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Original article

Selection of chickpea (*Cicer arietinum*) for yield and symbiotic nitrogen fixation ability under salt stress

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Abstract – This research was aimed at selecting chickpea for improved symbiotic nitrogen fixation (SNF) under saline conditions. 200 Moroccan lines were screened for nitrogenase activity, dry weight, nodule mass, plant colour and total nitrogen under 0 mM, 25 mM and 50 mM NaCl in screenhouse nitrogen-free sandbenches. Inoculum was a mixture of 4 Moroccan salt-resistant *Rhizobium* strains. Seven lines were selected as tolerant to salt for SNF. In winter 1998 these lines were tested on-station in a saline and a non-saline soil for salt tolerance with 2 checks. The salt reduced yield by 26 to 38% according to genotypes. Based on data from the 2 soils MCA103, MCA131 and MCA250 were selected as tolerant lines to the salt for SNF and yield. In 1999 these 3 tolerant lines were evaluated for production on-farm in 2 regions. The 3 lines over-yielded the checks. MCA250 was the most productive line on both sites.

***Cicer arietinum* / selection / nitrogen fixation / salt tolerance**

Résumé – Sélection du pois chiche *(Cicer arietinum)* pour le rendement et la fixation symbiotique de l'azote sous contrainte saline. L'objectif de cette recherche est de sélectionner des génotypes de pois chiche pour la capacité de fixation symbiotique de l'azote (SNF) sous des contraintes salines. Deux cents lignées marocaines ont été évaluées pour l'activité de la nitrogénase, la matière sèche, la masse des nodules, la couleur de la plante et l'azote total sous 0 mM, 25 mM et 50 mM NaCl en serre sur du sable dépourvu d'azote. L'inoculum comprend un mélange de 4 souches locales de *Rhizobium* tolérantes à la salinité. Sept lignées tolérantes à la salinité pour la SNF ont été sélectionnées. Les lignées sélectionnées ont été évaluées en hiver 1998 sur un sol salin et un autre non salin en station expérimentale. Les résultats ont confirmé la supériorité des lignées tolérantes au sel par rapport aux témoins pour le rendement et les caractères associés à SNF en sol salin. Le rendement a été réduit de 26 à 38 % en sol salin selon les génotypes. Sur la base des données des deux sols, les lignées MCA103, MCA131 et MCA250 ont été sélectionnées pour leur tolérance au stress salin pour la fixation symbiotique et le rendement. En 1999 ces 3 lignées ont été évaluées pour la production à la ferme dans 2 régions. Les résultats ont montré leur supériorité par rapport aux témoins Douyet et Rizky. MCA250 est la lignée la plus performante dans les 2 sites.

***Cicer arietinum* / sélection / fixation symbiotique de l'azote / tolérance au sel**

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1. INTRODUCTION

In Morocco, chickpea (*Cicer arietinum* L.) is produced on 77 000 ha and is the second most important grain legume crop after faba bean. It is mostly cultivated as a spring crop in well rain-fed areas and on deep soils with high capacity of water retention in the semi-arid zones. Chickpea production is characterised by low and unstable yields mainly because of the water stress at the end of the plant cycle. The low profitability of the crop induces a decrease in the chickpea area in favour of more profitable crops. Nevertheless when planted as a winter crop, chickpea produces nearly 200% more than spring planting [7]. Thus the extension of chickpea as a winter crop is an attractive alternative for improving productivity. However, this practice faces problems including diseases and the deficiency of symbiotic nitrogen fixation (SNF) by this crop. Indeed, a study on the limiting factors to winter and spring chickpea production showed that low SNF is an important constraint limiting production in many sites. Inoculation increases SNF by about 138 to 70% using local and foreign strains respectively. Additionally it was found that inoculation increases yield by 27 to 51% depending on the *Rhizobium* strain used [2].

Salt affects vast areas of Moroccan soils particularly in the semi-arid zones and irrigated areas. Furthermore localised spots of affected soils are continuously spreading in the southern part of the country as a result of drought. These soils are saline as their electrical conductivity (EC) is often greater than 2 ds/cm [1]. Different forms of soil salinity can affect crops. Such saline conditions may severely limit nitrogen fixation as they prevent symbiosis by affecting the survival and proliferation of rhizobia in the soil, inhibiting the infection process, directly affecting root nodule function, and/or reducing plant growth, photosynthesis, and demand for nitrogen.

Indeed salts of Na and Ca are known to be toxic to rhizobia at high concentrations. NaCl concentration of more than 1% inhibits the growth of the rhizobia [11]. However it was found that tolerant strains could grow with up to 120 mM of NaCI, indicating that the symbiosis processes from root hair infection are more sensitive than from rhizobia alone. Wilson (1970) stated that salts affect the host rather than the *Rhizobium* and the symbiosis resulting from the two partners is more sensitive to salinity.

Legumes are relatively salt-sensitive crops [10]. Chickpea, faba bean and pea are particularly salt-sensitive species [8, 10]. Germination of chickpea is relatively less affected by salinity than subsequent seedling growth [7, 13]. A significant variability was reported among chickpea lines for efficient nodulation under salt stress conditions [11].

The existence of genetic variability with the occurrence of genotypes withstanding critical values approaching the desired dose is the basis for the genetic improvement of salinity resistance. Genotypic variability has been demonstrated in chickpea but it occurs over a narrow range of salinity levels [6, 9].

Because chickpea is a very ancient crop in Morocco local populations have developed a large variability for many important traits and adaptation to various environmental stresses. Hence they offer a valuable germplasm source for breeding purposes. Local germplasm has not been analysed for SNF and associated traits [12]. Therefore, the objectives of this research were: (1) to screen local germplasm of chickpea for SNF capacity under salt stress conditions and select the most tolerant genotypes, (2) to evaluate the selected genotypes in the field on an experimental station, (3) to conduct on-farm field trials to evaluate the performance of the superior genotypes in farming conditions.

2. MATERIALS AND METHODS

This paper includes results of selection based on 3 different, yet dependent experiments: screenhouse screening, on-station evaluation and on-farm trial.

2.1. Plant material

For the screenhouse screening, the plant material included 200 lines previously selected for winter planting under natural conditions from a collection of 500 accessions collected throughout Morocco [12]. These core lines were previously characterised for morphological and agronomic traits [3, 14]. They generally cover the range of variability found in the collection for morphological and agronomic traits.

The screenhouse test resulted in selecting 7 salt-tolerant lines (MCA31, MCA45, MCA103, MCA131, MCA250, MCA301, MCA370) for nitrogen fixation. These lines were tested on-station for field confirmation of their tolerance.

The on-station field evaluation resulted in selecting 3 lines (MCA103, MCA131 et MCA250) as tolerant to salinity for symbiotic nitrogen fixation. These lines were evaluated in on-farm trials in 2 regions.

2.2. Inoculum

The inoculum used in all experiments of this research consisted of four strains of *Rhizobium* isolated from Moroccan soil and selected for tolerance to high level of salinity. In the screenhouse experiment the inoculation was applied as liquid solution immediately after sowing and at the seedling emergence to ensure the presence of *Rhizobium* strains in large number. The same strains were used to prepare inoculum for inoculating seeds before planting field trials on-station and on-farm.

2.3. Screenhouse experiment

The screening experiment was conducted in screenhouse nitrogen-free sandbenches purposely designed to screen for N_2 fixation [12]. The size of the benches was 4.00 m (length) × 2.00 m (width) × 0.20 m (depth). Seeds were planted in 5 cm × 5 cm × 20 cm (depth) plastic sleeves embedded in the sand benches to contain root growth. This prevented injury and loss of roots when plants were removed from the sand. Seeds were surface-sterilised with 10% chloride bleach for 2 min. Two seeds were planted per sleeve and randomly thinned to one after seedling emergence. Prior to planting, lime, macro and micronutrients were incorporated into the sand.

The treatments included 3 salinity levels: 25 and 50 mM created by adding NaCl as solution and the NaCl-free control. The experiment was set up as a split plot arrangement with 2 replications; NaCl treatments were allocated to main plots and chickpea lines to small plots. The saline solution was applied every two days starting at the plant emergence. Twice a week the experiment was heavily irrigated to drain salt and prevent its accumulation.

The plants were removed from the sand at flowering, identified by a numbered tag attached to the main stem and assayed for nitrogenase activity (NA) as measured by acetylene reduction activity (ARA) [12]. The same plants were used to measure top dry weight (mg), total nitrogen content in the top using the Kjeldahl techniques (%), and the nodule mass (1-9 class visual score: 1 = 0 to 30 mg, 2 = 31 to 50 mg, 3 = 51 to 70 mg, 4 = 71 to 90 mg, 5 = 61 to 75 mg, 3 = 76 to 90 mg, 7 = 91 to 105 mg, 8 = 106 to 120 mg, and 9 > 120 mg).

2.4. On-station evaluation

The 7 lines selected as tolerant to salinity for SNF in the screenhouse were tested in the field at Tadla Experimental Station, 300 km south of Rabat. The station is located in a semi-arid climate with an average annual rainfall of 300 mm. It was not possible to find adjacent salt-affected and non-affected plots to grow the lines in both situations in the same protocol for comparison purposes. Therefore, the 2 treatments were set up in two separated field plots but in the same station. The first plot is affected by salinity largely induced by saline water. The electrical conductivity (EC) of the soil is high and varies from 3.2 at the surface to 5.4 mmhos/cm in deep layers. The ground irrigation water is salty with total soluble salts exceeding 4.3 g/l. The other plot irrigated by water from a dam is not affected by salinity. The soil structure and water retention capacity of the 2 fields are comparable.

The trial was set up as a winter crop in the second week of November 1997. The experiment was laid out as a randomised complete block design with 4 replications. Plots consisted of 3 3-metre long rows. The varieties Douyet and Rizky were inserted as checks. The plants were treated using fungicides to prevent attacks of *Ascochyta*, the main disease of winter chickpea. All other cultural techniques were optimised to ensure favourable growth conditions. Plants were observed for the colour of leaves and stems during the vegetative growth. Yield and yield components were measured on the central row of each plot at harvest during the first week of June 1998.

2.5. On-farm trials

In the 1999 season, 3 tolerant lines selected in the on-station experiment were evaluated on-farm in 2 regions (Merchouche and Meknès) in close collaboration with extension agencies and farmers to assess the potential production under farm conditions. The field soils were not saline. The varieties Douyet and Rizky were used as checks. The trials were conducted as a winter crop planted the first week of January 1999. The trial was designed as a randomised complete block design with 3 replications. In each replication entries were sown manually in 5 m × 10 m plots. Farmers cultured the crop according to their common practices.

Observations were made on the vegetation status and on the disease symptoms. Nodulation was scored at flowering time and measurements were taken on the yield and its components at harvest.

3. RESULTS

3.1. Screenhouse experiment

Means and ranges of nodule mass, total dry weight, acetylene reduction activity, and % nitrogen measured in this experiment are presented in Table I. In the absence of salt stress, variations were larger than in the salt treatment for all traits. Similarly the average values decreased as the salt concentration increased. Figure 1 illustrates the effect of salt as percent reduction over all lines of each trait under salt treatments compared to the control (NaCl-free treatment). Treatment of 25 mM induced 30, 44, 71, and 23% reduction for NM, TDW, ARA and protein content respectively. Larger effects were induced by 50 mM (67, 92, 82, and 34% respectively). The most significant effect was recorded for ARA under 25 mM and for TDW under 50 mM. N content was the least affected trait under both treatments.

The differences between the lines were highly significant for all the traits. Superior genotypes were easily separated from the rest. Hence, 14 lines had a good ARA and nodulation under 25 mM. However among these lines only the 7 top continued to fix nitrogen under 50 mM. Thus, these lines were chosen as tolerant (Tab. II). The average values of all the 7 genotypes in both salt treatments were below the values observed in the salt-free treatment for all traits indicating that all entries were affected by both stresses. However the effect was different according to genotype (Tab. III).

Total nitrogen assessed under nitrogen-free conditions is the best effective measure of SNF. Nodule mass, plant dry weight, and ARA were significantly associated with total nitrogen (Tab. IV). Because NM and plant weight are reliable traits, easy and cheap to measure, they can be used as SNF indices for screening large germplasm collections for SNF potential under nitrogen-free conditions.

3.2. On-station field experiment

All plants analysed showed nodules in both saline and non-saline fields, demonstrating the presence of efficient

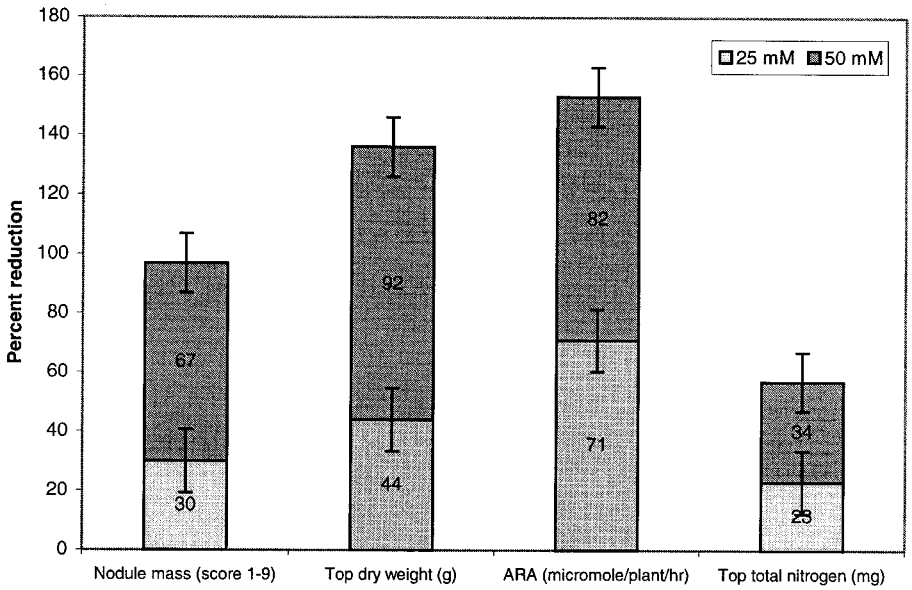

Figure 1. Effect of salt stress on BNF associated traits expressed as % reduction in the trait expression in salt treatments as compared to control (0 NaCl) for all lines tested (vertical lines represent standard errors).

Table I. Means and ranges of traits measured on all the chickpea lines tested in a screenhouse experiment under three NaCl treatments (0, 25 and 50 mM).

Trait	0 NaCl		25 mM		50 mM	
	Range	Mean	Range	Mean	Range	Mean
Nodule mass (score 1-9)	3–8	5.4	1–6	3.8	0–4	1.8
Top dry weight (g)	6.3–18.7	13.1	3–15.6	7.3	0–9.1	1.05
ARA (µmole/plant/h)	26.1–97.0	51.0	5–35.9	15.0	0–17.8	9.1
Top total nitrogen (mg)	16.3–24.2	19.8	12.0–19.1	15.3	10.0–16.0	13.1

Table II. Means and ranges of BNF traits measured on the 7 chickpea lines selected for the ability to fix nitrogen under salt stress conditions in the screenhouse.

No.	Lines	Nodule mass			Top dry weight (mg)			ARA			% N		
		Content NaCl in mM			Content NaCl in mM			Content NaCl in mM			Content NaCl in mM		
		0	25	50	0	25	50	0	25	50	0	25	50
1	MCA31	8	5	4	18.7	15.3	9.1	97.0	45.0	17.3	18.0	17.3	14.9
2	MCA45	7	6	3	16.5	14.2	6.8	80.0	35.0	13.4	17.0	16.7	14.5
3	MCA103	6	6	4	16.8	13.2	7.2	73.1	28.1	11.1	16.3	16.1	13.6
4	MCA131	8	5	3	17.8	15.3	5.5	68.1	16.3	6.3	16.4	15.3	14.3
5	MCA250	7	6	2	15.6	12.2	4.9	63.1	17.5	4.8	16.0	15.4	13.1
6	MCA301	7	5	3	18.5	15.6	8.7	75.2	52.1	12.3	16.9	16.5	13.0
7	MCA370	6	4	2	16.6	11.1	4.5	62.0	21.0	3.1	16.6	16.1	14.2
Mean		7.0	5.3	3.0	17.2	13.8	6.7	74.1	30.7	9.8	16.7	16.2	13.9

Table III. Comparison of the salt effect on BNF associated traits of 7 tolerant chickpea lines. The numbers indicate the percent reduction compared to the control (NaCl-free).

No.	Lines	Nodule mass		Top dry weight (mg)		ARA		% N	
		NaCl in mM		NaCl in mM		NaCl in mM		NaCl in mM	
		25	50	25	50	25	50	25	50
1	MCA31	38	50	18	51	54	82	4	17
2	MCA45	14	57	14	59	56	83	2	15
3	MCA103	0	33	21	57	62	85	1	17
4	MCA131	38	63	14	69	76	91	7	13
5	MCA250	14	71	22	69	72	92	4	18
6	MCA301	29	57	16	53	31	84	2	23
7	MCA370	33	67	33	73	66	95	3	14

Table IV. Correlation coefficients among traits measured in the screenhouse to assess BNF.

	Nodule mass	Plant dry weight	ARA
Total nitrogen	0.73	0.90	0.81
Nodule mass		0.75	0.66
Plant dry weight			0.57

nodulating rhizobia. Differences between lines were significant for nodule mass, yield components and total nitrogen. Mean values of the lines for these traits are presented in Table V. Generally the lines can be classified into three classes: tolerant (MCA103, MCA131, MCA250), moderately tolerant (MCA31, MCA45), and non-tolerant (MCA301, MCA370) (Tab. V).

The ranking pattern in the field evaluation was not firmly consistent with the ranking in the screenhouse. Hence the best nodulating genotypes in the screenhouse did not keep their superiority in the field for yield and yield components. In the saline soil all selected lines produced higher yield compared to the checks (Tab. V). However in non-saline soil only 3 lines maintained their superiority over the checks. This superiority was due to the differences in both seed weight and number of seeds per plant (Tab. V). Lines MCA103, MCA131, and MCA250 had the highest yield in saline soil and were tolerant.

The salt stress effect differed depending on plant genotypes. Hence, the yield reduction in saline soil compared to the performance in non-saline soil varied from 8.9 to 52.6% depending on lines. Nevertheless the genotypes ranked similarly in the two experimental fields for all traits. The checks were heavily affected with 59.9% average yield reduction. The largest effect of the salt was expressed by line 6 of which the yield was reduced by 52.6% particularly due to the reduction of number of

Table V. Means of the lines selected for BNF traits under salt stress in the screenhouse and tested in a non-salt affected (a) and a salt affected (b) field at Ben Slimane in 1996, and salt effect (c) as percent reduction.

a. Non-saline field

Line	Nodule mass (scale 1-5)	Number of seeds per plant	100-seeds weight (g)	Grain yield (g/m^2)
MCA31	7.2	40.5	54.2	130.1
MCA45	6.4	64.4	33.5	129.4
MCA103	8.5	144.8	24.2	210.2
MCA131	8.1	98.7	29.6	175.3
MCA250	7.4	79.7	35.2	168.3
MCA301	8.2	98.4	33.8	199.5
MCA370	6.6	87.4	29.9	156.8
Rizky	6.7	60.1	28.1	151.2
Douyet	7.4	65.3	29.5	149.3
Means	8.5	144.8	24.2	210.2
LSD (0.01)	1.2	15.2	9.5	13.5

b. Saline soil

Line	Nodule mass (scale 1-5)	Number of seeds per plant	100-seeds weight (g)	Grain yield (g/m^2)
MCA31	5.0	32.0	21.8	114.5
MCA45	5.4	57.0	24.7	113.2
MCA103	7.5	58.2	22.7	123.0
MCA131	7.8	72.1	21.6	128.0
MCA250	7.6	53.7	17.7	153.3
MCA301	3.2	47.0	24.5	94.6
MCA370	4.3	29.2	20.2	95.0
Rizky	3.5	29.1	15.1	65.2
Douyet	4.2	25.6	16.2	55.6
Means	4.5	43.7	15.6	70.8
LSD (0.01)	0.9	13.4	5.2	15.6

c. Salt effect (% reduction)

Line	Nodule mass (scale 1-5)	Number of seeds per plant	100-seeds weight (g)	Grain yield (g/m^2)	Reaction to salt
MCA31	30.6	21.0	59.8	12.0	Moderately tolerant
MCA45	15.6	11.5	26.3	12.5	Moderately tolerant
MCA103	11.8	59.8	6.2	41.5	Tolerant
MCA131	3.7	27.0	27.0	27.0	Tolerant
MCA250	–2.7	32.6	49.7	08.9	Tolerant
MCA301	61.0	52.2	27.5	52.6	Non-tolerant
MCA370	34.8	66.6	32.4	39.4	Non-tolerant
Rizky	47.8	51.6	46.3	56.9	Non-tolerant
Douyet	43.2	60.8	45.1	62.8	Non-tolerant

seeds per plant. Conversely the smallest effect was expressed by MCA250 (8.9%) that was the most stable genotype. MCA31 and MCA45 were also less sensitive to salinity concentration. Based on yield performance in saline soil and stability of performance, MCA250 is the best genotype. Line MCA103 that had the best yield in non-saline field ranked second after MCA250 in saline conditions. Over all genotypes number of seeds per plant and seed weight were the traits most affected by the salt (Tab. V).

3.3. On-farm evaluation

The means of the traits measured on the 3 lines at Merchouch and Meknès are given in Table V. Differences between lines were highly significant for nodule mass, grain yield and number of seeds per plant. The differences between lines were not different for the flowering date; they all flowered within one week starting 62 days after planting. Similarly the 3 tested lines did not differ from the 2 controls for this trait. Nodule mass was clearly higher at Merchouche than it was at Meknès for all entries. All analysed plants showed a good nodulation. According to their colour nodules were functional in both locations. Variations between lines were significant for nodule mass. Hence, line MCA250 produced the largest nodule mass in the 2 sites and is significantly different from the 2 other lines and the 2 controls (Tab. VI). At Merchouche MCA131 ranked second and is significantly different from the 2 controls and the 2 other lines. MCA103 is not significantly different from the 2 controls. However at Meknes, MCA131 and MCA103 form a uniform group with the 2 controls.

The combined analysis of variance showed significant differences of yield between the 2 locations. Generally the yields produced at Merchouche were higher than those registered at Meknès. Additionally the interactions of lines by locations were significant indicating that ranking of the lines on the yield basis was not similar in the 2 locations. The classification of lines based on the average yield of the 2 locations showed that MCA250 significantly over-yielded the 2 controls (Tab. VI). MCA103 and MCA131 were not different, but they produced significantly higher yield than the average yield of the controls. The 2 controls were not different. In the Merchouche trial, the ranking of entries revealed 3 groups. Line MCA250 is significantly isolated from the rest. MCA103 and MCA131 form one group. Finally the 2 controls form another different group. At Meknès, these groups overlap. MCA250 is not different from MCA131, which is not different from MCA103. This latter does not differ from the controls. The 2 controls do not differ significantly in the 2 locations although Rizky expressed a slight yield advantage in the 2 situations.

The entries were not significantly different for the seed size expressed by the weight of 100 seeds. However in both locations line MCA250 produced seeds of relatively larger size compared to the controls.

4. DISCUSSION

The screenhouse selection resulted in the identification of 7 lines tolerant to salt for nitrogen fixation traits. They showed a high nodulation score and high ARA under the 3 NaCl concentrations. Their ranking was consistent under both 25 mM and 50 mM treatments. Nevertheless the variability between lines decreased as the NaCl concentration increased. Therefore the discrimination of genotypes for selection purposes is better under mild salt stress (25 mM NaCl).

The on-station field test confirmed the screenhouse selection. In the saline soil all the 7 lines had higher values than the controls for all the traits measured.

Table VI. Means of traits measured on the 3 lines of chickpea evaluated on 2 farms in Merchouche and Meknès regions, 1998/99.

a. Merchouche

Line	Flowering (days from planting)	Nodule mass (scale 1-5)	100 seed weight (g)	Yield (qx/ha)
MCA250	65	8.8	38.1	32.8
MCA131	62	6.5	36.1	21.2
MCA103	65	4.3	29.4	24.7
Douyet	63	4.2	33.2	12.1
Rizky	66	4.6	34.1	17
Mean	64	5.7	34.2	21.4
LSD (0.05)	9.5	1.1	6.5	4.5

b. Meknès

Line	Flowering (days from planting)	Nodule mass (scale 1-5)	100 seed weight (g)	Yield (qx/ha)
MCA250	69	6.4	34.8	20.1
MCA131	67	4.3	33.3	18.7
MCA103	69	3.8	28.8	14.9
Douyet	66	4.1	31.0	11.2
Rizky	64	3.6	32.3	14.3
Mean	67	4.4	32.0	15.6
LSD (0.05)	8.7	0.9	5.1	4.9

Therefore selection in the screenhouse reflected the tolerance of the genotypes in the field. Hence, breeding can be reliably conducted in the greenhouse following the protocol developed that is suitable for screening large populations.

The data showed a significant line by salinity interaction for yield suggesting that it is difficult to associate salinity resistance to high production. Indeed yield was reduced by stress more for those genotypes with high values in favourable conditions. This result may be due to the metabolic cost imposed on the plant by stress resistance [4]. Among these 7 tested lines MCA103, MCA131, and MCA250 were selected as they produced significantly higher yield than the controls in both stressed and favourable conditions.

The on-farm evaluation showed that the 3 lines were more productive than the 2 controls that are the most recent released varieties in Morocco. Line MCA250, the most tolerant to the salt stress in on-station trials, maintained its production performance in the on-farm trials, in the 2 regions. It is appreciated by the farmers because of the seed size that is larger than that of the winter varieties registered in Morocco.

The implications of selection for SNF capacity under salt stress for developing new cultivars are two-fold. Chickpea can be extended as a winter crop to marginal salt-affected areas where few crops can be grown. Similarly improving SNF can be a successful approach to improving seed yield of the crops in traditional production areas recently affected by salt. Most N_2 fixation research is directed towards increasing legume productivity, although maximising the legume contribution to soil N in a system context has assumed greater importance with the prominence of sustainability issues. An active symbiosis under stress is necessary to contribute stabilising yields. Hence the selected lines are valuable genotypes that will be exploited in breeding research and developing new cultivars. Indeed these lines are locally adapted as they were derived from the local populations through a pedigree selection method for yield potential in winter cropping [3, 14]. The intensive evaluation of these lines was carried out on-farm in partnership with the extension and development services and the farmers in the 2 regions. This on-farm evaluation was used as support for an initial procedure of technology transfer.

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