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Genetic control of tolerance to salinity in Medicago truncatula

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Contrôle génétique de la tolérance à la salinité chez *Medicago trancatula*

Résumé :

Parmi les contraintes abiotiques la salinité est considérée comme un problème majeur, qui affecte le fonctionnement des plantes, en particulier leur croissance et leur rendement. Afin d'étudier le contrôle génétique de la tolérance à la salinité chez *Medicago truncatula*, plante modèle de la famille des légumineuses, deux expérimentations ont été réalisées. La première expérimentation visait à étudier l'effet de la contrainte saline sur différents paramètres morpho-physiologiques pour un panel de génotypes de *M. truncatula* afin de déterminer les traits de phénotypage pour la tolérance à la salinité. Les génotypes A17, TN1.11, DZA315.16, A20, TN1.12 et F83005.5 ont été sélectionnés parmi des lignées originaires de différents pays méditerranéens, qui ont été déjà séquencées (http://www1.montpellierinra.fr/BRC-MTR/mauguio/mauguio.php). Les génotypes ont été étudiés sous 6 traitements salins (0, 30, 60, 90,120 et 150 mM NaCl) dans un essai factoriel sous forme de blocs complets aléatoires en trois répétitions. L'analyse de la variance montre des différences significatives entre les niveaux de salinité et une interaction entre les génotypes et les traitements salins concernant la plupart des caractères étudiés. Le génotype « DZA315.16 » présente les valeurs les plus importantes concernant les effets principaux pour les caractères morphologiques alors que « TN1.11 » présente les valeurs les plus faibles. La projection verticale de la surface foliaire de la plante (Leaf Area=LA), significativement corrélée à la biomasse des plantes, apparaît comme un trait d'intérêt pour le phénotypage de la tolérance à la salinité. La concentration saline la mieux adaptée pour démontrer les différences parmi les lignes étudiées se situe entre 90 et 120 mM NaCl. Le génotype « TN1.11 » contrairement à « DZA315.16 » et à « Jemalong-A17 » présente un maintien de la surface foliaire de la plante en réponse à la salinité. Pour la deuxième expérimentation, une population de cent lignées recombinantes (Recombinant Inbred Lines=RILs) produite par le croisement entre « TN1.11 » et « Jemalong-A17 » a été retenue pour l'analyse du contrôle génétique de la tolérance à la salinité. Les RILs ont été développés par la méthode de descendant mono graines (Single Seed descent= SSD) jusqu' à la génération F6 à l'INP-ENSAT, France. Le plan d'experimentation est « Spli plots » , sous forme de blocs randomisés avec trois répétitions et deux conditions : traitement salin (100 mM NaCl) et témoin (eau). L'expérience a été menée pour déterminer la variabilité génétique et pour identifier les QTLs contrôlant les caractères morphologiques et physiologiques chez la population des lignées recombinantes (RILs). L'analyse de la variance a montré une large variation génétique et une ségrégation transgressive pour les caractères étudiés. La différence entre la moyenne des RILs et la moyenne de leurs parents n'est pas significative concernant tous les caractères étudiés dans les deux conditions, ce qui montre que les RILs utilisées dans notre expérimentation sont représentatives de toutes les lignées recombinantes possibles du croisement « TN1.11 x Jemalong-A17 ». 21 QTLs ont été détectés dans la condition témoin et 19 QTLs ont été identifiés sous contrainte saline (100 mM NaCl). Le pourcentage de la variance phénotypique expliqué par les QTLs varie entre 4.60% et 23.01%. Certains de ces QTLs sont spécifiques à la condition saline, ce qui démontre l'existence du contrôle génétique de la tolérance à la salinité chez *M. truncatula* ; tandis que les autres ne sont pas spécifiques et contrôlent un même caractère dans les deux conditions. Des QTLs superposés concernant différents caractères ont été aussi observés. Les résultats fournissent des informations importantes en vue de futures analyses fonctionnelles de la tolérance à la salinité chez *M.truncatula* et pour d'autres espèces voisines.

Abstract:

Among abiotic stresses salinity is considered as a serious problem affecting plant functions especially growth and yield. In order to study the genetic control of salt stress in the model legume *Medicago truncatula*, two experiments were performed. The first experiment was conducted to study the effect of salt stress on some morpho-physiological parameters in *M. truncatula* genotypes and to determine the eventual use of some traits as tolerance criteria. Genotypes including A17, TN1.11, DZA315.16, A20, TN1.12 and F83005.5 are selected through a sequenced lines collection (http://www1.montpellierinra.fr/BRC-MTR/mauguio/mauguio.php) which are originated from different Mediterranean countries. Genotypes were studied under 6 salinity treatments (0, 30, 60, 90,120 and 150 mM NaCl) in a factorial experiment based on randomized complete blocks with three replications. Analysis of variance show significant differences among genotypes, salinity levels and interaction between genotypes and salt treatments for most of studied traits. ~~"D~~ZA315.16" genotype presents the highest main effect values for morphological traits whereas"TN1.11" has low values. Vertically projected leaf area (LA); show the highest variability through all studied salt concentrations. The best concentration to find differences between parental lines is 90 to 120 mM Nacl. A segregating population of recombinant inbred lines (100 RILs) of *M.truncatula* derived from a cross between TN1.11 and Jemalong-A17 was used for the second experiment. RILs were developed by single-seed descent until F6 generation at the INP-ENSAT, France. The experiment was undertaken to determine the genetic variability and to identify QTLs controlling several traits related to plant growth and physiology, in the population of recombinant inbred lines (RILs). Analyses of variance showed a large genetic variation and transgressive segregation for the traits studied. The difference between the mean of RILs and the mean of their parents was not significant for all of the traits in both conditions, showing that the RILs used in our experiment are representative of the possible recombinant lines from the cross TN1.11 x A17. A total of 21 QTLs were detected under control and 19 QTLs were identified under 100mM salt stress conditions. The percentage of total phenotypic variance explained by the QTLs ranged from 4.60% to 23.01%. Some of the QTLs were specific for one condition, demonstrating that the genetic control of a traits differed under control and salt stress conditions. Some others are non-specific and control a trait in both conditions. Overlapping QTLs for different traits were also observed. The results provide important information for further functional analysis of salt tolerance in *M. truncatula*

Abbreviations:

RILs: recombinant inbred lines

SFW: Shoot fresh weight

SDW: shoot dry weight

RFW: root fresh weight

RDW: root dry weight

PDW: Plant dry weight

SL: shoot length

RL: root length

LA: leaf area

CC: Chlorophyll content

F0: Minimal fluorescence level

Fm: Maximal fluorescence level

Fv/Fm (φP): potential photochemical efficiency of PSII electron transport

φPSII: actual efficiency of PSII electron transport

NPQ: non- photochemical fluorescence quenching

1-qP: proportion of closed PSII traps

SNa: Shoot Na+ concentration

SK: Shoot K+ concentration

RNa: Root Na+ concentration

RK: Root K+ concentration

SNaK: Shoot Na+/K+ concentration

RNaK: Root Na+/K+ concentration,

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Introduction: background to the study and presentation of targets

Medicago truncatula is a small legume native to the Mediterranean region which is used in genomic studies. It is a low-growing, clover-like plant, 10–60 cm tall with trifoliate leaves. Each leaflet is rounded, 1–2 cm long, often with a dark spot in the center.

M. truncatula has been chosen as a model organism for legume biology because it has a small diploid genome (2n = 16) and a high synteny with widely cultivated species such as peas or alfalfa, is self-fertile, has a rapid generation time and prolific seed production, and is amenable to genetic transformation. Its genome (seven times smaller than pea) provides quick access to genomic sequences and produce mutants that are particular to identify the functional role of genes. The genome of *M. truncatula* is currently being sequenced.

Soil salinity is widely reported to be a major agricultural problem, particularly in irrigated agriculture, and research on salinity in plants has produced a vast literature. However, in most species there are not many cultivars developed, which are tolerant to saline soils. The limited success of selection, can be accounted for by the fact that salt tolernt is a complex character controlled by a number of genes or groups of genes. We anticipate that the importance of salinity as a breeding objective will increase in the future.

The objectives of the present research are to study the effects of different salinity concentrations on six *M. truncatula* genotypes, which are the parental lines of some crosses, to identify the favourable salinity concentration and suitable crosses for further genetic investigation. The recombinant inbred lines (RILs) population of a selected cross are used under the favourable salinity treatment to study the genetic variability of RILs under control and salinity conditions as well as to identify the QTLs controlling several plant growth and physiological traits which should be used in breeding programs of *M. truncatula* or other related species.

CHAPTER I

Bibliographic Study

1.1. *Medicago truncatula*

Medicago truncatula is an annual weedy species which belongs to the *Fabaceae* family, *Faboideae* sub-family, *Trifolieae* tribe. It is close to the genus *Melilotus* (sweet clover) and *Trigonella* (fenugreek). The particularity of this family is to develop a symbiotic relationship with bacteria that fix the nitrogen available in the air (http://www1.montpellier.inra.fr/BRC-MTR/accueil.php?menu=medicago&page=menu11). Several cultivated species belongs to the *Medicago* genus, the most famous member and the most extensively cultivated is alfalfa (*Medicago sativa*). *Medicago sativa* is a perennial, allogamous and tetraploïd species. This species is an interesting source of vegetal protein.

The genus *Medicago* also includes annual medics such as *M. truncatula* (barrel medic), *M. polymorpha* (burr medic) and *M. scutellata* (snail medic). Annual medics are utilized as cover crops, short season forage crops, and weed-suppressing smother crops (Castillejo et al., 2004).

Among the annual medics, *M. truncatula* has been developed as a model legume. *M. truncatula* shares many important characteristics with alfalfa, such as its symbiotic associations with *rhizobia* and *mycorrhizal* fungi, and its high forage quality (Dita et al., 2006).

A number of biotic (fungi, bacteria, nematodes, viruses, parasitic plants, insects) and abiotic (drought, freezing, salinity, water logging) stresses are severely affecting the yield of legumes. Successful application of biotechnology to legumes facing biotic/abiotic constraints will require both a good biological knowledge of the target species and the mechanisms underlying resistance/tolerance to these stresses. The large genome size and the polyploidy of some legumes have hampered this goal, but in order to solve some of these problems. *Medicago truncatula* has emerged as model plant to investigate the genetics of nodulation and other important processes such as tolerance to stresses (Dita et al., 2006). *M. truncatula* flowers are yellow, produced singly or in a small inflorescence of 2-5 together. The fruit is a small spiny pod (Figure 1).

Figure 1.1 Flower, leaf, pod and seed of *Medicago truncatula* as a model legume (http://mips.helmholtz-muenchen.de/plant/medi/).

1.2. Medicago truncatula growth and development cycle

M. truncatula seeds develop inside a pod that provides protection during development and is a source of nutrients, as well as possibly contributing to maintenance of dormancy (Baskin and Baskin, 1998). The spines on the resulting mature pod favour mechanical dispersal, and the compressed spiral structures presumably contribute to protection of the mature seeds against herbivores. *M. truncatula* displays a typical indeterminate growth habit, resulting in flower and seed set over a protracted period after the development of the primary inflorescence meristem. The plant is autogamous and sets seed efficiently in the absence of

insect pollination. The only effective method for maximizing seed yield is to grow the plants under optimal conditions of nutrition and illumination.

1.3. *Medicago truncatula* as a model legume

The so-called barrel medic, *M. truncatula* is a plant of Mediterranean origin which is well adapted to semiarid conditions and is a winter-growing annual. There are several hundred reported ecotypes of *M. truncatula,* including commercial varieties such as Jemalong, Cyprus and Ghor (Barker et al., 1990). Some of these cultivars are commonly grown in rotation with cereal crops in certain regions of Australia. *M. truncatula* also grows well in greenhouse and several growth cycles can be completed within a year (Barker et al., 1990). *M. truncatula* shows high synteny with other legume plants, especially with *M. sativa* (Choi et al., 2004). Genetic studies of resistance to biotic and abiotic factors in alfalfa are difficult due to its outcrossing and tetraploid nature. The closely related species *Medicago truncatula* has been developed as the first model legume plant. It is autogamous with a short life cycle (6 months), and diploid (2n=2x=16) with a small genome (500 Mbp), which simplifies studies on the genetic mechanisms of responses to abiotic and biotic agents, notably with regard to the nitrogen-fixing symbiosis with *rhizobia* (Barker et al., 1990). *M. truncatula* has not a great importance from an agronomic point of view, but it has a syntenic relationship with other legume plants, especially with *M. sativa* (Figure 1- 2**)**. The two species share conserved genome structure and content (Choi et al., 2004), and thus *M. truncatula* can serve as a surrogate for cloning the counterparts of many economically important genes in alfalfa (Yang et al., 2008).

M. truncatula is more attractive for transformation work and the associated genetic studies than allogamous auto tetraploid *M. sativa*. Using Jemalong 2HA, transformation (Thomas et al., 1992; Chabaud et al., 1996; Wang et al., 2008), regeneration from protoplasts, asymmetric somatic hybridisation (Tian and Rose, 1999) and transfer of agriculturally important genes such as viral resistance genes was feasible (Jayasena et al., 2001).

M. truncatula was a focus for several meetings and workshops in the United States and Europe in the 1990s to establish it as a model and initiate the development of the necessary genetic and genomic tools. There are 227 000 *M. truncatula* ESTs on the The Gene Index Project database (http://compbio.dfci.harvard.edu/tgi/). The first steps towards sequencing were taken when Nam et al. (1999) produced the first BAC clones from Jemalong A17. The first published genetic map of *M. truncatula* was produced by Thoquet et al. (2002) using

two homozygous lines selected from Jemalong (Jemalong 6 or J6) and the Algerian natural population DZA315.16. Thoquet et al. (2002) noted that the three Jemalong lines A17, J5 and J6 could be considered as closely related genotypes, but different to the highly regenerable Jemalong genotype 2HA (Ray, 2008).

The vast amount of sequence data available in *M. truncatula* makes it an excellent resource for translational genomics. Of course, *M. truncatula* genome sequence provides a ready source for easily accessible DNA markers across legume species, enabling pan-legume comparisons, facilitating linkage mapping, comparative genomics, and marker-assisted selection (Choi et al., 2004). *M. truncatula* genome also reveals the scale and scope of biologically important gene families, including disease resistance genes (Ameline-Torregrosa et al., 2008). *M. truncatula* sequence creates a platform for gene discovery and positional cloning. Finally, the essentially complete *M. truncatula* genome sequence accelerates physical mapping in related species and provides a scaffold for next-generation sequencing in close relatives like alfalfa (Young and Udvardi, 2009).

1.4. Breeding in *Medicago Truncatula*

In parallel to its model species statute, the medics are also cultivated in Australia, in a ley farming (alternate husbandry) model, and in France where French National Institute for Agricultural Research (INRA) selected 4 cultivars that can be used in three main areas:
(http://www1.montpellier.inra.fr/BRC-MTR/accueil.php?menu=medicago&page=menu11)

1. To provide feed for livestock in the dry Causses (ewe, for ewe‘s milk cheese –Roquefort”): to improve the quantity and quality of the rangeland for extensive ewe rearing.
2. To maintain soils around infrastructures: roadsides, railway lines embankments and disturbed areas.
3. To avoid soil erosion from rainfalls and winds, reduce the use of herbicides, limit infiltrations of pesticides in soils and enhance underground life in slopping vineyards.

The use of annual medics in France is limited to the very south, near the Mediterranean Sea, since frost is too important in the northern parts of France for the available cultivars to be grown there. Four cultivars were obtained at Montpellier plant breeding department and are under Plant Breeder‘s Right (UPOV= L'Union Internationale pour la protection des obtentions végétales). They belong to three species: *Medicago truncatula, Medicago polymorpha* and *Medicago rigidula.*

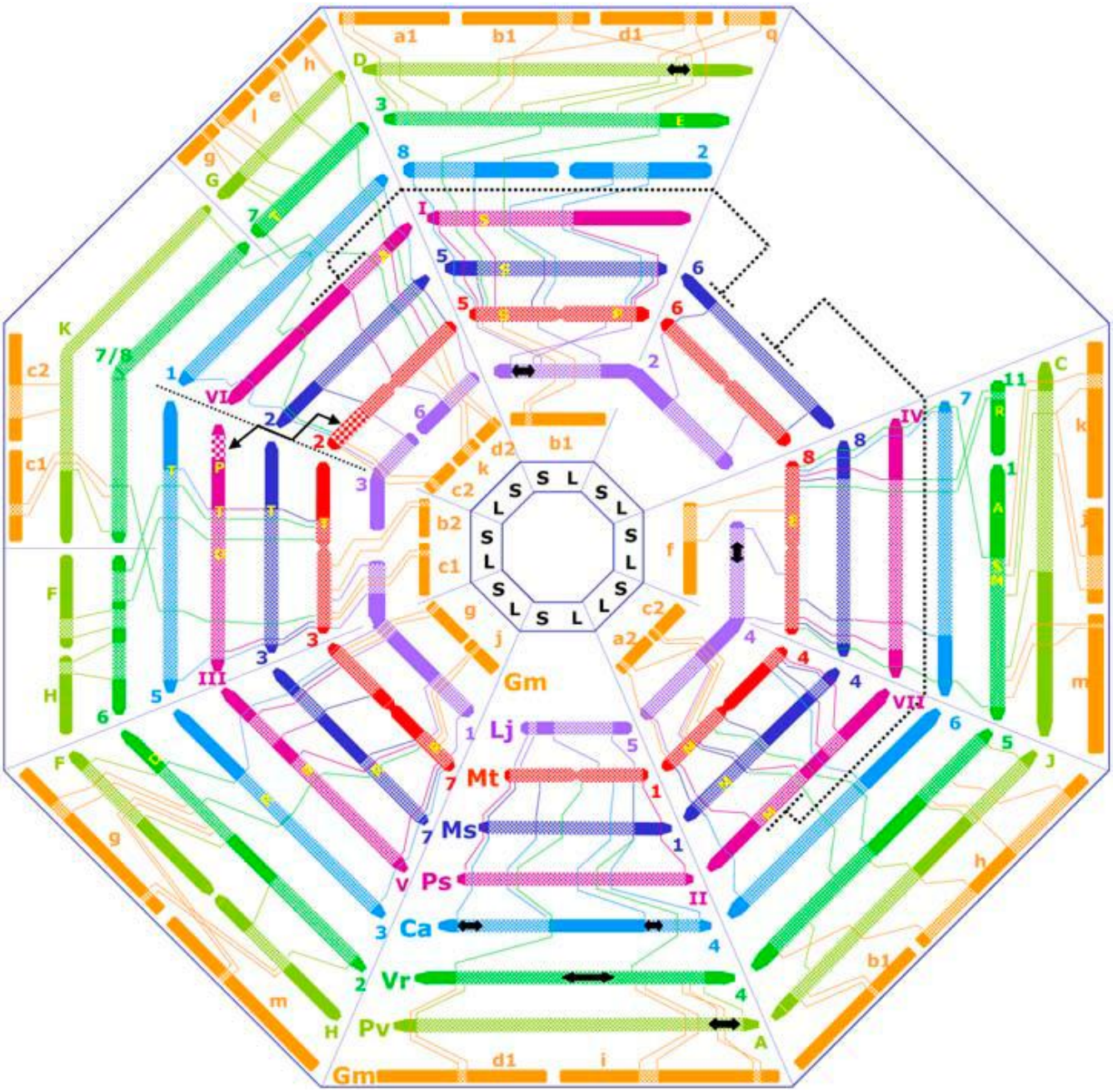

Figure 1-2 A simplified consensus map for eight legume species. Mt, *M. truncatula*; Ms, alfalfa; Lj, *L. japonicus*; Ps, pea; Ca, chickpea; Vr, mungbean; Pv, common bean; Gm, soybean. S and L denote the short and long arms of each chromosome in *M. truncatula*. Syntenic blocks are drawn to scale based on genetic distance. *M. sativa* in blue, *M. truncatula* in red. (Choi et al. 2004)

Breeding objectives have included improved seedling vigor, greater forage yield and seed production, greater resistance to pests and diseases.

1.5. Molecular markers, genetic mapping and identification of QTLs

Conventionally, plant breeding depends upon morphological/phenotypic markers for identification of agronomic traits. With the development of methodologies for the analysis of plant gene structure and function, molecular markers have been utilized for identification of traits to locate the gene(s) for a trait of interest on a plant chromosome and are widely used to study the organization of plant genomes and for the construction of genetic linkage maps. Molecular markers are independent from environmental variables and can be scored at any stage in the life cycle of a plant. There has thus been marked increase in the application of molecular markers in the breeding programmes of various crop plants. Molecular markers not only facilitate the development of new varieties by reducing the time required for the detection of specific traits in progeny plants, but also fasten the identification of desired genes and their corresponding molecular markers, thus accelerating efficient breeding of important traits into *Medicago truncatula* cultivars by marker assisted selection (MAS). Several genetic maps have been constructed in *M. truncatula*. A genetic map of the cross Jemalong-6 × DZA315.16 using a population of 199 RILs (LR4) was constructed which contains 72 SSR markers (Julier et al., 2007). A RIL population (LR5) was derived from the cross between Jemalong A17 and F83005.5 and the RILs population of the cross was genotyped by SSR markers and a map with 70 markers was reported (Arraouadi et al., 2012). The genetic map of the F83005.5 × DZA45.5 cross (LR3) was also constructed by Hamon et al. (2010). Genetic maps of –A17 × DZA315.16" (LR4), and –A17 × F83005.5" (LR5) were improved by Ben et al (2013).

1.5.1. Marker assisted selection (MAS).

MAS is a technology that changes the process of variety creation from traditional field based format to a laboratory format. It is the use of molecular markers to track the location of genes of interest in a breeding programme. MAS is a form of indirect selection and most widely application of DNA markers. Once traits are mapped a closely linked marker may be used to screen large number of samples for rapid identification of progeny that carry desirable characteristics. Several molecular marker types are available and each of them has is advantage and disadvantage. Restriction fragment length polymorphisms (RFLPs) have been

widely and successfully used to construct linkage maps of various species. With the development of the polymerase chain reaction (PCR) technology, several marker types emerged. The first of those were random amplified polymorphic DNA (RAPD), which quickly gained popularity over RFLPs due to the simplicity and decreased costs of the assay. However, most researchers realized the weaknesses of RAPDs and do not use them any more. Microsatellite markers or simple sequence repeats (SSRs) combine the power of RFLPs (codominant markers, reliable, specific genome location) with the ease of RAPDs and have the advantage of detecting higher levels of polymorphism.

Compared with other DNA-based markers, RFLP, single nucleotide polymorphisms (SNPs) amplified fragment length polymorphisms (AFLP), simple sequence repeat (SSR) markers occur frequently in plants and are multiallelic, co-dominant, highly reproducible and can function with low-quality DNA (Morgnate and Olivieri 1993; Roca and Wang. 1994). Many SSR markers have been developed and are widely used in plants for genetic mapping, genetic diversity assessment, population genetics and marker-assisted selection (Gupta and Varshney 2000).With the rapid development of expressed sequence tags (ESTs), a large number of SSR markers have been developed from the ESTs library of *Medicago truncatula* (Baquerizo-Audot et al. 2001; Eujayl et al. 2004). Eujayl et al. (2004) searched 147,000 *M. truncatula* ESTs and identified 455 SSR primer pairs which produced characteristic SSR bands of the expected length in *Medicago* species. Ellwood et al. (2006) used six SSR primers to analyze the genetic diversity and relationships between randomly selected specimens from 192 accessions in the core *M. truncatula* collection. It thus seems that SSR markers would be a powerful molecular approach for assessing genetic diversity and germplasm characterization in tetraploid alfalfa. Bernadette et al. (2003) used 87 SSR primer pairs, most from *M. truncatula* ESTs, for genotyping and mapping tetraploid alfalfa populations and SSR markers have been applied to alfalfa in several other studies (Diwan et al. 1997, 2000; Mengoni, 2000, b; Baquerizo et al. 2001; Eujayl et al. 2004; Flajoulot et al. 2005; Sledge et al. 2005; Ellwood et al. 2006). Marker assisted selection (MAS) is based on the identification and use of markers, which are linked to the gene(s) controlling the trait of interest. By virtue of linkage, selection may be applied to the marker itself. The advantage consists in the opportunity of speeding up the application of the selection procedure. For instance, a character which is expressed only at the mature plant stage may be selected at the plantlet stage, if selection is applied to a molecular marker. Selection may be applied

simultaneously to more than one character. Selection for a resistance gene may be carried out without needing to expose the plant to the biotic and abiotic agents. If linkage exists between a molecular marker and a quantitative trait locus (QTL), selection may become more efficient and rapid. The construction of detailed molecular and genetic maps of the genome of the species of interest is necssary for most forms of MAS.

1.5.2. **Use of molecular markers in *M.truncatula* improvement programmes: conservation of genetic resources**

Loss of genetic diversity has become a problem not only of the natural plant and animal population but also agriculturally important species. Ancient cultivars or landraces and wild relatives of domesticated species are being lost as modern varieties become adopted by farmers. Microsatellites are commonly used to study genetic relationships among genotypes within species because of their high level of polymorphism (Devos et al. 1995; Korzun et al. 1997). Microsatellites markers are currently used to identify quantitative trait loci (QTLs) and genetic diversity (Medini et al. 2005).

Molecular genetic markers have enabled the identification of quantitative trait loci (QTL) which are involved in the expression of agronomically important traits of *M. truncatula*, such as forage quality (Lagunes Espinoza et al. 2012), flowering date (Pierre et al. 2008) and components of biomass like shoot dry weight, root dry weight, length of roots , leaf area or leaf morphology (Veatch et al. 2004; Julier et al. 2007; Lopez 2008a; Espinoza et al. 2012, Pottorff et al. 2012) or physiological traits (Moreau et al. 2012). Genetic analyzes were reported for disease resistances (Ben et al. 2013) and abiotic stress such as tolerance to drought (Badri et al. 2011), seed germination and pre-emergence growth at xtreme temperatures (Dias et al. 2011) and water deficit (Vandecasteele et al. 2011). For salt tolerance, significant decrease in shoot biomass of *M. truncatula* genotypes under different saline irrigation was reported (Veatch et al. 2004, Lopez 2008a). Salinity stress could induce also changes in soluble sugars, amino acids and proline content in shoots (Lopez et al. 2008b). Arraouadi et al. (2011, 2012) reported the effect of moderate salt stress on aerial parts of *M. truncatula. S*alt treatment reduced significantly leaves and roots K^+ content, whereas Na^+ content increased in leaves and roots in *M. truncatula*.

1.6. Effects of salinity and adaptation strategies

1.6.1. Effects of salinity on the production

Salt stress has a major negative effect on food production and quality worldwide by limiting the growth, development, and yield of crops (Tester and Davenport. 2003). More than one-fifth of the world's arable lands are now under salt stress. As the global population increases, water resource management is deteriorating and salinity level of lands becomes more extreme which provoke decreases in development of agricultural economics.

Salt stress can damage plants by several mechanisms, including water deficit, ion toxicity, nutrient imbalance, and oxidative stress (Vinocur et.al, 2005). Plants respond and adapt to salt stress through a series of biochemical and physiological changes, involving expression and coordination of many genes (Bartels and Sunkar 2005; Chinnusamy et.al 2005). Gene expression in the model plant Arabidopsis *thaliana* in response to salt and other abiotic stresses has been studied extensively (Zhu et. al., 2001; Seki et.al, 2002).

Currently, 50% of all irrigation schemes are affected by salinity. Nutrient disturbances under salinity reduce plant growth by affecting the availability, transport, and partitioning of nutrients. However, salinity can differentially affect the mineral nutrition of plants. Salinity may cause nutrient deficiencies or imbalances, due to the competition of Na+ and Cl– with nutrients such as K+, Ca2+, and NO (Yuncai and Schmidhalter 2005).

In general, high NaCl concentrations affect plant morphology, at different levels such as changes in plant architecture, vegetative growth or variations in leaf thickness. They also modify physiology and plant metabolism and affects the overall cell metabolic activities like seed germination, nitrogen metabolism, ionic toxicity, stomatal regulation and photosynthesis rate (Edmeades et al. 2001; Santos et al. 2002; Vinocur and Altman 2005). Salt tolerance is thus usually assessed as the percent biomass production in saline versus control over a prolonged period of time.

1.6.1.1. Effects of salinity on photosynthetic activity

The stresses imposed by salinity relate to ion composition and to ion concentration within the plants. When dissolved salt concentrations in soil solutions increase, water energy gradients decrease, making it more difficult for water and nutrients to move through root membranes and into the plant. The rate of water and solute uptake slows, but does not cease. With time, the solute-rich soil water increases ionic concentrations within the plant‘s aqueous

transportation stream. This osmotic effect, encountered at the root membrane, applies at all the plant‘s internal membranes served by its conductive tissue. In addition to the osmotic effect of concentrated solutes, there are ionic effects that arise from the specific composition of the solute flowing through plant tissue. Internal excesses of particular ions may cause membrane damage, interfere with solute balances, or cause shifts in nutrient concentrations.

Salinization of soils is becoming an increasing problem in production systems where high rates of fertilization and irrigation are employed in climates with high evapotranspiration. The initial effects of increasing soil salinity are very similar to those observed when plants are exposed to drought. Reductions in leaf water potential will reduce stomatal conductance and eventually inhibit photosynthetic metabolism.

It has been widely reported that photosynthetic capacity of chloroplast is depressed due to salt stress because of the reasons that salt stress leads to instability of the pigment protein complexes, destruction of chlorophylls and changes in the quantity and composition of carotenoids (Dubey, 1997). Besides, plants growing under saline condition, high stress, and photo inhibition are known to damage PSII (Ashraf and Harris, 2004). The measurements of chlorophyll fluorescence provide quantitative information about photosynthesis through non invasive means (Lichtenthaler, 1996). Fv/Fm ratio gives an estimate of the maximum quantum efficiency of PSII photochemistry (Baker and Rosenqvist, 2004) and has been widely used to detect stress induced perturbations in the photosynthetic apparatus (Weng and Lai, 2005; Sixto et al. 2005)

1.6.2. Mechanisms of adaptation to salinity

During their life-cycle, plants acclimate to environmental constraints by a wide range of mechanisms that are classified as avoidance or tolerance strategies (Levitt, 1980). In case of lowered water availability in the environment, stress avoidance essentially aims at maintaining the initial plant water status and lowering the rate of stress imposed at the tissue or cellular level. Tolerance strategies aim at preventing damage and maintaining metabolism, once water deficit has been established. Avoidance and tolerance mechanisms are not active in a temporal sequence. Their distinction is conceptual, but useful when studying plant response to stress (Verslues et al., 2006).

Plant acclimatory responses are complex, exhibiting multigenic and interrelated properties. In addition, it should be hampered, due to heterogeneities in factors influencing stress responses such as plant age, growth conditions, and the experimental treatments, such as

severity, duration, and method of stress imposition (Aguirrezabal et al., 2006). Consequently, robust parameters for a specific definition of stress are still missing. Due to the complexity of plant stress response and its interlinked mechanisms and influencing factors, it becomes necessary to extend research to multilevel analyses (Jogaiah et al., 2012).

1.6.2.1. Plants Vary in Tolerance to salinity

Plants differ greatly in their tolerance to salinity, as reflected in their different growth responses. Through the cereals, rice (*Oryza sativa*) is the most sensitive and barley (*Hordeum vulgare*) is the most tolerant. The variation in salinity tolerance in dicotyledonous species is even greater than in monocotyledonous species. Some legumes are very sensitive, even more sensitive than rice (L¨auchli, 1984).To understand the physiological mechanisms responsible for the salinity tolerance of species, it is necessary to know whether their growth is being limited by the osmotic effect of the salt in the soil, or the toxic effect of the salt within the plant. In the simplest analysis of the response of a plant to salinity stress, the reduction in shoot growth occurs in two phases: a rapid response to the increase in external osmotic pressure, and a slower response due to the accumulation of Na+ in leaves.

In the first, osmotic phase, which starts immediately after the salt concentration around the roots increases to a threshold level, the rate of shoot growth falls significantly. The threshold level is approximately 40 mM NaCl for most plants, or less for sensitive plants like rice and Arabidopsis.

This is largely (but not entirely) due to the osmotic effect of the salt outside the roots. Figure 1-3a shows the effect on the rate of shoot growth, which is, the rate of increase in shoot dry matter or in leaf area over time. The rate at which growing leaves expand is reduced, new leaves emerge more slowly, and lateral buds develop more slowly or remain non active, so fewer branches or lateral shoots form. The second, ion-specific, phase of plant response to salinity starts when salt accumulates to toxic concentrations in the old leaves (which are no longer expanding and so no longer diluting the salt arriving in them as younger growing leaves do), and they die. If the rate at which they die is greater than the rate at which new leaves are produced, the photosynthetic capacity of the plant will no longer be able to supply the carbohydrate requirement of the young leaves, which further reduces their growth rate (Figure 1- 3*a*). The osmotic stress not only has an immediate effect on growth, but also has a greater effect on growth rates than the ionic stress. Ionic stress impacts on growth much later, and with less effect than the osmotic stress, especially at low to moderate salinity levels

(Figure 1-3a). The effect of increased tolerance to the osmotic stress, with no change in ionic stress tolerance, is shown by the dotted line in Figure 3a. A significant genetic variation within species exists in the osmotic response. An increase in ionic tolerance takes longer to appear (Figure 1- 3b). Within many species, documented genetic variation exists in the rate of accumulation of Na+ and Cl− in leaves, as well as in the degree to which these ions can be tolerated. An increase in tolerance to both stresses would enable a plant to grow at a reasonably rapid rate throughout its life cycle. This combined tolerance is shown in Figure 3*c*. For most species, Na+ appears to reach a toxic concentration before Cl− does, and so most studies have concentrated on Na+ exclusion and the control of Na+ transport within the plant. However for some species, such as soybean, citrus, and grapevine, Cl− is considered to be the more toxic ion (L¨auchli, 1984; Storey et al, 1999). The evidence for this is the association between genetic differences in the rate of Cl− accumulation in leaves and the

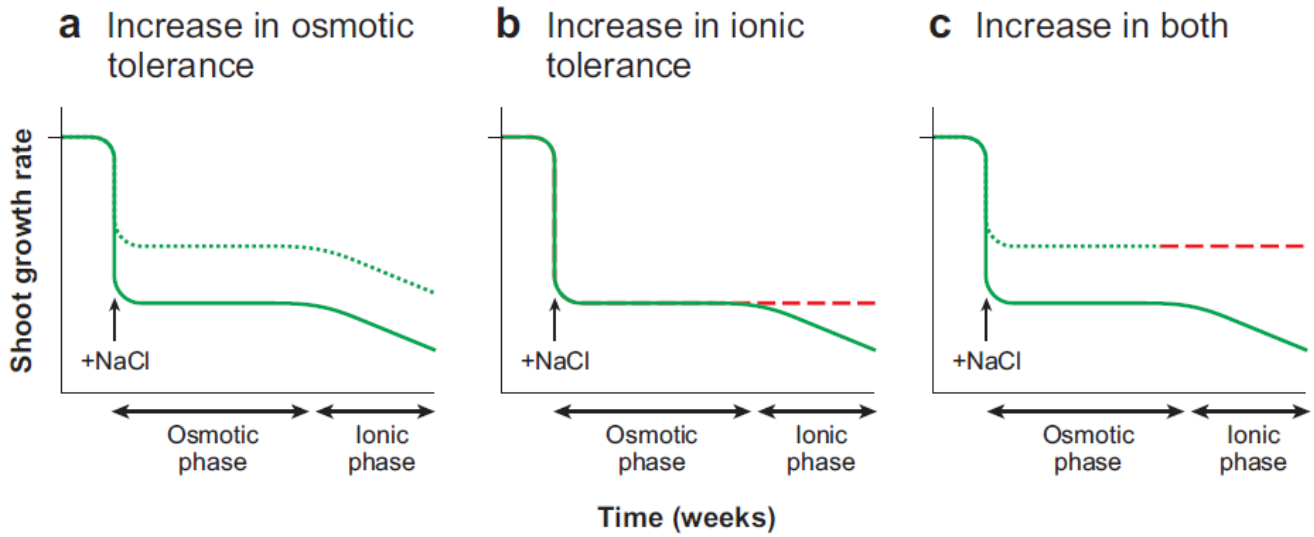

Figure 1.3. The growth response to salinity stress occurs in two phases: a rapid response to the increase in external osmotic pressure (the osmotic phase), and a slower response due to the accumulation of Na+ in leaves (the ionic phase). The solid green line represents the change in the growth rate after the addition of NaCl. (*a*) The broken green line represents the hypothetical response of a plant with an increased tolerance to the osmotic component of salinity stress. (*b*) The broken red line represents the response of a plant with an increased tolerance to the ionic component of salinity stress. (*c*) The green-and-red line represents the response of a plant with increased tolerance to both the osmotic and ionic components of salinity stress (Munns et al, 1995).

plant‘s salinity tolerance.This difference may arise because Na+ is not so effectively in the woody roots and stems that little reaches the leaves, and K+ becomes the major cation. Thus Cl−, which continues to pass to the lamina, becomes the more significant toxic component of the saline solution.

1.6.2.2. Osmotic stress tolerance

The decreased rate of leaf growth after an increase in soil salinity is primarily due to the osmotic effect of the salt around the roots. A sudden increase in soil salinity causes leaf cells to lose water, but this loss of cell volume and turgor is transient.Within hours, cells regain their original volume and turgor owing to osmotic adjustment, but despite this, cell elongation rates are reduced (Yeo et al, 1991; Passioura and Munns, 2000; Cramer, 2002 and Fricke and Peters, 2002). Over days, reductions in cell elongation and also cell division lead to slower leaf appearance and smaller final size. Cell dimensions change, with more reduction in area than depth, so leaves are smaller and thicker.

The mechanism that down regulates leaf growth and shoot development under stress is not precisely known. The reduction in leaf growth must be regulated by long distance signals in the form of hormones or their precursors, because the reduced leaf growth rate is independent of carbohydrate supply(Munns et al, 2000) and water status (Munns et al, 2000; Fricke and Peters, 2002). The reduction occurs in the absence of nutrient deficiency (Hu et al, 2007) and ion toxicity, as evidenced by very low concentrations of Na+ and Cl− in expanding cells or tissues that do not correlate with growth rates (Fricke, 2004; Hu et al, 2005; Neves-Piestun and Bernstein, 2005 ; Hu et al, 2007).

Root growth is usually less affected than leaf growth, and root elongation rate recovers remarkably well after exposure to NaCl or other osmotica (Munns, 2002). Recovery from a moderate stress of up to 0.4 MPa (megapascal) of mannitol, KCl, or NaCl (i.e., an osmotic shock that does not cause plasmolysis) is complete within an hour (Foyer and Noctor, 2005). Recovery from NaCl concentrations as high as 150 mM can occur within a day (Munns, 2002). In contrast to leaves, these recoveries take place despite turgor not being fully restored (Frensch and Hsiao, 1994). This indicates different changes in cell wall properties compared with leaves.

1.6.2.3. Photosynthesis and Stomatal Conductance

Rates of photosynthesis per unit leaf area in salt-treated plants are often unchanged, even though stomatal conductance is reduced (James et al, 2002). This paradox is explained by the changes in cell anatomy described above that give rise to smaller, thicker leaves and result in a higher chloroplast density per unit leaf area. When photosynthesis is expressed on a unit chlorophyll basis, rather than a leaf area basis, a reduction due to salinity can usually be measured. In any case, the reduction in leaf area due to salinity means that photosynthesis per plant is always reduced.

Cause-effect relationships between photosynthesis and growth rate can be difficult to unravel. It is always difficult to know whether a reduced rate of photosynthesis is the cause of a growth reduction, or the result. With the start of salinity stress, a reduced rate of photosynthesis is certainly not the only cause of a growth reduction because of the rapidity of the change in leaf expansion rates (Cramer and Bowman, 1991; Passioura and Munns ; 2000; Fricke, 2004), but also because of the increase in stored carbohydrate, which indicates unused assimilate (Munns, 2000). At high salinity, salts can build up in leaves to excessive levels. Salts may build up in the apoplast and dehydrate the cell, they may build up in the cytoplasm and inhibit enzymes involved in carbohydrate metabolism, or they may develop in the chloroplast and play a direct toxic effect on photosynthetic processes.

1.6.2.4. Accumulation of sodium ions in shoot

The main site of Na+ toxicity for most plants is the leaf blade, where Na+ accumulates after being deposited in the transpiration stream, rather than in the roots (Munns, 2002). A plant transpires 50 times more water than it retains in leaves (Munns, 2006), so excluding Na+ from the leaf blades is important, even more so for perennial than for annual species, because the leaves of perennials live and transpire for longer. Most Na+ that is delivered to the shoot remains in the shoot, because for most plants, the movement of Na+ from the shoot to the roots can likely recirculate only a small proportion of the Na+ that is delivered to the shoot. As such, the processes determining Na+ accumulation in the shoot are primarily the processes controlling the net delivery of Na+ into the root xylem.

1.6.2.5. K+ accumulation in cytoplasm.

The concentration of K+ in the cytoplasm relative to that of Na+ may be a contributing factor to salinity tolerance. In *Arabidopsis,* an additional supply of K+ alleviated the phenotype of

the *sos* mutants (Zhu, 2002), which may be due to an increase in cytoplasmic K+ concentrations. In barley Chen et al, (2005) found a negative correlation between the magnitude of K+ efflux from the root and salt tolerance of mature barley plants. This phenomenon may be related to root K+ status, although a strong relationship between leaf K+ concentrations and salinity tolerance has not been found. We can resume that accumulation of Na+ and K+ in shoots and roots are affected differently by salinity according to genotypes of species. The amounts of sodium (Na^+) and potassium (K^+) ions were measured in pooled tissues of 3 plants from each replication of each genotype just in our second experiment which was realized with 100mM NaCl and control (water). Genetic variability for Na+ and K+ content and also QTLs controlling the traits in both conditions were identified

1.7. Fluorescence

1.7.1. Fluorescence induction

During the induction of photosynthesis when a dark-adapted leaf is exposed to light, large changes in chlorophyll fluorescence occur. The rapid changes in fluorescence that occur during the rapid induction to a peak have been attractive for detecting differences in photosynthetic performance of plants. On immediate exposure to light, fluorescence rises to the minimal level of fluorescence, termed Fo level, which is the fluorescence level obtained when the PSII reaction centers are in the ‗open' state (capable of photochemistry since QA, the primary quinone acceptor of PSII, is maximally oxidized). It should be noted that if the actinic photosynthetic photon flux density (PPFD) being used to drive the fluorescence induction is saturating and effects maximal closure of PSII reaction centers (maximal reduction of QA) at Fp, then the maximal fluorescence level, defined as Fm will be attained. The difference between Fm and Fo is termed the variable fluorescence, Fv. Absolute fluorescence values, such as Fo and Fm, are dependent upon both the photochemical activities and the optical properties of the leaf and, consequently, it is essential to remove the variable of leaf optical properties when attempting to compare changes in fluorescence characteristics between different leaf samples. This can be achieved by comparing ratios of fluorescence values. The potential of the use of ratios of the fluorescence induction.

1.7.2. Modulated fluorescence measurements

The majority of fluorescence measurements are now made using modulated fluorometers with the leaf balance in a known state. The procedures for making such measurements are shown in Fig. 1-4, together with the fluorescence levels for a leaf in specific states. For a dark-adapted leaf, Fo is determined using a very low PPFD (generally considerably below 1 μmol m^{-2} s^{-1}), which ensures that almost all of the PSII reaction centers are in the open state (capable of photochemistry). When the dark-adapted leaf is exposed to a short actinic light pulse of very high PPFD (generally less than 1 s at several thousand μmol m^{-2} s^{-1}), a maximal level of fluorescence (Fm) is generated as the majority of the PSII reaction centers have been closed (Incapable of photochemistry). The ratio of Fv/Fm provides an estimate of the maximum quantum efficiency of PSII photochemistry (Butler, 1978). Fv/Fm has been widely used to detect stress-induced perturbations in the photosynthetic apparatus, since decreases in Fv/Fm can be due to the development of slowly relaxing quenching processes and photo damage to PSII reaction centers, both of which reduce the maximum quantum efficiency of PSII photochemistry. The potential for the application of fluorescence measurements to study changes in leaf photosynthetic performance increase dramatically with the development of the light addition technique which could resolve fluorescence quenching into photochemical and non-photochemical components (Bradbury and Baker, 1981, 1984). When a leaf in the light-adapted state is exposed to a saturating pulse of very high PPFD, there is an increase in fluorescence from the F' level to a maximal level, F' m (Fig. 1-4).

The difference between F' m and F' is termed F' q since this is the fluorescence that has been quenched from the maximal level. The saturating light pulse maximally closes the PSII reaction centers and consequently removes any photochemical quenching by open PSII reaction centers. For a healthy leaf operating at steady-state photosynthesis under moderate to high PPFDs, the F' m level generated by the saturating light pulse will be considerably less than the Fm level generated from a dark-adapted leaf by the same pulse (Fig.1-4). This difference is due to the development of light induced, non-photochemical quenching processes during the induction of photosynthesis in the leaf. The demonstration that the ratio F' q/F' m was an estimate of the quantum yield of PSII photochemistry for a leaf at any given

light condition (Genty et al., 1989) has led to this parameter, being widely used to estimate the operating quantum efficiency of PSII electron transport (hereafter termed the PSII operating efficiency).

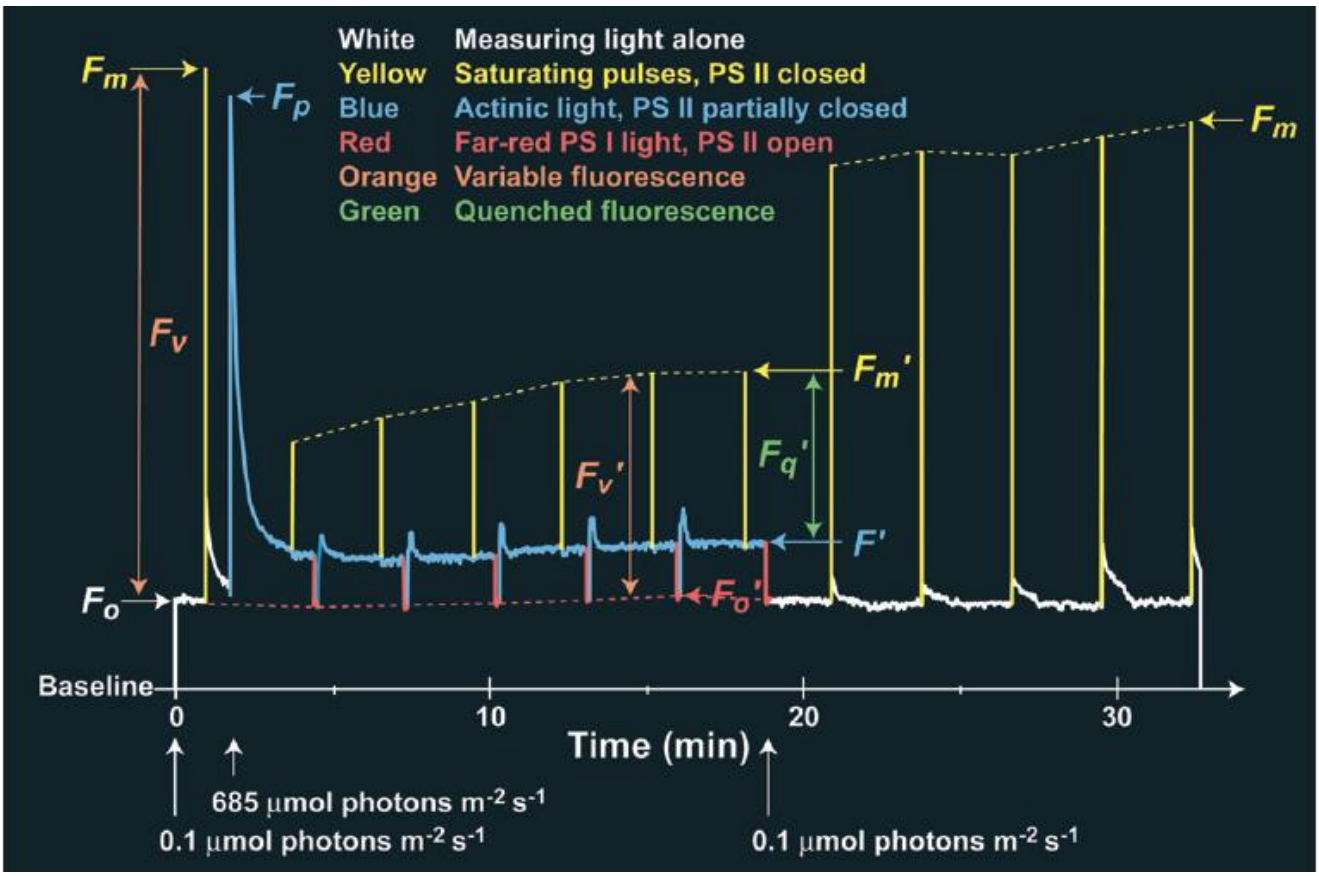

Fig. 1.4. Protocol for quenching analysis using modulated fluorescence. A dark-adapted leaf is exposed to various light treatments. The fluorescence parameters denoted with a prime originate from the illuminated leaf, where energy-dependent, non-photochemical quenching is present. The parameters without a prime are obtained from the leaf in the dark-adapted state, where there is no energy-dependent non-photochemical quenching. The different colours of the trace denote different light treatments. White, weak measuring light alone (0.1 µmol photons m^{-2} s^{-1}) that gives Fo. Yellow, saturating light pulse (<1 s duration, >6000 µmol photons m^{-2} s^{-1}) that gives Fm in darkness and F' m in light. Blue, actinic light that drives photosynthesis (in this case 685 µmol photons m^{-2} s^{-1}) that gives F' (if steady-state has been reached this has often been denoted by F' s). The actinic light can be produced from a range of sources, for example, sunlight, halogen lamp, light-emitting diodes. The initial peak of fluorescence is denoted as Fp (without prime, since it originates from the nomenclature of the rapid phase of fluorescence induction, see Fig. 1). Red, far-red light (30 µmol photons m^{-2} s^{-1} at 720–730 nm for 4 s) that excites PSI only, and thus oxidizes the plastoquinone and QA pools associated with PSII and gives F' o: Orange, variable fluorescence calculated as Fv/Fm-Fo from the dark-adapted leaf and

F' v = F' m - F' o from the illuminated leaf. Green, fluorescence that is quenched from F' m to F' by PSII photochemistry in the illuminated leaf, calculated as F' q = F' m - F' : All parameters except F' q; Fv, and F' v; are measured from the baseline. (Neil et al, 2004))

The PSII operating efficiency has been shown to be the product of two other important fluorescence parameters, F' v/F' m (the maximum efficiency of PSII under the given light conditions, generally determined by the level of quenching in PSII reaction centers and antenna) and F' q/F' v (the PSII efficiency factor which relates to the ability to maintain PSII reaction centers in the open state):

$$\frac{F'_q}{F'_m} = \frac{F'_v}{F'_m} \cdot \frac{F'_q}{F'_v}$$

F' v is the variable fluorescence of a light-adapted leaf defined as (F' m - Fo') (Fig. 1.4). Fo' is the minimal level of fluorescence when PSII centers are maximally open for the leaf in a light-adapted state. Fo' has frequently been measured by exposing the leaf at F' to weak far-red light in the absence of actinic light (van Kooten and Snel, 1990; Maxwell and Johnson, 2000), since it is assumed that far red light will preferentially excite PSI relative to PSII thus removing electrons from the PSII electron acceptors and opening the PSII reaction centers.

Non-photochemical quenching (heat dissipation) has been quantified using NPQ which compares the light induced F' m level to the dark-adapted Fm level (Bilger and Bjo¨rkman, 1990):

$$NPQ = (F_m/F'_m) - 1$$

It is important to recognize that NPQ assesses increases in non-photochemical quenching in a light-adapted leaf relative to the non-photochemical quenching occurring in the dark-adapted

state. Consequently, it is only valid to make comparisons between samples which have the same quenching characteristics in the dark-adapted state; similar values of Fv/Fm would be a good indication of this.

In the first experiment we have measured several fluorescence values (minimum fluorescence (*Fo*), maximum fluorescence (*Fm*), ratio of variable on maximum fluorescence (*Fv/Fm* where *Fv = Fm-Fo*) for the six genotypes with different saline concentrations and control to identify the effect of genotype an salinity on the fluorescence values. The same method was applied also on the recombinant inbred lines of the second experiment which was realized with 100mM NaCl and control (water). Genetic variability for fluorescence values and also QTLs controlling the traits in both conditions were identified

Chapter 2

Materials and methods

2.1 Plant materials

Two successive experiments were undertaken in order to identify the effect of different NaCl concentrations on six genotypes which are parental lines of some crosses and then based on the results of the first experiment, we realized the second one inorder to detect QTLs controlling several growth and physiological traits in a Recombinant Inbred Lines (RILs) population of *M. truncatula.*

2.1.1Genotypes

The six *Medicago truncatula* genotypes including TN1.11, A17, DZA315.16, A20, TN1.21 and F83005.5, used in the first experiment were selected through a sequenced lines collection (http://www1.montpellier inra.fr/BRC-MTR/mauguio/mauguio.php), coming from different Mediterranean countries and present a high genetic variability (Table 2.1). All genotypes are pure lines derived from one plant by single seed descendant method. Seeds were produced at INRA Montpellier and Ecolab Toulouse-France in the greenhouse.

Recombinant inbred lines populations for some combinations between different genotypes are available as presented in fig 2-1. According the results of the first experiment the population of recombinant inbred lines (100 RILs) of *M.truncatula* in F6 generation derived from the cross between TN1.11 and Jemalong-A17 (A17) was used in the second experiment.

Table 2.1. Medicago truncatula genotypes and their origin

Line	Origin
A17b	Unknown
F83005.5 a, b	France
TN1.11 b	Tunisia
TN1.21 b	Tunisia
A20 b	Morocco
DZA315.16 a,b	Algeria

a Lines from the core collection CC16

b Sequenced lines

2.1.2 Experimental conditions

In the first experiment the seeds of all genotypes were scarified with sand paper and then placed in Petri dishes on a piece of blotting paper imbibed with sterile water. The dishes were kept at 4°C in obscurity for 4-5 days, to overcome seed dormancy, and then for 24 h at 25°C to let them germinate. Seedlings with root length of about 4 mm were individually transferred to pots (10 cm diameter and 10cm deep) filled with 3:1 (V: V) of perlite and sand. Four plants of each genotype were grown in a given individual pot under controlled conditions at 25°C, with relative humidity of 80% and photosynthetic photon flux of 200 $\mu mol.m^{-2}.s^{-1}$ with 16 h photoperiod. The experiment was carried out in a factorial experiment based on randomized complete block design with three replications, 2 pots per replication and 4 plants per pot. Plants were grown in the phytotron under controlled conditions. During 35 days after planting, the plants were sub-irrigated once a week. For control treatment, a nutritive solution was used as described by Vadez *et al*., (1996) whereas the iron source was modified by adding 25ml of Fe-EDTA (8.2g /L) per litter. In salinity treatment, NaCl was added just one time to the nutritive solution according to different concentrations (0, 30, 60, 90,120 and 150 mM NaCl). Salt treatments were applied 7 days after planting. To avoid the overcoming of NaCl accumulation problem in the substrate, seedlings were sub-irrigated one time with NaCl concentrations for the first seven days and other irrigations were done only with nutritive solution. Irrigation was done with 2L nutritive solution in each tray of 12 pots per week.

The second experiment was also carried out in the same conditions as explained for the first one. The experimental design was split plot in 3 replications. The main factor was salinity treatment (100 mM NaCl and control) and sub factor consisted of 100 Rils and their parents.

2.2 Trait measurement

Several growth and physiological traits were measured in both experiments which are the following.

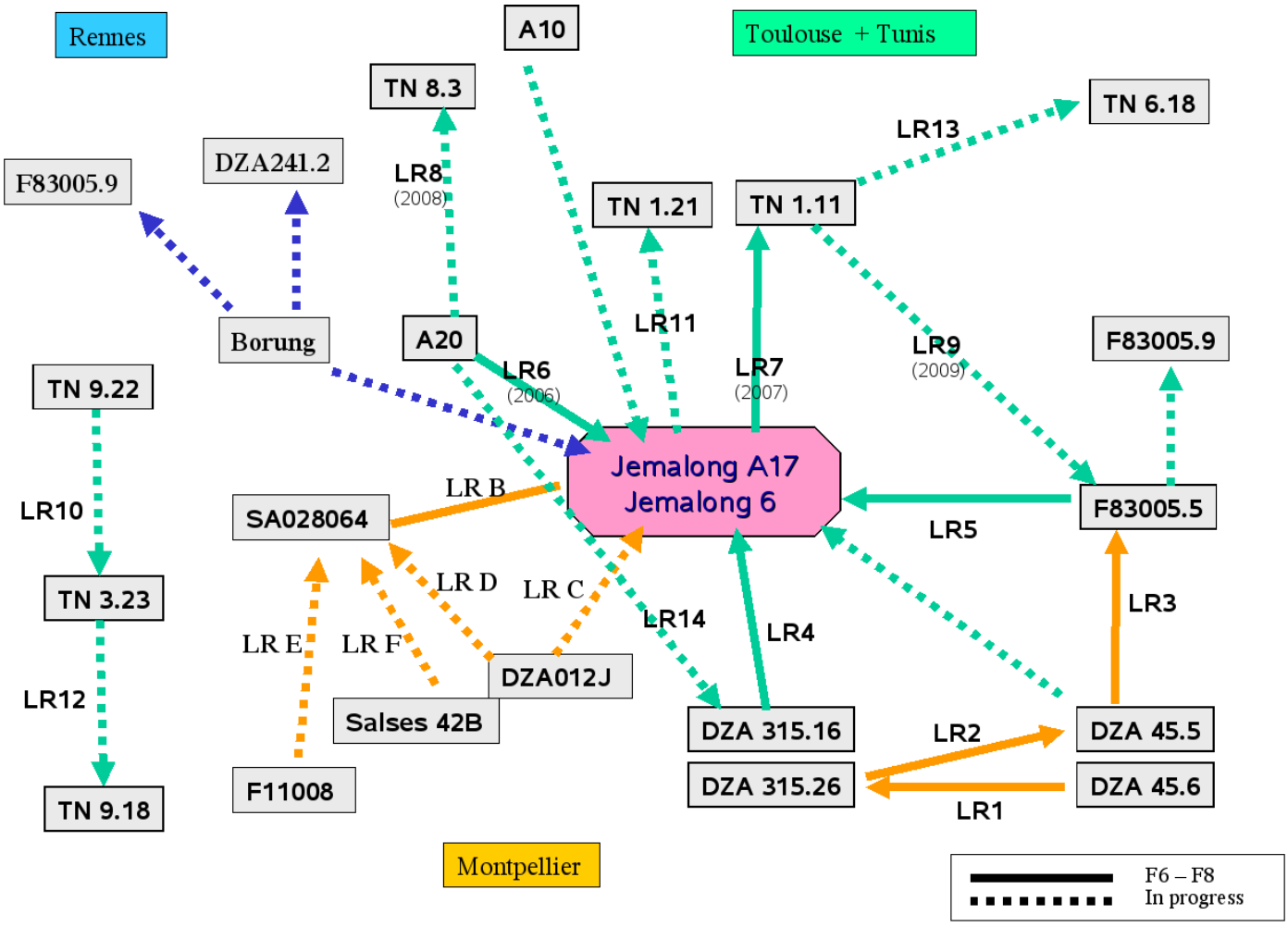

Figure 2.1. RILs populations (LR) obtained by crossing parental lines of *Medicago truncatula*. The full arrows mean that the RILs population is fixed (the lines are homozygous) and mapped; the dotted arrows mean that the RILs population is not fixed yet. This work is a collaboration with INRA Montpellier, INRA Rennes and the center of Biotechnology of Borj Cedria (Tunisia).

2.2.1 Plant growth

Plants were harvested 35 days after planting in the first experiment and 30 days after planting in the second one. Fresh weights of shoots and roots for each plant were measured at harvest. Plants were oven dried during 3 days at 70°C, and then dry weights of shoot and roots were measured. The plants dry weight was also calculated. The relative water content (RWC) was measured for 1 plant from each pot. Immediately after harvest, fresh aerial part of plants were weighed (FW) and placed in distilled water for 24h at 4°C and their turgid

weight (TW) was recorded. The samples were oven dried at 70°C for 3 days and weighed (DW). The RWC was calculated by the formula RWC (%) = ([FW − DW] / [TW − DW]) ×100. Vertically projected leaf area (LA) of each plant was determined by analyzing the photos of ~~–*in*~~ *situ*" plants by Image J software, 20 days after salt application in the first experiment and 14 days after salt treatment in the second one.

2.2.2 Chlorophyll fluorescence parameters

In both experiments chlorophyll content and chlorophyll fluorescence measurements were performed 20 and14 days after salt application for a young and fully expanded leaf from the middle section of the plant in the first and second experiments, respectively. Leaf chlorophyll content was measured by SPAD-502 chlorophyll meter (Konica Minolta, Osaka, Japan). Chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany) after 20-min dark adaptation of the plant. Several chlorophyll fluorescence parameters were studied as following: the potential photochemical efficiency of photosystem II electron transport (ΦP), the actual efficiency of PSII electron transport (ΦPSII), non-photochemical fluorescence quenching (NPQ) and the proportion of closed PSII traps (1-qP). The chlorophyll fluorescence parameter measurements were done according to method described in detail by Maury et al. 1996.

F_0 (minimal fluorescence) and Fm(maximal fluorescence) were determined upon excitation of leaves using a weak measuring light of 0.15 μmol quanta m^{-2} s^{-1} from a light emitting diode and in the form of a 800 ms pulse length at 600 Hz frequency of saturating white light. The fibre optics to sample distance and light intensity was chosen, such that the F_0 value remained under 500 fluorescence unit. The distance from fibre optics to sample was kept constant throughout the experiment. Variable to maximum fluorescence ratio was then calculated by $F_v/F_m = (F_m-F_0)/F_m$ (Schreiber et al., 1994) which represents the efficiency of open PS II. Fs at steady state was measured with active light at ~200 μmol quanta m^{-2} s^{-1}. Actinic light was applied for 2 min 30 sec. Fm‘ was determined by applying a saturation pulse of 10 000 μmol quanta m^{-2} s^{-1}. Fo' has frequently been measured by exposing the leaf at F' to weak far-red light in the absence of actinic light. Quenching coefficients were calculated using the following equations:

Proportion of open PSII, qP = (F_m□-Fs)/ (F_m□-F_0□)

Non-photochemical fluorescence quenching**,** NPQ = (F_m/F_m□)-1

The proportion of closed PSII traps ($1-q_p$)

2.2.3 Na^+ and K^+ content

The amounts of sodium (Na^+) and potassium (K^+) ions were measured in pooled tissues of 3 plants from each replication of each genotype just in the second experiment. Na^+ and K^+ were assayed by flame emission spectrophotometry (Perkin Elmer, Aanalyst 300, Waltham, MA, USA) after nitric acid extraction (HNO3, 0.5%) of the dry shoot and root tissue as described by Munns et al. (2010).

2.3 Molecular methods

2.3.1 DNA extraction

To extract the genomic DNA from a plant, three young leaves are harvested and placed in a well of a plate with 96 wells of 2.5 mL each. Then, 2 big glass beads and 2 small glass beads are added in each well. The leaves were dried at 65°C for 5 days, and ground with the "glass bead grinder" (1 min at maximum power). 500 µL of extraction buffer (2 mL Tris-HCl 1 M (PH=8)+ 0.5 mL NaCl 5 M + 0,5 mL EDTA 0.5 M (PH=8)+ sterile DNase-free water for 10 mL of buffer) are added to each well, the plate is shaken for 15 min and then centrifuged for 20 min at 4000 rpm. The supernatant from each well is transferred to a new plate and centrifuged 20 min at 4000 rpm; again, the supernatant is transferred to a new plate, and 300 µL of isopropanol are added to each well. The plate is shaken and centrifuged 30 min at 4000 rpm. The pellet is dried 5 min under the laminar flow hood and then re-suspended in 100 µL of DNase-free water. The plate should be kept at -20°C.

2.3.2 Polymerase Chain Reaction (PCR)

The PCR mixtures for 1 µL of genomic DNA (diluted 1: 10 in sterile DNase-free water) consists of: 2.5 µL of 10X PCR Buffer (*Interchim, France*), 1.25 µL of $MgCl_2$ 50 mM (*Interchim, France*), 1 µL of each dNTP 1.25 mM (*Promega, Madison*, WI, USA), 1 µL of each primers (*Invitrogen™, France*), suspended in sterile water (25 ng/µL), 0.25 µL of *Taq*-Polymerase and 14 µL of sterile water, for a total of 25 µL. The ordinary reaction conditions

are: 94°C for 4 minutes, followed by 38 cycles of 94°C for 30 seconds, 55°C for 30 sec and 72°C for 30 sec, followed by 72°C for 6 min.

2.3.3 Gel electrophoresis

The amplified PCR products are separated by gel electrophoresis on 3.5% Agarose (2.6% UltraPure Agarose + 0.9% "Low melting" Agarose) in 1XTAE buffer, and stained with ethidium bromide. The reading of the electrophoresis gel was used to design a table of the genotype of each line in the markers' zone, giving the A genotype to the lines that were equal to TN1-11 (the female line, by convention) and B genotype to the lines that were equal to A17 **(**the male line).

2.4 Statistical analysis

2.4.1 Statistical analysis for 6 genotypes in the first experiment

The complete set of data was involved in analysis of variance, using the Statistical Analysis System (SAS 7.02 Institute, Inc 1998), to determine the main effects of genotype, salinity and their interactions. Means comparison between the salt concentrations or genotypes for each studied trait was done with the SNK test. Correlations among different traits in 90 mM salinity conditions were calculated using the means of each genotype in all replications.

2.4.2 Statistical analysis in the second experiment

Data of our split plot design were analyzed, to determine genetic variability of 100 RILs in salinity and control conditions for the plant growth and physiological traits. Means comparison between genotypes for each studied trait was done with LSD test. SAS was also used to analyse the frequency distribution of RILs and their parents for tolerence to salinity. Correlations among different traits in both salinity and control conditions were calculated using the means of each genotype in all replications. Heritability for all the traits in both conditions was also calculated as:

$$h^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)$$

Where: σ_g^2 is the genetic variance and σ_e^2 is the variance of error in the experiments.

2.5 Map construction and QTL detection

A RIL population, named LR7, was derived from the cross between TN1.11 and Jemalong A17 (A17) pure lines. Genetic map of this cross using a population of 192 RILs was constructed using 146 SSR markers based on the *Medicago truncatula* genome sequence assembly (Young et al. 2011), (http://www.medicagohapmap.org/?genome) and unigene set of *Medicago* Gene Index at DFCI (http://compbio.dfci.harvard.edu/). The map covers 470 cM with an average interval between markers of 3.19 cM. PCR conditions, gel electrophoresis and genotype scoring were done as previously reported (Julier et al. 2007). Each linkage group was numbered according to the *M. truncatula* reference map (http://www.medicago.org/genome/map.php) and is presumed to correspond to one of the 8 chromosomes in the haploid *M. truncatula* genome (x=8). QTLs were detected by Multiple QTL Mapping (MQM) (Jansen, 1993; Jansen, 1994) using the ~~"~~qtl" package (Broman, 2003; Arends et al., 2010) of the R system. Threshold values for the LOD scores were empirically determined by computing 1,000 permutations (Churchill & Doerge, 1994). Additive effects of the detected QTL, the percentage of phenotypic variation explained by each one (R^2) as well as the percentage of total phenotypic variation explained (TR^2) were estimated using the MQM program of Rqtl. Map chart 2.1 was used for graphical presentation of linkage groups and map position.

Chapter 3

The effect of salinity stress on morpho-physiological traits of some *Medicago truncatula* genotypes

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Summary:

Among abiotic stresses salinity could be consider as a serious problem affecting plant growth and crop yield (Duzan *et al*., 2004). A major focus of plant breeding efforts in many areas has been to maintain or improve crop productivity in salt-affected environments by selecting salt tolerant genotypes (Epstein, 1985; Ashraf, 1994; Nuccio *et al*., 1999; Pakeeza, 2007). Salt stress can affect numerous plant functions in legume plants, such as seed germination, vegetative growth, and yield (Zhu, 2001). *Medicago truncatula* has an outcrossing rate of less than 3% allowing the generation of highly homozygous genotypes (Li *et al*., 2009). It could be an appropriate model for understanding salt response in legumes.

Significant decrease in shoot biomass of *M. truncatula* genotypes under different saline irrigation was reported (Veatch *et al*., 2004). Lopez (2008a) reported that plant biomass of some *M. truncatula* lines was markedly affected by salt stress conditions (25 and 50 mM NaCl). In susceptible genotypes of *M. truncatula*, salinity stress induces reduction in chlorophyll content and fluorescence parameters as; maximum fluorescence (Fm), variable fluorescence (Fv) and potential photochemical efficiency of photosystem II (Fv/Fm) (Lopez *et al*., 2008b).

To improve the reliability and selection efficiency for salt tolerance, it is necessary to identify the salt-induced characteristic changes in multiple traits among different genotypes. The objectives of this research involved evaluation of some morpho-physiological traits of six *M. truncatula* genotypes, irrigated with saline solutions ranging from 0 to 150 mM NaCl grown in controlled growth chamber conditions. Genotypes are parental lines of some crosses and we tried to identify the important parameters affected by salinity and to determine the convenient salinity concentration as well as the favorable crosses which should be used in genetic studies of tolerance to salinity programs.

The experiment was carried out in a factorial design based on randomized complete blocks with three replications, 2 pots per replication and 4 plants per pot. Six *Medicago truncatula* genotypes including TN1.11, A17, DZA315.16, A20, TN1.21 and F83005.5 used in our

experiment are from different mediterranean countries and present a high genetic variability. Seeds were produced at INRA Montpellier and Ecolab Toulouse-France in greenhouse.

During 35 days after planting, the plants were sub-irrigated once a week. For control treatment, a nutritive solution was used as described by Vadez *et al*., (1996) whereas the iron source was modified by adding 25ml of Fe-EDTA (8.2g /L) per liter. In salinity treatment, NaCl was added just one time to the nutritive solution.

Leaf chlorophyll content was measured by SPAD-502 chlorophyll meter (Konica Minolta, Osaka, Japan). Chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). Vertically projected leaf area (LA) of each plant was determined by analyzing the photos of ~~–in~~ situ" plants 20 days after salt application. The complete set of data was involved in analysis of variance, to determine the main effects of genotype, salinity and their interactions. Means comparison between the salt concentrations or genotypes for each studied trait was done with the SNK test. Correlations among different traits in 90 mM salinity conditions were calculated using the means of each genotype in all replications. Principal component analysis was also carried out for control and 90 mM, NaCl , based on the means of each genotype in all replications for the studied traits.

Abstract:

Among abiotic stresses salinity is considered as a serious problem affecting plant functions especially growth and yield. This research was conducted to study the effect of salt stress on some morpho-physiological parameters in *Medicago truncatula* genotypes and to determine the eventual use of some traits as tolerance criteria. Genotypes including TN1.11, A17, DZA315.16, A20, TN1.12 and F83005.5 are selected through a sequenced lines collection (http://www1.montpellierinra.fr/BRC-MTR/mauguio/mauguio.php) which are originated from different Mediterranean countries. Genotypes were studied under 6 salinity treatments (0, 30, 60, 90,120 and 150 mM NaCl) in a factorial experiment based on randomized complete block design with three replications. Each replication contains 2 pots with 4 plants. Analysis of variance show significant differences among genotypes and salinity levels for most of studied traits. The interaction between genotypes and salt treatments was also significant in most of the studied traits. "DZA315.16" genotype presents the highest main effect values for morphological traits whereas"TN1.11" has low values. Vertically projected leaf area measured 20 days after salt treatment (LA); show the highest variability through all studied salt concentrations. Genotype "TN1.11" has low values for LA in control and also for different salt levels, whereas "DZA315.16" presents the highest values for control and low ones for salt concentration. LA measurement with 90 mM salt concentration should be used for genotype discrimination and recombinant inbred lines of "DZA315.16"x "TN1.11" cross as the favorable population for genetic and genomic studies.

Key words: genotype variability, growth traits, leaf area, salt stress, Chlorophyll content

3.1. Introduction

Abiotic stresses represent the most limiting factors for plant productivity and play a major role in the distribution of plant species across different types of environments. Among abiotic stresses salinity could be consider as a serious problem affecting plant growth and crop yield (Duzan *et al*., 2004). Currently more than 20% of the world‘s irrigated lands are salt-affected. About 60% of salt-affected soils are sodic and alkali soils with limited productivity (Qadir *et al*., 2001). A major focus of plant breeding efforts in many areas has been to maintain or improve crop productivity in salt-affected environments by selecting salt tolerant genotypes (Epstein, 1985; Ashraf, 1994; Nuccio *et al*., 1999; Pakeeza, 2007). Among plant families, legumes contribute significantly to human and animal diets due to their high protein content. They also improve soil fertility through symbiosis with soil bacteria. Salt stress can affect numerous plant functions in legume plants, such as seed germination, vegetative growth, and yield (Zhu, 2001). The heterozygous and outcrossing nature makes it difficult to study the response of genotypes of alfalfa across a range of salinities (Holland and Bingham, 1994; Xuehui and Brummer, 2012). *Medicago truncatula*, an annual relative of alfalfa, has an outcrossing rate of less than 3% allowing the generation of highly homozygous genotypes (Li *et al*., 2009). It could be an appropriate model for understanding salt response in legumes. Significant decrease in shoot biomass of *M. truncatula* under different saline irrigation was reported (Veatch *et al*., 2004). Lopez (2008a) reported that plant biomass of *M. truncatula* was markedly affected by salt stress conditions (25 and 50 mM NaCl). Salinity stress could induce also changes in soluble sugars, amino acids, proline content and other inorganic solutes in shoots of *M. truncatula* (Lopez *et al*., 2008a). In susceptible genotypes of *M. truncatula*, salinity stress induces reduction in chlorophyll content and fluorescence parameters as; maximum fluorescence (Fm), variable fluorescence (Fv) and potential photochemical efficiency of photosystem II (Fv/Fm) (Lopez *et al*., 2008b). Salinity stress causes reduction in plant growth, leaf area and consequently dry matter in rice (Asch *et al*., 2000). Netondo *et al*. (2004) reported that photosynthetic activity decreases when sorghum varieties are grown under saline conditions leading to reduced growth and productivity.

Reduction in chlorophyll fluorescence due to salinity stress was related to the damage of chlorophyll under saline conditions which can be attributed to a decrease in chlorophyll content and activity of photosystem II (Ganivea *et al*., 1998). Salinity can affect chlorophyll content through inhibition of chlorophyll synthesis or an acceleration of its degradation (Reddy and Vora, 1986). The reduction of photosystem II activity induced a decrease in the potential photochemical efficiency of photosystem II (*Fv/Fm*) in sorghum (Netondo *et al*., 2004).

To improve the reliability and selection efficiency for salt tolerance, it is necessary to identify the salt-induced characteristic changes in multiple traits among different genotypes. The objectives of this research involved evaluations of some morpho-physiological traits of six *M. truncatula* genotypes, irrigated with saline solutions ranging from 0 to 150 mM NaCl grown in controlled growth chamber conditions. Genotypes are parental lines of some crosses and we tried to identify the important parameters affected by salinity and to determine the convenient salinity concentration as well as the favorable crosses which should be used in genetic studies of tolerance to salinity programs.

3.2. Materials and Methods

The experiment was carried out in a factorial experiment based on randomized complete blocks with three replications, 2 pots per replication and 4 plants per pot. Six *Medicago truncatula* genotypes including TN1.11, A17, DZA315.16, A20, TN1.21 and F83005.5, used in the experiment are through a sequenced lines collection (http://www1.montpellier inra.fr/BRC-MTR/mauguio/mauguio.php), coming from different Mediterranean countries and present a high genetic variability (Table 3.1). All genotypes are pure lines derived from one plant as single seed descendants. Seeds were produced at INRA Montpellier and Ecolab Toulouse-France in the greenhouse. Recombinant inbred lines populations for some combinations between genotypes are also avalable.

Seeds of these genotypes were scarified by fine sand paper and then cold treated by placing on moist filter paper in a Petri dish and incubated at 4°C for 4 days. After cold treatment, seeds were germinated in the dark at room temperature for 12h. Seedlings with root length of about 4 mm were individually transferred to pots (10 cm diameter and 10cm deep) filled with 3:1 (V: V) of perlite and sand. Four plants of each genotype were grown in a given individual pot under controlled conditions at 25°C, with relative humidity of 80% and photosynthetic photon flux of 200 $\mu mol.m^{-2}.s^{-1}$ with 16 h photoperiod. During 35 days after planting, the

plants were sub-irrigated once a week. For control treatment, a nutritive solution was used as described by Vadez *et al*., (1996) whereas the iron source was modified by adding 25ml of Fe-EDTA (8.2g /L) per liter. In salinity treatment, NaCl was added just one time to the nutritive solution according to different concentrations (0, 30, 60, 90,120 and 150 mM NaCl). Salt treatments were applied 7 days after planting. To avoid the overcoming of NaCl accumulation problem in the substrate, seedlings were sub-irrigated one time with NaCl concentrations for the first seven days and other irrigations were done only with nutritive solution. Irrigation was done with 2L nutritive solution in each tray of 10 pots per week.

Leaf chlorophyll content was measured by SPAD-502 chlorophyll meter (Konica Minolta, Osaka, Japan). Measurements were performed 14 days after salt application for a young and fully expanded leave from the middle section of the plant. Chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany) for the young fully expanded leave after 20-min dark period. The minimum fluorescence (*Fo*) and the maximum fluorescence (*Fm*) following a saturating light pulse were measured. The ratio of variable on maximum fluorescence (*Fv/Fm* where *Fv = Fm-Fo*) was calculated. The *Fv/Fm* ratio represents the potential photochemical efficiency of photosystem II electron transport (Krause, 1988).

Vertically projected leaf area (LA) of each plant was determined by analyzing the photos of ~~–in~~ situ" plants 20 days after salt application. The aerial part and also roots of each plant (35 days old) were collected at harvest, then, photographed using a camera. Fresh weights of aerial part and roots were measured and thus the samples were dried in the oven at 65-70°C for 5 days. Dry weight of aerial part and roots were also measured. Shoot length, root length and vertical projected leaf area (LA) were determined by analyzing the photos of each plant by Image J software. The complete set of data was involved in analysis of variance, using the Statistical Analysis System (SAS 7.02 Institute, Inc 1998), to determine the main effects of genotype, salinity and their interactions. Means comparison between the salt concentrations or genotypes for each studied trait was done with the SNK test. Correlations among different traits in 90 mM salinity conditions were calculated using the means of each genotype in all replications. Principal component analysis was also carried out for control and 90 mM, NaCl , based on the means of each genotype in all replications for the studied traits.

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Analysis of variance for 6 genotypes in different levels of salinity is presented in Table 3.2. Results show that the main effect of genotype is significant for all morpho-physiological traits and chlorophyll fluorescence parameters except maximum fluorescence (*Fm*). This indicates that some of studied genotypes are more tolerant to salinity than others. The main effect of different levels of salinity present significant effects for all of morpho-physiological traits studied. The interaction between genotype and treatment was also significant, for all morpho-physiological traits studied showing that the pattern of salinity effect on all genotypes is not the same.

The main effects of genotypes for all studied traits are presented in table 3.3. –ĐZA315.16"genotype shows the highest values for most of morphological traits whereas –ŦN1.11" has low values. Significant differences are also observed between other studied genotypes. Genetic variability for shoot biomass production in *M. truncatula* was also reported by Veatch *et al*. (2004). Leaf growth in length and area is reported to decrease in susceptible *Mungbean* varieties (Misra *et al*., 1997). In our experiment vertical projected leaf area (LA) which is very easy to measure has a high variability through studied traits and –ĐZA315.16" and –A20" genotypes present the maximum and minimum values respectively (Table 3.3). Our results concerning the main effect of salinity for all studied genotypes are resumed in table 4. Fresh and dry shoot and root weights are affected by salt concentrations from 60 mM to 150 mM. Vertically projected leaf area (LA) was significantly reduced 20 days after salt application with 90 to 150 mM NaCl concentrations (Table 3.4). In our experiment salt treatment present also significant effects on chlorophyll content. Leaf chlorophyll content was affected by salinity in *Brassica juncea* (Qasim, 1998), rice (Sultana *et al*., 1999), tetraploid wheat (Munns and James, 2003) and *Brassica oleracea* (Bhattacharya *et al*., 2004). Salinity can affect chlorophyll content through inhibition of chlorophyll synthesis or an acceleration of its degradation in naked oat (*Avena sativa L*.), (Zhao *et al*., 2007). Decreasing the chlorophyll content was reported as the result of increasing salinity in guar (*Cyamopsis tetragonoloba L*.), (Ashraf *et al*., 2005). In our experiment maximal fluorescence level (Fm) and the potential photochemical efficiency of PS II (Fv/Fm) are significantly reduced with different salinity concentrations compared with control (Table 3.4). Chlorophyll fluorescence could be modified by salinity stress in plants and it can be used for screening tolerant genotypes in plants non-invasively (Baker and Rosenqvist, 2004).

For the most severe salt stress, Fv/Fm decreases significantly in barley (Jiang et al., 2006). Fv/Fm reduced significantly at high salt level in sorghum (Netondo *et al*., 2004). Kafi *et al*., (2009) suggested that only slight inhibition of photosynthesis occurred at high salt level in wheat (*Triticum aestivum*).

Correlations between some of the morphological or physiological traits, for example; shoot fresh weight (SFW) with: shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW) and shoot length (SL) are positives and significant in 90 mM NaCl salt treatment condition (Table 5). Vertical projected leaf area (LA) also present significant correlations with: shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), and root length (RL). The results confirm again that leaf area (LA) which presents high variability for the main effects of genotypes and salt concentrations can be used as selection criteria in salt stress programs. Positives correlations between some morphological traits were also reported by Arraouadi (2012).

We have already mentioned (tables 3.3, 3.4 and 3.5) that vertically projected leaf area (LA) which is easy to determine present high variability for the main effects of genotypes and salinity concentration. Considering the interaction between genotypes and salinity concentrations for leaf area (LA), –TN1.11" has rather low values for control and also for different salt concentrations, whereas –DZA315.16" genotype has the highest values for control and low values for salt treatments (Fig 3.1). –DZA315.16" should be considered as a susceptible genotype, contrary –TN1.11" has not high values in the absence of salinity but it is tolerant to salt concentrations. Ninety mM salt concentration reduced 10% of LA compared with control for –TN1.11" whereas the reduction was about 65% for –DZA315.16" (Fig 3.1). Principal component analysis(PCA), revealed that the two principal components (PC 1and PC 2) represent 82% of the whole variance in control condition and 79% of the whole variance in 90 mM salt concentration(Fig 3.2) . In both conditions, the first component explaining 52% of the initial variance in control and 54% in salinity condition comprised of shoot fresh and dry weight (SFW and SDR), root fresh and dry weight (RFW and RDW) and leaf area (LA) . It shows that, on average, most of growth traits are independent from physiological parameters in both conditions. This association was also observed in the results of correlation too (Table 3.5).The second component explains 30% of the variance in control and 25% in salinity conditions and involved physiological traits. The situation of some traits changes according to salinity or control conditions. For example Fm, Fv/Fm, CC, and RL

present opposite positions respectively. The situation of genotypes is also changed according their resistance and susceptibility to salinity stress as shown in Fig 2.

We can resume that resistance to salinity is not due to one factor but to several morphological and physiological parameters. Leaf area (LA) is an important trait to increase total crop photosynthesis and hence biomass production through increase or extended light interception. Salt tolerant genotypes maintain leaf area (LA) at high levels of salt concentrations (Fig.3.1). Through measured traits vertically projected leaf area 20 days after salt application (LA) which is significantly correlated with growth parameters, should be considered a favorable trait (noninvasive and easy to determine) to select salt tolerant genotypes in *medicago truncatula* .Through studied genotypes "DZA315.16" present high values for control but low ones in salinity concentration where as "TN1.11" has low values in control but it is tolerant to salinity. Recombinant inbred lines (RILs) coming form the cross between these two genotypes with 90 mM NaCl concentration should be used in genetics and genomics programs.

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Table 1. Medicago truncatula genotypes and their origin

Line	Origin
A17b	Unknown
F83005.5 a, b	France
TN1.11 b	Tunisia
TN1.21 b	Tunisia
A20 b	Morocco
DZA315.16 a,b	Algeria

a Lines from the core collection CC16

b Sequenced lines

Table 2. Analysis of Variance (mean square values) for the effect of salinity stress in *M.trancatula* genotypes

Source	df	SFW	SDW	RFW	RDW	SL	RL	LA	CC	F0	Fm	Fv/Fm
Genotype	**5**	**4148.03***	**149.99****	**17836.52****	**161.47****	**158.56***	**1506.35****	**53154.05****	**71.51***	**0.03****	**0.08**	**0.003***
Salinity	**5**	**90345.45****	**1769.11****	**14905.25****	**268.61****	**2428.92****	**1428.69****	**67211.01****	**1452.58****	**0.07****	**3.30****	**0.01****
Genotype ×Salinity	**25**	**11229.08****	**220.40****	**18368.31****	**76.62****	**230.78****	**426.06****	**14304.54****	**84.98****	**0.02****	**0.11***	**0.007****
Error	**70**	**1715.82**	**33.04**	**1534.02**	**20.04**	**62.43**	**164.21**	**1792.09**	**33.03**	**0.008**	**0.05**	**0.001**

*, ** and *** significant at 0.05, 0.01 and 0.001 levels respectively. Shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), root length (RL), leaf area (LA), Chlorophyll content (CC), Minimal fluorescence level (F0), Maximal fluorescence level (Fm), potential photochemical efficiency of PSII (Fv/Fm, relative units)

Table 3. Main effect of six *M. truncatula* genotypes on some morphological and physiological traits in several salt concentrations (0 to 150 mM)

Trait / Genotype	SFW (mg)	SDW (mg)	RFW (mg)	RDW (mg)	SL (mm)	RL (mm)	LA-2 (mm^2)	CC	F0	Fm	Fv/Fm
A17	184.61 b	24.79 c	209.60 b	17.38 cd	57.43 ab	187.91 bc	208.62 b	31.36 b	0.34 b	1.06 a	0.76 b
A20	215.00 ab	27.94 bc	207.13 b	23.66 a	54.52 b	186.82 bc	130.09 d	35.75 a	0.34 b	1.73 a	0.79 a
DZA315.16	225.76 a	32.55 a	270.73 a	19.67 bc	59.61 ab	190.90 b	292.41 a	32.69 ab	0.35 b	1.67 a	0.78 ab
F83005.5	207.33 ab	30.73 ab	214.72 b	22.22 ab	62.66 a	177.51 d	228.36 b	33.01 ab	0.44 a	1.81 a	0.76 b
TN1.11	193.27 b	26.69 bc	191.10 b	16.08 d	56.93 b	203.45 a	229.68 b	33.94 ab	0.34 b	1.69 a	0.77 ab
TN1.12	196.53 ab	26.68 bc	257.45 a	17.49 cd	55.46 b	180.24 cd	180.38 c	30.02 b	0.35 b	1.65 a	0.77 ab

Means followed by the same letter within a column are not significantly different ($P \leq 0.05$). Shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), root length (RL), leaf area (LA), Chlorophyll content (CC), Minimal fluorescence level (F0), Maximal fluorescence level (Fm), potential photochemical efficiency of PSII (Fv/Fm, relative units

Table 4. Main effect of salinity stress on some morphological and physiological traits in *six M. truncatula* genotypes

Trait / salinity	SFW (mg)	SDW (mg)	RFW (mg)	RDW (mg)	SL (mm)	RL (mm)	LA (mm^2)	CC	F0	Fm	Fv/Fm
0	**287.18 a**	**41.18 a**	**244.84 ab**	**24.55 a**	**72.20 a**	**195.93 a**	**283.42 a**	**41.88 a**	**0.42 a**	**2.31 a**	**0.81 a**
30	**295.73 a**	**39.67 a**	**267.75 a**	**23.66 a**	**72.39 a**	**191.25 a**	**257.15 a**	**45.09 a**	**0.45 a**	**2.09 b**	**0.78 bc**
60	**194.55 b**	**27.23 b**	**229.88 b**	**18.30 b**	**55.63 b**	**194.74 a**	**257.15 a**	**32.37 b**	**0.34 b**	**1.68.c**	**0.78 b**
90	**159.20 c**	**19.56 cd**	**190.31 d**	**18.62 b**	**50.08 c**	**191.55 a**	**161.56 b**	**29.54 b**	**0.34 b**	**1.88 c**	**0.76 bc**
120	**132.81 c**	**22.88 c**	**199.53 cd**	**16.21 bc**	**46.74 c**	**174.56 b**	**159.94 b**	**24.98 c**	**0.29 b**	**1.24 d**	**0.76 cd**
150	**153.03 c**	**18.85 d**	**218.41 bc**	**15.17 c**	**49.57 c**	**178.79 b**	**148.33 b**	**22.90 c**	**0.30 b**	**1.30 d**	**0.74 d**

Means followed by the same letter within a column are not significantly different ($P \leq 0.05$). Shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), root length (RL), leaf area (LA), Chlorophyll content (CC), Minimal fluorescence level (F0), Maximal fluorescence level (Fm), potential photochemical efficiency of PSII (Fv/Fm, relative units)

Table 5. Coefficients of correlation between measured traits for six genotype under 90 mM NaCl conditions

	SFW (mg)	**SDW (mg)**	**RFW (mg)**	**RDW (mg)**	**SL (mm)**	**RL (mm)**	**LA (mm^2)**	**CC**	**F0**	**Fm**	**Fv/Fm**
SFW (mg)	1										
SDW (mg)	0.96**	1									
RFW (mg)	0.92**	0.92**	1								
RDW (mg)	0.68**	0.81**	0.84**	1							
SL(mm)	0.87**	0.82**	0.76**	0.46	1						
RL (mm)	0.63**	0.51*	0.42	0.04	0.71**	1					
LA	**0.94****	**0.94****	**0.85****	**0.66****	**0.88****	**0.61****	1				
CC	0.12	0.13	-0.06	0.07	-0.08	0.01	0.17	1			
F0	**0.54***	0.43	**0.63****	0.32	0.41	0.44	0.4	-0.34	1		
Fm	0.12	0.11	0.11	-0.03	0.29	**0.49***	0.29	-0.19	0.26	1	
Fv/Fm	-0.45	-0.34	-0.57*	-0.29	-0.25	-0.23	-0.22	0.27	-0.91**	0.09	1

* and ** significant at 0.05 and 0.01 levels, respectively. Shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), root length (RL), leaf area (LA), Chlorophyll content (CC), Minimal fluorescence level (F0), Maximal fluorescence level (Fm), potential photochemical efficiency of PSII (Fv/Fm, relative units

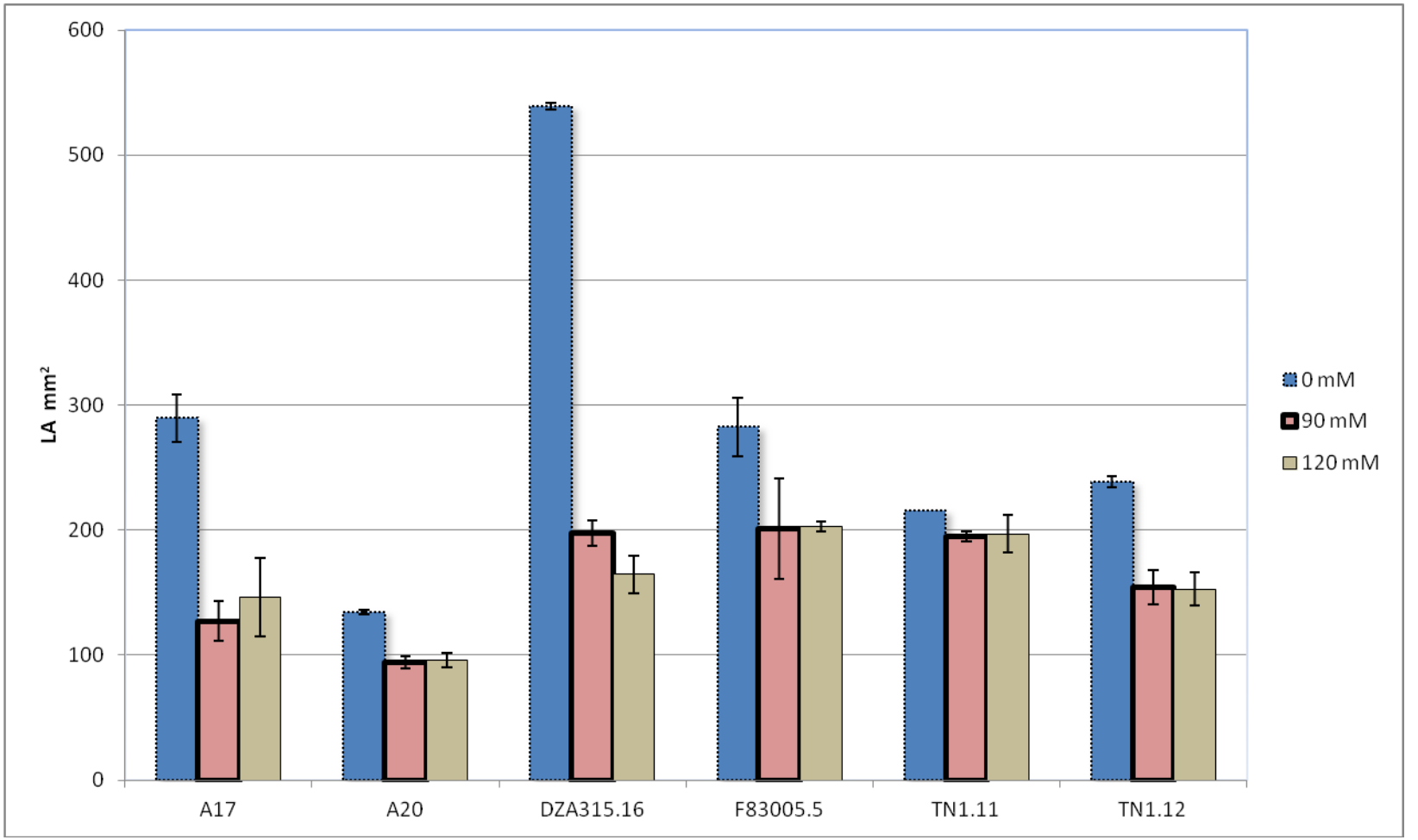

Figure 1. The effect of salt concentrations in six *M. truncatula* genotypes on vertical projected leaf area (LA) measured 20 days after salt treatments

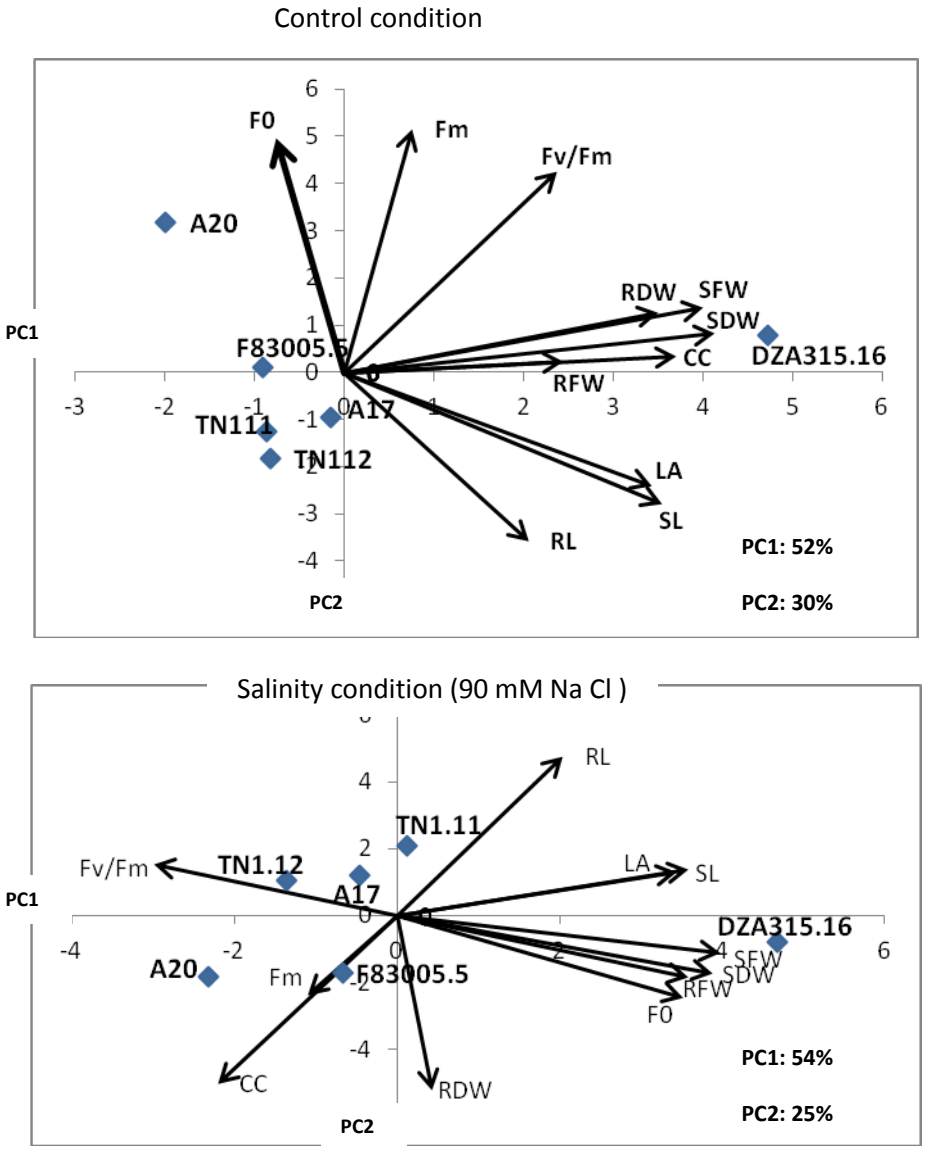

Figure 2. Principal components analysis of morphological and physiological traits for six M. *truncatula* genotypes, under control and salt condition (90 mM NaCl**) .**

PC1 and PC2: First and second principal components. Shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), root length (RL), leaf area (LA), Chlorophyll content (CC), Minimal fluorescence level (F0), Maximal fluorescence level (Fm), potential photochemical efficiency of PSII (Fv/Fm, relative units)

Discussion:

Analysis of variance for 6 genotypes in different levels of salinity show that the main effect of genotype, levels of salinity and their interaction is significant for most of morpho-physiological traits and chlorophyll fluorescence parameters. The interaction shows, that the pattern of salinity effect on all genotypes is not the same. –ÐZA315.16"genotype present the highest values for most of morphological traits whereas –ŦN1.11" has low values. Genetic variability for shoot biomass production in *M. truncatula* was also reported by Veatch *et al*. (2004). In our experiment vertical projected leaf area (LA) which is very easy to measure has a high variability through studied traits and –ÐZA315.16" and –A20" genotypes present the maximum and minimum values respectively. In our experiment maximal fluorescence level (Fm) and the potential photochemical efficiency of PS II (Fv/Fm) are significantly reduced with different salinity concentrations compared with control. Chlorophyll fluorescence could be modified by salinity stress in plants and it can be used for screening tolerant genotypes in plants non-invasively (Baker and Rosenqvist, 2004). Kafi *et al*., (2009) suggested that only slight inhibition of photosynthesis occurred at high salt level in wheat (*Triticum aestivum*).

Correlations between some of the morphological or physiological traits, for example; shoot fresh weight (SFW) with: shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW) and shoot length (SL) are positives and significant in 90 mM NaCl salt treatment condition. Vertical projected leaf area (LA) also present significant correlations with: shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), and root length (RL). The results confirm again that leaf area (LA) which presents high variability for the main effects of genotypes and salt concentrations can be used as selection criteria in salt stress programs. Positives correlations between some morphological traits were also reported by Arraouadi (2012).

Considering the interaction between genotypes and salinity concentrations for leaf area (LA), –ŦN1.11" has rather low values for control and also for different salt concentrations, whereas –ÐZA315.16" genotype has the highest values for control and low values for salt treatments. –ÐZA315.16" should be considered as a susceptible

genotype, contrary –TN1.11" has not high values in the absence of salinity but it is tolerant to salt concentrations. Ninety mM salt concentration reduced 10% of LA compared with control for –TN1.11" whereas the reduction was about 65% for –DZA315.16". Principal component analysis (PCA), revealed that the two principal components (PC 1and PC 2) represent 82% of the whole variance in control condition and 79% of the whole variance in 90 mM salt concentration.

We can resume that resistance to salinity is not due to one factor but to several morphological and physiological parameters. Leaf area (LA) is an important trait to increase total crop photosynthesis and hence biomass production through increase or extended light interception. Salt tolerant genotypes maintain leaf area (LA) at high levels of salt concentrations. Through measured traits vertically projected leaf area 20 days after salt application (LA) which is significantly correlated with growth parameters, should be considered a favorable trait (noninvasive and easy to determine) to select salt tolerant genotypes in *medicago truncatula* .Through studied genotypes –DZA315.16" present high values for control but low ones in salinity concentration where as –TN1.11" has low values in control but it is tolerant to salinity. Recombinant inbred lines (RILs) coming form the cross between these two genotypes with 90 mM NaCl concentration should be used in genetics and genomics programs.

Chapter 4

Genetic variability and identification of QTLs affecting plant growth and chlorophyll fluorescence parameters in the model legume *Medicago truncatula* under control and salt conditions

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Summary:

High NaCl concentrations affect plant morphology, at different levels such as changes in plant architecture, vegetative growth or variations in leaf cuticle thickness. Salinity strongly affects photosynthesis, (Santos et al. 2002), and reduces activity of photosystem II (PSII) (Pérez-López et al. 2012). Such physiological changes result in decreased plant growth and consequently decreased crop yield (Singla and Garg 2005; Tejera et al. 2006). In soybean following exposure to NaCl treatment for one month, the photon saturated photosynthetic rate (PN), the ratio of variable to maximum fluorescence (Fv/Fm), the quantum yield of PSII (ΦPSII), and the electron transport rate (ETR) decreased dramatically (Kao et al. 2003).

Medicago truncatula is widely used as a model plant for legume genetics and genomics. Molecular genetic markers have enabled the identification of quantitative trait loci (QTL) which are involved in the expression of agronomically important traits of *M. truncatula*, such as forage quality (Lagunes Espinoza et al. 2012), flowering date (Pierre et al. 2008) and components of biomass (Veatch et al. 2004; Julier et al. 2007; Lopez 2008a; Espinoza el et al. 2012, Pottorff et al. 2012) or physiological traits (Moreau et al. 2012). Arraouadi et al. (2011, 2012) reported effect of moderate salt stress on aerial parts of *M. truncatula. S*alt treatment reduced significantly leaves and roots K^+ content, whereas Na^+ content increased in leaves and roots.

As far as we know identification of QTLs controlling chlorophyll fluorescence parameters in *M. sativa and M. truncatula* ara not reported in the literature. The present research was undertaken to enlarge our understanding of mechanisms underlying response to salt stress tolerance in this species. The objectives of the research were to study genetic variability for plant growth and key physiological traits for salt tolerance in recombinant inbred lines (RILs) coming from the cross TN1.11 x A17, under control and salt stress conditions, and to map QTLs for the measured traits .

In our first experment, 6 genotypes were tested under salinity concentrations. Results showed that 100mM NaCl should be considered as a favorable concentration to study the tolerance to salinity in *M. truncatula.* TN.1.11 was more tolerant than A17comparing growth traits in control with salinity stress condition. Briefly, a population of 100 recombinant inbred lines (RILs) and their parents (TN1.11 and A17) were grown in the

phytotron under controlled conditions. The experiment was carried out in a Split plot design based on randomized complete blocks with three replications, 2 pots per replication and 4 plants per pot. During 30 days after planting, the plants were sub-irrigated once a week. For control treatment, a nutritive solution was used as described by Vadez et al. (1996). In salinity treatment, 100mM NaCl was added just one time to the nutritive solution. Salt stress was applied 7 days after planting. Irrigation was performed every 7 days. Plants were harvested 30 days after the beginning of the experiment. Fresh weights and dry weights of shoots and roots for each plant were measured. Vertically projected leaf area (LA) of each plant was determined by analyzing the photos of "*in situ*" plants 14 days after salt application by Image J software. Chlorophyll content and chlorophyll fluorescence measurements were performed 14 days after salt application. The amounts of sodium (Na^+) and potassium (K^+) ions were measured in pooled tissues of 3 plants from each replication of each genotype.

Data were analyzed, to determine genetic variability of RILs in salinity and control conditions for the studied traits. Correlations among different traits in both salinity and control conditions were calculated using the means of each genotype in all replications. Heritability for all the traits in both conditions were also calculated

A genetic map of the cross between TN1.11 and A17 using a population of 192 RILs was constructed using 146 SSR markers based on the *Medicago truncatula* genome sequence assembly (Young et al. 2011), (http://www.medicagohapmap.org/?genome) and unigene set of Medicago Gene Index at DFCI (http://compbio.dfci.harvard.edu/). QTLs were detected by Multiple QTL Mapping (MQM) (Jansen, 1993; Jansen, 1994) using the "qtl" package (Broman, 2003; Arends et al., 2010) of the R system.

Abstract

Salinity is one of the major stresses that limits crop production worldwide and affects most of physiological activities in plant. In order to study the genetic control of salt stress in the model legume *Medicago truncatula*, an experiment was undertaken to determine the genetic variability and to identify quantitative trait loci(**QTLs)** controlling several traits related to plant growth and physiology, in a population of recombinant inbred lines (**RILs**). Shoot and root dry weights, relative water content, leaf area, chlorophyll content, chlorophyll fluorescence parameters and $\mathbf{Na}^+$ and $\mathbf{K}^+$ in shoots and roots were measured. The experiment was carried out with three replications. Analyses of variance showed a large genetic variation and transgressive segregation for the traits studied, suggesting putative tolerance mechanisms. A total of 21 **QTLs** were detected under control and 19 **QTLs** were identified under 100mM salt stress conditions. The percentage of total phenotypic variance explained by the **QTLs** ranged from 4.60% to 23.01%. Some of the **QTLs** were specific for one condition, demonstrating that the genetic control of a trait differed under control and salt stress conditions. Some others are non-specific and control a trait in both conditions. Overlapping **QTLs** for different traits were also observed. The results should be helpful information in further functional analysis of salt tolerance in *M. truncatula.*

Keywords: *Medicago truncatula*, Salt stress, Quantitative trait loci, Chlorophyll fluorescence, Na^+ and $\mathbf{K}^+$ content, RILs

Introduction

Intensified by agricultural practices such as irrigation, salinity represents today the major cause of land degradation and plant yield decrease over all regions in the world (Graham and Vance 2003). Salinity in the arid and semi-arid regions of the world as well as in irrigated lands is a serious threat to agriculture, reducing plant growth and crop yields (Duzan et al. 2004). In general, high **NaCl** concentrations affect plant morphology, at different levels such as changes in plant architecture, vegetative growth or variations in leaf cuticle thickness. They also modify physiology and plant metabolism and affects the overall cell metabolic activities like seed germination, nitrogen metabolism, ionic toxicity, stomatal regulation and photosynthesis rate (Edmeades et al. 2001; Santos et al. 2002; Vinocur and Altman 2005). Salt tolerance is thus usually assessed as the percent biomass production in saline versus control condition. During initial exposure to salinity, the whole plant photosynthesis is decreased by restricting leaf area expansion (Netondo et al 2004). In addition, salinity affects photosynthesis per unit leaf area through both stomatal and non-stomatal limitations (Jeranyama et al. 2009, Pérez- López et al. 2012). During long-term exposure to salinity, the premature senescence of adult leaves reduces the photosynthetic area available to support continued growth (Cramer and Nowak, 1992). Such physiological changes result in decreased plant growth and consequently decreased crop yield (Singla and Garg 2005; Tejera et al. 2006). These effects also limit the ability to maintain defense mechanisms (Zheng et al. 2009). Chlorophyll *a* fluorescence parameters are commonly used to study the functioning of photosynthetic apparatus under salt stress. Chlorophyll *a* fluorescence is a sensitive indicator of photosynthetic efficiency in plants and has been proved as a rapid, noninvasive, and reliable method to assess photosynthetic performance under various environmental stresses (Krause and Weis 1991; Schreiber et al. 1994). Photosystem II (PSII) is

more sensitive to all types of stresses compared to PSI (Apostolova et al 2006). The data on the effects of salinity stress on photochemical efficiency of **PSII** are inconsistent. Some studies have shown that salt stress inhibits **PSII** activity in celery and sorghum respectively (Everard et al. 1994; Netondo et al. 2004), whereas others have indicated no effect on the potential photochemical efficiency of **PSII** (Fv/Fm), in hexaploid triticale (Morant-Manceau et al. 2004). Jimenez et al. (1997) reported no significant change in the potential photochemical efficiency of **PSII** (Fv/Fm) in response to **NaCl** treatments, and concluded that **Fv/Fm** was not a useful indicator of salt stress in roses. In contrast, Misra et al. (2001) suggested **Fv/Fm** was an early indicator of salt stress in mung bean and Brassica seedlings. In soybean following exposure to **NaCl** treatment for one month, the photon saturated photosynthetic rate (**PN**), the potential photochemical efficiency of **PSII** (Fv/Fm), and the **PSII** quantum efficiency (**ΦPSII**) decreased dramatically (Kao et al. 2003). Salinity provokes disorders in plant nutrition which may lead to $\mathbf{K}^+$ deficiency and high accumulation of $\mathbf{Na}^+$ in leaves (Mengel and Kirkby 2001). Salinity also delay development and promotes the accumulation of toxic ions ($\mathbf{N}a^+$ and $\mathbf{C}l^-$), that can lead to death of plants before the end of their development in wheat (Munns, 2002). Salt tolerance of the crop is the final manifestation of several components, such as $\mathbf{Na}^+$ and $\mathbf{K}^+$ content, ion balance and ion compartmentation. To keep the $\mathbf{Na}^+$ level low inside a plant cell is not an easy task especially when the external $\mathbf{Na}^+$ levels are high in saline soils. A major toxic ion from saline soil is $\mathbf{Na}^+$ that gets into plant cells through $\mathbf{Na}^+$ permeable transporters (Horie and Schroeder 2004).

Medicago truncatula is widely used as a model plant for legume genetics and genomics by virtue of being an annual, diploid (2n = 16) and autogamous legume with a moderate genome size (500–550 Mbp) (May and Dixon 2004). Molecular genetic markers have enabled the

identification of quantitative trait loci (**QTLs**) which are involved in the expression of agronomically important traits of *M. truncatula*, such as forage quality (Lagunes Espinoza et al. 2012), flowering date (Pierre et al. 2008) and components of biomass like shoot dry weight, root dry weight, length of roots , leaf area or leaf morphology (**Veatch et al. 2004;** Julier et al. 2007; Lopez et al 2008a; Espinoza el et al. 2012, Pottorff et al. 2012) or physiological traits (Moreau et al. 2012). Genetic analyzes were reported for disease resistances (Ben et al. 2013), abiotic stress such as tolerance to drought (Badri et al. 2011), seed germination and pre-emergence growth at extreme temperatures (Dias et al. 2011) and water deficit (Badri et al. 2011; Vandecasteele et al. 2011). For salt tolerance, significant decrease in shoot biomass of *M. truncatula* under different saline irrigation was reported (**Veatch et al. 2004, L**opez et al. 2008a). Salinity stress could induce also changes in soluble sugars, amino acids and proline content in shoots (Lopez et al. 2008b). Arraouadi et al. (2011, 2012) reported the effect of moderate salt stress on aerial parts of *M. truncatula. S*alt treatment reduced significantly leaves and roots $\mathbf{K}^+$ content, whereas $\mathbf{Na}^+$ content increased in leaves and roots in *M. truncatula*. As far as we know identification of **QTLs** controlling chlorophyll fluorescence parameters in *M. truncatula* and in its closely related cultivated crop *M. sativa* is not reported in the literature.

The present research was undertaken to enlarge our understanding of mechanisms underlying response to salt stress tolerance in this species. The objectives of the research were (i) to evaluate genetic variability for plant growth and key physiological traits for salt tolerance in our selected recombinant inbred lines (**RILs**) coming from the cross **TN1.11 x A17**, under control and salt stress conditions, and (ii) to map **QTLs** for the measured traits with the aim to compare the genetic control of traits that may putatively drive whole-plant response to salt stress.

Materials and methods

Plant materials and experimental conditions

In a preliminary study, six genotypes, which are parental lines of recombinant inbred line (**RIL**s) populations, were tested under salinity concentrations varying from 30 to 150 mM NaCl. Results showed that the RIL population coming from the cross **TN1.11xA17 (LR7)** with 100mM NaCl should be considered as a favorable combination to study the tolerance to salinity in *M. truncatula.* **TN1.11** was more resistance than **A17** when comparing growth traits in control with salinity stress. This finding was confirmed by previous data by Zahaf et al (2012) that identified **TN1.11**, an accession sampled in a salty Tunisian soil, as a resistant line with increased *in vitro* root growth under salt stress as well as a differential accumulation of sodium ions when compared to **A17**.

Briefly, a population of 100 recombinant inbred lines (**RILs**) and their parents (**TN1.11** and **A17**) were grown in the phytotron under controlled conditions. The experiment consisted of a split-plot design with three blocks. The main factor consisted of salinity treatment (control and 100mM NaCl) and sub factor consisted of genotypes (RILs and parental lines). The RILs and their two parents were randomized within each treatment-block combination. Two pots per genotype and four plants per pot were used in each bloc. Seeds were scarified by fine sand paper and then cold treated by placing on moist filter paper in a Petri dish and incubated at 4°C for 4 days. After cold treatment, seeds were germinated in the dark at room temperature for 12h. Seedlings with root length of about 4 mm were individually transferred to pots (10 cm diameter and 10cm deep) filled with 3:1 (V: V) of perlite and sand. Four plants of each genotype were grown in a given individual pot under controlled conditions at 25°C, with relative humidity of 80% and photosynthetic photon flux of 200µmol.m^{-2}.s^{-1} with 16 h photoperiod. During 30 days

after planting, the plants were sub-irrigated once a week. For control treatment, a nutritive solution was used as described by Vadez et al. (1996) whereas the iron source was modified by adding 25ml of **Fe-EDTA** (8.2g /L) per liter. In salinity treatment, 100mM **NaCl** was added just one time to the nutritive solution. Salt stress was applied 7 days after planting. To avoid the overcoming of **NaCl** accumulation problem in the substrate, seedlings were sub-irrigated one time with **NaCl** concentration for the first seven days and other irrigations were done only with nutritive solution. Irrigation was performed every 7 days.

Trait measurement

Plant growth

Plants were harvested 30 days after the beginning of the experiment. Fresh weights of shoots and roots for each plant were measured at harvest. Plants were oven dried during 3 days at 70°C, and then dry weights of shoot and roots were measured. The plants dry weight was also calculated. The relative water content (**RWC**) was measured for one plant from each pot. Immediately after harvest, fresh aerial part of plants were weighed (**FW**) and placed in distilled water for 24h at 4˚C and their turgid weight (**TW**) was recorded. The samples were oven dried at 70°C for 3 days and weighed (**DW**). The **RWC** was calculated by the formula **RWC (%) = ([FW − DW] / [TW − DW]) ×100.** Vertically projected leaf area (**LA**) of each plant was determined by analyzing the photos of ~~–~~*~~in~~ situ*" plants 14 days after salt application by Image J software.

Chlorophyll fluorescence parameters

Chlorophyll content and chlorophyll fluorescence measurements were performed 14 days after salt application for a young and fully expanded leaf from the middle section of the plant. Leaf chlorophyll content was measured by **SPAD-502** chlorophyll meter (Konica Minolta, Osaka, Japan). Chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorometer

(PAM-2000, Walz, Effeltrich, Germany) after 20-min dark adaptation of the plant. Four chlorophyll fluorescence parameters were studied as following: the potential photochemical efficiency of photosystem II electron transport (**ΦP**), the actual quantum efficiency of **PSII** electron transport (**ΦPSII**), non-photochemical fluorescence quenching (**NPQ**) and the proportion of closed **PSII** traps (**1-qP**). The chlorophyll fluorescence parameter measurements were done according to method described in detail by Maury et al. (1996).

Na^+ and K^+ content

The amounts of sodium (**N**a^+) and potassium (**K**$^+$) ions were measured in pooled tissues of 3 plants from each replication of each genotype. **Na**$^+$ and **K**$^+$ were assessed by flame emission spectrophotometry (Perkin Elmer, Aanalyst 300, Waltham, MA, USA) after nitric acid extraction (**HNO**3, 0.5%) of the dry shoot and root tissue as described by Munns et al. (2010).

Statistical analysis

Data of the split-plot experiment were analyzed and the mean squares of salinity and genotype effects as well as their interaction were determined. Mean of parents were compared in control and salinity stress separately for each trait and also the percent of each trait in saline versus control was calculated for parental lines and for the mean of RILs. Genetic gain which present the difference between the mean of parental lines and the mean of 10% selected **RIL**s was calculated for salinity and control conditions**.** Heritability for all the traits in both conditions was also calculated as:

$$h^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)$$

Where: σ_g^2 is the genetic variance and σ_e^2 is the variance of error in the experiments. Correlations among different traits in both salinity and control conditions were estimated as $r(xy)=Cov(xy) / (V(x) \times V(y))^{0.5}$, where Cov(xy) is the covariance between traits *x* and *y* and *V* is variance of each trait. Correlations were calculated using the means of each genotype in all

replications. Our results show that heritability is high for all the studied traits and environmental effect is very low as the experiment was realized in phytotron under controlled conditions, so correlations should be considered as genetic correlations.

Linkage map and QTL mapping

A genetic map of the cross between **TN1.11** and **A17** was constructed using a population of 192 **RIL**s and 146 **SSR** markers. Microsatellites (SSR) sequences, regularly positioned along *M. truncatula* 8 chromosomes were identified *in silico* using currently available *M. truncatula* A17 genome sequence data (http://jcvi.org/cgi-bin/medicago/) and SSR markers were developed as described previously (Mun et al 2006). The sequence of the primers used for SSR amplification is given in supplemented Table. A number of these markers had been previously mapped on different *M. truncatula* mapping populations (Ben et al 2013), allowing comparison of map positions between populations. The genetic map was built using the CarthaGene software (de Givry et al 2005). Each linkage group in our map was numbered according to previously published *M.truncatula* maps and is presumed to correspond to one of the eight chromosomes in the haploid *M.truncatula* genome(x=8). The map spans 470 cM with an average distance between markers of 3.28 cM (Table 5).

QTLs were detected by Multiple **QTL** Mapping (**MQM**) (Jansen 1993; Jansen and Stam 1994) using the ~~"~~qtl" package (Broman et al. 2003; Arends et al. 2010) of the R system. Threshold values for the **LOD** scores were empirically determined by computing 1,000 permutations (Churchill and Doerge 1994). Additive effects of the detected **QTL**, the percentage of phenotypic variation explained by each one ($\mathbf{R}^2$) as well as the percentage of total phenotypic variation explained ($\mathbf{TR}^2$) were estimated using the **MQM** program of **R**qtl. Mapchart 2.1 was used for graphical presentation of linkage groups and map position.

Results

Genetic variability for plant growth traits

Results of analysis of variance for the split-plot experiment data are summarized in table 1. Mean squares of genotype, salinity and their interaction effects are significant for all the studied traits.

In both conditions, significant difference between the two parental lines **TN1.11** and **A17** was observed for root weights and leaf area (Table 2). The difference between parents was also significant for shoot and plant dry weights in salinity stress. Salt tolerance, assessed as the percent of a trait in saline versus control condition summarized in Table 2, show that the values of **TN.1.11** for most of growth traits are higher than those of **A17**.The difference between the mean of the **RILs** and the mean of the parents was not significant for most of growth studied traits in both conditions, as expected for F6 RILs, due to the reduction of dominance effect (Table2). Shoot, root and plant dry weights and leaf area were reduced significantly by salt treatment in **RILs** population compared with control. The percent of these traits in saline versus control shows nicely this reduction (Table 2). Relative water content presents a high genetic variability in RIL population for salt treatment and it varies from 49.66% to 89.57% (Table1). Genetic gain expressed by the difference between 10% of selected RILs and the mean of parents for all growing traits is significant (Table 1).

To estimate the importance of measured traits in the description of the observed phenotypic variability between analyzed RILs, heritability was estimated for the traits. Heritability for the growth traits has high values as presented in Table 2. Correlation matrix between the studied traits in control and salinity conditions is shown in Tables 3 and 4. Correlations between all growth traits are positives and significant in both conditions.

Genetic variability for physiological traits

Results of our experiment summarized in Table 1 and 2, show significant effects for most of physiological traits studied. In control conditions, significant difference between the two parental lines **TN1.11** and **A17** was observed for chlorophyll content (**CC**), shoot and root $\mathbf{Na^+}$ and $\mathbf{K^+}$ concentrations. In salt conditions also significant differences between parents were observed for

most of the physiological studied traits. Salinity tolerance expressed by values of these traits *versus* control, presents high or low values. The difference between the mean of the **RILs** and the mean of the parents was not significant for the studied traits except for shoot and root $\mathbf{Na^+}$ concentrations under control conditions. Chlorophyll content (**CC**), potential photochemical efficiency of **PSII** electron transport (**ΦP**), actual quantum efficiency of **PSII** electron transport (**ΦPSII**) and shoot and root $\mathbf{K^+}$ concentrations were reduced significantly by salt treatment in **RILs** population compared with control. Salinity tolerance values [(salinity/control) x100] summarized in Table 2 confirms this reduction. Salt treatment in RILs increased the non-photochemical fluorescence quenching (**NPQ**). **NPQ** ranged from 0.10 to 0.87 in control condition, with the mean value of 0.41, whereas it ranged from 0.28 to 2.83 in salt treatment with the mean value of 0.77. Salt treatment increased the mean value of **RILs** in proportion of closed **PSII** traps (**1-qP**) from 0.22 in control to 0.42 in saline conditions. Salt treatment increased the mean value of **RILs** in shoot and root Na^+ concentrations, from 0.86µmol/mg and 1.64µmol/mg in control to 2.97µmol/mg and 2.51µmol/mg in salinity condition respectively (Table 2).

For most of the physiological traits heritability was high in both conditions (Table 2). Potential photochemical efficiency of PSII electron transport (**ΦP**) and proportion of closed PSII traps (1-**qP**) in control condition and non photochemical fluorescence quenching (**NPQ**), shoot $\mathbf{K^+}$ (**SK**) and shoot $\mathbf{Na^+/K^+}$ (**SNaK**) in salt stress condition presented rather low values for heritability compared with other traits. Genetic gain (10% selected lines- MP) for important physiological traits has significant, positive or negative value, depending on the trait (Table 1).

Correlations between physiological traits (Tables 3 and 4) are in some cases changed according to the two culture conditions. For example the relation between non photochemical fluorescence quenching (NPQ) and the proportion of closed PSII traps (**1-qP**) is not significant in control

(-0.090), but it is positive and significant in salinity condition (0.191*). Also correlation between RK and SK is significant in salinity (0.210*) and non significant in control condition (-0.060).

Quantitative trait loci for growth and physiological traits

The molecular genetic linkage map of ***M***. *truncatula* with eight linkage groups (**LG**) is constructed using **SSR** markers and 192 recombinant inbred lines (**RILs**) of the cross **TN1.11 x A17**. The number of markers in each linkage group, its length and average distance between markers are presented in Table 5. The map contains 146 **SSR** markers which were used for **QTL** identification. The markers with their positions are presented in the left side, and **QTLs** are presented in the right side of linkage groups (Fig. 1). **QTL** mapping showed the presence of several **QTLs** involved in all measured traits. The number of detected **QTLs** varied depending on the traits and salt treatments or control conditions. Some of the detected QTLs (Tables 6 and 7 and Fig 1) are specific and are revealed only in control or in saline conditions (*FP sl.5* and *FPct.4*). Some others are non-specific and control a trait in both saline and control conditions (*LA sl.7* and *LAcl.7, CCsl7 and CCct7 or FPSIIct6 and FPSIIsl6*). Overlapping **QTLs** are also observed which control more than one trait under saline treatment or both conditions (*PDWsl.8, 1-qPsl.8 and SDWsl.8*). A total of 21 **QTLs** were detected under control and 19 under salt condition. **QTLs** involved in tolerence to salt are located on all linkage groups (Table 6,7 and Fig. 1). The phenotypic variance explained by each **QTL** ($\mathbf{R^2}$) ranged from 3.14% to 10.77%, and the percentage of total phenotypic variance ($\mathbf{TR^2}$) varies from 4.62% to 23.01%. The signs of additive effects show that alleles having positive effects for **QTLs** come from both **TN1.11** and **A17** parents for different traits (Tables 6 and 7). The transgressive phenotypes presening genetic gain observed for some traits (Table 1) could be explained by the presence of **QTLs** of opposite sign in the two parents. Their recombination resulted in **RILs** with higher values than

those of their parents, which is explained by various positive gene effects having been accumulated.

Discussion

Genetic variation for plant growth traits

A large genetic variability was observed for all of plant growth traits measured 30 days after planting, under both control and salinity conditions across **RILs** (Table 1 and 2). Genetic variability was also observed in other ***M***. *truncatula* **RIL** populations for some morphological traits (Julier et al. 2007; Espinoza Ldel et al. 2012). This was also evidenced under salt treatment (Arraouadi et al. 2011). In the present study significant differences between the parents under salt stress for shoot, root, plant dry weights and leaf area (**LA**) indicated differential responses to salt stress (Table 2). Significant genotype x treatment interaction suggests that the parental lines respond differently to salt stress and may thus carry different genes for adaptation to salt stress. Tolerance to salinity (**TS**) for growth traits expressed by the percent of the value of a trait in salinity versus control condition is greater for **TN.1.11** than for A17. For example TS for plant dry weight is 86.19% for **TN.1.11** whereas it is only 55.08% in **A17**. This shows that parental line **A17** is more sensitive to salinity stress than **TN.1.11**. The difference between the mean of RILs and the mean of their parents was not significant for most of the traits in both conditions (Table 2), showing that the **RILs** used in our experiment are representative of the possible recombinant lines from the cross **TN1.11 x A17**. Our results show that, shoot and root dry weights for the mean of **RIL**s were reduced significantly in salinity stress (11.52mg and 10.61 mg respectively) when compared with control condition (15.05mg and 12.93mg). Similarly, the mean of RILs plant dry weight (PDW) was also reduced from 27.90mg in control condition to 22.10mg in salt treatment. This phenomenon shows that the relative performance of genotypes

for growth traits in early stage of development reduces on salinity condition. Similar results are reported in other ***M. truncatula*** RIL populations, (**Veatch et al. 2004;** Lopez et al. 2008a; Arraouadi et al. 2011**).** Palma et al, (2013) presented also a significant decrease of **PDW** under salt stress in *M. sativa*. As suggested by Ashraf and Foolad (2007), and Munns and Tester (2008), decrease of plant growth under saline conditions may be due to osmotic reduction in water availability or to excessive ion (particularly $\mathbf{Na^+}$) accumulation in plant tissues. Vertical projected leaf area (**LA**) was reduced by salt stress in our experiment (Table 1 and 2). For example salinity tolerance [(salinity/ control) x100] in RILs population for this trait is 83.59 (Table 2). In a preliminary experiment we found that LA is an important parameter for discrimination of genotypes in salt stress condition. Arraouadi et al. (2011) also reported that leaf area under salt condition decreases in *M. truncatula*. Relative water content (**RWC**) in RIL population ranged from 47.90 to 82.21 in control condition, and from 49.66 to 89.57 in salt treatment (Table 2), indicating that some of the RILs have the ability to sustain their water content under moderate stress. This phenomenon was also reported for the **RILs** population of *M. truncatula* in response to salinity (Arraouadi et al. 2011), and for tobacco in salt stress condition (Yadav et al. 2012).

Heritability for the growth traits (Table2) present high values compared with those reported by Arraouadi et al. (2011) under greenhouse condition. Our experiments were realized in controlled phytotron conditions where the temperature and light were measured and showed stable values, consecutively, with expected lower environment variances. Higher heritabilities, will be obtained compared with greenhouse if the RILs population presents a high genetic variability. As such, it may be also possible that the genetic variability for the traits in LR7 population is higher than those of the LR5 population used by Arraouadi et al (2011). Correlations between growth traits (SDW, RDW, PDW and LA) are positive and significant in both conditions suggesting that salt affect these growth

traits to the same extent. It also suggests that vigor in control conditions is a good predictor of plant vigor in salty environment.

Genetic variation for physiological traits

We reported for the first time several evaluations of photosynthetic parameters in *M.truncatula,* with the idea to better characterize the effect of salt stress onto that key process. Significant differences between the parents under salt stress for potential photochemical efficiency of PSII electron transport (**ΦP**), actual quantum efficiency of **PSII** electron transport (**ΦPSII**) and proportion of closed **PSII** traps (**1-qP**), as well as for shoot and root $\mathbf{Na^+}$ and root $\mathbf{K^+}$ concentrations indicated differential responses to salt stress (Table 2). Significant treatment x genotype interaction suggests that the parental lines carry different genes for adaptation that allow responding differently to salt stress. As described for growth traits, the difference between the mean of **RILs** and the mean of their parents was not significant for most of the physiological traits (Table 2), showing that the **RILs** used in our experiment are representative of the possible recombinant lines from the cross **TN1.11** × **A17** in both conditions. In our experiment Chlorophyll Content (**CC**) and the potential photochemical efficiency of PSII electron transport (**ΦP)** were reduced by salt treatment compared with control. Salt stress affects also photosynthetic components in *Populus euphratica* and *Atriplex centralasiatica* (Ma et al. 1997, Qiu et al. 2003). Misra et al. (2001) in mung bean and Brassica seedlings reported that **ΦP** is an early indicator of salt stress. Reduction in chlorophyll fluorescence due to salinity stress was related to the damage of chlorophyll under saline conditions which can be attributed to a decrease in chlorophyll content and activity of photosystem II in cotton (Ganivea et al. 1998). Actual quantum efficiency of **PSII** electron transport (**ΦPSII**) was reduced in **RILs** by salt treatment from 0.55 to 0.42 in our experiment. Netondo et al. (2004) reported that photosynthetic

activity decreases when sorghum varieties are grown under saline conditions leading to reduced growth and plant biomass. The reduction of **PSII** activity induced a decrease in the potential photochemical efficiency of photosystem II (**Fv/Fm**) in their experiment. Increased **1-qP** values under salt stress condition in our study could be due to closure of **PSII** reaction centers, which is associated to **PSII** inactivation, resulting from a saturation of photosynthesis and other electron sinks by light (Osmond et al. 1993). We have observed that even when thermal energy dissipation occurred via **NPQ**, over-excitation occurred in some **RILs** under salt stress condition resulting in a large genetic variation for **1-qP** (Table 2). Genetic gain expressed by the difference between 10% selected RILs and the mean of parents is positive or negative according the importance of the trait (Table1). Salinity reduced **ΦPSII** du to decrease in the rate of consumption of **ATP** and **NADPH** by photosynthesis (Mohammed et al 1995). These results, in saturation of photosynthesis and other electron sinks by light, and consequently **PSII** inactivation, and increased **1-qP** (Osmond et al. 1993). The chlorophyll fluorescence parameters suggested that the **RILs** genotype differed in their photochemical response to salt stress and this result enlarge our understanding of mechanisms underlying salinity tolerance in *M. truncatula*. As far as we know the genetic variability for the above mentioned fluorescence parameters in *M. truncatula* or *M. sativa* are not reported in the literature. Whether or not differences in chlorophyll fluorescence parameters may be linked to differential growth performances, these parameters have to be studied. The influence of salt stress on this putative relation is also a challenging question.

Shoot and root $\mathbf{K}^+$ concentration in RILs population were reduced significantly from 2.71 and 2.91µmol/mg in control to 1.41µmol/mg and 1.60 µmol/mg in salt condition respectively (Table 2). Salt stress significantly increased shoot and root $\mathbf{Na}^+$ concentrations in RILs from

0.86µmol/mg and 1.64 µmol/mg in control to 2.97µmol/mg and 2.51 µmol/mg in salt condition, respectively (Table 2). This shows that some lines of the RILs population have the capacity to regulate $\mathbf{K}^+$ transport during 30 days salt stress, despite increased $\mathbf{Na}^+$ concentrations. The same results are reported by Arraouadi et al. (2012) for $\mathbf{Na}^+$ and $\mathbf{K}^+$ contents in leaves, stems and roots in this species.

Heritabilities for most of the physiological traits were high in both conditions (Table 2). Potential photochemical efficiency of PSII electron transport (**ΦP**), proportion of closed PSII traps (1-**qP**) in control condition and non photochemical fluorescence quenching (**NPQ**), shoot $\mathbf{K}^+$ (**SK**) and shoot $\mathbf{Na}^+\mathbf{K}^+$ (**SNaK**) in salt stress condition presented rather low values for heritability compared with growth traits. Positive or negative correlations are observed between physiological traits (Tables 3 and 4) which are in some cases changed according the two culture conditions, revealing a strong modification of their relationships, due to salt stress. Positive correlation between potential photochemical efficiency of PSII electron transport (ΦP) and actual quantum efficiency of PSII electron transport (ΦPSII) was found in control (0.211*). Positive correlation between root $\mathbf{Na}^+/\mathbf{K}^+$ (**RNaK**) and root dry weight (**RDW**) suggests that tolerant lines are those which are able to maintain low levels of root $\mathbf{Na}^+/\mathbf{K}^+$ concentration in their roots. Similarly, shoot and root dry weight showed low correlation with $\mathbf{Na}^+$, $\mathbf{K}^+$ concentration and $\mathbf{Na}^+/\mathbf{K}^+$ ratio in rice, in salt conditions (Masood et al. 2004). In *M. truncatula,* Arraouadi et al. (2012) showed positive correlations between ion concentration ratio in root and stem, leaves and root dry weights.

Genetic map

Our genetic map of the cross **"TN1.11x A17"** was constructed using a population of 192 **RIL**s (LR7) which contains 146 **SSR** markers based on the *Medicago truncatula* genome sequence

(Table 5). A genetic map of the cross between **–Jemalong 6 × DZA315.16"** using a population of 199 **RIL**s, (**LR4**), was also constructed which contains 370 SSR markers (Julier et al. 2007,Ben et al,2013). A **RIL** population **(LR5**) was derived from the cross between Jemalong **A17** and **F83005.5** and the RIL population of the cross was genotyped by **SSR** markers and a map with **70 markers** was reported (Arraouadi et al. 2012, Ben et al. 2013). The genetic map of the **F83005.5** × **DZA45.5** cross (**LR3**) was also constructed by Hamon et al. (2010). Our RIL population (LR7) has not been used for mapping by other authors.

Quantitative trait loci analysis for plant growth traits

Results of our experiment concerning identification of **QTLs** controlling growth traits are summarized in Table 6, 7 and Fig 1. Several **QTLs** are involved in response to growth traits under control and salt conditions. In some cases one **QTL** was found to be associated with more than one trait for example on linkage group 8, QTLs for plant dry weight (PDW) and Shoot dry Weight (SDW) are overlapped (Fig 1).They may reflect the same genetic locus controlling components that show phenotypic correlations. Most of overlapping **QTLs** are located on linkage group 7. Julier et al. (2007) and Arraouadi et al. (2012) showed that linkage group 7 contains several **QTLs** involved in the control of important growth traits notably days to flowering. Some of the detected QTLs are specific and control a trait in one condition for example the QTL controlling root dry weight on linkage group 5, (*RDWst.5*). Non-specific or general QTLs are also identified in the present study which control one trait in both salinity treatment and control are identified, as for example the QTL on linkage group 7 for Leaf Area (***LAct.****7* and ***LAsl.7***) (Fig.1). These regions may contain different adaptive genes which are differentially expressed when salt treatment is applied. They may also contain allelic variants for regularity proteins that could exhibit different efficiencies under control or stress conditions.

Correlations between different growth traits confirmed also the existence of overlapping and non-specific QTLs for the traits. Notably the QTLs ***RDWsl.1*** and ***RDWsl.5*** controlling RDW on linkage group1 and 5 are on the same place as the QTL identified by Arraouadi et al. (2011) for RDW.

Quantitative trait loci analyses for physiological traits

Results of our experiment concerning identification of **QTLs** controlling physiological traits are summarized in Table 6, 7 and Figure 1. Several **QTLs** are involved in response of the physiological studied traits under control and salt conditions. In some cases one **QTL** was found to be associated with more than one trait. Under control conditions, the overlapping **QTLs** were located on linkage group 7 for photochemical efficiency of **PSII** electron transport (**ΦP**) and chlorophyll content (**CC**) (***FP****ct.7* and ***CC****ct.7*) (Fig.1). In some cases, one **QTL** is non-specific and controls a trait under two conditions. For example a QTL on linkage group 7 for chlorophyll content (***CCct****.7* and ***CCsl.****7*) and on linkage groups 6 for actual quantum efficiency of **PSII** electron transport (***FPSII****ct.6* and ***FPSII****sl.6*) (Fig.1). This reflects the role for some **QTLs** under different conditions, which may be a cause or consequence of the adaptation to salt stress condition. These regions may content different adaptive genes which are differentially expressed when salt treatment is applied, or allelic variation of important proteins. As far as we know identification of **QTLs** for the above mentioned traits in *M. sativa* and *M. truncatula* are not reported in the literature.

Our results showed that detected **QTLs** for $\mathbf{Na^+}$ and $\mathbf{K^+}$ traits measured in shoots and roots did not share the same map locations, suggesting that the genes controlling the transport of $\mathbf{Na^+}$ and $\mathbf{K^+}$ between the shoots and roots may be different or induced uncoordinatedly by salt stress. A similar conclusion is reported by Arraouadi et al. (2012). In rice, the uptake of $\mathbf{Na^+}$ and $\mathbf{K^+}$ are

independent as the major pathways of Na^+ and K^+ uptake occur in parallel and not directly in competition (Koyama at al. 2001). In control condition, the QTL (***RNaKct.1***) linked to *mte114* marker on linkage group 1 and ***SNact.2*** linked to *mte80* marker on linkage group 2, are also reported by Arraoudi et al (2012). Correlations between different physiological traits are in agreement with relations between identified QTLs.

Conclusions

Salinity reduced plant leaf area and affected photosynthetic apparatus, through a reduction in the potential photochemical efficiency of photosystem II (Fv/Fm). However, the relative importance of the reduction on growth performances of *M. Truncatula* remained to study. The plant shoot growth appeared to be more sensitive to salinity, in comparison with root growth, as indicated by the decrease in shoot to root dry weight ratio in response to salinity. The signs of additive effects show that alleles having positive effects for **QTLs** controlling growth and physiological traits come from both **TN1.11** and **A17** parents. The transgressive phenotypes observed for some traits (Table 1) could be explained by the presence of **QTLs** of opposite sign in the two parents. Their recombination resulted in **RILs** with higher values than those of their parents, which is explained by various positive gene effects having been accumulated. The present study enabled us to investigate the genetic basics of major growth and physiological traits in a RIL population (**TN1.11** x **A17**) where the parental line originated from *a priori* very distinct environments, in particular for salt content. Through 40 QTLs identified in salinity and control conditions, 23 located on linkage groups 1, 6 and 7 are overlapping controlling several traits. Some of these 23 overlapping QTLs are related to both growth and physiological traits. The major output of the **QTL** analysis is identification of relevant genomic regions that may be targeted in genetic and genomic programs to dissect functional traits under salinity in ***M. truncatula***.

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Table 1 Mean squares of analysis of variance and genetic gain for the effect of salinity stress on several traits in recombinant inbred lines (RILs) of *M.trancatula*

	Mean square			Control			Salt stress			
	S	G	I	MP	(M-10% S-RILs)	Genetic Gain	MP	(MP-S/MP-C)×100	M-10% S-RILs	Genetic gain
Shoot dry weight	2005.34**	108.13**	32.18**	16.81	27.31	10.50** (a)	11.55	68.71	18.33	6.78**(a)
Root dry weight	830.97**	55.03**	14.94**	15.47	20.49	5.02**	11.19	72.33	16.6	5.41**
Plant dry weight	5417.71**	299.39**	73.01**	32.38	46.43	14.05**	22.73	70.2	33.97	11.24**
Relative water content	3521.18**	214.58**	150.79**	66.78	78.45	11.67 **	68.85	103.1	86.46	17.61**
Leaf area	207.47**	18.10**	7.24**	176.92	296.07	119.15**	131.36	74.25	222.97	91.61**
CC	7308.76**	146.79**	40.16**	35.83	44.88	9.05**	29.75	83.03	38.87	9.12**
ΦP	0.38**	0.004**	0.004**	0.82	0.86	0.04**	0.76	92.68	0.83	0.07**
ΦPSII	2.59**	0.04**	0.02**	0.55	0.64	0.09**	0.41	74.55	0.59	0.18**
NPQ	19.20**	0.30**	0.21**	0.46	0.16	-0.30**	0.55	119.57	0.36	-0.19 ^{ns}
1-qP	5.84**	0.09**	0.03**	0.24	0.07	-0.17**	0.32	133.33	0.17	-0.15**
Shoot Na^{+} $^{(b)}$	676.96**	0.82**	0.79**	0.65	0.42	-0.23**	2.69	413.85	2	-0.69**
Root Na^{+}	114.18**	1.15**	0.85**	1.94	0.97	-0.97**	2.4	123.71	1.43	-0.97**
Shoot K^{+}	261.15**	0.59**	0.61**	2.55	3.9	1.35**	1.39	54.51	1.87	0.48**
Root K^{+}	52.82**	0.83**	0.61**	2.23	3.15	0.92**	1.6	71.75	2.29	0.69**
Shoot Na^{+}/k^{+}	556.89**	0.90**	1.00**	0.26	0.15	-0.11**	1.97	757.69	1.27	-0.70 ^{ns}
Root Na^{+}/k^{+}	116.48**	0.78**	0.62**	0.89	0.38	-0.51**	1.51	169.66	0.86	-0.65**

S effect of salinity, *G* effect of genotypes (RILs), *I* interaction between salt treatment and RILs **,** and NS* significant at 0.05, 0.01 probability levels and non-significant, *MP* Mean of Parents:(P1+P2)/2, *(M-10%SRILs)* Mean of 10% selected RILs, *(MP-S/MP-C)×100* Percentage of mean of Parents in saline condition versus mean of Parents in control condition, *CC* chlorophyll content, *ΦP* potential photochemical efficiency of PSII electron transport, *ΦPSII* actual quantum efficiency of PSII electron transport, *NPQ* non-photochemical fluorescence quenching, *1-qP* proportion of closed PSII traps, *a* Significant or non significant difference between mean of 10% selected RILs and Mean of Parents, *b* Na and K concentrations in µmol /mg of shoot or root dry weight

Table 2 -Genetic variability for growth and physiological traits in control and salt stress conditions in *M.truncatula* recombinant inbred lines (RILs)

	Control						Salt stress							
	A17	TN1.11	MP	Rils			A17	TN1.11	(A17-S/A17-C) ×100	(TN1.11-S/TN1.11-C) ×100	MP	Rils		
				Range	Mean	h2						Range	Mean	(Ril-S/Ril-C) ×100	h2
Shoot dry weight (mg)	17.76	15.86 $^{ns\ (a)}$	16.81	5.11-35.98	15.05 $^{**(b)}$	0.871	10.03	13.06 $^{**\ (a)}$	56.48	82.35	11.55	3.43-21.100	11.52 $^{ns\ (b)}$	76.54	0.764
Root dry weight (mg)	16.76	14.18 **	15.47	6.16-26.31	12.93 ^{ns}	0.843	9.53	12.84 **	56.86	90.55	11.19	4.86-19.53	10.61 ^{ns}	82.06	0.732
Plant dry weight (mg)	35.51	30.04 ^{ns}	32.38	12.15-62.28	27.99 ^{ns}	0.874	19.56	25.89 **	55.08	86.19	22.73	8.41-39.82	22.13 ^{ns}	79.06	0.811
Relative water content	67.46	66.09 ^{ns}	66.78	47.90-82.21	66.23 ^{ns}	0.682	65.13	72.56 ^{ns}	96.55	109.79	68.85	49.66-89.57	71.09 ^{ns}	107.34	0.684
Leaf area (mm2)	205.5	148.33 **	176.92	63.46-347.44	176.75 ^{ns}	0.913	141.46	121.26 **	68.84	81.75	131.36	55.45-242.27	147.75 ^{ns}	83.59	0.963
CC	33.39	38.26 **	35.83	24.68-46.70	36.16 ^{ns}	0.72	30.96	28.53 ^{ns}	92.72	74.57	29.75	12.00-44.98	29.24 ^{ns}	80.86	0.832
ΦP	0.82	0.81 ^{ns}	0.82	0.73-0.90	0.81 ^{ns}	0.571	0.8	0.72 **	97.56	88.89	0.76	0.62-0.87	0.76 ^{ns}	93.83	0.751
ΦPSII	0.54	0.56 ^{ns}	0.55	0.31-0.67	0.55 ^{ns}	0.934	0.45	0.36 **	83.33	64.29	0.41	0.19-0.61	0.42 ^{ns}	76.36	0.864
NPQ	0.49	0.43 ^{ns}	0.46	0.10-0.87	0.41 ^{ns}	0.732	0.57	0.53 ^{ns}	116.33	123.26	0.55	0.28-2.83	0.77 ^{ns}	187.8	0.381
1-qP	0.26	0.22 ^{ns}	0.24	0.04-0.52	0.22 ^{ns}	0.501	0.41	0.23 **	157.69	104.55	0.32	0.11-0.92	0.42 **	190.91	0.732
Shoot Na^{+} ^{c}	0.76	0.54 **	0.65	0.27-2.76	0.86 **	0.942	2.49	2.89 **	327.63	535.19	2.69	1.73-5.00	2.97 ^{ns}	345.35	0.923
Root Na^{+}	1.41	2.46 **	1.94	0.86-2.84	1.64 **	0.923	2.21	2.59 **	156.74	105.28	2.4	1.07-4.33	2.51 ^{ns}	153.05	0.952
Shoot K^{+}	2.86	2.24 **	2.55	1.59-4.16	2.71 ^{ns}	0.844	1.5	1.27 ^{ns}	52.45	56.70	1.39	0.66-2.17	1.41 ^{ns}	52.03	0.521
Root K^{+}	1.59	2.80 **	2.23	1.28-3.47	2.19 ^{ns}	0.881	1.44	1.75 **	90.57	62.50	1.6	0.50-2.38	1.60 ^{ns}	73.06	0.892
Shoot Na^{+}/k^{+}	0.27	0.24 ^{ns}	0.26	0.12-1.21	0.34 ^{ns}	0.93	1.66	2.28 ^{ns}	614.81	950.00	1.97	0.99-8.43	2.25 ^{ns}	661.76	0.413
Root Na^{+}/k^{+}	0.9	0.88 ^{ns}	0.89	0.31-1.88	0.81 ^{ns}	0.913	1.53	1.48 ^{ns}	170	168.18182	1.51	0.76-4.53	1.69 ^{ns}	208.64	0.852

MP Mean of Parents: (P1+P2)/2, *(A17-S / A17-C) ×100* Percentage of A17 in saline condition versus A17 in control condition, *(TN1.11-S/TN1.11-C) ×100* Percentage of TN1.11 in saline condition versus TN1.11 in control condition, *(Ril-S / Ril-C)×100* Percentage of Rils in saline condition versus Rils in control condition, h^2 broad sense heritability, *CC* chlorophyll content, *ΦP* potential photochemical efficiency of PSII electron transport , *ΦPSII* actual quantum efficiency of PSII electron transport, *NPQ* non-photochemical fluorescence quenching, *1-qP* proportion of closed PSII traps, *a* Significant or non significant difference between A17 and TN1.11. *b* Significant or non significant difference between Mean of Parents and Mean of Rills. *c* Na and K concentrations in µmol /mg of shoot or root dry weight

Table 3 Coefficients of correlation between measured traits in *M.truncatula* under control condition.

	SDW	RDW	PDW	RWC	LA	CC	ϕP	ϕPSII	NPQ	qP	SNa	RNa	SK	RK	SNaK	RNaK
SDW	1.000															
RDW	0.852**	1.000														
PDW	0.981**	0.941**	1.000													
RWC	-0.101	-0.022	-0.070	1.000												
LA	0.530**	0.621**	0.581**	0.241*	1.000											
CC	0.282**	0.380**	0.330**	0.040	0.222*	1.000										
ϕP	0.070	0.091	0.080	-0.110	0.002	0.221*	1.000									
ϕPSII	-0.020	0.002	-0.010	-0.040	-0.090	0.010	0.211*	1.000								
NPQ	-0.030	-0.011	-0.020	-0.020	0.080	-0.160	0.050	0.170	1.000							
qP	0.180	0.142	0.170	-0.010	0.170	0.130	0.070	-0.380**	-0.090	1.000						
SNa	0.002	0.090	0.040	0.212*	-0.050	0.191*	0.050	-0.110	-0.010	0.231**	1.000					
RNa	0.040	0.130	0.080	-0.080	0.191*	0.040	-0.210*	-0.19*	-0.090	0.140	-0.070	1.000				
SK	0.191*	0.190	0.192*	-0.080	0.110	0.080	0.010	-0.040	-0.040	-0.130	-0.040	0.040	1.000			
RK	-0.023	-0.160	-0.080	-0.150	-0.140	-0.080	-0.120	-0.170	0.050	-0.050	-0.280**	-0.030	-0.060	1.000		
SNaK	-0.071	0.010	-0.040	0.211*	-0.090	0.120	0.050	-0.020	0.040	0.221*	0.911**	-0.090	-0.411	-0.250**	1.000	
RNaK	0.032	0.190*	0.090	0.080	0.242**	0.060	-0.060	-0.040	-0.120	0.150	0.140	0.731**	0.090	-0.660**	0.090	1.000

SDW Shoot dry weight, *RDW* Root dry weight, *PDW* Plant dry weight, *RWC* Relative water content , *LA* Leaf area, *CC* Chlorophyll content, φ*P* potential photochemical efficiency of PSII electron transport, φ*PSII* actual quantum efficiency of PSII electron transport, *NPQ* non- photochemical fluorescence quenching, *1-qP* proportion of closed PSII traps, *SNa* Shoot Na^+ concentration , *SK* Shoot K+ concentration , *RNa* Root Na+ concentration, *RK* Root K+ concentration, *SNaK* Shoot Na^+/K^+ concentration, *RNaK* Root Na^+/K^+ concentration, *,** and NS: significant at 0.05, 0.01 probability levels and non-significant.

Table 4 Coefficients of correlation between measured traits in *M.truncatula* under salt stress condition.

	SDW	RDW	PDW	RWC	LA	CC	ϕP	ϕPSII	NPQ	qP	SNa	RNa	SK	RK	SNaK	RNaK
SDW	1.000															
RDW	0.771**	1.000														
PDW	0.950**	0.931**	1.000													
RWC	0.252**	0.170	0.230**	1.000												
LA	0.590**	0.461**	0.571**	0.020	1.000											
CC	0.261**	0.190*	0.250**	0.100	0.261**	1.000										
ϕP	0.190*	-0.010	0.100	-0.010	0.160	0.301**	1.000									
ϕPSII	-0.050	-0.100	-0.080	0.030	-0.080	0.010	0.090	1.000								
NPQ	0.020	-0.040	-0.010	-0.020	0.040	0.050	0.180	-0.080	1.000							
qP	0.040	-0.040	0.000	0.190*	0.060	0.110	0.060	-0.341**	0.191*	1.000						
SNa	0.090	-0.010	0.050	0.040	0.070	-0.030	-0.210*	-0.010	-0.060	0.080	1.000					
RNa	-0.070	0.010	-0.040	-0.070	-0.150	0.010	0.110	-0.160	0.000	-0.060	-0.070	1.000				
SK	0.170	0.241**	0.211*	0.050	0.060	0.090	0.170	-0.070	-0.080	-0.030	-0.230*	0.160	1.000			
RK	0.030	0.060	0.050	0.050	-0.030	-0.070	-0.050	-0.150	-0.180	-0.090	0.010	0.231**	0.210*	1.000		
SNaK	-0.070	-0.150	-0.110	0.010	0.040	-0.110	-0.211*	0.020	0.020	0.080	0.750**	-0.070	-0.751**	-0.140	1.000	
RNaK	-0.070	-0.010	-0.040	-0.040	-0.090	0.110	0.050	0.060	0.130	0.020	-0.060	0.491**	-0.030	-0.660**	0.050	1.000

SDW Shoot dry weight, *RDW* Root dry weight, *PDW* Plant dry weight, *RWC* Relative water content , *LA* Leaf area, *CC* Chlorophyll content , φ*P* potential photochemical efficiency of PSII electron transport , φ*PSII* actual quantum efficiency of PSII electron transport , *NPQ* non- photochemical fluorescence quenching , *1-qP* proportion of closed PSII traps, *SNa* Shoot Na^+ concentration , *SK* Shoot K+ concentration , *RNa* Root Na+ concentration , *RK* Root K+ concentration, *SNaK* Shoot Na^+/K^+ concentration, *RNaK* Root Na^+/K^+ concentration, *,** and NS: significant at 0.05, 0.01 probability levels and non-significant.

Table 5 Marker distribution among the linkage groups

Linkage group	Marker number	Length (cM)	Average distance (cM)
1	28	80.70	2.88
2	17	61.60	3.62
3	15	53.40	3.56
4	14	47.50	3.39
5	25	73.60	2.94
6	13	44.20	3.40
7	18	55.60	3.09
8	16	53.40	3.34
Total	146	470	3.28

Table 6 QTLs detected for growth and physiological traits by Multiple QTL Mapping (*MQM*) in RILs from the cross A17× TN1.11 under control condition

Trait	QTL	Linkage group	Marker	Position (cM)	LOD	Additive effect	R^{2a}	TR^{2a}
Shoot dry weight	SDWct.1	LG1	mt12	32.70-35.00	5.01	2.170	9.00	13.36
Root dry weight	RDWct.1	LG1	mt10	29.50-31.60	4.82	1.550	6.77	14.02
	RDWct.2	LG2	mt44	58.70-61.00	3.38	0.080	8.10	
Plant dry weight	PDWct.1	LG1	mt12	32.70-35.00	5.99	-1.590	8.56	11.77
Relative water content	RWCct.7	LG7	mt114	6.40-10.00	3.88	-1.260	5.77	12.20
Leaf area	LAct.6	LG6	mte39	0-6.14	4.26	-0.660	9.62	16.67
	LAct.7	LG7	mt113	0-7.20	3.56	-0.450	6.24	
CC	Ccct.7	LG7	mt125	48.20-50.50	3.27	1.320	6.35	7.95
ΦP	FPct.4	LG4	mt68	26.60-30.00	5.94	-0.007	5.55	23.01
	FPct.7	LG7	mt125	48.00-50.50	6.26	-0.018	7.57	
ΦPSII	FPSIIct.6	LG6	mt104	17.60-20.50	6.08	-0.020	5.01	14.13
NPQ	NPQct.1	LG1	mt1	0-2.30	3.47	0.031	6.91	13.26
	NPQct.3	LG3	mt50	15.90-200	5.54	0.039	4.06	
1-qP	1-qPct.1	LG1	mt6	8.00-10.10	4.04	0.044	5.95	8.61
Shoot $Na^{+©}$	SNact.2	LG2	mte80	49.20-50.50	4.83	0.130	5.77	13.13
	SNact.6	LG6	mt109	32.00-35.90	4.14	0.180	5.00	13.20
Root Na^+	RNact.1	LG1	mt28	78.00-80.70	3.38	0.150	4.14	7.25
Shoot K^+	SKct.1	LG1	mt8	17.50-20.00	3.62	-0.470	4.97	5.38
Root K^+	RKct.8	LG8	mt133	4.10-7.00	4.80	-0.200	6.89	13.21
Shoot Na^+/k^+	SNaKct.6	LG6	mt109	32.20-35.90	3.51	0.090	4.41	9.53
Root Na^+/k^+	RNaKct.1	LG1	mte114	78.00-80.70	6.31	0.090	8.98	12.98

CC chlorophyll content, Φ*P* potential photochemical efficiency of PSII electron transport, ΦPSII actual quantum efficiency of PSII electron transport, NPQ non-photochemical fluorescence quenching, *1-qP* proportion of closed PSII traps, a: Percentage of individual phenotypic variance explained, b: Percentage of individual phenotypic variance explained by the QTLs given all the covariates, c: Na and K concentrations in µmol /mg of shoot or root dry weight

Table 7 QTLs detected for growth and physiological traits by Multiple QTL Mapping (*MQM*) in RILs from the cross A17× TN1.11 under salt stress condition

Trait	QTL	Linkage group	Marker	Position (cM)	LOD	Additive effect	R^{2a}	TR^{2a}
Shoot dry weight	SDWsl.8	LG8	mte55	42.80-46.10	9.39	1.030	5.39	9.50
Root dry weight	RDWsl.1	LG1	mt8	17.50-20.00	6.13	-0.750	7.15	18.61
	RDWsl.5	LG5	mt87	30.00-39.60	4.00	-0.430	10.39	
Plant dry weight	PDWsl.8	LG8	mt143	45.00-46.10	8.86	1.210	4.20	7.83
Relative water content	RWCsl.3	LG3	mt50	13.00-15.90	6.11	-1.920	8.36	14.00
	RWCsl.5	LG5	mt86	24.00-26.4.00	5.98	-1.420	6.96	
Leaf area	LAsl.7	LG7	mt114	3.00-6.400	5.22	-0.420	6.84	7.19
CC	Ccsl.7	LG7	mt125	48.60-50.50	3.16	1.290	9.75	11.69
ΦP	FPsl.5	LG5	mt94	57.60-60.50	8.68	-0.020	10.77	19.68
	FPsl.7	LG7	mt113	0-6.40	4.48	-0.010	7.23	
ΦPSII	FPSIIsl.6	LG6	mt103	14.00-17.20	12.37	0.034	8.77	11.68
NPQ	NPQsl.4	LG4	mte23	0-4.20	5.27	-0.040	6.46	8.67
1-qP	1-qPsl.8	LG8	mt143	43.00-46.10	4.53	-0.050	3.14	6.61
Shoot $Na^{+©}$	SNasl.7	LG7	mt119	28.70-31.00	4.72	-0.060	6.54	9.35
Root Na^{+}	RNasl.6	LG6	mte67	23.00-26.70	4.90	-0.190	6.71	8.86
Shoot K^{+}	SKsl.1	LG7	mt122	34.90-38.00	4.13	-0.070	5.00	11.45
Root K^{+}	RKsl.4	LG4	mt64	9.00-11.40	4.00	-0.080	4.10	4.93
Shoot Na^{+}/k^{+}	SNaKsl.6	LG6	mte90	4.00-7.20	8.17	0.330	6.93	12.86
Root Na^{+}/k^{+}	RNaKsl.7	LG7	mt122	34.90-38.50	4.36	0.120	3.72	4.62

CC chlorophyll content Φ*P* potential photochemical efficiency of PSII electron transport, ΦPSII actual quantum efficiency of PSII electron transport, NPQ non-photochemical fluorescence quenching, *1-qP* proportion of closed PSII traps, a: Percentage of individual phenotypic variance explained b: Percentage of individual phenotypic variance explained by the QTLs given all the covariates, c: Na and K concentrations in µmol /mg of shoot or root dry weight

LG1
NPQct.1
1-qPct.1
RDWsl.1
SKct.1
RDWct.1
SDWct.1
PDWct.1
RNact.1
RNaKct.1
mt1 0.0
mte75 2.3
mt3 3.8
mte4 4.1
mt5 6.8
mt6 10.1
mt7 15.0
mt8 17.5
mte61 23.9
mt10 31.6
mt11 32.1
mt12 32.7
mt13 37.4
mt14 48.4
mt15 49.4
mt16 51.7
mt17 53.6
mt18 54.8
mt19 56.2
mt20 61.5
mt21 63.0
mt22 67.2
mte6 68.5
mt24 72.8
mt25 73.1
mte88 74.2
mt27 76.0
mte114 80.7
LG2
mt29 0.0
mte10 0.3
mt31 1.5
mte9 5.1
mt33 9.4
mt34 16.3
mte63 20.1
mt36 27.9
mte14 33.3
mt38 39.7
mt39 41.7
mte80 49.2
mt41 50.5
mt42 51.5
mt43 56.6
mt44 58.7
mte16 61.6
SNact.2
RDWct.2
LG3
mte17 0.0
mt47 0.8
mt48 4.3
mte89 11.0
mt50 15.9
mt51 25.3
mt52 28.3
mt53 31.5
mte117 33.0
mt55 33.5
mte20 37.8
mt57 43.5
mt58 48.8
mt59 53.1
mte22 53.4
NPQct.3
RWCsl.3

LG4
LG5
LG6
mte23 0.0
mte24 4.2
mt63 7.1
mt64 11.4
mt65 17.7
mt66 18.8
mt67 25.8
mt68 26.6
mt69 38.2
mte95 41.6
mt71 45.6
mt72 45.8
mt73 46.2
mt74 47.5
NPQsl.4
RKsl.4
FPct.4
mte30 0.0
mt76 0.5
mt77 0.8
mt78 1.6
mt79 2.7
mt80 4.6
mte31 6.2
mt82 8.5
mt83 12.0
mt84 19.6
mte33 22.4
mt86 26.4
mt87 30.0
mte36 39.6
mt89 40.2
mt90 41.3
mt91 41.8
mte238 49.8
mte37 56.5
mt94 60.5
mt95 64.8
mt96 69.4
mte38 71.1
mt98 71.7
mte66 73.6
RWCsl.5
RDWsl.5
FPsl.5
mte39 0.0
mte90 7.2
mt102 10.5
mt103 17.2
mt104 20.5
mt105 20.7
mte67 26.7
mt107 27.5
mt108 33.8
mt109 35.9
mt110 39.0
mt111 40.7
mt112 44.2
LAct.6
SNaKsl.6
FPSIIsl.6
FPSIIct.6
RNasl.6
SNact.6
SNaKct.6

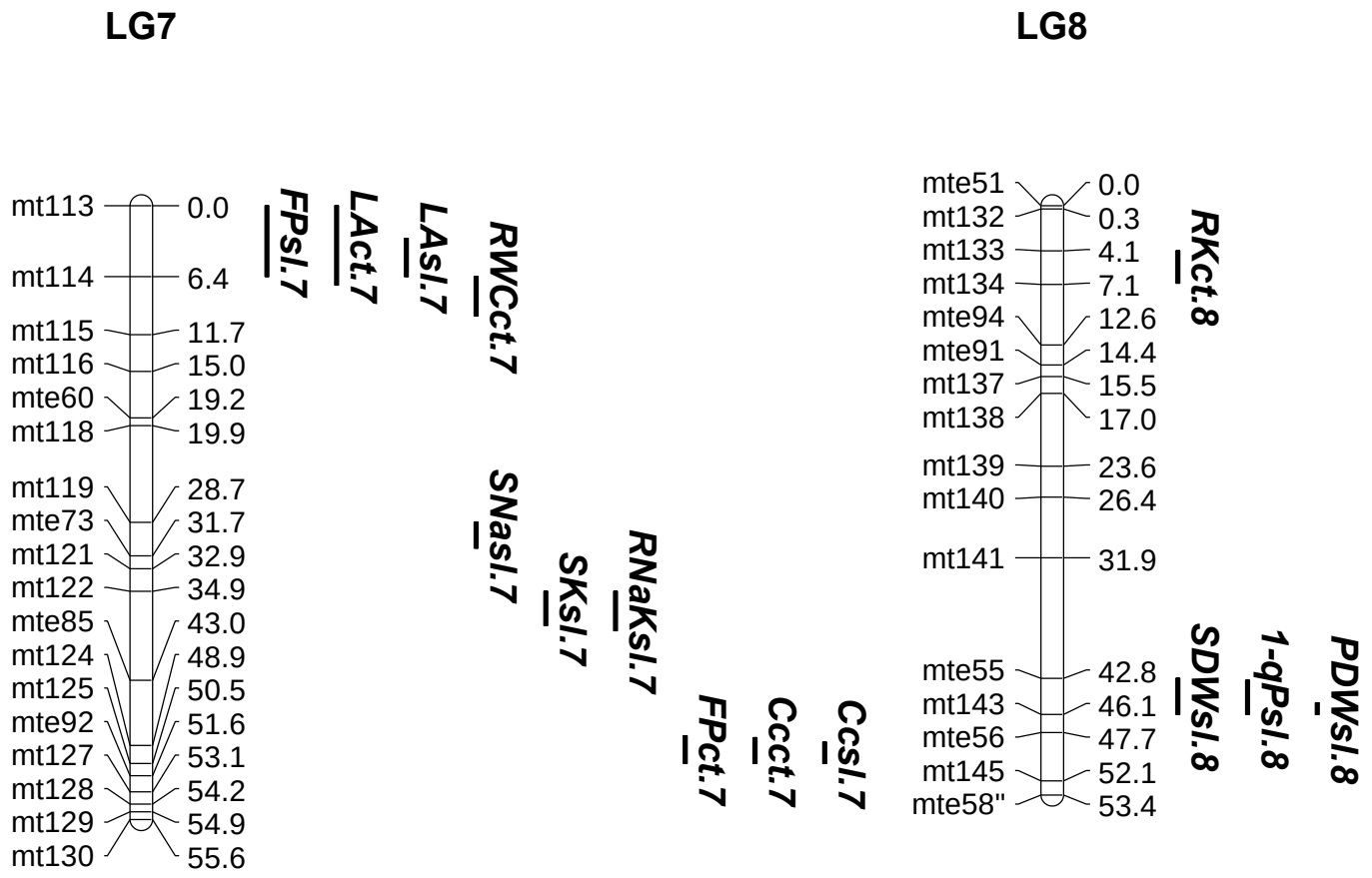

Fig. 1 Linkage map of *M. truncatula* based on 146 SSR markers using *RILs* recombinant inbred lines. The markers with their positions are presented in the left side and QTLs are presented in the right side of linkage groups. The QTLs are designated as *SDW* Shoot dry weight, *RDW* Root dry weight, *PDW* Plant dry weight, *RWC* Relative water content , LA Leaf area, *CC* Chlorophyll content , *FP* potential photochemical efficiency of PSII electron transport , *FPSII* actual quantum efficiency of PSII electron transport , *NPQ* non-photochemical fluorescence quenching , 1-qP proportion of closed PSII traps, *SNa* Shoot Na^+ concentration , *SK* Shoot K+ concentration , *RNa* Root Na+ concentration , *RK* Root K+ concentration, *SNaK* Shoot Na^+/K^+ concentration, *RNaK* Root Na^+/K^+ concentration following treatment (ct, control or sl, salt stressed) and linkage group. Bars represent intervals associated with the QTLs

Supplementary Table SSR markers used for mapping TN1.11 x A17 recombinant inbred lines population.

Name	Position	Linkage group	Alternate name		Forward Primer	Reverse Primer
mt1	0	LG1			TGGTGTCAACGGATCCTACC	ACTTTTTAACCAAGCTAACACACA
mt2	2.3	LG1	mte75	MTE75_mtic981	ATTGTAGGCCACATCATTTC	AACTTCTCAAGTAAAGCCTTTTT
mt3	3.8	LG1		MTE2_mtic68	AATTGCAGCAGCAACAATCA	TCCAAACCTCCCATGGTATC
mt4	4.1	LG1	mte4	MTE4_mtic70	GGTACCACTTTGGTATGGTCGT	TGTCAGGGACGAAGTTGATG
mt5	6.8	LG1		mtic262	CAAATCAAATCCAACAAACA	GTGATCTCTTCCTGGACTGA
mt6	10.1	LG1			CGGCTTCGTTTTTAAGATCC	CCCATGATTTCATCGCAATA
mt7	15	LG1			CTCCACCGCTTTTGACCTAA	TTTGCACATCCAAATCGAAA
mt8	17.5	LG1			TGCATTTTTGCTAACTTGGACA	TGCAGCTTGCTAACTTGCAC
mt9	23.9	LG1	mte61	MTE97_mtic1354	CCATGCAATTGAACAAATAA	CATGTGTCAACAGATCCTGA
mt10	31.6	LG1			AACCAAACAATCTTTGCAGGA	TTTTTGAAGTGTTTCACGGATT
mt11	32.1	LG1			CGCAACAGTCTAAACGGAAG	TCTCTCTCCCCGAGATTCAA
mt12	32.7	LG1			TCCCTCTCATTATTCTCAAAACC	GATCAAACCAGTGCTCACGA
mt13	37.4	LG1			TTTTCCTTAAAACATGGTGCAAT	CGATTGTTGGAGAAAAACAACT
mt14	48.4	LG1			GGCACTCAGTGTCAGTGGTT	CCCACCCCCAGAAATAACTT
mt15	49.4	LG1			TCATTTCCTCGGAAAACTCG	GTTCCAATGCCAAGCTTCTG
mt16	51.7	LG1			AAGTGGGTGTATGTTAGCAAATCTC	GGCGAGTCAACTCCTTACCA
mt17	53.6	LG1			AGGTGAATTGGTGGAGATCG	GAGGTATCCATTTTATGACAAACG
mt18	54.8	LG1			CAAACATGATTGACGACGAGA	TTGAGTGTGAAACGCATTACG
mt19	56.2	LG1			CACCGTACGGAACAAAGACA	TGTGTGATGAGAGAATGAGACTG
mt20	61.5	LG1			CAATCTTCCCACAACCACCT	CCGGATCTACCAAAGAACGA
mt21	63	LG1			GGAATGGAAGCAAGGTCAAA	ATCCCTTCCAACTCCGACTC
mt22	67.2	LG1		mtic640	CATAACTTCCAATAACTGCCA	GGCCCAACCACAATTTC
mt23	68.5	LG1	mte6	MTE6_mtic125	CATTCTTCTGCACCCAATCC	TGAAATTTGAACGCAGAAATCA
mt24	72.8	LG1			CCTCTCCTTCCCCTCATTCT	TCCTCCTCATTCTCCCTCCTA
mt25	73.1	LG1			ATCGTCCCCACTGTGTCTTC	GTGGGGTTGGTGAGAGTGTT
mt26	74.2	LG1	mte88	MTE88_mtic999	CTATAGCCGTTAATCTTGATGATCT	GCATCATCCCCACATCCTAA
mt27	76	LG1		mtic713	AGGGTCGCCTCAACTATTA	TCAACACCATTTTCTCAATG
mt28	80.7	LG1	mte114		CGCTACCAAACCACCTAAGC	TGGATGGTTGAAATGGAGAAG
mt29	0	LG2			ACCAAGCCCCAACTGGTTAT	TTCTTGTCTGGTTGTGTGAGC

Supplementary Table (Continued)

Name	Position	Linkage group		Alternate name	Forward Primer	Reverse Primer
mt30	0.3	LG2	mte10	MTE10_mtic210	CCAAACTGGCTGTGTTCAAA	GCGGTAAGCCTTGCTGTATG
mt31	1.5	LG2		mtic19	TCTAGAAAAAGCAATGATGTGAGA	TGCAACAGAAGAAGCAAAACA
mt32	5.1	LG2	mte9	mtic643	CGCAACACTTTTACATCTTCACTC	TTCGATTTCAGTTCCACCAA
mt33	9.4	LG2		mtic361	AGCTGAAGTGGAACCACCAG	CCCCTAGCTTGAGGAGAGGA
mt34	16.3	LG2		mtic513	TGATGCGATATGAAGAGAGA	AAAATCGGACAAAAGATTAAA
mt35	20.1	LG2	mte63	MTE63_mtic515	GCCCTTAGACACTGGTTATC	TGACGTTGAAGGTAATATTGG
mt36	27.9	LG2			TCATGTTTGTGTCATCAACACTTT	GATCACGCGTTCTCAATCCT
mt37	33.3	LG2	mte14	MTE14_mtic526	CTCCCTTCATAATAGAAAAATAGG	CTCCCATCCATAAATCCTTT
mt38	39.7	LG2			CCCAGCACAAAACAATATCAAA	AGCGCCTCGGTTTCTCAG
mt39	41.7	LG2			CCAACCATCGGTTCCTATTTT	TAGGGCAATTTAGGCTTCCA
mt40	49.2	LG2	mte80	MTE80_mtic1002	TTACAATATTGGGCAAGTCC	AATTTTGGCAATGTCATCTT
mt41	50.5	LG2			CATGCACTTCTTGGAGAGGA	CGTAAGGCATGTGTCATGGT
mt42	51.5	LG2			GATTTGTGGGAGGAACCAAT	TCTGAAATAGAGTTGAGTACATCGAAA
mt43	56.6	LG2			TGTGAACGGAATAAGCACTCA	CCTTGTGTTCATCGAATTTGG
mt44	58.7	LG2			TTAGCAGTACCGTCGGTTTTC	TGCAACAACTCTAGCCACACT
mt45	61.6	LG2	mte16	MTE16_mtic127	ACCATGACAACCCTCCAGTC	TTTGATCTTGTTGCCGAAAT
mt46	0	LG3	mte17	mtic706	CTGATCAATTGAAACAAACG	CTCTCACCCTCTCATTTGAA
mt47	0.8	LG3			TGCACATTAAATTGTGTCAATCA	GGTTCAACTGATGATGAAATGG
mt48	4.3	LG3		mtic742	CGCGAGTTTATACCATGACT	TTCCAGAATGTTCACAATGA
mt49	11	LG3	mte89	MTE89_mtic1050	GATTAAGCATTTATTGGATGTG	ATCATGATCAAAACTATTGC
mt50	15.9	LG3			CATCCAACAATCCCAAGACA	TAGCCGCTGGTTTGATCTTT
mt51	25.3	LG3			CGTCAAATAAATGAGCCGAAC	TTTTTACTCGGTTGAACATTTTT
mt52	28.3	LG3		MTE84_mtic1029	TGGAAGAAGGTTTTTGATTG	CATCTTCCTCTTCCCTCTCT
mt53	31.5	LG3			GTGGTGGTGGTGAGAGAGGT	TCCGACGATGACAACGTAAA
mt55	33.5	LG3		MTE117_mtic1260	TTTAGCTAGTGGCTCTTTGG	TTGGAGGGTCCTACAACTAT
mt56	37.8	LG3	mte20		TTTCATGGGGTAATTGATGAAAC	GAGGCATTTGCCTAGGGTCT
mt57	43.5	LG3		mtic66	CGATCTTCTTCCGCCATAGA	ATTCGTCTGTCCCGACTCTG
mt58	48.8	LG3			TTATTCCACTACGTGGGGTTG	GAGGGCAAACAAACGATGAT
mt59	53.1	LG3			TGCTCCCAATCTAGCATTCC	ATGCGTTTGAGGTTTGAACA
mt60	53.4	LG3	mte22	MTE22_mtic757	AAGCAACATTATCCCCTTTT	CTTCTCTGAGATTTTGAGTGC
mt61	0	LG4	mte23	MTE23-mtic559	GGGTTTTTGATCCAGATCTT	AAGGTGGTCATACGAGCTCC

Supplementary Table (Continued)

Name	Position	Linkage group		Alternate name	Forward Primer	Reverse Primer
mt62	4.2	LG4	mte24	MTE24_mtic703	CTTGGCAAAATGTCAACTCT	GGAAAGGGGTTAGGTGAGTA
mt63	7.1	LG4			TCAACCCGAACCAGATAAGC	TCAAACCTCAACCACAAAACA
mt64	11.4	LG4			GGACTCAGGTCGCAAGAAAA	TTTGGGAAGGTTAAAGCAGGT
mt65	17.7	LG4		mtic89	GGAACAAGAAAGATTTGATTTTG	TTCGAACGAATGAACCAAGA
mt66	18.8	LG4			TGAGTCCGATCAGAGGTTGA	GGAAGTGCAAGGGAACTGAA
mt67	25.8	LG4			GATCGAATCAATTTGAAGAAAAA	CCTGAAAGAATGGAAAAGGTTG
mt68	26.6	LG4			CAGATGCATAGGAGGTGCAA	AGCGGTCGAAATTTCTCTCA
mt69	38.2	LG4			TCGGTGAAAATTTATTGGAGA	AAGCTCACGGGGATAAAAGG
mt70	41.6	LG4	mte95	MTE95_mtic1133	TACGGCTCAACTTTGAGTTT	AGGATGTAGTATCCCTAGATTAGTTC
mt71	45.6	LG4			TTGGTTCAACAATCAAATGAGC	AGAACCTGTGAGCAGAAGCA
mt72	45.8	LG4		MTE93_mtic1267	ACTCCTTTATGCATGCTGTT	ATCAGTTGAAGCACCAATTT
mt73	46.2	LG4		mtic37	AAGAGCAAGCAAGAGGATGC	TCTTGGCCATTACAATATCATCA
mt74	47.5	LG4			CGCAATAATATTTGTTGTTTCTTAAA	CAACTCCTTATCCGCTGTCA
mt76	0.5	LG5			CGTACCATTCACCACATTCG	TGGTGTTTGTGACGGTGTTT
mt77	0.8	LG5			CCACAAGGCACCTAAGAAGG	TGGATGACAAAAAGTGCTGAA
mt78	1.6	LG5			TAGCAAATACAGGGACCAAT	ATGGCTTCATGCGTTAATAC
mt79	2.7	LG5			TTGACCTACCATGAGTTTGACG	AAGCAACAATTAAATTTGTACGTGA
mt80	4.6	LG5			TACCTCGCAAATCAAGCTCA	TCCAAATCCTATTGCCCGTA
mt81	6.2	LG5	mte31	mtic148	TAGTCTCTCTAGTACATGACTAATCT	CTCCCACACAATTTTTCG
mt82	8.5	LG5		mtic562	GCTTGTTCTTCTTCAAGCTC	ACCTGACTTGTGTTTTATGC
mt83	12	LG5			GCAACTTCACGGAATCAAAA	TGATTTGCTGAAGACCCACA
mt84	19.6	LG5			TGCAATAATCGAATTTCAAACAA	TTTGTATGTGCGTGTCTAAATGG
mt85	22.4	LG5	mte33	mtic932	GTTTTGTCAATTTTCGAAGG	TGGGATAAAATTACGACACA
mt86	26.4	LG5			AGTGGAAATCACACCTTGAG	AAAAATGTGGATTGAACCAT
mt87	30	LG5			TGAGCAGTACTCAGAAAATGG	TCTGAACCCGACTCAGAT
mt89	40.2	LG5			AGTGGCCGGGAAATATATGG	GAGTTCAAGGAAGCAATTACCAA
mt90	41.3	LG5			TGACACCAACAACAATCTTT	TGAGGATCTGGTTTCACCTA
mt91	41.8	LG5			GTCCCAAATTGTCAGAAAAA	TCGTCCCAAAATATAAGGAA
mt92	49.8	LG5	mtic238		TCTTACCTTCTCTGGCTCATCA	CAGAAGGGACATCCACACCT
mt93	56.5	LG5	mte37	MTE37_mtic836	ACCCACAAGTGCTATCCTCG	CGCGCCCACATACATACATA
mt94	60.5	LG5			ATGGGTTGTGAAACTCCATA	ATCTCTTTGTGTGGTTTTGC

Supplementary Table (Continued)

Name	Position	Linkage group	Alternate name		Forward Primer	Reverse Primer
mt95	64.8	LG5			GAATTGGGCTATTTTTCTTG	GGTACTTCTTAACATGGTTCCT
mt96	69.4	LG5			TCTTGCCTATCAAAAAGGTGTG	TGGACAATACAATGCGCTCT
mt97	71.1	LG5	mte38	MTE38_mtic732	CTTTTACACGTGCGCTTAAT	GTTTCAAAAACATCTCAACAAA
mt98	71.7	LG5		MTE38_mtic980	GAAGGCATTGATGAAAAGAC	TGTACTTCAAAACCGATCCT
mt99	73.6	LG5	mte66	mtic536	TCACATTAATTATCTTTTCACAA	GGCCAAAACATAAAAATTG
mt100	0	LG6	mte39	MTE39_MTIC831	CTTCACTTGCTTAGCCCTTA	TTCACACTATGTGCACCAAC
mt101	7.2	LG6	mte90	MTE90_mtic1039	CCCTCTCATCTCTCACCATA	GCCACACATTTTAATCTAACG
mt102	10.5	LG6		MTE86_mtic1040	ACCGTACGTGCAGTTATGA	TGGGGGATTGTTGTATTTTA
mt103	17.2	LG6			CGCGGTTTTAGTCACAACCT	TCACAACCGTCTTGCATTTC
mt104	20.5	LG6			TCTGGTACCTCTCTGCATTTGA	TCAAAACACGAAAATGACATGA
mt105	20.7	LG6			TGAAAGATTTAACAATGGTTTCCA	TGATGTATGTGGTTTATAATATGGACT
mt106	26.7	LG6	mte67	mtic759	TCATCACAACCCTAAAGGAC	AACCGGAATCATAAAATCCT
mt107	27.5	LG6			ATGCCAGCAACAAATGGAAT	GTTTGCAAGAGCATCAACCA
mt108	33.8	LG6			GGGAGTTGCGTGCCTATCT	TGCGTGTATCCTAGCAAACCT
mt109	35.9	LG6		mtic711	TTCGGTGTAGCACTTTTTCT	ATGGTTTGGTGTGACAAAAT
mt110	39	LG6			AAACCTCACTCATCTCGCATAG	TTCATTGATAGTGGATTAAAGAGTTG
mt111	40.7	LG6			CCTCGTGGATACGAGTGAGG	CTGCCCTCCCAGTCTCAATA
mt112	44.2	LG6		MTE41_mtic1379	AAGCACGCGAGTCTATAAAA	CCACAATTTCTTTTTCCTTG
mt113	0	LG7			GGAAATTCAATCGCGACCTA	CGGAAAATCGATCGAAAGAG
mt114	6.4	LG7		MTE45_MTIC503	AGCCTGCTCATTTGTATTGC	CAATCACTGGAAGCAAGGT
mt115	11.7	LG7		MTIC147	AATCCTTGATTGCATGGTAG	AAGAGAGTATTCATTCTCACTCTAA
mt116	15	LG7			CCAAAGTGTCAACAGCCAAA	TTTTTCTGAGGAACAAATGACTT
mt117	19.2	LG7	mte60	MTE60_mtic972	AATGACATTTTTCCGTCTTG	TTGATTTCAAGCACAAGCTA
mt118	19.9	LG7			TGGAACAAAGTTTTGTCATTGATT	TCAAAACAATCCTAACCGTCA
mt119	28.7	LG7			CCGAATTGGGAAAGGAACAT	GCATGCACTGAAGGTGAGAA
mt120	31.7	LG7	mte73	MTE73_mtic232	TAAGAAAGCAGGTCAGGATG	TCCACAAATGTCTAAAACCA
mt122	34.9	LG7		mtic635	CCCCAAATCAAACAACACATC	GGGCCACCACTATAAACACC
mt123	43	LG7	mte85	MTE85_mtic1240	CGGAAGAATCAACTTTTTGT	TCATCAAAACATGACGATTG
mt124	48.9	LG7		MTE119_mtic183	AAATGGAAGAAAGTGTCACG	TTCTCTTCAAGTGGGAGGTA
mt125	50.5	LG7		MTE124_mtic509	TTGTGGTGACTAGTGATTGG	ATGTGAAGTAAATCCCTTGC
mt126	51.6	LG7	mte92	MTE92_mtic502	GGATGAAATTGAAAGGAAAACAA	CAACAATCAACTAAGCATACTATTCG

Supplementary Table (Continued)

Name	Position	Linkage group		Alternate name	Forward Primer	Reverse Primer
mt127	53.1	LG7		mtic723	ACCCCTGAACCTTACAGAGT	TATTTCCTGAACCTTTGGTG
mt128	54.2	LG7		MTE48_mtic516	CAAGCTTCAATTCCACAAGT	TGGTGAATTACATGCTCAGA
mt129	54.9	LG7			GAAACCGCCATTGTTAAACC	TCTGTTATTCATCACAATGAGAATTT
mt130	55.6	LG7			GCCAATATATGCTTGGGTAGC	CCACCACCAATCACATACCA
mt131	0	LG8	mte51	MTE51_mtic86	ATGGCAGCTGCTTCAACTTT	CCTCCCCCAAATAACACAAA
mt132	0.3	LG8			GACTAGCGAACTAACAGGTCAATTA	CCACATGTCATTCAAAAAGCA
mt133	4.1	LG8		mtic762	TCGTCTTCTTCCATCATCCC	AATCCACCTCACCAATTCCA
mt134	7.1	LG8			CTTCATGGGTGGCTTCTAGC	TGGAATGATGAAAGTGGGTGT
mt135	12.6	LG8	mte94	MTE94_mtic1130	CCGTAGAACTCCCCTAAAGA	TCCGAATTTTAAAACCATGT
mt136	14.4	LG8	mte91	mtic75	CCGTCCCTCCACGAAACT	TGACATGTATTGTTTATTTTCGTAACA
mt137	15.5	LG8			CACATCGGTGTTCGATCAAG	TCTTGATCAGATGGTCCAAAAA
mt138	17	LG8			ATGGCATGATTGAACCGAAT	TGCAGCTAAATTCAAGGGAGA
mt139	23.6	LG8			CGTATTGCCAGGAAAACACC	GGACTGCTCAAAAGCCTACG
mt140	26.4	LG8		mtic492	GCGAGCCGAAGTGATGATGA	CAACATAAGCCACACACGAAC
mt141	31.9	LG8			ATCAATGGTCCGAATGGTGT	ACCACCGTTCATTCAATCGT
mt142	42.8	LG8	mte55	MTE55_mtic537	CATCAATTTGTCAGTACTTCGGTCAG	TGGGTTCAAGAAGTGGAAGTAAATAAT
mt143	46.1	LG8		mtic750	GGTTCAGGATATGATCCTCTT	ATCCTCTGCTGACAACACTT
mt144	47.7	LG8	mte56	MTE56_MTIC523	TCGACAGTAATACACGCTCA	GTCTGAAAATCATCCAAAGC
mt145	52.1	LG8		mtic1194	GGCAATCATTAGCTTTCAAC	CCTTTTACGAAGCTCTTTGA
mt146	53.4	LG8	mte58	MTE58_mtic80	TGTACATTTTCAACAGACAAAGCA	GCCAAGACTGTGTTTGGTTTC

Discussion:

Genetic variation for plant growth and physiological traits

Analysis of variance for RILs and their parents TN1.11 and A17 in both salinity and control conditions show significant effects for all of growth and physiological traits studied. In both conditions, significant difference between the two parental lines TN1.11 and A17 was observed for most of the studied traits. The difference between the mean of RILs and the mean of the parents was not significant for most of the studied traits. Shoot, root and plant dry weights, leaf area, Chlorophyll content (CC), potential photochemical efficiency of PSII electron transport (ΦP), actual quantum efficiency of PSII electron transport (ΦPSII) and shoot and root K^+ concentrations were reduced significantly by salt treatment in RILs population compared with control. The same results for plant growth traits are reported in other *M. truncatula* RIL populations, **(**Veatch et al. 2004; Lopez 2008a; Arraouadi et al. 2011**).** Palma et al, (2013) presented also a significant decrease of **PDW** under salt stress in *M. sativa.* Arraouadi et al. (2011) also reported that leaf area under salt condition decreases in *M. truncatula***.** Salt treatment increased the relative water content, non photochemical fluorescence quenching (NPQ) and proportion of closed PSII traps (1-qP). Increased **1-qP** values under salt stress condition in our study could be due to closure of **PSII** reaction centers, which is associated to **PSII** inactivation, resulting from a saturation of photosynthesis and other electron sinks by light (Osmond et al. 1993). As far as we know the genetic variability for the fluorescence parameters in *M. truncatula* or *M. sativa* are not reported in the literature. Wheter or not differences in chlorophyll fluorescence parameters may be linked to differential growth performances remained to be studied. The influence of salt strees on this putative relation is also a challinging question.

Heritability for the traits has high values. Correlations between all growth traits are positives and significant in both conditions. For example vertical projected leaf area (LA), is correlated positively with SDW, RDW and PDW in salinity and control conditions. Correlations between physiological traits are in some cases changed according the two culture conditions. For example the relation between RWC and the proportion of closed PSII traps (1-qP) is not significant in control (-0.010), but positive and significant in salinity (0.0.190*). Also correlation between RK and Sk is significant in salinity (0.210*) and non significant in control condition (-0.060).

Quantitative trait loci for growth and physiological traits

The molecular genetic linkage map of *M. truncatula* with is constructed using SSR markers and 192 recombinant inbred lines (RILs) of the cross TN1.11 x A17. The map contains 146 SSR markers. QTL mapping showed the presence of several QTLs involved in all measured traits. Some of the detected QTLs are specific and are revealed only in control or in saline conditions (*FP sl.5* and *FPct.4*), some others are non-specific and control a trait in both saline and control conditions (*LA sl.7* and *LAcl.7, CCsl7 and CCct7 or FPSIIct6 and FPSIIsl6*). Overlapping QTLs are also observed which control more than one trait under saline treatment or both conditions (*PDWsl.8, 1-qPsl.8 and SDWsl.8*). Most of overlapping **QTLs** are located on linkage groups 7 (Fig.1). Julier et al. (2007) and Arraouadi et al. (2012) showed that linkage group 7 contains several **QTLs** involved in controlling important traits. A total of 21 QTLs were detected under control and 19 under salt condition. The phenotypic variance explained by each QTL (R^2) ranged from 3.14% to 10.77%, and the percentage of total phenotypic variance (TR^2) varies from 4.62% to 23.01%. The signs of additive effects show that alleles having positive effects for QTLs come from both parents for different traits. The transgressive phenotypes observed for some traits could be explained by the presence of QTLs of opposite sign in the two parents. Their recombination resulted in RILs with higher values than those of their parents, which is explained by various positive gene effects having been accumulated. Our results showed that detected QTLs for Na^+ and K^+ traits measured in shoots and roots did not share the same map locations, suggesting that the genes controlling the transport of Na^+ and K^+ between the shoots and roots may be different or induced uncoordinatedly by salt stress. A similar conclusion is reported by Arraouadi et al. (2012).

Chapter 5

CONCLUSION ET PERSPECTIVES

Conclusion:

Afin d'améliorer la fiabilité et l'efficacité de la sélection pour la tolérance à la salinité, il est nécessaire d'identifier les changements induits par les solutions salines sur les caractères agronomiques et physiologiques de génotypes d'une espèce végétale. Cette étude a été menée dans le but d'enrichir notre compréhension des bases génétiques de la tolérance à la salinité chez *Medicago truncatula*. Dans la première étape, l'objectif a été d'étudier l'effet d'une large gamme de concentrations 0 à 150 mM - NaCl, sur des traits morphologiques et physiologiques majeurs chez six génotypes de *M. truncatula* cultivés dans des conditions contrôlées (chambre de culture). Ces génotypes d'origines géographiques variées sont des lignées parentales de différentes populations de lignées recombinantes disponibles dans notre laboratoire. Cette première expérimentation nous a permis d'identifier des traits de tolérance à la salinité ainsi que les conditions de phénotypage (concentration optimale en NaCl) et un croisement d'intérêt pour étudier les bases génétiques de la tolérance à la salinité chez cette espèce.

Dans une deuxième expérimentation, nous avons étudié la variabilité génétique des traits morpho-physiologiques clefs pour la tolérance à la salinité chez une population de lignées recombinantes (RILs) issues du croisement « TN1.11 x A17 », dans les conditions témoin (eau) et en stress salin. En utilisant notre carte génétique, nous avons ensuite identifié et cartographié les QTLs associés à ces traits. Les résultats les plus importants sont :

1-L'effet du stress salin sur les paramètres morphologiques et physiologiques des génotypes de *Medicago truncatula.*

Les résultats des analyses de variance sur 6 lignées parentales traitées avec différentes concentrations de NaCl (0 à150 mM) montrent que certains génotypes sont plus tolérants à la salinité que d'autres. L'effet de la salinité est significatif pour tous les traits étudiés. L'interaction entre les génotypes et les traitements est également significative. La lignée ―DZA315.16" a montré les valeurs les plus élevées pour les traits morphologiques alors que ―TN1.11" a les valeurs les plus faibles. La variabilité génétique pour la production de biomasse foliaire en réponse à la salinité chez *M. truncatula* a été également observée par Veatch et al. (2004). La gamme de concentration saline la plus adaptée pour identifier des différences génotypiques est comprise entre 90 et 120 mM NaCl. La surface foliaire - verticalement projetée de la plante, qui est facile à mesurer (mesure rapide et non

destructrice), montre une forte variabilité. Ce trait est également significativement corrélé à la production de biomasse et constitue un trait d'intérêt pour le phénotypage de la tolérance à la salinité. –DZA315.16" et –A20" ont respectivement les valeurs maximales et minimales pour ce caractère. Une variabilité importante de la teneur en chlorophylle des feuilles selon les concentrations de NaCl a été observée. A notre connaissance, l'effet de la salinité sur ce caractère chez *M. truncatula* n'a pas été étudié. La teneur en chlorophylle foliaire est affectée par la salinité chez *Brassica juncea (Qasim, 1998*), le riz (Sultana et al., 1999), le blé (Munns and James, 2003) et chez *Brassica oleracea* (Bhattacharya et al., 2004). La salinité peut affecter la quantité de chlorophylle en inhibant la synthèse de chlorophylle ou en accélérant sa dégradation, comme cela a été observé chez l'avoine (*Avena sativa L*.) (Zhao et al, 2007). Le niveau de fluorescence maximal (Fm) et l'efficience photochimique potentielle du PS II (Fv/Fm) sont significativement diminués par les différentes concentrations de NaCl utilisées dans notre étude par rapport au témoin. Le Fv/Fm est significativement diminué chez l'orge (Jiang et al., 2006) et le sorgho (Netondo et al., 2004) pour des stress salin marqués. A notre connaissance, la réponse de ces caractères physiologiques au stress salin n'a pas été étudiée *chez M. truncatula.*

Les corrélations entre quelques traits morphologiques et physiologiques sont positives et significatives dans la condition de culture avec 90 mM NaCl. La surface foliaire de la plante montre une corrélation significative avec la production de biomasse: les poids frais et sec de la partie aérienne et les poids frais et sec des racines. Des corrélations positives entre certains traits morphologiques chez *M. truncatula* ont été également observées par Arraouadi (2012). En considérant les interactions entre génotype et concentration de NaCl pour la surface foliaire, la lignée –TN1.11" montre des valeurs faibles dans la condition contrôle (eau) mais aussi dans les différentes concentrations salines, alors que –DZA315.16" présente la valeur la plus élevée pour la condition contrôle et une faible valeur pour les traitements salins. La lignée A17 présente une réponse à la salinité comparable à –DZA315.16". Ces deux génotypes peuvent être considérés comme des génotypes « sensibles » en terme de réponse au stress salin, en revanche, –TN1.11" est « tolérant » à la salinité. Les populations de RILs issue de croisements entre lignées contrastées (sensible vs tolérante) peuvent être des populations d'intérêts pour des études génétiques et génomiques sur la tolérance au stress salin chez

M. truncatula. L'analyse en composantes principales montre que la plupart des traits morphologiques sont indépendants des paramètres physiologiques, et ceci dans les deux conditions. Ce résultat suggère qu'il est nécessaire de prendre en compte ces différents traits une évaluation plus complète des performances d'un génotype en réponse à la salinité.

2-Développement de la carte génétique

Une carte génétique du croisement entre TN1.11 et A17, en utilisant une population de 192 RILs, a été construite grâce à 146 marqueurs SSR. Les marqueurs SSRs ont été sélectionnés sur la base de l'assemblement de séquences du génome (Young et al. 2011), (http://www.medicagohapmap.org/?genome) et l' unigène de "*Medicago* Gene Index" (http://compbio.dfci.harvard.edu/). Chaque groupe de linkage a été numéroté selon la carte référence *de M. truncatula* , présumé pour correspondre aux 8 chromosomes du génome haploide de *M. truncatula* (x=8).

3- La variabilité génétique de traits physiologiques et morphologiques chez la plante modèle de la légumineuse *Medicago truncatula* dans les conditions contrôle et de stress salin.

Une grande variabilité génétique a été observée pour tous les traits morpho-physiologiques étudiés chez les RILs dans les conditions témoin et de stress salin. La variabilité génétique a été observée chez d'autres populations de RILs de *M. truncatula* pour des traits morphologiques en l'absence de stress salin (Julier et al. 2007, Espinoza Ldel et al. 2012) et en condition saline (Arraouadi et al. 2011). Des différences significatives entre les parents dans la condition de stress salin suggèrent que les lignées parentales ont des gènes différents pour l'adaptation au stress salin. La différence entre la moyenne des RILs et celle des parents n'est pas significative pour tous les traits étudiés dans les deux conditions, ce qui montre que la population de RILs utilisées dans notre étude est représentative de toutes les lignées recombinantes issues du croisement « TN1.11 x A17 ». Les poids secs foliaire et racinaires moyens de la population de RILs sont significativement diminués en réponse au stress salin. Des résultats similaires ont été reportés sur d'autres populations de RILs (Veatch et al. 2004**;** Lopez 2008a; Arraouadi et al. 2011).

La surface foliaire verticalement projetée de la plante est réduite en réponse à la contrainte saline. Dans notre première expérimentation, nous avons observé que ce

caractère est un trait important pour différencier les génotypes en condition de stress salin. Arraouadi et al. (2011) ont aussi observé une diminution de la surface foliaire en condition de stress salin chez *M. truncatula*. Les paramètres de fluorescence de la chlorophylle suggèrent que les RILs diffèrent dans leur réponse photochimique à la salinité. Ces résultats ont permis d'élargir notre compréhension des mécanismes impliqués dans la tolérance au stress salin chez *M. truncatula*. A notre connaissance, la variabilité génétique des paramètres de fluorescence chez *M. truncatula* ou *M. sativa* n' est pas rapportée dans la littérature. Le stress salin augmente significativement la concentration en ions Na^+dans les racines et la partie foliaire. Les résultats montrent aussi la capacité de certaines lignées de la population de RILs à réguler la teneur en ions K^+ en condition de stress salin, malgré l'augmentation de la concentration en ions Na^+. Les mêmes résultats concernant Na^+ et K^+ dans les feuilles, la tige et les racines ont été obtenu par Arraouadi et al. (2012). L'héritabilité pour les traits étudiés présente des valeurs élevées comparées à celles observées par Arraouadi et al. (2011). Dans notre étude, les expérimentations ont été menées dans un phytotron avec des conditions contrôlées et sous une faible variabilité environnementale, comparativement aux travaux conduits en serre par Arraouadi et al. (2011 et 2012).

4- L'analyse QTL de traits morpho-physiologiques chez *Medicago truncatula*

Les signes des effets additifs montrent que les allèles qui, ont un effet positif pour les QTLs contrôlant les traits morpho –physiologiques, sont issus des deux parents « TN1.11 » et « A17 ». Les phénotypes transgressifs observés pour certains traits pourraient expliquer la présence de QTLs de signes opposés chez les deux parents. Leur recombinaison chez certaines lignées de la population de RILs aboutit à des lignées ayant des valeurs plus élevées par rapport à celle de leurs parents. Ce résultat peut être expliqué par l'accumulation des allèles favorables venant des deux parents chez les recombinants. Nous avons détecté 40 QTLs au total dans les conditions témoin et en stress salin. La plupart des QTLs qui se chevauchent sont situés sur le groupe de linkage 7. Julier et al. (2007) et Arraouadi et al. (2012) ont montré que ce groupe de linkage contient plusieurs QTLs contrôlant des caractères importants chez *M. truncatula*. De nombreuses co-localisation de QTLs sont observées, et 23 QTLs localisés dans les groupes de linkage 1, 6 et 7 se chevauchent. Parmi ces 23 QTLs, certains contrôlent deux ou trois traits

différents. En revanche, nos résultats ont montré que les QTLs détectés pour les concentrations de Na^+ et K^+ dans les feuilles et les racines ne sont pas localisés aux mêmes endroits sur les groupes de liaisons. Des résultats similaires ont été obtenus par Arraouadi et al. (2012).

Notre étude a été réalisée à partir d'une population de RILs chez laquelle les lignées parentales (TN1.11 et A17) sont originaires d'environnements très différents, en particulier pour la salinité du sol. Les résultats nous ont permis d'identifier les bases génétiques de traits majeurs de la croissance et de la physiologie chez cette espèce. Le résultat majeur de l'analyse des QTLs est l'identification de régions génomiques qui peuvent être ciblées par des études génétiques ou génomiques pour étudier des traits fonctionnels en condition de stress salin. Nos résultats fournissent une importante information pour de futures analyses fonctionnelles des gènes de la tolérance à la salinité chez *M. truncatula*.

Perspectives :

Ce travail pourrait être développé par :

1- Application à la sélection de génotypes d'intérêts :

Dans notre première expérimentation, la Lignée _DZA315.16' présente des valeurs élevées pour les traits étudiés (en particulier la croissance foliaire) en absence de salinité, mais des valeurs faibles pour toutes les concentrations de NaCl utilisées. La lignée "TN1.11" présente un comportement contrasté, avec de faibles valeurs pour ces traits dans la condition de contrôle, mais peu affectées par la salinité, ce qui montre que cette lignée peut être considérée comme tolérante à la salinité. La création d'une population de lignées recombinantes (RILs), à partir de croisements entre ces deux lignées « résistante » et « sensible », est en cours dans notre laboratoire. L'étude génétique de cette population permettra d'identifier des lignées nouvelles possédant les caractères intéressants de leurs deux parents. Les lignées sélectionnées peuvent être à leur tour utilisées dans des travaux génétiques et génomiques de *M. truncatula*.

2- Compréhension des différences génotypiques pour la tolérance à la salinité

2-1. Afin de vérifier la stabilité des zones du génome impliquées dans l'expression des caractères liés à l'adaptation à la salinité, il serait nécessaire de réaliser des expérimentations complémentaires dans différents environnements pédoclimatiques et

dans d'autres fonds génétiques, ce qui permettrait de valider les QTLs constitutifs et les QTLs spécifiques aux environnements.

2-2. Le croisement entre des RILs, présentant des comportements contrastés en situation de salinité et un polymorphisme pour des QTLs intéressants, permettrait, par la recombinaison génétique, d'identifier les marqueurs étroitement liés aux QTLs d'intérêt.

2-3.La cartographie fine et la réalisation de contig de BAC au voisinage des QTL permettrait d'envisager le clonage positionnel.

2-4.L'identification de gènes candidats pour les caractères d'adaptation étudiés, leur co-localisation avec les QTLs et enfin, leur validation à partir d'études d'association en populations naturelles fourniront des outils de grand intérêt pour l'étude de la diversité adaptative intra et interspécifique chez *Medicago truncatula.*

2-5. L'analyse de l'expression d'un nombre de gènes à l'aide de technique microarray peut aider à mieux comprendre les mécanismes impliqués dans la réponse au stress salin, ainsi qu' au développement de modèles de cultures à paramètres génétiques et d'outils pour l'aide à la sélection de génotypes d'intérêt chez cette espèce et principalement l'espèce voisine *Medicago sativa*.

Conclusion:

To improve the reliability and selection efficiency for salt tolerance, it is necessary to identify the salt-induced characteristic changes in multiple traits among different genotypes. The present research was undertaken to enlarge our understanding of mechanisms underlying response to salt stress tolerance in *Medicago truncatula*. In the first step, the objectives of the research involved evaluations of some morpho-physiological traits of six *M. truncatula* genotypes, irrigated with saline solutions ranging from 0 to 150 mM NaCl grown in controlled growth chamber conditions. Genotypes are parental lines of some crosses and we tried to identify the important parameters affected by salinity and to determine the convenient salinity concentration as well as the favorable crosses which should be used in genetic studies of tolerance to salinity programs. In the second step, the objectives were to determine genetic variability for plant growth and key physiological traits for salt tolerance in our selected recombinant inbred lines (RILs) coming from the cross TN1.11 x A17, under control and salt stress conditions, and to map QTLs for the measured traits with the aim to

compare the genetic control of the traits that may putatively drive whole-plant response to salt stress. The most important results are:

The effect of salt stress on some morpho-physiological parameters in *Medicago truncatula* genotypes

The results of Analysis of variance for 6 genotypes at different levels of salinity show that some of studied genotypes are more tolerant to salinity than others. The main effect of salinity was significant for all morpho-physiological traits studied. The interaction between genotype and treatment show that the pattern of salinity effect on all genotypes is not the same. –DZA315.16"genotype shows the highest values for most of morphological traits whereas –FN1.11" has low values. Genetic variability for shoot biomass production in *M. truncatula* was also reported by Veatch et al. (2004). The best concentration to find differences between parental lines is situated between 90 and 120 mM NaCl. Vertical projected leaf area (LA) which is very easy to measure has a high variability through studied traits and –DZA315.16" and –A20" genotypes present the maximum and minimum values respectively. Salt treatment present also significant effect on chlorophyll content in our experiment. As far as we know the effect of salinity on chlorophyll content is not reported in this species. Leaf chlorophyll content was affected by salinity in *Brassica juncea* (Qasim, 1998), rice (Sultana *et al*., 1999), tetraploid wheat (Munns and James, 2003) and *Brassica oleracea* (Bhattacharya *et al*., 2004). Salinity can affect chlorophyll content through inhibition of chlorophyll synthesis or an acceleration of its degradation in naked oat (*Avena sativa L*.), (Zhao *et al*., 2007). Maximal fluorescence level (Fm) and the potential photochemical efficiency of PS II (Fv/Fm) are significantly reduced with different salinity concentrations compared with control. For the most severe salt stress, Fv/Fm decreases significantly in barley (Jiang *et al*., 2006) and Fv/Fm reduced significantly at high salt level in sorghum (Netondo *et al*., 2004), whereas no report is available about these traits in *M. truncatula*.

Correlations among some of the morphological or physiological traits were positives and significant in 90 mM NaCl salt treatment condition. Vertical projected leaf area (LA) also present significant correlations with: shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), and root

length (RL). Positives correlations between some morphological traits in *M. truncatula* were also reported by Arraouadi (2012).

Considering the interaction between genotypes and salinity concentrations for leaf area (LA), ―TN1.11" has rather low values for control and also for different salt concentrations, whereas ―DZA315.16" genotype has the highest values for control and low values for salt treatments. ―DZA315.16" should be considered as a susceptible genotype, contrary ―TN1.11" doesn't have high values in the absence of salinity but it is tolerant to salt concentrations. The RILs coming from the cross between these two lines can be a good material for genetic and genomic investigations. Principal component analysis (PCA) showed that, on average, most of growth traits are independent from physiological parameters in both conditions. We can resume that resistance to salinity is not due to one factor but to several morphological and physiological parameters.

Development of the genetic map

A genetic map of the cross between TN1.11 and A17 using a population of 192 RILs was constructed which has 146 SSR markers. SSRs were selected based on the *Medicago truncatula* genome sequence assembly (Young et al. 2011), (http://www.medicagohapmap.org/?genome) and unigene set of Medicago Gene Index at DFCI (http://compbio.dfci.harvard.edu/). Each linkage group in our map was numbered according to the *M. truncatula* reference map (http://www.medicago.org/genome/map.php) and is presumed to correspond to one of the 8 chromosomes in the haploid *M. truncatula* genome (x=8). A genetic map of the cross between Jemalong-6 × DZA315.16 using a population of 199 RILs, (LR4), was also constructed which contains 72 SSR markers (Julier et al., 2007). A RIL population (LR5), was derived from the cross between Jemalong A17 and F83005.5 and the RIL population of the cross was genotyped by SSR markers and a map with 70 markers was reported (Arraouadi et al., 2012). The genetic map of the F83005.5 × DZA45.5 cross (LR3) was also constructed by Hamon et al. (2010).

Genetic variability for morpho-physiological traits in *Medicago truncatula* RILs population under control and salt conditions

A large genetic variability was observed for all of plant growth traits under both conditions across RILs. Genetic variability was also observed in other *M. truncatula* RIL

populations for morphological traits (Julier et al. 2007, Espinoza et al. 2012). This was also evidenced under salt treatment (Arraouadi et al. 2011). Significant differences between the parents under salt stress suggested that the parental lines carry different genes for adaptation to salt stress. The difference between the mean of RILs and the mean of their parents was not significant for all of the traits in both conditions, showing that the RILs used in our experiment are representative of the possible recombinant lines from the cross TN1.11 x A17. Shoot and root dry weights for the mean of **RILs** were reduced significantly. The mean of RILs plant dry weight was also reduced. The same results are also reported in other *M. truncatula* RIL populations, (Veatch et al. 2004**;** Lopez 2008a; Arraouadi et al. 2011**).** Vertical projected leaf area (LA) was reduced by salt stress. In our first experiment we found that LA is an important parameter for discrimination of genotypes in salt stress condition. Arraouadi et al. (2011) also reported that leaf area under salt condition decreases in *M. truncatula*. The chlorophyll fluorescence parameters suggested that the RILs genotypes differed in their photochemical response to salt stress. As far as we know the genetic variability for fluorescence parameters in *M. truncatula* or *M. sativa* are not reported in the literature. Salt stress significantly increased shoot and root Na^+ concentrations in our experiment. Results show also that some lines of the RILs population have the capacity to regulate K^+ transport during salt stress, despite of increased Na^+ concentrations. The same results are reported by Arraouadi et al. (2012) for Na^+ and K^+ contents in leaves, stems and roots in this species. Heritability for the studied traits present high values compared with those reported by Arraouadi et al. (2011). Our experiments were realized in controlled phytotron conditions where environmental variance is low. In that case, a high heritability, compared to green house conditions, will be obtained if RILs population presents a high genetic variability. Indeed heritability values represent the RILs population variability.

QTL analysis for morpho-physiological traits in *Medicago truncatula*

The signs of additive effects show that alleles having positive effects for QTLs controlling growth and physiological traits come from both TN1.11 and A17 parents. The transgressive phenotypes observed for some traits could be explained by the

presence of QTLs of opposite sign in the two parents. Their recombination resulted in RILs with higher values than those of their parents, which is explained by various positive gene effects having been accumulated. The present study enabled us to investigate the genetic basics of major growth and physiological traits in a RIL population (TN1.11 x A17) where the parental line originated from *a priori* very distinct environments, in particular for salt content. According to the results of the present study, 40 QTLs were identified in salinity and control conditions. Most of overlapping QTLs are located on linkage groups 7. Julier et al. (2007) and Arraouadi et al. (2012) showed that linkage group 7 contains several QTLs involved in controlling important traits. Twenty-three of these QTLs located on linkage groups 1, 6 and 7 were overlapping. Some of these 23 overlapping QTLs are related to both growth and physiological traits. Our results showed that detected QTLs for Na^+ and K^+ traits measured in shoots and roots did not share the same map locations. A similar conclusion is reported by Arraouadi et al. (2012). The major output of the QTL analysis is identification of relevant genomic regions that may be targeted in genetic and genomic programs to dissect functional traits under salinity. Our results shoud be used for further functional analysis of salt-tolerance genes in *M. truncatula*.

Perspectives:

This work could be developed by:

1.Application for selection of the genotypes of interest*:*

–DZA315.16" present high values for control but low ones in salinity concentration where as –TN1.11" has low values in control but it is tolerant to salinity. Recombinant inbred lines (RILs) coming form the cross between these two genotypes should be studied under 100 mM NaCl concentration in genetics and genomics programs. New lines with interesting characters of both parents should be identified in RILs population.

2.Comprehension of genotypic differences in the tolerance to salinity :

2-1. To verify the stability of regions, the genome involved in the expression of traits associated with adaptation to salinity, it would be necessary to conduct further experiments in different pedoclimatic environments and other genetic backgrounds, which would validate the constitutive QTLs and environment-specific QTLs.

2-2. The cross between RILs, with contrasting behavior in situations of salinity and polymorphism for interesting QTLs, would allow us to identify the markers closely linked to QTLs of interest, by genetic recombination.

2-3. Fine mapping and construction of BAC contig in the vicinity of QTLs would consider positional cloning.

2-4. The identification of candidate genes for adaptive traits studied, their co-localization with QTLs and finally, validation from association studies in natural populations provide tools of great interest for the study of diversity adaptive intra and interspecific *Medicago truncatula*.

2-5. Analysis of the expression of a number of genes using microarray technique may help to better understanding the mechanisms involved in the response to salt stress, and the development of models of crop genetic parameters and tools to aid in the selection of genotypes of interest in this species and especially the related species *Medicago sativa*.

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