Special issue of the 2nd International Conference on Computational and Experimental Science and Engineering (ICCESEN 2015)

Effects of Gamma Ray Irradiation and NaCl on Induced Somaclonal Variation in Arnavutköy Strawberry Cultivar

K. Kepenek*

Agricultural Biotechnology Department, Faculty of Agriculture, Suleyman Demirel University, 32260, Isparta, Turkey

Salinity stress impacts the growth and development of strawberry crop significantly in tropical and temperate regions across the world developing somaclonal variant which offers an option to develop salt stress tolerant genotypes. Somaclonal variants of Arnavutköy strawberry cultivar were produced via organogenesis by irradiating axillary shoots with gamma rays (20 and 40 Gy with ^{60}Co) along with imposing different NaCl concentrations (0, 250, 500, and 750 ppm). Increasing total doses of ^{60}Co together with NaCl concentrations significantly affected the percentage of survival rate, axillary shoots proliferation, length of axillary shoots, fresh weight of axillary shoots, leaf deformation scores and necrotic area of leaves. The variants with less deformation were selected for strawberry breeding. A gamma irradiation dose of 40 Gy was found to be the most effective dose together with 500 ppm of NaCl for to obtain the somaclonal variants without having much lethal effects and deformations of the strawberry breeding *in vitro* condition. If successfully applied, this methodology can lead to the identification of new strawberry cultivars resistant to NaCl within a relatively short period of time.

DOI: 10.12693/APhysPolA.130.337

PACS/topics: 61.80.Ed

1. Introduction

According to Food and Agriculture Organization [1] of the United Nations, world production of strawberries has exceeded 4 million tons since 2007 [2]. Currently, Turkey stands second in the world for strawberry production with an annual production of 353 173 tonnes [1]. Strawberry cultivation is affected by abiotic stress in tropical and temperate climate. Therefore, breeding for abiotic stress tolerance in strawberry is of high economic importance for Turkey and worldwide. Salinity is the biggest abiotic stress factor in Turkey and thus it is important to choose a suitable cultivar for it. Even though salinity is not a mutation causing agent, it may increase mutation sensitivity due to abiotic stress it causes [3]. Arnavutköy cultivar is a domestic cultivar that is aromatic, fragrant and resistant to biotic stress such as *Phytophthora infestans* and thus is preferred in breeding programmes in Turkey. Conventional breeding methods have been highly ineffective for obtaining resistance or tolerance to biotic and abiotic stresses conditions [4]. In contrast, tissue culture techniques can be used to create somaclonal variations (by organogenesis or somatic embryogenesis) through a process called somaclonal variation, which causes changes similar to those induced by physical and chemical mutagenic agents, and which can be incorporated into genetic breeding programmes [5–8]. *In vitro* cultivation of mutants and selection results in a quick dissolution of chimeras and the recovery of genetically stable mutants [9, 10]. Mutants can be selected for biotic and abiotic stress tolerance over a short period of time under *in vitro* conditions [11, 12]. Variation and mutations can be induced through a combination of radiation and chemical mutagens *in vitro* culture [13]. The *in vitro* mutation frequencies are much higher than conventional cultivation for somaclonal variation [9]. Radiation breeding has been proven to be a viable method for strawberry breeding [14]. Strawberry plants are very sensitive to gamma rays from ^{60}Co levels in the vegetative stage. Jain (2001) suggests that the optimal dose for *in vitro* irradiation of shoot is typically higher than that is used for meristem and axillary shoot irradiation. Tissue culture studies in strawberry are being done as a tool for mutation induction and as a means of micropropagation [15].

In the present study, we conducted tests for radiosensitivity of *in vitro* axillary shoots to gamma radiation; and *in vitro* selection of somaclonal variants and establishment of an assay method for evaluating the degree of somaclonal variation to NaCl.

2. Material and methods

The axillary bud shoots of strawberry were surface-sterilised. The meristems were excised and placed on solid Linsmaier and Skoog (LS) basal medium [16] for *in vitro* culture. These meristems and axillary shoots were then transferred onto LS medium supplemented with 6-benzylaminopurine (BAP; 2 ppm), indole-3-acetic acid (IAA; 0.1 ppm), ascorbic acid (80 ppm), TDZ (2.0 ppm), giberrellic acid (GA_3; 0.2 ppm), phloroglucinol (100 ppm), sucrose (30 g/l) and gelrite (3 g/l) at pH 5.6. Axillary microshoots were cultivated in solid LS medium. Fourty five axillary shoots were placed onto a fresh proliferation medium and subcultures were obtained in 30 days.

*e-mail: kahramankepenek@sdu.edu.tr

For the radiosensitivity assay, non-irradiated and irradiated axillary shoots were used [17]. The irradiation doses were applied eight days after planting the explants in the solid LS medium. Six different doses of gamma rays were tested [0 (control), 20, 40, 60, 80 and 100 Gy] and each sample was irradiated at a dose rate of 5 Gy/h irradiation with gamma rays in a ^{60}Co irradiation unit (gamma-cell). The experiment included 3 replicates with consisting of 15 axillary shoots per replication. The irradiated axillary shoots along with the control was subcultured thrice to increase enough shoots numbers for irradiation studies. After three subcultures, the axillary shoots were used for assessing somaclonal variations and *in vitro* screening for NaCl stress tolerance. Generation advancement was done in order to eliminate chimerism until M1V3 generation by subculturing somaclonal variation in the axillary shoots of M1V3 generation which were observed for the desired selection. For fast micropropagation, during post-irradiation, the explants were transferred to multiplication solid medium (same as above) by dividing the axillary shoot of each sample. Regenerated axillary shoots were irradiated with various doses (0, 20, and 40 Gy) of gamma rays. After irradiation, axillary shoots of the surviving plants were allowed to elongate, and each axillary shoot was divided and transferred onto a proliferation medium. Multiple axillary shoots were produced one month after culture. These axillary shoots were each separated, and sub-cultured onto a fresh solid LS medium. This procedure was repeated thrice (until M1V3 generation) to propagate axillary shoots and eliminate chimeras. Finally, 45 axillary shoots (≈2 cm long) were cut off and transferred onto a rooting medium [LS basal medium containing 3-indolebutyric acid (IBA) (1.0 ppm), sucrose (30 g/L) and agar (7 g/L), pH 5.6]. Various concentrations of NaCl (0, 250, 500, and 750 ppm) together with different doses of gamma rays (0, 20, 40 Gy) were applied, in order to select the most suitable somaclonal variants.

Rooted somaclonal variants plantlets were transferred to greenhouses and maintained at 25 ± 2 °C, 60–70% relative humidity and 7000 lx light intensity. The acclimatized plantlets were transferred to the soil after 1 month and advanced to M1V3 generation for further selection and evaluation. After 30 days of cultivation, M1V3 axillary shoots were irradiated with 20 and 40 Gy gamma rays and were observed for their survival rate (%), total number of axillary shoots per axillary shoot, length of axillary shoots (cm), leaf deformation scores and necrotic area of leaves (the leaf necrotic area = LNA; %), fresh weight of axillary shoots (mg), total number of somaclonal variants, DNA content (relative nuclear DNA content, RNDC; ng/μL) and purity (R280/R260), factor of effectiveness (FE, %), number of morphological variants and number of different character of somaclonal variants, number of morphological variants of axillary shoots, DNA contents of somaclonal variants of Arnavutköy strawberry which included several variants for morphological characters were evaluated.

Fifteen leaves of the first, second and third leaves from the top axillary shoots with a 2.5–5.0 cm length of axillary shoot were collected for each irradiation dose and NaCl concentration. Leaves were cut out from the axillary shoots for evaluating extended leaf deformation scores and necrosis induced by NaCl and gamma rays. Non-irradiated *in vitro* derived axillary shoots, bearing 15 leaves and with a 2.5–5.0 cm length of axillary shoot, were used as control. Control samples contained only solid LS basal medium without NaCl. At the end of four week period, axillary microshoots were assessed according to their regeneration capacity (number of axillary shoots). Leaf samples were classified into four groups by the degree of leaf deformation scores and necrotic area (LNA) grade as follows: grade 1 = 0.0–1.0: leaf deformation scores and necrotic areas on $< 5\%$ of the leaf; grade 2 = 1.1–2.0: 5–10% of the leaf; grade 3 = 2.1–3.0: 10–20% of the leaf; grade 4 = 3.1–4.0: 20–30% of the leaf; grade 5 = 4.1–5.0: 30–40% of the leaf [18, 19]. Leaves of cultivars were screened with leaf deformation scores and necrotic area of leaves, and the corresponding somaclonal variants were selected. Subsequently, somaclonal variants were elongated and rooted, resulting in the formation of somaclonal variants.

The leaf deformation scores and necrotic area (LNA) was measured (%) using GNU Image Manipulation Program (GIMP, version 2.2.10, 2005) software [20] by counting the number of pixels of the full leaf and the number of pixels of the green, brown and yellow area. RNDC of strawberry leaf samples was measured using flow cytometry. The samples were prepared from young leaves of *in vitro* propagated axillary shoots and its somaclonal variants. Non-irradiated strawberry leaf samples were used as controls to compare with the irradiated material. The obtained DNA was diluted to 50 ng/μl. The somaclonal variants derived from axillary shoots and one somaclonal variant derived from clone of each were analysed. Flow cytometry analysis of gamma ray-irradiated axillary shoots and somaclonal variants was carried out using 10 leaves for each dose, plus 10 non-irradiated as control plants.

Putative somaclonal variants (mutants) and factor of effectiveness-FE (%) analyses: FE (%) was calculated with a modified formula by Walther [8] cited by Bhagwat and Duncan [4] and described as follows:

$$\text{FE}(\%) = \text{Total number of variations} \times 100 / \text{Total number of plants treated.}$$

The test was performed in an entirely randomised experimental design with three replications, each replication represented by a tube with ten axillary microshoots and 45 axillary microshoots per dose were used. The statistical one-way analysis of variance was used to analyse data. The mean values were subjected to analysis using the Tukey–Kramer model (JMP, Version 5) to determine the dose of gamma radiation necessary to reduce or increase the above parameters for a comparison of the averages. The radiosensitivity together with NaCl were evaluated and analysed.

3. Results and discussion

Effect of radiation doses and salt concentrations on survival rate, number of axillary shoots in M1V1 generation; and on number of axillary microshoots, length of axillary shoot, weight of axillary shoot, and leaf deformation scores at axillary shoots in M1V3 generation are provided in Table I. In the irradiated axillary shoots, survival rate under non-saline conditions performed better than those grown under salinity conditions in M1V1 generation. Generally, there was a higher survival rate in the number of axillary shoots from control experiments than in the number of axillary shoots from irradiated explants. The survival rate decreased gradually as the dose of irradiation together with concentration of NaCl increased. At 750 ppm of NaCl concentration and the irradiation dose of 40 Gy, the survival rate was 57.0%. These results are supported by Weimin et al. [19]. The lethal dose 50% (LD_{50}) for inducing somaclonal variants was 40 Gy gamma rays together with 750 ppm of NaCl (Table I).

TABLE I

Effect of radiation doses and salt concentrations on survival rate, number of axillary shoots in M1V1 generation; and on number of axillary microshoots, length of axillary shoot, weight of axillary shoot, and leaf deformation scores at axillary shoots in M1V3 generation.

Radiation dose [Gy]	NaCl concentration [ppm]	Survival rate number of axillary shoots [%] ($n = 40$)	Number of axillary shoots ($n = 40$)	Length of axillary shoots [cm] ($n = 20$)	Weight of axillary shoots [mg] ($n = 20$)	Leaf necrotic area (LNA*) [%] ($n = 15$)
0 (control)	0	$98.7^{a} \pm 0.1$	$19.3^{a} \pm 0.7$	$3.0^{a} \pm 0.4$	$25.3^{b} \pm 0.8$	$4.9^{h} \pm 0.1$
	250	$97.5^{ab} \pm 0.4$	$18.2^{b} \pm 0.9$	$3.0^{a} \pm 0.8$	$23.9^{c} \pm 1.1$	$5.4^{h} \pm 0.2$
	500	$94.2^{abc} \pm 0.6$	$18.0^{b} \pm 1.3$	$2.7^{c} \pm 1.3$	$22.1^{d} \pm 1.2$	$6.1^{g} \pm 0.4$
	750	$91.4^{b} \pm 0.9$	$17.3^{c} \pm 1.2$	$2.5^{de} \pm 1.2$	$22.3^{d} \pm 1.3$	$6.8^{ef} \pm 0.9$
20	0	$80.1^{d} \pm 0.4$	$17.0^{c} \pm 0.9$	$3.0^{a} \pm 0.7$	$25.7^{ab} \pm 0.8$	$5.8^{gh} \pm 0.8$
	250	$78.6^{e} \pm 0.5$	$16.5^{cd} \pm 1.0$	$2.7^{c} \pm 0.9$	$26.8^{a} \pm 1.3$	$6.5^{f} \pm 1.0$
	500	$74.5^{e} \pm 0.7$	$15.7^{d} \pm 0.9$	$2.6^{cde} \pm 1.5$	$23.9^{c} \pm 1.2$	$10.4^{d} \pm 1.0$
	750	$73.1^{f} \pm 1.0$	$15.1^{e} \pm 1.3$	$2.3^{e} \pm 1.7$	$22.7^{cd} \pm 1.4$	$13.6^{d} \pm 1.3$
40	0	$67.4^{g} \pm 0.7$	$16.7^{cd} \pm 0.9$	$2.6^{cde} \pm 0.8$	$22.4^{cd} \pm 1.2$	$6.9^{e} \pm 1.0$
	250	$64.6^{gh} \pm 1.0$	$15.9^{d} \pm 1.0$	$2.3^{e} \pm 1.0$	$21.4^{e} \pm 1.3$	$10.7^{d} \pm 1.2$
	500	$61.2^{h} \pm 1.1$	$14.5^{ef} \pm 1.1$	$2.4^{e} \pm 1.4$	$18.9^{f} \pm 1.4$	$13.9^{b} \pm 1.4$
	750	$57.0^{i} \pm 1.3$	$13.8^{f} \pm 1.0$	$1.9^{h} \pm 1.7$	$15.0^{i} \pm 1.3$	$16.3^{a} \pm 1.4$
overall mean		78.1	16.5	2.5	22.5	9.4

^{a,b,c,d,e,f,g,h,i} — means with different superscript in the same column differ ($P < 0.05$);
n — number of samples used for analysis; * — leaf deformation score.

The number of axillary shoots was affected under concentration rates of NaCl together with doses of irradiation, and was lower than the controls. The number of axillary shoots decreased gradually as the dose of irradiation together with concentration of NaCl increased.

The number of axillary shoots separately had distinctive difference under salinity stress condition of 500–750 ppm NaCl than on non-saline medium. Generally, the numbers of axillary shoots were inhibited by all radiation doses (Table I). As related to the other recorded parameters, the doses of ^{60}Co together with concentrations of NaCl caused reduction in length of axillary shoots. These cultures included dwarf somaclonal axillary shoots and dwarf plantlets with small leave (Table I). Weight of axillary shoots generally decreased as NaCl concentration and irradiation dose increased and decrease was more prevalent in radiation dose of 40 Gy (Table I).

Axillary shoots receiving no irradiation and no NaCl showed a little or no leaf deformation scores and necrotic area. The axillary shoots grown at 750 ppm NaCl concentration and the irradiated with 40 Gy irradiation showed 16.3% necrotic areas (Table I). Thus, for somaclonal mutants, gamma rays together with NaCl were successfully evaluated *in vitro* by measuring leaf deformation scores. Increasing ^{60}Co doses together with NaCl concentrations increased necrotic spots, chlorosis and subjective leaf deformation score. The reason for the injurious effect of an elevated ^{60}Co doses together with NaCl concentrations is probably due to the increase in sensitivity of somatic mutagenesis. Under conditions of high irradiation doses together with high NaCl concentrations, many of the surviving plants showed rosette-type axillary microshoots or abnormal leaf morphology and the treatment with this application caused high mortality in cultures.

Effect of radiation doses and salt concentrations on total number of somaclonal variants and FE, RNDC of somaclonal variants and number of different character of somaclonal variants and FE from propagated the axillary shoots in M1V3 generation are given in Table II. Total number of axillary shoot, number of axillary microshoots with variation and FE in somaclonal variants showed randomness and did not show a trend with irradiation doses and NaCl concentration (Table II). FE of total number of somaclonal variants ranged from 0 to 3.06% depending on irradiation dose together with NaCl concentrations. The results of the screening with NaCl and gamma rays were mostly consistent with those obtained using the NaCl concentrations and gamma rays doses and the grade of leaf deformation scores of leaf properly corresponded to the number and FE of somaclonal variants, when applying NaCl concentrations of 500–750 ppm and gamma rays doses of 20–40 Gy. For this purpose, it is suggested that: (1) a dose of 40 Gy of gamma rays be applied for *in vitro* somaclonal variation induction of axillary shoots from *in vitro* irradiated axillary shoots; (2) generation advancement be done at least three times by subculturing (until M1V3 generation) in order to eliminate chimerism, and (3) *in vitro* selection be carried out with a 500 and 750 ppm NaCl.

TABLE II

Effect of radiation doses and salt concentrations on total number of somaclonal variats and FE, RNDC of somaclonal variats and number of different character of somaclonal variats and FE from propagated the axillary shoots in M1V3 generation.

Radiation dose [Gy]	NaC1 conc. [ppm]	Total Number of Somaclonal Variants			RNDC*		FE [%] and total number of shoot of morphological variants of axillary shoots							
							Characters							
		Total number of axillary shoot	Number of axillary microshoots with variation	FE [%]	[ng/μl]	Ratio R_{280}/R_{260}	Length of axillary microshoots		Number of axillary microshoots		Weight of axillary microshoots		Survival number of axillary microshoots	
							A	B	A	B	A	B	A	B
0 (control)	0	185	0.0	0.00	321	1.9	0.0	0.00	3.0	1.62	2.0	1.08	3.0	1.62
	250	176	1.0	0.56	212	2.0	2.0	1.13	2.0	1.13	3.0	1.70	3.0	1.70
	500	154	3.0	1.94	296	1.8	2.0	1.29	2.0	1.29	1.0	0.64	1.0	0.64
	750	180	2.0	1.11	132	1.6	2.0	1.11	1.0	0.55	0.0	0.00	1.0	0.55
20	0	131	1.0	0.76	407	1.7	2.0	1.52	2.0	1.52	2.0	1.52	3.0	2.29
	250	128	2.0	1.56	303	1.9	3.0	2.34	3.0	2.34	3.0	2.34	2.0	1.56
	500	110	2.0	1.81	191	1.7	1.0	0.90	1.0	0.90	1.0	0.90	2.0	1.81
	750	102	3.0	2.94	359	1.8	2.0	1.96	2.0	1.96	2.0	1.96	1.0	0.98
40	0	126	2.0	1.58	156	1.6	3.0	2.38	3.0	2.38	3.0	2.38	2.0	1.58
	250	134	1.0	0.74	174	1.7	2.0	1.49	2.0	1.49	3.0	2.23	3.0	2.23
	500	98	3.0	3.06	193	1.7	1.0	1.02	1.0	1.02	1.0	1.02	2.0	2.04
	750	104	3.0	2.88	205	1.8	2.0	1.92	2.0	1.92	2.0	1.92	1.0	0.96
overall mean		135.6	1.9	1.5	245.7	1.76	1.8	1.42	2.0	1.51	1.9	1.47	2.0	1.49

*Each figure is a mean of three observations; A: Total number of axillary shoot, B: Factor of effectiveness — FE [%].

By flow-cytometry analysis, no significant changes in RNDC level and its somaclonal variants were found in tested *in vitro* samples derived from axillary shoots. Isolated RNDC revealed similar quantity, which detected the concentrations ranged (in the control) from 132 ng/μl (or 1.60 ratio R280/R260) to 321 ng/μl (or 1.90 ratio R280/R260). In the same way, the analysis in RNDC of somaclonal variants showed that there was no significant changes in RNDC other than control somaclonal variants (Table II).

The reduction in length of axillary shoots was found; the more leaf deformation and necrotic area of leaves were found (Table II). The FE increased with an increase in doses of radiation together with concentrations of NaCl that is the more FE; in terms of above mentioned characters, somaclonal variants were different than their controls. These abnormalities might be partially due to the physiological stress caused by the extended and continuous culture of the plant material under different concentrations of NaCl together with doses rates of irradiation *in vitro* conditions [21]. Further studies are in progress to produce somaclonal variants in the strawberry from axillary shoots under salinity stress conditions from the promising somaclonal variants, especially from somaclonal variants obtained under high salinity (i.e. 750 ppm). This would also establish the

stability of somaclonal variants, which will then be multiplied through tissue culture for large-scale testing and release.

This study has demonstrated the useful application of gamma ray irradiation doses together with NaCl concentrations causing somatic mutagenesis *in vitro* on the LS medium for somaclonal variations, in breeding of strawberry plants. Several somaclonal variants were produced by gamma ray irradiation together with NaCl application. The somaclonal variants were selected for somaclonal variation and genetic variability. We described a method to screen for resistance in strawberry, from both irradiated and non-irradiated explants by using abiotic stres factor of various NaCl concentrations in *in vitro* conditions. Depending on our findings, a gamma irradiation dose of 40 Gy was found to be the most effective dose together with 500 ppm of NaCl for to obtain the somaclonal variants without having much lethal effects and deformations of the strawberry breeding *in vitro* condition. If successfully applied, this methodology can lead to the identification of new strawberry cultivars resistant to NaCl within a relatively short period of time.

References

[1] FAO, *Food and Agricultural Organization*, accessed on 3 April 2014, 2012.

[2] F. Wu, G. Zhengfei, A. Whidden, *Strawberry Industry Overview and Outlook*, accessed on 3 April 2014, 2012.

[3] E. Werker, H.R. Lerner, R. Weimberg, A. Poljakoff-Mayber, *Am. J. Botany* **70**, 222 (1983).

[4] B. Bhagwat, E.J. Duncan, *Euphytica* **101**, 143 (1998).

[5] S.M. Jain, *Euphytica* **118**, 153 (2001).

[6] S.M. Jain, *J. Plant Cell Tiss. Organ Cult.* **82**, 113 (2005).

[7] S. Vezzulli, L. Leonardelli, U. Malossini, M. Stefanini, R. Velasco, C. Moser, *J. Exp. Botany* **63**, 6359 (2013).

[8] F. Walther, in: *Induced Mutations in Plants. Proc. IAEA/FAO Symp. on the Nature, Induction and Utilization of Mutations in Plants, Pullman (USA)*, IAEA/FAO, Vienna 1969, p. 261.

[9] S.M. Kaeppler, H.F. Kaeppler, Y. Rhee, *Plant Mol. Biol.* **43**, 179 (2000).

[10] J.S. Mohan, *Roman. Biotechnol. Lett.* **15**, 88 (2010).

[11] S.S. Bidabadi, S. Meon, Z. Wahab, S. Subramaniam, M. Mahmood, *Austral. J. Crop Sci.* **6**, 391 (2012).

[12] T.B. Dao, P.D. Nguyen, Q.M. Do, T.H. Vu, T.L. Le, T.K.L. Nguyen, H.D. Nguyen, X.L. Nguyen, *Plant Mutat. Rep.* **1**, 26 (2006).

[13] S. Predieri, *J. Plant Cell Tiss. Organ Cult.* **64**, 185 (2001).

[14] S.M. Jain, *Acta Horti.* **447**, 69 (1997).

[15] Gu Di-Zhou, Jun-Yi Zhu, Yun-Tian Jiang, Ying Feng, Yu Liang, Cunying Ba, Li-Na Zhang, *South China Fruits* **2010**, 15 (2010).

[16] E.M. Linsmaier, F. Skoog, *Physiol. Plant.* **18**, 100 (1965).

[17] P. Donini, A. Sonnino, in: *Somaclonal Variation and Induced Mutations in Crop Improvement*, Eds. S.M. Jain, D.S. Brar, B.S. Ahloowalia, Kluwer Academic Publ., London 1998, p. 255.

[18] N. Delhomez, *Hortic. Sci.* **30**, 592 (1995).

[19] W. Weimin, Z. Mizhen, W. Zhuangwei, Q. Yaming, Y. Ji, *ISHS Acta Horti.* **842**, 597 (2009).

[20] *GIMP, GNU Image Manipulation Program* (created by S. Kimball and P. Mattis), ver. 2.2.10, 2005.

[21] Liu Yan-Meng, Xue-Ying Zhang, Hui-Bo Ge, Li-Jin Zheng, Chuan Wu, *J. Agricult. Univ. Hebei* **2008**, 26 (2008).