Molecular And Physiological Characterization Of Local Lebanese Barley (Hordeum Vulgare L.) Genotypes

Mostafa Shehadi, Ghayas Lakkis, Kassem Hamze, Hussein Abou Hamdan, Ahmad Kobaissi,

Abstract: Improving different Lebanese genotypes is based on analyzing local genotypes and estimating genetic dissimilarity between them. As a result this study aim to detect the salinity tolerant genotypes based on two culture systems, the in situ sand based system and the stagnant nutrient solution system and assessing the phylogenetic relationships among the five different genotypes under study, based on 16 SSR loci. The study illustrated the tolerance of the genotype Tairaya at the germination stage while its sensitivity was emphasized at later stages of development. Moreover, Kamed El Louz, Aaitit and Bebnine were considered as tolerant at later stages as they possessed the lowest percentage of reduction of the parameters under study in the two culture systems. Eventually, the genotypes didn't reflect the same tolerance pattern although the genetic differentiation between them was not significant.

Key words: Barley, genotypes, salinity, growth rate, chlorophyll, proline, SSR

1 INTRODUCTION

Abiotic stress is the principal detrimental stress dipping the average yield of most crops by more than 50% (Ehab .M.R. Metwali, 2012). Among these, soil and irrigation water, salinity is one of the most effective environmental parameters that determine the success or failure of the plants' establishment (Ayalewa Ligaba and Maki Katsuara, 2010). Salt effects on plants' behavior differ between genotypes and growth stages. Yield and growth parameters, height, weight, photosynthetic rate and others, show differential responses to salinity stress, as a result, investigators impose the stress over the entire plant growth cycle (Frans J. M. Maathius and Anna Amtmann, 1999).

- *Mostafa Shehadi is currently pursuing his phD thesis at the Doctoral School of Science and Technology, Lebanese University, Rafic Hariri Campus, Hadath, Lebanon*
  E-mail: mostafa_shehadi@hotmail.com
- *Ahmad Kobeissi: is currently an associate professor, head of the Biology Department at the Lebanese University, Faculty of Sciences and Doctoral School of Sciences and Technology, Rafic Hariri Campus, Hadath, Lebanon, Tel: +961 3 612 566. E-mail: ahkobeissi@ul.edu.lb*
- *Ghayas Lakkis is currently an associate professor at the Lebanese University, Faculty of Sciences and Doctoral School of Sciences and Technology, Rafic Hariri Campus, Hadath, Lebanon.*
  E-mail: ghayas.lakis@hotmail.com
- *Kassem Hamzeh is currently an associate professor at the Lebanese University, Faculty of Sciences and Doctoral School of Sciences and Technology, Rafic Hariri Campus, Hadath, Lebanon.*
  E-mail: kassem_hamze@hotmail.com
- *Hussein Abou Hamdan is currently an associate professor at the Lebanese University, Faculty of Sciences, Rafic Hariri Campus, Hadath, Lebanon, Tel: +961 3 612 566. E-mail: ah.hussein@yahoo.fr*

Among cereals, rice (Oryzae sativa) is the most sensitive one and Barley (Hordeum vulgare), the fourth widely distributed crop (Ehab .M.R. Metwali, 2012), is considered to be relatively tolerant, such that it is cultivated in a wide range of environmental conditions, exhibiting wide morphological, physiological and genetic diversity (Forster et al., 2000). As a result improvement of barley production and cultivation becomes a great need. Indeed, the development of tolerant varieties to salinity and water stress with high potential of production, yield and good seed quality could be achieved by different crop improvement approaches. Whatever the approach used, collection and characterization of local varieties could be useful for directing crosses, evaluating and conserving the available germplasm. Accordingly, characterizing varieties is highly needed. As a result, the current study aims to compare the growth and physiological responses of five cultivars in different Lebanese regions under salinity using two culture systems. Furthermore, genetic diversity was assessed and the phylogenetic analysis was done and its relation with the physiological responses was discussed.

2 MATERIALS AND METHODS

2.1 Collection of Plant Material

Five Barley cultivars were collected from five different Lebanese regions. The seeds were randomly collected from, Tairaya, Kamed El Laouz, Blida, Aaitit and Bebnine and the analysis was done based on both in situ sand based system and stagnant nutrient solution systems (SNS), and genetic diversity was evaluated using simple sequence repeats (SSRs) markers.

2.2 Germination Test

48 h cold pretreated seeds at 4°C were allowed to germinate on filter papers under different NaCl concentrations, 5, 10, 15 and 20 g/L, along with the control where no salt was added. 90 mm petri dishes were used for the seed culture where 3 replicates per treatment per genotype were randomly designed. These were exposed daily for 7 days to the different treatments. The percentage of germination was recorded over the 7 days, using the following equation:

$$\text{Germination Percentage} = \frac{\text{Number of seeds forming radicle or coleoptile}}{\text{Total number of seeds}} \times 100$$

2.3 Plant Growth Systems

In Situ – Sand Based System 48 h cold pretreated seeds were allowed to germinate in a plastic pot such that 100 pots were randomly designed, filled with sands and irrigated with Hoagland nutrient solution. After 10 days of growth, the seedlings were subjected to salinity stress. Sodium chloride was added to the nutrient solution to obtain four different salt concentrations, 5, 10, 15 and 20g/L as well as the control deprived of NaCl, each tested using four replicates. The average temperature for day/night was 22/18°C, relative humidity was 50%−60%, and the photoperiod for the day/night cycle was 16/8 h. After a month of the treatment, the analyses were done and the measurements were taken. Stagnant Nutrient Solution System Stagnant nutrient solution (SNS) with inert gravel as a substrate was designed. The seeds were allowed to geminate in dark pots (5 cm × 10 cm) filled with inert gravel and irrigated with Murashige and Skoog (MS medium) solution. The plants were sown under controlled conditions 16/8 h light/dark regime with a relative humidity of 50 to 60% where the temperature ranges between 18 and 22°C. Seedlings were grown for 10 days in a half MS solution, changed every 2 days. On day 10, a salt stress treatment was initiated such that four different salt concentrations were added to the irrigation solution (5, 10, 15, 20 g/L) in addition to the control where NaCl wasn't added. After a month of treatment, the analyses were done and measurements were taken.

2.4 Physiological Analysis

Quantitative Estimation of Chlorophyll

Chlorophyll estimation was done according to (Arnon, 1949), where the total chlorophyll was calculated using the formula suggested by (Arnon, 1949). The chlorophyll was expressed as mg/g fresh tissue (Arnon, 1949). Total Chlorophyll = 20.2 (A645) + 8.2 (A663) V/1000 x W Where, A663 - Absorbance at 663 nm, A645 - Absorbance at 645 nm, V - Volume of extract, W - Weight of tissue

Proline Content

Proline content in the plant parts was estimated according to the method of (Bates et al., 1973), where proline estimation was done based on the following Equation:

$$\text{mmoles per gram tissue} = \frac{\frac{\text{mg}}{\text{ml}}\text{proline} \times \text{ml toluene}}{115.5} \times \frac{5}{\text{g sample}}$$

where 115.5 is the molecular weight of proline.

2.5 Statistical Analysis

A completely randomized system was used in all experiments and the data were collected, extracted and the statistical analysis was done by SPSS 17.0 software. Analysis of variance and Duncan's Multiple Range Test was used for comparison of means at a significance level of 5%.

2.6 Molecular Analysis

DNA extraction

Genomic DNA was isolated from the leaves collected after a month of sowing (three plants per genotype) using the Gen-Elute Plant Genomic DNA Miniprep Kit.

SSR primers

Sixteen (16) microsatellite primer pairs used for genotyping assays were selected on the basis of their chromosomal location and as indicated by several publications (Chaabane et al. 2009; El-Awady et al. 2012). Primer names and sequences are listed in table 1.

PCR amplification and electrophoresis

PCR amplification was performed in a volume of 25 µl containing approximately 30 ng of template DNA, 1 µl of each forward and reverse primer, suitable quantity of dNTPs, MgCl2 and Taq DNA Polymerase and PCR buffer. Reactions were conducted in Eppendorf PCR system (Germany) with initial denaturation step for 5 min at 94°C followed by 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 2 min; followed by a final extension at 72°C for 5 min. The PCR reaction products were evaluated for polymorphisms on 3% agarose gel. After staining with 8 µl Nancy (revelation dye) for 60 min, the gels were photographed by gel documentation system.

Band scoring and cluster analysis

The SSR gel images were scanned using the Gel Doc 2000 Bio- Rad system. The bands were sized and then coded by 1, 2, and 3 for their presence or absence in each genotype. The software Genetix was used for data entry. Cluster analysis was based on Cavalli – Sforza and Edwards similarity matrix and done by Populations software program.

Table 1: Primers for the 16 SSR Markers Used in assessing the genetic diversity among the studied genotypes

	Primer	Primer Sequences
1	Ebmac0874	Forward: AACCATTCCTCACCCAGG Reverse: GTGAATGATGTTGAGGACATTG
2	MGB391	Forward: AGCTCCTTTCCTCCCTTCC Reverse: CCAACATCTCCTCCTCCTGA
3	Bmag13	Forward: AAGGGGAATCAAAATGGGAG Reverse: TCGAATAGGTCTCCGAAGAAA
4	Ebmac0715	Forward: GCGAACATTGTCATGTTAGTA Reverse: TGTCATGCCAGACCTATG
5	HV13GEIII	Forward: AGGAACCCTACGCCTTACGAG Reverse: AGGACCGAGAGTGGTGGTGG
6	HVITR1	Forward: CCACTTGCCAAACACTAGACCC Reverse: TTCATGCAGATCGGGCCAC
7	MGB402	Forward: CAAGCAAGCAAGCAGAGAGA Reverse: AACTTGTGGCTCTGCGACTC
8	MGB371	Forward: CACCAAGTTCACCTCGTCCT Reverse: TTATTCAGGCAGCACCATTG
9	EBmac624	Forward: AAAAGCATTCAACTTCATAAGA Reverse: CAACGCCATCACGTAATA
10	GMS1	Forward: CTGACCCTTTGCTTAACATGC Reverse: TCAGCGTGACAAACAATAAAGG
11	HVB23D	Forward: GGTAGCAGACCGATGGATGT Reverse: ACTCTGACACGCACGAACAC
12	MGB318	Forward: CGGCTCAAGGTCTCTTCTTC Reverse: TATCTCAGATGCCCCTTTCC
13	MGB357	Forward: GCTCCAGGGCTCCTCTTC Reverse: AGCTCTCTCTGCACGTCCTT
14	HVGLUEND	Forward: TTCGCCTCCATCCCACAAAG Reverse: GCAGAACGAAAGCGACATGC
15	Bmac0576	Forward: CAATTGTAGCCTAGCTGGTCG Reverse: GGGTGTATGCAAGTGGGC
16	Bmac0577	Forward: TCATACAGAAGCCCACACAG Reverse: TGCATGTTCATTCTAGACAGG

3 RESULTS AND DISCUSSION

3.1 Effect of Salinity on Germination

For better cropping, highest plant population is required, which is only possible if seed germination is satisfactory under saline conditions (Martinez-Cob et al. 1987), as a result germination under salinity is widely used as a screening criterion to select salt tolerant genotypes, The study revealed a decrease in the percentage of germination with the increase in the concentration of the salt. The maximum percentage of germination and the highest germination rate were recorded in Taraiya cultivars under all salinity levels. whereas the minimum was recorded in Kamed El Louz cultivar.

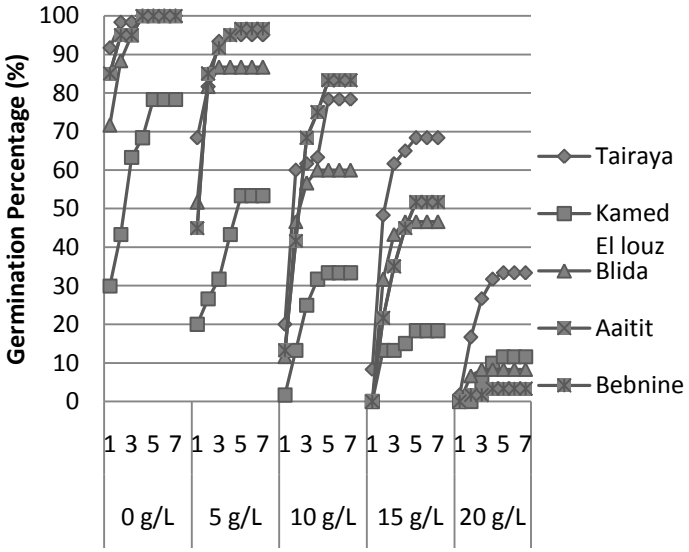

Figure 1: Germination Percentage of the Studied genotypes when exposed to different sat concentrations

Several investigators reported the sensitivity of the germination stage to salinity, where they find a decrease and delay in the germination of most crops upon salinity treatment (Yousofinia et al., 2012). Such effect is attributed to both the osmotic and ionic phases of stress, where salt stressed seeds are desiccation sensitive, physiologically injured reducing the seed germ inability (Yousofinia et al., 2012). Furthermore, ion toxicity alters nucleic acid and protein metabolism, disturbs hormonal balance, and reduces the utilization of seed reserves (Hasanuzzaman et al., 2013). It may also negatively affect the ultra-structure of cell, tissues and organs.

3.2 Morphological Analysis

In our study, high NaCl concentrations lead to stunted development of the plants, as well as a reduction in its growth rate, height and number of leaves. Salinity treated plants showed chlorosis and the plants lost their wealth and possessed necrosis. However, the growth rate over time is the most effective parameter of study. Based on the statistical analysis done, Taraiya and Blida are categorized into the mostly affected cultivars, while the other three genotypes are categorized in the same less affected group at 10, 15 and 20 g/L salt concentrations. Such clustering of the genotypes can be emphasized by comparing their percentages of growth rate reduction. Where 80 and 90% growth rate reduction can be recognized in both Tairaya and Blida respectively, however this percentage decreased in Bebnine and Kamed El Louz and Aaitit, as they possessed a 20 to 30% fluctuation range proposing the tolerance of the latters (Figure 2).

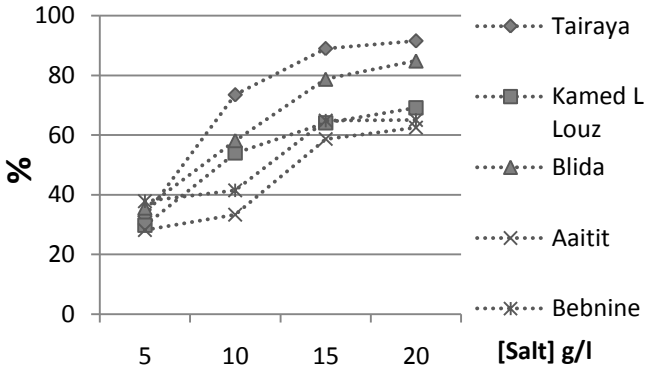

Figure 2: Percentage of Reduction of growth rate of the genotypes grown on the sand based system when exposed to the different salinity level

As for the SNS, Blida and Tairaya showed the highest percentage of growth rate reduction indicating the sensitivity of these two genotypes. On the other hand, Bebnine reduction percentage ranged from 20% to 50% while Aaitit showed a 30% reduction between 5g/L and 20g/L. Moreover, Kamed El Louz reduction ranged from 40 to 80%.

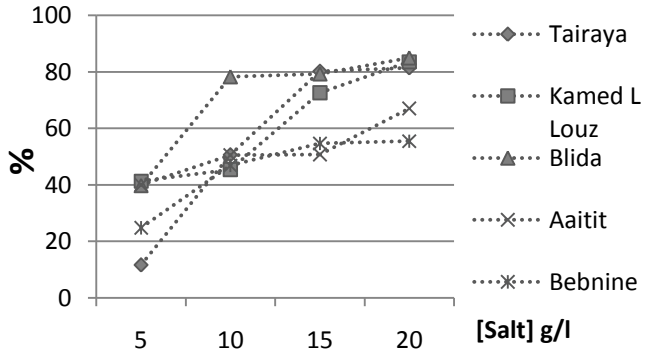

Figure 3: Growth rate reduction percentage of the stagnant nutrient solution (SNS) sown plants under variable levels of salt stress

The reduction in shoot length is due to excessive accumulation of salts in the cell wall losing its elasticity. Furthermore, secondary cell appears sooner and wall becomes rigid, as a consequence the turgor pressure efficiency in cell enlargement decreases. These processes may decrease the shoot length (Farhad Taghipour and Mohammad Salehi, 2008). In addition, the growth of young leaves is not inhibited directly since cells are elongating and their expanding vacuoles can accommodate the salt arrived. However, the rate at which old cells die is greater than the rate at which new leaves are produced, thus the photosynthetic capacity of the plant will no longer be able to supply the carbohydrate requirement of the young leaves,

which reduces their growth rate (Rana Munns and Mark Tester, 2008).

3.3 Physiological Analysis

Physiologically, the produced reactive oxygen species destabilize and misbalance the growth and development potential of the plant by destructing its photosynthetic ability. The study under analysis, proved the negative impact of salt treatment on the chlorophyll content. The reduction in the chlorophyll content is correlated with the sensitivity of the plant, where a tolerant plant can withstand the stress and tend to perform well and produce its primary metabolites. The five different genotypes showed a decrease in their chlorophyll concentration however it differs owing to the different tolerance ability of the plant, where Kamed El Louz and Aaitit were the least affected while the others especially Bebnine possessed higher sensitivity to NaCl amount (Figures 4 and 5). It can be concluded from what was mentioned above that Kamed El Louz and Aaitit showed a high tolerance due to a lower reduction in the chlorophyll content based on the sand based system, moreover, several studies indicated that production of osmoprotectants such as proline under salinity is achieved by the diversion of the biochemical pathway from the primary metabolite production (Asish Kumar Paridaa and Anath Bandhu Dasa, 2005) and the shift of the cell's energy demand toward salinity tolerance mechanisms thus decreasing the photosynthetic rate which might be the case of Bebnine which can be considered tolerant as well (shown later).

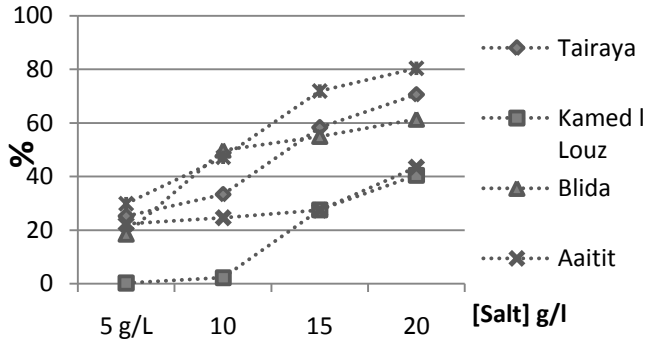

Figure 4: Percentage of reduction of the chlorophyll content of the plants grown on the sand based system under different salt concentrations

Similar to the sand based system, the reduction in the chlorophyll content was significantly different between the genotypes, where Kamed El Louz showed higher chlorophyll content at high salinity levels, 0.32 mg/g of fresh tissue. Thus, Kamed El Louz is more tolerant. Furthermore, this data approves the tolerance of Aaitit and Kamed El Louz, along with Bebnine (shown later).

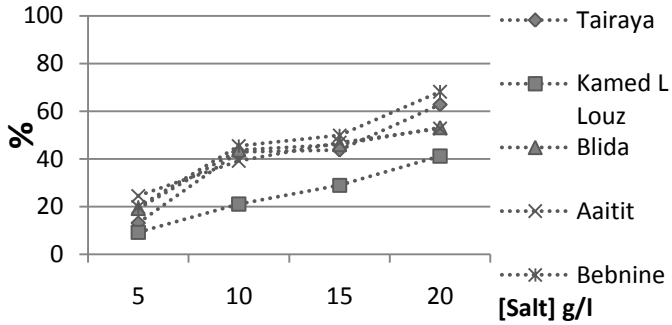

Figure 5: Percentage of chlorophyll content reduction for the plants exposed to salinity under the SNS

The photosynthetic electron transport is unaffected by the salt concentrations, so any reduction in the photosynthetic rate might be due either to a decrease in the carbon metabolism as it reduces the availability of CO2 (Hasanuzzaman et al., 2013). Kalaji et al. (2011) indicates that the first stage of salinity effect on photosynthesis of barley plants is related to stomatal conductance limitation rather than to photosystem II (PSII) activity reduction. They added that salinity stress negatively influenced PSII activity in barley plants, and its effect was dependent on the duration of stress application and on the cultivar used. Moreover, NaCl increases the activity of chlorophyllase, a chlorophyll degrading enzyme. Furthermore, salinity enhances the plant senescence, alters membrane integrity and changes the enzymatic activity as a result it negatively impacts photosynthesis. The decrease in chlorophyll contents under saline condition is also reported by Nazarbeygi et al. (2011) and several other studies; where chlorophyll reduction can be further attributed to changing Nitrogen metabolism direction toward forming compounds such as proline used to regulate osmosis. Accumulation of solutes especially proline, glycine-betaine and sugars is a common observation under salinity condition. Our results reveal a significant increase in the proline following an increase in the salinity levels; however, Kamed El Louz produced the highest proline amount, 75.86 mmol/g fresh tissue, at high NaCl levels.

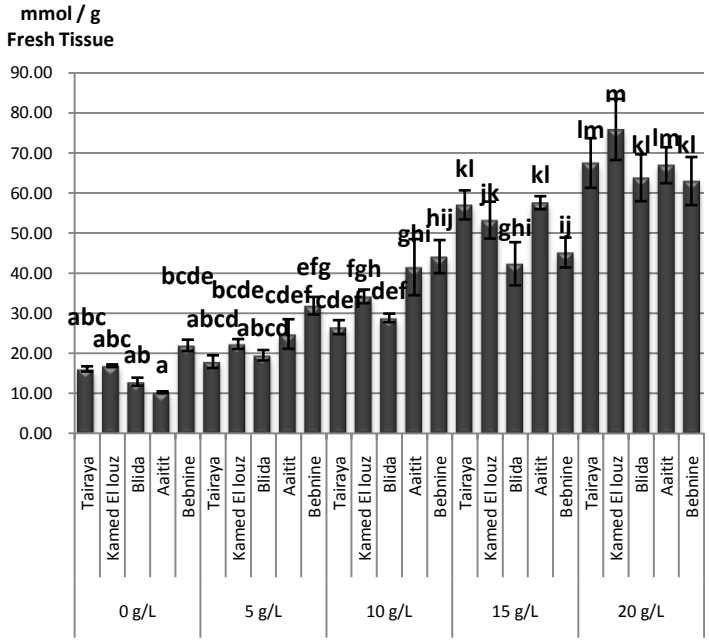

Figure 6: Proline content of the plants grown under salt stress in the in situ sand based system

Similarly, the genotypes' response differs for the SNS. Kamed El Louz produced the highest amount of proline at high NaCl levels, 71.32 mmol/g fresh tissue while the others produced approximately the same amount of proline.

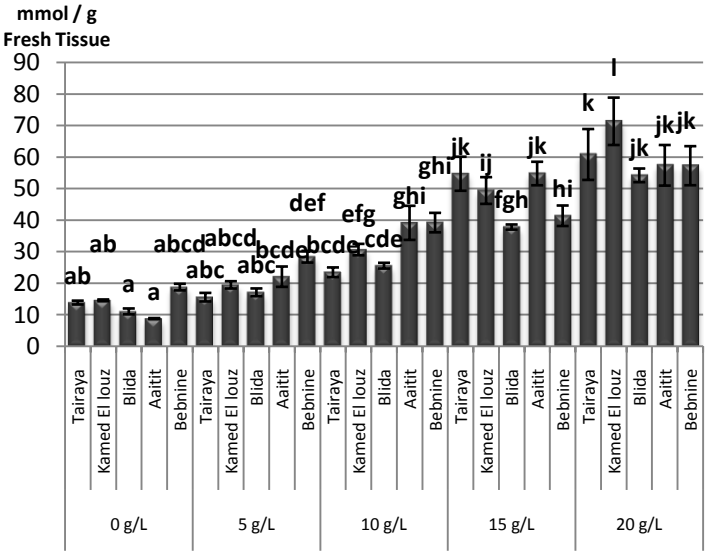

Figure 7: Proline content of the salt tress plants grown in the SNS

Proline accumulating in plants exposed to salinity stress is an osmotic regulator preserving the dynamic carbon uptake by the chloroplasts. The increase in proline content illustrates the high decrease in chlorophyll content and growth rate, as the carbon demand was shifted toward producing the required osmoprotectant for the plant survival.

All the results aforementioned approve the tolerance of Aaitit and Kamed El louz where they maintained their growth and physiology, while the Blida and Tairaya are sensitive. Bebnine is considered tolerant as it possessed a relatively high growth rate, while the decrease in chlorophyll is justified by the increase in the proline concentration.

3.4 Molecular Analysis

The characterization and assessment of genetic diversity among the barley genotypes would be important at the short term for detecting genetic diversity and differentiation among the studied genotypes and at the long term for designing breeding strategies for quantitative and qualitative traits. In the present study, 16 primer pairs (Table 1) flanking simple sequence repeats were employed to investigate the level of polymorphism among the five barley genotypes cultivated at different regions of Lebanon. Thirteen primers produced amplicons and were reproducible. Four of these produced aspecific bands (Ebmac0874, HV13GEIII, MGB402, MGB371), where they were discarded. The remaining nine primers generated polymorphic banding patterns used for the analysis of genetic diversity.

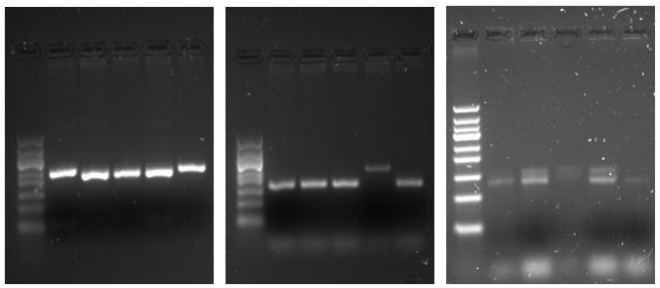

Figure 8: Agarose Gel Documents showing the different bands of the markers; each gel corresponds to one of the polymorphic markers used where all the genotypes are revealed

The number of alleles per marker ranged from 2 to 3. The highest number of alleles as an average of all the loci was attained by Blida. The aforementioned fact was also approved by calculating the expected heterozygosity, an indication of the genotype diversity. It can be recognized from the histogram above the high heterozygosity of Blida, 0.4028. On the other hand, Kamed El Louz and the other populations showed a low heterozygosity, half that of Blida, which was 0.1790. Blida's heterozygosity might be due to that the farmer uses a mix of different varieties considering them as the same population or was able to develop a more diverse homogenous population using different origins or sources. The low heterozygosity possessed by the others might be due to selection efforts done by the farmer on a certain trait such as the grain yield or others, thus the population will tend to uniformity especially that Barley are mainly self breeding plants.

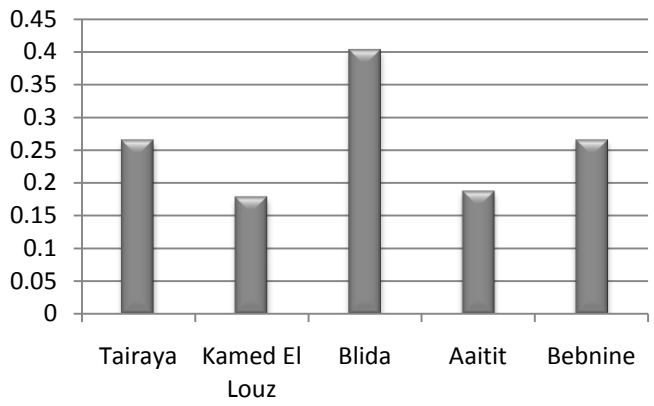

Figure 9: Heterozygosity of the different genotypes studied based on the polymorphism of the markers within the population

To examine the genetic relationships among the five barley cultivars under study, the data scored from the nine primers were compiled and analyzed using the cavalli and Sforza coefficient. The results obtained for both the phylogenetic tree as well as the Fst value were not significant at a confidence level of 5%, One of the explanations could be the fact that these cultivars belong to the same landrace, or the data taken for analysis weren't significant. Furthermore, the genotypes do not reflect the pattern of tolerance since they might have same genetic backgrounds at the whole genome level, but differs at the loci controlling salinity tolerance.

4 CONCLUSION

The study approves the tolerance of Tairaya at the germination stage; on the other hand, the tolerance of Kamed El Louz, Aaitit and Bebnine at the adult stage was detected. Therefore, the study potentially selected two genotypes tolerant at two different developmental stages paving the way toward several other breeding studies. Moreover, Blida possessed the highest intra population diversity; however, whole genome differentiation between genotypes wasn't significant, though a difference in their tolerance potential was detected. As a result, more genetic studies are to be done on the genes controlling salinity tolerance in a way to detect potential QTLs.

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