

Full Length Research Paper

Early seedling development of *Medicago truncatula* genotypes under salt stress in relationship with seed dry weight and storage protein content

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In order to have more information on early seedling development under salinity stress (one of the important factors limiting plant productivity and also crop growth), it will be interesting to examine radicle growth in comparison to plumule elongation under different salinity stress conditions. In this study, we assessed the variability of four genotypes of the plant model *Medicago truncatula* under four levels of salt stress (distilled water as control 0, 68, 102 and 137 mM) of sodium chloride (NaCl) solution. Several studies are focalised in root or radical development because is the most sensitive part of the plant and controls rapid transmission information to other plant parts. In order to evaluate the degree of radicle sensitivity, we measured the plumule length to radicle length ratio (PL: RL ratio). Also, we investigated the relationship between storage protein content, seed weight and salinity tolerance degree. The results show that Tru 131 and Tru 673 genotypes, with a low ratio (radicle more resistant (longer) than plumule) and high storage protein content, are more tolerant to salinity stress than the sensitive genotypes Tru 26 and *Jemalong* that had a high ratio (radicle more sensitive (shorter) than plumule) and low storage protein content. We concluded that the most tolerant genotype (Tru 131) with rich protein and high seed weight can be cultivated in saline and semi-arid areas in Algeria and Mediterranean regions to improve the legumes productivity. Although, the genotype (Tru 673) that had a low seed vigour, appear tolerant (high PL:RL ratio) despite coming from 'aged seeds'. This additional data shows the influence of 'aged seeds' on seed vigour and root growth because seeds contain all of the genetic resources of the plant.

Key words: *Medicago truncatula*, salt stress, seed weight, storage protein content, seed vigour, aged seeds.

INTRODUCTION

Salinity is an important abiotic stress which significantly affects seedling growth and seed quality. Legumes are very important plants both ecologically and agriculturally because they are able to interact symbiotically with rhizobia for biological nitrogen fixation (Spaink, 2000; Perret et al., 2000) which avoids the use of chemical

fertilizers, affecting the rhizosphere and polluting the atmosphere. Among the *Medicago* annual species, *Medicago truncatula* is widely used as a model legume plant for understanding tolerance to abiotic stress (Young and Udvardi, 2009). This kind of legume is of great interest for sustainable agriculture and ecology. Early

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Abbreviations: RL, Radicle length; PL, plumule length; SL, seedling length; FGP, final germination percentage; VS, seeds vigour; SPC, storage protein content; SDW, seed dry weight; SDS, sodium dodecyl sulphate; LEAs, late embryogenesis abundant; HSPs, heat shock proteins; ABA, abscisic acid.

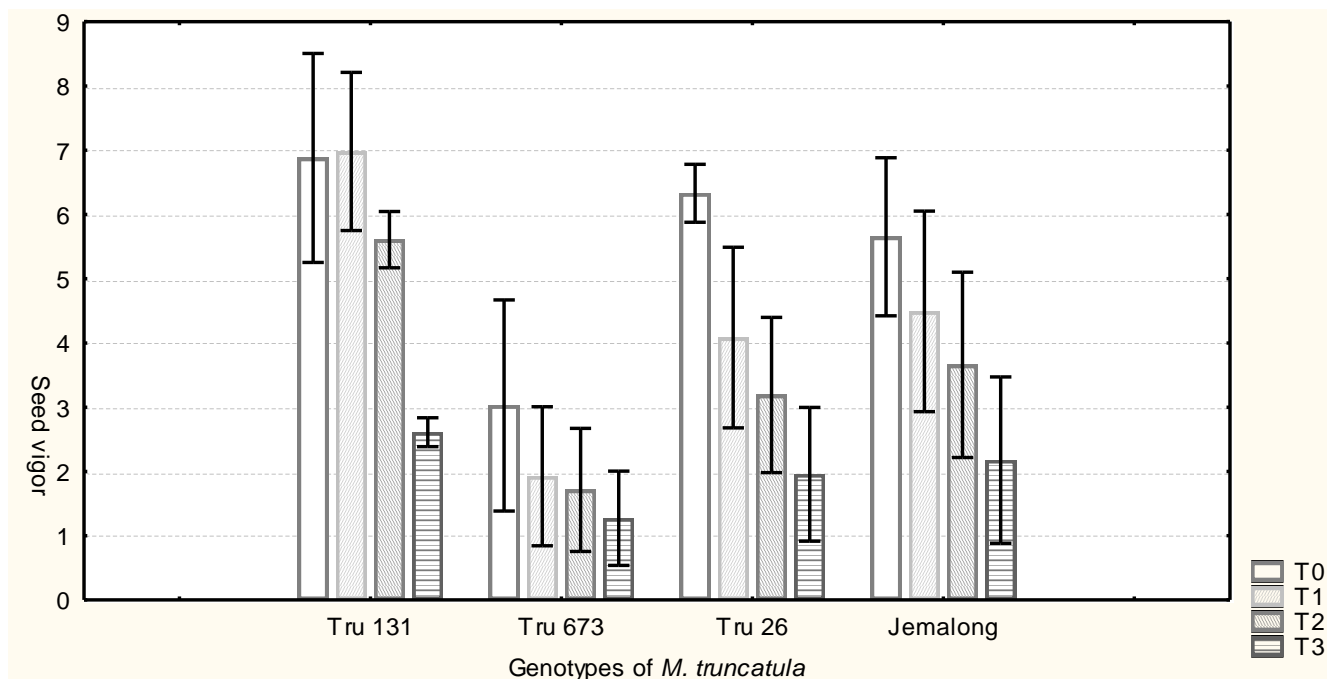


Figure 1. Seed vigor of *M. truncatula* genotypes under different salt stress conditions. Results are means \pm S.E (n = 4 replicates). Aged seeds: Tru 673.

seedling growth of many crop plants is the most sensitive to environmental stresses (Penmetsa and Cook, 1997). Root and shoot length provide important indications of a plant's response to salt stress (Jamil and Rha, 2004). In certain plants, the radicle may be longer than the plumule and consequently this ratio may be lower compared to other plants (Snapp and Shenman, 1992). The understanding of the relationship between seedling development, environmental conditions and seed quality at the physiological and agronomical level are basic aims of seed science (Bláha and Pazderů, 2013). Proteins that accumulate in plants under saline conditions may provide a storage form of nitrogen that is re-utilized later (Singh et al., 1987) and may play a role in osmotic adjustment; they may be synthesized *de novo* in response to salt stress or may be present constitutively at low concentration (Pareek et al., 1997), and it is very interesting to find an association between storage protein content and salt stress tolerance. The aim of this study was to assess the response of seedling growth and especially radicle (root) growth to salt stress between four genotypes of *M. truncatula* in relationship with seed weight and storage protein content in order to find a correlation between the accumulation of storage protein and growth parameters of salt stress tolerance. The determination of the most tolerant genotype with high protein content is a great interest for sustainable agriculture and ecology in order to improve the productivity of this legume very important economically and environmental (nitrogen fixation). A second approach

for this study was to use one genotype of *M. truncatula* of aged seeds in order to evaluate the influence of seed quality on different growth parameters in a relation with salinity tolerance.

MATERIALS AND METHODS

Plant material, growth conditions and salt stress treatment

Recently, harvested seeds (2010) of *M. truncatula* genotypes provided by different institutes were studied. *Jemalong*, is a reference genotype from the Australian collection, Tru 131 and Tru 26 genotypes from IDGC BelAbes, ENSA El Harrach, Algeria, respectively, and the genotype Tru 673, aged seeds (Old collection since 24/06/1977) from ICARDA Aleppo, Syria. These four genotypes were used in four levels of salinity treatment (distilled water as control 0, 68, 102 and 137 mM) of sodium chloride (NaCl) solution (Amouri and Fyad-Lamèche, 2012). The Tru 673 genotype, characterized by a low seed vigour (Figure 1), was used to study the impact of the aged seed on the response of radicle and plumule growth to salinity. Seeds were manually scarified and sterilized for 10 min in sodium hypo-chloride (6%) and then rinsed 3 times with distilled water for 2 min. Ten seeds were sown in Petri dishes and germinated on filter papers imbibed in distilled water or in sodium chloride solutions. The seeds of *M. truncatula* genotypes were germinated in Petri-dishes (50 mm) on two layers of filter paper in an incubator maintained in the dark at $25\pm 2^{\circ}\text{C}$. The filter papers were changed after 48 h in order to avoid salt accumulation (Rahman et al., 1996). To evaluate the effect of different levels of NaCl concentrations on different genotypes, the experimental design was a randomized plot with four replications and an emerged radicle was the criterion for germination. During ten days of germination, radicle length (RL), plumule length (PL) and

Table 1. Two-way ANOVA of the effect of Salt stress on different parameters of seedling development in *M. truncatula* genotypes.

Source of variation	RL		PL		PL/RL		SL		FGP		SV		
	df	F	P	F	P	F	P	F	P	F	P		
Genotypes (G)	3	78.44	0.00**	70.59	0.000**	9.280	0.000*	74.31	0.000**	3.118	0.035*	5.469	0.003*
Treatments (T)	3	43.70	0.00**	65.55	0.000**	1.897	0.428	62.84	0.000**	0.112	0.999	5.564	0.002*
Interaction (G x T)	9	3.45	0.00**	4.33	0.000**	0.712	0.695	3.40	0.000**	1.161	0.334	0.372	0.943

RL, Radicle length; PL, plumule length; SL, seedling length; FGP, final germination percentage; SV, seeds vigour. Significance levels; *P < 0.05, ** P < 0.001; ns, non significant; F: coefficient of Snedecor-Fisher.

Table 2. One-way ANOVA of the effect of salt stress on different parameters of seedling development in *M. truncatula* genotypes.

Source of variation	RL (cm)		PL		PL/RL		SL (cm)		FGP		SV		
	NaCl (mM)	df	F	P	F	P	F	P	F	P	F	P	
0	3	9.855	0.000**	26.95	0.000**	2.60	0.100	13.50	.000**	3.476	0.051	1.488	0.268
68	3	39.55	0.00**	23.13	0.000**	5.36	0.014*	32.24	.000**	0.881	0.478	2.184	0.143
102	3	32.60	0.000**	17.57	0.000**	1.56	0.200	26.83	.000**	0.382	0.768	0.337	0.799
137	3	10.47	0.000**	9.734	0.000**	1.83	0.100	10.18	.000**	1.033	0.413	2.008	0.167

RL, Radicle length; PL, plumule length; SL, seedling length; FGP, final germination percentage; SV, seeds vigour. Significance levels; *P < 0.05, **P < 0.001; ns, non significant; F: coefficient of Snedecor-Fisher.

seedling length (SL) were measured. Final germination percentage (FGP), plumule length to radicle length ratio (PL / RL) and seeds vigour (SV) were calculated as: PL: RL ratio = Plumule length (cm) / radicle length (cm) and seeds vigour (SV) = FGP(%) * SL (cm). In this study, we considered tolerant genotypes that have a low ratio, that is, their radicles (roots) are more resistant than their plumules.

Determination of seed protein content (SPC)

Protein was determined by the method described by Bradford (1976) using bovine serum albumin as standard. Four individual seed samples for each genotype, were homogenized with extraction buffer (50 mM Tris-HCl (pH: 6.8), 2% sodium dodecyl sulphate (SDS), 2 5% beta-mercaptoethanol, 10% glycerol). Samples were centrifuged at 14000 rpm for 15 min and supernatants were isolated and used for protein assay. The intensity of blue colour developed was recorded at 595 nm and protein concentration was measured using bovine serum albumin as standard. Seed protein content (SPC: mg. g⁻¹) was calculated in relation to individual seed dry weight (SDW: mg seed⁻¹) for each genotype.

Statistical analysis

All statistical tests were carried out using the Statistical Analysis System Statistica 6.1 version. Statistical analysis was performed using two-way ANOVA (for P < 0.01 and P < 0.05). Based on the ANOVA results, a Duncan's multiple range tests for means comparison was performed, for a 95% confidence level, to test for significant differences among treatments (Duncan, 1955). In the tables, different letters after the values within the same column for each trait express significant differences.

Investigated traits (PL to RL ratio and SPC) were subjected,

respectively to hierarchical cluster analysis using the procedure of Wards minimum variance method as a clustering algorithm. Wards minimum method is a hierarchical clustering procedure in which similarity is used to join clusters and is calculated as the sum of squares between the two clusters summed over all variables and minimizes them within cluster sums of squares across all partitions (Hair et al., 1998). Clustering of genotypes gives information on their similarity and dissimilarity in responses to salt stress which facilitate the choice of genotypes to be involved in future breeding programs.

RESULTS AND DISCUSSION

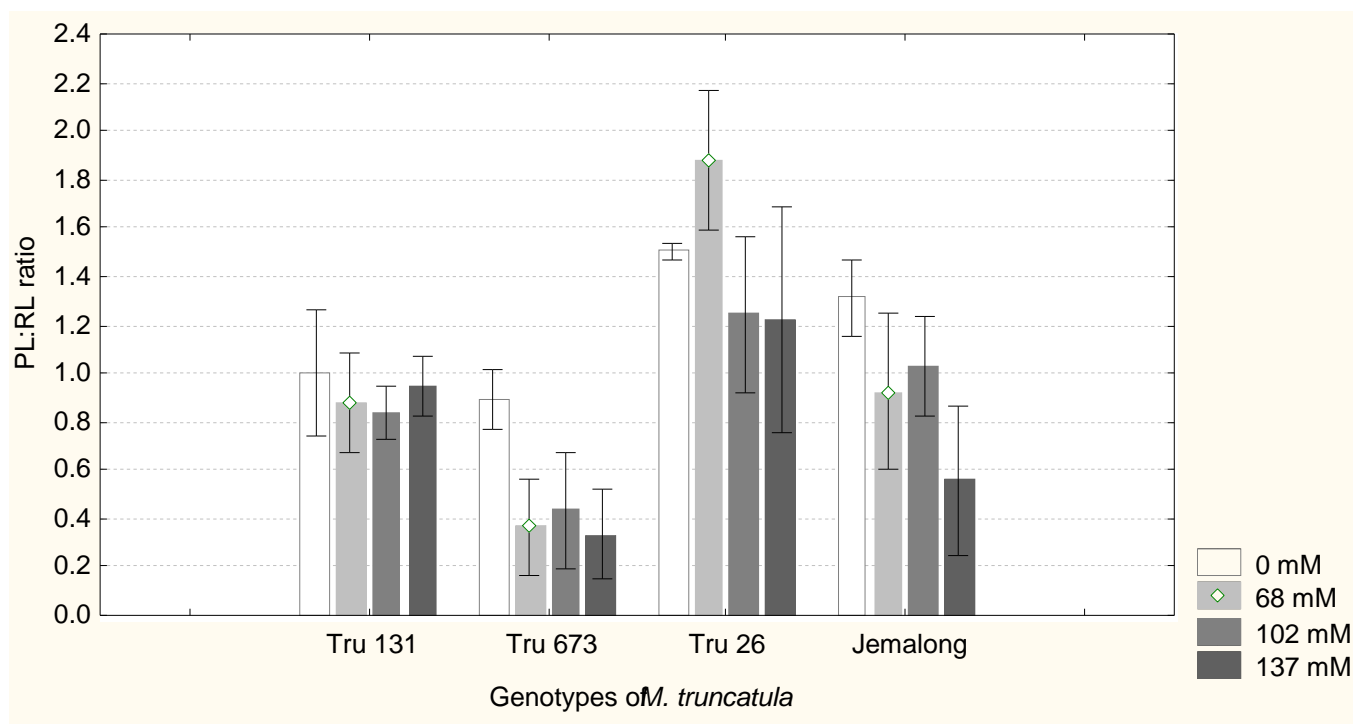
Influence of salt stress on different parameters of seedling development in *M. truncatula* genotypes

Early seedling growth is a critical stage for the establishment of plant populations under saline conditions (Bosque-perez et al., 1998). The research presented here indicated differences in the seeds vigour, radicle and plumule lengths of the four genotypes studied. The data of two-way and one way ANOVA shown in Tables 1 and 2, showed a highly significant difference between genotypes in interaction with salinity treatment for all the growth parameters studied that decrease with augmentation of NaCl concentration (Table 3). Compared to the control, radicle length and plumule length decreased with same speed (2.9 to 1.17 cm and 3.1 to 1.15 cm), respectively. PL: RL ratio decreased (1.17 to 0.76) along the salinity gradient and vigour seeds also (5.47 to 2).

Table 3. Classification of means simple effect of different salt stress levels on different parameters of seedling development in *M. truncatula* genotypes.

NaCl (mM)	RL (cm)	PL (cm)	PL/RL	SL (cm)	FGP	SV
0	2.908 a	3.104 a	1.179a	6.012 a	0.882 a	5.471a
68	2.289 a	2.372 a	1.012b	4.661 b	0.743 a	4.369 ab
102	1.923 b	1.964 b	0.885b	3.888 c	0.746 a	3.542 bc
137	1.175 c	1.154 c	0.765c	2.327 d	0.647 a	2.00 c

RL, Radicle length; PL, plumule length; SL, seedling length; FGP, final germination percentage; SV, seeds vigour. Means with similar letter(s) in each trait is not significantly different at 5% probability level according to Duncan's Multiple Range Test.

**Figure 2.** Mean values of plumule: radicle ratio of *M. truncatula* genotypes under different salt stress condition. Results are means \pm S.E (n = 4 replicates).

In general, all development traits measured decreased as salinity increased. High salinity may inhibit root and shoot elongation by slowing down the water uptake by the plant (Werner and Finkelstein, 1995). The capacity of plants to tolerate salt stress varies with the stage of development in their life cycle (Khan et al., 2002) and seedling emergence is critical for the establishment of plant populations (Khan and Gulzar, 2003). The majority of the research indicates that most annual crops are tolerant at germination stage but sensitive during emergence and early vegetative development (Maas and Grattan, 1999). In this study, we used the parameter (PL: RL ratio) in order to have more information on reduction of radicle length compared to plumule length (Figure 2).

The roots are the most sensitive part of the plant and have the role as control centre with rapid transmission information to other plant parts (Bláha and Pazderů, 2013). So, it is interesting to examine radicle growth compared to plumule elongation under different salinity stress conditions. The results showed a low ratio in the genotypes Tru 131 and Tru 673 for the majority of treatments (Figure 2). This data confirm that the radicle part was more resisted to salinity than the plumule in the tolerant genotypes. However, a high PL: RL ratio was observed in the sensitive genotypes Tru 26 and *Jemalong* (Figure 2), that is, the radicle part was more sensitive than the plumule in the sensitive genotypes.

Zahaf et al. (2012) analyzed the root transcriptome of

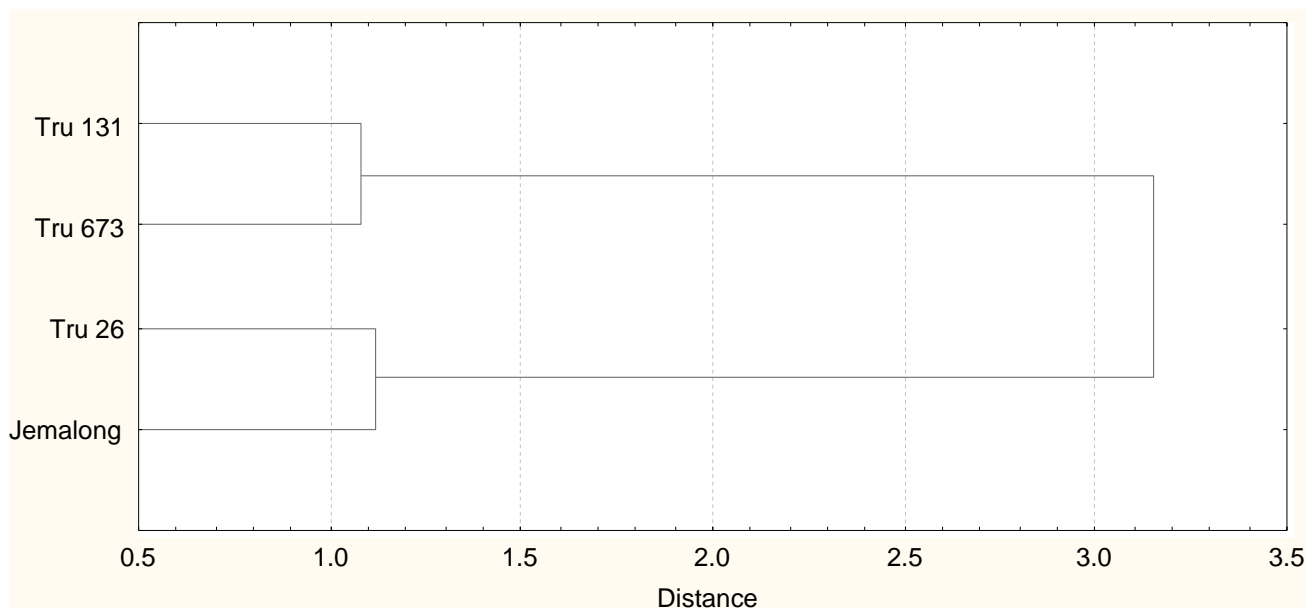


Figure 3. Cluster analysis of *M. truncatula* genotypes under different levels of salinity stress for the plumule to radicle ratio (PL / RL) using Ward's minimum variance method with Statistica 6.1 version software.

Table 4. Distances matrix between *M. truncatula* genotypes under different levels of salinity stress for the plumule to radicle ratio (PL / RL).

Genotype	Tru 131	Tru 673*	Tru 26	Jemalong
Tru 131	0			
Tru 673*	1.08	0		
Tru 26	2.07	1.38	0	
Jemalong	3.00	2.05	1.12	0

*Aged seeds

two *M. truncatula* genotypes having contrasting responses to salt stress: TN1.11 (tolerant), sampled in a salty Tunisian soil, and the reference *Jemalong* A17 genotype (sensitive). So, the tolerant genotypes showed increased root growth under salt stress as well as a differential accumulation of sodium ions when compared to *Jemalong* A17. The same authors found that the root lengths of these two contrasting genotypes were significantly reduced after 90-150 mM NaCl treatments in comparison to non-treated plants. In our studies, we observed a significant reduction of radicle length after 68-102 mM NaCl concentrations compared to control plants.

The results of cluster analysis (Ward's minimum variance method) of different genotypes of *M. truncatula* for the parameter (PL: RL ratio) under different salt stress levels, arranged genotypes into two groups (Figure 3). The first cluster includes the two tolerant genotypes (Tru 131 and Tru 673) with low mean values for PL: RL ratio and a minimum distance ($d = 1, 08$) (Table 4). This result

confirms that Tru 673 (aged seeds) is associated with the tolerant genotype Tru 131. Bláha and Pazderů (2013) reported that during a long term of seed conservation rapidly lose germination energy (low vigour) and aged seeds can influence optimal seedling growth. The most important changes during a long term of seed conservation, affected by environmental conditions is damage of storage proteins.

On the other hand, high quality seeds are undamaged seeds that have a high level of germination, which will produce uniform, vigorous seedlings (Dickson, 1980). The second group of clustering includes the sensitive genotypes (Tru 26 and *Jemalong*) that have a high mean values of the PL: RL ratio with a minimum distance ($d = 1,12$). The same result in the sensitive genotype *Jemalong* A17 under salt stress was found by (Zahaf et al., 2012).

Seed dry weight and storage protein content analysis

The data of seed dry weight (SDW) represented in Figure 4 shows that the tolerant genotype (Tru 131) had a highest SDW ($5.67 \text{ mg seed}^{-1}$) and the sensitive one (Tru 26) had a lowly SDW ($2.27 \text{ mg seed}^{-1}$). Dornbos and Mullen (1991) found that severe water stress reduced the average seed weight by 15% at 27°C, and by 22% at 33°C. Also, Erskine and Ashkar (1993), explained that the reduction in mean seed weight of lentil with increasing water limitation, could be resulted from the stimulation of seed maturity under stress. Seed size is considered a significant factor only during the early

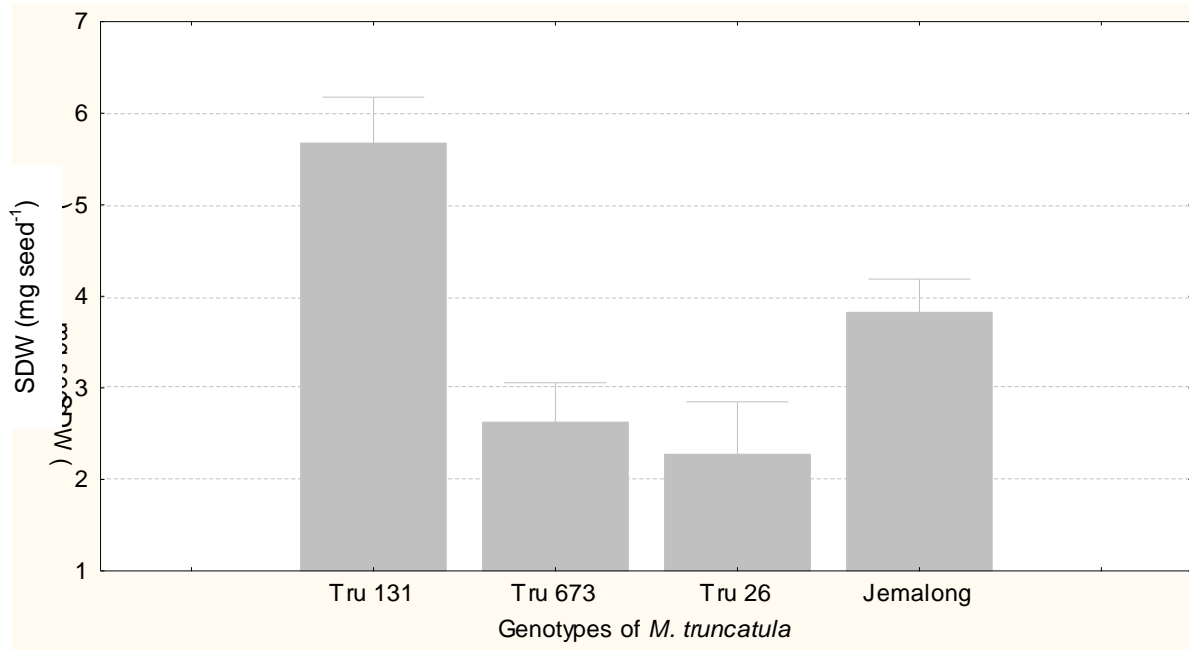


Figure 4. Seed dry weight (SDW) of different genotypes of *M. truncatula*. Results are means \pm S.E (n = 4 replicates).

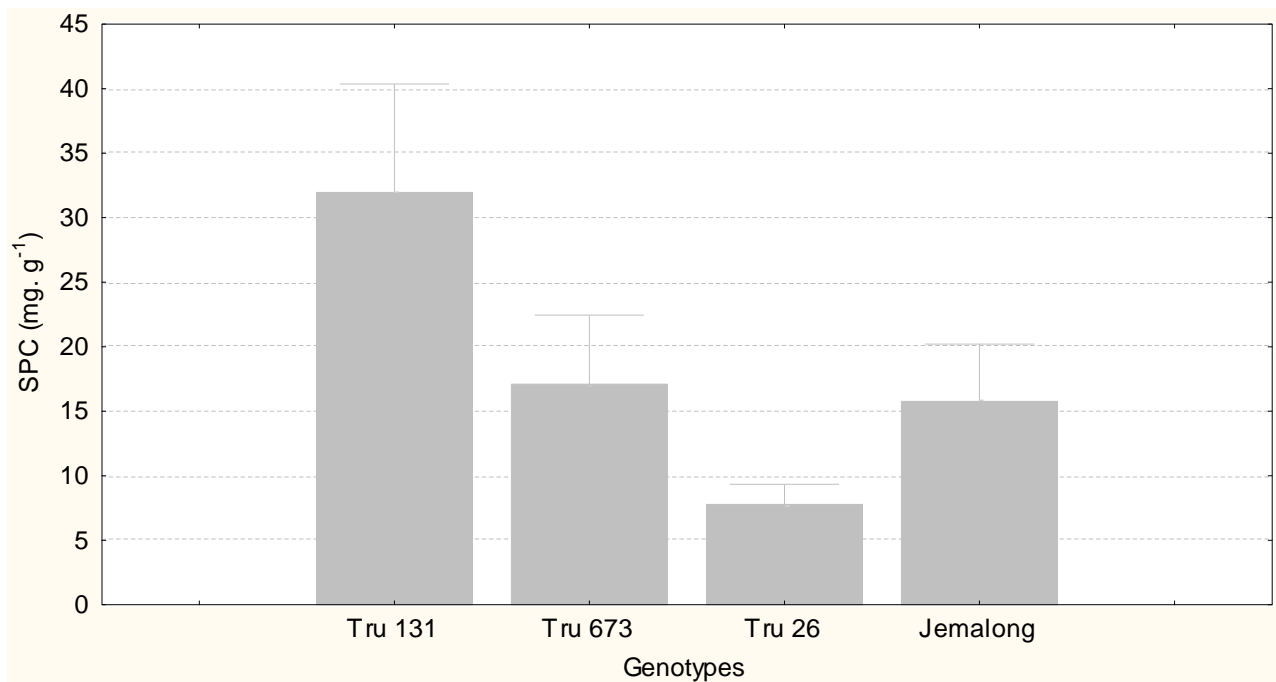


Figure 5. Storage protein content (SPC) in different genotypes of *M. truncatula*. Results are means \pm S.E (n = 4 replicates).

stages of growth (Ghassemi-Golezani, 1992) and it is interesting to use high seeds weight like seeds of the tolerant genotype (Tru 131) in lay farming. On SPC, the tolerant genotype (Tru 131) had a higher storage protein content (SPC = 0.31 mg.g⁻¹) than the sensitive ones (*Jemalong* and Tru 26) (SPC = 0.15 mg.g⁻¹ and 0.077

mg.g⁻¹), respectively (Figure 5). Djemel et al. (2005) affirmed that *M. truncatula* seeds accumulate large quantities of protein at maturity until 32-42% of dry weight. This suggest that the most tolerant genotype (Tru 131) with a high (SPC) had a genetic potential (genes) to resist salinity stress. Proteins that accumulate in plants

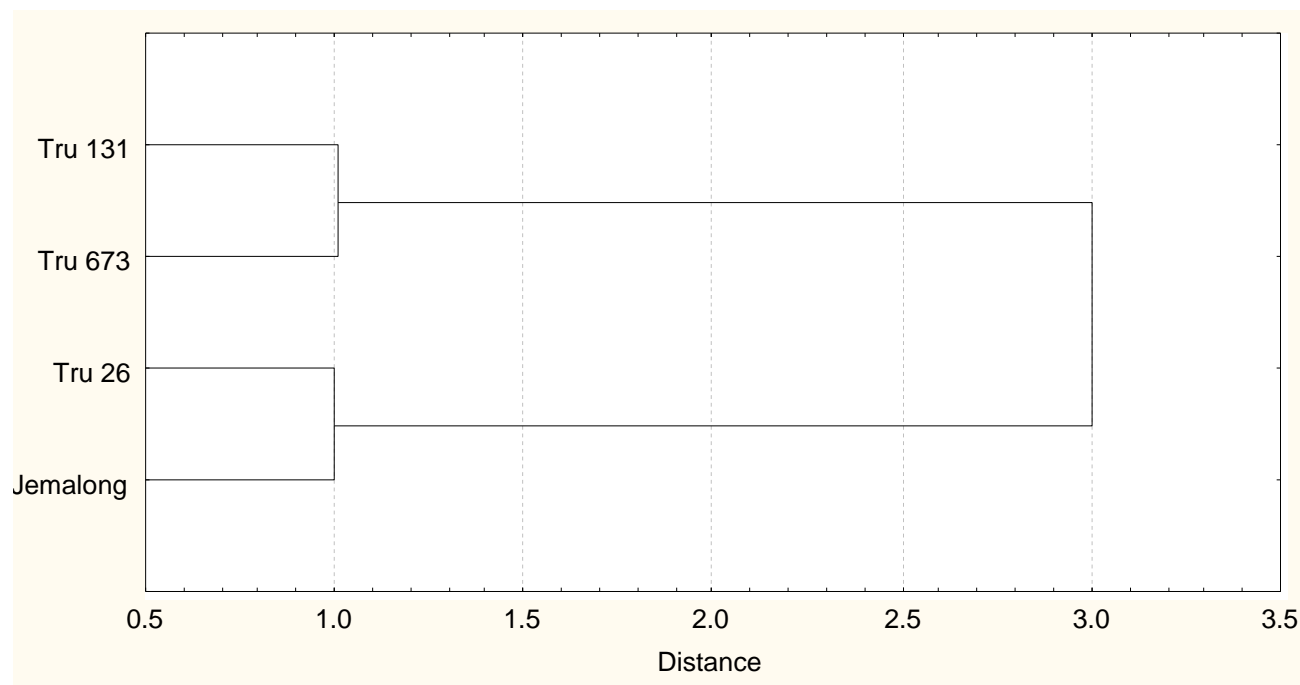


Figure 6. Cluster analysis of *M. truncatula* genotypes for storage protein content (SPC) using Ward's minimum variance method with Statistica 6.1 version software.

Table 5. Distances matrix between *M. truncatula* genotypes for storage protein content (SPC).

Genotype	Tru 131	Tru 673*	Tru 26	Jemalong
Tru 131	0			
Tru 673*	1.01	0		
Tru 26	2.01	1.00	0	
Jemalong	3.00	2.00	1.00	0

under saline conditions may provide a storage form of nitrogen that is re-utilized later (Singh et al., 1987) and may play a role in osmotic adjustment. Their protective function consists in stabilization of membrane structures (Tolleter et al., 2010). Priming-induced stress tolerance correlates with the occurrence of late embryogenesis abundant (LEAs) proteins and heat shock proteins (HSPs) that accumulate during seed late maturation under abscisic acid (ABA) control (Gallardo et al., 2001). These proteins which are involved in drying tolerance and seed longevity in orthodox seeds are also induced by salinity and can act as osmoprotectants and antioxidants (Kalemba and Pukacka, 2007). The result of cluster analysis of SPC arranged genotypes of *M. truncatula* into two groups (Figure 6). This result was the same obtained for the clustering of PL: RL ratio (Figure 3). The first cluster include the two tolerant genotypes (Tru 131 and Tru 673) that a minimum distance is ($d = 1.01$). The second group includes the sensitive ones (Tru 26 and

Jemalong) that a minimum distance is ($d = 1.00$) (Table 5). These data, suggest that the genotypes with high storage protein content were more tolerant than the sensitive ones.

Relationship between different parameters of seedling development under salt stress with seed dry weight and storage protein

Positive and highly significant relationship existed between SDW and RL with a correlation coefficient ($r = 0.95^*$) (Table 6). Figure 7 illustrates this association, for example the tolerant genotype (Tru 131) that had a highest SDW had a highest (RL) and the best growth of radicle or root. Toon et al. (1990) showed that bigger seed size indicated better quality and genetic potential for germination but quality of the seeds may be related to variation in seed nutriment content (Abideen et al., 1993) and time of seed collection (Bellari and Tani, 1993). In post anthesis, water deficiency, a positive correlation was found between grain weight and harvest index in Wheat. It means that increasing grain weight is accompanied with increasing harvest index (Koocheki et al., 2006). The roots are influenced by seeds quality on the start of plant growth. Generally, it is known that the biological quality of seed is also one of the basic factors, which influences growth and development of the roots at the beginning of the filial generation and during vegetation period (Bláha and Pazderů, 2013). Also, we noted a positive correlation

Table 6. Correlation coefficients (r) for the relationships between different parameters of seedling development under various level of salt concentration, seed dry weight and storage protein content in *M.truncatula*.

Trait	SV	PL/RL	RL	PL	FGP	L	SPC	SDW
SV	1							
PL/RL	0.76	1						
RL	0.92	0.10	1					
PL	0.92	0.72	0.70	1				
FGP	0.97*	0.63	0.84	0.94	1			
SL	1.00*	0.42	0.93	0.91	0.96*	1		
SPC	0.55	-0.45	0.84	0.20	0.41	0.59	1	
SDW	0.79	-0.17	0.95*	0.53	0.64	0.82	0.92	1

RL, Radicle length; PL, plumule length; SL, seedling length; FGP, final germination percentage; SV, seeds vigour. Means with similar letter(s) in each trait is not significantly different at 5% probability level according to Duncan's Multiple Range Test. SPC, Seed protein content; SDW, seed dry weight. Significant correlation levels; *P < 0.05.

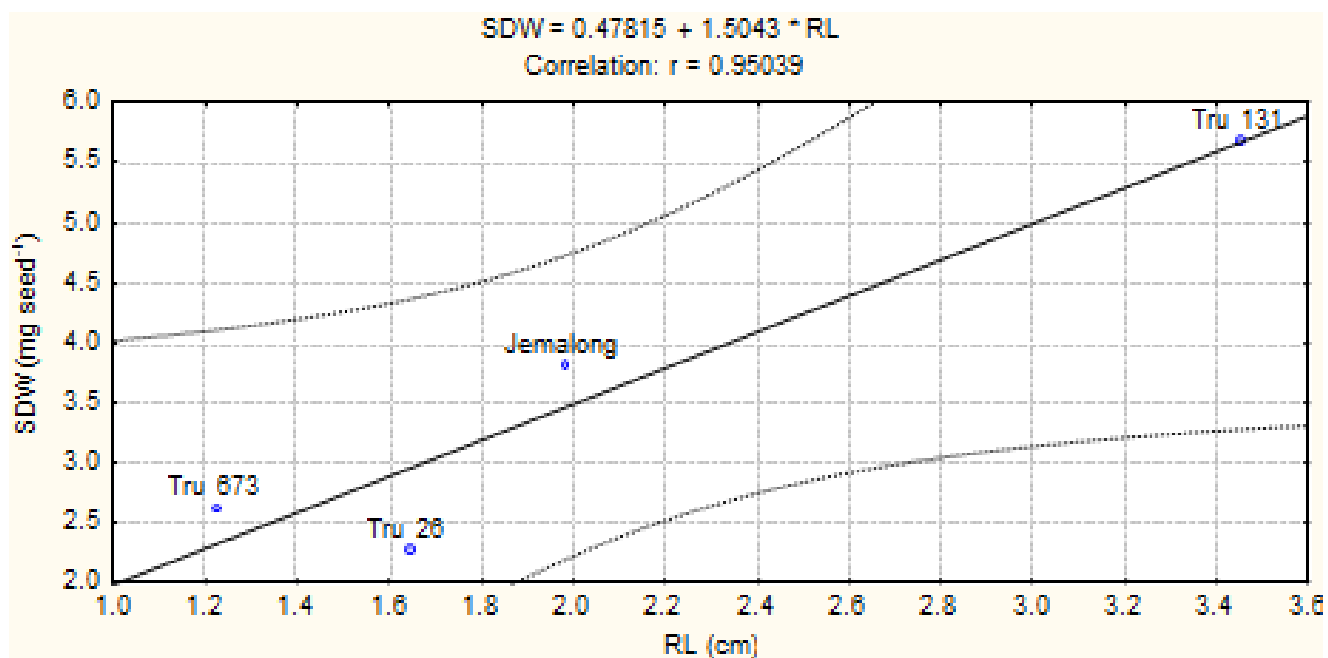


Figure 7. Relationship between root length (RL) under various level of salt concentration and seed dry weight (SDW) in *M.truncatula* genotypes.

coefficient between SPC and SDW ($r = 0.92$) (Table 6). Figure 8 illustrates this relationship and the most tolerant genotype (Tru 131) had a higher values of (SPC and SDW) than the most sensitive one (Tru 26). Another interesting data, concerning the association between SPC and RL with a correlation coefficient ($r = 0.84$) is shown in Table 6. Figure 9 shows this association for the most tolerant genotype (Tru 131) that had higher radicle length and higher storage protein content than the

sensitive one (*Jemalong*) under different levels of salinity. Ingram and Bartels (1996) affirmed that in higher plants, osmotic stress induces several proteins in vegetative tissues, which are related to LEA proteins and HSPs. The correlation between these proteins accumulation and salt stress tolerance indicated its protector role under dehydration stress and seed quality increased during the late stages of seed development after seeds have attained maximum dry weight (Sanhewe et al.,

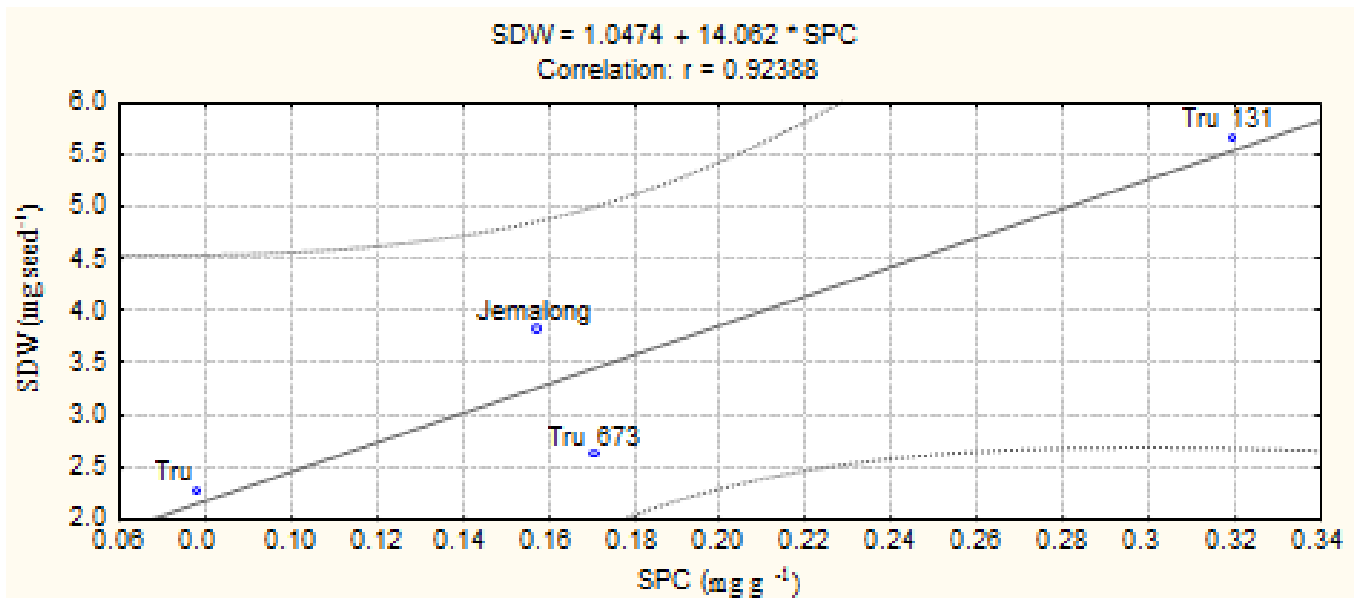


Figure 8. Relationship between storage protein content (SPC) and seed dry weight (SDW) in *M.truncatula* genotypes.

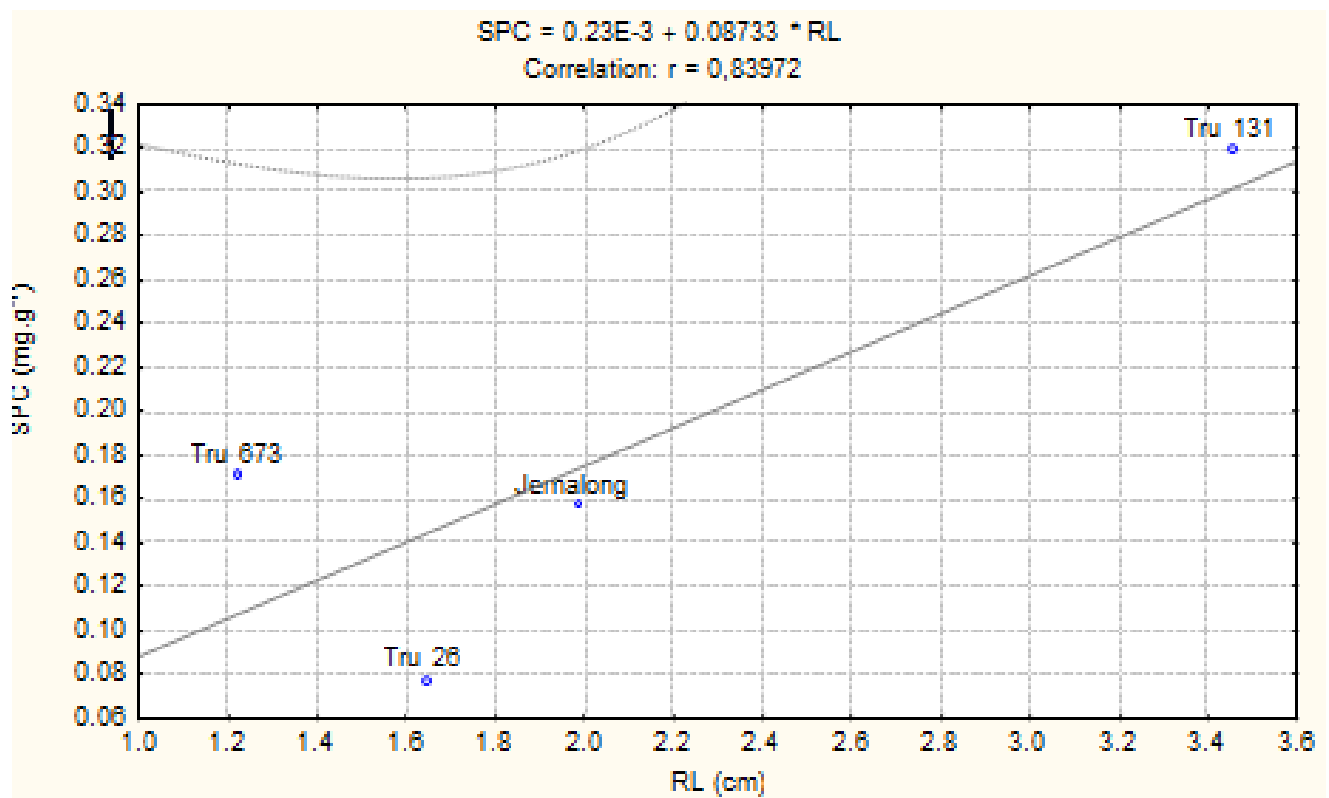


Figure 9. Relationship between storage protein content (SPC) and radical length (RL) in *M.truncatula* genotypes.

1996).

In conclusion, the investigation of the effect of salt stress on seedling development of four genotypes of *M.*

truncatula, showed a significant variation between different genotypes. A lower (PL: RL ratio) was observed in the tolerant genotypes like (Tru 131), this was explained

by resistance of radicle to salinity than plumule. Also, this study showed the influence of 'aged seed' of the genotype (Tru 673) on radicle and plumule growth, separately, but not on the PL: RL ratio and the ability to tolerate salt stress, this can be explained by the presence of genes involved to salinity stress tolerance. The same clustering was obtained for SPC, this permitted to conclude that the genotypes with a higher PL: RL ratio and higher storage protein content (proteins involved in salt stress tolerance) were more tolerant (Tru 131 and Tru 673) than the sensitive ones (Tru 26 and *Jemalong*) and further investigations are required in order to assess the accumulation of this kind of protein in relation to salinity tolerance for breeding of *M. truncatula*.

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