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CHANGES OF PROTEINS AND OXIDATIVE ENZYMES IN SEEDS AND *IN VITRO* REGENERATED PLANTS OF THREE IRANIAN CULTIVARS OF WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

Somaclonal variation is one of the possible sources of variation in plant breeding. To evaluate the usefulness of somaclonal variation for creating variation in some Iranian cultivars of wheat, changes in proteins and oxidative enzymes in regenerated and seed-produced plants of wheat were studied. Three populations of each plant were used each of which belong to one embroyogenic callus or one ear. Immature embryos from three cultivars of wheat (Alamout, Hyrmand and Maroon),14 days after anthesis were cultured on solid MS medium with $1mgl^{-1}$ 2,4-D. The embryogenic calli obtained were then transferred to solid hormone-free MS medium to produce plantlets. Seeds of these three cultivars of wheat were also cultured on solid hormone free MS medium. Plantlets obtained from the two above sources were collected and used for the study of proteins, peroxidases, polyphenoloxidases and superoxidedismutases. There was no significant difference in the banding pattern SDS-PAGE and PAGE systems.Comparison of proteins between different regenerated,or between different seed plants and the resulting dandrogram showed that the plants derived from seed culture belong to one group and those derived from tissue culture belong to another group. The level of peroxidase isozymes were lower in regenerated plants while superoxidedismutase represented additional isozyme in regenerated plants. Polyphenoloxidase had equal isozymes in both kinds of plant.

Introduction

Wheat is one of the most important agricultural crops. Thus it has always been important for investigators to develop strains with desirable genotypes. But these attempts specially to increase yield, have failed to give results. For such crop breeding it needs alternative techniques. The techniques such as recombinant DNA technology has been suggested as possible alternative (Maheshwari *et al.*, 1995). This technology requires regeneration of cells or tissue cultured *in vitro*. By using this method, variation caused by tissue culture is significant. In spite of low variation from regenerated Graminae of embryogenic culture, there is no assurance that genetic variation can not occur in regenerated somatic embryo (Lindsey & Jones, 1989).

Variation and unstability of regenerated plants have been shown. The usefulness of these changes depends on selected desirable mutants, but the unstability of this variation is one of the biggest problem. Scowcroft & Larkin (1981) for the first time carried out studies on somaclonal variation induced by tissue culture indicating the ability of somaclonal variation to cause genetic variation in wheat, resulting in variation of plant height, protein content, amylase amount, tolerance to aluminum and freezing conditions, change of flowering time, variation of ploidy level, decline in chromosome number and activation of transposable elements have been reported (Maheshwari *et al.*, 1995). Changes in isozyme patterns represented a wide variation. Ryan & Scowcroft (1987)

analyzed β-amylase isozymes and showed five additional isozymes in regenerated plants of wheat (Smith, 1984). Bapat *et al.*, (1992) studied peroxidase isozymes in different developmental stages of somatic and germinated embryos and reported three peroxidase isozymes in germinated embryo of wheat, one of which was exclusively formed in germinated embryo while the other two were found in callus as well.

Phenotypic and genetic methods are common for evaluating somaclonal variation. We selected electrophoresis of proteins and enzymes and also chromosome number from the above two methods and investigated changes in proteins, peroxidase, superoxidedismutase and polyphenoloxidase isozymes in regenerated wheat plants and compared them with the results obtained from intact plants.

Material and Methods

1. Culture of immature embryos and its regeneration: Seeds of three cultivars of bread wheat (Alamout, Maroon and Hyrmand) were grown in the fields of Seed and Plant Improvement Institute Karadj, Iran. Caryopses were dissected out of the spikes, 10-14 days after anthesis, washed with soap and water and then sterilized with 3% NaOCl for 15 min., and rinsed four times with sterile distilled water. Under sterile conditions the , embryos were excised and scutellum placed on MS medium with $1mgl^{-1}$ 2,4-D and 2% agar. The callus obtained were subcultured on MS medium supplemented with 2mg 2,4-D, 500 mg glutamine and 100 mg casein hydrolysate l^{-1}, after one month interval. The embryogenic calli were then transferred to hormone-free MS medium, for a 16 hr photoperiod and 2000 Lux (light intensity) for developing embryos to whole plants.

2. Culture of seeds: Seeds of the same cultivars were again washed with soap and water then soaked in 0.2% benomyl for 5 hr and after washing with water the seeds were sterilized with 3% NaOCl and 0.1% $HgCl_2$ for 15 min., each. They were then rinsed four times with sterile distilled water and cultured on hormone-free MS medium and incubated for a 16 hr photoperiod with 2000 Lux intensity light and collected after 10 days.

3. Proteins and enzymes electrophoresis

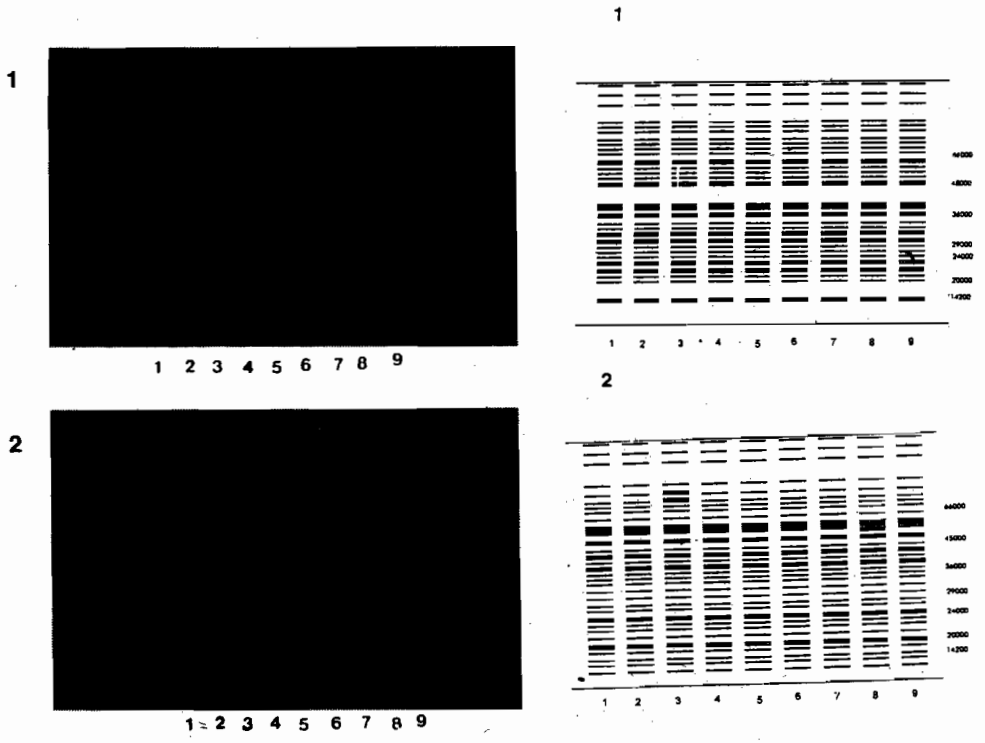
Protein extraction: For protein extraction, Tris-glycine buffer (pH 8.3) was used. The ratio of buffer to dry material was 3v/1w. SDS - PAGE and PAGE electrophoresis techniques utilized 10% acrylamide gel and electrode buffer in PAGE system consisting of 25mM Tris, 192 mM glycine, but the buffer in SDS-PAGE system was supplemented with 1% SDS (Smith, 1984; Hames & Rickwood, 1990).

The reaction mixtures used for visualization of banding pattern of enzyme in the gel were as follows:

Peroxidases (EC 1.11.1.7): 0.2 M benzidine in 27ml 50° methanol, 90ml 14.7% H_2O_2 in 36ml 0.2M acetate buffer (pH 5) (Van Loon, 1971).

Polyphenoloxidases (EC 1.14.18.1): 0.2 M phosphate buffer (pH 6.8) 100ml, 73.5% $CaCl_2$. $2H_2O$ 104 ml, 0.5% L- dopa 20ml. The gel was kept in this solution for 1hr (Van Loon, 1971).

Superoxidedismutases (EC 1.15.1.11): The gel was first immersed in a solution that consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.03 mM riboflavin, 1.25mM NBT, 326% v/v (TEMED) for 30 min., and then illuminated on light box for 20 min., (Constantine & Stanley, 1971).



Figs. 1-2. Electrophoresis pattern of proteins of leaves, seed-produced and regenerated plants by SDS-PAGE electrophoresis method.
Lanes 1,2,3 Alamout cv. Lanes 4,5,6 Hyrmand cv., and lanes 7,8,9 Maroon cv.

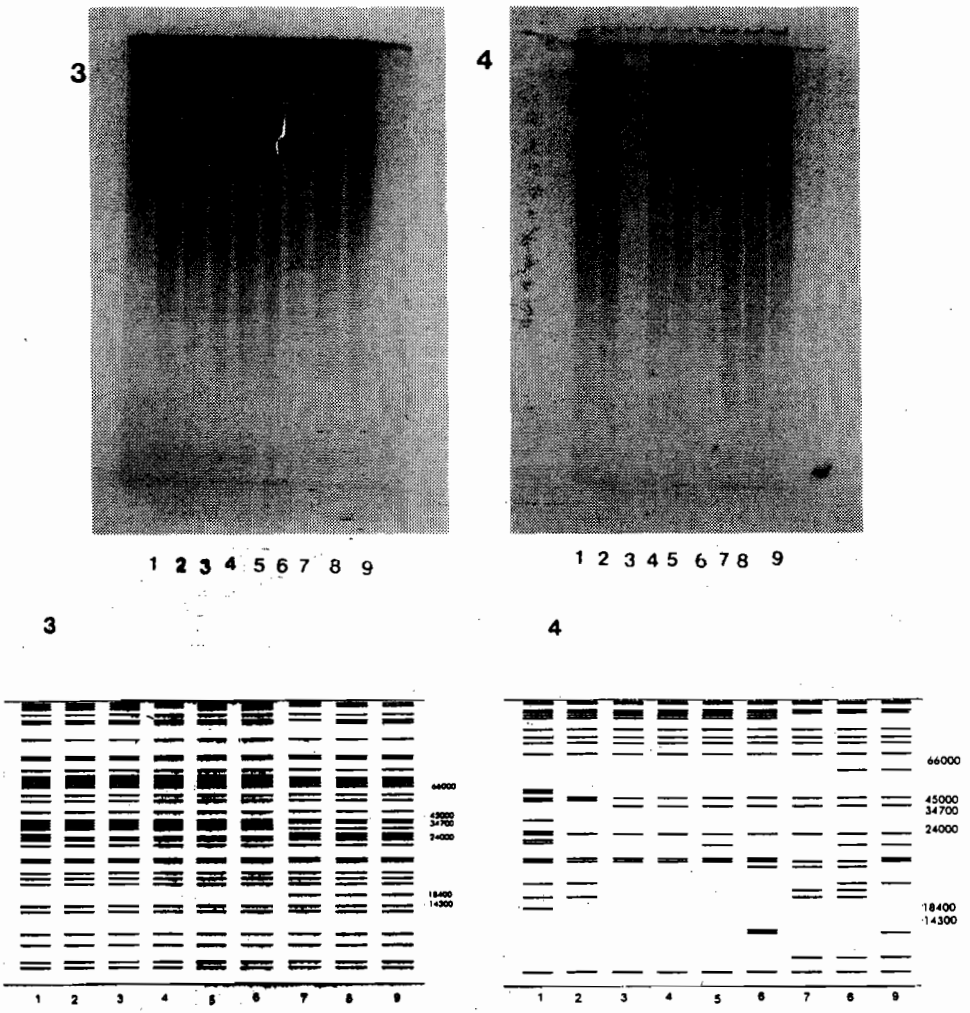
For protein determination, Bradford method was utilized (Bradford, 1976) and the gel was loaded with equivalent amount of protein from each extract.

4. Proteins: SDS - PAGE and PAGE gels were stained with Coomassi Blue R-250 (Simth, 1984; Hames & Rickwood, 1990).

Results

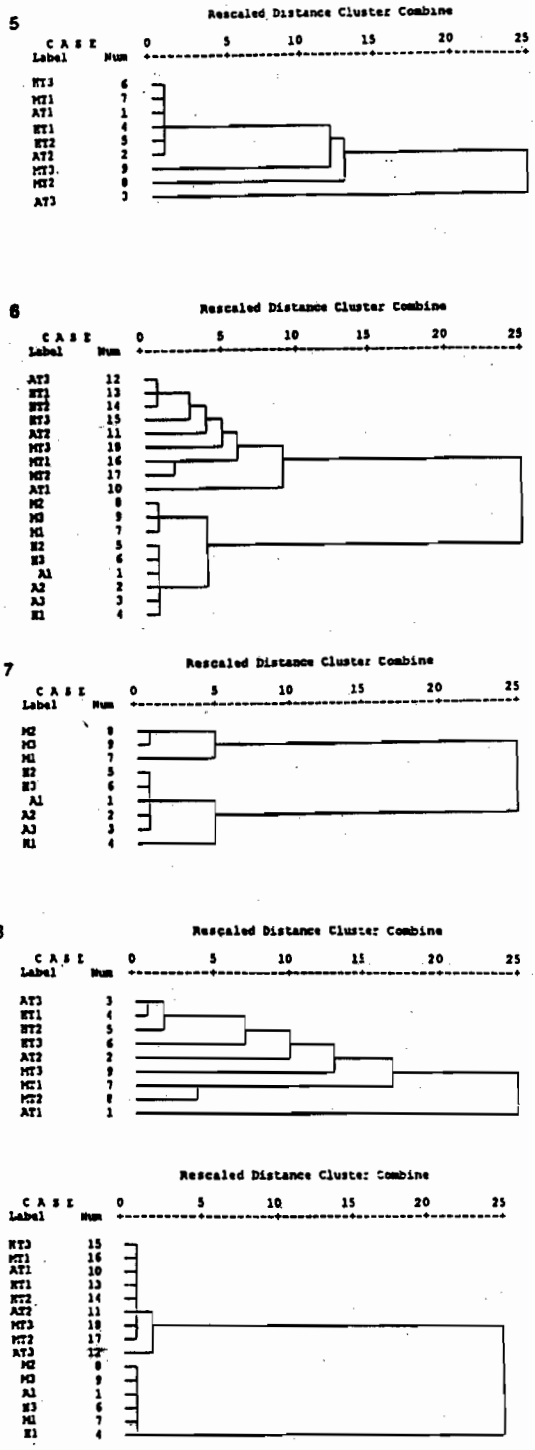
Study of proteins in SDS-PAGE and PAGE system

In SDS - PAGE system, the study of proteins in leaves of seed plants showed 39 protein bands which displayed no difference between three cultivars and different population of each cultivar (Fig. 1). Thus the similarity percentage amongst these samples was 100. In extracts from leaves of regenerated plants, 42 protein bands were observed most of which showed similarity between different populations and different cultivars. Only two bands observed in AT3 and one band exclusively in MT2. Thus AT3 and MT2 had 41 and 40 protein bands, respectively and other samples had 39 protein bands (Fig. 2).



Figs. 3-4. Electrophoresis pattern of proteins of leaves, seed-produced and regenerated plants by PAGE electrophoresis method.
Lanes 1,2,3 Alamout cv. Lanes 4,5,6 Hyrmand cv., and lanes 7,8,9 Maroon cv.

The comparison of SDS-PAGE electrophoresis pattern of the proteins between the leaves of plants developed from the cultivation of seed and leaves from plants produced by *in vitro* regeneration indicated that there were 23 common protein bands between the regenerated plants and there were 24 common protein bands in M2. The resulting dandrogram showed that such regenerated plants would be allocated in two groups, AT3 in one group and the remaining regenerated plants in the other two groups. MT2 and MT3 showed a great degree of difference with the other groups (Figs. 5, 6).



Figs. 5-9. The resulting dandrogram from relation comparison of relationship between different plants produced from seed (7), *in vitro* regenerated plants (5,8) and between different seed produced and *in vitro* regenerated plants (6,9) by SDS-PAGE (5,6) and PAGE (7,8,9) electrophoresis methods respectively.
(Lanes 1, 2, 3 Alamout cv, Lanes 4, 5, 6 Hyrmand cv and Lanes 7, 8, 9 Maroon cv).

In seed produced plants from the above cultivars, electrophoresis pattern of proteins by PAGE system represented 38 bands with less difference amongst them. The number of protein bands in Alamout and Hyrmand cultivars were 38 and 37, respectively (Fig. 3).

The resulting dandrogram represented two groups comprising Maroon in one group and the two other cultivars in another group. In the Maroon group, M2 and M3 showed extended similarity and M1 is closer to M2. In the later, H1 was more distance to H2 , H3 and Alamout (Fig. 7). In PAGE, the banding pattern of proteins from regenerated plants showed presence of 33 bands with some differencs amongst the three cultivars (Fig. 4). Thus the number of protein bands in these plants was: 28 (AT1), 30 (AT2, AT3), 20 (HT1, HT3, MT1, MT3), 21 (MT2) and 22 (HT2). The resulting dandrogram placed one population of Alamout cv (AT1) in one group and the remaining in another group, in the latter group, AT3 showed the most similarity with HT1 and in Marron cv MT1 and MT2 are the most related ones (Fig. 8). The comparison of proteins between different population of seed plants and *in vitro* regenerated plants indicated that the number of common protein bands between the plants resulted from seed and regenerated plants of AT1 was 10, with 5 AT2, and 4 with AT3, HT1, HT2, HT3 and MT2 and 4 between the regenerated plants of MT1 and MT3. The resulting dandrogram indicated that the different plants produced from seed, could be grouped in one and the different plants produced by regeneration grouped in another. Amongst the regenerated plants AT3 is most similar to HT1 and HT2 and could be allocated to one group and MT2 is most close to MT1. The plants produced from seeds M1, M2 and M3 are placed in one group and the Alamout and Hyrmand cultivars in another group (Fig. 9).

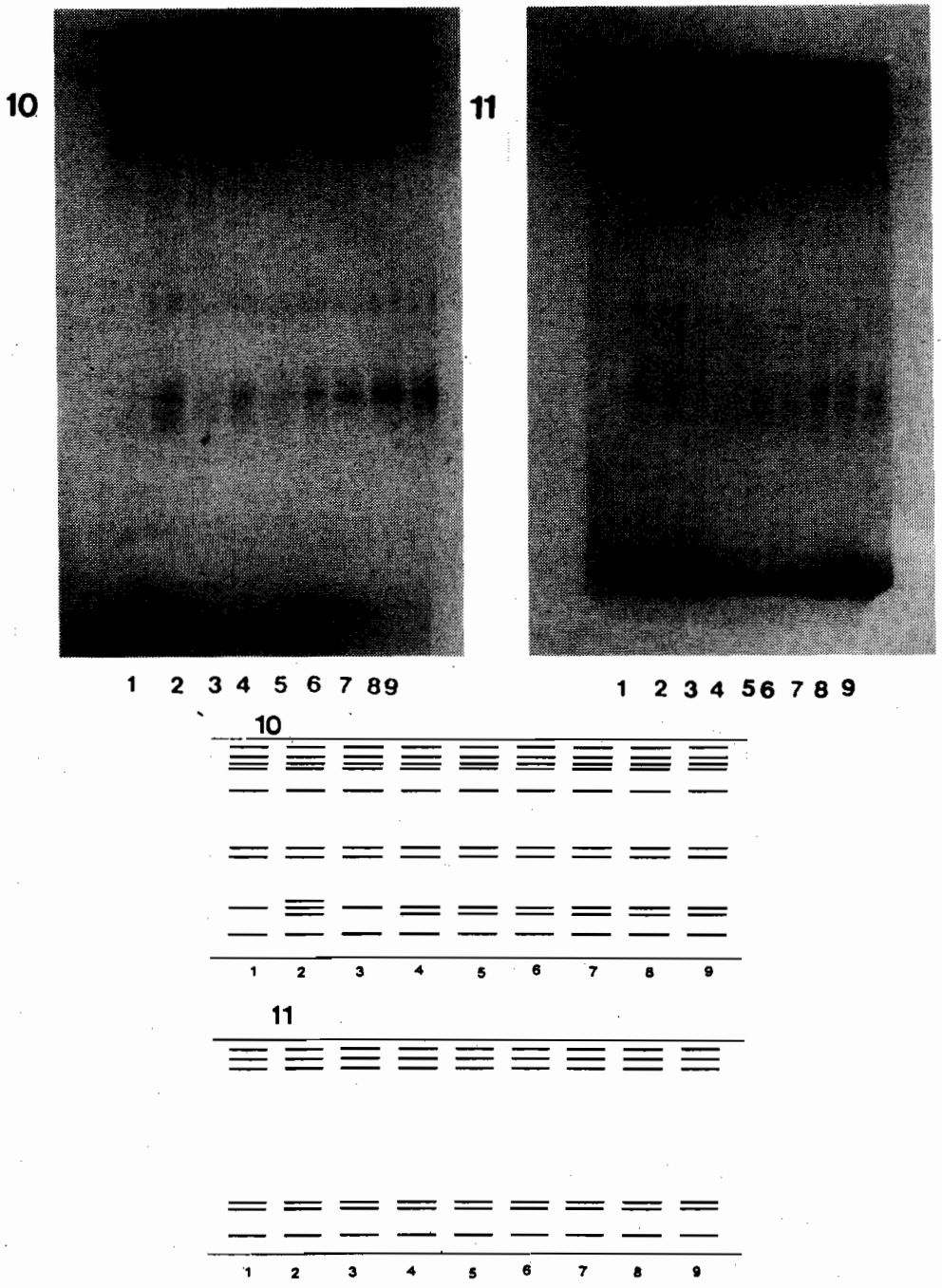
Peroxidases: The comparison of electrophoresis pattern of peroxidase in plants produced by cultivation of seed represented 11 protein bands (R_m= 0.05, 0.08, 0.10, 0.11, 0.18, 0.35, 0.42, 0.51, 0.53, 0.55 and 0.61). Isozyme eight was observed only in Alamout cultivar. The above cultivar of *in vitro* regenerated plants had six isozymes and isozymes three, five, six, seven and ten were absent in these plants (Figs. 10, 11).

Polyphenoloxidases: In leaves of seed plants and *in vitro* regenerated plants of wheat there were 10 isozymes of polyphenoloxidase (R_m = 0.015, 0.11, 0.22, 0.37, 0.42, 0.45, 0.46, 0.48, 051 and 0.58). The plants did not exhibit any differences regarding isozyme banding patterns (Fig. 12).

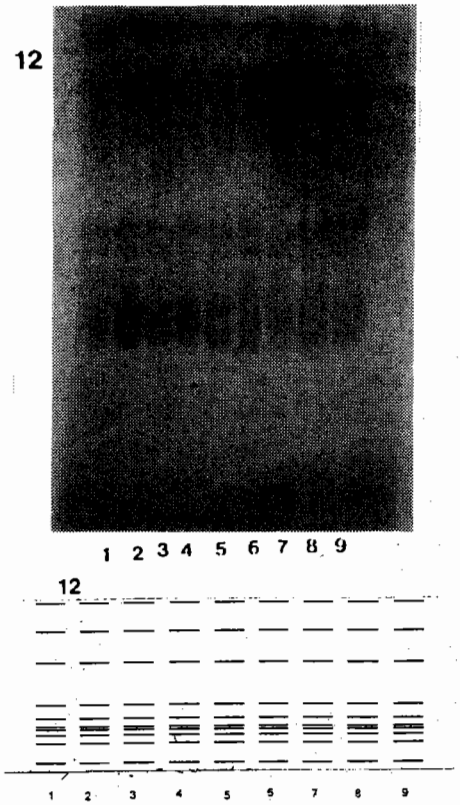
Superoxidedismutase: This enzyme represented six isozymes in leaves of seed produced plants and seven isozymes in leaves of regenerated plants (R_m = 0.011, 0.2, 0.41, 0.45, 0.57, 0.6 and 0.64) whereas the two types of plants showed a difference in banding of isozyme with R_m of 0.41. This isozyme was detected only in leaves of plants produced by regeneration (Figs. 13, 14).

Conclusion

The SDS-PAGE electrophoresis pattern of the proteins from leaves of seed produced plants showed no differences between the three cultivars and different population of each cultivar. But the study of *in vitro* regenerated plants showed that the one of Alamout cultivar population (AT3) displayed a vast difference compared to other regenerated plants. Two Maroon cultivar population (M3, M2) showed some degree of difference with other plants produced by *in vitro* regeneration.



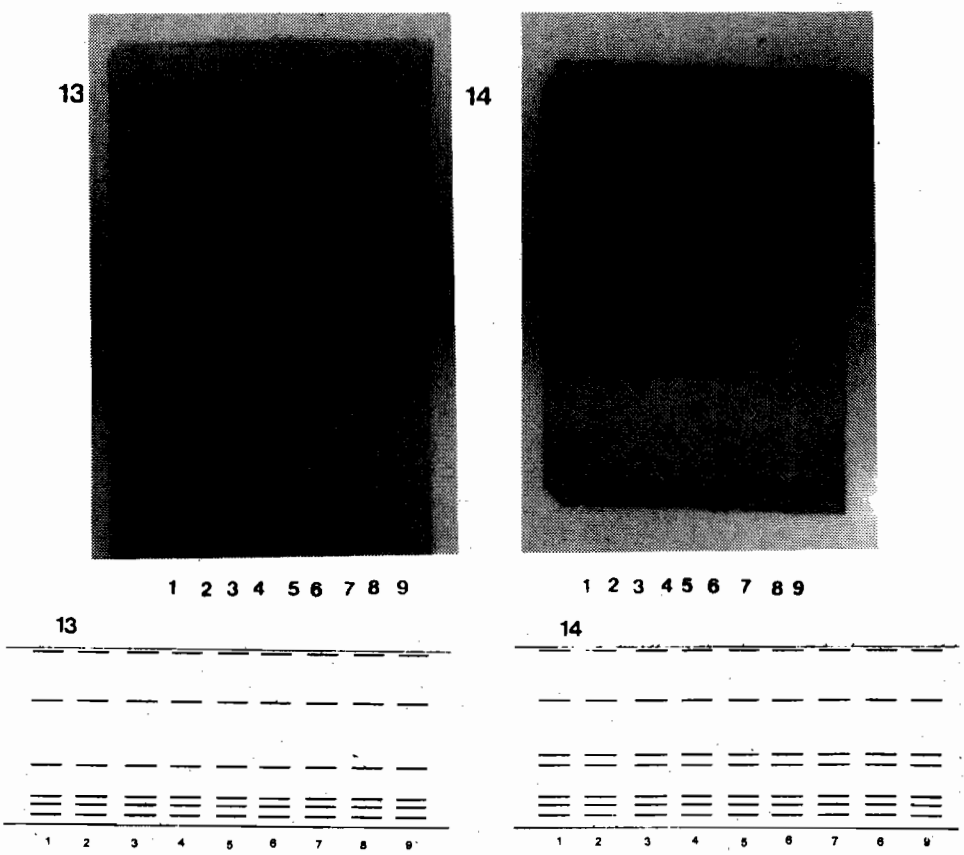
Figs. 10-11. Electrophoresis pattern of peroxidase in seed plants and regenerated plants respectively. Lanes 1, 2, 3 Alamout cv, lanes 4, 5, 6 Hyrmand cv and lanes 7, 8, 9 Maroon cv.



Figs. 12. Electrophoresis pattern of polyphenoloxidase in seed plants and regenerated plants respectively. Lanes 1, 2, 3 Alamout cv, lanes 4, 5, 6 Hyrmand cv and lanes 7, 8, 9 Maroon cv.

The PAGE electrophoresis pattern of protein in the plants from seeds showed a much less difference between various cultivars. In the dandrogram of such proteins, the Maroon cultivar exhibited some distance to Alamout and Hyrmand cultivars. In the leaves of plants produced by *in vitro* regeneration, the distance within different cultivars and between different cultivars and population varied from 25 to 100% and the amount of proteins were lower (33 in regenerated plants compared to 38 in seed plants). In the dandrogram of such proteins, AT1 is located more distant to others.

In the electrophoresis pattern of isozymes some differences was observed between plants produced from seed and plants produced by regeneration. There were 11 peroxidase isozymes in the leaves of plants produced from seed and six isozymes in the leaves of *in vitro* regenerated plants, in contrast to *in vitro* regenerated plants, a degree of difference was seen. The number of polyphenoloxidase isozymes in the leaves of plants produced from seed and *in vitro* regenerated plants was 10. There were six superoxidedismutase isozymes in seed-produced plants and seven isozymes in regenerated plants. Thus, the polyphenoloxidase isozymes in the plants produced by regeneration has not varied, while number of the superoxidedismutase isozymes and particularly peroxidase isozymes is reduced.



Figs. 13-14. Electrophoresis pattern of superoxidedismutase in seed plants and regenerated plants respectively. Lanes 1, 2, 3 Alamout cv, lanes 4, 5, 6 Hyrmand cv and lanes 7, 8, 9 Maroon cv.

So far, no comparison has been reported between SDS-PAGE and PAGE banding pattern of proteins from seed-produced and regenerated wheat but there has been a report of five additional β-amylase isozymes in plants produced by tissue culture (Bapat *et al.*, 1992). There is a report of three peroxidase isozymes in germinated embryos of wheat one of which is specific to germinated embryos. Due to the uniformity of the occurring variation of isozymes of the three cultivars, no statement can be made regarding their somaclonal state. Presumably such variations are due to their cultivation *in vitro*. Results of protein electrophoresis in *in vitro* regenerated plants and the seed-produced plants showed variations between the three cultivars that can be related to somaclonal variations. This variation can not be readily reproduced and possibly shows different frequency of variation in the different genotypes and different groups of *in vitro* regenerated plants. This explains the occurrence of variation in regenerated plants. Maroon cultivar seems to show a higher variation than other cultivars, and such difference is obvious in the dandrogram obtained by PAGE method for protein electrophoresis.

Reference

Ahmed, K. and F. Sagi. 1993. Culture of fertile plant regeneration from regenerable embryogenic suspension cell - derived protoplasts of wheat (*Triticum aestivum*). *Plant Cell Reports*, 12: 175-179.

Bapat, S.A., S.K. Rawal and A.F. Mascarenhas. 1992. Isozyme profiles during ontogeny of somatic embryos in wheat (*Triticum aestivum*). *Plant Science*, 82: 235-242.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities in utilizing the principle of protein - dye binding. *Analytical Biochemistry*, 72: 254-284.

Constantine, N. and K. Stanley. 1977. Superoxidedismutase occurrence in higher plants. *Plant Physiol*, 59: 309-314.

Hames, B.D. and D. Rickwood. 1990. *Gel electrophoresis of proteins. A practical approach.* Oxford University Press.

Lindsey, K. and M.G.K. Jones. 1989. *Plant Biotechnology in Agriculture.* Open University Press.

Maheshwari, N., K. Rajyalakshmi, K. Bawejak, K. Dhris, N. Chowdhry and S.C. Maheshwari. 1995. *In vitro* culture of wheat and genetic transformation, retrospect and prospect. *Critical Review in Plant Sciences*, 14: 149-178.

Ryan, S.A. and W.R. Scowcroft. 1987. A somaclonal variation of wheat with additional β amylase isozymes. *Theor. & Applied Genetics*, 73: 459.

Scowcroft, W.R. and PJ. Larkin. 1981. Somaclonal variation, a normal source of variability from cell culture for plant improvement. *Theor. & Applied Genetics*, 73: 459.

Smith, B.J. 1984. SDS-polyacrylamide gel electrophoresis of proteins. In: *Method biology*, Hamana press.

Van Loon, L.C. 1971. Tobaco polyphenoloxidase: A specific staining method indicating non - identity with peroxidase. *Phytochemistry*, 10: 503-597.

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