ejpmr, 2016,3(12), 68-73

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

SCREENING FOR GENETIC MUTATIONS TO SALT TOLERANCE OF *PHASEOLUS VULGARIS* L. *IN VITRO* TREATED WITH SALT STRESS USING ELECTROPHORESIS TECHNIQUE

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Article Received on 06/10/2016 **Article Revised on 26/10/2016** **Article Accepted on 15/11/2016**

ABSTRACT

Sterilized embryos excised from mature seeds of kidney bean (*Phaseolus vulgaris* L.) were cultured on (MS) medium supplemented with vitamins and growth regulators. Results showed that 2,4-D at (3) mg/l was the best for callus production. Callus was then exposed to different levels of salt mixtures, which were (NaCl, CaCl2 and MgCl2) in a ratio of 2:2:1 at concentrations of(0, 50, 100, 150, 200and250) mM added to the culture medium. To produce genetic variation we soaked the callus in SA at concentration of (0.1)mM for (30) min. Callus cultures treated with(0.1) mM of SA for(30) min were cultured on MS medium containing (0 – 250) mM salt mixtures. An experiment of total protein analysis for callus treated with screening and selection or treated with SA in different concentrations was carried out by using electrophoresis to prove any possible differences may be happened in callus tissues which undergo the previous mentioned stresses. Differences were noticed in protein bands: in migration distance, number of bands intensity of appearance and in the molecular weight , and these results were compared with un exposed treatments .

KEYWORDS: *Phaseolus vulgaris*, *In Vitro*, salt tolerance, electrophoresis.

INTRODUCTION

Protein electrophoresis is the movement of proteins within an electric field. Popular and widely used in researches, it is most commonly used to separate proteins for the purposes of analysis and purification. The term electrophoresis refers to the movement of charged molecules in response to an electric field, resulting in their separation. In an electric field, proteins move toward the electrode of opposite charge. The rate at which they move (migration rate, in units of cm2/V sec) is governed by a complex relationship between the physical characteristics of both the electrophoresis system and the proteins. Factors affecting protein electrophoresis include the strength of the electric field, the temperature of the system, the pH, ion type ,and concentration of the buffer as well as the size ,shape, and charge of the proteins (Garfin ,1990) . Proteins come in a wide range of sizes and shapes and have charges imparted to them by the dissociation constants of their constituent amino acids. As a result, proteins have characteristic migration rates that can be exploited for the purpose of separation. Protein electrophoresis can be performed in either liquid or gel-based media and can also be used to move proteins from one medium to another (for example, in blotting applications). Over the last 50 years, electrophoresis techniques have evolved as refinements have been made to the buffer systems, instrumentation, and visualization techniques used. Protein electrophoresis can be used for a variety of applications such as purifying proteins, assessing protein purity (for example, at various stages during a chromatographic separation), gathering data on the regulation of protein expression, or determining protein size, isoelectric point (pI), and enzymatic activity. In fact, a significant number of techniques including gel electrophoresis, isoelectric focusing (IEF), electrophoretic transfer (blotting), and two- dimensional (2-D) electrophoresis can be grouped under the term "protein electrophoresis" (Rabilloud , 2010). (Oliver and Martine-Zapater ,1985) use this technique to classify 74 varieties of potatoes based on an analysis of similar enzymes and determine the convergence of these varieties. (Vinter-Halter, 1986) found distinction between the originally 37 apples ancestries depending on the similarities in the enzymes Peroxidase for those ancestries. (Al-Jibouri and Mahdi, 1993) identified genetic differences in the 30 complex cultivars of barley dependent on enzymes Got, LAP,

Peroxidase and EST using this technique .(Al-Jibouri *et al*., 1992) used electrophoresis technique for enzymes and proteins to determine genetic differences in some mutated wheat *Triticum aestivum* that were resistance to rust disease these mutations produced using physical mutagens based on enzymatic systems Got, LAP, EST and ACP for the both sensitive genotypes and resistance genotypes to the disease. As well as the(Al-Jibouri,1989) used this technique to distinguish between local oranges varieties grown in the country . they were able to distinguish between the varieties of date palm by examining four enzymatic systems on multi-gels acrylic amide, clear differences between varieties appeared in the number of bands and their location and intensity appear on gels (Al-Jibouri and Dham, 1990).

MATERIALS AND METHODS

Explants sterilized (embryos) of *phaseolus vulgaris* L. were excised and cultured in universal tubes containing MS medium (Murashige and Skoog,1962) with concentration of the auxin 2,4-D (3) mg/l, which incubated in dark at a temperature 23 ± 1 °C . Callus tissue grown on MS medium was directly exposed to different concentrations of NaCl,CaCl2 and MgCl2 mixture (50, 100, 150 , 200 and 250 mM) and the one concentration at which growth was completely inhibited was determined. Cell survival was nonexistent at 250 mM. Consequently a concentration of 150 mM was used for selection experiments. Solution SA were prepared at a concentration of 0.1 mM and immersion callus in it for 30 minutes . and then we took the constant weight of callus (300 mg) and were cultured on culture media containing salt levels (0, 50,150,200 or 250)mM and by 10 replications for each level of salt.

The total protein in callus

the method of Laemmli (1970) was used to prepare gels

The first solution

Acrylamide – Biss Acrylamide

We dissolved 14.6 g of Acrylamide and 0.4 g of Biss Acrylamide and completed the volume with Distilled water to 50 ml.

The second solution

Tris – HCl – buffer 8.8

1.57 g of hydrochloric acid dissolved in distilled water and completed the volume with distilled water to 50 ml, adjust the pH to 8.8 using HCl and NaOH (1N).

The third solution

Tris – HCl – buffer 6.8

1.5 g of Tris – HCl was dissolved in distilled water and completed the volume with distilled water to 25 ml, the pH adjusted to 6.8 using HCl and NaOH.

The fourth solution

Sodium Dodecyl sulphate 10% (SDS)

5 g of sodium dodecyl sulfate was dissolved in 50 ml of distilled water.

The fifth solution

Ammonium persulphate 10%

We dissolved 0.5 gm of ammonium persulfate in 5 ml of distilled water were used directly after preparation.

The sixth solution

Buffer solution

Prepared by dissolving 12 g of Tris and 57 g of glycin and added to 40 ml of Sodium Dodecyl Sulfate solution concentration of 10% (fourth solution) and added distilled water to one liter.

The seventh solution

Staining Stock

dissolved 1 g of (Commassi) R - 250 in distilled water and and completed the volume with distilled water to 100 ml.

The eighth solution

Staining Solution

Prepared by mixing 31.25 ml of staining stock solution (seventh solution) with 125 ml of methanol with 20 ml acetic acid and then completed the volume with distilled water to 250 ml**.**

The ninth solution

Destaining Solution I

10% solution of acetic acid - 50% methanol

Prepared by mixing 125 ml of methanol with 25 ml acetic acid and then completed the volume with distilled water to 250 ml.

The tenth solution

Destaining Solution II

7% solution of acetic acid - 50% methanol

Prepared by mixing 35 ml acetic acid with 250 ml of methanol and then completed the volume with distilled water to 500 ml.

Extracting protein from callus

We used Al - Jibouri and Dham (1989) method in extracting protein from callus, we took 100 mg of callus tissue for each sample and added 1 ml of extraction solution ,the extraction solutions components was showed in the table (1). Samples crushed fully in a ceramic bowl and then centrifuged the solution to speeds of 18,000 rev / min for 15 minutes. We collected the Supernatant and put it in 5 ml tubes and stored in the degree of - 20 °C.

Table (1): Components of the extracting solution:

No.	Material	Quantities
1	Tris – HCl	0.985 gm
2	Sodium Dodecyl	2 gm
3	Glycerol	10 ml
4	α - Mercapto	10 ml
We completed the volume with distilled water to 100 ml		

Electrophoresis

1. **Preparing Separating Gel 10%.**
   Preparing by 60 ml of separating gel , which consists of 20 ml of the first solution, 15 ml of the second solution, 0.6 ml of the fourth solution and 24.1 ml of distilled water. Mix the ingredients well to a magnetic stirrer and after the adjournment added 300 microliter from the fifth solution and 20 microliter from TEMED then the mixture placed in the wells of the electrophoresis device and left for 30 minutes to solidifies and then wash the upper surface of the gel with distilled water and pulled the water layer**.**

2. **Stacking Gel 4%**
   Prepared by 20 ml of the gel by adding 2.66 ml of the first solution, 5 ml of the third solution, 0.2 ml of fourth solution and 12.2 ml of distilled water. Then the solutions mixed by a magnetic stirrer and then we added 100 microliter from the fifth solution and 10 microliter of TEMED then add this mixture over the gel in the wells of the electrophoresis device.

3. **Loading device and operating.**
   We prepared protein samples by mixing 50 microliter of protein samples with 0.1% of promophenol pigment, then we took 50 microliter of this mixture and placed inside each well and then connect the device with Power supply and operate the system with voltages on 100 volts at a temperature of 2 C and the process lasted for 4 hours

4. **staining and destaining**
   After the process was complete we took the gel and soaked it with a solution of the stain (eighth solution) for an hour and then removed the excess stain that was non-reactive with protein by immersing the gel with Destaining Solution I. (ninth solution) for 3 hours and then a solution of Destaining Solution II (tenth solution) the latter solution is replaced every hour to get blue bands and clear.

Calculation of Rf values of protein bands

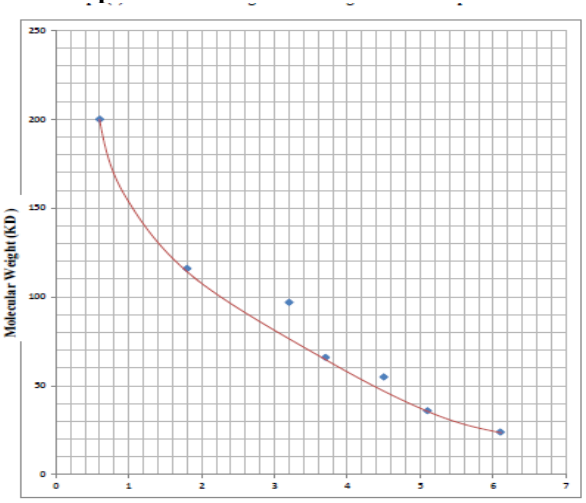
we used the method of (Harborne ,1976) to determine the values of Rf depending on the following equation:

$$Rf = \frac{\text{the distance that the protein band migrated from the beganing point of migration}}{\text{the total distance of migration}}$$

Calculating the Molecular weights of migrated proteins

For the purpose of determining the molecular weights of migrated proteins ,protein sample which were containing standard proteins known molecular weights were migrated with the other proteins , and then draw a standard curve determining the vertical axis of (the molecular weights of standard proteins) and horizontal axis (the migrated distance for each band of standard proteins) and molecular weights of proteins were estimated on depending on this curve (Laemmli, 1970).

Shape (1) The molecular weights for the migrated standard proteins



Migration distance (cm)

RESULT AND DISCUSSION

Total protein analysis for the callus of *Phaseolus vulgaris*

We used the electrophoresis technique to see if there was a genetic variations after using SA , also to infer the occurrence or non-occurrence of genetic variation in callus cells which were cultured on salts medium without exposing them to a chemical mutagen. These protein alterations based on changes in polypeptides molecular weights (MWs), bands intensities, appearance of new bands (unique bands), migration distance, and disappearance of some bands (polymorphic bands). The table (2) showed the migration distance , the molecular weight and the Rf values of the seven standard proteins that were migrated, as noted that these standard proteins had molecular weights between(24-200) kD asymptotic range of molecular weights of proteins that appeared in electrophoresis with different treatments of *Phaseolus vulgaris* L.

Table (2) The number of bands, migration distance (cm), molecular weight (KD) and Rf values of the standard proteins:

Standard protein	Bands	Migration distance (cm)	Molecular weight (KD)	Rf Value
Myosin	**1**	**0.6**	**200**	**0.08**
B- glycosidase	**2**	**1.8**	**116**	**0.24**

Phosphorylase B	**3**	**3.2**	**97**	**0.42**
Albumin	**4**	**3.7**	**66**	**0.49**
Glutamice dehydrogenase	**5**	**4.5**	**55**	**0.60**
eDehydrogenase	**6**	**5.1**	**36**	**0.68**
Trypsinogin	**7**	**6.1**	**24**	**0.81**
The total migration distance = 7.5 cm				

Tables (3) and Figure (1) demonstrate the effect of sodium azide and salt stress on the SDS- electrophoretic patterns of callus total protein fractions for *Phaseolus vulgaris* L. The total numbers of protein bands recorded were six bands for the seeds treatment. we compared the other treatments (cont.,SA, salt stress) with the seeds treatment , for the cont. treatment, it had the same number of protein bands as the seeds treatment, but there were differences in the bands intensities between the two treatments . As for the effect of the SA and salt stress, the same table showed that there were differences in the total protein for callus when compared with seeds treatment , differences appeared in a number of protein bands , the SA treatment had only four bands, there were differences in bands intensities . while the salt stress treatment had five protein bands ,and there were differences appeared in the bands intensities.

Table (3): The number of bands and locations of protein for the different treatments:

Rf values	Treatments			
	Seeds	**Cont**	**SA**	**Screenin and selection**
0.12			**	
0.13				**
0.15	**	**		
0.24				*
0.27	*			
0.29		**		
0.36				**
0.38			**	
0.40	**	**		
0.53	*			
0.55		**		
0.66				**
0.67			**	
0.69	*			
0.70		**		
0.83			**	
0.84				**
0.86	**			
0.87		**		
Light * dark **				

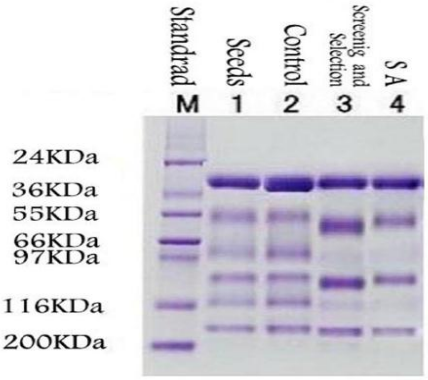


Fig (1) Migrated proteins for different treatments

Table (4) inclusively showed the migration process results for the different treatments the table showed the number of the bands, migration distances

Rf values and molecular weight . when we compared the cont. treatment with seeds treatment we saw that there were differences in the migration distances but both treatments had the same numbers of bands ,and when we compared the SA treatment and salt stress treatment we obviously realized the differences in the number of the bands and the migration distances .the differences in the location of the bands caused differences in the molecular

weight. The disappearance of protein bands in both SA and salt stress treatments and the different in the migration distances and bands intensities, proved the presence of genetic variations.

Table (4) The number of bands and migration distance (cm), estimated molecular weight (KD) and Rf values for different treatments

Treatments	Bands	Migration distance (cm)	Molecular weight (KD)	Rf Values
Seeds	1	1	180	0.15
	2	1.8	113	0.27
	3	2.6	94	0.40
	4	3.5	70	0.53
	5	4.5	47	0.69
	6	5.6	30	0.86
Cont	1	1	180	0.15
	2	1.9	112	0.29
	3	2.6	94	0.40
	4	3.6	68	0.55
	5	4.6	45	0.70
	6	5.7	27	0.87
SA	1	0.8	175	0.12
	2	2.5	96	0.38
	3	4.4	50	0.67
	4	5.4	31	0.83
Screening and selection	1	0.9	170	0.13
	2	1.6	130	0.24
	3	2.4	100	0.36
	4	4.3	51	0.66
	5	5.5	31	0.84
The total migration distance = 6.5 cm				

The disappearance of some protein bands which led to formation of polymorphic bands could be attributed to the loss of genetic material which may be due to the breaking of a small number of peptide bonds to form polypeptides of shorter length than the original protein. Furthermore, the changes in band intensity could be interpreted on the basis of gene duplication or point mutation that leads to production of shorter and longer polypeptide chains and alteration in the structural genes which may be due to the changes in regulator gene(s) expression (Abdel-Hameid *et al* ., 2011). Under stress conditions, some proteins that specifically respond to stress are induced in many plants. Although both the expression and function of such protein is unclear, it is suggested that there is a relationship between some forms of plant adaptation and tolerance to stresses and the expression of stress induced proteins. (Bekheet *et al*., 2000) found a positive correlation between protein content of callus cultures and salt stress level in culture medium. (Poljakoff-Mayber ,1982) reported that osmotic adaptation under salinity stress may be achieved by ion uptake or by internal synthesis and accumulation of organic solutes. According to (Mansour,2000) many amino acids including proline, alanine, arginine, glycine, serine, leucine, and valine and the non-protein amino acids (citrulline and ornithine) and amides (glutamine and asparagines) accumulate in plants exposed to salt stress. Proline is a major amino acid that accumulates in plant at a higher rate than other amino acids (Torabi and Halim ,2010). (Ketchum *et al*., 1991) showed that accumulation of proline occurred in the cytosol and accomplished osmotic adjustment. Proline accumulation affects on membrane maintenance and also alleviated the effects of NaCl on cell membrane interruption (Mansour, 1998). (Maggio *et al*., 2002) noted proline as a signaling/regulatory molecule able to activate multiple responses that are components of the adaptation process. There are different reports in terms of effects of salinity on proline in tolerant and sensitive genotypes among species.(Kaymakanova and Stoeva ,2008) indicated that with increasing salinity the amount of proline increased in bean (*Phaseolus vulgaris* L.).

CONCLUSIONS

Differences appeared in total protein of callus tissue which was exposed to salt stress and SA, the differences were in protein bands number ,migration distance ,bands appearance intensities and in the molecular weight. which may indicate to the occurrence of genetic variations in the callus tissue cells as a result of exposing the callus to salt stress and SA.

RECOMMENDATIONS

conducting studies in fields on plants that have been regenerated from callus tissue after being planted in the soil by carrying out the process of proteins Electrophoresis of plants tolerant to salinity that resulted from the screening and selection processes or exposing

callus to SA and irrigated with the salt level at which it was cultured during selection process and compare it with plants that came from unexposed callus to see the genetic differences between them.

REFERENCES

1. **Abdel-Hameid**, N. R.; Elanany, M. A. M.; Haiba, A. A. A. and AbdEl- Hady, E. A. A. (2011). "Relative mutagenicity of some food preservatives on plant cells," *Australian Journal of Basic and Applied Sciences*, vol. 5, no. 12, pp. 2817–2826.
2. **Al – Jibouri**, A. A. M. (1989). Isozyme analysis in *Citrus sinensis* L. Osbeck grown in Iraq. J. Agric. Sci. 20: 127 – 136.
3. **Al – Jibouri**, A. A. M. and A. A. Mahdi. (1993). Purity determination in *Hordeum vulgare* L. mutants by using isozymes and protein analysis Proc. of the first. Sci. Conf. of Field Crop Research. 72: 83. Baghdad. Iraq.
4. **Al – Jibouri,** A. A. M.; I. F. Ibrahim and A. A. Mahdi. (1992). Isozyme and protein analysis of induced rust resistant wheat mutants. J. of Islamic Academy of Sciences. 5: 115 – 117.
5. **Al – Jibouri**, A. A. M. and K. M. Dham. (1990). Biochemical classification of date palm cultivars. J. Hort. Sci. 5: 725 – 729.
6. **Al-Jibouri**, A. A. M. and K. M. Dham. (1989). Isosymes differences in barley mutant. Proc. 6th Intenatl. Congr. Of SABRAO. Japan. 805 – 808.
7. **Bekheet** ,S.A.;Taha, H.S.; Sawires, E.S. and El-Bahr, M.K. (2000). Salt stress in tissue cultures of *Asparagus officinalis*. Egyp. J. Hort., 27: 275-187.
8. **Garfin**, D.E .(1990). One-dimensional gel electrophoresis. Methods Enzymol 182: 425–441.
9. **Harborne**, J. B. (1976). Phytochemical Methods. A guide to Modern Techniques of Plant Analysis. Cox and Wyinan Ltd. Fakenham, Norfolk. 1 - 32.
10. **Kaymakanova** ,M. and Stoeva, N. (2008) Physiologycal reaction of bean plant (*Phaseoluse Vulg L.*) to salt stress. Gen. Appl. Plant Physiology 34:177-188.
11. **Ketchum**, R.; Warren, R.; Klima, L.; Lopez-Gutierrez F. and Nabors, M. (1991). The mechanism and regulation of proline accumulation in suspension cell cultures of the halophytic grass *Distichlis spicata*. Journal of plant physiology 137:368-374.
12. **Laemmli**, U. K.(1970). Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature. 227: 680 – 685.
13. **Maggio**, A.; Miyazaki, S.; Veronese, P.; Fujita, T.; Ibeas, J.; Damsz, B.; Narasimhan, M.;Hasegawa, P.; Joly R. and Bressan R. (2002). Does proline accumulation play an active role in stress-induced growth reduction? The Plant Journal 31:699-712.
14. **Mansour**, M. (1998).Protection of plasma membrane of onion epidermal cells by glycinebetaine and proline against NaCl stress.Plant Physiology Biochemistry 36: 767-772.
15. **Mansour**, M. (2000) Nitrogen containing compounds and adaptation of plants to salinity stress. Biologia Plantarum 43: 491-500.
16. **Murashige**, T. and Skoog, F. (1962).A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.
17. **Oliver**, J. L. and Martine-Zapater, J. M. (1985). A genetic classification of potato cultivar based on isozyme patterns. Theor. Appl. Genetic. 69(3): 305 – 11.
18. **Poljakoff-Mayber,** A. (1982). Biochemical and physiological responses of higher plants to salinity stress. In: San Pietro, A. (ed), Biosaline research. A look to the future. pp. 245-270. Plenum Press,New York.
19. **Rabilloud,** T. (2010). Variations on a theme: changes to electrophoretic separations that can make a difference. J Proteomics 73(8): 1562–1572.
20. **Torabi**, M. and Halim ,R. A. (2010). Variation of root and shoot growth and free proline accumulation in Iranian alfalfa ecotypes under salt stress. Journal of Food, Agriculture & Environment 8 (3&4): 323-327.
21. **Vinter-Halter**, D. V. (1986). The use of peroxidase polymorphism in the identification of malling and malling merton apple rootstocks. J. Hort. Sci. 61(2): 147 – 152.