J.Agric.Chem.and Biotechn., Mansoura Univ.Vol. 7 (9): 249 - 255, 2016

Screening of Cowpea (*Vigna unguiculata* L. Walp) genotypes for salinity tolerance using field evaluation and molecular analysis

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ABSTRACT

Salinity is one of the major abiotic stress which seriously affect cowpea yield production. Thus, developing of new salinity tolerant lines is one important factor to face the harmful effect of salinity. In this study five cowpea genotypes were evaluated for their response to salinity stress using field characterization and inter simple sequence repeat (ISSR) molecular marker analysis. The behavior of some agronomic traits was screened under both normal and saline water irrigation conditions. The results revealed the presence of highly significant differences among all genotypes in both normal and salinity stress conditions whereas saline water reduced the performance of all evaluated traits. The genotypes, Sudany and Chinese red were the most salinity tolerant although they had the lowest yield production. On the other hand, Cream7 was the highest for yield performance with low salinity tolerance. The genetic distance among genotypes was measured by ISSR marker, the percentage of polymorphism was 82.08%. while, genetic similarities ranged from 0.48 to 0.67. Moreover, seven out of nine primers were able to produce many salinity specific unique bands which would be used as salt tolerance markers. The dendrogram separated the genotypes into two main clusters, the first one only consisted of the lowest salinity tolerant genotype while the two highest salinity tolerant genotypes were coupled in one group. The high capacity of some genotypes for salinity tolerance suggested that these genotypes could be used in breeding programs to produce more salinity tolerant varieties having a higher yield production.

Keywords: Cowpea, salinity stress, ISSR, dendrogram.

INTRODUCTION

Cowpea (*Vigna unguiculata* L. Walp.) is one of multifunctional legume crops, which plays an important role in both human food and animal feed. Furthermore, it has a high proportion of proteins in its dry seeds with natural tolerance to biotic and abiotic stress to grow in poor soil due to its high ability for nitrogen fixation (Eloward and Hall, 1987; Hall, 2004). In Egypt, cowpea cultivation area according to Agricultural economic bulletin, 2013 was about 14830 feddan with production of about 17248 tons with (an average yield of 1.163 ton/feddan). In fact, salinity is one of abiotic stress which severely limited cowpea productivity. Whereas in Egypt 33% out of total cultivated land is suffering from salinity (Khatab and Samah, 2013). Additionally, in most of the newly reclaimed lands ground water is used for irrigation that has a higher content of Na and Cl, and its salinity increasing gradually from year to year. Salinity has harmful effects on germination, plant growth, crop productivity and plant survival (Parida *et al*., 2004; Ashraf and Foolad, 2007). Through damaging many physiological and biochemical pathways like photosynthesis, transpiration and others (Kendirli *et al*., 2005 and Tiwari *et al*., 2010). To face salinity effects, must produce genetically improved varieties that have both highly yield and salt tolerance.

Beside morphological characterization molecular markers have been successfully used to study genetic diversity. several PCR based molecular markers have been extensively used during the last decade such as Simple Sequence Repeats (SSR; Akkaya *et al*., 1992), Inter Simple Sequence Repeats (ISSR; Zietkiewicz *et al*., 1994), Amplified Fragment Length Polymorphism (AFLP; Vos *et al*.,1995) and Randomly Amplified Polymorphic DNA (RAPD; Williams *et al*., 1990).

In this study, five cowpea genotypes were evaluated for their ability to salinity stress tolerance and genetic variability using field evaluation and ISSR molecular marker analysis.

MATERIALS AND METHODS

Plant material:

Five cowpea genotypes were used including Cream 7, Chinese red, IT82C-16, Sudany and Black Crowder as presented in Table 1.

Table 1. The description and origin of the five cowpea genotypes.

Genotype	Origin	Source	Seed color	Growth habit
Cream 7	USA	a	Yellowish-white	Determinate
Chinese Reds	USA	b	Dark Brown	Indeterminate
IT82C-16	ITTA	b	Dark Brown	Determinate
Sudany	Sudan	b	Black	Indeterminate
Black Crowder	USA	b	Black	Indeterminate

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Field trials: The field experiment was performed during two growing summer seasons (2014 and 2015), at the Experimental Farm of Faculty of Agriculture, South Valley University, Qena, Egypt. Seeds were planted on 1st and 3rd of April in summer season of 2014 and 2015, respectively. Experimental layout was split-plot in randomized complete blocks design with three replications, irrigation treatments were allocated to the main plots, whereas the genotypes were arranged in sub-plots. The long of the row was 3 m, 60 cm apart and plants were spaced at 20 cm from each other. Sown seeds were irrigated with water that has salinity (Ec) 153 ppm as normal condition and saline water with Ec 4800 ppm as stress condition as showing in Table 2. The different agricultural practices i.e. fertilization and pest management were applied as commercial cowpea production. At harvest time, for each treatment, ten plants from each plot (genotype) were randomly taken to determine, plant height (cm), Number of pods per plant, Pod length (cm), Fresh weight (gm) (average in gm for five plants 38 days-old), 100-seeds weight (gm) and Seed yield per plant (gm) (Average of pods collected from ten plants at 50% of plants developing dry pods). Additionally, the stress susceptibility index (SSI) was calculated using method of Fischer and Maurer, (1978). Stress tolerance index (STI) was estimated according to the formula used by Farshadfar *et al*., (2001).

Table 2. Analysis of two water types used for irrigation.

Irrigation Type	pH	Ec (ppm)	Water sample Meq/l (ppm)						
			Ca^{++}	Mg^{++}	Na^{++}	K^{+}	Cl^{-}	$CO_3^{-} + HCO_3^{-}$	SO_4^{--}
Normal Water	7.15	153	0.65	0.62	0.95	0.05	0.95	0.55	1.05
Saline Water	7.94	4800	10.7	10.5	47.15	0.9	31	3.2	15.5

Molecular characterization

DNA extraction: Genomic DNA was extracted from young leaves as described by Torres *et al*., (1993).

PCR amplification and electrophoresis: Nine Primers of ISSR markers (UBC 840, UBC 834, UBC 815, UBC 846, UBC 808, UBC 807, UBC 810, UBC 811 and UBC 816) were utilized in this work (EZBiolab-USA). PCR amplification reactions were applied according to instructions of Ben El Maati *et al*., (2004) and produced products which were immigrated on 1.5% agarose gels, followed by ethidium bromide staining for visualization. The identified bands were counted as 1 (present) and 0 (absent). Genetic similarity was calculated using Nei-Li's similarity index (Nei and Li, 1979). A dendrogram was constructed according to similarity matrix data by unweighted pair group method with arithmatic average (UPGMA), The MEGA program software was utilized in achievement of cluster analysis.

Statistical analysis.

All recorded data were subjected to statistical analysis of variance for each season separately and combined analysis over the two seasons according to Snedecor and Cochran, (1980). Treatment means were compared using least significant difference (LSD) test at 0.05 level of significance, using MSTAT-C statistical software package (Michigan State University, 1983).

RESULTS AND DISCUSSION

A. Field screening:

Combined analysis of variance for plant height, number of pods per plant, pod length, fresh weight, 100-seeds weight and seed yield traits are presented in Table 3. The effect of years was highly significant for all studied traits except seed yield which was insignificant. Concerning irrigation treatments, they showed highly significant effect on all of studied traits and all genotypes.

Concerning the mean performances of all genotypes for all studied traits were presented in Table 4. For plant hight, under normal irrigation, the mean values ranged from 51.18 to 93.53 cm for Chinese red and Black Crowder, respectively. While under saline irrigation the mean values were decreased to be 45.2 cm and 80.16 cm for the two varieties, respectively. this result indicated that salinity stress significantly reduced plant height and this result was in full agreement with the result that obtained by El-Hefny, (2010) who reported that using saline water at 5500 ppm reduced plant height by 15.22% for Kafr El-Shaikh, and 34.62% for Cream 7. Also, Islam *et al*., (2012) and Tesfaye, (2014) in lentil and Mehmood *et al*., (2009) in rice, reported that increasing NaCl salinity level decreased plant height. However, decreased plant height would be due to inhibition of cell division or cell enlargement by salinity stress (Raza *et al*., 2014).

Number of pods per plant significantly varied and reduced under salinity stress conditions among all genotypes. Whereas Sudany genotype was the best one in normal and stress treatment of 66.50 and 62.67, respectively. On the other hand, IT82C-16 genotype was the worst under both conditions. Similar result was reported by Patil *et al*., (1996), Zaki *et al*, (2009), El-Hefny, (2010), Islam *et al*., (2012) and Tesfaye, (2014) who found that number of pods per plant was decreased by increasing salinity level in both lentil and cowpea crops. They indicated that this reduction may be due to the accumulation of salt at high level in cells which in turn affecting many of biochemical process in plants such as translocation of assimilates towards organ regeneration and photosynthesis of the plant. For pod length, clear variation among genotypes in both normal and stress conditions. The lowest value (10.92 and 9.8 cm) was given by Sudany genotype in both normal and stress conditions respectively. IT82C-16 genotype displayed the highest value (17.92 cm) under normal condition as well as in the stress condition (16.47 cm). This results in agreement with those findings by El-Hefny, (2010), he reported that Kafr El-Shaikh cultivar more tolerant for high salt concentration compared with Cream7 cultivar, for yield and its components included pod length.

Fresh weight significantly varied among genotypes under normal stress and ranged from 27,32 for Sudany to 35.52 gm for IT82C-16. Salinity stress has reduction effect on the fresh weight trait for all genotypes, Chinese red gave the lowest value of 18.5 gm but Black Crowder gave the highest value of 28.76 gm. Obviously, fresh weight was decreased by applying saline water and this reduction may be due to limitation of metabolites substance that supply little tissues of plants, because high salt concentration decreased productivity of metabolic substance in the leaves, decrease water absorption and ions toxic effect on plants (Munns, 2002, Hussain *et al*., 2009 and Taffouo *et al*., 2009). The findings of this study are in agreement with those of El-Hefny, (2010), Soliman and El-Shaieny (2014) and El-Shaieny (2015).

About 100-seeds weight, IT82C-16 genotype showed the highest value of 16.16 and 15.43 gm in both non and stress conditions. While Chinese red and Sudany genotypes showed the lowest values in both conditions. Seed yield was significantly changed among all genotypes in normal and stress conditions and decreased with irrigation by saline water. In normal condition, seed yield was in between 28.76 gm (Chinese red) and 73.14 (cream7). While ranged from 27.27 (chines red) to 57.25 (Black Crowder) under stress condition. Both 100-seeds weight and seed yield were reduced by using saline water in irrigation, Similar results were reported by El-Hefny, (2010) in cowpea, Islam *et al*., (2012) and Mohammad, (2012) in lentil and Kazemand and Minoo, (2011) in soybean.

To discriminate between salinity tolerance and susceptibility genotypes, salt susceptibility index and stress tolerance index were estimated based on the difference in yield performance between non-stress and stress conditions as presented in Table 4. Accordingly, all genotypes were classified into three groups, high salinity tolerance (HST) group which consisted of two genotypes (Sudany and Chinese red) had lower rate for SSI (0.18 and 0.26) respectively, and higher rate of STI (96.45 and 94.82 %), respectively. Although they were the less genotypes for seed yield production under both normal and stress conditions, but in contrast they were the best in the stability of seed yield production over all genotypes under salinity stress. The second group was moderate salinity tolerance (MST) contained genotypes displayed moderate values in previous parameters. one genotype (Cream7) had the highest value for salinity sensitivity index (1.69) and the lowest value of STI (66.94 %) was existed in the last low salinity tolerance (LST) group. These results indicated that Sudany or/and Chinese red genotype can be crossed with Cream7 which had high seed yield and low salinity tolerance to create new varieties having both high seed yield and salinity tolerance. However, SSI and STI were an efficient indications for selection of tolerant genotypes under saline conditions, which are in agreement with many researchers, Goudarzi and Pakniyat, (2008), Khodarahmpouret *et al*., (2011) and Sbei *et al*., (2014).

Table 3. Mean squares for the field evaluated traits of 5 cowpea genotypes in response to salinity stress.

S.O.V.	D.F.	Mean squares					
		plant height	No. of Pods/ plant	pod length	Fresh weight	100-seeds weight	Seed yield
Year (Y)	1	4.571	0.104**	0.033**	66.507**	5.364	25.445
Y x R	4	0.464**	8.629**	2.522	1.893 **	1.836**	6.198**
Irrigation (I)	1	1295.862**	283.837**	23.313**	845.176**	8.214	1723.529**
Y x I	1	29.624	1.504**	1.873	180.579**	5.081**	34.333
Error (a)	4	4.466	10.696	0.944	3.862	3.521	10.778
Genotypes (G)	4	2982.686**	1291.850**	88.805**	161.677**	67.105**	2885.963**
Y x G	4	28.643**	4.250**	0.430**	16.409**	0.940	41.299**
I x G	4	58.848**	3.067**	0.420**	31.790**	3.619**	276.540**
Y x I x G	4	4.799**	2.733**	0.420**	33.408**	0.331**	45.610**
Error (b)	32	8.490	1.975	0.689	4.659	0.551	8.186

*** and ** indicate significant at 5% and 1% levels of probability, respectively**

Table 4. Mean performance of five cowpea genotypes for all evaluated traits under normal (N) and salinity stress (S) conditions, salinity susceptibility index (SSI), stress drought index (STI) and tolerance (Tol).

Genotypes	plant height cm		No. of Pods/ plant		pod length cm		Fresh weight gm		weight of 100 seeds (gm)		Seed yield/ plant (gm)		SSI	STI	Tol
	N	S	N	S	N	S	N	S	N	S	N	S			
Cream 7	66.37	51.43	53.50	48.67	14.92	14.13	33.94	23.10	14.00	12.68	73.14	48.96	1.69	66.94	L
Chinese red	51.18	45.20	49.00	46.17	12.30	10.53	25.67	18.50	10.12	10.78	28.76	27.27	0.26	94.82	H
IT82C-16	55.05	48.83	38.25	33.33	17.92	16.47	35.52	24.72	16.16	15.43	64.59	52.19	0.98	80.8	M
Sudany	54.72	48.67	66.50	62.67	10.92	9.80	27.32	22.41	10.20	10.20	39.51	38.11	0.18	96.46	H
Black-Crowder	93.53	80.16	55.83	50.00	14.92	13.80	32.58	28.76	14.45	12.32	69.37	57.25	0.89	82.53	M
L.S.D 5%	4.59		2.83		NS		3.52		1.62		3.761				

Capital leters H, M and L refers to high, moderate and low tolerance degree, respectivally.

B. ISSR marker characterization

To investigate the relationships and genetic diversity among the five examined cowpea genotypes, nine ISSR primers have been utilized in amplification of various reproducible DNA bands from genomic DNA (Fig. 1). A total of 80 bands were scored at size ranged from 150 bp to 1123 bp with an average 8.9 bands per primer (Table 3). UBC808 primer recorded the maximum bands number (14 bands) while the minimum bands number (6) was detected by the three UBC840, UBC810, and UBC816 primers. From 80 generated bands only 68 were polymorphic with an average of 7.56. The polymorphism ratio among primers ranged from 66.7% to 100% with an average of 82.08% (Table 5). Using ISSR marker analysis for studying genetic diversity among cowpea genotypes was also achieved by Ajibade *et al*., (2000), Ghalmi *et al*., (2010) and Gajera *et al*., (2014). clearly, in this study the polymorphism rate was high among genotypes and it is approximately close equal with several studies, Ajibade *et al*., (2000) obtained 96.8 % polymorphism rate, and 62.5 was detected by Ghalmi *et al*., (2010), while 100 % were observed by Gajera *et al*., (2014). Additionally, many of specific unique bands for salinity tolerance were detected by seven out of all primers, whether were only present in tolerant genotype but were absent in susceptible genotypes (as positive markers) or only found in susceptible genotype without others (as negative markers). UBC840 primer produced one as positive marker band and another one as negative band at size (345 bp and 494 bp), respectively. Other negative bands were shown, one by each UBC834, UBC815, UBC808 and UBC816 primers at size (902, 198, 394 and 1038 bp) respectively, and two by UBC811 primer at size (262 and 327 bp).on other hand, UBC807 primer produced one positive band at 403 bp. These results are in harmony with finding of Younis *et al*., (2007) who obtained several positive and negative specific bands associated with salt tolerance by using ISSR marker. Moreover, these salinity tolerance markers are helpful for selection of salinity tolerant genotypes.

The resulted data from ISSR analysis were used in the estimation of genetic diversity among the five evaluated cowpea genotypes through a UPGMA cluster analysis of genetic similarity matrices, cluster analysis was achieved based on the Nei-Li's similarity coefficient matrices. The results revealed that the highest similarity value (0.67) was noted between Sudany and Chinese red genotype, and both Crem7 and Chinese red genotypes showed the lowest value (0.48) (Table 4). Furthermore, the all genotypes were distributed by the dendrogram of genetic distant into two main clusters, the first one consisted only the lowest salinity tolerant genotype (Crem7). However the second cluster splitted into two sub-clusters, IT82C-16 genotype which had MST were categorized in the first sub-cluster. While the second sub-cluster subdivided into two main groups, the first one contained Black Crowder genotype, the other group included the two HST genotypes (Sudany and Chinese red) (Fig. 2). In the present work dendrogram represented good clustering system for salinity tolerance which showed that ISSR primers were able to recognize tolerant genotype and group genotypes according their origin genetic background. Similar results were recorded in barley by (Khatab and Samah 2013).

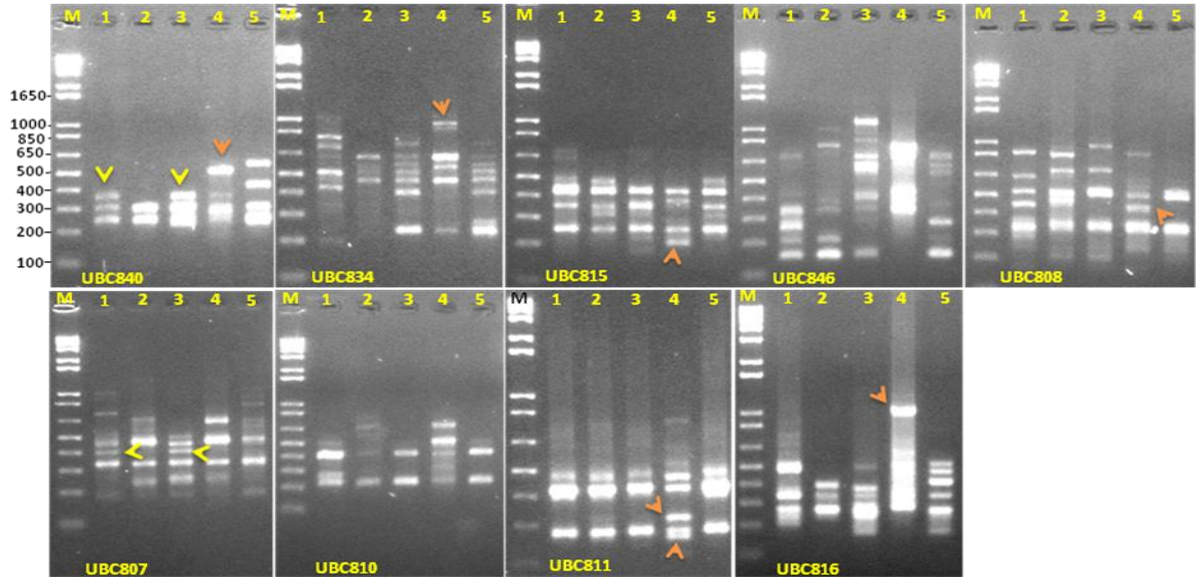

Fig. 1. ISSR-PCR amplified fragments produced by nine primers for five cowpea genotypes (1, Chinese red; 2, Black Crowder; 3, Sudany; 4, Cream 7; 5, IT82C-16). M, 1kbp DNA marker, yellow arrows shows positive salinity tolerance specific bands, orange arrows shows negative salinity tolerance specific bands.

Table 5. Polymorphism obtained by nine ISSR primers in five cowpea genotypes.

	Primer sequence	Range of fragment size bp	Total No. of fragments	Monomorphic fragments	Polymorphic fragments	Polymorphism %
UBC 840	$(GA)_8YT$	225-541	6	1	5	83.3
UBC 834	$(AG)_8TT$	198-958	12	1	11	91.7
UBC 815	$(CT)_8G$	198-470	7	2	5	71.4
UBC 846	$(CA)_8RT$	336-958	10	1	9	90
UBC 808	$(AG)_8C$	150-1123	14	0	14	100
UBC 807	$(AG)_8T$	255-1033	10	2	8	80
UBC 810	$(GA)_8T$	225-690	6	2	4	66.7
UBC 811	$(GA)_8C$	270-1038	9	1	8	88.9
UBC 816	$(CA)_8T$	262-497	6	2	4	66.7
Total		150-1123	80	12	68	
Average			8.9	1.3	7.56	82.08

Table 6:The similarity index among five cowpea genotypes based on ISSR

Genotypes	Chinese red	Black Crowder	Sudany	Cream 7	IT82C-16
Chinese red	1.00				
Black Crowder	0.64	1.00			
Sudany	0.67	0.61	1.00		
Cream 7	0.48	0.55	0.59	1.00	
IT82C-16	0.56	0.58	0.63	0.49	1.00

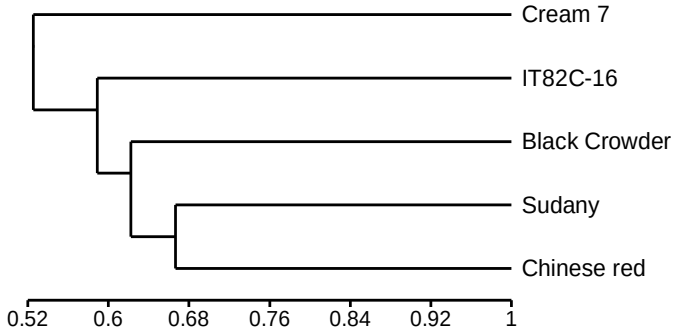

Fig. 2. The dendrogram of genetic distances among five cowpea genotypes using UPGMA cluster analysis of Nei-Li's similarity coefficient based on ISSR markers.

CONCLUSION

In the current study, five cowpea genotypes were evaluated for their capacity to tolerate salinity through field evaluation and ISSR molecular marker analysis. The varieties Sudany and Chinese red were identified as more tolerant genotypes but they had the lowest value of yield production. While, Cream7 genotype was the best one in the yield performance and had low salinity tolerance. These genotypes could be utilized in breeding approaches for improving salinity tolerant genotypes. Furthermore, specific salt tolerance bands have been detected which can be also serve in selection of tolerant genotypes.

Acknowledgments

We are grateful to The New Valley Agriculture Faculty, Assiut University, Egypt and Faculty of Agriculture, South Valley University, Qena, Egypt for the financial support of this work.

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فحص بعض التراكيب الوراثية للوبيا (*Vigna unguiculata* L. Walp) لمدي تحملها للملوحة باستخدام التقييم الحقلي والتحليل الجزيئي

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الملوحة هي أحد عوامل الإجهاد البيئي التي تؤثر بشكل خطير علي إنتاج محصول اللوبيا و حيث أن إنتاج أصناف جديدة أكثر تحملا للملوحة من أهم العوامل المساعدة لمواجهة آثارها الضارة. لذلك، في هذه الدراسة تم تقييم خمسة أصناف من اللوبيا لمدي استجابتها للإجهاد الملحي بواسطة التقييم الحقلي و تحليل الواسمات الجزيئية (ISSR). فقد تم تقييم سلوك بعض الصفات المحصولية تحت ظروف كل من مياه الري العادية والمالحة. وكشفت النتائج عن وجود فروق معنوية بين جميع التراكيب الوراثية في كل من الظروف العادية و الإجهاد الملحي. وتسببت المياه المالحة في خفض جميع الصفات المدروسة في كل التراكيب الوراثية. حيث كانت الأصناف Sudany و Chinese red الأكثر تحملا للملوحة و لكنها كانت أقل إنتاجية للمحصول. في حين كان Cream7 أفضلهم إنتاجا للمحصول وأكثر حساسية للملوحة. و كذلك استخدمت الواسمات الجزيئية (ISSR) لدراسة التنوع الوراثي بين هذه السلالات و كانت نسبة تعدد الأشكال المظهرية (٨٩.٨٦٪) وتراوحت قيمة التشابه بين التراكيب الوراثية من ٠.٤٨ إلي ٠.٦٧. وعلاوة على ذلك أظهرت سبعة بادءات القدرة علي إنتاج العديد من حزم الـDNA المرتبطة بالملوحة والتي يمكن أن تستخدم كعلامة للكشف عن السلالات الأكثر تحملا للإجهاد الملحي. ولقد قسم التحليل العنقودي جميع الأصناف إلي عنقودين رئيسيين يحتوي الأول منها علي الصنف الأقل تحملا للملوحة بينما يجتمع الصنفان الأكثر تحملا للملوحة في مجموعة واحدة. القدرة العالية التي أظهرتها بعض التراكيب الوراثية لتحمل الملوحة تشير إلى أن هذه التراكيب الوراثية يمكن استخدامها في برامج التربية لإنتاج المزيد من الأصناف الأكثر تحملا للملوحة وذات إنتاج محصولي عالي.