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Genetic Improvement of Salinity Tolerance in Wheat

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In vitro **mutagenesis for selected salt tolerant wheat cultivars was carried out, by exposing embryogenic calli of Kharj A and Kharj B to gamma rays at different doses (0, 40, 80 and 120 Gy) and then treated with 0, 0.9 and 1.2 % NaCl. Salt stressed calli exhibited higher levels of proline content and the 1.2 % NaCl stressed calli exhibited 9 and 8 folds proline accumulation in Kharj B and Kharj A, respectively. The non-salinized irradiated calli revealed successive increasing of proline content and combining the effects of gamma rays and salinity stress showed progressively increasing in proline content that reached the maximum at 120 Gy under 1.2 % NaCl. SDS-PAGE analysis of total proteins revealed 11 variable bands among a total of 28 bands. Non-salinized calli irradiated with gamma ray showed 3 disappeared bands at the 3 different gamma doses in Kharj A, while 2 bands disappeared only at 120 Gy in Kharj B. Conversely, two protein bands were induced in Kharj A at 80 and 120 Gy, respectively. Combining the effects of salinity stress and gamma rays showed 8 of the variable bands were affected by gamma rays, whereas some were induced at different gamma doses under 0.9 and 1.2 % NaCl. The non-irradiated calli displayed some isozyme bands of the 4 enzymes that appeared under either 0.9 or 1.2 % NaCl. The effects of non-salinized calli irradiated with different gamma ray doses induced some distinctive bands either similar or different to the bands appeared under salinity stress at 40, 80 and 120 Gy.**

Key words: *In vitro* embryonic culture, Wheat varieties,
Gamma rays and salinity stress, protein and isozyme markers.

The mutagenesis technology has been applied to plant breeding comprehensively, which allowed crops to produce beneficial varieties with good traits[1,2]. In recent years, *in vitro* mutagenesis technology has been applied more frequently to the development of quality and to improve resistance traits, which has accelerated crop improvement and germplasm innovation[3]. *In vitro* techniques mainly include microspore culture, anther culture, shoot organogenesis, somatic embryogenesis, and protoplast fusion and so on. *In vitro* culture, especially microspore culture, in combination with induced mutations such as using physical mutagens (UV, gamma, X-ray, and so on), chemical mutagens (EMS, $NaNO_3$, colchicines, herbicides, salinity, silver nitrate, and so on), and plant growth regulators (GA, IAA, BAP, JA, and so on) has been extensively used to speed up breeding programs, from the generation of variability, through selection and multiplication of the desired genotypes[1]. The *in vitro* culture of propagated crops in combination with induced mutations has proved to be a valuable method to produce desired variation, and to rapidly multiply the selected mutants and parental material in a disease-free condition[4]. Crop production is greatly inhibited by numerous biotic and abiotic stresses. *In vitro* mutagenesis techniques have been extensively applied in plant breeding. These

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methods induce point mutations, deletions, or insertions and have been useful in breeding for biotic[5,6] and abiotic[7,8] stresses in crops. Biotechnology tools such as marker-assisted breeding, tissue culture, *in vitro* mutagenesis, and genetic transformation can contribute a lot to solve or reduce these problems[9]. Among these abiotic stresses, drought, water logging, salinity, ozone exposure, UV irradiation, heat, wounding, and heavy metals are very crucial and limit crop production. Soil salinity affects total nitrogen uptake and soil nitrogen contribution[10] resulting in reduced yield. The transgenic, mutagenic, and genetic approaches have improved strongly the understanding of the genetic and molecular mechanisms of salinity tolerance in plants, and this will help to develop crops with improved tolerance[11]. Excessive salt accumulation in the soil has devastating effects on plant growth, which results in huge losses in terms of yield. Salinity tolerance by plants depends primarily on genotype together with metabolic and physiological events[12]. Improvement of salt tolerance in crops through conventional breeding methods has provided very limited success[13]. This study aims to genetic improve salinity tolerance in wheat and develop induced mutant cell lines via *in vitro* gamma mutagenesis, as well as develop low-cost biochemical genetic markers for salinity tolerance.

MATERIALS AND METHODS

Callus Induction

Immature grains of main tiller spikes (14 days post anthesis) of the two wheat varieties were surface sterilized in 70 % ethanol and in 20 % commercial bleach, followed by several washing several times of sterile distilled water. Immature embryos aseptically dissected from the grains and placed with embryo axis upward on MS medium[14] supplemented with 2 $mg.L^{-1}$ 2,4–D and 30 $g.L^{-1}$ sucrose. All media were solidified with 7.L^{-1} agar after adjusting pH to 5.8. Five embryos were excised from similar age of each spike and cultured in each jar[15].

Gamma Irradiation and in Vitro Salt Treatment

One month growing calli were irradiated with 40, 80 and 120 Gy gamma doses with a dose rate of 1.64 $Kr.min^{-1}$. Calli were immediately transferred to the callus culture medium containing 0,9 and 12.L^{-1} NaCl for 30 days stress period[16].

Estimation of Free Proline Content

Free proline contents of calli of two the wheat varieties were determined according to Bates[17]. 500 mg calli exposed to 0, 40, 80 and 120 Gy gamma rays and treated with 0, 0.9 and 1.2 % NaCl were used for the study.

Total Proteins and Isozyme Electrophoretic Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[18]. Calli of mature embryo cultures of the two wheat varieties were ground in liquid nitrogen and 1 ml of extraction buffer was added. After centrifugation for 10 min at 12,000 rpm under 4°C, the supernatant was collected. Electrophoresis was carried out at 4°C and the gel was stained with silver nitrate according to Goldberg and Warner[19] until the bands were clearly observed. Gel bands were scanned and analyzed using Gel Doc Bio-Rad system. Four enzymes; esterase (est), polyphenoloxidase (PPO), acid phosphatase (acp) and peroxidase (PRX) were extracted from calli of the two wheat varieties under control, gamma doses and NaCl concentrations with 1.5 ml extraction buffer (pH 8.9). Samples were centrifuged for 10 minutes at 10,000 rpm at 4°C. Polyacrylamide gel electrophoresis (PAGE) was performed according to Stegemann[20]. Gel preparation and enzyme staining solutions were performed according to Wendel and Weeden[21].

RESULTS AND DISCUSSION

Data in Table (1) showed the free proline content percentage of the two varieties at three gamma doses and under different NaCl conc. Salt stressed callus cultures exhibited higher levels of free proline content as compared to the controls of the two wheat varieties. The 1.2 % NaCl stressed calli exhibited nine and eight folds proline accumulation in Kharj B and Kharj A, respectively than that of the control. The non-salinized irradiated calli of Kharj B and Kharj A revealed successive increasing of free proline content (8.2, 12.1 and 14.8 %) in Kharj B, while Kharj A showed (6.3, 9.1 and 13.7 %) at 40, 80 and 120 Gy gamma rays, respectively in the two varieties. Combining the effects of gamma rays and salinity stress showed progressively increasing in free proline

Table 1. The variable bands of total proteins extracted from the calli, as well as the free protein content of two wheat varieties at different gamma ray doses (Gy) under 0.0, 0.9 and 1.2 % NaCl

Band No.	MW (KDa)	Kharj B													Kharj A											
		Control			40 Gy			80 Gy			120 Gy			Control			40 Gy			80 Gy			120 Gy			
		0	0.9	1.2	0	0.9	1.2	0	0.9	1.2	0	0.9	1.2	0	0.9	1.2	0	0.9	1.2	0	0.9	1.2	0	0.9	1.2	
1	234						+			+			+										+				
3	+	+3	+	+	+	+	+	+							+												
5	140	+	+		+	+		+			+				+		+		+			+				+
8	110	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+		+				+			
10	100	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+		+				+			
12	90		+	+		+	+		+	+					+	+		+	+	+							
14	76		+	+		+	+		+	+		+	+			+			+				+				+
16	53		+	+		+	+		+	+					+	+		+									
22	34	+	+	+	+	+	+	+	+					+	+	+		+									
25	25	+	+	+	+	+		+			+				+	+		+	+		+	+	+	+			
28	15	+	+	+	+	+	+	+	+		+				+	+		+	+		+	+	+	+			
proline content (100 %)	2.4	8.4	18.2	8.2	12.9	21.2	12.1	16.1	24.3	14.8	20.3	28.5	2.2	9.5	16	6.3	11.7	21.2	9.1	12.3	25.4	13.7	25.9	32.8		

(+) = the presence of protein band

Table 2: Isozyme electrophoretic analysis of four enzymes extracted from the calli of two wheat varieties at different gamma rays doses under different NaCl concentrations

Isozyme effect	Enzyme	Wheat cultivar	Salinity (100 %)			Gamma rays (Gy)				Gamma rays on salinity						
			Band No.	0.9	1.2	Band No.	40	80	120	Band No.	40		80		120	
											0.9	1.2	0.9	1.2	0.9	1.2
Disappearance	EST	Kharj B	8	+	+	8		+	+	2,6,8	41	+				
			6		+	6			+							
		Kharj A	8	+	+	2	+	+	+	2		+				
			2.3		+	2				8					+	+
	PPO	Kharj A	8,10	+	+	8,10		+	+	8,10,5					+	+
			2		+	5			+							
	ACP	Kharj B	5	+	+	5,13	+	+	+	3,5,13	+	+				
		Kharj A	3	+	+	3		+	+	3	+	+	+	+		
			9,11		+	11	+		+	11					+	+
	PRX	Kharj A	1,6	+	+	1	+	+	+	1,6,5	+	+	+	+		
			5		+	5			+							
Appearance	EST	Kharj B								11			+	+	+	+
										6					+	+
	PPO	Kharj A	9,11	+	+	9	+			11	+	+	+	+	+	+
										4		+	+	+	+	+
										9			+	+		+
	ACP	Kharj B				12		+		12	+		+	+	+	
						6,7			+	6,7			+	+	+	
		Kharj A	10		+	13	+	+	+	10		+	+	+	+	+
										13			+	+	+	+
	PRX	Kharj B	6,10	+	+	9	+			10	+	+	+	+	+	+
										6		+	+	+	+	+
										9				+		
		Kharj A	10	+	+	10	+	+	+	10		+	+	+	+	+
			3		+	9	+			9						

content in the two varieties. Whereas, the irradiated calli of Kharj B under 0.9 % NaCl exhibited 12.9, 16.1 and 20.3 % at 40, 80 and 120 Gy gamma rays, respectively and Kharj A displayed 11.7, 12.3 and 25.9 % at the previous three gamma doses. It is interesting to note that, free proline content in the irradiated calli of the two varieties increased at 1.2 % NaCl than that of 0.9 % NaCl, as the gamma doses increased from 40 to 120 Gy. The maximum values of proline contents were observed at 120 Gy and under 1.2 % NaCl, where showed 28.5 % in Kharj B vs. 32.8 % in Kharj A. Consequently, proline accumulation increased significantly as salinity level raised in the culture media and also more accumulated at the high gamma ray doses. These results are in agreement with several reports, for example[22,23]. Proline accumulation in plant cells exposed to salt or water stress is widespread phenomenon[24]. It has been speculated that it can serve as an osmotic regulator[25]. Proline contents in the M3-403-6 line treated by 1.25 % salt increased significantly compared to the original variety. It is confirmed that the differences in the accumulation patterns of proline in Dongjinbyeo and its mutation line indicate the involvement of different proline mechanisms related to salt tolerance[26]. Accumulation of organic solutes such as proline under stress conditions are among those nonspecific mechanisms[27]. Proline found to reduce the toxic effects of NaCl for helical destabilization at DNA replication[28].

SDS-PAGE analysis of total proteins extracted from the calli of Kharj B and Kharj A, which irradiated with 40, 80 and 120 Gy gamma doses and treated with 0.9 and 1.2 % NaCl revealed 28 protein bands with different molecular weights ranged from 234 to 15 KDa as presented in figure (1). Among such protein bands, eleven showed high variability, while the other residual bands were commonly detected in wheat calli under different gamma rays and NaCl treatments (Table 1). The non-irradiated wheat calli treated with 0.9 and 1.2 % NaCl revealed some distinctive bands that disappeared at each of 0.9 and 1.2 %. For instance, two bands with molecular weights 210 and 140 KDa were disappeared at 0.9 % NaCl in Kharj A, while two bands with 140 and 110 KDa were disappeared at 1.2 % in Kharj B and in Kharj A, respectively. On the other hand, four other protein bands were induced at either 1.2 % or at both of two NaCl levels, such as band with 234 KDa at 1.2 % in Kharj B and two bands with 90 and 53 KDa in the two wheat varieties. Moreover, band with 76 KDa was induced in Kharj B at the two NaCl levels. When non-salinized calli irradiated with gamma ray, three protein bands (210, 140 and 34 KDa) were disappeared at the three different gamma doses in Kharj A, while two bands with 210 and 34 KDa were disappeared only at 120 Gy in Kharj B (Table 1). Conversely, two other protein bands with 90 and 25 KDa were induced in Kharj A at 80 and 120 Gy, respectively. However, Kharj B revealed no induced protein bands at any of gamma ray doses. The genetic variability among the *in vitro* mutagenized wheat calli that treated with different NaCl revealed outstanding differences in the banding profiles represented by their presence and absence as shown in figure (1-a). Eight of the variable protein bands were significantly affected by gamma irradiation. Hence, they uniquely detected in the controls of the two wheat varieties under 0.9 and 1.2 % NaCl, while disappeared at different doses of gamma ray (Table 1). For example, at 40 Gy, four bands with molecular weights 110, 100, 53 and 34 KDa were disappeared at 1.2 % NaCl in Kharj A, while one band with 25 KDa was disappeared a 1.2 % NaCl in Kharj B. At 80 Gy gamma dose, seven of the eight inhibited protein bands were disappeared under 0.9 and 1.2 % NaCl in Kharj A and four bands were affected by gamma irradiation in Kharj B under 1.2 % NaCl, whereas two of them were disappeared under 0.9 %. At 120 Gy gamma dose, six and seven bands were disappeared under 0.9 % NaCl in Kharj B and Kharj A, respectively. While all the eight inhibited bands were disappeared at 1.2 % NaCl in the two varieties. On the other hand, three protein bands were induced at different gamma doses, which disappeared in the controls. For instance, band with 234 KDa appeared only under 1.2 % NaCl at 40, 80 and 120 Gy in Kharj B, while it appeared only at 80 Gy gamma dose under 0.9 % NaCl in Kharj A. Two other bands with 140 and 76 KDa were induced at 40, 80 and 120 Gy in Kharj A under the high NaCl dose (1.2 %).

The electrophoretic profiles of four enzymes; EST, PPO, ACP and PRX of Kharj B and Kharj A at 40, 80 and 120 Gy gamma ray and under 0.9 and 1.2 % NaCl are presented in figure (1). Esterase (EST) isozyme analysis displayed eight

bands; six of them were variable with different genetic responses in the two wheat varieties at different gamma ray doses and under salinity stress as shown in figure (1-b). The appearance of new EST bands revealed no any genetic changes in the two wheat varieties, when each of salinity and gamma-radiation treated individually. However, combining the genetic effects of gamma rays and salinity induced two band numbers 11 and 6 in Kharj B at 80 and 120 Gy, respectively under 0.9 and 1.2 % NaCl (Table 2). The non-irradiated Kharj B calli revealed two disappeared EST bands (8 and

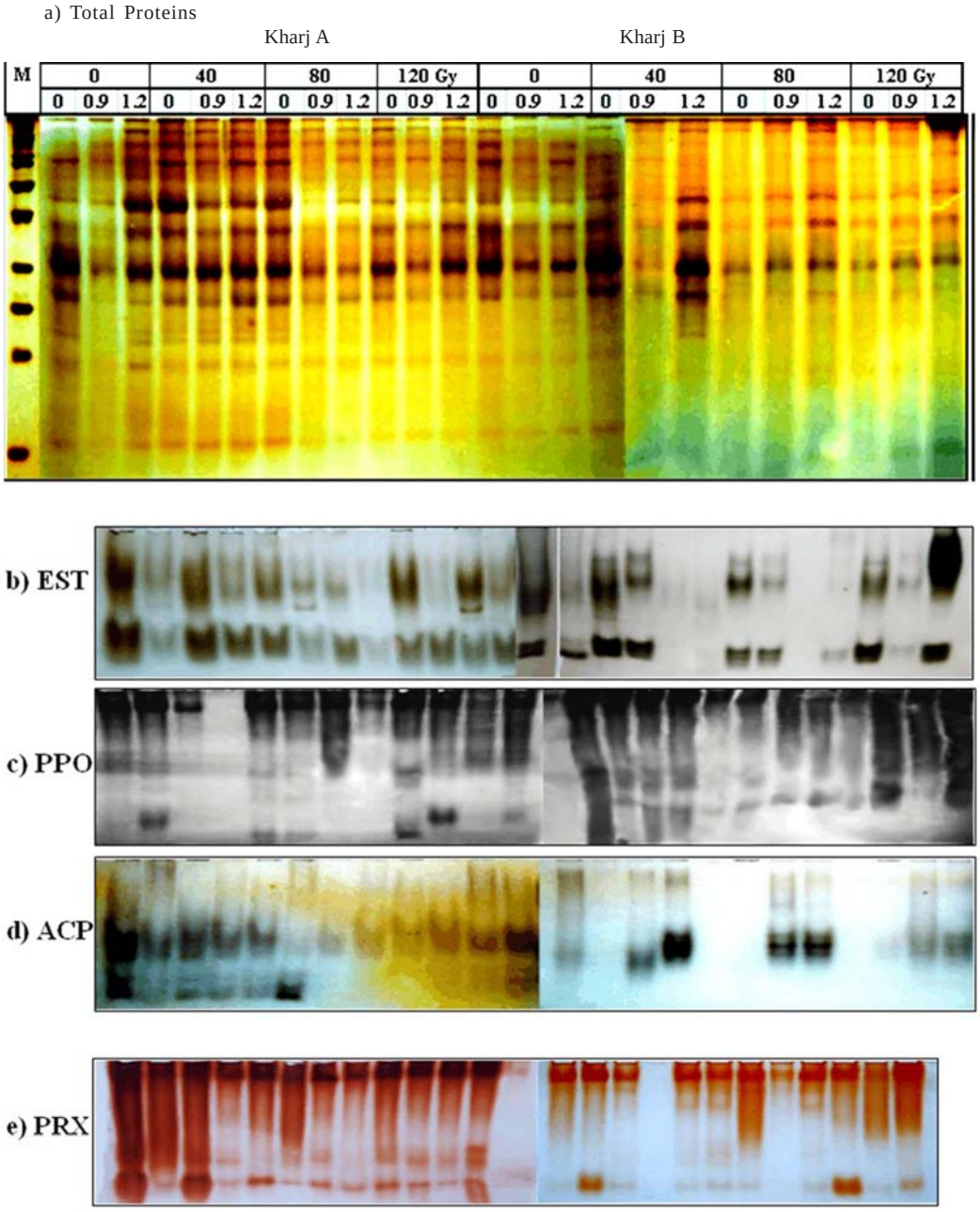

Fig. 1. SDS-PAGE profile of total proteins (a) and isozyme electrophoretic zymograms of four enzymes; EST (b), PPO (c), ACP (d) and PRX (e) of calli derived from two gamma irradiated wheat varieties at 40, 80 and 120 Gy doses and under 0.9 and 1.2 % NaCl. M= protein marker with 13 bands from 205 to 6.5 KDa

6) at 0.9 and 1.2 % NaCl and at 1.2 %, respectively. While, non-irradiated Kharj A showed three bands, two of them (2 and 3) disappeared under 1.2 % NaCl and band number 8 disappeared under the two NaCl levels. The non-salinized Kharj B calli showed the two similar bands that disappeared under salinity and were also disappeared at 80 and 120 and at 120 Gy gamma doses, respectively. However, one EST band number 2 disappeared under the three gamma doses in the non-salinized Kharj A. When combining the effects of both gamma and salinity stress, one additional EST band number 2 was disappeared in Kharj B under 40 Gy at the two NaCl levels, besides the two disappeared bands (8 and 6). In Kharj A, two bands disappeared, one of them that disappeared under salinity band 8 was disappeared at the high gamma dose 120 at 0.9 and 1.2 % NaCl, and band 2 disappeared at 40 Gy only under 1.2 % NaCl (Table 2). Polyphenoloxidase (PPO) isozyme analysis revealed a total of eleven bands, whereas three of them are variable and exposed the effect of gamma ray and salinity with different genetic response of the two wheat hybrids as shown in figure (1-c). Salinity stress induced two PPO band numbers 9 and 11 in Kharj A at both of 0.9 and 1.2 % NaCl. While, the effect of gamma ray alone induced one band number 9 at 40 Gy. it is interesting to note that, a new PPO band number 4, beside the two previous bands, was appeared in Kharj A at 40 Gy under 0.9 % NaCl, when combining the effects of gamma-radiation and salinity stress together. However, Kharj B showed no appearance of PPO bands at either salinity or radiation (Table 2). On the other hand, in Kharj A, two PPO bands (8 and 10) of three were disappeared under 0.9 and 1.2 % NaCl and disappeared also at 80 and 120 Gy gamma rays. They also showed the similar disappearance when combining the gamma and salinity stresses at the higher gamma dose under the two NaCl levels, with an additional band (number 5), which disappeared under the high gamma ray dose. Acid phosphatase (ACP) profile showed 12 bands with three appeared new band numbers (6, 7 and 12) in Kharj B and two (10 and 13) in Kharj A as shown in (Figure 1-d and Table 2). No any appeared ACP bands could be detected in Kharj B under the different NaCl conc., while band number 10 appeared at 1.2 % NaCl in Kharj A. However, one band number 12 appeared at 80 Gy and disappeared at 120 Gy and two bands (6 and 7) appeared only at 120 Gy in Kharj B, while Kharj A displayed new isozyme band (no. 13) that appeared at the three gamma ray doses.

Combining salinity and radiation effects displayed all the previous ACP bands at different gamma ray doses and salinity stress levels, without any additional new bands in both wheat varieties. One ACP band (number 5) was disappeared in Kharj B under the two salinity levels and also disappeared under the three gamma doses, with an addition band number 13. The salinized-irradiated Kharj B displayed the two bands at 40 Gy under 0.9 and 1.2 % NaCl. In Kharj A, two bands (numbers 3 and 11) were disappeared under salinity concentration and resumed at different gamma rays. The salinized-irradiated calli of Kharj A displayed band number 3 at 40 and 80 Gy under all NaCl conc., while band number 11 disappeared at the high gamma dose under the two NaCl conc. (Table 2). The analysis of peroxidase (PRX) isozymes revealed ten bands as shown in figure (1-e). Some common isozyme bands appeared in the two varieties with different genetic responses under salinity and radiation stresses. Under salinity stress, band number 10 appeared in the two varieties at 0.9 and 1.2 % NaCl, while two other bands (6 and 3) appeared at the two NaCl conc. in Kharj B and at 1.2 % NaCl in Kharj A, respectively. However, at 40 Gy gamma dose, band number 9 appeared due to radiation in both of the two wheat varieties and band number 10 that induced under salinity was also appeared at the three gamma doses in Kharj A. The combination of gamma rays and salinity stress effects revealed the two induced bands (numbers 9 and 10) in the two varieties at different gamma ray doses and salinity levels, with an additional salinity induced band number 6 in Kharj B that appeared at 40 Gy under 1.2 % NaCl (Table 2). In Kharj B, three PRX bands (1, 6 and 5) were disappeared under the two NaCl conc. and in the irradiated-salinized calli at 120 Gy gamma rays under all NaCl conc. The results from the present study are in conformity with the study by Rashid[29] on the detection of genetic variation using molecular techniques among the irradiated and salt stress (200 mM NaCl) calli. El-Sayed[30] detected biochemical genetic markers of some gamma irradiated maize hybrids *in vitro* culture. Moreover, the obtained results concerning the effect of

gamma irradiation upon isozymes are in agreements with many reports. For example, Yoshiba and Yamaguchi[31] compared peroxidase isozyme patterns between two gamma radiation-induced dwarf mutants and their parental variety, and they found difference in isozyme patterns. Changes in peroxidase isozymes due to treatment with radiation had also been reported as marked increase activity of leaves from plants originated by irradiated nodal segments of sweet potato[32]. Moreover, Lage[33] emphasized that gamma radiation as an agent able to induce changes in peroxidase isozyme profiles when compared with the sweet potato control. Maluszynski[34] pointed out that gamma radiation is the preferred choice of radiation for inducing mutations in plants. He also reported that, mutations provide a rapidly inducible source of genetic variation that potentially expands the genetic repertoire and adaptation of crops.

CONCLUSION

In vitro mutagenesis for wheat cultivars Kharj A and Kharj B was carried out by using gamma rays to improve soil salinity resistance. Proline content as a potential osmoregulant highly increased with genetic mutagenesis that reached to 120 Gy under 1.2 % NaCl. The mutation induced by gamma rays led to production of new protein which improved salinity tolerance in wheat.

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