Development of Doubled Haploid Wheat Genotypes Using Chromosome Eliminating Technique and Assessment under Salt Stress

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Abstract: The chromosome elimination technique is an efficient method by which beneficial characters for salt tolerance can be combined within a short time and a large number of doubled haploid (DH) genotypes with desirable variability can be produced. In the present study 120 spring wheat DH genotypes has been developed using the wheat (*Triticum aestivum L.*) x millet (*Pennisetum glaucum*) crosspollination method with the F1 cross between Kharchia (Indian cultivar) one of the most cultivar recorded as salt tolerance worldwide and Sakha 93 (Egyptian breeding cultivar) cultivated in saline soil and recommended for newly reclaim lands, north area of Egypt. Under normal conditions the DHs agronomical traits (i.e. flowering time, number of spikelets per spike (NSPS), plant height, spike length and 1000 grain weight) distribution was normal and significant transgressive segregation was observed. ANOVA analysis showed significant differences among DH genotypes for all agronomical traits, and the DH 11, 22, 57, 98, 106, 111 and 118 lines found to have better yield characters SNPS, spike length and 1000 grain weight than the parents under non-saline condition. The 120 DHs and both parents were grown in hydroponics culture medium, with the concentration of NaCl : $CaCl_2$ 4:1 being 150mM. Some of those DHs showed much high in responses to growth under salinity than both parents. The variances between DH lines were significant for Na^+ and Cl^- ions, leaf Fresh weight (lfw), leaf dry weight (ldw), leaf 2 extension rate before salt additions (LE-b), leaf 4 extension rate after salt additions (LE-a), number of spikes per plant (SNPP), number of spikelets per spike (NSPS), number of grains per plant (GN) and grain weight per plant (GW), and non-significant for K^+ ion and water percentage (W%). Overall the mean values for the DHs were higher than the parents values under salt stress, for the DH 3, 12, 38, 57 and 96 genotypes mid-values of LE-a were close to the average of LE-b under non-saline conditions. A significant negative correlation was determined between Na content and yield parameters i.e. SNPP, NSPS, GN and GW. In contrast it was positively correlation with W%, which might indicates that better yield characters of DH lines i.e. 10, 25, 42, 57, 68, 96, and 114 than parents under salt conditions may be due mainly to better exclusion of Na from the shoots. [Journal of American Science 2010;6(7):139-148]. (ISSN: 1545-1003).

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1. Introduction

Changing climatic conditions and misuse of agricultural lands over the last few years have lead to a rapid increase in the area of soil under salinity. There is, therefore, an urgent need for more salt tolerant varieties of various crops, in particular wheat. By using the utilization of conventional plant breeding techniques, the selection intensity and number of selection cycles necessary to improve tolerance effectively needs years. Obviously, any method that can accelerate and increase the efficiency of a programme to produce salt tolerant crop plants will be beneficial. The use of chromosome elimination in wheat DH production is one technique which can contribute to this development. By simplifying and accelerating breeding programmes, this is the quickest possible way of achieving homozygosity. From a breeding point of view, in self pollinated species, such as the small grained cereals, doubled haploids can be used directly for the production of varieties, since each DH has the potential to become a new variety.

In Wheat, the production of uniform homozygous lines enhances selection efficiency, allowing a better discrimination between genotypes in breeding nurseries within only one generation, making it a useful technique for both wheat breeding and genetical studies. Plant anther culture is one of the plant breeding techniques that have been developed to produce new homozygous cultivars within a relatively short time (Flavell, 1981). Progress has been made in the anther culture technique through modifying the culture medium (Ghaemi et al., 1994). Nevertheless, this method may cause unpredictable genetic alterations due to gametoclonal variation (Huang 1996; Raina 1997). These factors could affect the means and genetic variances of a breeding population, thus affecting selection (Ma et al., 1999).

Moreover, wheat DHs can be produced by inter-specific crosses. In this technique, haploid embryos appear as a result of selective elimination of the alien chromosomes during embryogenesis. The first use was by barely (***Hordeum bulbosum***) as the pollen parent for this purpose (Barcley 1975). However, most wheat varieties show no crossability with ***Hordeum bulbosum*** due to the presence of the Kr1 locus on chromosome 5B and/or Kr2 locus on chromosome 5A (Snape et al., 1979). The dominant alleles of these genes, Kr1 and Kr2, are responsible for the poor crossability.

In contrast, wheat x maize cross-pollination has been used for producing wheat haploid plants, and no recalcitrant genotypes have been reported because the maize chromosomes are eliminated at early embryonic stages (Laurie and Bennett, 1988a), and the efficiency of haploid production is not affected by ***Kr*** genes (Laurie and Bennett 1988b).
Several studies have been proven that different species such as sorghum (***Sorghum bicolor***) and pearl millet (***Pennisetum glaucum***) are also possible pollinators for wheat haploid production (Ahmad and Comeau 1990; Inagaki and Mujeeb-Kazi 1995).

In Egypt, wheat is the most important daily food cereal, however, only 40% of its annual domestic demand can be produced (Salam, 2002). In present, cultivated land, comprises only 3% of total land area in Egypt, is already salinized. The government strategic plain is to increase the total agriculture land by adding newly reclamation land irrigation with saline underground water, due to limitation of other water resource from Nile river and low precipitation (less than 25mM annual rainfall) (Ghassemi et al., 1995). Therefore, it is necessary to disseminate newly released cultivars with more salt tolerance to be introduced for the newly recrimination land irrigated by underground water, which affected by access salt from Mediterranean and Red Seas (Shannon, 1997; Pervaiz et al., 2002). According to results by Kingsbury and Epstein, (1984) it was found that among spring wheat (***Triticum aestivum***) cultivars, Kharchia showed relatively high salt tolerance in respect of growth and grain yield. However, as compared to other relatively salt tolerant varieties it accumulated more Na^+ in the shoot when grown under salt conditions (Mahmood 1991). Similarly, the spring wheat breeding genotypes Sakha 93 was obtained from Agricultural Research Centre, Giza, Egypt is usually cultivated in saline areas in Egypt for restricted Na^+ accumulation in the shoot, and salt tolerance in respect of growth and grain yield (El-Hendawy et al., 2005), indicating that the two parents might contain a different set of genes with different strategy to survive under salt stress conditions.

Due to observed differences in the negative effect of salinity on growth processes among and within species, many of the traits like plant height (Chauhan and Singh, 1993), fresh and dry weight of shoots and roots (Ashraf and Noor, 1993), leaf area (Romero and Maranon, 1996), and number of leaves per plant (Yang et al., 1990) are now widely used as measures of the degree of plant salt tolerance. In wheat, the total grain yield from a plant is a combination of number of spikes per plant, weight per grain, and grain number per spike. Often, salinity affects the traits contributing to yield structure. Thus, total grain yield in wheat was reduced under irrigation with water with an EC 16 dSm^{-1}, to about 60% (Chauhan and Singh, 1993).

Disturbances in grain yield under salinity are caused by a ranges of factors, e.g., a decrease in spikelet numbers per spike (Grieve et al., 1993), disturbed pollen viability (Sacher et al., 1983), and a reduction in the number of fertile tillers per plant (Khatun and Flowers, 1995). The aim of this research was to develop of doubled haploid lines that might contain individuals having a better performance under salt stress, due mainly to recombination of desirable characters from both parents Sakha 93 and Karchia. Because of the importance of biomass and grain yield indices for assessing plant response to salinity, this paper describes work done to assess and establish the relationship between some characteristics during early and later stages of growth including yield components under non-saline and salin conditions, which will help in deciding the optimal genotype for more studies on wheat.

2. Material and Methods

Development DHs Genotypes

Two spring wheat were chosen as parents on the basis of highly response to salinity. The parent varieties were Kharchia (Indian cultivar; relatively salt tolerant) and Sakha 93 (Egyptian genotype; salt tolerance and high exclusion of Na^+). A cross between these two genotypes was made and the resulting F_1s were crossed to produce a haploid wheat using pearl millet (***Pennisetum glaucum***) pollen as the male parent, which were doubled by following the procedure described by Amin (2002). Wheat spikes were emasculated 1-2 day(s) before anthesis and pollinated 1 day later with fresh pearl millet pollen. Twenty-four hours after pollination the florets were injected with a drop of 50 ppm Dicamba. In total 75 spikes were pollinated with fresh millet pollen and covered with polyethylene bags. Haploid embryos were excised and cultured on Gamborg's medium with B5 vitamin following the procedure described by Amin (2002). When seedlings had developed an adequate shoot and root, they were gently pulled out of the culture tube and potted into very wet seedling compost in 6 cm pots. When the seedlings were about 15-20 cm high, one-third of the roots and shoots were cut. Root haploids were then immersed into colchicine solution (12ml distilled 0.5% colchicine, with 2% dimethyl sulfoxide and few drop of tween-20), and left for 5.5 h at room temperature. After treatment the roots of the plants were washed in running tap water to remove chemicals before being re-potted into 15 cm pots of compost. The setting of seed on the colchicine-treated plants was the indicator of the success of chromosome doubling. Ten replicates of each DH line and the two parents Kharchia and Sakha 93 were grown to maturity. Five parameters were measured i.e. flowering time, NSPS, spike length, 1000 grain weight and plant height.

Hydroponics Culture Medium

Four seedlings of each DH line were planted one per pot, with three replicates; these pots were placed randomly. The hydroponics was set up as described by Amin (2002). For the first week, plants were irrigated with only water, after that solution with half strength Hoagland nutrient medium was replaced the water. When leaf 2 was half emerged, NaCl and $CaCl_2$ (4:1) were added gradually by 50mM a day to give a final concentration of 150mM. The nutrient solution was added daily to the initial level in the tank, and replaced twice weekly. The experiment was performed in a greenhouse conditions, at about 20^{o}C with 16/8 day/night natural daylight supplemented with lamps.

Measurements of Inorganic Ions

Two weeks after the addition of salt, the leaf 3 was collected. Leaf samples were ashed in porcelain crucibles in an oven at 500 ^{o}C for 5 h. After cooling down, 2ml of concentrated nitric acid was added to each crucible to dissolve the ash. The solution was transferred into 50 ml plastic tubes and made up to 50 ml with ddH_2O. Na^+ and K^+ ions were measured using a flame photometer (Corning 410). Cl^- was determined using a Russell combination Cl^- ion selective electrode directly in the tubes after the determination of cations.

Growth Characteristics

Fresh weight (lfw), dry weight (ldw) and percentage of water (W%) traits were measured in the leaf 3 for determination of ions under salt stress. For determining leaf extension rate before salt additions (LE-b), the length of leaf 2 was measured from its tip to the sand surface and to the ligule of leaf 1. Same thing after salt addition, the length of leaf 4 was scored (LE-a) day by day from the moment it protruded from the subtending leaf sheath, until no increase in length could be detected. At maturity, plants were harvested and the following traits were measured: number of spikes per plant (SNPP), number of spikelets per spike (NSPS), total number of grains per plant (GN) and grain weight per plant (GW).

3. Results

In total, of 150 haploid embryos (Figure 1) that were rescued and cultured, only 125 haploid seedlings have survived. Finally, 120 doubled haploid plants reached the flowering stage and developed seeds. A combined analysis of variance of DHs under normal conditions showed significant differences between DH lines for flowering time, NSPS, plant height, spike length and 1000 grain weight (Table 1). The range of expression of differences amongst the DH lines under normal conditions was determined in the form of frequency distributions, shown in Figures (2). The DH 11, 22, 57, 98, 106, 111 and 118 lines were found to have better yield characters than the parents under normal conditions. These also showed that Sakha 93 has a better performance comparing with Kharchia over all parameters. The normality was tested using the MINITAPE soft wear. Overall, the distribution of above traits was normal and significant transgressive segregation was observed. A comparison for various parameters between DH means and parental means values is shown in Table (2). Under non-saline conditions, DH means were higher than Kharchia and Sakha 93 means.

Hydroponics culture

Some of the data for yield parameters under salt conditions were skewed from the normal distribution, as 25 DHs did not developed seed under salt stress. Such data were transformed by natural logarithms prior to statistical analyses (i.e. SNPP, NSPS, GN, and GW). Normality of frequency distributions was tested using the normality test in MINITAB software.

Ion contents

Kharchia accumulated 1.5 times more sodium than Sakha 93 and its Na and K concentrations were very close to the population means (14.59 mg gDW^{-1} and 9.12 mg gDW^{-1} respectively) (Table 3). The parents contrasted with respect to Cl concentrations. Sakha 93, on average,

Fig. 1 Wheat haploid embryo from wheat x millet cross pollination

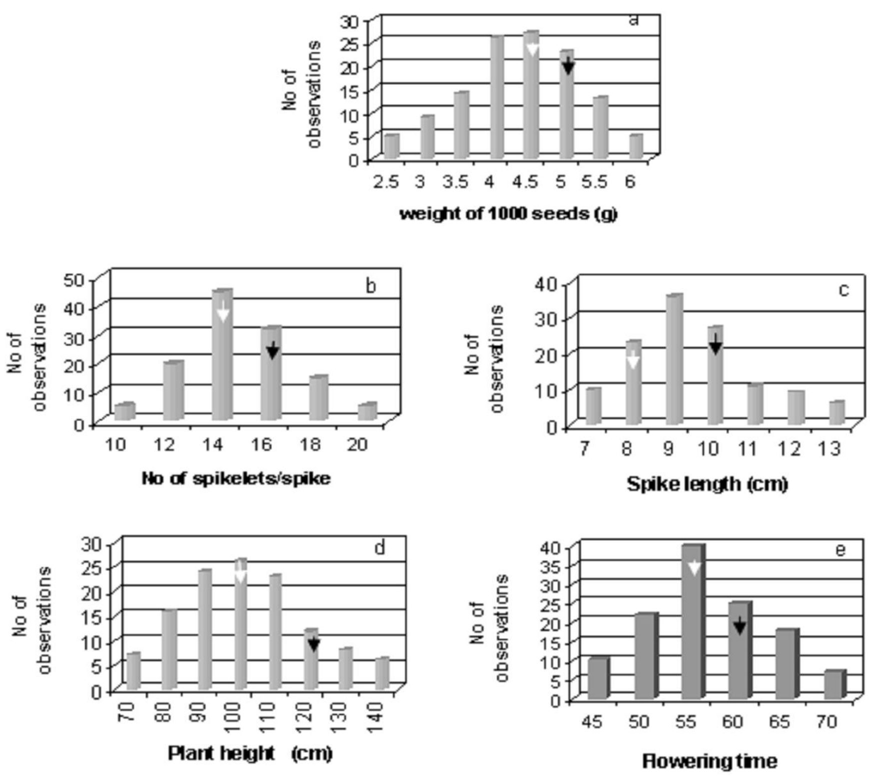

Fig. 2 Distribution of the doubled haploid lines and the two parents Kharchia (whit arrow) and Sakha 93 (black arrow) for five characters (a)1000 grain weight (b)number of spikelets per spike (c) spike length (d) plant height (e) flowering time

Table 1 Analysis of variance of agronomics traits under non-saline conditions

Trait	Source of variation between DHs		F-test	*P*
	df	MS		
weight of 1000 seeds (g)	119	1925	3.79	<0.001**
plant height	119	704	1.04	0.207
No. of spikelets/spike	119	1590	2.39	0.003**
Spike length	119	867	1.4	<0.001**
flowering time	119	2384	4.67	<0.001**
grain number / ear	119	1590	2.4	<0.001**

Table 2 Mean values for agronomics traits under non-saline conditions

Trait	N	Mean	Min.	Max.	Kharchia	Sakha 93	Std. Deviation
weight of 1000 seed (g)	120	4.6	2.8	6.2	3.7	5.0	0.60
Plant height (cm)	120	107	75	152	92	115	10.18
No of spikelets/spike	120	15.7	11.2	21.9	12.7	18.0	2.36
Length of spike (cm)	120	10	7.4	14.2	8.6	11.2	1.15
Flowering time	120	62	48	83	52	63	5.51
No of grain/ear	120	40	7	70	28	45	7.77

Table 3 Analysis of variance and means values for all characteristics of doubled haploid population described in the hydroponics culture

Trait	Means	Minimum	Maximum	Kharchia	Sakha 93	Sdve.	Source of Variance Between DHs			
							D.F.	M.S.	F-test	***P***
Leaf Na content (mg/g DW)	15.49	2.68	35.81	14.59	9.12	4.144	119	164.07	3.99	<0.001***
Leaf K content (mg/g DW)	1.24	0.41	2.4	1.15	1.10	0.442	119	0.91	1.45	0.076
Leaf Cl content (mg/g DW)	8.11	0.77	37.80	4.26	6.98	3.992	119	2.312	3.88	<0.001***
leaf 2 extension rate (cm/day)	4.34	3.06	5.608	3.550	5.34	1.464	119	0.196	2.57	0.033*
leaf 4 extension rate (cm/day)	2.06	0.87	4.344	2.436	1.08	0.131	119	0.204	3.60	0.014*
Water content	79.14	68.17	83.243	81.354	74.4	4.003	119	0.018	1.91	0.094
Leaf fresh weight FW (gm)	0.145	0.056	0.269	0.189	0.082	0.017	119	0.8061	4.99	<0.001***
Leaf dry weight (gm)	0.042	0.018	0.073	0.054	0.026	0.005	119	0.593	4.47	<0.001***
No. of ears per plant	0.174	0.283	0.991	0.850	0.765	0.435	99	0.592	3.09	0.0012**
No. of spikelets per spike	1.004	0.425	6.232	4.532	4.079	2.268	99	0.156	1.67	0.043*
No. of grains per plant	0.361	0.283	2.974	1.133	1.020	0.496	84	1.557	2.80	0.024*
Grain weight per plant (g)	0.026	0.059	0.016	0.032	0.029	0.011	84	2.981	3.42	0.017*
Total dry weight (g)	0.228	0.068	0.589	0.269	0.197	0.026	119	3.316	2.12	0.04*

**:p < 0.01 *:p <0.05

accumulated almost 1.6 as much chloride as Kharchia though still less than the mean value of Cl for the DHs (8.11 mg gDW^{-1}). Large variations in the concentrations of Na, K, and Cl were observed among the doubled haploid population (Table 3) with genotypic values ranging from 2.68 to 35.81 mg gDW^{-1} for Na, 0.41 to 2.4 mg gDW^{-1} for K, and 0.77 to 12.8 mg gDW^{-1} for Cl. Analysis of variance among DH lines for the Na, K and Cl under salt conditions is presented in Table 3. The differences between DH lines were significant for sodium, chlorine, and non-significant for potassium content.

Growth Characters

In saline conditions, lfw, ldw and water percent (W%) were affected by the transgressive segregation of beneficial traits coming from both parents for salt tolerance (Table 3). Among the parents, Kharchia had a higher W% average than Sakha 93 (81.354% and 74.4%, respectively) as well as almost twice as much lfw and ldw (Table 3). The lfw of doubled haploids ranged from 0.056 to 0.269 per plant (average 0.145), ldw varied from 0.018 to 0.073 per plant (average 0.042), and W% from 68.17 % to 83.243 % (average 77.14 %) (Table 3). Leaf extension rate is expected to be correlated with traits determining plant growth under normal or saline conditions. Under salt stress, Kharchia had a better extension rate (2.436 cm/day) than Sakha 93 (1.08 cm/day) and than the average of DH lines (2.06 cm/day), in contrast with normal conditions, when Sakha 93 (5.34 cm/day) had a better performance than Kharchia (3.55 cm/day). Among the DH lines there were differences in Leaf extension rate between contrasting conditions reflect the transgressive segregation (Table 3). Under salt stress the leaf extension rate ranged from 0.87 to 4.344 cm per day (Table 3), whereas with the non-stressed condition the value of this trait ranged between 3.06 to 5.608 cm per day. The leaf extension rate of DH line 3, 12, 38, 57 and 96 under salinity was close to the average for plants under non-saline conditions. Analysis of variance showed the differences between genotypes were significant for all growth characteristics except for W% (Table 3).

Yield Characters

The distribution for all yield parameters values are shown in table 3. Overall, Kharchia was found to be more tolerant than Sakha 93 under salt stress. Moreover, the distributions indicate that about 20% of the DH lines performed better than the parents under salt conditions. The traits such SNPS, NSPS, GN, and GW were examined; there was a skewed distribution toward zero values. As Eighteen DHs affected by salinity and did not produce any spike, add to that 7 DHs produced ears were completely sterile. These zero values were eliminated from the datasets for analyses of variance. A separate analysis of variance of DHs and parents for each character was carried out, and showed that DHs differed significantly for all yield characters.

Correlations between all characters

A simple correlation between the all parameters for DHs under salt conditions were calculated on a genotype mean basis. As we expected, the SNPP, NSPS, GN, and GW had significantly negative correlation with Na and Cl content (e.g. Figure 3), and were non-significant with K content.

As expected the W% was positively correlated with lfw, however, it was unexpected to be as well positively correlated with Na and Cl contents (e.g Fig 4). Other significant correlations are presented in figure (5a and 5b) between the leaf size with leaf extension rate, which showed opposite direction under normal (positive correlation) and saline condition (Negative correlation).

4. Discussion

Although DH production for wheat has been available for over two decades, there is a few reports in the literature (Amin., 2002; Mahmood and Baenziger, 2008) where this technique has been used for the production of progenies differing in salt tolerance. Nevertheless, many reports exist where the DH system has proven better than other breeding methods in respect of efficiency and for the production of genotypes with improved agronomic characters. In the present work the better performance of some DH lines (11, 22, 57, 98, 106, 111 and 118) compared with that of the parents in normal conditions may be due to the recombination of desirable characters in DH lines.

In agreement with this, Laurie and Snape (1990) and Snape et al. (1988) reported that a large number of DH lines with varying degrees of ariation in agronomic characters may be the result of gametoclonal variation. They also observed that within a population of DH lines derived using chromosome eliminating technique, there was significant genetic variation for biomass production and grain yield. Similar results were found and presented in Table 1 and 2, with the differences between DH lines being significant for all the characters studied under normal conditions except plant height. In barley, Bozorgipour and Snape (1991) observed that DH progenies of the haploid plantlets which had bigger embryo size and faster

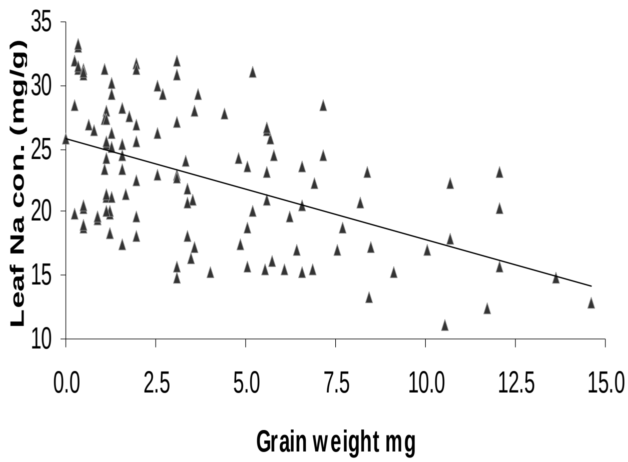

Fig 3 Relationships between leaf Na content and grain weight per plant

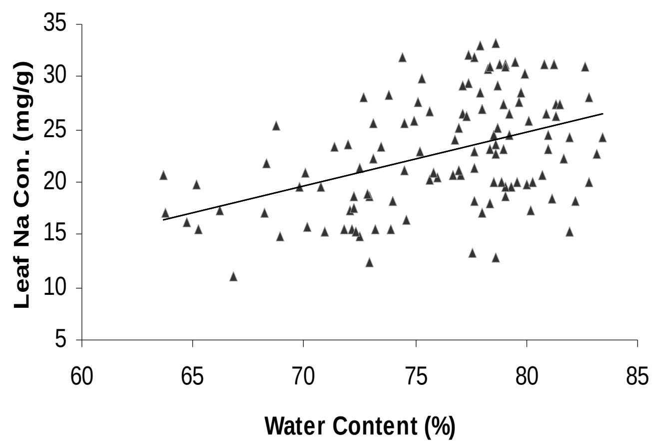

Fig 4 Relationships between leaf Na content and water content (%)

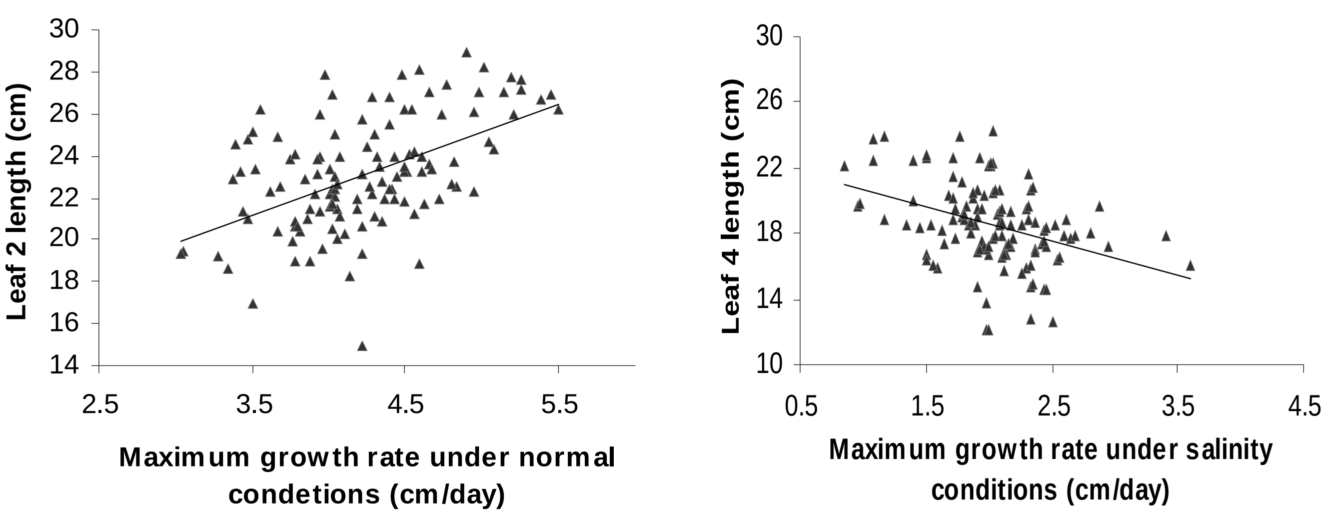

Fig. 5 Relationships between leaf length and maximum growth rate under normal condition (a), and under salinity conditions (b).

growth rate at germination, were superior to the other lines with respect to a number of agronomic characters including plant biomass, plant height and grain yield. According to that DHs DH 11, 22, 57, 98, 106, 111 and 118 may have developed from the vigorous embryos, which ultimately produced more vigorous DH lines.

This experiment was designed to improve salt tolerance of the existing wheat cultivars by the appropriate combination of desirable characters from a good background parents i.e. Sakha 93 and Kharchia for salt tolerance within a short time and a large number of homozygous genotypes. In this research work, the 120 DH genotypes exhibited large variation in all the traits described in Table 3 under saline conditions. Forster et al. (1990) mentioned, in terms of growth, that higher salt tolerance of some of the DH lines than either of the parents can occur through transgressive segregation over the better parents for growth. Absence of the typical bimodal distribution indicates that single major genes with large effects controlled none of the traits studied. The transgressive segregation observed in almost all the traits (Table 3) strongly supports this deduction. Evidently, these traits were controlled not by a single gene with a large effect but rather by a combination of several genes with smaller effects.

On the basis of previous research by Amin (2002), the better performance of some DH lines compared with the mid performance of the parents (similar to the result obtained in Table 3) more likely to be due its ability of use different mechanisms for salt tolerant, which is provided by both parents Sakha 93 and Kharchia. As the mechanisms of salt tolerance are still not fully understood, all the sources of these variations detected between the DH lines for all the characters studied in this work remain unclear. It is also difficult to identify which processes had the greater effect on growth under salt conditions or which of them worked together to increase the plant salt tolerance.

Sodium affects, mostly negatively, on plant physiological and biochemical systems (Murthy et al., 1979). Similar to the result presented in Figure 3, wheat grain yield was found to decrease with increased Na concentrations in leaves. On anther study, salt tolerance of wheat plants, defined as relative grain yield, was found to be negatively correlated with the concentration of Na in leaf 5 (Schachtman et al., 1992). Out of 120 DHs only 95 DHs eventually produced one or more ears with a highly significant variation among DHs (Table 3) seven of them were not developed any grain. In many cases under salt stress, this was attributed to a decrease in pollen viability and pollen fertilization (Khatun and Flowers, 1995), was considered to be sufficient to retard the germination of a pollen grain and growth of the pollen tube (Sacher et al., 1983). This might indicates that better growth performance of DH lines i.e. 10, 25, 42, 57, 68, 96, and 114 than parents under salt conditions may be due mainly to better exclusion of Na from the shoots. For Cl measurements under salt stress, a significant variation in the amount of Cl absorbed was detected within the DHs (Table 3). However, non-significant negative correlation was found between this trait and grain yield characters. It might be related to some kind of gene, who can restrict Cl influx into cells, thus avoiding its negative effects. So far, only a soybean gene, Ncl, involved in chlorine uptake has been described (Abel, 1969).

According to the difference between both results for the correlation between leaf length and plant growth rate under normal and salt conditions (Fig 5), it seems that under salt stress, phenological escape is one of the strategies employed by plants to survive such environmental conditions, when plants decrease leaf size as well as the life cycle smaller leaves maintain higher growth rates. In agreement with this, the reduction in leaf area reduces the evaporative surface and by doing so inhibits gas exchange (Gale et al., 1967), the rate of transpiration and ultimately the efficiency of photosynthesis (Cramer et al., 1990), which is one of the mechanisms plants use to tolerate the reduced ion uptake effects of the soil salinity (Flowers and Yeo, 1989).

The water percent showed significant differences amongst doubled haploids (Table 3). Surprisingly, the relationship between percentage of water and Na accumulation in the leaf was significantly positive (Fig. 4). This result contrasts with findings by Hoffman and Jobes, (1978). In their experiment, the severe reductions in growth of the plant in salt conditions were thought to be at least partly due to the negative correlation with water content. For these unexpected results the obvious explanations, it may have happened because the older leaf, still green, that was sampled for the ion measurements, was accumulating ions in that leaf to protect the youngest leaf from the effects of toxicity. This is one of the mechanisms plants employ to survive the adverse effects of the salt stress.

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