First Report of Obtaining Haploid Plants Using Tissue Culture Techniques in Spinach

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Abstract. Monoic, dioic, and hermaphrodite flower types complicate spinach breeding and cultivar development. The availability of haploid plants will definitely accelerate spinach breeding; however, there are currently no reports in the literature about the use of tissue culture techniques to obtain spinach haploids. Therefore, in this study, pollination with irradiated pollen and anther culture methods were used to obtain haploid plants in spinach. For anther culture, three spinach varieties (Koto F1, Favorit F1, and Greenstar F1) and four different nutrient media were tested to obtain haploid embryos. Significant outcomes were not achieved from anther culture, and only two plants were obtained from all the experiments. Gynogenesis studies using irradiated pollen were performed with the same three spinach varieties and six gamma ray doses (100, 150, 200, 300, 400, and 500 Gy) from (cobalt⁶⁰) Co⁶⁰. Murashige and Skoog (MS) nutrient medium containing 1 mg·L⁻¹ indoleacetic acid (IAA) was used for embryo germination. Current findings revealed that the varieties produced responded differently to the various doses of radiation. A total of 3414 embryos and 1710 plants were obtained from experiments carried out for 2 years. Considering the numbers of embryos and plants per 100 seeds, the Favorit F1 variety provided better results than the other two varieties. However, significantly different outcomes were not achieved with regard to irradiation doses. Embryos were observed at all doses tested. Flow cytometry analyses that were carried out on regenerated plants revealed whether the plants were diploid or doubled haploid, and molecular analyses revealed that diploids resulted from spontaneous chromosome doubling. The current findings offer significant results for spinach breeding using haploids.

Spinach (*Spinacia oleracea* L.), which belongs to the Chenopodiaceae family, is a cool-season vegetable. After China, the United States, and Japan, Turkey has

the fourth place in spinach production (FAOSTAT, 2013).

In cultivar development, haploidization offers a great advantage by shortening the breeding cycle. Haploid plants can be obtained using various tissue culture techniques and full homozygosity can be achieved in quite a short time using these methods. Since the heterozygosity rate is high in open pollinated species, inbreeding should be performed for 10–12 generations to obtain homozygous lines. Such inbreeding should be performed for 5–7 generations in self-pollinated species and is impossible in dioic species. Dihaploidization allows development of homozygous lines in a single generation (Ellialtıoğlu et al., 2002). Anther culture is one of the techniques that are used widely to obtain haploid plants.

The advantage of this technique over other methods to obtain in vitro haploid plants is the existence of thousands of microspores in an anther, and the possibility to obtain numerous haploid plants from an anther. Several factors affect embryo regeneration in anther culture. These factors include genotype, age of donor plant, pollen development stage, pretreatments made to buds or anthers, and composition of nutrient media. Successful outcomes were achieved with anther culture in pepper (Al Remi et al., 2014; Buyukalaca et al., 2004; Comlekcioglu et al., 2001; Ercan et al., 2006; Gémesné Juhász et al., 2001; Keleş et al., 2015; Niklas-Novak et al., 2012; Olszewska et al., 2013; Taşkın et al., 2011), eggplant (Başay and Ellialtıoğlu, 2013; Başay et al., 2011), and *Brassica* spp. (Achar, 2002; Arnison et al., 1990a, 1990b; Biddington and Robinson, 1990; Górecka et al., 2007; Phippen and Ockendon, 1990; Roulund et al., 1990).

Haploidization studies about pollination with irradiated pollen were initiated in the 1980s, and significant results were obtained for melon (Baktemur et al., 2013; Cuny et al., 1993; Godbole and Murthy, 2012a, 2012b; Gursoz et al., 1991) and squash (Berber, 2009; Kurtar et al., 2002, 2009), and promising outcomes were achieved for cucumber (Chun et al., 2006; Claveria et al., 2005; Faris et al., 1999; Nikolova and Alexandrova, 2001) and watermelon (Sarı et al., 1994; Taşkın et al., 2013). Factors such as genotype, environmental conditions, irradiation dose, size and shape of pollen grain, and thickness of pollen wall have significant impacts on the success of pollination with irradiated pollen. Gamma rays obtained from Co⁶⁰ source are commonly used for pollen irradiation. When a flower is pollinated with irradiated pollen, the pollen's vegetative nucleus starts karyokinesis and germination occurs on the stigma. This activity results in stimulation of the embryonary sac and then the ovule, syngonite or antipode cells start to divide. As a result of these divisions, irregular cell masses (callus) or embryos are formed. To provide the necessary stimulation to the embryonary sac, the pollen should not lose its vigor and ability to germinate and, at the same time, should be in a state in which it is unable to perform normal pollination. Thus, the suitability of irradiated pollen for haploidization is determined in great part by the dose of radiation to which the pollen is exposed (Ellialtıoğlu et al., 2002).

Spinach is a long-day plant and flowering starts when daylength exceeds 12 h. Germination is accelerated at dry and hot temperatures. Bolting usually is earlier in male plants than female plants. Both male and female flowers bloom at the same time; male flowers die earlier than female flowers. Female plants live longer than male plants and have larger and thicker leaves (Sneep, 1958). Earliness is a commonly desired characteristic in spinach and daylength requirement, growth rate, and the balance between growth and development play important roles in earliness. Spinach varieties differ in terms of earliness, and such variation is necessary

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Fig. 1. (A) Male spinach flowers in glass petri dishes prepared for irradiation, (B) pollination of female spinach plants with irradiated pollen, (C) closing female plants with pollen-proof pouches to prevent open pollination before pollination with irradiated pollen, (D) closing female plants after pollination with pollen-proof pouches to prevent contamination with different pollen until the fruit set.

Fig. 2. (A, B) Callus formation from anther, (C, D) different types of regeneration in anther culture.

Table 1. Results for anther culture of Koto F1 variety (second year).

MN	RN	NA	NDA	NC	NE	NP
1	1	730	708	—	1	1
1	2	624	614	—	—	—
1	3	421	410	—	—	—
1	4	482	477	—	—	—
1	5	504	486	—	—	—
2	1	532	514	—	—	—
2	2	537	512	1	1	1
2	3	501	460	—	—	—
2	4	500	475	—	—	—
2	5	383	379	—	—	—
3	1	429	416	5	—	—
3	2	367	355	3	—	—
3	3	316	300	—	—	—
3	4	267	259	1	—	—
3	5	253	248	2	—	—
4	1	476	470	1	1	—
4	2	407	402	—	—	—
4	3	430	414	—	—	—
4	4	323	312	1	1	—
4	5	321	307	1	1	—

MN = medium no.; RN = replication no.; NA = number of anthers cultured; NDA = number of anthers developed; NC = number of anthers produced callus; NE = number of embryos; NP = number of plants.

Table 2. Results for anther culture of Favorit F1 variety (second year).

MN	RN	NA	NDA	NC	NE	NP
1	1	200	176	—	—	—
1	2	200	191	—	—	—
1	3	135	132	—	—	—
1	4	124	114	—	—	—
1	5	100	95	—	—	—
2	1	101	100	—	—	—
2	2	94	94	—	—	—
2	3	125	124	—	—	—
2	4	100	100	—	—	—
2	5	77	76	—	—	—
3	1	267	264	2	—	—
3	2	260	256	4	—	—
3	3	194	193	1	—	—
3	4	220	220	—	—	—
3	5	125	120	—	—	—
4	1	50	49	—	—	—
4	2	50	50	—	—	—
4	3	50	45	1	—	—
4	4	55	55	1	—	—
4	5	65	65	—	—	—

MN = medium no.; RN = replication no.; NA = number of anthers cultured; NDA = number of anthers developed; NC = number of anthers produced callus; NE = number of embryos; NP = number of plants.

for successful spinach culture under different growing conditions. Therefore, earliness is a significant trait for breeders (Parlevliet, 1967). Spinach breeding was initiated by Snee (1958) for resistance to spinach mildew [*Peronospora spinaciae* (Mont.) de By] and mosaic virus (*Cucumis virus 1*). Breeding studies are difficult in spinach since monoic, dioic, and hermaphrodite flower patterns are encountered in the crop. Developing a haploidization protocol will greatly accelerate and make spinach breeding more widespread. Therefore, the aim of the present study was to develop an efficient method for haploid plant production in spinach. To reach this aim, anther culture (androgenesis) and pollination with irradiated pollen (parthenogenesis) techniques were used. The

Table 3. Results for anther culture of Greenstar F1 variety (second year).

MN	RN	NA	NDA	NC	NE	NP
1	1	238	203	—	—	—
1	2	180	179	—	—	—
1	3	175	165	—	—	—
1	4	135	107	—	—	—
1	5	150	147	—	—	—
2	1	105	83	—	—	—
2	2	50	44	—	—	—
2	3	50	50	—	—	—
2	4	50	50	—	—	—
2	5	50	44	—	—	—
3	1	50	45	—	—	—
3	2	50	50	—	—	—
3	3	50	44	—	—	—
3	4	50	44	—	—	—
3	5	50	50	—	—	—
4	1	146	146	—	—	—
4	2	150	150	—	—	—
4	3	125	125	1	—	—
4	4	110	110	2	—	—
4	5	90	90	1	—	—

MN = medium no.; RN = replication no.; NA = number of anthers cultured; NDA = number of anthers developed; NC = number of anthers produced callus; NE = number of embryos; NP = number of plants.

effects of the different methods on haploid plant acquisition were compared as were the effects of nutrient media, genotype, and irradiation dose. The primary objective of the present study was to set up a substructure for more economical, shorter, and more efficient production of high quality and quantities of spinach plants using modern breeding techniques.

Materials and Methods

Experiments were carried out at the Alata Horticultural Research Institute (Erdemli, Mersin, Turkey) and the Horticulture Department of Çukurova University (Adana, Turkey). Among the methods used for obtaining haploid plants, anther culture and pollination with irradiated pollen techniques were used to obtain haploid plants for breeding studies in spinach. Experiments were replicated for 2 years.

Plant materials. Greenstar F1 (May Agro Seed Company, Bursa, Turkey), Favorit F1 (May Agro Seed Company), and Koto F1 (CN Seeds Ltd., Cambrigeshire, UK) spinach varieties were used as plant material. Greenstar F1 is a mid-early, high-yield variety. It has smooth, large, oval leaves with bright green color. Leaf tips are bumped, petioles are short, and leaf blades are large and fleshy. The variety is suitable for spring, autumn, and winter sowings. It is resistant to seven races of spinach downy mildew. It has either hermaphrodite flowers or is dioic. Favorit F1 is also a mid-early, high-yield variety with slightly wavy and dark green leaves. Leaf tips are again bumped, petioles are short, and leaf blades are large and fleshy. The variety is suitable for autumn and especially spring sowing. It is resistant to four races of spinach downy mildew. It has either hermaphrodite flowers or is dioic. Koto F1 is an early, high-yield variety. The leaves have pointed tips and are dark green color. The variety is suitable for autumn and especially

Table 4. Number of embryos and plants obtained through pollination with irradiated pollen and 100 seed ratios (first year).

Variety	ID	PNo	NS	NE	NP	NE/100 S	NP/100 S
Koto F1	100	4	1,098	55	17	5.01	1.55
Koto F1	100	10	310	132	25	42.58	8.06
Koto F1	100	42	187	25	2	13.37	1.07
Koto F1	100	5	203	23	10	11.33	4.93
Koto F1	100	12	245	30	6	12.24	2.45
Koto F1	100	14	960	35	7	3.64	0.73
			3,003	300	121	9.99	4.03
Koto F1	150	8	20	10	5	50.00	25.00
Koto F1	150	11	218	120	13	55.04	5.96
Koto F1	150	1	857	194	5	22.64	0.58
Koto F1	150	2	145	139	93	95.86	64.14
Koto F1	150	6	1,524	52	15	3.41	0.98
Koto F1	150	9	1,410	55	6	3.90	0.42
Koto F1	150	11	230	20	7	8.69	3.04
			4,404	590	144	13.40	3.27
Koto F1	200	3	974	57	20	5.85	2.05
Koto F1	200	10	332	22	4	6.63	1.20
Koto F1	200	13	1,208	30	7	2.48	0.58
			2,514	109	31	4.33	1.23
Favorit F1	150	34	256	16	2	6.25	0.78
Favorit F1	150	36	103	33	5	32.04	4.85
Favorit F1	150	39	154	63	5	40.91	3.25
			513	112	12	21.83	2.34
Favorit F1	200	9	330	99	5	30.00	1.51
Favorit F1	200	38	92	65	7	70.65	7.61
Favorit F1	200	40	40	36	5	90.00	12.50
			462	200	17	43.29	3.68
Greenstar F1	100	14	246	75	10	30.49	4.06
Greenstar F1	100	28	143	41	10	28.67	6.99
Greenstar F1	100	31	39	14	5	35.90	12.82
Greenstar F1	100	58	408	35	10	8.58	2.45
			836	165	35	19.74	4.19
Greenstar F1	150	4	21	5	2	23.81	9.52
Greenstar F1	150	5	27	9	4	33.33	14.81
Greenstar F1	150	18	337	93	5	27.60	1.48
Greenstar F1	150	29	39	14	5	35.90	12.82
			424	121	16	28.54	3.77
Greenstar F1	200	25	12	6	4	50.00	33.33

ID = irradiation dose; PNo = plant no.; NS = number of seeds; NE = number of embryos; NP = number of plants; NE/100 S = number of embryos/100 seeds; NP/100 S = number of plants/100 seeds.

spring sowings. It is dioic and resistant to two races of spinach downy mildew.

Anther culture. Proper anther stages were identified for Koto F1, Greenstar F1, and Favorit F1 varieties before the initiation of anther culture procedures. For this purpose, male flower buds of different sizes were collected, classified based on their sizes, and numbered separately. Anthers were removed from flower buds using sterile forceps and scalpels, squashed over a microscope slide, and stained with acetocarmine. Because immature anthers including late-uninucleate or early-binucleate phase at the beginning of the first mitotic division are the best material for initiation of anther culture, flower buds at this stage were selected using a stereo microscope, and were used for anther culture. Flower buds were sterilized in a laminar flow cabinet using 20% sodium hypochlorite solution for 15 min and rinsed five times with sterile water. After sterilization, anthers were removed from flower buds with sterile forceps and scalpels,

and were placed into 6-cm diameter sterile glass petri dishes containing nutrient medium. For anther culture experiments, four MS [Murashige and Skoog (1962)] nutrient media with different auxin and cytokinin levels were tested: 1) MS + 2 mg·L⁻¹ naphthaleneacetic acid (NAA) + 0.5 mg·L⁻¹ gibberellic acid (GA₃); 2) MS + 4 mg·L⁻¹ NAA + 0.5 mg·L⁻¹ GA₃; 3) MS + 2 mg·L⁻¹ NAA + 1 mg·L⁻¹ 6-benzylaminopurine (BAP); and 4) MS + 4 mg·L⁻¹ NAA + 1 mg·L⁻¹ BAP.

Cultured anthers were incubated at 25 °C and 12:12 h dark:light photoperiod. The embryos obtained from anthers were removed with forceps and scalpels under the microscope and transferred to 15 cm long sterile glass culture tubes. Experiments were arranged in a complete randomized design with five replications.

Pollination with irradiated pollen. Spinach is a long-day plant and flower bud initiation begins when the days are longer than 12 h. Therefore, with the initiation of flower bud

formation, female flowers were closed with pollen-proof pouches to prevent open pollination. Male flower clusters were collected the day before anthesis, and were placed into glass petri dishes for irradiation. Male flowers were irradiated with gamma rays coming from a Co⁶⁰ source. Irradiation doses were 100, 150, and 200 Gy in the first year and 200, 300, 400, and 500 Gy in the second year. After irradiation, the male flower clusters were kept at room temperature during the night for flower buds to open and for pollen anthesis. On the following day, isolated female plants were pollinated with irradiated pollen for 3 d, and then were closed with pouches until fruit set to prevent contamination (Fig. 1). In the following days, female plants were checked, and seeds reaching maturity were harvested. Flower inflorescences taken from the female plants were surface sterilized in 15% sodium hypochlorite solution for 15 min and rinsed using sterile distilled water five times. Embryos were identified using a stereo microscope and placed into sterile glass culture jars containing 0.1 mg·L⁻¹ IAA MS nutrient medium. The cultures were incubated at 25 °C under 12 h dark–12 h light photoperiod. Since Favorit F1 and Greenstar F1 varieties had more hermaphrodite flowers, fewer plants were pollinated in these varieties than in Koto F1.

Ploidy analyses. The ploidy level of plants was detected using a flow cytometer (Partec, Münster, Germany) and molecular analyses. The method employed in Keleş et al. (2015) was used for flow cytometry analyses. For molecular analyses, genomic DNA was extracted from young fresh leaves of donor plants and progenies using CTAB protocol (Doyle and Doyle, 1990). Amount of DNA was quantified on a Gene Quant pro RNA/DNA spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ). A set of ten primer pairs (SO4, SO7, SO10, SO14, SO17, SO22, SO29, SO32, SO48, and SO51) amplifying spinach microsatellites and polymorphic between the parents was used in the present study. One of these previously described microsatellite primer pairs [SO29 (GT)⁵ and TAGGGTACTGTAGAGGAAGTCG] was used to test progenies (Groben and Wricke, 1998). Polymerase chain reaction (PCR) mixtures for microsatellite analysis were prepared as described by Khattak et al. (2006) with minor modifications and contained 50 ng template DNA, 0.2 μM of each primer, 0.2 mM of each dNTP, 1× reaction buffer (Thermo Fisher Scientific, MA), and 1U Taq Polymerase (Thermo Fisher Scientific) in a volume of 10 μL. PCR products were electrophoresed using metaphor agarose in 1× TAE buffer, stained with ethidium bromide, and visualized by a gel image system (Kodak Gel Logic 200; Eastman Kodak Company, Rochester, NY).

Results

Anther culture studies. For anther culture, three different varieties were tested in four different nutrient media with five replications for 2 years, and no results were achieved in the first year of experiments.

Table 5. Number of embryos and plants obtained through pollination with irradiated pollen and 100 seed ratios (second year).

Variety	ID	PNo	NS	NE	NP	NE/100 S	NP/100 S
Koto F1	200	6	68	18	3	26.47	4.41
Koto F1	200	78	230	30	15	13.04	6.52
Koto F1	200	144	219	24	10	10.96	4.57
Koto F1	200	162	260	25	9	9.61	3.46
Koto F1	200	166	368	15	14	4.08	3.80
Koto F1	200	170	730	16	4	2.19	0.55
Koto F1	200	221	324	15	1	4.63	0.31
Koto F1	200	223	243	23	2	9.46	0.82
Koto F1	200	227	126	5	3	3.97	2.38
Koto F1	200	263	438	27	4	6.16	0.91
Koto F1	200	267	592	30	14	5.07	2.36
Koto F1	200	265	62	19	3	30.64	4.84
			3,660	247	82	6.75	2.24
Koto F1	300	38	44	23	10	52.27	22.73
Koto F1	300	74	424	9	1	2.12	0.23
Koto F1	300	76	35	8	6	22.86	17.14
Koto F1	300	83	168	16	11	9.52	6.55
Koto F1	300	117	725	29	22	4.00	3.03
Koto F1	300	132	51	26	7	50.98	13.72
Koto F1	300	155	330	10	3	3.03	0.91
Koto F1	300	167	320	6	7	1.87	2.19
Koto F1	300	173	228	25	12	10.96	5.26
Koto F1	300	176	116	18	1	15.52	0.86
Koto F1	300	222	162	27	16	16.67	9.88
Koto F1	300	235	93	13	2	13.98	2.15
Koto F1	300	295	98	30	15	30.61	15.31
Koto F1	300	319	210	7	3	3.33	1.43
Koto F1	300	330	76	25	19	32.89	25.00
Koto F1	300	333	280	30	11	10.71	3.93
Koto F1	300	370	245	10	1	4.08	0.41
			3,605	312	147	8.65	4.08
Koto F1	400	79	372	28	2	7.53	0.54
Koto F1	400	80	55	45	43	81.82	78.18
Koto F1	400	85	662	19	8	2.87	1.21
Koto F1	400	91	15	10	2	66.67	13.33
Koto F1	400	135	287	30	29	10.45	10.10
Koto F1	400	145	386	54	33	13.99	8.55
Koto F1	400	149	261	28	3	10.73	1.15
Koto F1	400	151	383	28	4	7.31	1.04
Koto F1	400	159	385	25	11	6.49	2.86
Koto F1	400	177	428	36	13	8.41	3.04
Koto F1	400	191	276	32	6	11.59	2.17
Koto F1	400	220	1,520	24	11	1.58	0.72
Koto F1	400	224	383	25	13	6.53	3.39
Koto F1	400	316	378	24	6	6.35	1.59
			5,791	408	184	7.04	3.18
Favorit F1	200	122	294	28	9	9.52	3.06
Favorit F1	300	30	122	28	14	22.95	11.47
Favorit F1	300	46	186	25	6	13.44	3.22
			308	53	20	17.21	6.49
Favorit F1	400	394	40	31	6	77.50	15.00
Favorit F1	400	411	224	25	3	11.16	1.34
Favorit F1	400	461	56	21	5	37.50	8.93
			320	77	14	24.06	4.37
Favorit F1	500	314	359	25	3	6.96	0.83
Favorit F1	500	489	108	40	17	37.03	15.74
			467	65	20	13.92	4.28
Greenstar F1	200	15	681	18	13	2.64	1.91
Greenstar F1	200	247	77	17	2	22.08	2.60
Greenstar F1	200	328	119	27	16	22.69	13.44
Greenstar F1	200	383	148	10	2	6.76	1.35
Greenstar F1	200	388	132	25	7	18.94	5.30
Greenstar F1	200	417	450	25	3	5.55	0.67
Greenstar F1	200	455	359	25	7	6.96	1.95
Greenstar F1	200	459	147	27	14	18.37	9.52
Greenstar F1	200	499	349	27	5	7.74	1.43
			2,462	201	69	8.16	2.80

(Continued on next page)

Table 5. (Continued) Number of embryos and plants obtained through pollination with irradiated pollen and 100 seed ratios (second year).

Variety	ID	PNo	NS	NE	NP	NE/100 S	NP/100 S
Greenstar F1	300	14	372	28	5	7.53	1.34
Greenstar F1	300	24	265	40	7	15.09	2.64
Greenstar F1	300	245	54	12	4	22.22	7.41
Greenstar F1	300	370	98	27	7	27.55	7.14
Greenstar F1	300	398	830	30	1	3.61	0.12
Greenstar F1	300	399	372	33	4	8.87	1.07
Greenstar F1	300	415	112	10	2	8.93	1.78
Greenstar F1	300	441	48	16	3	33.33	6.25
			2,151	196	33	9.11	1.53
Greenstar F1	400	13	569	12	1	2.11	0.17
Greenstar F1	400	244	51	19	12	37.25	23.53
Greenstar F1	400	361	250	8	1	3.20	0.40
Greenstar F1	400	385	216	22	5	10.18	2.31
Greenstar F1	400	400	486	25	4	5.14	0.82
Greenstar F1	400	447	321	28	11	8.72	3.43
Greenstar F1	400	448	378	25	4	6.61	1.06
Greenstar F1	400	501	608	33	8	5.43	1.31
Greenstar F1	400	506	113	22	10	19.47	8.85
			2,992	194	56	6.48	1.87
Greenstar F1	500	507	607	30	20	4.94	3.29

ID = irradiation dose; PNo = plant no.; NS = number of seeds; NE = number of embryos; NP = number of plants; NE/100 S = number of embryos/100 seeds; NP/100 S = number of plants/100 seeds.

Promising outcomes were achieved from the anther culture studies in the second year. Callus formation was observed in medium number 2 of Koto F1 variety (Fig. 2) and a plant was formed from that callus (Table 1). This plant was placed into a glass culture tube containing hormone-free MS medium. The plant was monitored at certain intervals, propagated, and transferred into tubes. Callus formation was also observed in the first, second, fourth, and fifth replications of number 3 nutrient medium, and the first, fourth, and fifth replications of number 4 nutrient medium of Koto F1 variety. Callus formation was also observed in Favorit F1 and Greenstar F1 varieties [first and second replications of number 3 nutrient medium of Favorit F1 and third, fourth, and fifth replications of Greenstar F1 variety (Tables 2 and 3)].

Pollination with irradiated pollen. As there was no previous work in spinach using pollination with irradiated pollen, doses of 100, 150, and 200 Gy were tested in the first year, and doses of 200, 300, 400, and 500 Gy were tested in the second year. Gamma rays coming from Co⁶⁰ were used as irradiation source, and the results are provided in Tables 4 and 5. Considering the results of the first year, it was observed that 150-Gy dose yielded better outcomes for Koto F1 variety with regard to embryo formation (number of embryos/100 seeds), and 100-Gy dose yielded better outcome with regard to regeneration to plant (number of plants/100 seeds). Among the varieties used in this study, only Koto F1 exhibited completely dioic sexual reproduction. Since both hermaphrodite and dioic flowers were observed in Favorit F1 and Greenstar F1, some of the pollinated plants were not taken into consideration. Therefore, in Favorit F1, the plants pollinated with pollen irradiated at 100 Gy were not taken into consideration, only the 150- and

200-Gy doses were compared, with the 200-Gy dose yielding better results with regard to embryo formation and plant regeneration. Only one dioic plant was obtained for Greenstar F1 variety irradiated with 200 Gy, and the high results were observed (50.00% embryos/100 seeds, 33.33% plants/100 seeds) in this plant (Table 4). However, successful outcomes were also achieved with 150-Gy dose (Fig. 3).

Experiments were conducted with 200-, 300-, 400-, and 500-Gy doses in the second year. In the Koto F1 variety, the 300-Gy dose yielded successful outcomes with regard to both embryo formation and plant regeneration. In Favorit F1, the 400-Gy dose yielded better results for embryo formation, whereas 300 Gy was better for regeneration into plant. In Greenstar F1 variety, 300 Gy gave better results for embryo formation and 500 Gy for regeneration into plant. However, only one plant was obtained with the 500-Gy dose in Greenstar F1 variety, and a similar result was observed from the 200-Gy dose of Favorit F1. Despite such deficiencies, the 300-Gy dose yielded superior outcomes in the second year.

Considering the averages for the irradiation doses of the first year, the 100-Gy dose yielded 465 embryos from 3839 seeds, and 156 of these embryos were transformed into plants (12.11 embryo/100 seed and 4.06 plant/100 seed). For the 150-Gy dose, 823 embryos and 172 plants were obtained from 5341 seeds (15.41 embryos/100 seeds and 3.22 plants/100 seeds). For the 200-Gy dose, 315 embryos and 52 plants were obtained from 2988 seeds (10.54 embryos/100 seeds and 1.74 plants/100 seeds). Thus, in the first year, the 150-Gy dose yielded better results with regard to embryo formation. Considering the averages for the irradiation doses of the second year, the 200-Gy dose gave 476 embryos and 160 plants from 6416 seeds (7.42 embryos/100 seeds and 2.49 plants/100

seeds). For the 300-Gy dose, 561 embryos and 200 plants were obtained from 6064 seeds (9.25 embryos/100 seeds and 3.30 plants/100 seeds). For the 400-Gy dose, 679 embryos and 254 plants were obtained from 9103 seeds (7.46 embryos/100 seeds and 2.79 plants/100 seeds). For the 500-Gy dose, 95 embryos and 40 plants were obtained (8.84 embryos/100 seeds and 3.72 plants/100 seeds).

When the experimental results of the 2 years were assessed with regard to varieties, it was observed that better results were achieved for the Favorit F1 variety in both years. In the first year, 312 embryos and 29 plants were obtained from 975 seeds, with ratios of 32 embryos/100 seeds and 2.97 plants/100 seeds. In the second year, 223 embryos and 63 plants were obtained from 1389 seeds with ratios of 16.05 embryos/100 seeds and 4.53 plants/100 seeds. For the Koto F1 variety, 999 embryos and 296 plants were obtained from 9921 seeds in the first year (10.07 embryos/100 seeds and 3.72 plants/100 seeds); 967 embryos and 413 plants were obtained from 13056 seeds in the second year (7.41 embryos/100 seeds and 3.16 plants/100 seeds). For the Greenstar F1 variety, 292 embryos and 55 plants were obtained from 1272 seeds in the first year (22.95 embryos/100 seeds and 4.32 plants/100 seeds); and 621 embryos and 178 plants were obtained from 8212 seeds in the second year (7.56 embryos/100 seeds and 2.17 plants/100 seeds).

Different embryo types were observed including those which were solid inside, slightly solid inside, liquid inside, almost empty inside, and empty inside. In all three varieties, empty inside and almost empty inside embryos were more common at high irradiation doses (300, 400, and 500 Gy). The plants with such embryo types exhibited slower growth than the other embryo types.

The significance of haploid plants, as well as the fully homozygotic lines of double haploids originating from them, has been increasing for numerous plant species in modern breeding programs (Nowaczyk and Kisiala, 2006). Flow cytometer was used to determine if the regenerated plants were haploid, doubled haploid, or diploid. In this study, all plants obtained from anther culture and irradiated pollen and screened via flow cytometer, were diploid or double haploid (Fig. 4). Flow cytometry results had no peak for haploid plants. Simple sequence repeat (SSR) markers from the spinach genome were used to control homozygosity or heterozygosity of the plants to determine if they were normal diploids or doubled haploids. A set of ten primer pairs (SO4, SO7, SO10, SO14, SO17, SO22, SO29, SO32, SO48, and SO51) were used, which yielded two bands for heterozygotes. Of these primer pairs, nine were monomorphic and one (SO29) was heterozygous for Koto variety. Then, 66 Koto progenies were screened using SO29 SSR primer pairs. Of these 66 Koto progenies, 23 yielded one band like the homozygote, and therefore should be spontaneous doubled haploids (Fig. 5; Table 6).

Fig. 3. (A) Female spinach flowers pollinated with irradiated pollens, (B–D) in vitro plants obtained from pollination with irradiated pollen technique.

Fig. 4. (A) Flow cytometer results for donor plant of Koto variety, (B) flow cytometer results for donor plant of Koto progeny.

Discussion

Monoic, dioic, and hermaphrodite flowering in spinach make breeding difficult. Therefore, most companies abstain from spinach breeding. However, optimization of a proper haploidization technique will definitely accelerate spinach breeding. In this study, anther culture and pollination with irradiated pollen haploidization techniques were investigated, and a basis was established for the generation of haploids for spinach breeding. To the best of our knowledge, there are no previous studies on haploidization using in vitro techniques in spinach. Thus, current findings will enlighten future research in spinach breeding.

Although no results were achieved through anther culture in the first year, some successful outcomes were obtained in the second year. During the second year, callus development and embryo formation were observed in all three varieties tested, and a plant was obtained in the Koto F1 variety. Such developments experienced in the second year indicated that further nutrient medium and genotype experiments might reveal successful combinations for anther culture procedures in spinach.

Better results were achieved from pollination with irradiated pollen than from anther culture, and the average number of embryos per 100 seeds even reached the 50s in irradiation treatments. All genotypes and irradiation

doses yielded embryo formation and regeneration into plants. Although experimental results did not clearly indicate the optimum irradiation dose, the haploidization response of spinach to pollination with irradiated pollen was considered as a remarkable outcome. With the use of this method, 3414 embryos and 1710 plants were obtained. Slow growth and yellowing were identified as the greatest problems after the regeneration into plant. Pretests revealed that BAP and FeEDHA supplementation to nutrient media may reduce the incidence of such problems.

Van Geyt et al. (1987) carried out a study on sugar beet (*Beta vulgaris* L.), which is in the same family as spinach, to obtain haploid plants, and obtained more than 2.2% haploid plants with an ovule culture method. The researchers also reviewed previous studies and indicated that successful outcomes could not be achieved in sugar beet with anther culture and pollination with irradiated pollen techniques. Bossoutrot and Hosemans (1985) were able to obtain two haploid plants from 1000 ovules in sugar beet. Doctrinal et al. (1989), Lux et al. (1990), Baranski (1996), and Gürel et al. (2000) carried out studies on sugar beet and investigated the effects of nutrient medium, genotype, and pretreatments on gynogenesis. As was seen in those studies, commercially available haploid plants were not obtained in sugar beet through tissue culture techniques. Even if haploid plants were obtained, the same response would not be observed in different species despite belonging to the same family. In other words, a protocol successfully implemented in sugar beet may be unsuccessful in spinach. For instance, although anther culture is routine in pepper and eggplant of the Solanaceae family, success has not yet been achieved in tomato, another member of the same family. Similarly, although successful outcomes are achieved through pollination with irradiated pollen in melon and squash of the Cucurbitaceae family, commercial success has not yet been achieved in watermelon of the same family.

Another interesting outcome of the present study was the fact that embryos obtained through pollination with irradiated pollen did not have endosperm, and had different shapes. In other words, although they appeared to be haploid, they were diploid according to flow cytometry results. Such a case suggests that spontaneous chromosome doubling was occurring as is commonly observed in pepper and cabbage. Spontaneous diploids were also reported by Keleş et al. (2015) in pepper (over 50%), by Gémesné Juhász et al. (2001) in sweet pepper (29.8%), by Gu et al. (2003) in *Brassica rapa* ssp. *chinensis* (over 70%), by Mohammadi et al. (2007) in maize (7%), and by Vanous (2011) again in maize varying with genotypes (2.8% to 46%). El-Hennawy et al. (2011) indicated varying spontaneous doubling in wheat with genotypes, Stober and Hess (1997) reported such rates in German spring wheat as between 15% to 44%, and Barnabas (2003) reported the rates in central and eastern European winter wheat as between 25% to 68%. Kahrizi and Mohammadi

Fig. 5. (A, B) Electrophoresis results obtained from SO29 simple sequence repeat primer pairs (order and description of samples in Table 6).

Table 6. Description of samples used in Fig. 5.

Order	Donor no.	ID (Gy)	OD	ES
For Fig. 5A				
1	Koto parent	—	—	—
2	Koto parent	—	—	—
3	Koto parent	—	—	—
4	Koto parent	—	—	—
5	191 Koto	400	29 July 2015	Liquid inside
6	191 Koto	400	29 July 2015	Solid inside
7	135 Koto	400	30 July 2015	Solid inside
8	135 Koto	400	30 July 2015	Solid inside
9	135 Koto	400	21 July 2015	Almost empty inside
10	220 Koto	400	23 June 2015	Slightly solid inside
11	220 Koto	400	27 May 2015	Empty inside
12	151 Koto	400	8 June 2015	Solid inside
13	85 Koto	400	3 Aug. 2015	Solid inside
14	221 Koto	200	16 June 2015	Solid inside
15	145 Koto	400	27 July 2015	Solid inside
16	145 Koto	400	3 Aug. 2015	Almost empty inside
17	38 Koto	300	21 July 2015	Almost empty inside
18	38 Koto	300	1 June 2015	Solid inside
19	149 Koto	400	9 June 2015	Solid inside
20	333 Koto	300	9 June 2015	Solid inside
21	333 Koto	300	3 Aug. 2015	Liquid inside
22	333 Koto	300	9 June 2015	Almost empty inside
23	267 Koto	200	13 July 2015	Solid inside
24	316 Koto	400	28 July 2015	Solid inside
For Fig. 5B				
1	221 Koto	200	3 Aug. 2015	Solid inside
2	173 Koto	300	17 Aug. 2015	Solid inside
3	235 Koto	300	10 July 2015	Almost empty inside
4	162 Koto	200	18 June 2015	Solid inside
5	265 Koto	200	30 July 2015	Almost empty inside
6	74 Koto	300	17 Aug. 2015	Solid inside
7	117 Koto	300	8 June 2015	Empty inside
8	117 Koto	300	8 June 2015	Slightly solid inside
9	235 Koto	300	31 July 2015	Liquid inside
10	6 Koto	200	21 July 2015	Slightly solid inside
11	83 Koto	300	28 July 2015	Almost empty inside
12	83 Koto	300	27 July 2015	Solid inside
13	166 Koto	200	5 June 2015	Solid inside
14	177 Koto	400	15 June 2015	Solid inside
15	144 Koto	200	3 June 2015	Solid inside
16	330 Koto	300	9 June 2015	Solid inside
17	330 Koto	300	9 June 2015	Almost empty inside
18	330 Koto	300	9 June 2015	Liquid inside
19	76 Koto	300	22 July 2015	Solid inside
20	165 Koto	300	29 June 2015	Slightly solid inside
21	224 Koto	400	18 June 2015	Solid inside
22	316 Koto	300	14 June 2015	Solid inside
23	144 Koto	200	31 July 2015	Liquid inside
24	80 Koto	400	27 May 2015	Slightly solid inside

ID = irradiation dose; OD = observation date; ES = embryo status.

(2009) reported spontaneous doubling rates in wheat as between 65% to 76%, and Mirzaei et al. (2011) reported the rates in

barley as between 63% to 72%. All these findings are from anther culture procedures carried out on different plant types. Spontaneous

doubling was not reported in plants obtained through pollination with irradiated pollen. However, this does not necessarily mean that such cases will not exist, since there are limited studies about pollination with irradiated pollen method. It was surmised according to SSR marker analysis that single-band individuals might come from homozygote double haploid plants and double-band individuals might come from somatic cells. Therefore, further molecular studies should be carried out to identify if the chromosome doubling occurred through spontaneous doubling or if plants were already diploid from the beginning.

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