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Development of Salt-Tolerant Alfalfa Clones by In Vitro Culture †

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Abstract: In this research, in vitro culture was applied to evaluate five alfalfa lines for salt tolerance and to select and multiply the most resistant clones. The pregerminated seeds were tested on a semisolid culture medium enriched with four different NaCl concentrations. Saline stress response was estimated by evaluating the survival capacity, growth, sensitivity indexes and electrolyte leakage. The most salt-tolerant plantlets for each line were multiplied to clone true-to-type plants. At the same time, the selected clones were evaluated again in vitro on middle salt stress conditions to confirm the salt tolerance. In conclusion, in vitro culture allowed the rapid selection of alfalfa genotypes to develop salt-tolerant clones.

Keywords: salinity test; in vitro selection; NaCl; multiplied clones

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1. Introduction

Alfalfa is an important forage crop that exhibits moderate sensitivity to salt levels in irrigation water and soil. Since alfalfa is polyploid and highly heterozygous due to cross-pollination, populations vary widely in their salt tolerance; furthermore, there is enormous variability within populations. Therefore, this genetic variation can be exploited through screening by allowing the selection of plants with superior performance when exposed to such stress.

In vitro tissue culture can be applied to large and diverse populations for screening vigorous salt-tolerant individuals, uncovering desired phenotypes in a short time, limited space and low cost while other factors (nutrients, lighting, temperature) are kept constant and optimally controlled [1,2].

The objective of this study was the evaluation of five alfalfa lines for salt tolerance and the selection and multiplication of the most resistant clones.

2. Materials and Methods

2.1. In Vitro Screening Selection for Salt Tolerance of Five Alfalfa Lines

The experimental trials were carried out on five alfalfa lines (A, B, C, D, E) of the Jouffray-Drillaud Company (now become Cérience)-RN 147-4 Av de la CEE-La cour d'Hénon CISSE, FR 86170, thanks to a research agreement with the Department of Agricultural and Environmental Science (DAES)-University of Bari Aldo Moro. The seeds were surface sterilised (70% ethanol for 1 min followed by NaOCl (active chlorine 14–15%) for 20 min) two times and then rinsed with deionised and sterile water. Then they were placed in sterile conditions on wet filter paper in Petri dishes and germinated for five days at 20 °C in a growth chamber. Germinated seedlings of each line were transferred on a growth

medium (basal medium- BM) [3] enriched with sucrose (20 g L^{-1}) and different concentrations of sodium chloride (0, 100, 150, 200 mM NaCl). The values corresponding to electrical conductivity (EC) were about 6, 15, 20 and 25 mS respectively, measured by Model 120 Microprocessor Conductivity Meter. Seedlings were grown on the selective medium for ten weeks in a growth chamber at 23 °C, with a photoperiod of 16 h and under a light intensity of 50 μE s^{-1} m^{-2}. Then on 20 samples per line and treatment survival capacity (%), shoot and root length (g), shoot number node, fresh and dry weight of shoot and root system were evaluated. The salt influence on the plant growth was expressed through sensitivity index calculated for height, node number and fresh weight of shoots and length, number and fresh weight of roots, according to the formula: IS = (Ps − Pt)/Pt × 100, in which Ps is the value of each parameter of the salt-stressed plants and Pt is the value of the same parameter in non-stressed plants [4]. Moreover, Electrolyte leakage was determined on the fresh leaves according to Lutts et al. [5], using the formula: EL = (Lt/L0) × 100, where Lt and L0 are the values of conductivity after and before the release of electrolytes in the solution, respectively, measured by conductimeter WTW (LF330 model-Weilheim, Germany).

2.2. In Vitro Establishment of Clones from Salt Tolerant Genotype

The salt-tolerant selected genotypes were micropropagated using BM enriched with sucrose (20 g L^{-1}) and 6- Benzilaminopurine (BAP) 0.5 mg L^{-1} to induce shoot proliferation and the same medium hormone free for the rooting. Each micropropagated clone, obtained from the corresponding selected genotype, identified as being salt stress tolerant in in vitro screening, was transferred in Jiffy pots of 8 cm^2 containing a commercial peat mixture soil (organic carbon 46%, organic nitrogen 1–2%, organic matter 80%) and mixed with perlite at a 2:1 (*v*/*v*) ratio. Acclimatisation took place in a climatic greenhouse at 18–25 °C with mist, reducing the humidity level from 85–90% to 50–60% over 20 days in natural daylight. After four weeks, the plants were trimmed and managed in natural climatic conditions before shipping to the Jouffray-Drillaud Company.

2.3. Evaluation of Salt Tolerance of Selected and Multiplied Clones

To evaluate the salt tolerance of selected and multiplied clones, plant samples were grown for 30 days on BM added with 0.75 mM NaCl or on the salt-free control medium, according to the method developed by Dziadczyk et al. [6]. The increase of shoot fresh weight, expressed as relative growth rate [RGR: (ln Fwfinal − ln FWinitial) × 100/total days of growth (where total days of growth are 30 and FW is the fresh weight of the plants)], and proline content were measured in both experimental conditions. Proline content was determined following the protocol described by Bates et al. [7].

3. Results and Discussion

3.1. Evaluation of the Response of Five Alfalfa Varieties to Salt Stress

Salt presence in the growth media interfered with both physiological and biochemical processes even if a different establishment of the lines to the in vitro condition was shown. Indeed, regarding the survival capacity of the in vitro seedlings measured after 10 weeks of growth on the salt media, LC and LD seemed the lines more sensitive about survival, although they were also the same ones that showed a reduced survival on the control medium (Figure 1). Instead, LB showed the best performance on the salt media. Considering the total values, LB showed the highest survival (72.7%), while the others raised values between 57.8 % (LD) and 47.37 % (LC).

The morphological parameters evaluated showed an obvious reduction of the growth of the plantlets, depending from the salt stress. It is known that slower growth is an adaptive feature for plant survival under stress. Because the behaviors are also linked to the genotypes that had different responses in term of enhancement, for standardising the data, IS was used to discuss the effect of salt stress on seedlings of five alfalfa lines. In Table 1 the indexes of salt sensitivity (IS) estimated, based on the total fresh biomass, are reported.

These data showed LA and LD the most sensitive to salt stress at all the concentrations of NaCl, while LB and LC were the less sensitive.

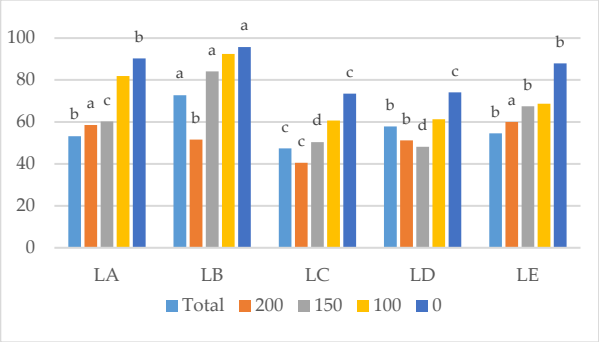

Figure 1. Survival of alfalfa five lines measured after 10 weeks on culture medium enriched with 0, 100, 150 and 200 mM NaCl. The histograms represent the average of 20 replicates. The different letters show the statistical differences among the varieties for each salt treatment. (Test SNK-$p \leq 0.01$).

Table 1. Indexes of salt sensitivity (IS) estimated based on the total fresh biomass of five alfalfa lines grown for 10 weeks on a basal medium enriched with different salt concentrations. Data represent the average of 20 replicates.

NaCl (mM)	Alfalfa Lines				
	LA	**LB**	**LC**	**LD**	**LE**
200	−64.66 a	−55.77 b	−54.48 b	−69.09 a	−65.33 a
150	−52.12 b	−36.54 c	−33.29 c	−61.82 a	−38.49 c
100	−50.86 a	−32.69 b	−29.77 b	−49.09 a	−31.93 b

The different letters show the statistical differences among the varieties for each salt treatment. (Test SNK-$p \leq 0.01$).

To better understand how the salt stress caused the growth reduction and the tissues and organs more involved, IS of shoot height, node number and fresh weight and IS of root length, number and fresh weight were compared among the lines. To simplify the comparison, IS values were divided into three classes: 0 to −33% (low stress); >−33% to −66% (moderate stress); >−66% to −100% (high stress) and represented respectively with *; **; *** (Table 2). One of the strongest effect of salt stress on the growth of all five lines was on the elongation of shoots and roots. At 200 and 150 mM NaCl concentrations, only LC responded with moderate stress to the shoot height, while the other lines resulted highly stressed. A comparison between the IS weight of shoots and roots showed a higher sensitivity to salt stress of the roots than the shoots that showed IS representing moderate or low classes of stress. Therefore, the most sensitive organ appeared to be the root. Moreover, a simple consideration on the frequencies of the high class stress, for all the sensitive indexes considered and independently from the concentration of NaCl, confirmed LA and LD the most sensitive, followed by LE, LB and LC.

The effect of salt stress on membrane permeability, a physiological measurement often used to differentiate the genotypes that can grow at different salinity levels, was estimated according to the electrolyte leakage (EL). Figure 2 showed that the salt stress caused a high increase of EL in all the tested lines. At severe stress, LB was the least affected (83.7%) and LC and LD were more affected by this treatment (about 92%). The results of electrolyte leakage confirmed the high sensitivity to salt stress of LA and LD lines, and indicated as most tolerant LB, the line that already showed the best performance for the survival on the salt media and for the low IS.

Table 2. Effect of salt stress, expressed as classes of IS values, on the different morphological parameters of five alfalfa lines grown for 10 weeks on a basal medium enriched with different salt concentrations.

		LA			LB			LC			LD			LE		
		200	150	100	200	150	100	200	150	100	200	150	100	200	150	100
Shoot	Height	***	***	***	***	***	**	**	**	*	***	***	**	***	***	**
	Nodes	**	**	*	**	**	*	**	**	*	***	***	***	**	**	*
	Fresh weight	*	*	*	**	*	*	**	*	*	**	**	*	**	*	*
Root	Length	***	***	***	***	**	*	**	**	*	***	***	***	***	**	**
	Number	***	***	**	*	*	*	**	**	*	**	*	**	**	**	*
	Fresh weight	***	***	***	***	**	**	**	**	**	***	***	***	***	***	*

*: 0 to −33% (low stress); **: >−33% to −66% (moderate stress); ***: >−66% to −100% (high stress).

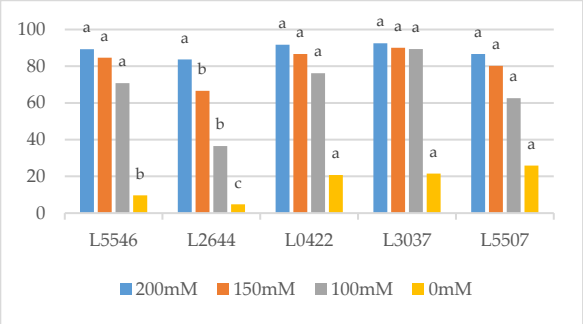

Figure 2. Electrolyte leakage of five alfalfa lines measured after 10 weeks on culture medium enriched with 0, 100, 150 and 200 mM. The histograms represent the average of 20 replicates. The different letters show the statistical differences among the varieties for each salt treatment. (Test SNK-$p \leq 0.01$).

3.2. Micropropagation of the Salt Tolerant Plants

All the lines showed a good response to the micropropagation technique, obtaining 4/5 shoots for each subculture starting from each selected shoot. The clones raised a high percentage of rooting. The rooted clones were transferred to acclimatisation in the climatic greenhouse and then hardened in natural climate conditions before sending to Jouffray-Drillaud Company.

3.3. Salt Tolerance of Selected Clones

The results of RGR showed an excellent capacity of the selected salt tolerant clones to grow both on mild salt stress (75 mM NaCl) and on control (0 mM NaCl) (data not reported). Taking into consideration that the rapid accumulation of proline in vegetal tissues as response to salt stress has been associated with the ability of proline to act as an osmolyte to adjust the plant under drought/saline condition, the greater proline accumulation in the leaves of all alfalfa selected clones grown on mild salt stress in comparison of the control proline content (Table 3), confirmed the effectiveness of this protocol.

Table 3. Proline content of salt tolerant selected clones of five alfalfa lines measured after 30 days of growth on culture medium enriched with 0 and 75 mM NaCl. Data represent the average of 20 replicates.

NaCl (mM)	Proline Content (μmol g^{-1} $_{FW}$)				
	LA	LB	LC	LD	LE
0	32.1 b	27.3 b	40.1 b	32.2 b	33.6 b
75	50.0 a	52.4 a	49.7 a	50.0 a	50.5 a

The different letters show the statistical differences among the salt treatments for each line. (Test SNK-$p \leq 0.01$).

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