol., 110: 101-107.

Niroula, R.K. and H.P. Bimb, 2009. Effect of genotype and callus induction medium on green plant regeneration from anther of Nepalese rice cultivars. Asian J. Plant Sci., 8: 368-374.

Pechan, P.H. and W.A. Keller, 1989. Induction of microspore embryogenesis in *Brassica napus* L. gamma irradiation and ethanol stress. *In vitro* Cell Dev. Biol. Plant., 25: 1073-1074.

Prem, D., K. Gupta, G. Sarkar and A. Agnihotri, 2008. Activated charcoal induced high frequency microspore embryogenesis and efficient doubled haploid production in *Brassica juncea*. Plant Cell Tissue Organ Cult., 93: 269-282.

Sangwan, R.S. and B.S. Sangwan, 1986. Effets des Rayonsgamma sur Lembryogenese Somatique et Landrogenesechez Divers Tissue Vegetaux Cultives *in vitro*. In: Nuclear Techniques and *in vitro* Culture for Plant Improvement, IAE. Agency (Eds.). International Atomic Energy Agency, Vienna, pp: 181-185.

Shariatpanahi, M. E., U. Bal, E. Heberle-Boors and A. Touraev, 2006. Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. Physiol. Plant., 127: 519-534.

Shtereva, L.A., N.A. Zagorska, B.D. Dimitrov, M.M. Kruleva and H.K. Oanh, 1998. Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.) II. Factors affecting induction of androgenesis. Plant Cell Rep., 18: 312-317.

Touraev, A., O. Vicente and E. Heberle-Bors, 1997. Initiation of microspore embryogenesis by stress. Trends Plant Sci., 2: 297-302.

Wan, G.L., M.S. Naeem, X.X. Geng, L. Xu, B. Li, G. Jilani and W.J. Zhou, 2011. Optimization of microspore embryogenesis and plant regeneration protocols for *Brassica napus*. Int. J. Agric. Biol., 13: 83-88.

Zhang, W., Q. Fu, X. Dai and M. Bao, 2008. The culture of isolated microspores of ornamental kale (*Brassica oleracea* var. *acephala*) and the importance of genotype to embryo regeneration. Sci. Hort., 117: 69-72.