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Effect of salt stress on pollen tube growth in two *Medicago truncatula* ecotypes

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Abstract: Annual Medicago species contribute significantly to improved fodder production in Algeria. Leguminous plant model is *Medicago truncatula* Gaertner. Because of its high protein content, this plant is essential for natural soil fertilization as well as good quality nutrition for animals and humans. However, abiotic stresses such as salinity are the leading cause of land degradation and crop productivity limitations worldwide, and they have an impact on legume physiology and metabolism. We investigated the pollen tube growth behavior in two contrasting *Medicago truncatula* ecotypes under salt stress (Tru 42, tolerant, and Tru 242, sensitive) with different NaCl concentrations (68, 102, and 137 mM) because pollen tube growth mechanisms can be affected by environmental stresses such as water and salt stress. According to the data, the Tru242 ecotype has a pollen tube elongation of 1.41 to 8.45 um, whereas the high pollen tube elongation of the Tru 42 ecotype is between 2.8 and 18.83 um. It's crucial to comprehend the physiological reactions of male gametophytes in order to reproduce, as salinity stress can hinder their ability to do so. It is intriguing to include the tolerant genotype in a selection program for leguminous breeding, as the analysis revealed that the tolerant ecotype has better pollen tube elongation than the sensitive one. Actually, not enough knowledge existed about Medicago truncatula's or other Medicago species' pollen tolerance mechanism to salt stress.

Keys word: *Medicago truncatula*; male gametophyte**;** salinity; legume

1. Introduction

Annual species of '*Medicago*' genus play an important role in improving fodder production in Algeria. They are often used in "cereal-alfalfa" rotation systems, regenerate by self-sowing, and help maintain soil fertility because of its atmospheric nitrogen-fixing capacity. Salinity is one of the environmental stresses that reduce the crops productivity and quality in the world (1) and is one of the factors limiting the distribution of these plants in arid and semi-arid areas. In addition, an increase in precipitation raises the saline shallow groundwater (2). The high salt content of soil water considerably reduces the water potential of this solution and imposes water stress conditions on plants (3). Double fertilization in flowering plants requires the targeted release of sperm from the pollen tube. After depositing on compatible stigmatic cells, the pollen germinates and the tube develops deep inside the pistil, penetrating various tissues to arrive precisely at the micropylar end of the ovule and release or deliver the sperm (4). This mechanism can be affected by various abiotic stresses such as water stress and salinity and it is Important to understand the male gametophyte physiological responses to accomplish reproduction. In this case, it is interesting to study the behaviour of pollen tube development in the model leguminous of *Medicago truncatula* in order to analyze the effect of salt stress on pollen tube growth in the tolerant ecotype compared to the sensitive one because male gametophyte selection has an impact on sporophystic stage in order to obtain an offspring of seeds tolerant to salt stress. This technique can improve legume productivity in arid land.

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2. Methods

The plant material used for the morphometric study for the gametophytic phase consists of two ecotypes of the annual model species *Medicago truncatula* Gaertner (Table 1; Figure 1), provided by the National Agronomic Institute of El-Harrach (I.N.A) in Algiers-Algeria. This legume is an annual self-pollinating species characterized by a diploid complement 2n = 16. For the analysis of the male gametophyte of each ecotype, tolerant (Tru 42) and sensitive (Tru 242), pollen grains are collected from flowers at a well-defined stage, corresponding to a well-opened corolla (5). In vitro pollen germination assay, mature pollen grains were collected from male flowers at anthesis.

2.1. Pollen condition of germination

Pollen germination is carried out on four microscope slides, containing four media differing in their NaCl concentration, and then incubated in an oven at 25 ± 2°C for half an hour (30 min). The observations are made under an optical microscope, objective x10. The culture medium used is composed of 100 ppm boric acid, 100 ppm potassium carbonate, 300 ppm calcium nitrate and 10% sucrose in distilled water at pH= 6.8 (6). The duration of pollen grains germination is 30 min under different NaCl concentrations (0; 68; 102 and 137 mM), after that, the length of the pollen tube was measured. Observations are carried out using a low magnification objective (10X) allowing the entire image to be observed. The chosen NaCl concentrations are based on a study of pollen > germination rates in different Medicago truncatula ecotypes under > salt stress compared to the sporophytic stage under the same NaCl concentrations, because of the existence of structural overlap between haploid and diploid stages (11). The experimental device used is a block device, staggered over time, completely random, with three repetitions (n=3). In each replicate, each ecotype is represented by five plants.

2.2. Data analysis

A two-factor analysis of variance test (ecotype and treatment) was performed on data obtained for pollen tube length. The relative data for each treatment, as well as those of the control, were analyzed independently by a one-way analysis of variance test (ecotype). All statistical tests were performed with Statistica software.

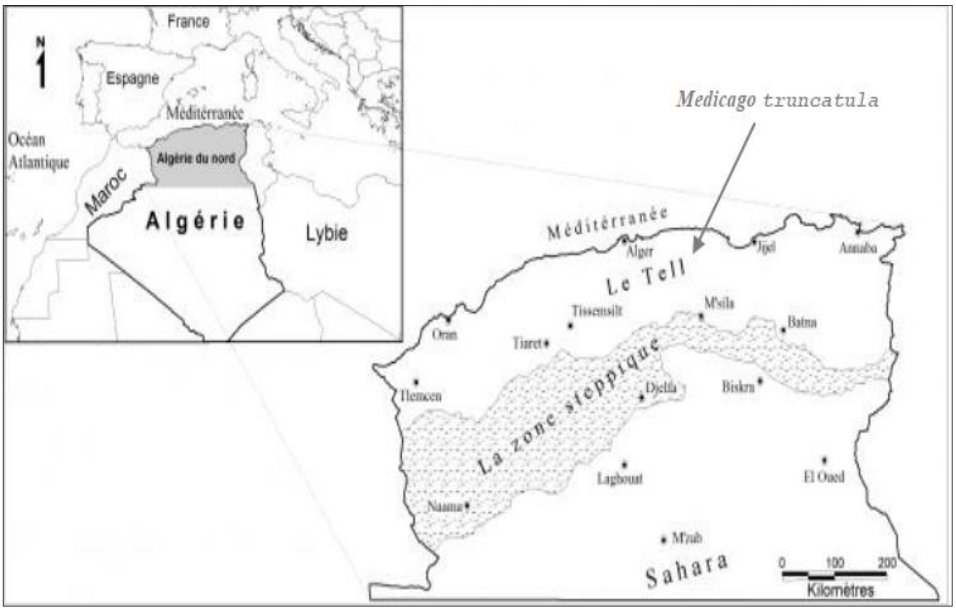

Figure 1. Location of *Medicago truncatula* Gaertner in Algeria

Table 1. Characteristics of the two genotypes of *Medicago truncatula* used for salt stress tolerance.

Species	Ecotypes	Origin	Altitude (m)	Pluviometry (mm)	Degree of Tolerance
Medicago truncatula Gaertn.	Tru 42	Fetzara (Annaba) 36°47'50.2"N 7°30'44.5"E	100	660	Tolerant
	Tru 242	Kaïs (Khenchela) 35°29'30.1"N 6°55'42.2"E	980	450	Sensitive

3. Results and Discussion

The pollen tubes lengths without treatment are between 55.18 and 65.22 um (Figure 2). The application of salt stress reduced the growth of the pollen tube in both ecotypes compared to the control, as the salt concentration increased. This reduction varies according to the ecotype and the treatment (Figure 3). In this case, the Tru 42 ecotype has a high pollen tube length under different treatments and considered the tolerant genotype, which is between 2.8 and 18.83 um, while the Tru242 genotype is less tolerant regardless of the treatment applied and pollen tube length is between 1.41 and 8.45 um.

After variance test analysis, ecotype effect was found to be significant at a 95% confidence level. The analysis of variance on pollen tube elongation shows a significant ecotype effect ($P < 0.01$) for the three treatments applied (68, 102 and 137 mM) of NaCl. It is noted that the treatment with 68 mM of NaCl presents the highest statistical value of F, which suggests this saline concentration discriminates best between the two ecotypes of *Medicago truncatula* studied (Table 2).

Table 2. Results of analysis of variance tests for ecotype effect by treatment for pollen tube elongation after 30 minutes pollen germination in *Medicago truncatula* ecotypes.

Source of variation	ddl	F	P
Ecotypes (Ec)	1	28,6	0,000**
Treatment (T)	3	515,6	0,000**
Interaction (Ec xT)	3	3,7	0,011*

Level of significance: * = $p < 0.05$, ** = $p < 0.01$, ns: no significant.

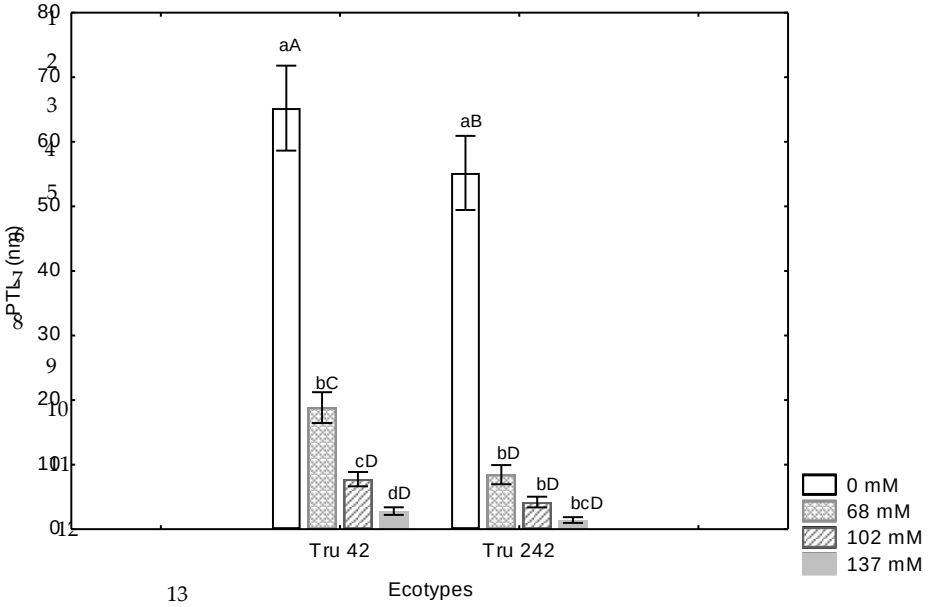

Figure 2. Pollen tube length (PTL) of two Medicago truncatula ecotypes under different NaCl concentration. Bars represent average ± s.e. (n = 3). Same letters, lower case among treatments and upper case between genotypes do not significantly differ ($p < 0.05$).

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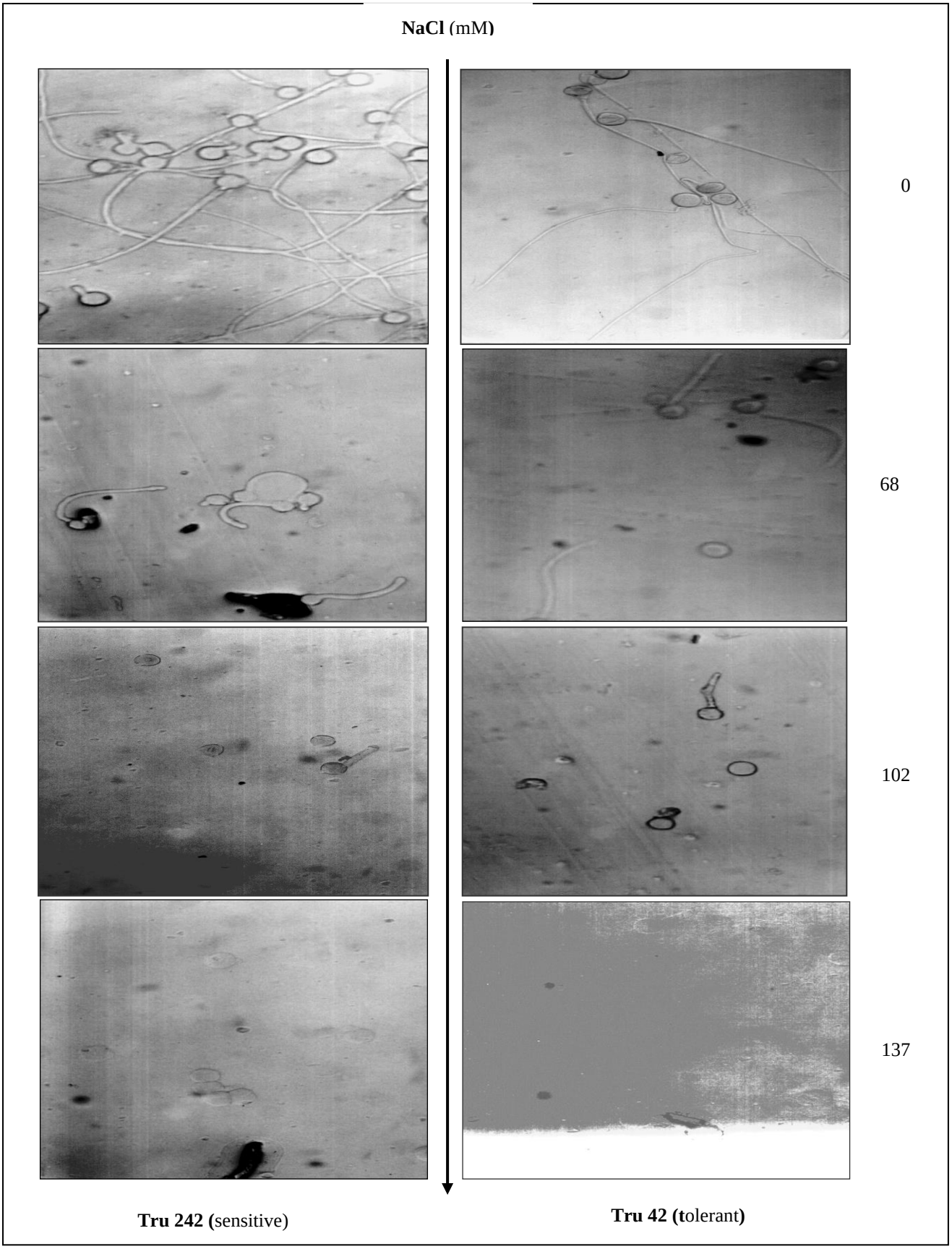

Figure 3. Some Photographs of *Medicago truncatula* pollen exposed to increasing levels of salt stress.

The study of the response to salt stress for the post-germination growth of pollen grains (male gametophyte) at different concentrations of salinity induce leads a weak elongation of the pollen tube. The Tru 42 ecotype has a high pollen tube elongation compared to the Tru 242 ecotype under salt stress. The analysis of genetic variability showed a significant variation between the two contrasting ecotypes at the 68 mM NaCl concentration and this concentration can be used to selection a tolerant pollen for in vitro fecundation in *Medicago truncatula* and also in haplodiploidisation technique for obtaining new tolerant plants to salt stress . According to (7), if there is water stress the growth of the pollen tube inside the silk in maize is slowed down and the pollen tube cannot reach the ovule and fertilization was not possible. This shows the importance of pollen tube elongation in the mechanism of fertilization. According to (8), NaCl reduced pollen viability in rice and Na+ and Cl− ions are responsible for poor pollen viability, which also appear during in vitro pollen germination. An accumulation of toxic ions within pollen grains was observed also in tomato (9**)**. It was also reported that pollen viability decreased at flowering (10). A study investigating the effect of salt stress on pollen grain germination ability in six ecotypes of annual *Medicago* species (*Medicago truncatula, Medicago ciliaris, and Medicago polymorpha*) showed that ecotype Tru 42 had the highest germination ability and concluded that male gametophytic selection could be an alternative for legumes breeding which is more efficient and more economical compared to selection during the diploid phase (11). In the carrot, pollen viability was also affected by salt stress (12). The salinity can inhibit photosynthesis at vegetative stage, and induce floret sterility in relationship with decrease pollen viability (13). Pollen salinity stress generates osmotic stress that its resistance was used as a parameter for screening tolerant ecotypes (14). However, changes in pollen tube osmotic pressure showed that this male gametophyte can adapt to environmental stress (15; 16). It was showed that salinity decrease pollen viability in tomato during the flowering stage (17) and Seed production, a key determinant for the commercial success, essentially depends on pollen viability (18)

Actually, no information was available on pollen tolerance mechanism to salt stress in *Medicago truncatula* or other *Medicago* species.

4. Conclusion

The gametophytic selection study of pollen tube elongation in *Medicago truncatula* permitted to select the vigorous pollen from the most salinity-tolerant ecotype "Tru 42" and this pollen can be used to fertilize sensitive ecotypes, giving an hybrid offspring of tolerant plants introduced in arid and saline lands, for enhancing legumes productivity and natural soil fertilization by nitrogen fixation. This technique is important in legumes breeding programs, which is efficient and rapid technique that allows interesting agronomic characters selection. The male gametophytic selection is an economical method and can be applied in other legumes species. Also, is useful in haplodiploidization technique applications.

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