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Effects of salt stress on leaf protein patterns of rapeseed (*Brassica napus* L.) genotypes

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Key words: *Brassica napus* L., Cluster analysis, Salinity, SDS-PAGE.

Abstract

Environmental stresses caused a protein disorders in plants and plants overcome to stress by altering the expression of genes which necessary for the synthesis of metabolites, structural proteins and enzymes of some metabolic pathways. To study the effect of NaCl salinity on rapeseed's protein patterns, twelve spring genotypes were evaluated by hydroponic culture at seedling stage. Experiment was performed on three levels of salinity (0, 175, 350 mM) in three replications. In order to study protein patterns by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method, four weeks after salt stress performing and at the end of the seedling stage, leaf samples were collected. For each genotype in each stress level, proteins were extracted in three samples and electrophoresis was performed for them. 30 reproducible bands were identified by SDS-PAGE, in which 18 were polymorphic. Polymorphic bands divided to two groups. The first group had genetic origins and showed the differences among genotypes and the second one called stress-dependent and were affected by salinity. The 20.1 kDa protein bands were induced by salinity in all genotypes while the protein bands with an approximate 56.7 kDa weight were not shown under salt stress. Cluster analysis of data which obtained from protein patterns based on polymorphic bands were used to classification, which had shown high similarity with genotype grouping basis of physiological, biochemical and agronomical data.

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Introduction

Oil seeds have the third place after cereals and beans in the human needs of food (Ashraf and Mc Neilly, 2004); besides, the species belongs to the *Brassica* have the third place among oil seeds (Shirazi *et al*., 2011). Plants due to immobility in their life are exposed to various environmental stresses such as drought, salinity, flooding, high and low temperatures, which water deficit, salinity and high temperature damages are more extensive than other environmental stresses (Parvaiz and Satyawati, 2008). Plants respond to stresses by increment or decrement the expression of their genes (Purty *et al*., 2008). One way to understand the ability of plants to tolerate environmental stresses is to study and identify the changes at specific protein levels caused by stress (Sha Valli Khan *et al*., 2007). Changes in gene expression and production of secondary metabolites in plants as a result of environmental stresses are for the reduction of structural proteins or enzymes in specific metabolic pathways (Vahdati and Leslie, 2013). Products of genes that induced by stress are divided into two main groups (Seki *et al*., 2003): 1-The group that directly protect plants against environmental stresses. 2- A group that regulates the expression of genes and signal transduction in response to stress.

Based on studies of the effects of salinity, it is indicated that the amount of soluble proteins of leaves increase or reduce in response to stress (Parida and Das, 2005). One method to study protein patterns is SDS-PAGE which is an inexpensive, rapid and reproducible mechanism in protein's study. The most use of this system is to evaluate molecular weight of peptides by using standard proteins with certain molecular weight (Rossignol *et al*., 2006). Protein patterns of plants under normal conditions in comparison to the plants under salt stress can identify peptides that their increment, decrement, appearance and disappearance are observed by applying salt stress (Joseph and Jini, 2010). In different species of plants, reduction in the proteins level that are affected by salinity, have been attributed to the reduction in the production of proteins and enzymes which interfere the production of amino acids and proteins (Turan *et al*., 2012).

Furthermore, over expression of proteins due to salinity, resulted by an increment in the synthesis of routine proteins and number of new proteins which are involved in salt stress tolerance (Sobhanian *et al*., 2011). This study was performed to investigate the effects of salinity stress on protein pattern of leaf tissues on spring rapeseed at seedling stage by using SDS-PAGE method.

Material and methods

Plant material and salt treatments

In this research, effects of salinity on twelve spring rapeseed genotypes (Olga, Wild Cat, Sarigol, Heros, Cracker, Option500, Comet, Hyola308, Amica, Eagle, SW500l and RGS003) were studied by asplit plot based on randomized complete block design in three replications. Three levels of NaCl (0 (control), 175 and 350 mM) were performed in hydroponic culture. A week after the transfer of plantlets, salt stress depends on the salinity level was applied over 3-5 days.

SDS-PAGE and gel analysis

To investigate changes in protein pattern, four weeks after applying salt and at the end of the seedling stage just before the start of flowering stage, leaf samples were packed in the aluminum foil and transported to the lab in liquid nitrogen tanks and then were stored at-80°C freezer. This was for keeping the leaf tissues fresh and preventing protease activity. To determine and evaluate the protein pattern of leaf, SDS-PAGE method based on Laemmli method (LAEMMLI, 1970) was used. In order to compare protein patterns, the gels were divided from 1 to 100 from the beginning to the end. Then, protein bands with accuracy of one millimeter were specified and, molecular weights of bands were determined by using standard protein marker (Fermentas #SM0431).

Genotypes Clustering

For grouping genotypes based on presence or absence of bands (1,0) cluster analysis by using UPGMA method based on Jaccard similarity coefficient for each level of stress were done separately. Discriminant analysis was performed to determine the cutting point by using IBM SPSS Statistics 21.

Result and discussion

For each genotype in each stress level, three or four protein samples were extracted and electrophoresed. The bands which were repeated in two or more gels were known as reproducible bands (Fig. 1). Bands numbering were done based on the relative movement (RM) of proteins in main gels (Dolatabadi *et al.*, 2012). Thus, 30 identified bands which had a high reproducibility were determined for conclusion and interpretation. Among them, 18 polymorphic bands were used for classification based on presence or absence of them. Bands 15, 19, 25, 30, 40, 42 and 50 were in all genotypes under control condition, but bands presence or absences were different between genotypes under salt stress. Actually, these bands showed different responses of genotypes to salinity stress. On the other hand, bands 17, 41 and 22 were not shown in any of the genotypes in control condition but they were induced in some genotypes by salinity. Band 85 was induced in all genotypes by the effect of salinity stress and was not shown in non-stress environment. Band 32 was observed in all genotypes under salt stress, but it was seen just in some genotypes and not in all of them under control condition. Bands 70 and 83 were not observed in stressful environments, but they were shown in some of the genotypes in control condition. Bands 5, 38, 64 and 75, unlike previous bands showed genetic variation among genotypes and did not respond to salt stress. In general, the protein bands can be divided into three groups: In the first group, bands were monomorphic and no differences were observed among genotypes and stress levels. The second group showed differences between genotypes and they had a genetic origin and the third group showed the effects of salt stress on different genotypes and got influenced by salinity. In this experiment, it was found that low molecular weight (20.1 kDa) polypeptide (85) under salt stress was induced in all genotypes and it could be mentioned that this polypeptide had a general role in the development of tolerance to salinity. Another protein band with weight about 56.8 kDa (40) was severely affected by salinity and was not expressed. Study of protein patterns showed that for some bands difference between control and stress levels were about the presence or absence of bands, and for some bands difference were in the intensity of their expression. Many studies have reported the appearance of new protein bands or lack of some other under salinity stress (Joseph and Jini, 2010; Sobhanian *et al.*, 2011; Turan *et al.*, 2012).

Another important effect of salinity is the intensity of band's expression which has been reported in many researches (Gan *et al.*, 2010; Bandehagh *et al.*, 2011; Parihar *et al.*, 2014). In this experiment protein bands 36, 53 and 80 with molecular weights 60.1, 41.2 and 21.8 kDa were monomorphic, but under salt stress their expressions were decreased.

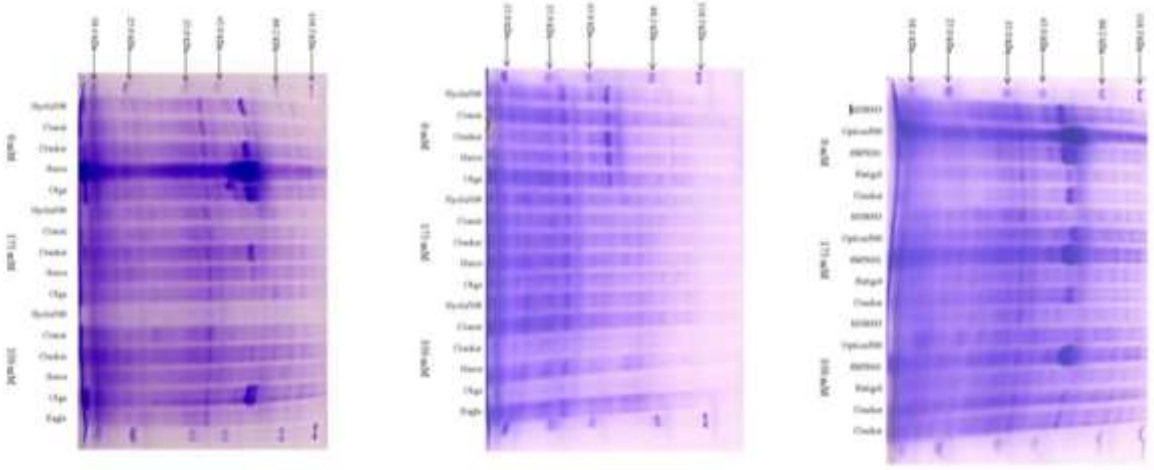

Fig. 1. Protein pattern of rapeseed genotypes in different salinity stress (0, 175, 350 mM) using SDS-PAGE method.

Cluster analysis based on qualitative data which obtained from protein patterns of genotypes under control condition, divided them into four groups (Fig. 2 and Table 1). In the first group, five genotypes of Wild Cat, Amica, Eagle, Comet and Hyola 308 were graded which these five genotypes based on agronomical, morphological and biochemical data ranked as 7, 2, 4, 3 and 1, respectively (Dolatabadi, 2008). Therefore this group had higher tolerance to salinity than others. In the second group, Sarigol and RGS003 were placed. These two genotypes were respectively marked as 6 and 5 and they ranked as moderately tolerant genotypes in comparison to others. Olga, Cracker, Option 500 and Heros were located in the third group which ranked respectively as 10, 9, 11 and 8. Thus, the genotypes of this group were more sensitive than previous groups. Finally, the fourth group included SW 5001 which was ranked 12 identified as the most sensitive genotype. Thus, the grouping of genotypes based on protein patterns data and ranking the genotypes based on biochemical, physiological and agronomical data mostly matched and genotypes which had close rank were located in same groups. Fig. 2 shows dendrograms resulted by cluster analysis under 175 mM NaCl stress. Discriminant analysis to determine the best cutting point divided genotypes into four groups (Table 1). The first group included Comet, Hyola 308, Eagle and Cracker had 3, 1, 4 and 9 ranks. In the second one, two genotypes of Sarigol and RGS 003 with ranks of 6 and 5 were located. The third group included two genotypes of Heros and SW 5001 with ranks of 8 and 12, and four genotypes Olga, Wild Cat, Option 500 and Amica with ranks of 10, 7, 11 and 2 included in fourth group. This grouping based on morphological, biochemical and agronomical data partially matched, but it was not as well as control condition. This shows that in classifying under salt stress, only absolute data cannot be reliable (Ashraf *et al.*, 2001). Fig. 2 has shown the dendrogram of cluster analysis of genotypes under 350 mM NaCl. The genotypes were divided into four groups based on discriminant analysis (Table 1). In the first group, four genotypes Sarigol, RGS 003, Wild Cat and Olga were located. The second group included three genotypes Heros, SW 5001 and Amica, the third group had Comet, Eagle and Cracker and finally, in the fourth group Option 500 and Hyola 308 were placed. An important point in this classification was the locating of Option 500 and Hyola 308 in the same group. Ranking based on absolute data identified these two genotypes as the lowest and the highest-ranking genotypes respectively. However, based on relative data (percentage of control), these two genotypes got the first rank. Grouping based on protein patterns data showed that it is better to use relative data under stress conditions, this issue had been mentioned in some previous studies too (Ashraf *et al.*, 2001; Ashraf and McNeilly, 2004; Bandehagh *et al.*, 2013).

Table 1. Discriminant analysis to identify the dendrograms's cutting point.

Salinity Levels	0 mM		175 mM		350 mM	
Number of Groups	Wilk·s Lambda	Probability	Wilk·s Lambda	Probability	Wilk·s Lambda	Probability
2	0.246	0.062	0.066	0.038	0.063	0.086
3	0.062	0.035	0.011	0.019	0.019	0.152
4	0.031	0.019	0.003	0.012	0.001	0.016

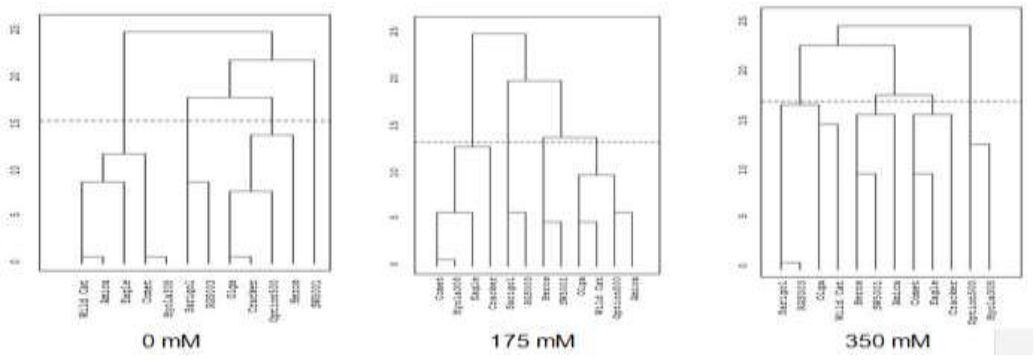

Fig. 2. Cluster analysis of rapeseed genotypes based on protein patterns under NaCl treatments (0, 175, 350 mM).

Conclusion

By studying the protein patterns can conclude that the protein bands 5, 38, 64 and 75 indicated the genetic differences between genotypes, unlike other detected polymorphic bands these bands were not responded to salt stress. A 20.1 kDa molecular weight protein band was induced under salt stress in all genotypes. On the other hand, a 56.7 kDa protein band was identified which was severely affected by stress and was not shown under salinity stress. To determine the exact type and structure of proteins involved in plant tolerance to salinity, advanced isolation methods like two-dimensional electrophoresis can be used. By use of this technology and mass spectrometry analysis of polymorph spots and by sequencing amino acids, proteins can be identified precisely.

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