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Mme ZAHRA PAKBAZ

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Directeur(s) de Thèse :

MME MARTINA RICKAUER

Rapporteurs :

M. MOUNAWER BADRI, C. BIOTECH. BORJ CEDRIA

M. MUHAMMAD ARSHAD, National Univ of Sciences & Technology

Membre(s) du jury :

M. CHRISTOPHE DUNAND, UNIVERSITE TOULOUSE 3, Président

MME CARMEN SOFIA PEDRO DOS SANTOS, UNIVERSITE NOVA DE LISBONNE, Membre

Université Fédérale
Toulouse Midi-Pyrénées

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Zahra PAKBAZ

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Genetic control of plant tolerance to heavy metal pollution: a study on the legume model plant *Medicago truncatula* and cadmium stress

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Thesis supervised by
Mrs. Martina RICKAUER, Mrs. Asa EBRAHIMI and Mrs. Cecile BEN

Jury

Mr. Mounawer BADRI, Reviewer
Mr. Muhammad ARSHAD, Reviewer
Mr. Christophe DUNAND, Examiner
Mrs. Carmen Sofia PEDRO DOS SANTOS, Examiner
Mrs. Martina RICKAUER, Thesis director
Mrs. Cecile BEN, Thesis Co-director

Continuous effort not strength or intelligence
is the key to unlocking our potential.

Winston Churchill

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Résumé

Le cadre général de cette étude est d'explorer le contrôle génétique de *Medicago truncatula* au stress cadmium. Un métal lourd courant, le cadmium (Cd) est rejeté dans l'environnement par les centrales électriques, les systèmes de chauffage, l'industrie métallurgique, les installations d'incinération des déchets, le trafic urbain, les cimenteries et comme sous-produit des engrais phosphatés. La contamination au cadmium a un impact néfaste sur la productivité des plantes ainsi que sur la santé animale et humaine. La légumineuse annuelle *Medicago truncatula* L., parfois connue sous le nom de "barrel medic", est considérée comme une plante modèle ainsi qu'une culture fourragère. La petite taille du génome de *M. truncatula* (2n = 16 avec environ 550 Mbp), la structure diploïde, la nature autogame, la forte synténie avec *M. sativa* et d'autres légumineuses économiquement importantes en font une plante modèle idéale pour étudier le développement, les interactions avec des microorganismes et d'autres aspects de la biologie des légumineuses. Afin d'obtenir des données phénotypiques pour une analyse QTL 147 Recombinant Inbred Lines de la population LR4 de la génération F8 et les 2 lignées parentales (A17 et DZA315.16) ont été cultivées au stade plantule pendant 6 jours sous exposition à ± 15 µM de Cd sur de l'eau gélosée en trois répétitions. Le plan d'expérimentation en split plot a été mis en place en incluant dans chacun de ces réplicats les accessions de la lignée parentale A17, DZA315.16 comme accessions « de contrôle ». La longueur, le diamètre, le poids frais et le poids sec des racines, ainsi que le poids frais et le poids sec des parties aériennes ont été mesurés. Le modèle mixte linéaire a été utilisé pour analyser les traits de croissance des racines. L'exposition au Cd a considérablement réduit la longueur des racines. La variation phénotypique de la longueur des racines a montré un schéma continu parmi les RILs. Les résultats de l'analyse de la variance (ANOVA) de la longueur des racines ont montré un effet significatif du génotype, des conditions (±15 µM Cd), ainsi que de leur interaction. L'analyse de la variance de la biomasse des racines et des pousses a montré des effets génotypiques hautement significatifs pour les caractères de poids des feuilles fraîches (FLW), de poids des racines fraîches (FRW) et de poids des feuilles sèches (DLW). L'effet de la condition était significatif pour FLW et FRW mais l'interaction du génotype et de la condition n'était pas significative pour ces caractères. Une analyse des locus de traits quantitatives (QTL) a été réalisée avec les paramètres longueur des racines et poids des feuilles fraîches en utilisant une cartographie d'intervalle simple et composite. La carte génétique de la population RIL contient 316 marqueurs de répétition de séquence simple (SSR). L'analyse a été réalisée avec le package

QTL dans l'environnement R (Citr R). Un QTL pour la réponse au Cd a été détecté au groupe de liaison 8 avec la longueur de la racine. La variance phénotypique expliquée par le QTL est de 9,34 %. Un autre QTL a été identifié pour le poids des feuilles fraîches sous stress Cd sur le groupe de liaison 7.

Dix lignées parmi les RILs les plus sensibles et dix parmi les plus tolérantes ont été regroupées pour des analyses d'expression génique. Cinq gènes ont été choisis dans deux catégories pour ces études : une catégorie de gènes liés au stress oxydatif et aux voies de défense antioxydantes, notamment la superoxyde dismutase (*SOD*), la peroxydase (*PRX*), la glutathion réductase (*GR*) et la *glutathion S transférase* (*GST*), et un lié à la nutrition glucidique, à savoir *M. truncatula monosaccharide transporter1* (*MST1*). L'expression de ces gènes a été examinée dans les pools sensibles et tolérants. Le profilage d'expression de *GR*, *GST*, *MST1*, *PRX* et *SOD* a révélé une régulation à la hausse après 24h dans le pool sensible, mais après 48 et 96h, il est revenu à des niveaux de base. Le profilage de l'expression de *GR*, *GST* et *PRX* dans le pool tolérant a révélé une régulation à la hausse après 96h, tandis que *MST1* et *SOD* n'ont présenté aucun changement à aucun moment.

Abstract

The general framework of this study is to explore the genetic control of *Medicago truncatula* in response to cadmium stress. Cadmium (Cd), a common heavy metal, is discharged into the environment by power stations, heating systems, the metalworking industry, waste-burning facilities, urban traffic, cement factories, and as a byproduct of phosphate fertilizers. Cadmium contamination harms plant productivity as well as animal and human health. The annual legume *Medicago truncatula* L., sometimes known as barrel medic, is regarded as a model plant as well as a forage crop. The unique features of *M. truncatula* such as its small genome size (2n = 16 with about 550 Mbp), diploid structure, self-pollinating nature, close genetic relationship, genetic tractability, natural diversity, and high genome similarity with *M. sativa* and other economically important legumes make it an ideal model plant for studying legume biology, including root development, symbiotic nitrogen fixation, and responses to biotic and abiotic stress. In order to obtain phenotypic data for a QTL analysis of tolerance to Cadmium 147 Recombinant Inbred Lines of the LR4 population in the F8 generation and the 2 parental lines (*A17* and *DZA315.16*) were grown at the seedling stage for 6 days under exposure to ±15 µM Cd on a water agar medium, in three independent experiments. The split-plot experimental plan was set up by including the parental line accessions *A17*, and *DZA315.16* as the "check" accessions in each of these replicates. Length, diameter, fresh weight, and dry weight of the root, as well as fresh weight and dry weight of the aerial parts, were measured. The linear mixed model was used for analyzing root growth traits. Cd significantly decreased root length. The phenotypic variation of root length showed a continuous pattern among the RILs. The results of root length analysis of variance (ANOVA) showed a significant effect of genotype, conditions (±15 µM Cd), and their interaction. ANOVA of root and shoot biomass showed highly significant genotype effects for Fresh Leaf Weight (FLW), Fresh Root Weight (FRW), and Dry Leaf Weight (DLW) traits. Condition effect was significant for FLW and FRW but the interaction of genotype and condition was not significant for these traits. Quantitative trait loci (QTL) analysis was performed with the parameters root length and fresh leaf weight using simple and composite interval mapping. The genetic map of the RIL population, LR4 contains 316 simple sequence repeat markers (SSRs). The analysis was conducted with the QTL package in the R environment (Citr R). One QTL for Cd response was mapped to linkage group

8 with root length under Cd stress. The phenotypic variance explained by the QTL is 9.34 %. Another QTL was identified for fresh leaf weight under Cd stress on the linkage group 7. Ten of the most susceptible and ten of the most tolerant lines were pooled for gene expression analyses. Five genes were chosen for gene expression studies from two categories: one category of genes related to oxidative stress and antioxidant defense pathways including superoxide dismutase (*SOD*), peroxidase (*PRX*), glutathione reductase (*GR*), and glutathione S-transferase (*GST*), and one related to carbohydrate nutrition including *M. truncatula* monosaccharide transporter1(*MST1*). The expression of these genes was examined in the susceptible and tolerant pools. Expression profiling of *GR*, *GST*, *MST1*, *PRX*, and *SOD* revealed an up-regulation after 24h in the susceptible pool, but after 48 and 96h it returned to basic levels. Expression profiling of *GR*, *GST*, and *PRX* in the tolerant pool revealed an up-regulation after 96h, while *MST1* and *SOD* exhibited no change at any time.

Abbreviations

ACC: Aminocyclopropane-1-Carboxylic Acid

AFLPs: Amplified Fragment Length Polymorphisms

AM: Arbuscular mycorrhizal

ANOVA: The analyses of variance

AOS: Active oxygen species

As: Arsenic

AsA: Ascorbate

BACs: Bacterial artificial chromosome

BBSRC: The Biotechnology and Biological Sciences Research Council

Ca: Calcium

Cd: Cadmium

CIM: Composite interval mapping

Co: Cobalt

Cu: Copper

DEPC: Diethyl pyrocarbonate

DLW: Dry Leaf Weight

DRW: Dry Root Weight

EF1: Elongation factor 1

FAAS: Free amino acid

Fe: Iron

FLW: Fresh Leaf Weight

FRW: Fresh Root Weight

GHG: Greenhouse gas

GPOX: Glutathione peroxidase

GR: Glutathione reductase

GSH: Reduced glutathione

GSSG: Oxidised glutathione

GST: Glutathione S-transferase

GWAS: Genome-wide association studies

H_2O_2: Hydrogen peroxide

H_3L: Core histone

HapMap: The haplotype map

Hg: Mercury,

HK: Haley-Knott regression,

IARC: The International Agency for Research on Cancer

IMP: Multiple imputation

INRA: The Institute for Agricultural Research

IRT: Iron-regulated transporters

K: Potassium

LG: linkage groups

LOD: The log-likelihood

LRT: likelihood ratio test

LSMEANS: The least-square means

MAS: Marker-assisted selection

Mg: Magnesium

MIM: Multiple interval mapping

ML: Maximum Likelihood

Mn: Manganese

MQM: Multiple QTL mapping

MR: Marker regression

MSTs: Monosaccharide transporter

MTP: Metal tolerance protein

MTs: Metallothioneins

NRAMP: The natural resistance-associated macrophage protein

NSF: The National Science Foundation

O_2^-: Superoxide

OH: Hydroxyl radical

Pb: Lead

PCS: PCs synthetase

PCs: Phytochelatins

PRX: Peroxidase

qPCR: Quantitative real-time PCR

QTLs: Quantitative trait loci

RAPDs: Random Amplified Polymorphic DNA

RFLPs: Restriction fragment length polymorphisms

RILs: Recombinant inbred lines

ROS: Reactive Oxygen Species

RT-PCR: Reverse transcription polymerase chain reaction

SA: Salicylic acid

Si: Silicon

SIM: Simple interval mapping

SNPs: Single nucleotide polymorphisms

SOD: Superoxide dismutase

SSD: Single-seed descendants

SSRs: Simple sequence repeat markers

SUTs: Sucrose transporters

SWEETs: Sugar will eventually be exported to transporters

The EM algorithm: The expectation-maximization algorithm

TSS: Total soluble sugars

ZIP: Zinc-regulated transporter-like proteins

Zn: Zinc

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I - Introduction

I.1. Heavy metals

Heavy metals is a term that refers to metals that have a density of more than 5 g/cm3 (Jaishankar et al., 2014). The concentration of these metals in the main rocks of the Earth's crust is less than 100 mg/kg, so they are often called trace elements (Zaikov et al., 2017). Some heavy metals, such as Zinc (Zn), Cobalt (Co), Iron (Fe), Manganese (Mn), and Copper (Cu), are also known as "potentially toxic elements." because they have critical biological performance at low levels but can cause toxic effects in excessive amounts (Liu et al., 2019; Zaikov et al., 2017). In contrast, non-essential elements such as cadmium (Cd), mercury (Hg), lead (Pb), and arsenic (As) are toxic to all living organisms, even at low concentrations. They have negative impacts on their growth and development (Liu et al., 2019; Shirazi et al., 2019). Heavy metals enter the environment both naturally and through human activities, such as soil erosion, mining, weathering of the earth's crust, industrial evacuation, urban runoff, sewage effluents, and pesticides and chemical fertilizers used in agriculture (Morais et al., 2012). The chemical coordination and oxidation-reduction properties of heavy metals enable them to bind to protein sites specific to other metals and cause these proteins to malfunction leading to cell toxicity and damage in cell control mechanisms such as homeostasis, transport, and compartmentalization. The binding of heavy metals to the DNA and nuclear proteins causes biological macromolecule oxidation (Flora et al., 2008). In general, the first human exposure to heavy metals is through diet (water and food). Complications of exposure to heavy metals depend on the concentration and duration of exposure to these metals, *i.e*. acute or chronic. Various disorders and damage caused by oxidative stress and the production of free radicals are among the consequences of heavy metal toxicity (Jaishankar et al., 2014; Morais et al., 2012). Lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) are broadly scattered in the environment. They are the most dangerous to humans and animals and there is no known homeostasis system for them; the negative human health effects linked with their exposure, even at low concentrations, are broad and include, but are not limited to, neurotoxic and carcinogenic actions. Two major methodologies can be used to estimate human exposure to hazardous chemicals in the environment. The first is environmental monitoring, or determining the chemical concentration scenario, which is one method. The second methodology is based

on exposure estimations using biomarkers. Biomarkers can be applied at any level of a biological system (eg. molecular, cellular, or organ levels). These technologies can be used to identify and quantify exposed individuals or groups, estimate health risks, and aid in the diagnosis of environmental or occupational diseases. DNA and protein adducts, mutations, chromosomal abnormalities, inducible genes, and a slew of other "early" cellular or subcellular processes hypothesized to link exposure and effect are all potential biomarkers (Morais et al., 2012). In summary, soil pollution by heavy metals such as cadmium emphasizes two major issues: On the one hand, they disrupt plant life cycles, reducing agricultural production; on the other hand, once adsorbed and stored in plant tissues, they infiltrate the food chain, poisoning both humans and animals. Plants are now subjected to rapid environmental changes, primarily as a result of human activities such as air and soil pollution, acid precipitation, climate change, and so on (Dalcorso et al., 2008). When discussing the toxic effects of heavy metals, one should be aware that the toxicity of metals to plants depends on the external bioavailable metal concentration, the exposure time, the plant genotype, and the general condition of the plant (Morais et al., 2012).

1.1. Cadmium

Cadmium is typically a metal of the 20th century and is known as a by-product of zinc production (Benavides et al., 2005; Bernard, 2008). It belongs to group IIB of the Mendeleev periodic table placed between the two elements zinc (an essential element) and mercury (a toxic elements). It is a divalent cation having a very strong affinity to ligands containing oxygen, nitrogen, or sulfur. According to Greek mythology, Cadmus is the son of one of the Phoenician kings who founded Thebes and gave them the letters of the Greek alphabet, but he and his wife, Harmonia, became serpents at the end of their lives. This duality of success and mortality is reminiscent of the role of cadmium in industry and biology. Cadmium is used in the production of special alloys and rechargeable batteries, and also as a semiconductor in solar cells, as an anti-corrosion agent in steel, and as a stabilizer in plastics. Phosphate fertilizers also show a big cadmium load. The widespread use of cadmium in industry and phosphate fertilizers in agriculture has increased its entry into the environment (Bernard, 2008; Rahimzadeh et al., 2017; Sigel et al., 2013a). Although not essential for plant growth, cadmium is readily absorbed by the roots and transported to the aerial parts (Wagner, 1993). Its accumulation in "edible" parts is the main route for this toxic metal to enter the food chain (Gupta et al., 1999;

McLaughlin & Singh, 1999). Organisms have been exposed to cadmium in various ways since the beginning of evolution. However, no specific system has developed to treat cadmium in living organisms, and cadmium trafficking and transport. cadmium within eukaryotic cells usually relies on biological molecules that are specific for other purposes than cadmium handling. This is related to the specific properties of cadmium as well as the diversity of potential ligands in the biological system (Sigel et al., 2013a). The anthropogenic contributions of cadmium to soils are significant compared to other trace elements. In soils, cadmium is less strongly adsorbed than other divalent metals. It is, therefore, more mobile and more easily bioavailable. The phytoavailability of Cd is mainly governed by the edaphic characteristics of the soil, such as the pH, the content of organic and mineral matter, or the redox potential and the temperature. It is rare to find soils polluted only by Cd since it is often linked to Pb and/or Zn. Slightly polluted soils contain cadmium concentrations between 0.04 and 0.32 mM. Moderately to heavily contaminated soils reach concentrations above 0.32 mM and up to 1 mM (Daud et al., 2009). Its tolerable threshold in cereals and edible legumes is 0.1 mg / Kg (Harris & Taylor, 2001). Cadmium contamination has a harmful impact on plant productivity as well as animal and human health. It can disrupt a variety of plant physiological systems, including water relations, nitrogen metabolism, photosynthesis, respiration, and mineral feeding, causing obvious damage symptoms in plants such as chlorosis, growth inhibition, browning of root tips, and eventually death. Furthermore, Cd disrupts the antioxidant defenses of plants, resulting in oxidative stress. Cd does not engage in Fenton-type reactions, but it might indirectly stimulate the generation of various ROS, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), and hydroxyl radical (.OH), resulting in an oxidative burst (Rahoui et al., 2017). Root browning has been observed in many plants that have been exposed to Cd. Cadmium toxicity is frequently characterized by decreased root length and dry mass, as well as increased root diameter. Root elongation inhibition has been identified as one of the earliest and most identifiable indicators of Cd toxicity. Cadmium-induced root elongation inhibition could be related to the depolymerization of cell cytoskeleton microtubules and the production of chromosome abnormalities, which cause meristematic cells to have decreased mitotic activity. The effects of Cd on root morphology, however, may differ amongst plant species, ecotypes, and cultivars. Toxic signs of Cd stress in plant foliage include stunting, chlorosis, necrosis, and desiccation. For most plant species, the typical Cd content in the leaf varies from 0.05 to 0.2 mg kg^{-1}. Reduced nodule development and poor nodule function were reported in Cd-treated leguminous plants. According to research on the effects of Cd on seed development, when plants are exposed to Cd during their growth stages, they have lower seed output and

germination rates (HE et al., 2017). Cadmium toxicity is thought to be caused by interactions between zink (Zn), iron (Fe), calcium (Ca), potassium (K), manganese (Mn), copper (Cu), silicon (Si), and magnesium (Mg) in plants. Zn^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Si^{2+} are cations that compete with Cd for exchange sites in soils. The presence of competing ions in soils can lower Cd adsorption in general (Nedjimi & Daoud, 2009). The cell wall is the initial line of defense against external aggressions at the cellular level. Its composition varies during cell growth and also depending on the species. The wall consists of a middle lamella formed soon after mitosis and a primary wall deposited during cell elongation (Wang et al., 2008). During cell differentiation, a secondary wall is put in place (Popper, 2008). The primary wall is approximately 30% cellulose, 30% hemicelluloses such as xyloglucans, 35% pectic compounds, and 5% glycoproteins. Pectins are a very diverse group of branched complex polysaccharides, with a backbone of galacturonic acids linked in α − 1,4 which can be methylated on the C6 carboxylic function or acetylated in C2 and/or C3. Regions where the carboxylic functions are not substituted can associate via calcium bridges thus creating a rigid structure known as an “egg box” (Caffall & Mohnen, 2009). Pectins play a privileged role in the trapping of metals in the wall. In fact, 70 to 90% of metals are trapped by pectic compounds. This binding efficiency is due to the cation exchange capacity: metal cations can replace calcium ions (Matsunaga & Ishii, 2004). The capacity of walls to fix cadmium has been demonstrated by Wang et al. (2008); Exposure of ramie (*Boehmeria nivea*) plants for 20 days to increasing concentrations of cadmium showed approximately 50% metal uptake in the cell walls of roots, stems and leaves. Uptake of heavy metal by the plant can take place either by the roots directly from the soil or by the aerial organs in the event of air pollution. Indeed, the leaves are able to ensure the incorporation of heavy metals such as Cd from polluted atmospheres. These metals can then be redistributed within the plant. Nevertheless, the roots are considered to be the main source of entry of heavy metals which will subsequently be redistributed within the plant (Benavides et al., 2005; Sigel et al., 2013a). The absorption kinetics of several heavy metal including cadmium, at the root level have shown the existence of active and passive absorptions, the extent of each of which is a function of the external concentration of the metal. Indeed, many studies have shown the preponderance of active absorption in the case of low metal concentrations as opposed to relatively high doses where passive absorption prevails. The primacy of passive flow in the event of a high concentration of heavy metals could be explained, among other things, by the deterioration of the membrane integrity (Kabała et al., 2008). Cadmium is believed to borrow cation transport systems

normally involved in the uptake of essential elements, such as zinc (ZIP, IRT) or iron (Nramp) transporters (Wei et al., 2009).

1.2. Plant behavior towards heavy metal

In terms of heavy metal uptake, plants are divided into three categories: indicator, excluder, and hyperaccumulator plants (Maret & Li, 2009). The majority of heavy metal resistant plants are classified as "excluders," meaning they do not allow heavy metals to accumulate in their tissues. Metal exclusion can be used in a variety of ways. The most straightforward method is to reduce cells' non-selective permeability (Williams, 2002). The lignification of plant cell walls is a common way to achieve this, and increasing lignifying enzymes is a well-known response to cadmium toxicity. Metals can also be excluded via precipitation or binding in the apoplast (cell walls) before they enter through the plasma membrane. Finally, plants can actively lower undesired metal concentrations in their cells by pumping them out. Indicator plants possess very little defense against uptake of toxic metals so their internal metal content follows the metal concentration in their environments in a roughly linear way. Hyperaccumulators are plants that actively take up and store enormous amounts of potentially harmful metals in their above-ground tissues (Sigel et al., 2013b). There is no consensus on what level of Cd accumulation, under what conditions, is sufficient to qualify a Cd-accumulating plant as a real hyperaccumulator. However, the southern French ecotype of *Thlaspi caerulescens* = *Noccaea caerulescens* (from the Ganges region) has the highest naturally occurring Cd hyperaccumulation. Shoots of this plant easily reach >2000 ppm Cd on natural soil and >20000 ppm grown until mature state in nutrient solution (Sigel et al., 2013a); the Cd bioaccumulation coefficient of this ecotype in its natural habitat is >70 (Krezel et al., 2007). Hyperaccumulators are a topic of tremendous interest for a reason; they can be exploited biotechnologically for two different objectives, which we will describe below: phytoremediation, dealing with the cleaning of anthropogenically contaminated soils as well as phytomining, i.e., the use of plants for commercial metal extraction (HE et al., 2017). They produce less biomass because they spend a large portion of their metabolism energy adapting to the high-metal concentration environment (Seregin & Kozhevnikova, 2020).

1.2.1. Plant response to heavy metals

Plants have evolved two key ways to cope with high levels of heavy metal exposure. The first is the excluder approach, in which plants attempt to prevent heavy metals from entering the roots by, for example, limiting soil metal bioavailability or decreasing the expression of metal uptake transport proteins. The second technique is the tolerance strategy, which is based on the controlled confinement and detoxification of metals (Lin & Aarts, 2012). The creation of stress-related proteins and signaling molecules is the plant's molecular response to Cd stress. Chelating substances generated by roots include phytosiderophores, nicotinamide, and organic acids, which impact heavy metal intake (Kutrowska & Szelag, 2014). Roots are the principal entry point for heavy metals in plants. Indeed, due to their negative charge, cell walls have a high potential for heavy metal binding and retention (Polle & Schützendübel, 2003). As a result, it is assumed that the phosphorylation cascade is involved in Cd signaling to the nucleus. Calcium ions and calmodulin are well-known external stimuli second messengers, and this system's involvement in the calmodulin system is also engaged in sensing other heavy metals (Dalcorso et al., 2008; Suzuki et al., 2001). The reduced glutathione (GSH)-oxidized glutathione (GSSG) ratio (GSH/ GSSG) is another mechanism that is thought to be involved in Cd sensing. Glutathione can control the differential expression of antioxidant enzymes, such as chalcone synthase, phenylalanine ammonia-lyase, superoxide dismutase, or glutathione reductase, usually induced by heavy metal stress (Li et al., 2017). Different plant species have shown a drop in the GSH/GSSG ratio during Cd stress, resulting in the activation of response genes. Also, Cd is chelated in the cell by glutathione and its product phytochelatins (PCs) to allow ATP-dependent membrane pumps to transfer Cd-complexes into the vacuole or to the apoplast. The synthesis of PCs necessitates the presence of GSH. The cytosolic PC synthetase (PCS) catalyzes this reaction (Cobbett, 2000). Metallothioneins (MTs) are Cys-rich low-molecular-weight peptides that could also bind metal ions like Cd. MTs, unlike PCs, are the result of mRNA translation, which is induced in response to heavy metal stress (Cobbett & Goldsbrough, 2002). Hormone synthesis has also been reported to be regulated during heavy metal stress. Treatments with Cd or Cu, for example, increase the amount of jasmonic acid. Treatment with Cd, Cu, Fe, and Zn increases ethylene synthesis, and in the case of Cd and Cu, this is due to an elevation of 1-Aminocyclopropane-1-carboxylic acid synthase (ACC synthase) transcription and higher activity. Another well-known hormone implicated in plant stress signaling is salicylic acid (SA), which has been demonstrated to promote SA accumulation in

roots when exposed to Cd. Hormone signaling isn't the only example of heavy metal and (a) biotic stress redundancy (Maksymiec, 2007). It has also been suggested that Reactive Oxygen Species (ROS) have a role in signaling. Heavy metal stress, in fact, causes the formation of ROS both directly, through the Fenton or Haber-Weiss reactions, and indirectly, through the unbalanced activity of antioxidative enzymes, as in the case of Cd (Yousuf et al., 2012).

1.2.2. ROS production and scavenging in response to heavy metal

When plants are exposed to cadmium ROS such as superoxide radicals, hydroxyl radicals, and hydrogen peroxide are rapidly produced in plant cells and can promote the oxidation of proteins and lipids membrane, leading to inhibition of plant growth and cell death. Therefore, an important aspect of plant detoxification is the reduction of ROS concentrations through biochemical reactions within the cells (Tarhan & Kavakcioglu, 2016). Considering that Cd has no redox activity, it can enhance ROS production by inhibiting free radical scavengers such as GSH and inhibiting detoxifying enzymes such as SOD, catalase (CAT), and GSH peroxidase and /or by other indirect mechanisms. The formation of free radicals such as superoxide ions, hydrogen peroxide, and hydroxyl radicals is associated with GSH depletion and altered antioxidant enzyme activity (Matović et al., 2011). The enzymatic antioxidants are SOD, CAT, APX, and GR. Non-enzymatic antioxidants include ascorbate (AsA) and reduced glutathione (GSH) and form (AsA-GSH) a pathway that helps reduce ROS levels to maintain proper cellular homeostasis upon exposure to stress (Asgher, Per, Anjum, Khan, et al., 2017). In a cell, SODs form the first line of defense against ROS. $O_2^{\bullet-}$ is produced wherever the electron transport chain is present, and thus O_2 activation can occur in different compartments of the cell (Alscher et al., 2002). The imbalance of $O_2^{\bullet-}$ results in the generation of H_2O_2 spontaneously or in a reaction catalyzed by the enzyme SOD. Superoxide produced in different compartments of plant cells is rapidly converted to H_2O_2 by its action. Three classes of SOD have been described in plants based on the metal cofactor present at the active site: (i) CuZn-SOD containing Cu and Zn in the active site is present in the plastid, peroxisome, cytosol, and extracellular space, (ii) MnSOD containing Mn in the active site is present in mitochondria and peroxisomes, and (iii) FeSOD containing Fe in the active site is mainly present in plastids (Mishra & Sharma, 2019) (Figure 1).

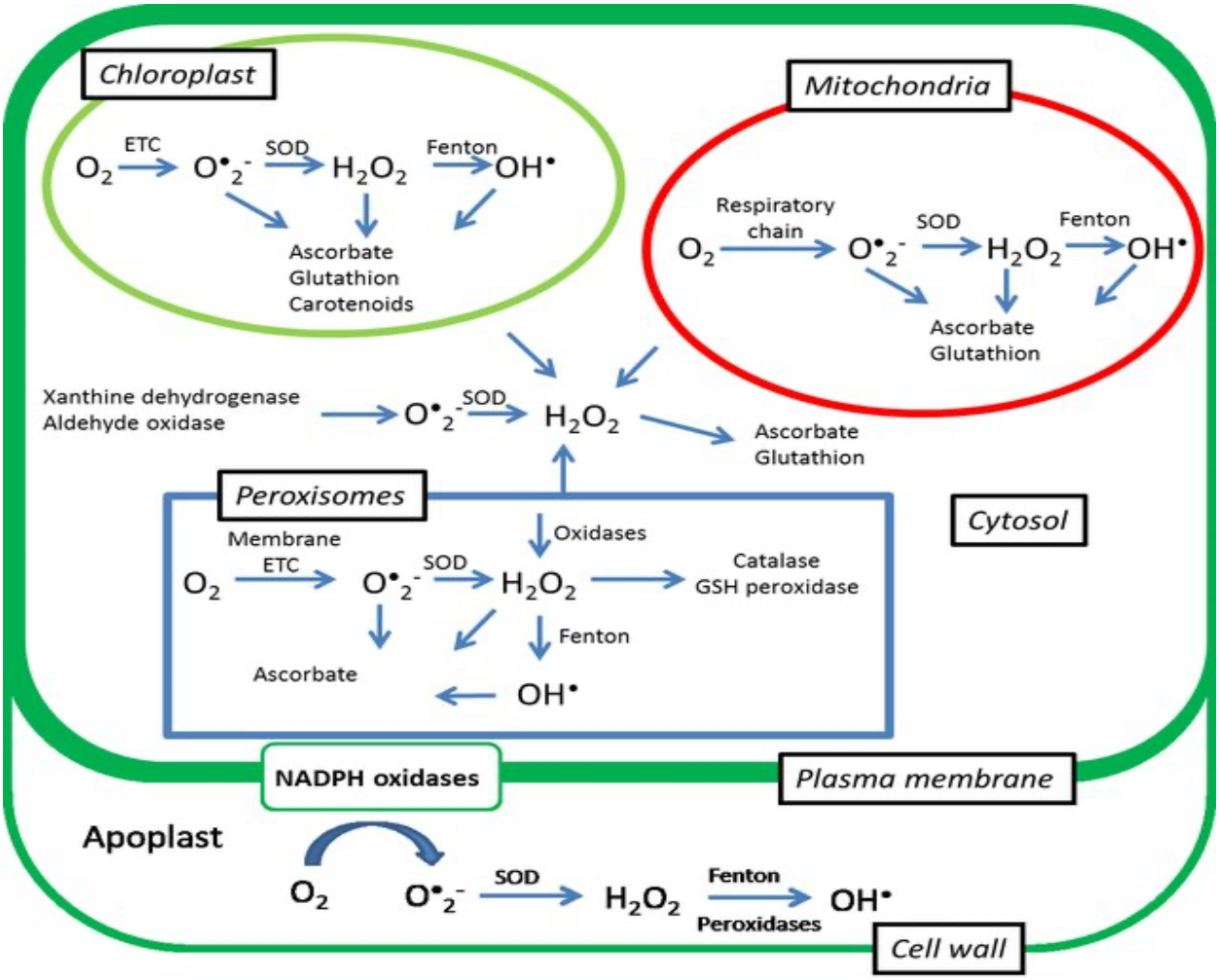


Figure 1- Production and scavenging of ROS in a plant cell.

SOD (Superoxide dismutase); Fenton (decomposition of hydrogen peroxide to highly reactive hydroxyl radical in presence of iron); ETC (electron transport chain)

Taken from : (Jajic et al., 2015)

H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups, so it should not be excessively accumulated in organelles such as chloroplasts, where photosynthesis depends on thiol-regulating enzymes. CAT converts H_2O_2 to water and molecular oxygen. These enzymes have very high maximal catalytic rates but low substrate affinities because the reaction requires two H_2O_2 molecules to access the active site simultaneously. In addition, the absence of CAT in chloroplasts precludes a role in the protection of Calvin cycle thiol-regulatory enzymes. Another mode of H_2O_2 destruction is through peroxidase, which is found throughout the cell and has a much higher affinity for H_2O_2 than CAT cells (Jiménez et al., 1998). However, peroxidases require reducing agents, as they reduce H_2O_2 to H_2O. In plant cells, ascorbate is the main reducing substrate for H_2O_2 detoxification. Ascorbate peroxidase (APX) uses two molecules of ascorbic acid to reduce H_2O_2 to water, simultaneously producing two molecules of mono-dehydroascorbic acid. MDHA is a short-lifetime free radical that, if not rapidly reduced, is disproportionate to ascorbate and dehydroascorbate. This reaction produces GSSG, which is then reduced back to GSH by NADPH, a reaction catalyzed by GR. GR also plays an essential role in maintaining a reduced glutathione pool during stress, allowing regulation of cellular redox reactions. The expulsion of H_2O_2 through this arrangement of responses is known as the ascorbate-glutathione cycle. Ascorbate and glutathione are not expended in this pathway but take part in a cyclic exchange of lessening reciprocals, including four enzymes that grant the decrease of H_2O_2 to H_2O utilizing electrons inferred from NAD(P)H. Most of the enzymes of the ascorbate-glutathione cycle are located in the stroma of chloroplasts, the cytosol, mitochondria, and peroxisomes. The physiological importance of glutathione in plants falls into two categories. Sulfur metabolism and defense. GSH is the major non-protein thiol and regulates sulfur uptake at the root level (CONTOUR-ANSEL et al., 2006; Rennenberg, 1980)(Figure 2).

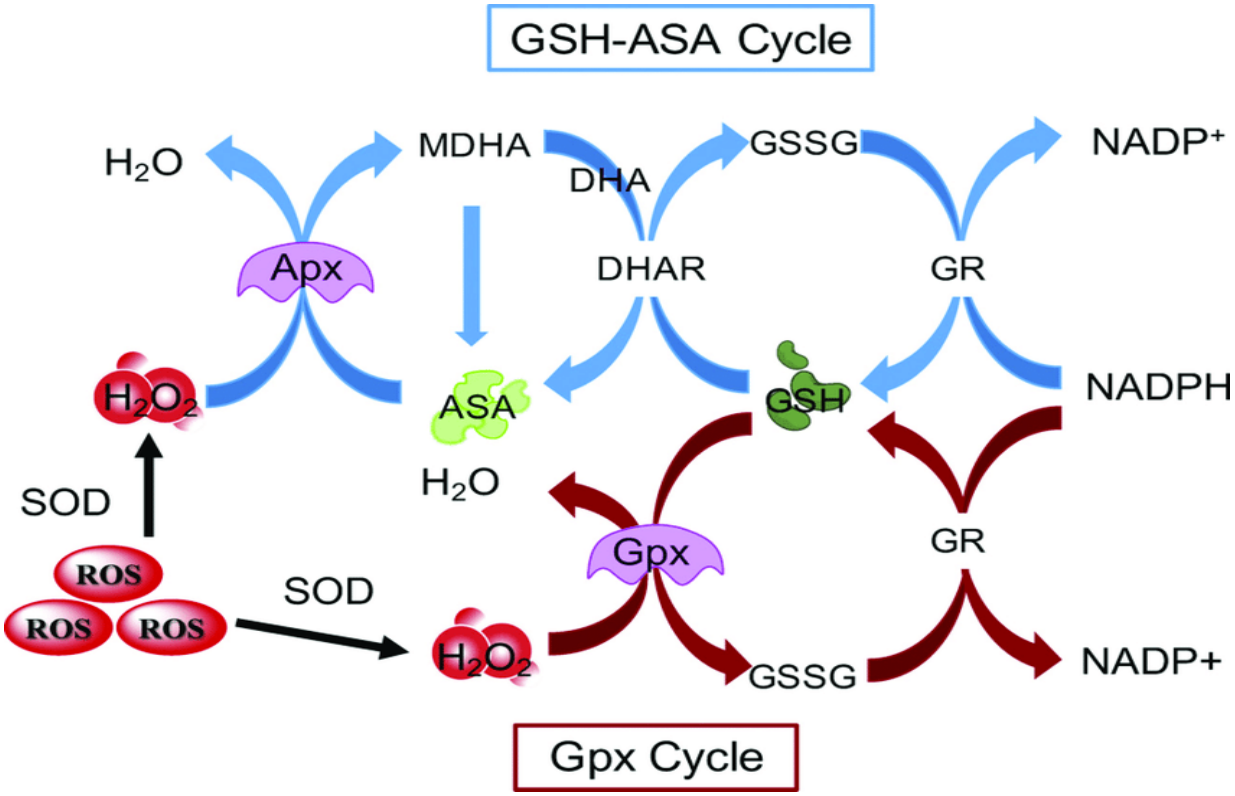


Figure 2- Schematic diagram of glutathione-ascorbate (GSH-ASA) cycle and glutathione peroxidases (Gpx) cycle.

ASA, ascorbate; APX, ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; MDHA, monodehydroascorbate; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; ROS, reactive oxygen species; MDA, Malondialdehyde; NADP +, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate H.

Taken from: (Li et al., 2020)

It is utilized by the GSH S-transferases within the detoxification of xenobiotics and could be a precursor of PCs, which are vital in controlling cellular heavy metal concentrations (Noctor & Foyer, 1998). The well-recognized role of GST is to detoxify toxic substances by conjugation with glutathione, reduce oxidative stress, and participate in hormone transport. Some GSTs exhibit glutathione peroxidase activity, and these GSTs can detoxify toxic lipid hydroperoxides that accumulate during stress. Notably, the expression of some GSTs was significantly activated by salicylic acid, and several GST enzymes have been shown to be receptor proteins for salicylic acid (Gullner et al., 2018) (Figure 3).

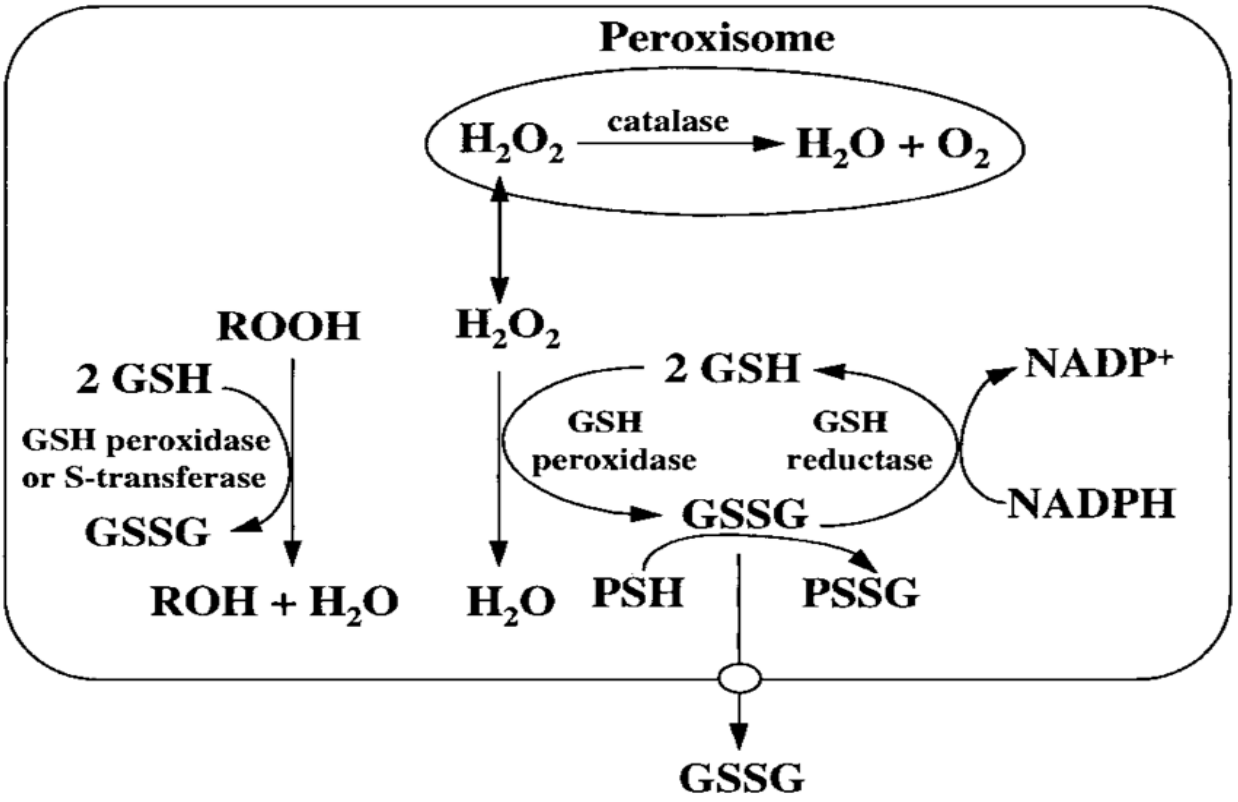


Figure 3- Antioxidant function of GSH.

Hydrogen peroxide, which is generated as a result of aerobic metabolism, can be metabolized by GSH peroxidase in the cytosol and by catalase in the peroxisome. To maintain the cellular redox equilibrium, GSSG is reduced back to GSH by GSSG reductase at the expense of NADPH, thereby forming a redox cycle. Organic peroxides can be reduced by either GSH peroxidase or GSH S-transferase. Under severe oxidative stress, the ability of the cell to reduce GSSG to GSH may be overcome, leading to accumulation of GSSG within the cytosol. To avoid a shift in the redox equilibrium, GSSG can either be actively transported out of the cell or react with a protein sulfhydryl (PSH) to form a mixed disulfide (PSSG).

Taken from: (Lu, 1999)

Cadmium's use by humans is new, and it is only in the last few decades that substantial consideration has been paid to cadmium as a probable pollutant (Gungsat et al., 2018). Food products account for the majority of human exposure, for non-smokers and non-occupationally exposed workers, to cadmium. Only inorganic cadmium salts are found in food because Organic cadmium compounds have a high degree of instability (Morais et al., 2012). Agricultural soil introduces cadmium into the food chain (Bañuelos & Ajwa, 1999). Metal concentrations are typically higher in roots than in shoots, however, Cd is stored in the leaves of leafy vegetables (e.g. lettuces and endives) due to its rapid absorption and translocation (Peijnenburg et al., 2000). These plants are Cd accumulators, and they are absorbed into the body as part of a human diet. The main sources of dietary Cd, according to predictions of Cd

exposure levels in food items, are vegetables and grains. Human expos are influenced by the consumption of staple foods like wheat and rice. It is estimated that the daily consumption of food material in an average adult is 10.0–30.0 μg in different countries. Its concentration in vegetables ranged from 0.001 to 0.124 mg kg^{-1} in some reference areas (Huang et al., 2017). Cadmium is also found in milk and fatty tissues of animals. Seafood, such as mollusks and crustaceans, can be also a source of cadmium. As a result, when individuals eat plant- and animal-based meals, they are exposed to cadmium (Castro-González & Méndez-Armenta, 2008). In addition, tobacco smoke is one of the most common sources of cadmium exposure in humans. Tobacco includes significant levels of the metal in all of its forms. Cadmium is absorbed far more readily through the lungs than from the gastrointestinal tract so, smoking adds greatly to the total body load (Figueroa B., 2008). Since one cigarette contains approximately 1 to 2 μg Cd, smoking one pack per day results in a daily uptake of Cd that approximates that derived from food (Bernard, 2008). Safe daily levels of Cd intake should be kept below 30 μg per person. Cd is most harmful to the kidney, particularly the proximal tubular cells, which are the primary site of accumulation (Satarug et al., 2000). Cd can also result in bone demineralization, either directly or indirectly as a result of renal dysfunction. Excessive exposure to airborne Cd in the workplace, mines metal smelters, and also industries that use cadmium compounds, may damage lung function and raise the risk of lung cancer. The International Agency for Research on Cancer has also classed it as a human carcinogenic component (IARC). The most compelling evidence comes from studies that demonstrate higher lung cancer risks in workers exposed to Cd through inhalation, as well as animal studies that show Cd delivered through various ways can cause cancer at multiple places, including the lungs (Bernard, 2008; Huff et al., 2007; Mezynska & Brzóska, 2018; Rahimzadeh et al., 2017). Cd is competently stored in the kidney (half-life 10–30 years) and the concentration is proportionate to that in urine. Some studies showed that in mature male rats, Cd poisoning reduced sexual activity, aggression, fertility, and anxiety-like behavior. Cd exposure can damage women's reproductive systems as well, however, there is less data on Cd exposure and female reproductive health than there is on Cd exposure and male reproduction. This could be due to the fact that male reproductive endpoints are easier to study than female reproductive endpoints. Because of elevated gastrointestinal uptake of Cd due to reduced iron stores, which is reported to be a common phenomenon in childbearing women, the toxicity of chemicals and environmental pollutants may be expressed differently in women and men as these toxicants affect specific time periods of a woman's life and maybe a greater threat to women than men (Kumar & Sharma, 2019). The most severe form of chronic Cd poisoning is known as itai-itai

disease reported in the Cd-polluted Jinzu River basin of Toyoma, Japan. Itai-itai occurred due to Cd waste discharge from the industry into a river. Residents of the river's surrounding communities who drank the tainted water felt severe pain in the spine and joint. The disease process is characterized by renal tubular dysfunction, osteoporosis, bone pain, and waddling gait leading to disabling condition. Following the government's official recognition (in 1968) that Cd exposure was the cause of the itai-itai sickness cases, efforts were made to eliminate Cd contamination in the region (Zwolak, 2020). Therefore, it is essential to pay attention to the health risks and exposure pathways of Cd in the human body.

I.2. Legume plants

In the years ahead, food security, reducing the risk of climate change, and meeting the rising need for energy will become increasingly important challenges. As a result, in agricultural and food systems, sustainable production is becoming increasingly important. Legume crops could play a key role in this setting by providing a variety of functions while adhering to sustainability principles (Carneiro da Silva et al., 2019). In addition to providing a source of high-quality food and feed, legumes help to minimize greenhouse gas (GHG) emissions by emitting 5–7 times less GHG per unit area than other crops (Stagnari et al., 2017). Legumes can create symbiotic partnerships with soil-borne bacteria known as rhizobia, which fix atmospheric nitrogen. This symbiosis leads to the release of high-quality organic matter into the soil, protection of the plant from fungal infections, and improvement of soil nutrient circulation and water retention. Legumes offer a lot of potential for conservation agriculture because of their numerous uses. They can be used as a growing crop or as crop leftover (Meena et al., 2018). In summary, legumes play central roles: (I) at the food-system level, as a source of plant proteins for human and animal use, and with an increasing role in enhancing human health;(II) at the production system level, because to their ability to fix atmospheric nitrogen, making them potentially highly suitable for inclusion in low-input cropping systems, and due to their involvement in reducing GHG emissions;(III) at cropping- system levels, in agroecosystems focused on a few large species, as diversification crops, breaking pest and disease cycles and helping to balance the shortfall in plant protein production in many parts of the world, including Europe (Mahmood et al., 2017; Tharanathan & Mahadevamma, 2003). The *Leguminosae*

family is the third biggest flowering plant family, with 800 genera and 20,000 species. Some legumes are weeds in cereal crops, while others are important grain crops; the latter are known as grain legumes or pulses (Kenicer, 2005). Legumes are second only to the *Gramineae* in terms of human importance, with their distinctive floral structure, podded fruit, and the ability of 88 percent of the species investigated to date to form nodules with rhizobia. The 670 to 750 genera and 18,000 to 19,000 species of legumes include important grain, pasture, and agroforestry species. Domestication of lentils at a site in Iran dating to 9,500 to 8,000 BP was reported; *Hymenaea* was used as a food source in the Amazonian prehistory (Cohen, 1977). Some common forage species are found in the *Papilionoideae* subfamily. In temperate climates, the genera *Trifolium* and *Medicago* are particularly popular in sustainable agricultural systems. However, only a few species in the subfamily have been used in grassland farming, despite the fact that alfalfa or lucerne (*Medicago sativa*), white clover (*Trifolium repens*), and red clover (*Trifolium pratense*) all play an important role in grazing and conservation (Graham & Vance, 2003). **Error! Not a valid bookmark self-reference.** shows the taxonomy of legumes. The benefits of forage legumes have been advocated firstly because of their contribution to the nitrogen (N) economy of grasslands and, secondly, their superior feeding value for ruminant livestock nutrition compared with grasses. Biological N_2-fixation accounts for about 65 percent of the N used in world agriculture, of which a high proportion is rhizobially fixed by forage legumes in grasslands (Meena et al., 2018). However, a variety of biotic (fungi, bacteria, nematodes, viruses, parasitic plants, insects) and abiotic (drought, cold, salt, waterlogging, heavy metal) challenges are wreaking havoc on these crops' yields. Abiotic stresses can cause crop damage that is equal to or greater than that caused by biotic stresses, depending on the nature and degree of the stress. Furthermore, crops exposed to abiotic stress are more sensitive to weeds, insects, and diseases, resulting in significant losses. Biotechnology and plant breeding technologies can help solve or lessen these issues effectively. A good biological understanding of the target species as well as the mechanisms behind resistance/tolerance to these pressures will be required for the successful application of biotechnology to biotic/abiotic restrictions affecting legume crops. Although the huge genome size and polyploidy of some legumes have complicated this goal, two species, *Medicago truncatula,* and *Lotus japonicus*, have emerged as model plants to explore the genetics of nodulation and other essential processes such as resistance or tolerance to stressors. Their small and diploid genomes, autogamous nature, short generation durations, and prolific seed production were all crucial factors in their selection (Dita et al., 2006).

Table 1- The legume taxonomy

Kingdom	Plantae – plantes, Planta, Vegetal, plants
Subkingdom	Viridiplantae – green plants
Infrakingdom	Streptophyta – land plants
Superdivision	Embryophyta
Division	Tracheophyta – vascular plants, tracheophytes
Subdivision	Spermatophytina – spermatophytes, seed plants,phanérogames
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae – peas, legumes

(https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=183623#null)

2.1. *Medicago sativa* (Alfalfa)

Alfalfa (*Medicago sativa* L.) appears to be the only forage crop that was cultivated before recorded history. Most experts agree that alfalfa most likely originated in Persia (an arid region that is now mainly Iran) and then from Persia this forage plant found its way to Arabia, where it was named, "alfalfa", which simply means "the best forage". True Persia alfalfa also spread to Greece in about 490 B.C. and later to the Roman Empire in about 200 B.C. The Romans made use of it in Italy and many other parts of the Mediterranean region. *Medicago* is a large genus with over 87 species, two-thirds of which are annuals and one-third of which are perennials (Brough et al., 1977; Quiros, 2015, Small and Jomphe 1989). *Medicago sativa* L. (Lucerne or alfalfa) is a species that is widely considered a low-cost source of good protein not only for animal fodder but also for nutrition in the world's poorest and developing countries. It was once known as alfa facah (the father of all food) and is currently referred to as "the queen of forages" due to its great nutritional value among the legumes it has the highest crude protein yield: 2000–3000 kg/ha, which is three times that of soya and four times that of wheat. Furthermore, this herb has a long history of usage in folk medicine (Rafińska et al., 2017). Aside from its high protein content and great amino acid profile, this extract provides a

significant amount of beta-carotene, iron, folic acid, calcium, magnesium, and vitamins E, B_2, B_6, and B_{12}. The European Food Safety Authority (EFSA) has approved alfalfa leaf extract as a safe protein and vitamin supplement. Several studies have demonstrated that alfalfa is a rich source of biologically active chemicals and secondary metabolites in addition to its nutritional value. Also, as alfalfa can fix atmospheric nitrogen, it does not require any nitrogen fertilizer to reach its full potential production, and it also works well as a crop for other plants (Lemaire et al., 2019; Rafińska et al., 2017). *Medicago*'s fundamental genomic number is x=8. Except for a few annual species with an x = 7 genomic number. Annual species of *Medicago* are autogamous. Annual plant progenies are impressively homogeneous in accordance with their breeding technique. Most perennial species, on the other hand, are allogamous, with varying degrees of self-incompatibility. Because of their high degree of outcrossing, the majority of these species are very polymorphic. Numerous bee species serve as pollinators for them (Quiros & Bauchan, 2015).

2.2. *Medicago truncatula*

Over the last two decades, an increase in the number of legume research programs has resulted in the introduction of model plants for legume species. Model plant species are valuable not just for advancing basic biology, but also for providing resources for improving (other) economically important crops through translational biology. Models appeal to plant scientists because of their simplicity of manipulation, simple genome organization, quick life cycles, and the availability of a variety of different genetic and genomic techniques (Bruijn, 2020). In general, crop and pasture legumes are poor models for genetic and genomic research. Some cultivated legumes (e.g., peanut) are tetraploid, many have big genomes (e.g., peas and faba beans), and many are resistant to transformation or difficult to regenerate (e.g., common bean, pea, and soybean) (Barker et al., 1990). Most grain legumes produce large but few seeds per plant, as well as huge seedlings, which limits high-density cultivation (e.g., chickpea, black-eyed pea, mung bean, pea, bean, and soybean). Some legumes, such as soybeans, have duplicated genomes, whereas others are self-incompatible or have a protracted generation period (Kang et al., 2016). For a long time, *Arabidopsis thaliana* has been the model plant, but it lacks interactions with beneficial organisms like symbiotic nitrogen-fixing rhizobia, which provide fixed nitrogen to legume plants, or mycorrhizal fungi, which colonize 85 percent of land plants and provide phosphorus and nitrate to the host plant in a symbiotic relationship.

Because rhizobia and arbuscular mycorrhiza interact with legume plants, a versatile legume species was sought to study both symbiotic nitrogen fixation and mycorrhization, as well as other plant developmental processes (Bruijn, 2020). The alfalfa relative, *Medicago truncatula*, commonly known as barrel medic is considered both as a model plant and a forage crop. It was first offered as a model plant for legume biology because it has a variety of fascinating molecular and classical genetic traits. It is native to the Mediterranean basin and is grown as an annual fodder, particularly in Australia. *M. truncatula* has several advantages for plant genomic research: diploid genome (2n = 16), autogamous, relatively small genome (∼550 Mbp), which was sequenced and annotated (Young et al., 2011), and a relatively short generation time (around four months seed to seed), a high level of biodiversity and a well-characterized nitrogen-fixing symbiont, *Sinorhizobium meliloti* (Colditz & Braun, 2010; Saeidi et al., 2012). It is also utilized as a model plant for mycorrhizal interactions research. Furthermore, the genus *Medicago* belongs to the Galegoid phylum and is thus linked to a variety of key crop legumes, including pea, faba bean, chickpea, lentil, and clover, in addition to alfalfa (Figure 4). Members of this phylum are likely to have high levels of nucleotide sequence conservation and similar genetic architecture, implying the possibility of relatively straightforward gene transfer across member species. *Lotus japonicus*, a diploid legume, has also been considered a model legume. This plant possesses characteristics similar to *M. truncatula*, but it is phylogenetically distant from the Galegoid phylum and other legumes such as soybeans or beans (Bruijn, 2020).

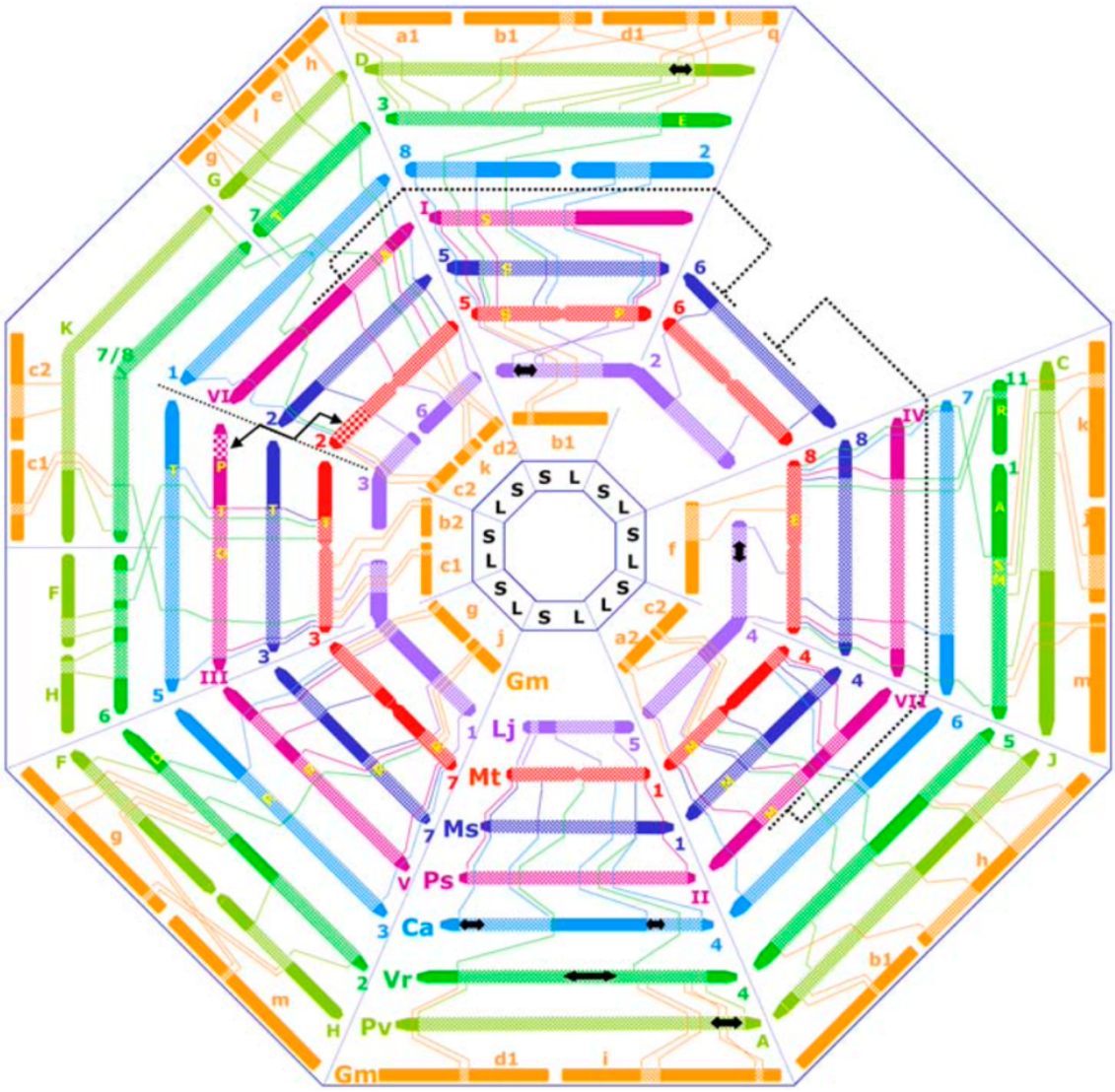


Figure 4- Synteny between *M. truncatula* and seven other important legume species.

Mt, *M. truncatula*; Ms, *M. sativa*; Lj, *L. japonicus*; Ps, pea; Ca, *Cicer arietinum*; Vr, *Vigna radiata*; Pv, *Phaseolus* vulgaris; Gm, *Glycine max*. Syntenic blocks are illustrated to scale based on genetic distance. Taken from: (Zhu et. al.,2005) , TGIL database

2.2.1. *Medicago truncatula* sequencing

The sequencing of *M. truncatula* began in 2003, with the National Science Foundation (NSF) and the European Union's Sixth Framework Program giving initial funding to complete the euchromatic section of the genome. Six of *Medicago*'s eight chromosomes were sequenced in the United States by NSF-funded projects, one (chromosome 5) was sequenced in France by Genoscope with funding from the European Union and the Institute for Agricultural Research (INRA), and one (chromosome 3) was sequenced in the United Kingdom with funding from the European Union and the Biotechnology and Biological Sciences Research Council (BBSRC). In terms of the sequencing line, it was decided to use the Jemalong A17 line, which was already being used as the parental line of RILs in 2003 (Young et al., 2003). These funds then enabled the complete sequencing of the euchromatin portion of the *M. truncatula* genome (ie between 280 and 300Mb). Indeed, preliminary analyses of the *M. truncatula* genome revealed that the majority of the plant's genes were discovered in the euchromatic portions of the arms of the chromosomes, rather than in the heterochromatin of the centromeres and pericentromers (Kulikova et al., 2001). The Mt3.5 version of the *M. truncatula* genome was collected and assembled in 2011 utilizing the genetic map generated from 93 individuals in F2 resulting from a cross between A17 and A20 (Choi et al., 2004) as well as by sequencing mainly by the Sanger method 2,536 BACs: 246 Mb were assembled thanks to the overlaps of the BACs (Bacterial Artificial Chromosomes), 104.2 Mb were not anchored in the pseudomolecule and were sequenced by sequencing Illumina at a depth of 40X. Thus, 62,388 genes predicted by the IMGAG group (using the Eugene pipeline), including 14,322 annotated genes, were sequenced, accounting for almost 94 percent of the genes expressed (Young et al., 2011). The new genome assembly of *M.truncatula* (Mt4.0) was constructed in 2014 using the genetic map of the population of LR4 RILs (A17xDZA315.16) (Figure 5), the population developed in the laboratory, and contains sequences obtained by the Illumina (> 50X), Sanger, and 454 technologies. This new assembly made it possible to anchor 75.8 Mb of the non-anchored 104.2 Mb of the Mt3.5 version : the total length of the genome obtained is 384.5 Mb (i.e. an anchoring of 93% of the sequences against 71% for version Mt3.5). The EVM and MAKER pipelines were used to annotate this genome, and the results revealed the presence of 50,894 genes, or 82 percent of the genes annotated on the Mt3.5 version of the genome (31,661 genes at high confidence and 19,233 at low confidence) (Tang et al., 2014) (Figure 6).

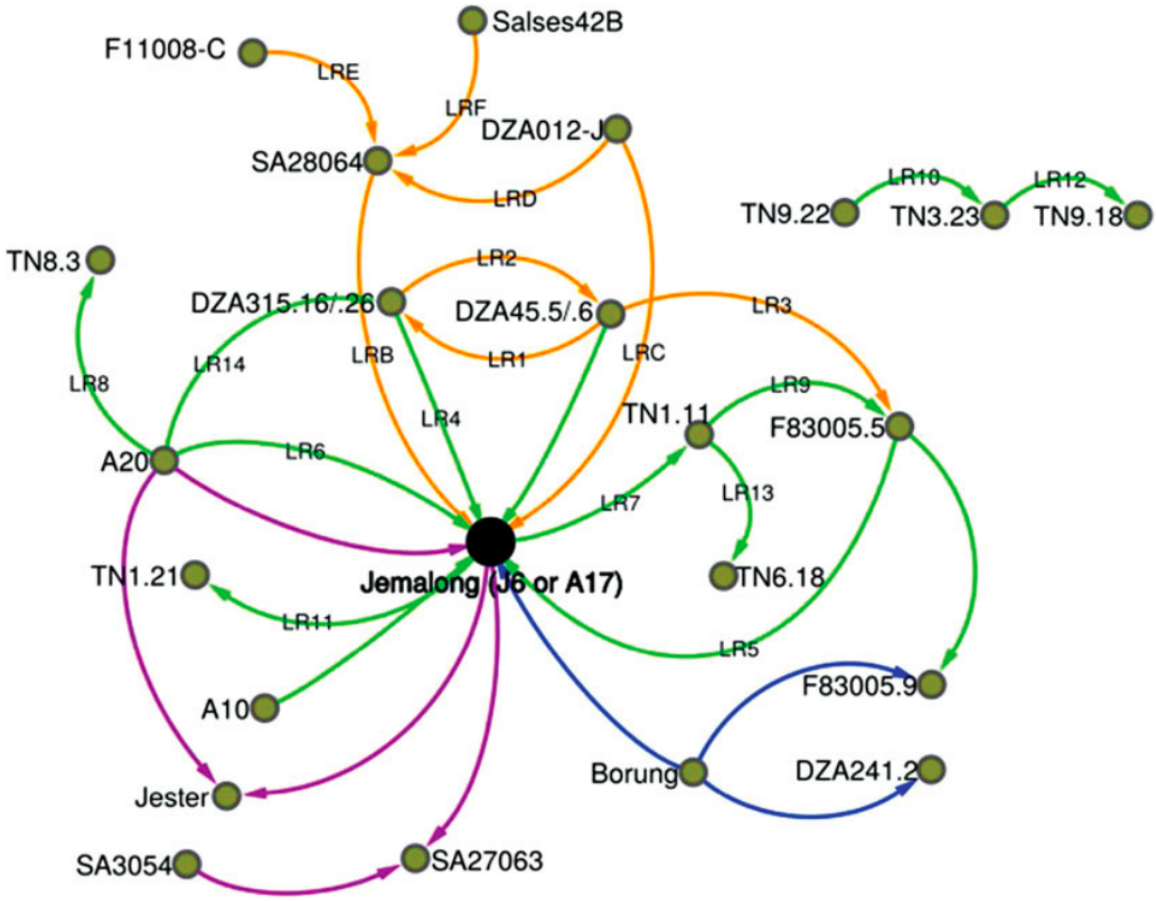


Figure 5- The *Medicago truncatula* RIL populations of different crosses.

Purple lines: crosses done by Australian groups; Orange lines: crosses done by INRAe Montpellier; Blue lines: crosses done by INRAe Rennes; Green lines: Crosses including Tunisian lines (TN1.11; TN1.21; TN6.18) where performed by the CBBC in Tunisia in collaboration with CNRS/INRA.

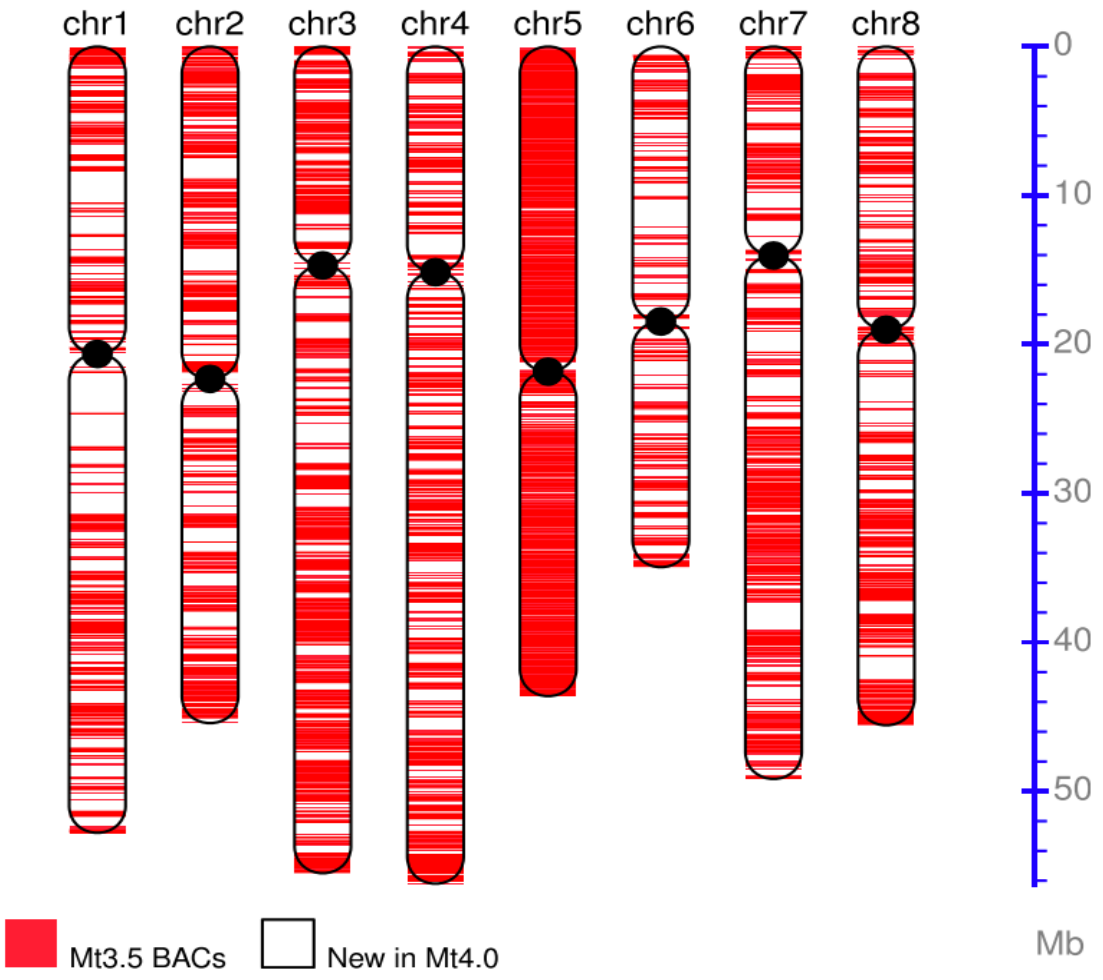


Figure 6- The *Medicago truncatula* genome sequence Mt4.0 compared with Mt3.5 in terms of increased number of chromosomal anchor sequences.

Red-colored segments of the chromosomes represent BAC sequences used in Mt3.5 and the white areas on the chromosomes represent newly anchored sequences in Mt4.0. Taken from: (Tang et al., 2014)

I.3. QTL mapping

Many characters of organisms show a continuous variation in the progeny of specific parents. The investigation of these "metric" traits led to the employment of biometric or "quantitative" approaches, which postulate that variation is generated by the effects of several gene loci, environmental impacts, and genotype-environment interactions. As a result, individual discontinuities induced by Mendelian genes are included in the continuous variation (Geldermann, 1975). Before the rediscovery of Mendel's work, at the end of the nineteenth century, a method for analyzing quantitative qualities called biometrical genetics was created, which looked at quantitative variation. For a long time, the biometrical and Mendelian techniques seemed irreconcilable. Johannsen (1909), who proved that both heritable and non-heritable factors contributed to variance in bean seed weight and that these factors could be identified by breeding studies, made a big step toward reconciling conflicting methods (Hackett, 2002). Many agriculturally essential features, including yield, quality, and some forms of disease resistance, are controlled by multiple genes and are referred to as quantitative traits (sometimes referred to as polygenic, multifactorial, or complex traits). Quantitative trait loci (QTLs) are areas inside genomes that include genes associated with a specific quantitative trait (Collard et al., 2005). QTL analysis is a statistical tool that connects two types of information—phenotypic data (trait measurements) and genotypic data (typically molecular markers)—in an attempt to understand the genetic basis of variation in complex characteristics. QTL analysis enables researchers in domains as diverse as agriculture, biology, and medicine to associate complicated characteristics with specific chromosomal regions (Miles & Wayne, 2008). QTL can be a single gene, or it may be a cluster of linked genes that affect the traits. QTL mapping studies have reported in most crop plants for diverse traits like yield, quality disease and insect pest resistance, abiotic stress tolerance and environmental adaptation. Two things are required for scientists to begin a QTL study. First, they need two or more strains of organisms that differ genetically with regard to the trait of interest. For example, they might select lines fixed for different alleles influencing egg size (one large and one small). Second, researchers also require genetic markers that distinguish between these parental lines. For genotyping, molecular markers are preferred since they are unlikely to change the trait of interest. Single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs, or

microsatellites), restriction fragment length polymorphisms (RFLPs), and transposable element locations are among the markers employed (Miles & Wayne, 2008). The basic problem with studying a quantitative trait has always been that the phenotype of a given genotype tells us little about the genotype itself; two plants could be 1 m high but have very different genotypes. The discovery of extensive and easily recognizable molecular variation has opened up the possibility of studying individual QTL. The principle is simple. Molecular markers give unambiguous, single-site genetic differences that can easily be scored and mapped in most segregating populations. It is not difficult in populations of most species to identify and map 10 to 50 segregating markers per chromosome. Most will be in non-coding regions and will not affect any trait directly, but some at least will be linked to QTL which does affect the trait of interest. QTL analysis depends on the fact that where such linkage occurs, the marker locus and the QTL will not segregate independently and so differences in those marker genotypes will be associated with different trait phenotypes. Situations, where genes fail to segregate independently, are said to display 'linkage disequilibrium'(Kearsey, 1998).

3.1. QTL mapping principles

Identifying a gene or QTL within a plant genome is like finding the needle in a haystack. QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. The process of QTL mapping involves four major steps, which are discussed below under the following subheadings.

3.1.1. Developing of mapping populations

QTL mapping requires an appropriate mapping population derived from phenotypically dissimilar parents (E.g.: highly resistant and susceptible lines). The mapping population's paternal lines should be genetically varied, increasing the chances of finding a high number of polymorphic markers that are evenly dispersed across the genome. Several alternative populations may be used for mapping within a given plant species, each with advantages and disadvantages. The mapping population may differ depending on the study's purpose, time

frame, and resources available for QTL mapping (Broman & Sen, 2009). Almost any type of population is amenable to such analysis. The most useful are those derived from a cross between two inbred lines (F2, backcross, recombinant inbred lines, doubled haploid lines) because the marker-QTL linkages in the F1 cause the derived populations to be in linkage disequilibrium (Kearsey, 1998). The F2:3 families have the advantage that it is possible to measure the effects of additive and dominant gene actions at specific loci. RILs are essentially homozygous, and only additive gene action can be detected. The benefits of employing RILs for QTL analysis are self-evident. First, numerous selfing cycles can increase the amount of recombination events, resulting in more precise QTL mapping. More crucially, once RILs are produced with all genotypes fixed as homozygotes, these lines can be utilized repeatedly to investigate QTLs of diverse traits under varied environments. As a result, establishing a comprehensive set of RILs will make a significant contribution to the species' QTL mapping (Takuno et al., 2012). The size of the mapping population for QTL analysis depends on several factors, type of mapping population used for QTL analysis, genetic nature of the target trait, objective of the study, and resources available for handling a sizable mapping population in terms of phenotyping and genotyping. From a practical standpoint, the goal of QTL mapping is to find QTL with major effects, which is only attainable when a large number of people, say 500 or more, are utilized for QTL analysis. As a result, the mapping population is roughly 200-300 people in total (Singh & Singh, 2015).

3.1.2. Generating a saturated linkage map

Mapping entails arranging the markers in order, identifying their relative genetic distance, and assigning them to linkage groups based on recombination values obtained from all pairwise combinations of the markers. The position and relative genetic distance between markers along chromosomes are shown on the linkage map. We can analyze the segregation patterns for each of the markers by genotyping the mapping population using polymorphic molecular markers. A variety of molecular markers, such as RFLPs, RAPDs, SSRs, AFLPs, and SNPs, have been employed to identify individual QTLs and determine their effects and positions (Collard et al., 2005).

3.1.3. Phenotyping of a mapping population

The quantitative qualities to be measured must be as precise as feasible. Strictly speaking, no dates should be missing, but small amounts of missing data can be accepted. The data is pooled over location and replication to obtain a single quantitative value for the line. It is also necessary to measure the target traits in experiments conducted in multiple locations to have a better understanding of the QTL x Environment interaction (Kearsey, 1998).

3.1.4. QTL detection using statistical tools

The goal of QTL mapping is to detect QTL while avoiding false positives (Type I Errors), which occur when a marker is associated with a QTL that does not exist. The tests for QTL or trait association are often performed by approaches including the Single marker approach, Simple interval mapping, Composite interval mapping, and Multiple interval mapping (Hackett, 2002).

3.1.5. QTL mapping strategies in summary

The requirements of QTL mapping are mapping population, saturated linkage map, phenotypic screening, and statistical package. Ideally, markers should be less than 5 cm from a gene or QTL. Using a pair of flanking markers can greatly improve reliability but increases time and cost. The QTL mapping strategies are choosing divergent parents, Screening for marker loci polymorphism to create mapping populations for generating a saturated linkage map, phenotype screening, and Comparing the mean of the marker attached and pair of genes of interest at every marker locus (Chandra & Pandey, 2017).

3.2. Single–marker analysis by linear regression

Testing for relationships between trait values and marker genotypes is a good way to start an examination of the genetics of a quantitative trait. The simplest way to analyze QTL mapping data is to look at each marker separately, divide the individuals into groups according to their genotypes at the marker, and compare the phenotype averages of the groups. This method was developed by Sax (1923) and has been widely employed in maize by numerous groups, notably Edwards et al. (1987). We can construct a regression line for each marker individually without knowing where it is on the genome, but rearranging the markers into map order first provides a lot more information (Hackett, 2002). A suitable first step in the study is regression on marker genotypes. A score, which compares the hypothesis that there is a QTL at the marker to the hypothesis that there is no QTL elsewhere in the genome, has traditionally been used to assess evidence for linkage to a QTL. Large LOD values indicate the presence of a QTL, although the statistical significance of LOD scores must be considered in light of the multiple tests conducted. One significant disadvantage is that people with lacking marker genotypes must be excluded. Furthermore, positions between markers cannot be examined, and information concerning QTL placement is limited (Broman & Sen, 2009).

3.2.1. Problems with the regression approach

Although regression on marker genotypes provides a wealth of information regarding marker trait associations, it is not without flaws. Only the marker sites are used in this method, hence it has less power to discover a QTL between the markers. The QTL effect and the recombination frequency cannot be estimated separately. Within each marker class, there are a lot of variances, and part of it is attributable to other QTLs affecting the trait. This must be taken into account in order to perform a more reliable test for the presence of a QTL (Hackett, 2002).

3.2.2. Single–marker analysis strategy

DNA markers can be used to map useful genes using recombination frequencies of linked genes (Figure 7):

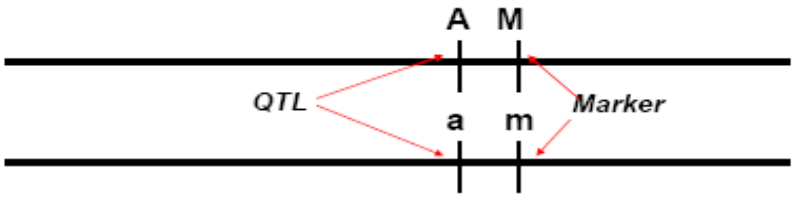


Figure 7- Schematic representation of QTL mapping through a single marker analysis.

Markers near QTLs co-segregate with them, Markers tightly linked to QTL detected by ANOVA, if crossover between marker & QTL, Am & aM gametes will be produced

Single marker analysis can be conducted using a variety of statistical analyses, including t-tests, ANOVA, regression, maximum likelihood estimations, and log-likelihood ratios. The fact that molecular marker genotypes can be classified into groups means that marker genotypes can be used as classifying variables for a t-test or ANOVA, or as variables for regression analysis. The null hypothesis tested is that genotypic classes do not differ in phenotype for a given molecular marker. Single marker analysis calculates whether phenotype values differ among genotypes for a given molecular marker. For example, do resistant and susceptible individuals have different genotypes at a given molecular marker? Significant differences suggest that the marker genotype and phenotype are connected.

3.3. Simple interval mapping method

Interval mapping methods estimate QTL position based on the values of two flanking markers. Standard interval mapping assumes the presence of a single QTL once again, but now considers a grid of sites along the genome as candidate QTL locations. The most popular and extensively used interval mapping approaches are Simple Interval Mapping (SIM), Maximum Likelihood

(ML), and regression-based SIM methods. These approaches, like ANOVA, assume the presence of a single QTL and use a genetic map of the typed markers. Each locus is analyzed one at a time in SIM, and the logarithm of the chances ratio for the model that the particular locus is a true QTL is calculated. The Pearson correlation coefficient between the phenotype and the marker genotype for each individual in the experimental cross is related to the odd ratio. SIM uses two neighboring markers to perform a likelihood ratio test (LRT) at each place in the interval to see if there is a QTL inside the interval (Akond et al., 2019). The management of missing genotype data varies between interval mapping approaches. Under a mixed model, standard interval mapping uses maximum likelihood estimation, whereas Haley–Knott regression methods use approximations to the mixture model. The multiple imputation approach uses the same mixing model as the maximum likelihood method, but instead of maximum likelihood, multiple imputation is used (Broman & Sen, 2009).
Interval mapping methods use information on values of 2 flanking markers (Marker interval = the segment between 2 markers) to estimate QTL position. The probability that data could be obtained assuming a QTL at several positions between the markers is calculated and QTL analysis is declared where the probability of obtaining the observed data is highest which leads to finding the position of QTL with molecular markers. DNA markers can map useful genes using recombination frequencies of linked genes.

3.4. Composite interval mapping method

One popular method for dealing with QTL mapping where multiple QTLs contribute to a trait is to scan the genome iteratively and add known QTL to the regression model as QTLs are identified. This method, known as Composite Interval Mapping (CIM), determines the location and magnitude of QTL effects more precisely than single-QTL approaches Particularly in small mapping populations where the effect of genotype correlation in the mapping population may be problematic (Akond et al., 2019). Multiple regression analysis and interval mapping are combined in composite interval mapping (CIM). CIM regulates the effects of QTLs found in other marker intervals on the same chromosome as the QTL under investigation, as well as on other chromosomes. The precision of QTL detection is improved as a result of this. CIM performs single-marker analysis first and then uses stepwise or forward regression to build the model as a multiple QTL model. By accounting for linked QTLs and reducing residual

variation, these markers serve as proxies for other QTLs, increasing the resolution of interval mapping. The CIM method selects appropriate marker loci to serve as covariates. The benefits of considering multiple QTLs at the same time are that (a) residual variation is reduced, making it easier to detect loci with smaller effects, (b) linked QTLs are separated, and (c) interactions between QTLs are identified. As an initial exploratory step, a marker near a putative QTL may be considered a covariate in the search for additional QTL. The main advantage of using a marker as a covariate is that it reduces residual variation and thus clarifies evidence for additional QTL (Broman et al., 2003; Singh & Singh, 2015).

I.4. Gene expression analysis

4.1. Quantitative real-time PCR (qPCR)

For confirming results from assays that quantify gene expression patterns, real-time PCR (quantitative PCR or qPCR) has become the primary recommended approach. Real-time quantitative PCR is the accurate detection and quantification of products produced during each cycle of the PCR process, which is exactly proportional to the amount of template used before the PCR process begins. To assess differences in transcriptome levels between samples, the procedure employs reverse transcription polymerase chain reaction (RT-PCR) in conjunction with fluorescent chemistry. Quantitative real-time PCR (qPCR) is a type of reverse transcription PCR that determines the number of transcriptomes in a sample (Figure 8) (Thornton & Basu, 2011). The PCR reaction produces copies of a DNA template at an exponential rate. As a result, there is a quantitative relationship between the amount of starting target sequence and the amount of PCR product accumulated at each cycle. Because of polymerase reaction inhibitors found in the template, reagent limits, or pyrophosphate molecule accumulation, the PCR reaction eventually ceases to generate the template at an exponential rate (i.e., the plateau phase), rendering endpoint quantification of PCR products inaccurate. As a result, duplicate reactions may produce different amounts of PCR product. Extrapolating back to identify the starting quantity of the template sequence is only achievable during the exponential phase of the PCR reaction. The detection of PCR products as they accumulate (i.e., real-time quantitative PCR) allows quantification during the exponential phase of the reaction,

eliminating the uncertainty associated with traditional PCR (Figure 8) (Arya et al., 2005). Unlike other methods for quantifying mRNA (such as Northern blotting and ribonuclease protection tests), qPCR uses less RNA, needs less effort, and produces vast amounts of data in a short amount of time. Its sensitivity and relative ease of use have made it a significant tool in bioinformatics, virology, and molecular diagnostics. To amplify small amounts of DNA, qPCR, like conventional PCR, employs Taq polymerase, buffer, dNTPs, and primers. It varies from the traditional method in that it employs a fluorescent signal that is monitored by a specific, computerized thermocycler. Depending on the experiment's purpose, different fluorescent reporters may be utilized. Molecular Beacons (Public Health Research Institute Properties, Inc., USA), Scorpions1 (Sigma-Aldrich, USA), SYBR1 Green (Invitrogen, USA), and Taq- Man1 (Invitrogen, USA) are the four most frequent (Applied Biosystems, USA). Probes like TaqMan1, Molecular Beacon, or Scorpions1 can be employed if the purpose of the experiment is to analyze one or a few genes. Testing, on the other hand, could be prohibitively expensive due to the fact that probes must be tuned to a specific target sequence.SYBR1 Green may be a more cost-effective choice for experiments involving numerous genes or laboratories with multiple researchers performing qPCR analysis.SYBR1 Green is a nonspecific binding dye that produces a fluorescence signal in the presence of double-stranded DNA (Thornton & Basu, 2011).

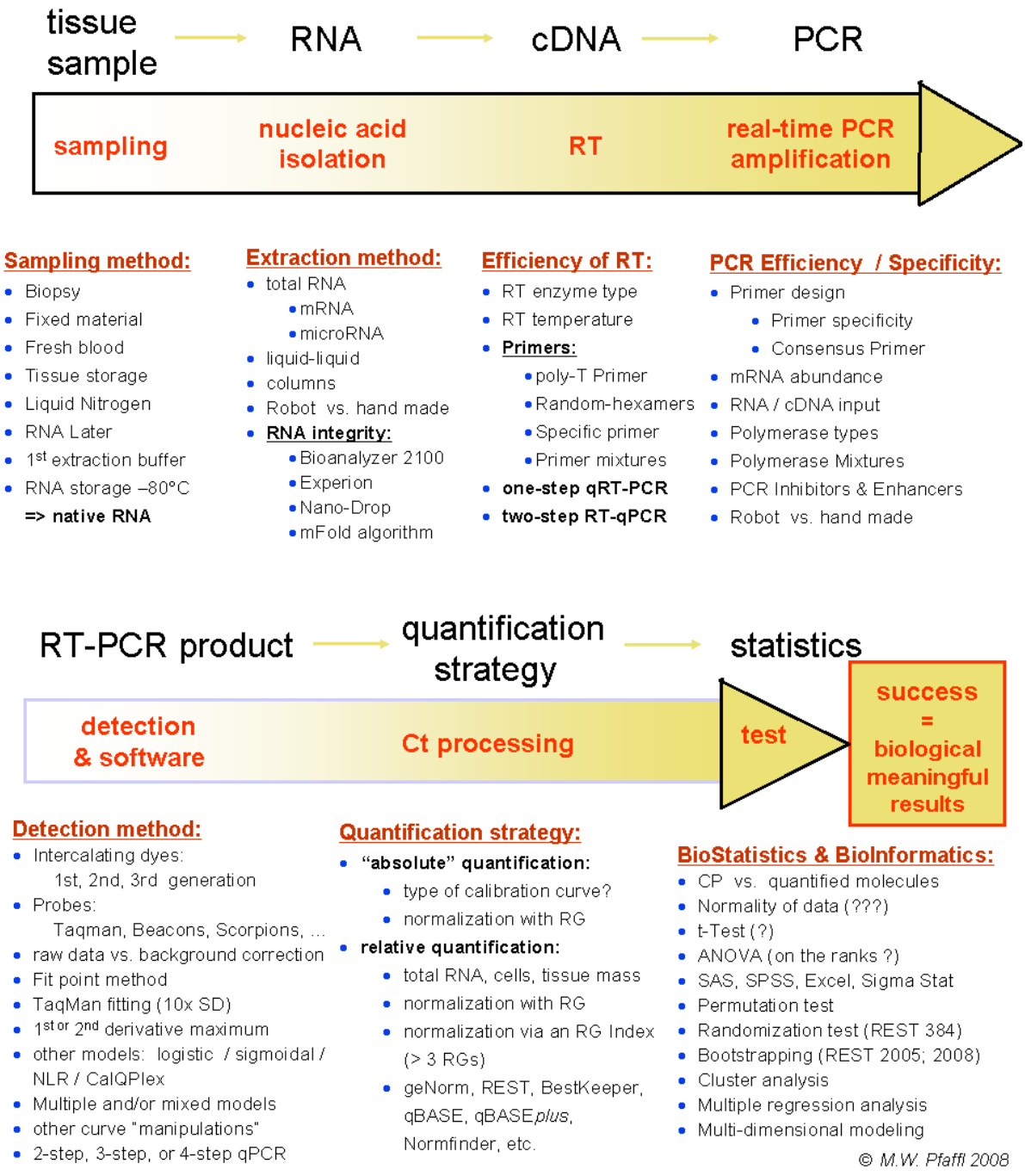


Figure 8- Steps and variables of a successful mRNA quantification using real-time RT-PCR.

Taken from: (Pfaffl, 2008)

II - Objectives of the study

Earlier investigations in our group on the tolerance of *M. truncatula* to cadmium stress have demonstrated that *M. truncatula* is a Cd-sensitive plant, and that Cd has a rapid and severe effect on seedling growth. The highly sensitive response to this stress was the inhibition of root growth, which can be utilized as a reliable indicator of Cd toxicity and tolerance for screening breeding materials. Additionally, high genetic variability was reported for Cd tolerance across the accessions of the core collection that were used at the seedling stage, which suggests the possibility of selection for Cd toxicity tolerance in *M. truncatula* (Saeidi et al., 2012). Another study reported that when Cd toxicity was present throughout the early seedling growth of six *M. truncatula* genotypes, oxidative disorders were observed. Root growth inhibition, cadmium uptake, and the presence of oxidative damage all interact to imply differing responses between genotypes, depending on whether the accessions are susceptible or tolerant. Target genes for ROS-scavenging enzymes like superoxide dismutase (*SOD*) and peroxidase (*PRX*), reduced glutathione (GSH) metabolism like Glutathione S-transferase (*GST*) and glutathione reductase (*GR*), and metal-chelating metabolism like PCS were examined for transcriptional changes in response to cadmium treatment. Their findings provided an overview of the negative impacts and oxidative damage caused by cadmium stress in *M. truncatula* and contributed to the understanding of how relatively tolerant *M. truncatula* seeds can survive and grow in cadmium-contaminated media by preventing a potential oxidative burst, reducing metal uptake by cadmium chelating within roots, and inducing antioxidant defenses during germination (Rahoui et al., 2014).It was also reported that cadmium treatment altered macronutrients (Ca and K), microelements (Fe, Zn, and Cu), carbohydrates (total soluble sugars (TSS), glucose, fructose, and sucrose), and free amino acid (FAAS) accumulations. The tested genotypes showed variability in these mobilization changes. Under control conditions, carbohydrates governed the proliferation of susceptible lines, while free amino acids allowed tolerant lines to resist cadmium intrusion. MtMST1, a gene encoding a monosaccharide transport protein, was studied for transcriptional alterations in response to cadmium treatment. The most susceptible line, TN1.11, showed a considerable down-regulation. Since tolerant lines consumed excessive amounts of glucose, glucose metabolic integrity appears to be crucial for preserving growth while exposed to cadmium. Their results provided an overview of the harmful effects of cadmium on membrane integrity and biomass mobilization. Under cadmium stress, nitrogen metabolism was found to be essential for establishing seedling growth, while carbon

metabolism was demonstrated to be important for enhancing early embryonic growth. The avoidance of cadmium stress in moderately tolerant germinating *M. truncatula* lines has been achieved by maintaining a safe respiration process and inducing defensive mechanisms through amino acid conversion (Rahoui et al., 2015). In another study, *M. truncatula* seedlings' roots have been used to highlight defense mechanisms and stress reactions induced by Cd stress. Except for A17 and TN1.11, Cd initially has elevated reactive oxygen species and boosted antioxidative (total SOD, CAT, and PRX) and ascorbate-glutathione-related metabolic enzymes (APX and MDAR). Their enzyme activity analysis has revealed that Cd increased total SOD in four of the five genotypes while decreasing line TN1.11. In four out of five lines exposed to cadmium, PRX, and CAT activities have been increased. It was demonstrated that Cd stress causes an increase in PRX, CAT, and total SOD activity to fight ROS and oxidative burst. Also, Cd treatment has had an impact on APX and MDAR; divergent outcomes have been found across various genotypes. APX has marginally reduced in A17 and unaffected in TN1.11, but has been elevated in the other genotypes. Cd, on the other hand, induced increased MDAR activity in all of the genotypes examined. The amount of total phenolic compounds and their induction by Cd treatment varied between accessions, with the more sensitive lines (A17 and TN1.11) showing the greatest increase (Rahoui et al., 2017). According to the results of these past studies, there is a true genetic variation in the response to cadmium stress in *M. truncatula.* However, no genetic study related to this stress had been done in this species despite the availability of several RIL populations and their genetic maps. The LR4 population has parents with contrasting responses to cadmium and other stresses. Hence, we made use of this population for a thesis project entitled "Genetic control of plant tolerance to heavy metal pollution: a study on the legume model plant *M. truncatula* and cadmium stress". This study addressed 2 general objectives:

(I) Identification of *loci* involved in *M. truncatula* cadmium tolerance in early stages after germination in the recombinant inbred lines (RILs) and exposure to cadmium on water-agar plates by using several growth traits to identify QTLs involved in Cd tolerance.

(II) Identification of mechanisms involved in cadmium tolerance in *M. truncatula* by assessing the expression of genes known to be involved in responses to Cd, in tolerant and susceptible RILs.

III - Material and Methods

III.1. Material

1.1. Plants

1.1.1. *Medicago truncatula* LR4 population

A set of 147 Recombinant Inbred Lines (RILs) of the LR4 population of *M. truncatula* at F8 generation, resulting from the cross of two parental lines A17 and DZA315.16 was used in this study. A17 (from the Australian cultivar Jemalong6) is used as female and the line DZA315.16 (from a population collected in Algeria) was used as the male parent. All the accessions (including the parental lines are resources available in our laboratory (Ecolab, ENSAT, Toulouse, France).

1.1.2. Iranian *Medicago* species

A set of four *M. truncatula* accessions and one *M. scutellata* that were available in our collection were used in this study (Table 2). The Iranian accessions were fixed genetically by selfing in our greenhouse. In order to fix them, they were planted in the greenhouse for two successive generations by means of the single-seed descendants (SSD) method.

Table 2- Iranian *Medicago* species used in this study.

***Medicago* species**	**Accession Numbers**	**Province**	**City**	**Height**	**Latitude**	**Longitude**
M. truncatula	1724	Golestan	Gorgan	70	-	-
	7321	Qom	Qom	-	-	-
	7543	Golestan	Minoo dasht	420	37/54/00	55/56/00
	7600	Golestan	Minoo dasht	300	37/48/00	55/58/00
M. scutellata	Scu	-	-	-	-	-

1.2. Cadmium solution

Cadmium was purchased from Sigma Aldridge Company (Sigma Aldrich, USA; CAS number: 7440-43-9, Ref: 265365-100G). To prepare a 15 µM cadmium solution, a 1 M cadmium stock solution was prepared at the first step in deionized water and subsequently diluted to a solution of 15 µM.

1.3. Bioinformatics data

Simple sequence repeat markers (SSR) data were obtained from the *Medicago truncatula* genetic map. On the *Medicago truncatula* genome, there are 316 simple sequence repeat markers (SSRs), and covers 910 cM of all 8 haploid chromosomes with a 3 cm average distance between markers (Julier et al., 2007; Yu et al., 2012).

III.2. Methods

2.1. Phenotypic evaluation

2.1.1. Seed germination

To germinate, *M. truncatula* seeds were taken out from pods and were sacrificed by rubbing them on sandpaper (P180), then placed in Petri dishes on absorbent paper soaked in water. After that, the Petri dishes were covered with aluminum foil and were placed in the dark at 4° C for 2 to 4 days, and finally at 25° C overnight.

2.1.2. Plant treatment

Water agar medium (agar hp696, 0.8% w/v in deionized water) containing or not 15µM Cadmium final concentration was prepared and autoclaved. 50 ml medium was poured into each square Petri dish (12x12 cm) and left to solidify in a way to create a slanted surface (Figure 9A). Seed coats of germinated seeds were smoothly removed (this should be done carefully and calmly to prevent damage to the roots) (Figure 9B) and seedlings were put onto the medium. Each Petri dish was divided into two parts and in each part, one genotype was placed (approximately five seeds per genotype, two genotypes per Petri dish). Petri dishes were then sealed with parafilm tape and put into envelopes of brown paper to shade the developing roots. Placed in a plastic box and were incubated in a phytotron with (25/20) °C, 16/8h (day /night) photoperiod, and 75% relative humidity (Figure 10).

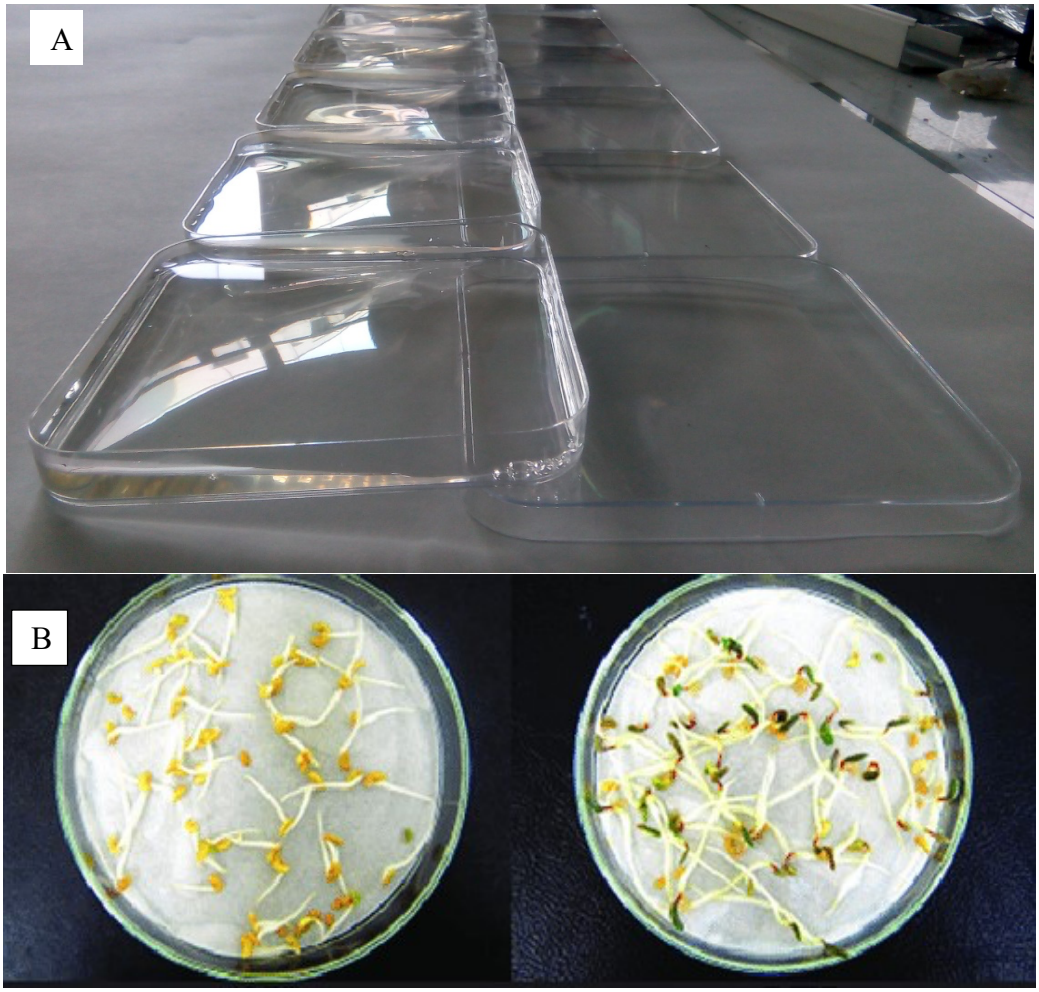


Figure 9- Germination of *M. truncatula* seeds.

A) Petri dishes containing slanted water agar medium, B) *M. truncatula* germinated seeds, on the left panel with seed coat, on the right panel seed coat removed.



Figure 10- Seedling incubation.

A) Petri dishes covered by paper to simulate dark situation for root part, B) Arrangement of boxes and Petri dishes in the phytotron.

2.1.3. Experimental design

In order to obtain three biological repeats of the phenotypes for each of the 147 accessions, 23 experiments were done. The split-plot experiment plan was set up by including in each of these replicates the parental line accessions A17, and DZA315.16 as the "check" accessions. Using two "Check" lines made it possible to estimate the block effect of each of the repetitions and to correct all the data by the method of marginal means (or LSMEANS), calculated using the LSMEANS package (Lenth, 2018). Each replication contained 4-5 experiments, each experiment contained 2 blocks, and each block contained 2 conditions (control and stress with +15µM Cd, final concentration) (Figure 11). Petri dishes of each whole plot were placed into one box. Boxes were incubated in a phytotron in a way that 2 boxes of each block were placed near each other but far from the other block. Petri dishes of each whole plot were placed into one box. Boxes were incubated into the phytotron with (25/20) °C, 16/8h (day /night) photoperiod, and 75% relative humidity, in a way that 2 boxes of each block are placed near each other but far from the other block. Also to simulate the environment of the Petri dishes with the soil environment in terms of darkness, the surface of the Petri dishes where the roots were located was covered with paper.

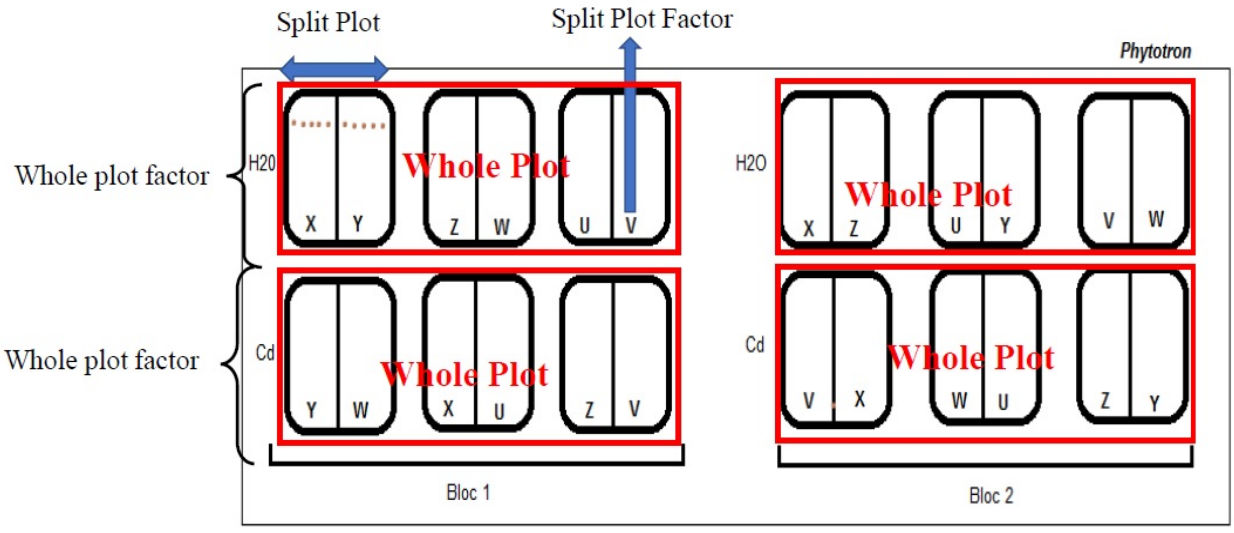


Figure 11- Experimental design used in this study.

Physical experimental design Split plot design. Whole Plot: Box, Whole Plot Factor: Condition, Split Plot. Petri dish, Split Plot Factor : Genotype.

2.1.4. Phenotyping

Root growth traits were recorded by scanning (MFPs & copier e-STUDIO3515AC machine) the Petri dishes, immediately as 0 days. To obtain images on a black background, the scanner was used with an open lid with a resolution of 200 dpi and in grayscale mode. Petri dishes were scanned after 1, 2, 4, and 6 days, approximately at the same time as the 0th day (Figure 12A). Root length, diameter, and surface were measured using ImageJ and Smart root software. Data were analyzed and visualized with the R software (Agricola, ggplot2, lmerTest, reshape2, ….). The images are then processed with the SmartRoot V4.21 module (Lobet & Draye, 2013) implemented in ImageJ V1.50i (Schneider et al., 2012) (Figure 12B).

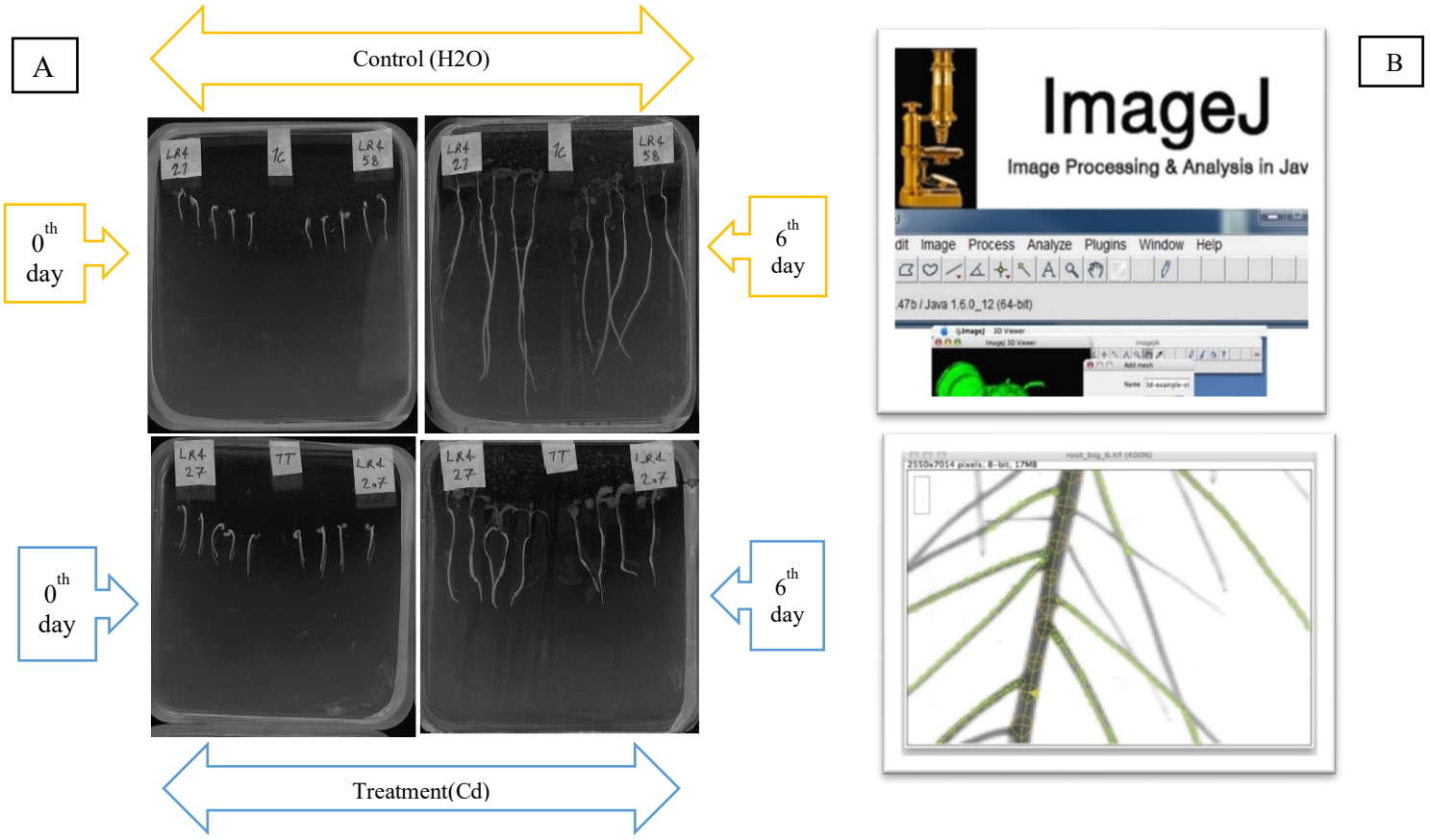


Figure 12- Root length measurement.

A) Scans of petri dishes on the 0^{th} and 6^{th} day(s) in two control and Cd-treated conditions, B) ImageJ and Smart root software.

The required number of aluminum sheets in the appropriate size was prepared and the weight of each was measured and recorded. After scanning the Petri dishes on the 6th day, roots and aerial parts of plants are harvested and placed in aluminum sheets (roots and aerial parts pooled respectively for each accession) then their fresh weight is determined. Dry weight was determined after 2 days in the oven at 60 ˚C.

2.1.5. Phenotyping data analysis

The experiment was organized as a split-plot design with 147 RILs and their parents in three replications. The diameter, surface, length, fresh weight, and dry weight of roots as well as the fresh and dry weight of aerial parts were measured. Root growth development from the first day until the end of the experiment (0th, 1st, 2nd, 4th, and 6th day) under Cd stress was plotted for each of the inoculated plants using the agricolae package (Mendiburu, 2021) of the R software (The R Project for Statistical Computing, version 4.2.1). In total 39375 root lengths were measured. The linear mixed model was used for analyzing root growth traits. The analyses of variance (ANOVA), the Shapiro tests (verification of the postulate of normality of the residues), and the heritability tests of the lmer4 package were carried out with the R software. The least-square means (LSMEANS) for each genotype were calculated due to the effect of the condition on the genotype and the data were adjusted based on the two check lines (A17 and DZA315.16).

2.2. QTL analysis

Quantitative trait loci analysis was performed using the genetic map of the LR4 RILs population. The map was explained as low-density by Julier (2007) and then was improved by Ben et. al. (2013). It contains 316 simple sequence repeat markers (SSRs) and covers 910 cM of all 8 haploid chromosomes with 3 cM average distance between markers (Figure 13). QTL analysis was conducted with root length and Fresh leaf weight (FLW) traits with the QTL package (Arends et al., 2010; Broman et al., 2003) in the R environment (R Core Team, 2020).

Root length data in the treatment condition was used as a variable while the root length in the control condition was considered a covariate. Single marker analysis (Marker regression method, Mr), simple interval mapping (the EM algorithm, Haley-Knott regression, Multiple imputation), Composite interval mapping (CIM), and following that, multiple QTL mapping (MQM) methods were used for analysis to compare and improve the reliability of the QTL detection. Threshold values for the log-likelihood (LOD) scores were determined by computing 1000 permutations (Broman & Sen, 2009).

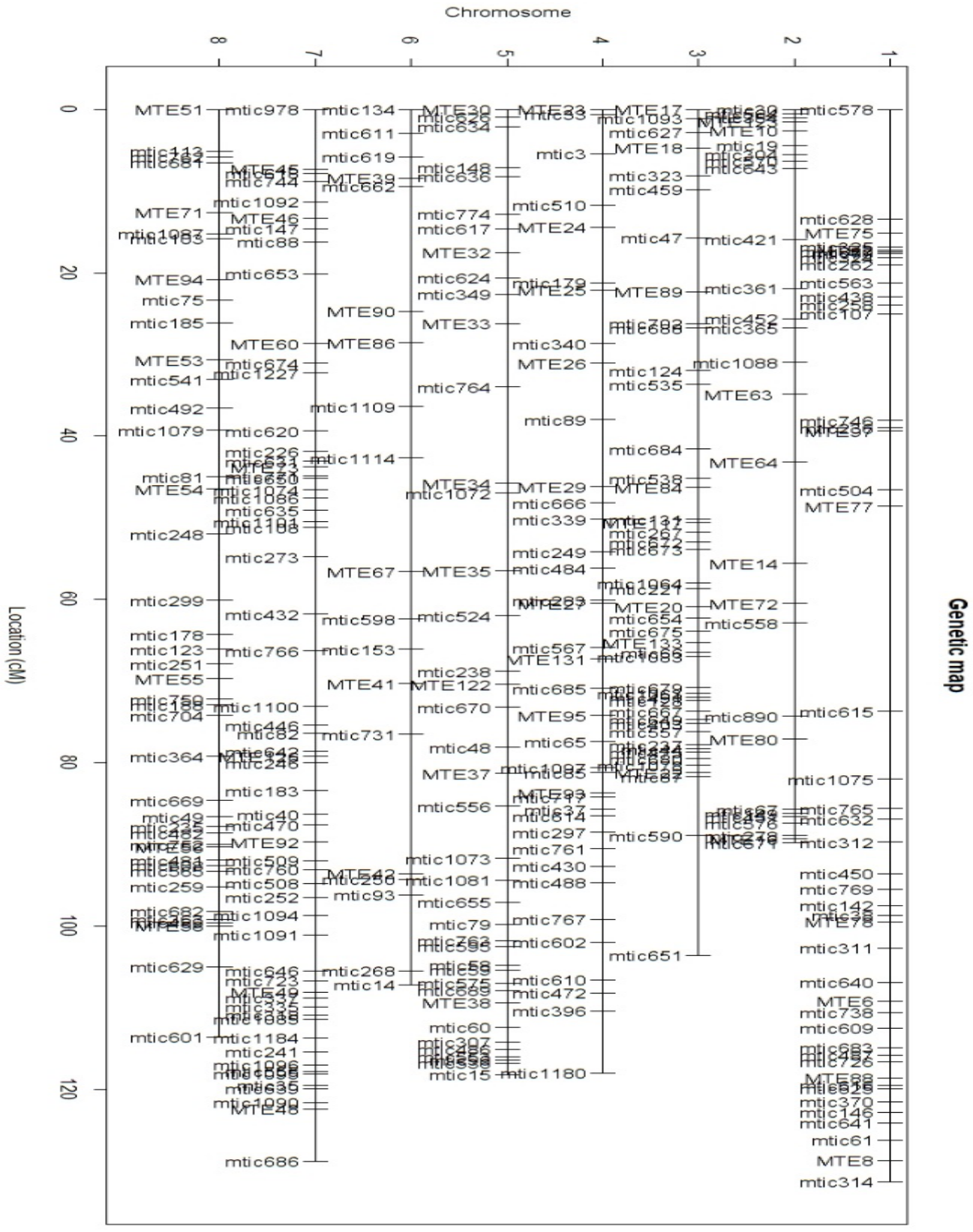


Figure 13- LR4 Genetic map of 316 SSR marker position on the 8 linkage groups of *M. truncatula*.

Source: (Julier et al., 2007; Yu et al., 2012).

2.3. Gene expression study

2.3.1. Selection of susceptible and tolerant genotypes

The least-square means (LSMEANS) for each genotype were calculated due to the effect of condition on genotype using root length data in both control and Cd stress conditions, separately. The data were adjusted based on the two check lines (A17, DZA315.16) that were included in all experiments. Then the difference in root length in two conditions was calculated. Finally, 10 genotypes were chosen as susceptible, and 10 genotypes were chosen as tolerant among those that had the most and least difference, respectively.

2.3.2. Preparation of plant samples

Germinated seeds of the selected tolerant and susceptible lines were incubated on water agar containing or not 15 µM Cd as described. After 24, 48, and 96h roots were collected and wrapped in aluminum foil cooled with liquid nitrogen to prevent any RNA degradation before being stored at -80 °C. They are then ground in liquid nitrogen with a pestle in mortars previously cooled in liquid nitrogen and stored at -80° C in powder form in 15 mL tubes. Three independent biological repeats were performed.

2.3.3. RNA extraction

The RNA extraction was carried out with TRIzol® Reagent (Invitrogen), under a hood and on ice. All the reagents used were stored in the refrigerator. Plant samples were frozen with liquid nitrogen, 1 ml of TRIzol (Invitrogen) to 250 mg of powdered plant tissue was added and mixed on a vortex for 15s, and incubated for 15 min at 37 ° C to allow dissociation of nucleoprotein

complexes. Then 200 µl of chloroform/250 mg of plant tissue were added to each sample and were homogenized on a vortex for 15 seconds. Samples were centrifuged 15min at 4 ° C at 12000g. The aqueous phase was transferred to a new 1.5 ml tube (previously cooled in ice) and 500 µl of isopropanol (per 250 mg plant tissue) was added and incubated on ice for 15 minutes. The samples were then centrifuged 20min at 8000g at 4 ° C and the supernatant was removed. To purify the RNAs (the roots are rich in polysaccharides), the pellet was resuspended in 100 µL in a 2.5 M lithium chloride solution (stock solution consisting of 7.5 M LiCl2 and 50 mM EDTA, pH 8). The samples were then left overnight on ice, then centrifuged for 20 minutes at 12,000 g at 4 ° C. The supernatant was removed. The final step of the extraction was a washing step by adding 1 mL of cold 70% ethanol to the samples and centrifuging for 10 minutes at 8000 g at 4 ° C. The ethanol was removed and the pellets were then dried in air for 20 minutes before being re-suspended in 30 µL of DEPC (diethylpyrocarbonate) treated water per 250mg of sample. RNA extractions were stored at -80 °C (Cathala et al., 1983).

2.3.4. Treatment of RNA with DNase

To get rid of contaminating DNA before proceeding with the reverse transcription reaction, the extracted RNAs were treated with DNase (RQ1 RNase-Free DNase, Promega). The reaction mixture used for processing 1 µg of RNA is shown in (Table 3). The samples were incubated for 30 minutes at 37° C, 1 µL of Stop Solution (20 mM EDTA at pH 8.0) was added and then they were incubated for 10 minutes at 65° C in order to denature the DNase. The concentration and quality of extracted RNA were assessed with a nanodrop (NanoDrop nd-1000 Spectrophotometer) and samples were stored at -80 °C.

Table 3- Reaction mixture for the treatment of 1 µg of RNA with DNase.

Product	**Initial Concentration**	**Volume (µL)**
RQ1 DNase Reaction Buffer	10X	1
RQ1 RNase-Free DNase	0.35 µM	1
RNA	-	8

2.3.5. cDNA synthesis

The ImProm-II ™ ReverseTranscription System kit (Promega, reference A3800) was chosen to carry out the reverse transcription reaction. The 10 µL of RNA treated with DNase were mixed with 1 µL of Oligo dT15, incubated for 5 minutes at 70° C, then on ice for at least 5 minutes. To each sample 9 µL of the reaction mixture as shown in Table 4 was then added. The synthesis of complementary DNAs (cDNA) was then carried out by incubating the samples at 25°C for 5 minutes, at 42°C for 60 minutes, and then at 70°C for 15 minutes. The concentration and quality of cDNA were assessed with a nanodrop (NanoDrop nd-1000 Spectrophotometer) and samples were stored at -20 °C.

Table 4- Reaction mixture for the synthesis of complementary DNA for one sample. (ImProm-II ™ Reverse Transcription System kit, Promega, reference A3800).

Material	**Initial concentration**	**Volume (µl)**
RNA + Oligo (dT) 15 primer	-	11
ImProm-II™ Reaction Buffer	5 X	4
$Mgcl_2$	25mM	1.2
d NTP Mix	10 mM	1
H_2O DEPC	-	1.3
ImProm-II™ Reverse Transcriptase	-	1
Recombinant RNasin® Ribonuclease Inhibitor	-	0.5

2.3.6. Analysis of candidate gene expression

qPCR reactions were performed in the QuantStudio 6 and 7 Flex Real-Time PCR System (Thermo Fisher). The EurobioGreen Mix ® qPCR Kit REF: GAEMMXO2L-8T) was used with 16.6 ng of cDNA per reaction (Table 5). All samples were subjected to two technical repeats. Two normalizing genes were used: elongation factor 1 (*EF1*) and core histone (*H3L*) . In addition to the expression of these normalizing genes, the expression of the following genes was studied: superoxide dismutase (*SOD*), peroxidase (*PRX*), glutathione S-transferase (*GST*),

glutathione reductase (*GR*), and monosaccharide transporter encoding gene (*MtMST1*). The primers used during these qRT-PCRs are shown in Table 6, and the qPCR program is shown in Table 7. The variation in expression of candidate genes for tolerance to Cd responses is estimated by the 2-ΔΔCT where ΔΔCT = (CT Target -CT Standard) Treatment - (CT Target – CT Standard) Control. The CT standard corresponds to the harmonic mean of the CT of the two normalizing genes (Livak & Schmittgen, 2001).

- ΔC_T control = C_T Target - C_T Standard
- ΔC_T Cd-treatment = C_T Target - C_T Standard
- $\Delta\Delta C_T$ = ΔC_T Cd-treatment - ΔC_T Control
- Fold change = $2^{-\Delta\Delta CT}$

Table 5- Reaction mix for qPCR and RT-qPCR for one sample.

Product	**Volume per 1 sample (µl)**
EurobioGreen Mix	5
Primer F (4µM)	1
Primer R (4µM)	1
cDNA matrix* (16,6ng/µl)	3
Total	10

Table 6- Primers used in real time PCR.

Gene	Gene name	Direction	Sequence (5′→ 3′)	Reference
EF1	Elongation Factor 1-α (*EF1*-α)	F	ATGAACCACCCTGGACAGAT-3′	Ramírez-Suero (2010)
		R	GTCCAAGAGGAGGATATTCAGC 3′	
H3L	*Core histone* H2A/H2B/H3/H4	F	ATTCCAAAGGCGGCTGCATA	Ben et al.(2013)
		R	CTTTGCTTGGTGCTGTTTAGATGG	
GR	*Glutathione reductase*	F	TCAGACAGATAAAGTTCTTGGAGCAT	Rahoui et al. (2014)
		R	TGCAATAGCAATACCCTGAACAAT	
GST	*Glutathione-S-transferase*	F	CAAAGCAAGAAGCTTTTTGAATCA	Rahoui et al. (2014)
		R	CCAAGCTGGTCTTGCAGTGA	
SOD	*Superoxide dismutase*	F	AGCCTTAGTGGTTCATGAGC	Rahoui et al. (2014)
		R	CAACATGGCTGGTACAAAAGC	
MtMST1	*M. truncatula MonoSaccharide Transporter*	F	GCTGCATAAACTACGCCACG	Rahoui et al. (2015)
		R	TTGCGTAAGGCTTGTTTGGC	
PRX	*Peroxidase*	F	ACCAAGGACTTAGTCGCATTGTC	Rahoui et al. (2014)
		R	GGTCTCATTGTAGATTCGTACCCAAA	

F: stands for forward; R: stands for reverse

Table 7- RT-qPCR program.

Cycles	T˚C	Times	Notes
1	95˚C	2 min	Activation of polymerase
40	95˚C	5 Seconds	Denaturation
	60˚C	30 Seconds	Annealing/Elongation
1	95˚C	15 Seconds	For melting curve
	95˚C	15 Seconds	
	60˚C	15 Seconds	

IV - Results

IV.1. Genetic control of tolerance to cadmium in *Medicago truncatula*

1.1. Effect of Cadmium on Root Growth Parameters

1.1.1. Root Length

147 genotypes of the *M. truncatula* LR4 RIL population along with their parental lines, A17, and DZA31.16, were exposed to ±15µM of Cd during 6 days in the early stage of germination. First, the effect of ±15µM cadmium exposure on the parental lines was examined. Root length growth as evaluated by the root growth index was inhibited, with an inhibition that differed between them (Figure 14). Phenotypic assays showed that root length is the first trait that is affected by cadmium even at low concentrations. Inhibition was visible after the second day (Figure 15 and, Figure A1, Figure A2, Figure A3, Figure A4 in annex). The normality of root length data was checked with the Shapiro test and visualized with Q-Q PLOT (Figure 16).

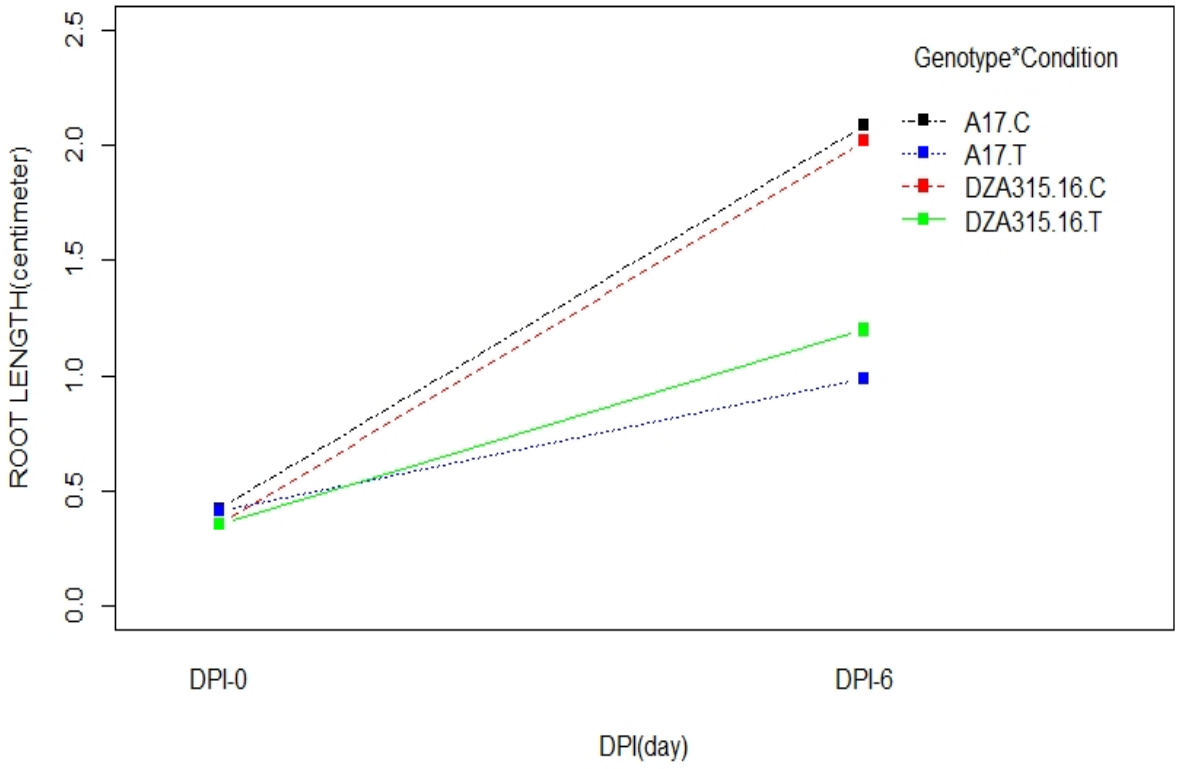


Figure 14- Interaction plot of root length of the two parental lines treated with water (C) or Cadmium at 15 µM (T) at day 0 and 6 after incubation (DPI).

Values are the means of 3 biological repetitions (each with 40 plants, per repetitions).

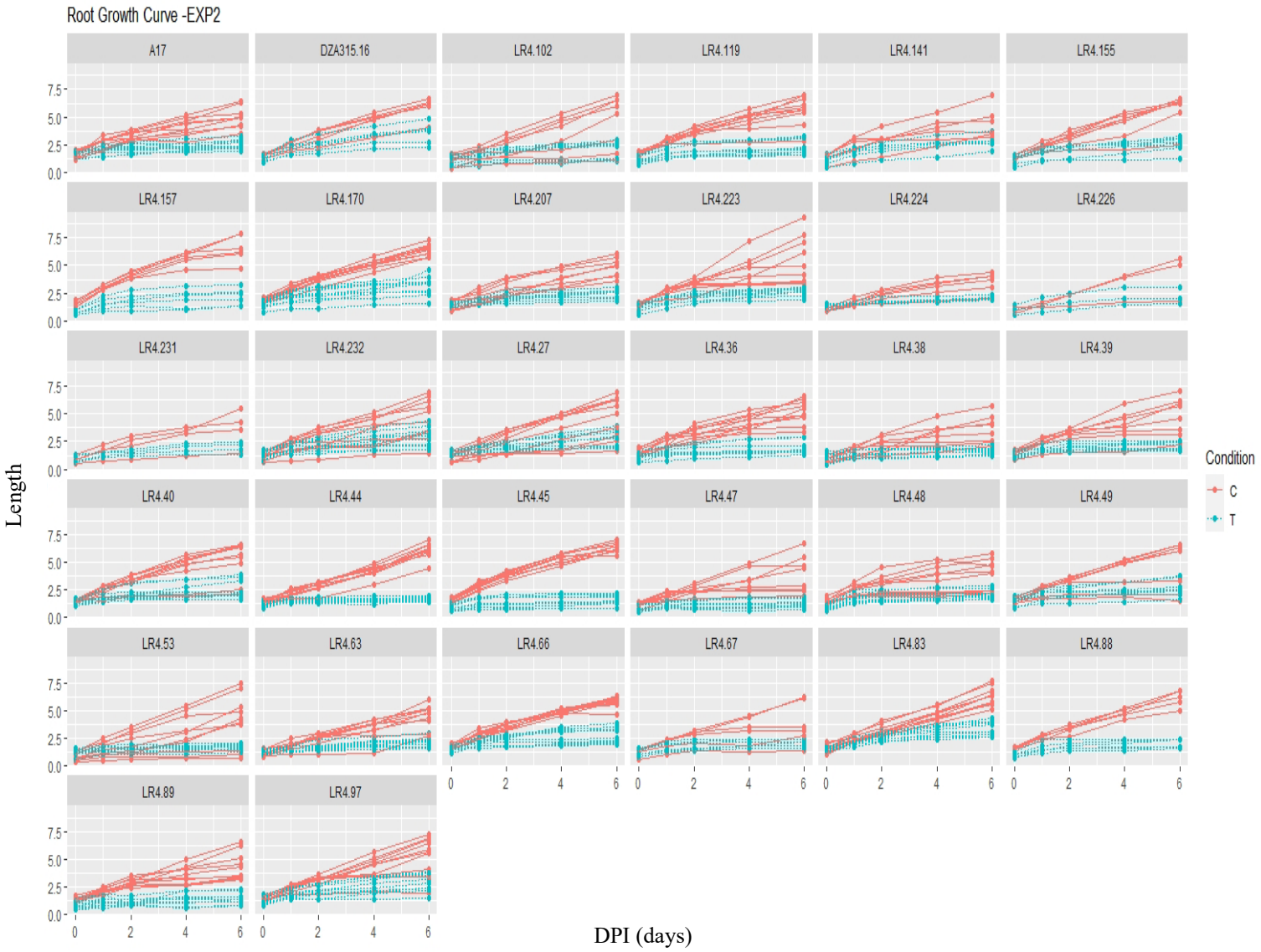


Figure 15- Root growth curves of *M. truncatula* LR4 genotypes.

Seedlings were incubated without (C, red curves) or with (T, blue curves) 15µM cadmium (T) for 6 days and root length was recorded daily. Shown is only part of the second repetition, curves of all experiments are shown in annex figures A1, A2, A3 and A4.

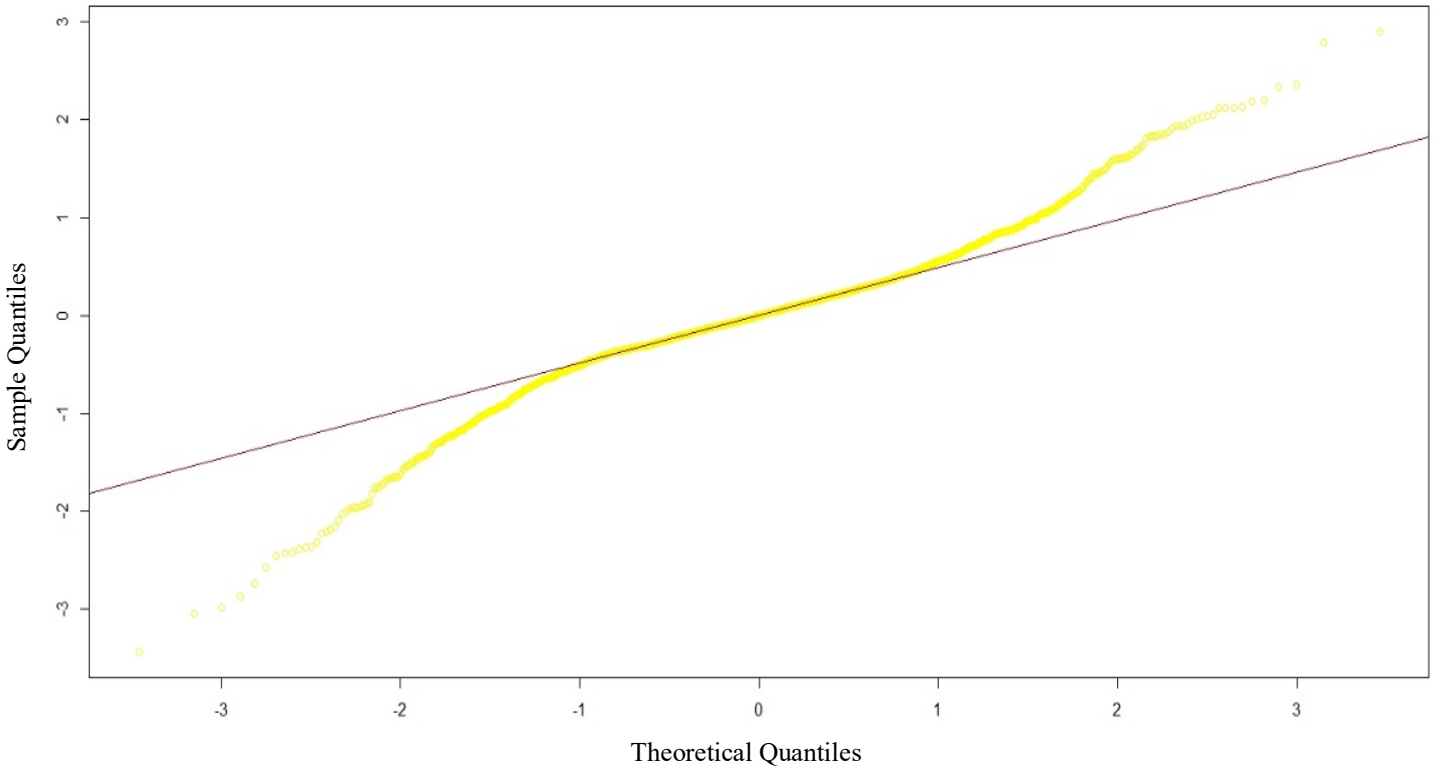


Figure 16- normality plot of root length data of 149 genotypes of the *M. truncatula* LR4 population.

The response showed a great degree of variation. Genotypes with high root growth rates under control conditions were affected more by cadmium than genotypes with less vigorous growth. Differences in root growth under control and cadmium-treated conditions were considered as an indicator of tolerance. A high degree of transgressive segregation was observed among the LR4 population (Figure 17). The results of root length analysis of variance (ANOVA) showed a significant effect of genotype, conditions (±15 µM Cd), and also their interaction (Table 8). Other measured root traits such as root surface and root diameter did not show stable and significant results.

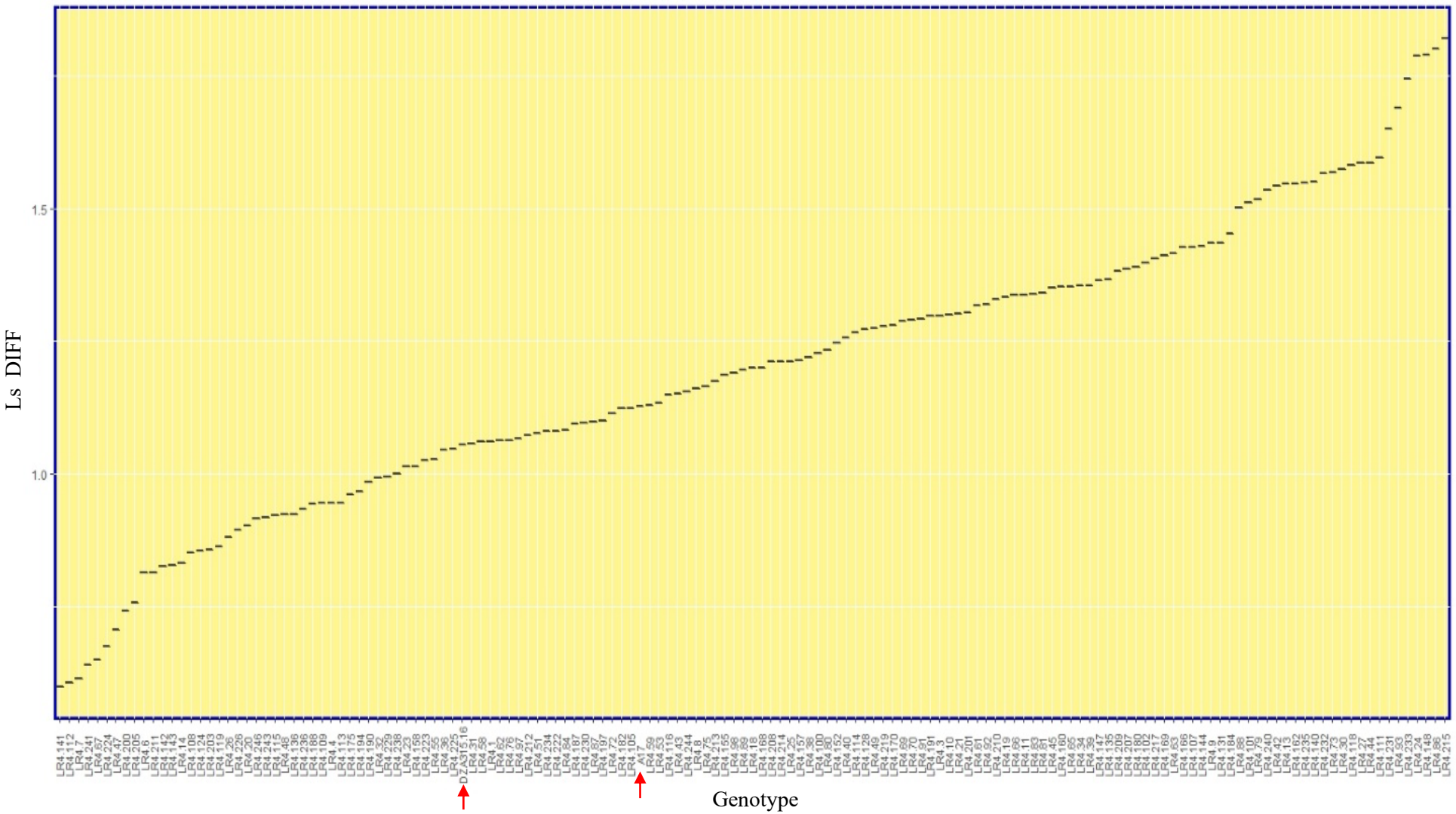


Figure 17- Root growth differences after 6 days of Cadmium treatment for 147 RILs and parental lines A17 and DZA315.16 (red arrows).

Parental lines are marked with red borders. LSMEANS adjustment based on the Tukey method. LS_DIFF : (Control root length) – (Cd- treated root length).

This proved the existence of genetic variability between the LR4 population against cadmium exposure and is considered a good point for QTL detection. The block and biological repeats didn't have significant effects. The root data of all experiments of three repeats were adjusted with the LSMEANS function based on the Tukey methods according to genotype and condition. Then adjusted data of root growth inhibition by cadmium were used to identify the most susceptible and tolerant genotypes for further studies of gene expression (Table 9, Figure 18). Analysis of variance (ANOVA) for genotypes of pools showed a significant difference between genotypes against cadmium treatment (Table 10).

Table 8- Analysis of variance for root growth in control and Cd-treatment (15µM Cd) conditions for 147 genotypes of RILs and 2 parental lines (A17 and DZA315.16).

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Length_0	190.76	190.76	1	1248.14	321.8994	< 2.2e-16 ***
Condition	339.93	339.93	1	55.92	573.6335	< 2.2e-16 ***
Genotype	197.30	1.33	148	1456.67	2.2496	4.743e-14 ***
Condition: Genotype	152.04	1.03	148	1458.38	1.7335	5.010e-07 ***
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1						

Table 9- LR4 susceptible and tolerant genotypes pooled for gene expression studies.

Pool 1	**Genotype**	**Pool 2**	**Genotype**
Tolerant	LR4.14	Susceptible	LR4.9
Tolerant	LR4.47	Susceptible	LR4.24
Tolerant	LR4.67	Susceptible	LR4.27
Tolerant	LR4.112	Susceptible	LR4.30
Tolerant	LR4.141	Susceptible	LR4.44
Tolerant	LR4.188	Susceptible	LR4.140
Tolerant	LR4.203	Susceptible	LR4.148
Tolerant	LR4.205	Susceptible	LR4.231
Tolerant	LR4.224	Susceptible	LR4.232
Tolerant	LR4.241	Susceptible	LR4.233

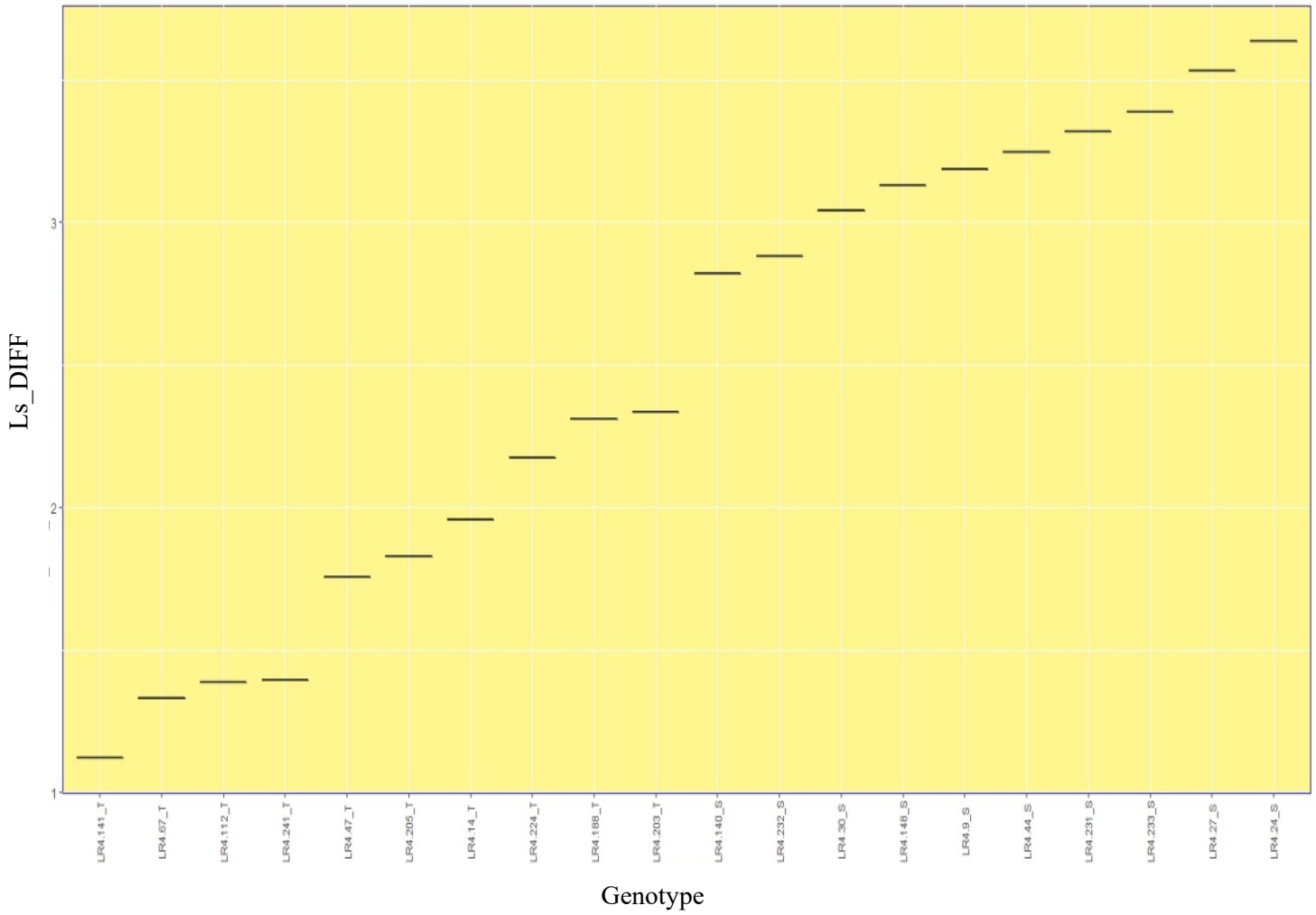


Figure 18- Root growth differences in control and Cd stress conditions for chosen genotype as susceptible (S) and tolerant (T).

LSMEANS adjustment based on the Tukey method. LS_DIFF: (Control root length) – (Cd- treated root length).

Table 10- Analysis of variance for root growth of 10 susceptible and 10 tolerant lines at control and cadmium treatment conditions.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
length_0	25.105	25.105	1	29.637	39.9870	5.978e-07 ***
Condition	140.040	140.040	1	47.070	223.0546	< 2.2e-16 ***
Genotype	48.243	2.539	19	119.711	4.0443	1.132e-06 ***
Condition: Genotype	33.829	1.780	19	149.800	2.8359	0.000214 ***
--- Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

1.1.2. Root and shoot biomass

The fresh and dry weight of roots and leaves were measured for 147 genotypes of LR4 and the two parental lines, A17 and DZA315.16 in 3 repetitions. Analysis of variance showed highly significant genotype effects for fresh leaf weight (FLW), fresh root weight (FRW), and dry leaf weight (DLW) traits (Table 11, Table 12, Table 13). Condition effect was significant for FLW and FRW but the interaction of genotype and condition was not significant for these traits. Dry root weight (DRW) analysis of variance didn't show any significant effects in genotype or condition or their interactions (Table 14). This is due to the fact that DRW values were too low and near 0 so it was not possible to detect significant differences between genotype and condition.

Table 11- Analysis of variance of Dry leaf weight at different conditions; control and Cd-treatment for 147 genotypes of LR4 +2 parental lines, A17 and DZA315.16.

	Type III Analysis of Variance DLW Table with Satterthwaite's method					
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	0.0000225	2.2511e-05	1	48.67	2.0306	0.1605
Genotype	0.0046946	3.1720e-05	148	1488.42	2.8612	<2e-16 ***
Condition: Genotype	0.0009897	6.6870e-06	148	1443.42	0.6032	0.9999
---Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1						

Table 12- Analysis of variance of Fresh leaf weight at different condition. Control and Cd-treatment for 147 genotypes of LR4 +2 parental lines, A17 and DZA315.16.

Type III Analysis of Variance FLW Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	**0.000729**	**0.00072852**	**1**	**49.09**	**6.4558**	**0.01427 ***
Genotype	**0.073831**	**0.00049886**	**148**	**1489.34**	**4.4206**	**< 2e-16 *****
Condition: Genotype	**0.007636**	**0.00005160**	**148**	**1454.11**	**0.4572**	**1.00000**
---Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**						

Table 13- Analysis of variance of Fresh Root Weight at different condition. Control and Cd-treatment for 147 genotypes of LR4 +2 parental lines, A17 and DZA315.16.

Type III Analysis of Variance FRW Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	0.0025378	0.00253780	1	49.01	58.5969	6.459e-10 ***
Genotype	0.0221491	0.00014966	148	1446.40	3.4555	< 2.2e-16 ***
Condition:Genotype	0.0049897	0.00003371	148	1421.77	0.7784	0.9743
---Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1						

Table 14- Analysis of variance of Dry Root Weight at different condition. Control and Cd-treatment for 147 genotypes of LR4 +2 parental lines, A17 and DZA315.16.

Type III Analysis of Variance DRW Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	29.3	29.296	1	1478.3	0.1449	0.7035
Genotype	29770.6	201.153	148	1388.0	0.9948	0.5040
Condition: Genotype	30810.9	208.182	148	1478.0	1.0295	0.3926
---Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

1.2. QTL analysis to detect *loci* involved in tolerance to cadmium treatment

Following the demonstration of variability in the response to Cd and the presence of sources of tolerance to Cd within the LR4 population, the primary objective of this thesis was to identify loci involved in *M. truncatula* Cd tolerance in the early stages after germination. This identification was made using a quantitative treat loci (QTL) Study. Root length and Fresh Leaf Weight indexes were used to find QTLs that might be involved in cadmium tolerance. In total, 39375 root length and 1647 fresh and dry weight values were evaluated.

1.2.1. QTL analysis based on root length under cadmium treatment

QTL analysis was conducted with Single marker analysis (Marker regression method, Mr), simple interval mapping (the EM algorithm, Haley-Knott regression, Multiple imputation), and Composite interval mapping (CIM) to compare and improve the reliability of the QTL detection. All the used methods showed the presence of one significant QTL on linkage group 8 (LG8) with LOD scores of 3.51, 3.41, 3.36, and 3.39 respectively for MR (Figure 19), EM, HK, and IMP methods (Figure 20). Results obtained with the CIM method also demonstrated the existence of the same QTL on LG8 with a LOD score of 3.10 (Figure 21). The properties of these QTLs are summarized in Table 15.

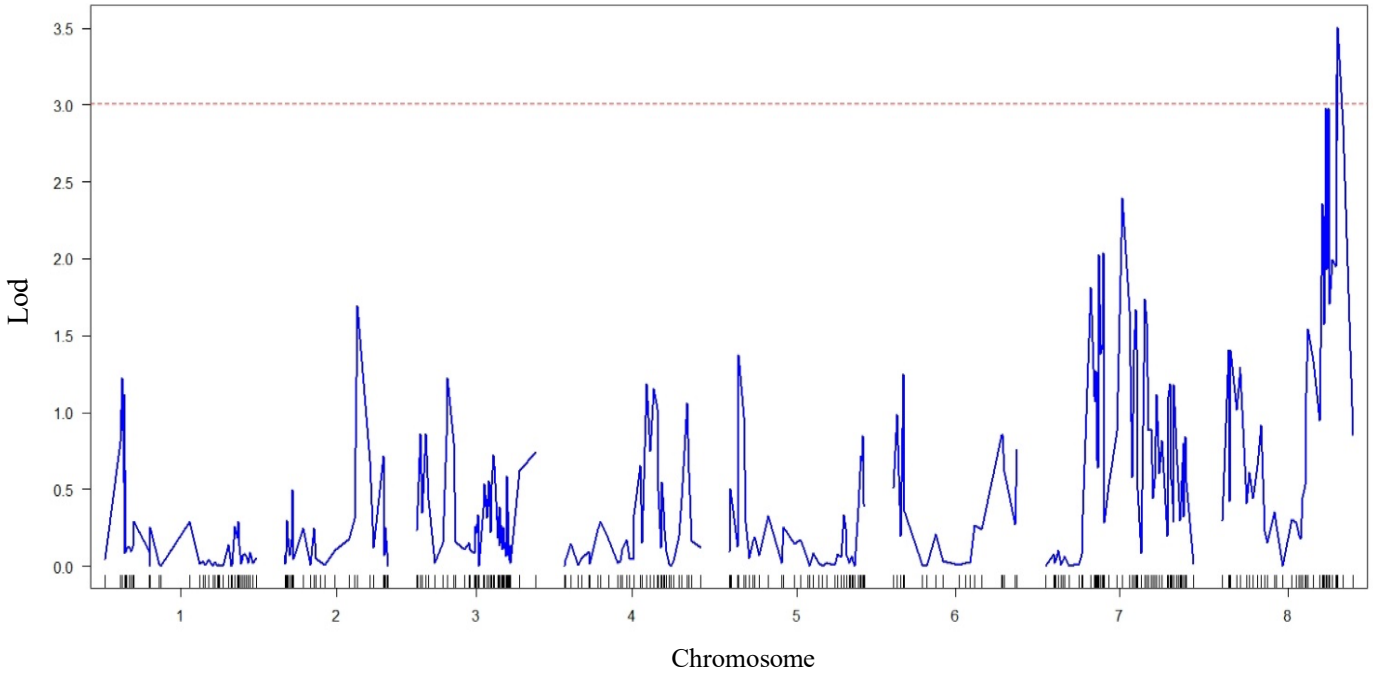


Figure 19- QTL analysis of LR4 population by Single-marker analysis, marker regression (MR) method.

Detection of QTL on the eight linkage groups (LG), by using root length trait in Cd stress condition.

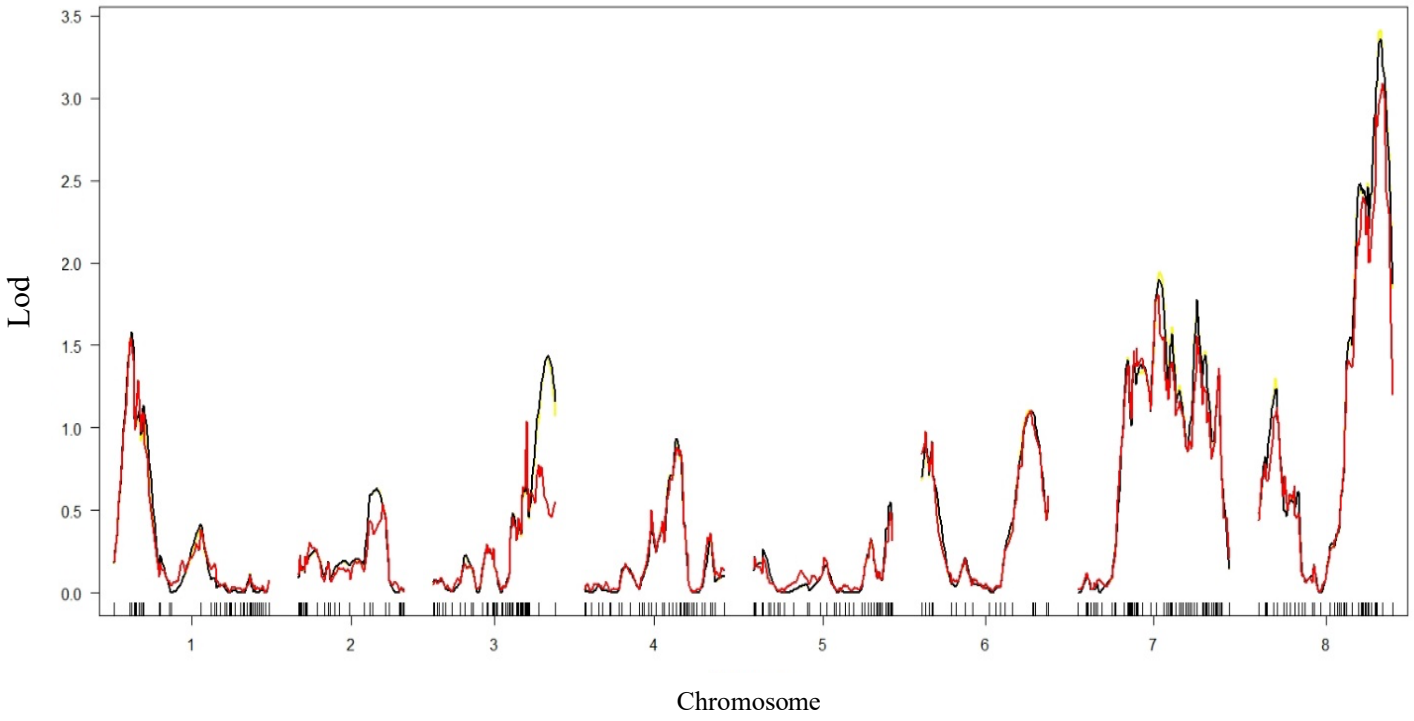


Figure 20- QTL analysis of LR4 population by Simple interval mapping.

Detection of QTL on the eight linkage groups (LG), by using root length trait in Cd stress condition. EM method: yellow line, IMP method: red line, HK method: black line.

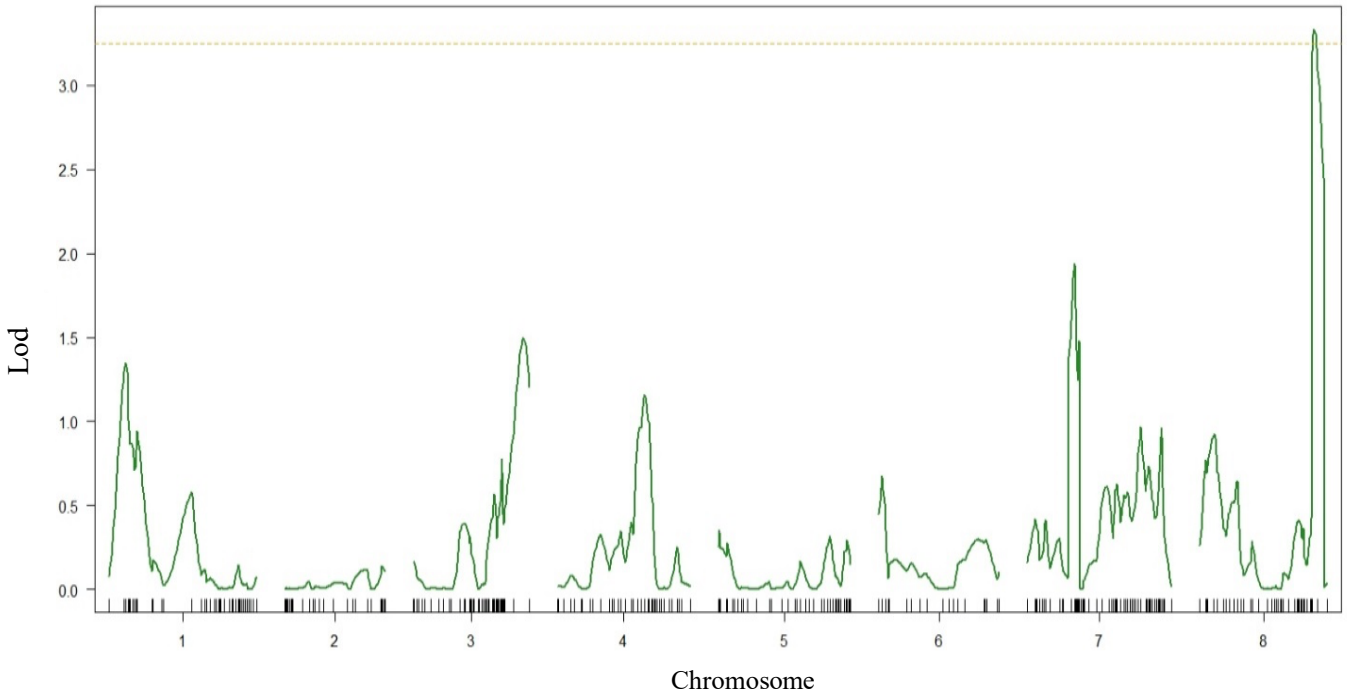


Figure 21- QTL analysis of LR4 population by Composite interval mapping (CIM) method.

Detection of QTL on the eight linkage groups (LG), by using root length trait in Cd stress condition.

Table 15- Map positions and genetic effect of putative QTLs detected for root length in RILs of the *M. truncatula* LR4 population under 15µM of Cadmium treatment condition.

Cd treatment root length data								
QTL	**Method**	**Linkage group**	**Nearest Marker**	**Position**	**Peak LOD Score**	**Threshold 0.05**	**R^2**	**est .eff**
MTE58	MR	LG 8	MTE 58	100	3.51	3.01	8.1360	12.91
c8.loc103	EM	LG 8	mtic629	103	3.41	2.69	7.9137	13.77
c8.loc103	HK	LG 8	mtic629	103	3.36	2.8	7.8023	13.77
c8.loc104	IMP	LG 8	MTE 58	104	3.39	2.61	7.8691	13.35
c8.loc103	CIM	LG 8	mtic629	104	3.10	3.03	7.2209	12.844

R^2: Coefficient of determination of regression, est .eff: estimated effects.

1.2.2. Evaluation of the effect and location of root length QTL

The root length phenotype was plotted against the genotype at marker MTE58 in (Figure 22). At MTE58, the BB individuals exhibit a larger average phenotype than the AA, indicating that this marker is linked to a QTL. The range of possible QTL locations was checked by the interval mapping that is supported by the root length data. The most possible location is between the two markers MTE58 and mtic629 (Figure 23).

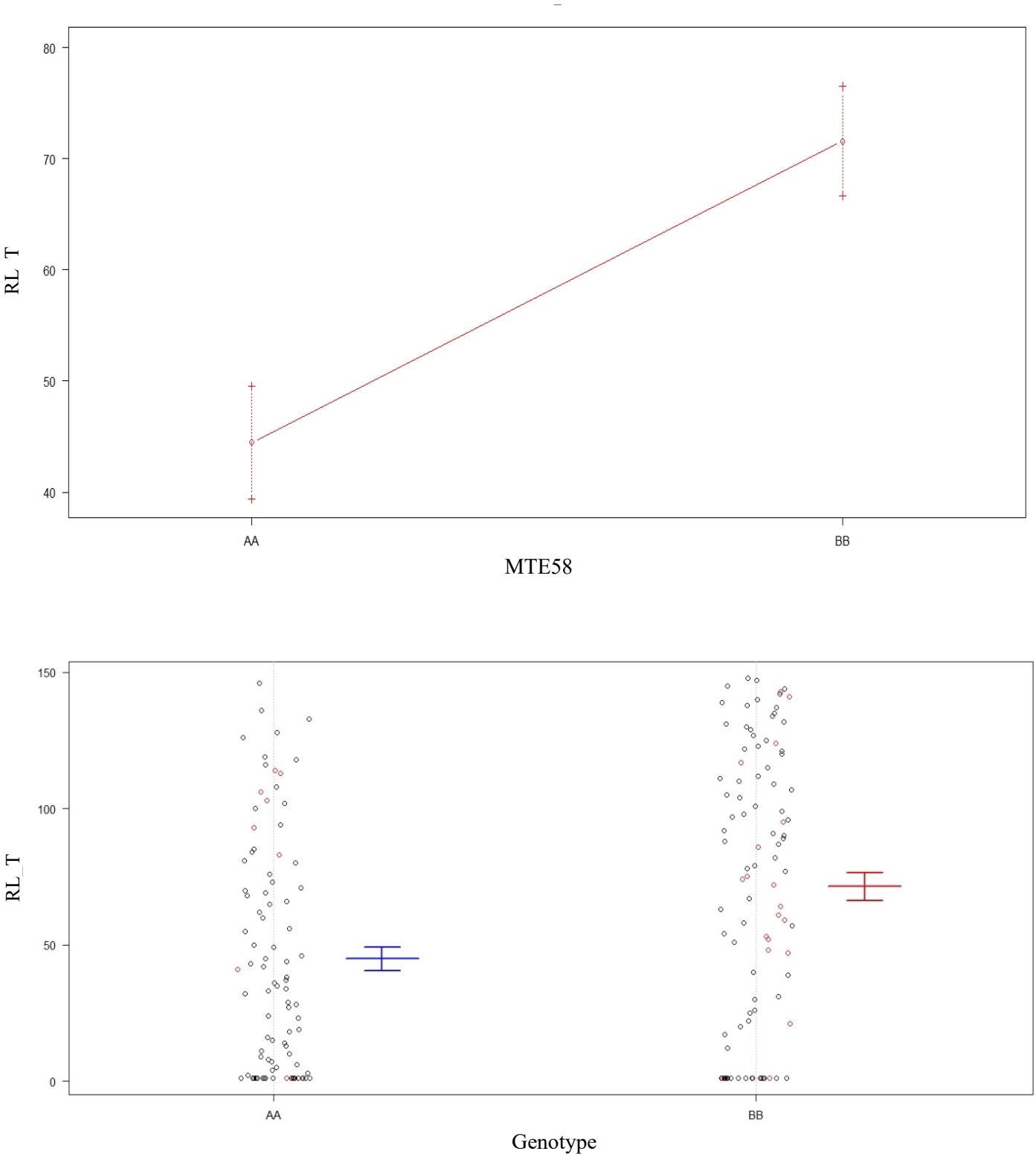


Figure 22- Plot of the root length against the genotype at marker MTE58.

The upper panel was produced by effect plot. The lower panel was produced by plot.pxg ; RL_T : root length of Cd treated data. Error bars are ± 1SE.

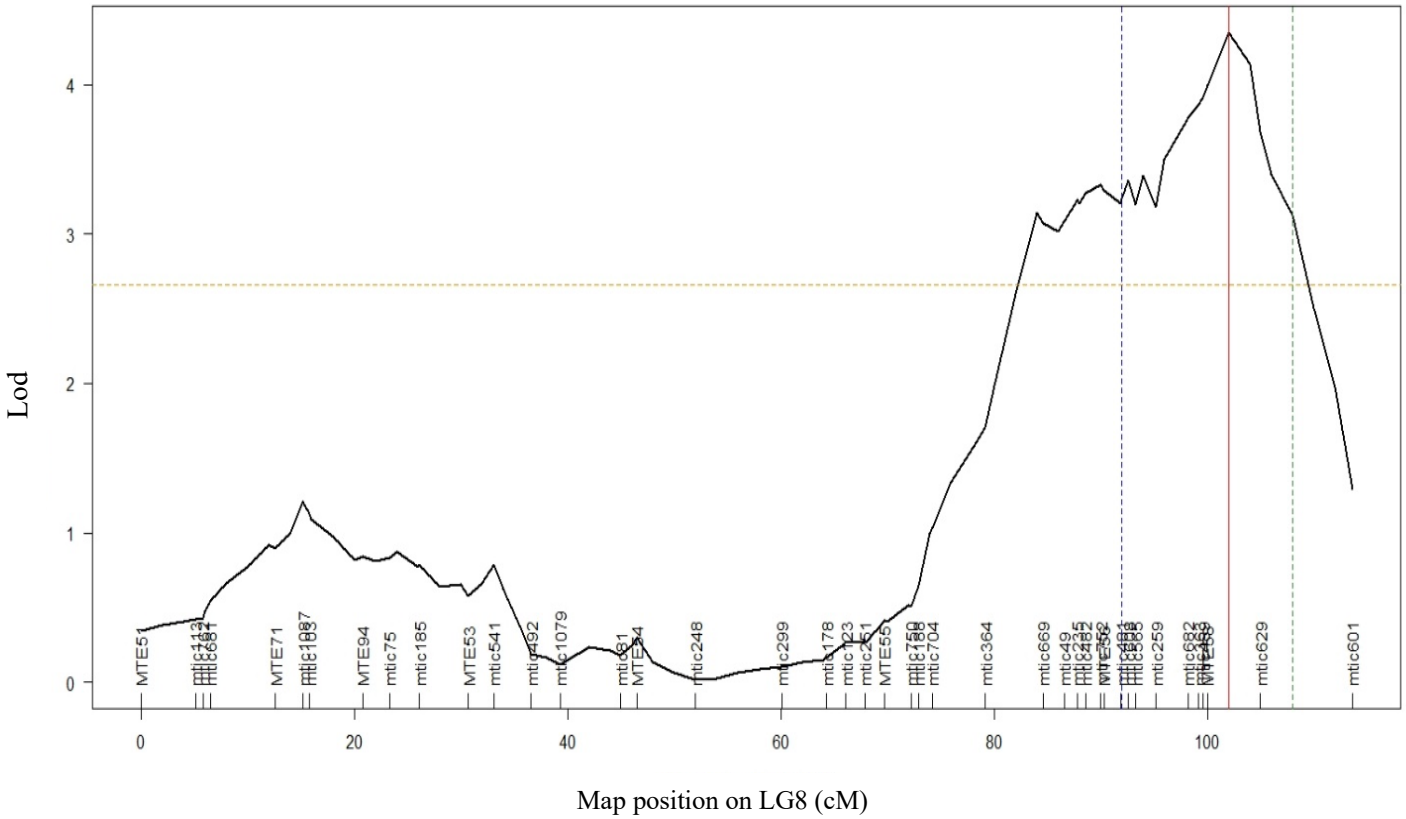


Figure 23- Interval estimate of QTL location.

The maximum likelihood estimates of QTL location, of root length trait on linkage group 8 of *M. truncatula* LR4 population.

1.2.3. Simple interval mapping (SIM) analysis by considering control data as a covariate

When root length data in the control condition were considered as a covariate for QTL detection, the power was improved. LOD scores increased to 4.34, 4.31, and 4.06 for EM, HK, and IMP methods, respectively, and were well above the thresholds (Figure 24). Additive effects of detected QTL were estimated by the fitqtl() function (Broman & Sen, 2009), and were found to be positive around 13.8 (Table 16).

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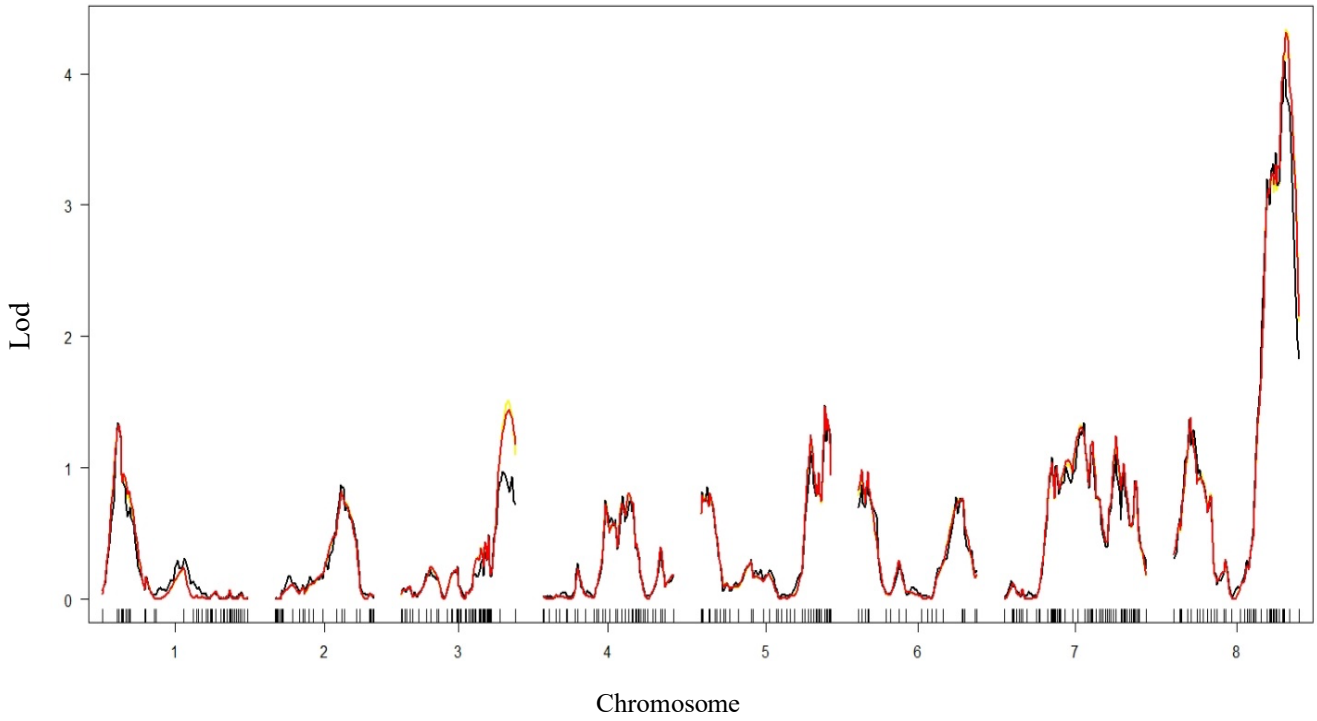


Figure 24- QTL analysis of LR4 population by Simple interval mapping.

The EM, IMP and HK methods while control data was considered as covariate. Detection of QTL on the eight linkage groups (LG), by using root length trait in Cd stress condition. EM method: yellow line, IMP method: red line, HK method: black line.

Table 16- Map positions and genetic effect of QTLs detected for root length inhibition by cadmium treatment in RILs of *M. truncatula* LR4 population Cd treated data wasused.as variable while control data was considered as covariate.

Cd-treated root length data while control data as Covariate								
QTL	**Method**	**Linkage group**	**Nearest Marker**	**Position**	**Peak LOD Score**	**Threshold 0.05**	**R2**	**est .eff**
c8.loc102	EM	LG 8	MTE58	102	4.341	2.63	9.9633	13.829
c8.loc102	HK	LG 8	MTE58	102	4.315	2.71	9.9066	13.829
c8.loc102	IMP	LG 8	MTE58	102	4.06	2.52	9.3495	13.829

R^2: Coefficient of determination of regression, est. eff: estimated effects

1.2.4. QTL analysis based on fresh leaf weight (FLW)

Since FLW showed a significant effect of genotype under cadmium treatment, this trait was also used for QTL detection. Analysis by Single Marker analysis showed a significant QTL on LG7 using the MR method with a LOD score of 3.50 (threshold LOD score =3.1) (Figure 25**Error! Reference source not found.**). The CIM method also was performed and showed a putative QTL on LG7 with an LOD score of 3.15, while the LOD score of thresholds was estimated at 3.57 (Figure 26). Additive effects of the QTL were estimated as large and negative around -11 (Table 17).

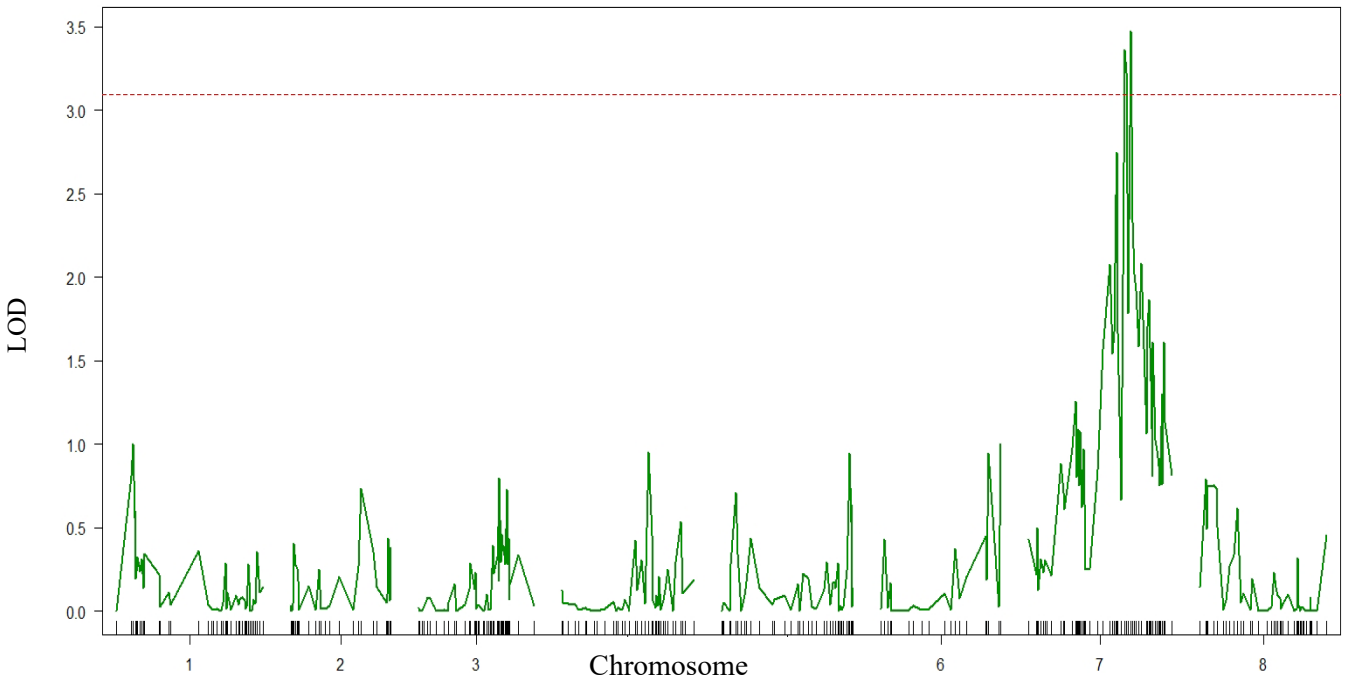


Figure 25- QTL analysis of LR4 population by Single-marker analysis, marker regression (MR) method.

Detection of QTL on the eight linkage groups (LG), by using fresh leaf weight trait in Cd stress condition.

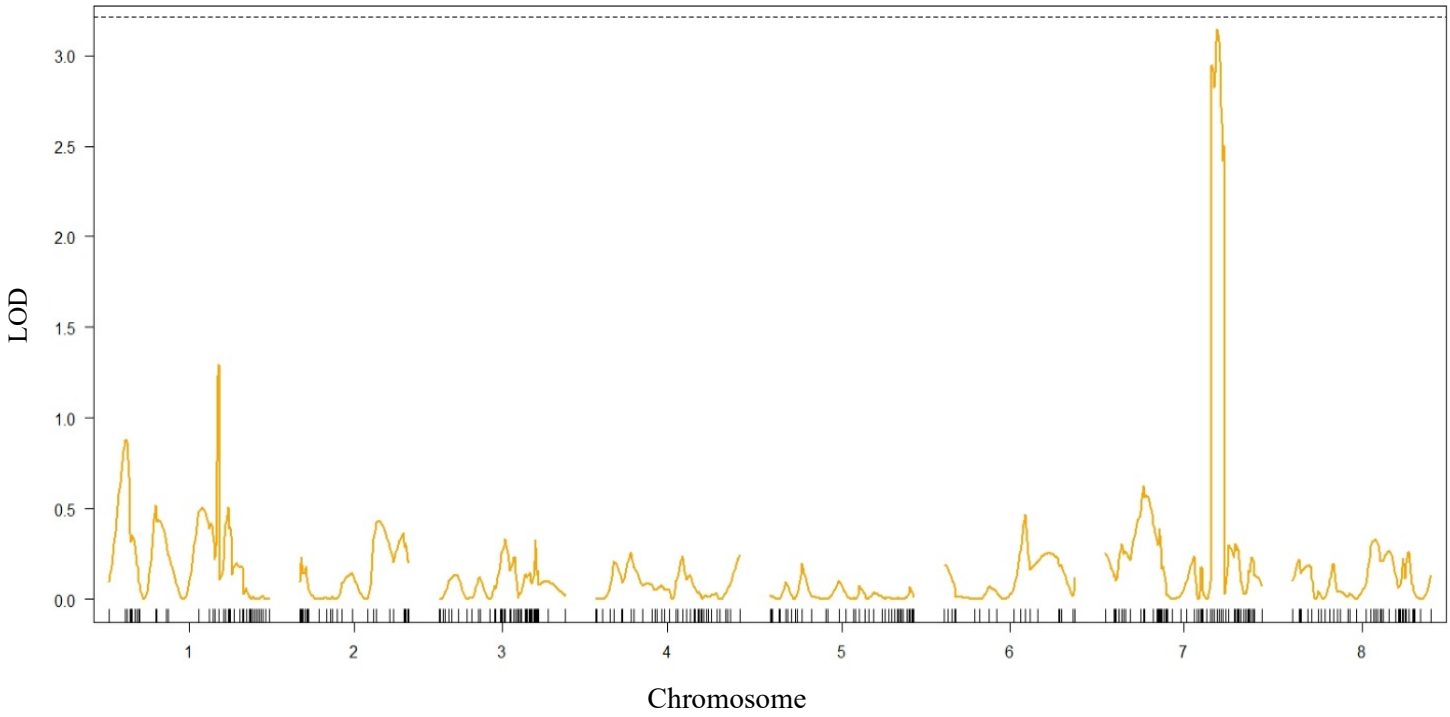


Figure 26- QTL analysis of LR4 population by Composite interval mapping (CIM) method.

Detection of QTL on the eight linkage groups (LG), by using fresh leaf weight trait in Cd stress condition.

Table 17- Map positions and genetic effect of putative QTLs detected Fresh Leaf Weight (FLW) data in RILs of *M. truncatula* LR4 population in 15µM of Cadmium treatment condition.

QTL	Method	Linkage group	Nearest Marker	Position	Peak LOD Score	Threshold 0.05	R^2	est. eff
mtic509	MR	LG 7	mtic509	92	3.5	3.1	7.7577	-11.9
mtic509	CIM	LG 7	mtic509	92	3.15	3.57	7.333	-11.9

R^2: Coefficient of determination of regression, est. eff: estimated effects.

1.2.5. Evaluation of the effect and location of fresh leaf weight QTL

Interval estimation of QTL position showed the most likely position of QTL at 92 cM related to Marker mtic509 (Figure 27). The FLW phenotype is plotted against the genotype at markers mtic509 in (Figure 28). At mtic509, the AA individuals exhibit a larger average phenotype than the BB, indicating that this marker is linked to a QTL.

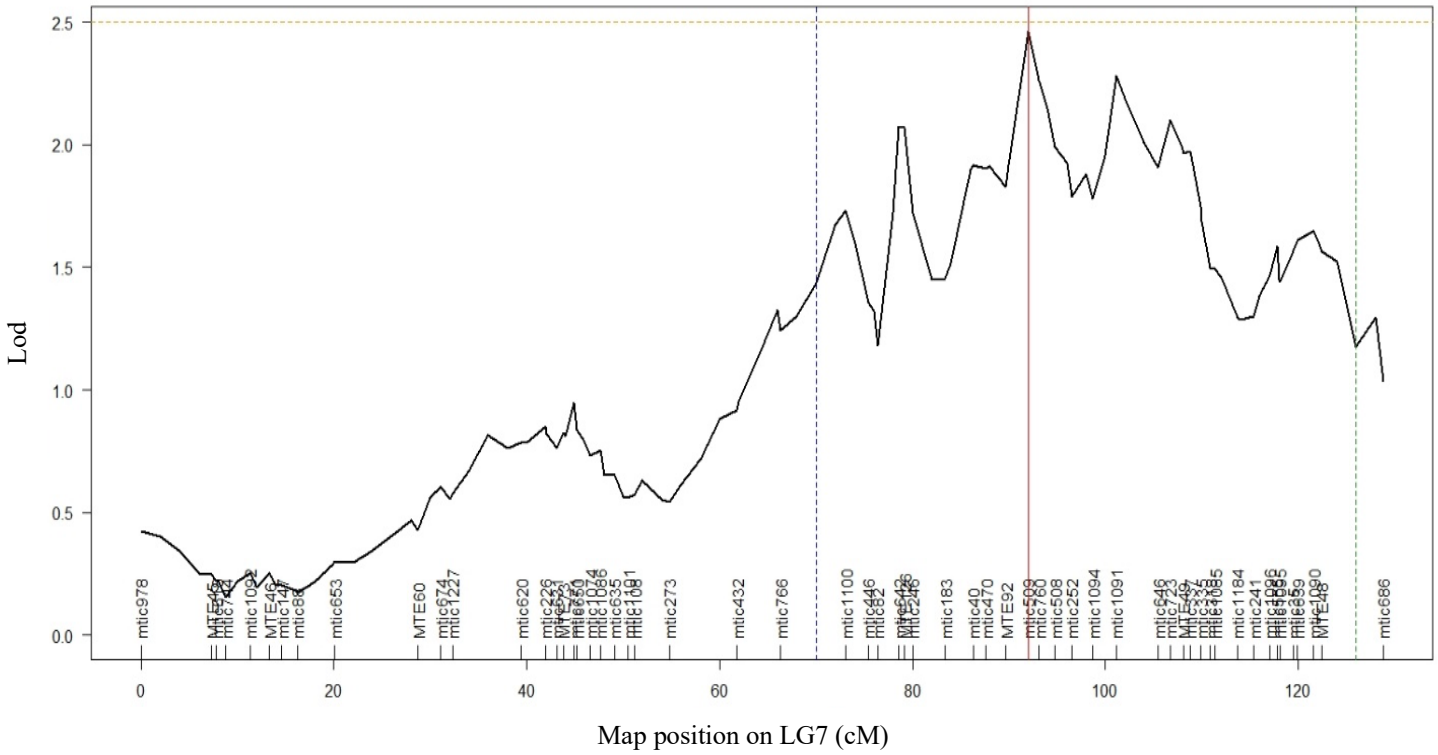


Figure 27- Interval estimate of QTL location.

The maximum likelihood estimates of QTL location, of fresh leaf weight trait on linkage group 7 of *M. truncatula* LR4 population.

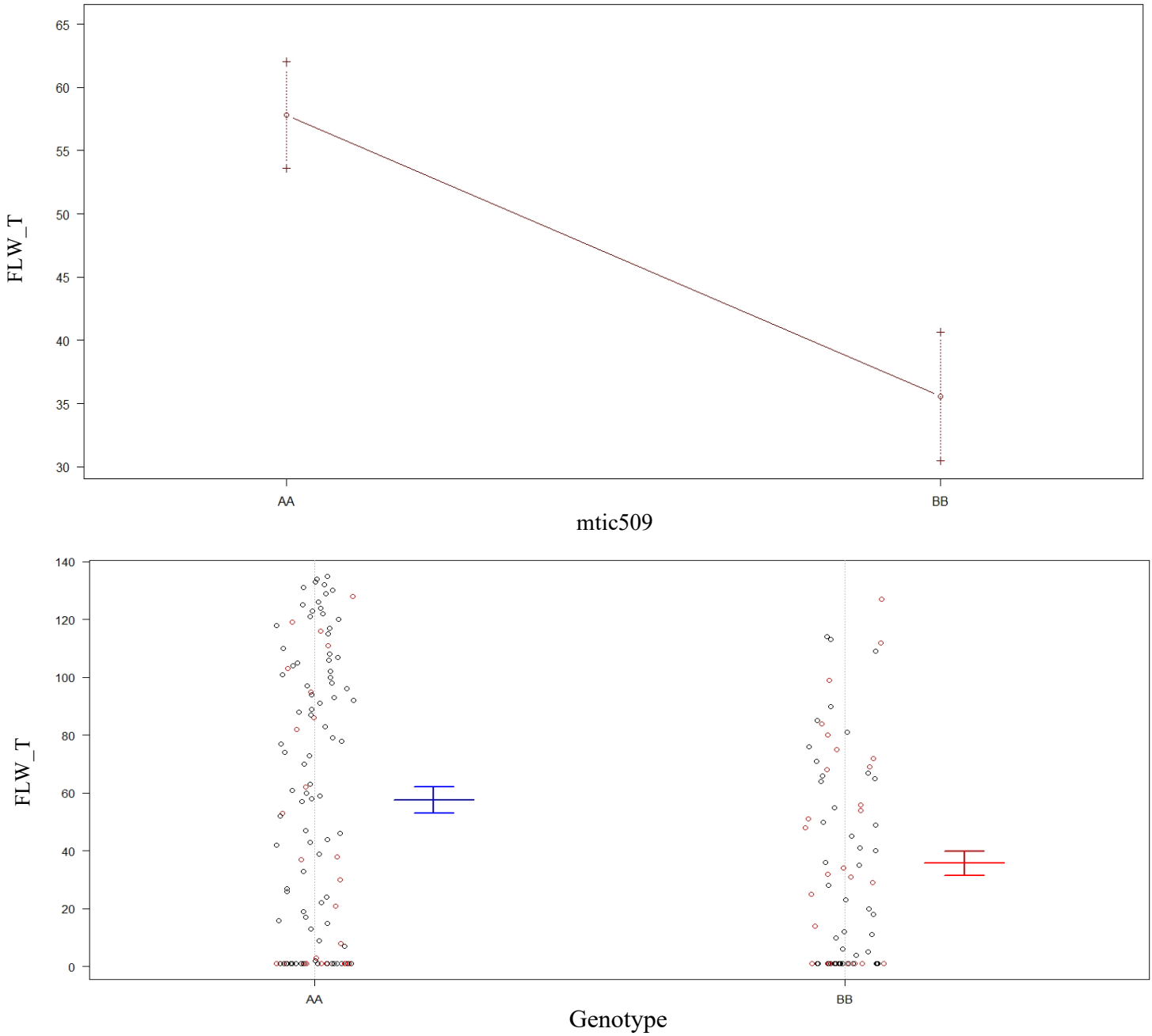


Figure 28- Plot of the FLW against the genotype at marker mtic509.

The upper panel was produced by effect plot. The lower panel was produced by plot. pxg; Error bars are ± 1SE.

1.3. Expression of genes involved in stress response

Since the QTLs identified above were too large to identify putative candidate genes, genes known to be involved in stress response were chosen for expression studies. Five genes were chosen for gene expression studies from two categories: one category of genes related to oxidative stress and antioxidant defense pathways including *SOD, PRX*, *GR*, and *GST,* and one related to carbohydrate nutrition including *MST1*. These genes were reported to be involved in the response of *M. truncatula* to cadmium stress (Rahoui et al., 2014, 2015, 2017; Sakouhi et al., 2021; Zheng et al., 2018). Cd can indirectly favor the production of different ROS, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radical. *SOD, PRX*, glutathione-ascorbate cycle enzymes, or *GR* reduced deleterious effects of ROS. *GST* plays a protective role in oxidative stress initiated by hydroxyl radicals which causes lipoperoxides and membrane damage (Rahoui et al., 2014). Cadmium treatment also caused alteration in the content of total soluble sugars (glucose, fructose, and sucrose). *MST* genes are involved in various processes of plant growth and development as well as in response to abiotic stresses (Benavides et al., 2005; Rahoui et al., 2015). 20 RILs were chosen for gene expression studies, 10 as susceptible and 10 as tolerant genotypes as described previously. In general, the expression of the genes in the pool of susceptible plants showed an upregulation after 24 hours and then decreased to a basic level but in plants of the tolerant pool upregulation was observed at 96h for most genes. In other words, gene expression in the susceptible pool showed an earlier upregulation which was transient, whereas the tolerant pool showed later upregulation, from 24h to 96h.

1.3.1. Genes involved in reduced glutathione metabolism

1.3.1.1. *Glutathione reductase* gene

GR gene expression showed upregulation by cadmium treatment with an induction factor of 4 at 24h in the susceptible pool and then returned to basic levels with induction factors 1.35 and 1.25. Expression in the tolerant pool didn’t show significant up-regulation at 24 and 48h (1.50

and 1.72, respectively), and reached an induction factor of 4 at 96 h (Figure 29).**Error! Reference source not found.**

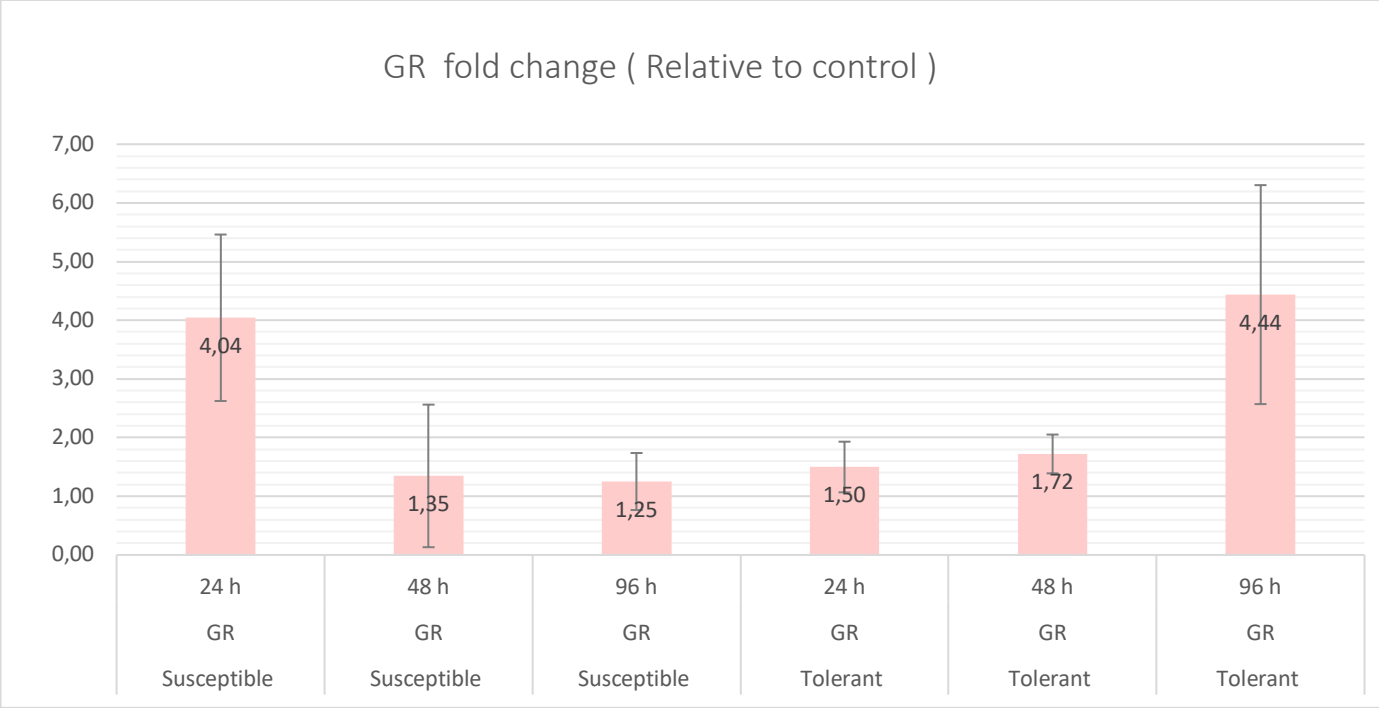


Figure 29- GR gene expression in *M. truncatula* roots of susceptible and tolerant pools.

Seedlings were treated with H2O (control) or Cd (15 µM) and roots harvested at 24, 48, and 96 h. Data show the expression level of Cd-treated roots over control roots, and are mean values of three independent experiments.

1.3.1.2. *Glutathione S-transferase* gene

The highest *GST* gene expression in susceptible pools was equal to an induction factor of 2.74 after 24 hours then returned to a lower level after 48h and 96h with induction factors of 1.37 and 1.67, respectively. In the tolerant pool, *GST* gene expression levels were upregulated significantly only at 96 hours with a factor of 2.74 (Figure 30).

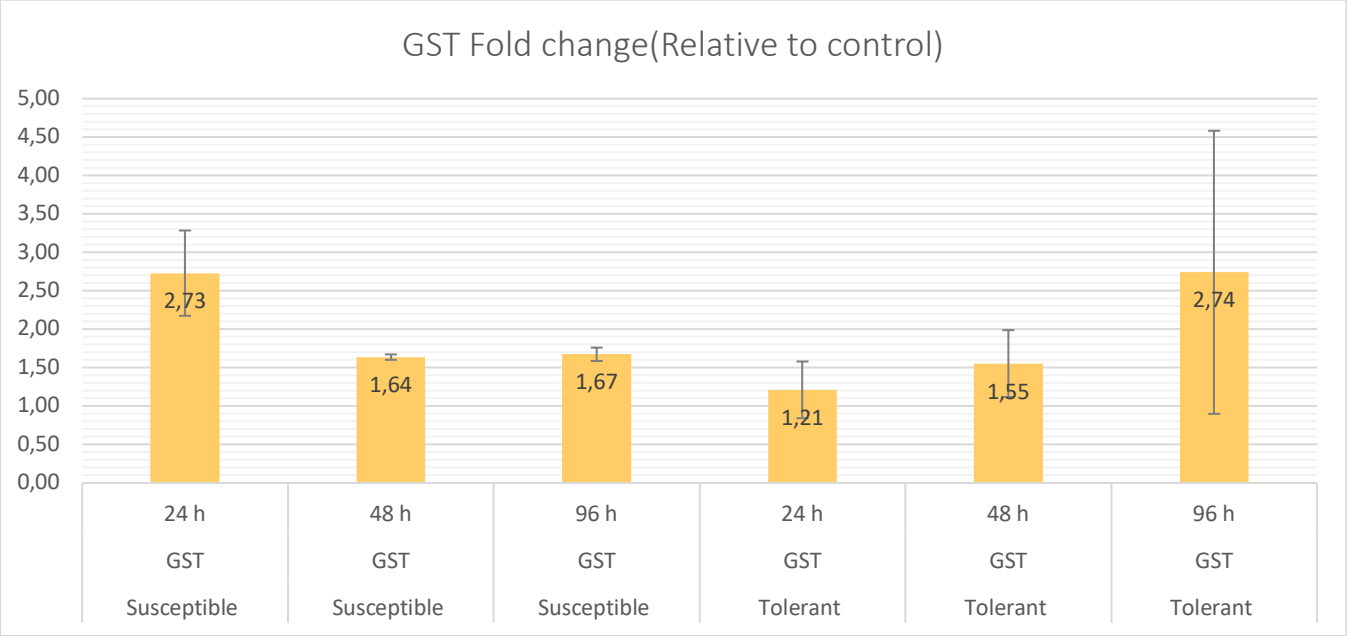


Figure 30- GST gene expression in *M. truncatula* roots of susceptible and tolerant pools.

Seedlings were treated with H2O (control) or Cd (15 µM) and roots harvested at 24, 48, and 96 h. Data show the expression level of Cd-treated roots over control roots, and are mean values of three independent experiments.

1.3.2. Genes encoding ROS scavenging enzymes

1.3.2.1. *Peroxidase* gene

PRX gene expression was strongly upregulated by cadmium treatment in the susceptible pool after 24h where it reached an induction factor of 6.26 and then decreased after 48h and 96h to 1.25 and 1.03, respectively. Gene expression in the tolerant pool was upregulated significantly but weakly only at 96 hours with a factor of 2.28 (Figure 31).

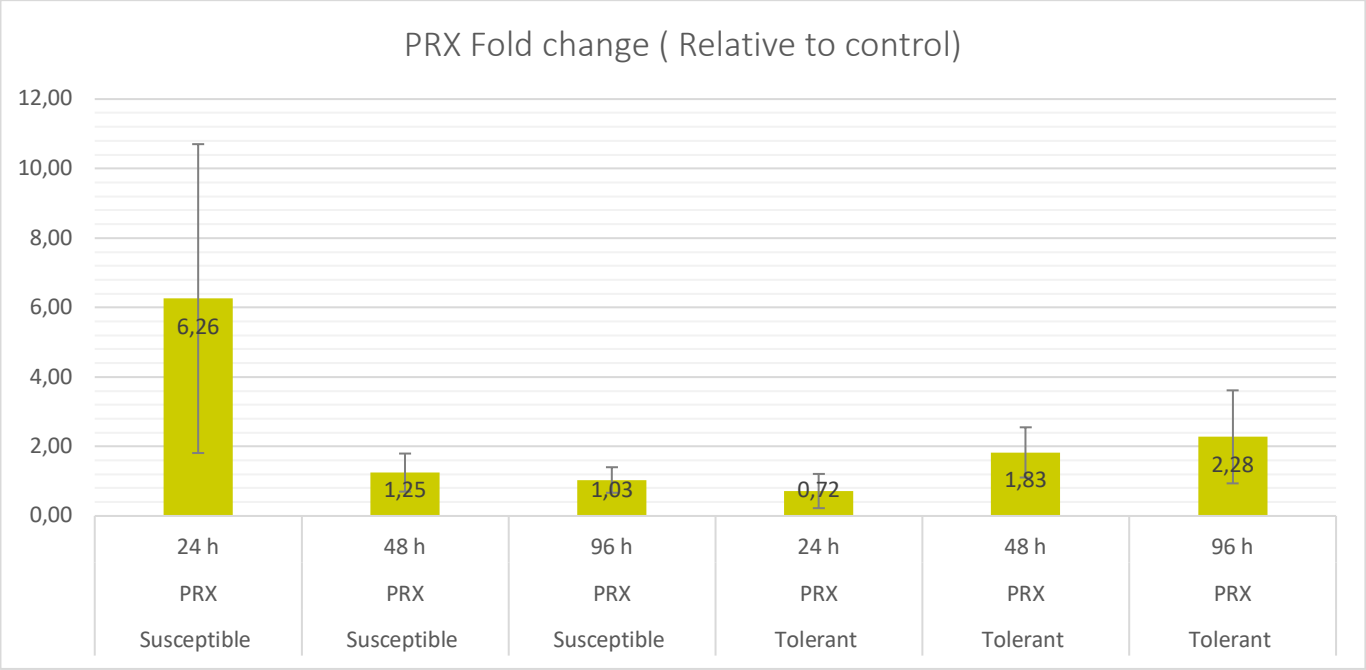


Figure 31- PRX gene expression in *M. truncatula* roots of susceptible and tolerant pools.

Seedlings were treated with H2O (control) or Cd (15 µM) and roots harvested at 24, 48, and 96 h. Data show the expression level of Cd-treated roots over control roots, and are mean values of three independent experiments.

1.3.2.2. *Superoxide dismutase*

SOD gene expression in the susceptible pool was upregulated after 24h with an induction factor of almost 3 after which it decreased to factor 1.71 after 48h and was downregulated after 96h with a factor of 0.23 (fold change). Gene expression in the tolerant pool did not show significant upregulation at all-time points (Figure 32).

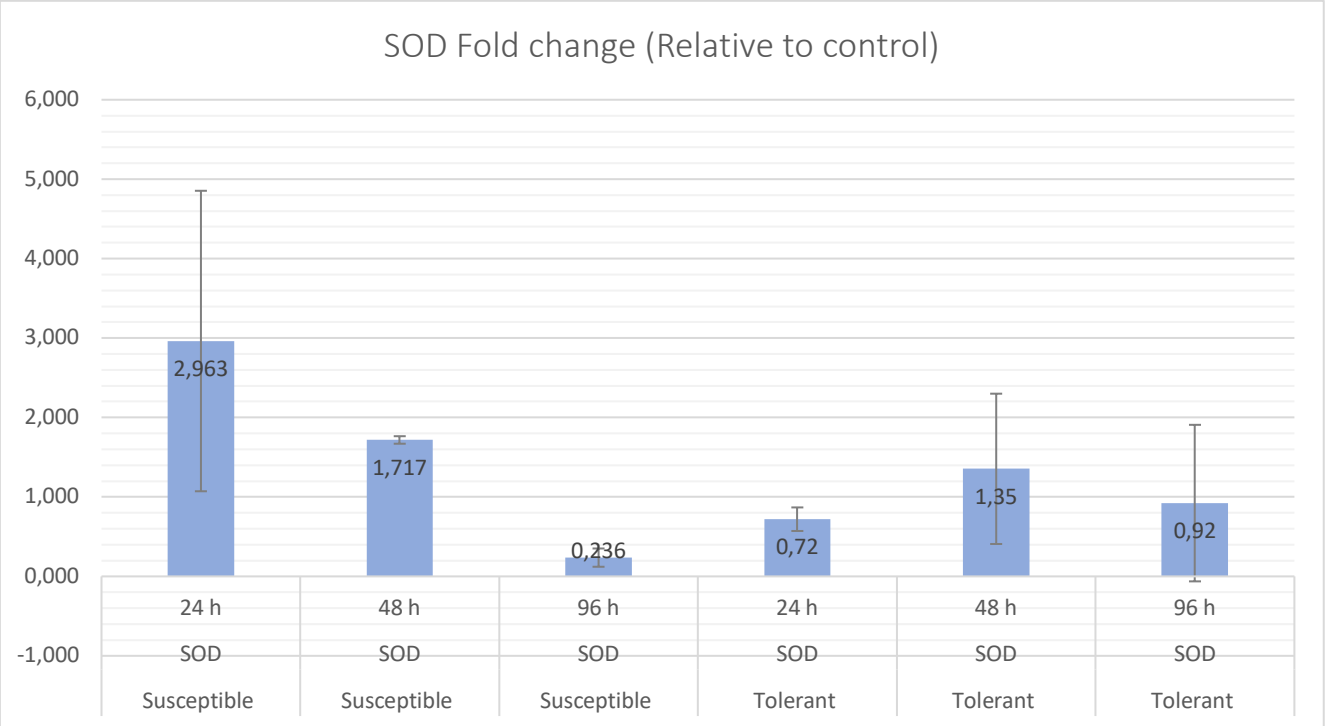


Figure 32- SOD gene expression in *M. truncatula* roots of susceptible and tolerant pools. SOD gene expression in *M. truncatula* roots of susceptible and tolerant pools.

Seedlings were treated with H2O (control) or Cd (15 µM) and roots harvested at 24, 48, and 96 h. Data show the expression level of Cd-treated roots over control roots, and are mean values of three independent experiments.

1.3.3. A gene implicated in monosaccharide transportation

1.3.3.1. *Medicago truncatula Monosaccharide Transferase 1* gene

MtMST1 gene expression in the susceptible pool showed upregulation after 24 h with an induction factor of 3.23 after which it decreased to basal levels. In the tolerant pool, no significant change in expression levels was observed at all three-time points (Figure 33).

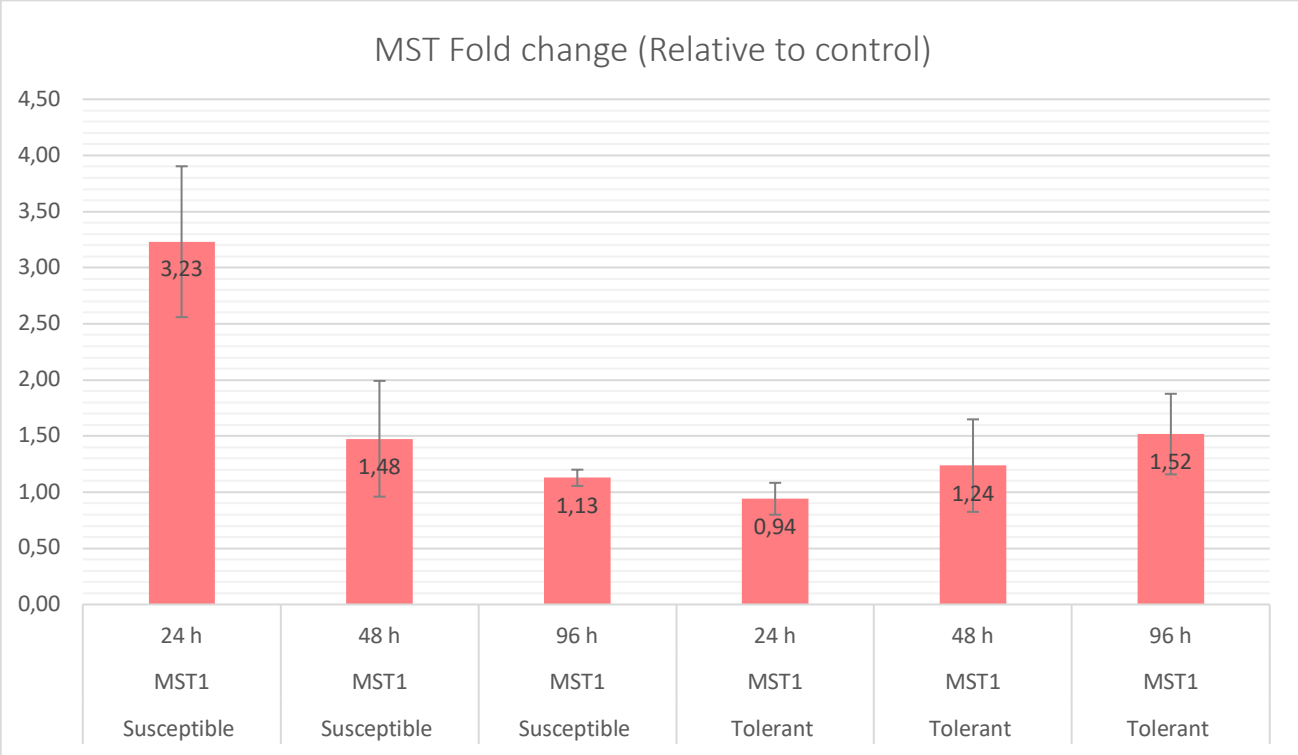


Figure 33- MST1 gene expression in *M. truncatula* roots of susceptible and tolerant pools.

Seedlings were treated with H_2O (control) or Cd (15 µM) and roots harvested at 24, 48, and 96 h. Data show the expression level of Cd-treated roots over control roots, and are mean values of three independent experiments.

IV.2. First assessment of cadmium response in Iranian accessions of two *Medicago* species

2.1. Effect of Cadmium on root growth

Four genotypes of the Iranian *M. truncatula* and one genotype of Iranian *M. scutellata* accessions along with two LR4 parental lines, A17, and DZA31.16, were exposed to ±15µM of Cd during 6 days in the early stage of germination. All the experimental steps were done similarly to the LR4 population phenotypic experiments. Root length growth as evaluated by the root growth index was inhibited, with an inhibition that differed between them (Figure 34). Phenotypic assays showed that root length is the first trait that is affected by cadmium even at low concentrations. The least square means (LSMEANS) for each genotype was computed based on a function of genotype and condition and the grouping was performed by the Tukey method Differences in root growth under control and cadmium-treated conditions were considered as an indicator of tolerance (Figure 35, Figure 36). The results of root length analysis of variance (ANOVA) showed a significant effect of genotype, conditions (±15 µM Cd), and also their interaction (Table 18).

Table 18- Analysis of variance of root growth in control and Cd-treatment (15µM Cd) conditions for four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* + two LR4 parental lines, A17

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
length_0	12.539	12.539	1	55.832	29.0472	1.471e-06 ***
Condition	33.469	33.469	1	8.213	77.5312	1.843e-05 ***
Genotype	64.385	10.731	6	54.120	24.8581	6.324e-14 ***
Condition: Genotype	24.827	4.138	6	53.020	9.5854	3.954e-07 ***
--- Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

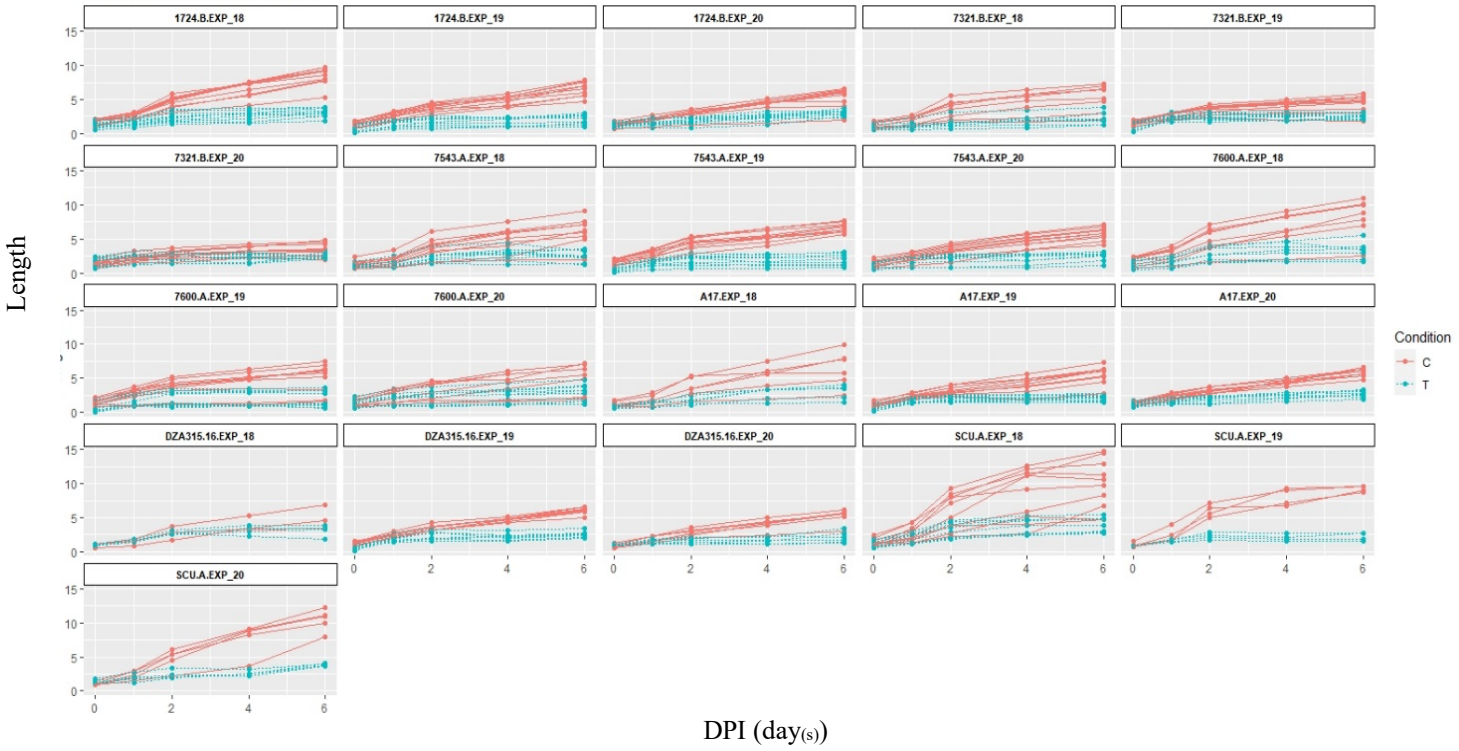


Figure 34- Root growth curves of four Iranian *M. truncatula* accessions, one Iranian *M. scutellata* accession and 2 LR4 parental lines, A17 and DZA315.16.

Seedlings were incubated without (C, red curves) or with (T, blue curves) 15µM cadmium (T) for 6 days and root length was recorded daily.

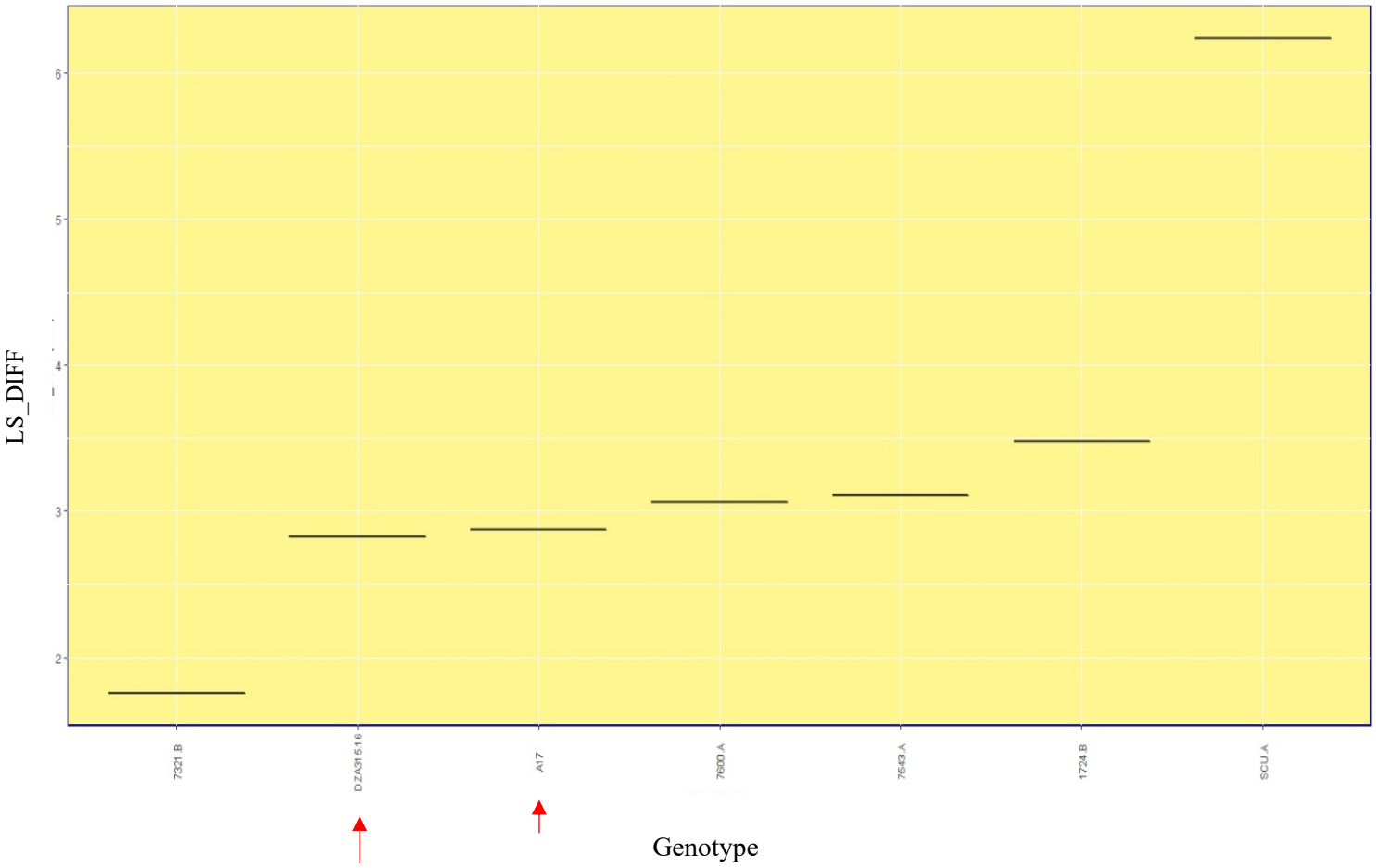


Figure 35- Root growth differences in control and Cd stress conditions for four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* + two LR4 parental lines, A17 and DZA315.16.

LSMEANS adjustment based on the Tukey method. LS_DIFF: (Control root length) - (Cd-treated root length).

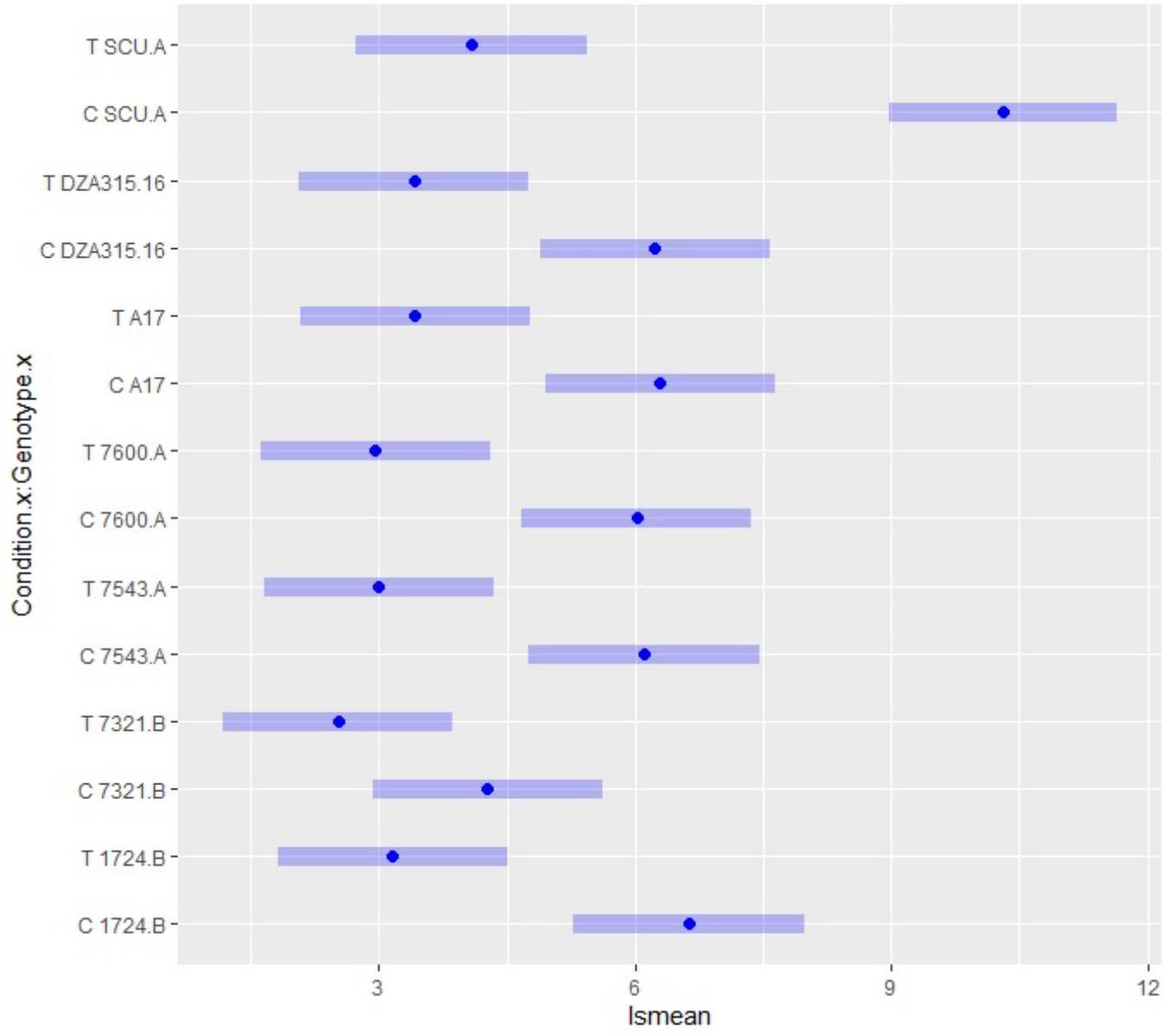


Figure 36- LSMEANS value of root growth Boxplot of the mean values of three repeats of four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* + two LR4 parental lines, A17 and DZA315.16.

2.2. Root and shoot biomass

Fresh and dry weight of roots and leaves were measured for four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* and the two LR4 parental lines, A17 and DZA315.16 in 3 repetitions. Analysis of variance showed highly significant genotype effects for fresh leaf weight (FLW), fresh root weight (FRW), and dry leaf weight (DLW) traits and dry root weight (DRW) (Table 19, Table 20, Table 21 and Table 22). Condition effect was significant for FRW but the interaction of genotype and condition was not significant for it.

Table 19- Analysis of variance of Fresh leaf weight at different condition; control and Cd-treatment for four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* and +two LR4 parental lines, A17 and DZA315.16.

Type III Analysis of Variance FLW Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	0.0005170	0.0005170	1	40	0.8336	0.366706
Genotype	0.0195475	0.0032579	6	40	5.2530	0.000454 ***
Condition:Genotype	0.0007004	0.0001167	6	40	0.1882	0.978423
--- Signif. codes : 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

Table 20- Analysis of variance of Fresh Root Weight at different condition; control and Cd-treatment for four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* + two LR4 parental lines, A17 and DZA315.16.

Type III Analysis of Variance FRW Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	0.00070710	0.0007071	1	41	12.5221	0.001016 **
Genotype	0.00106620	0.0001777	6	41	3.1469	0.012461 *
Condition:Genotype	0.00026282	0.0000438	6	41	0.7757	0.593611
--- Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

Table 21- Analysis of variance of Dry leaf weight at different conditions; control and Cd-treatment for four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* and + two LR4 parental lines, A17 and DZA315.16.

Type III Analysis of Variance DLW Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	2.2820e-06	2.2820e-06	1	37.139	0.4016	0.5301668
Genotype	2.0720e-04	3.4533e-05	6	37.441	6.0756	0.0001637 ***
Condition:Genotype	2.3403e-05	3.9010e-06	6	37.139	0.6862	0.6618434
--- Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

Table 22- Analysis of variance of Dry Root Weight at different condition; control and Cd-treatment for four Iranian genotypes of *M. truncatula* and one Iranian genotype of *M. scutellata* + two LR4 parental lines, A17 and DZA315.16.

Type III Analysis of Variance DRW Table with Satterthwaite's method						
	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Condition	8.9000e-09	8.9100e-09	1	2.791	0.0174	0.90411
Genotype	8.8296e-06	1.4716e-06	6	35.182	2.8666	0.02222 *
Condition: Genotype	9.4740e-07	1.5790e-07	6	35.182	0.3076	0.92877
--- Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1						

V -Discussion

V.1. Cadmium stress and related QTL study

1.1. Cadmium concentration in the environment

The natural concentration of Cd in non-polluted areas is reported to be < 1 ppm (8.917 μM) in the soil although it can differ between different soils (Haghiri, 1973; Lugon-Moulin et al., 2004). Liu et. al. (2013) reported that arable soils may contain 0.42 to 42 ppm (3.74-374.5 μM). According to a large survey, Cd soil levels varied throughout different places in the United States (Burt et al., 2003), with a mean value of 0.32 ppm, which was equivalent to European (0.30ppm) (Alloway, 2013), Spanish (0.30–0.53ppm) (Cal-Prieto et al., 2001), and Italian (0.30ppm) values. The Netherlands, Denmark, and New Zealand had average quantities of 0.20 ppm in their soils, which was slightly lower than the United States (Wakelin et al., 2016). According to the National Soil Inventory of the United Kingdom, Cd-polluted soils were found in 45 percent and 20 percent of soils in England and Wales, respectively (Rawlins et al., 2012). Cd levels in soil have risen by 7%–43% as a result of fertilizer use in several European countries during the last few decades (Huang et al., 2017). In Isfahan, a city in Iran, the total concentration of Cd in 75% of soil samples is more than 0.8 ppm and more than 1 mg kg−1 in 67% of the samples. About 7% of the samples have more than 2 ppm Cd and more than 1% have a Cd concentration higher than 3 ppm (Salehi et al., 2013). Cd concentration in the topsoil of 31 Chinese metropolises ranged from 0.088 ppm to 2.087 ppm (Cheng et al., 2014). The 73rd report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2011) reported that the regional mean Cd concentrations in vegetables (0.006–0.1ppm) were higher than those in other crops including rice (0.004–0.02ppm), root and tuber vegetables (0.006–0.04ppm), pulses and legumes (0.003–0.03ppm), and fruits (0.001–0.007ppm). In general, metal concentrations are normally higher in roots than shoots, but in numerous leafy vegetables (e.g. lettuces and endives), Cd is stored in the leaves due to its high uptake and translocation (Huang et al., 2017).

1.2. Cadmium concentrations used in experiments with plants

Different concentrations of Cd induce varying degrees of inhibition of plant growth (Li et al, 2019). Fifty μM was selected as the sublethal dosage for Cd treatment in *M. truncatula*, by Xu et. al. (2010) and in rice by Yang et. al. (2017) and Shilin et. al. (2021), who reported that when the concentration of Cd was 50 μM, the differences of shoot length, shoot dry weight, root dry weight and total dry weight between their genotypes were the most significant. It is also worth noting that plants' tolerance to varying Cd concentrations differs even within the same species (Lunáčková et al., 2003). García de la Torre et al.(2022) reported that treatment with 10 μM $CdCl_2$ for 48 h had a clear effect on *M. truncatula* Cd-sensitive cultivar without completely arresting root growth and this concentration was selected for *M. truncatula* germplasm screening. According to our goal, which was to investigate the genetic control of tolerance mechanisms towards low and natural concentrations of cadmium, 15 μM was selected as a suitable concentration for cadmium treatment. This concentration can be found in many fields that are not declared as polluted but nonetheless with the potential to affect plant growth and crop performances.

1.3. The effects of cadmium on plants

Generally, germination and the early seedling growth stages are more sensitive to Cd toxicity than older plants, due to lack of some defense mechanisms at this stage (Das et al., 1997; Prasad, 1995). Root elongation inhibition has been identified as one of the earliest and most identifiable indicators of Cd toxicity (Lux et al., 2011). Manifestations of Cadmium toxicity are often described as decreased root length and dry mass, as well as enlarged root diameter. Cadmium has also been observed to affect the architecture of root systems in a variety of plant species. As a result, root morphological features have been proposed as a Cd toxicity indicator. However, the effects of Cd on root morphology might vary across plant species, ecotypes, or cultivars. Root browning has been observed in many plants subjected to Cd exposure (He et al., 2017; Lux et al., 2011). Lunáčková et. al. (2003) reported that the roots of willow (*Salix alba*) and poplar (*Populus* × *euramericana* cv. Robusta) became shorter and thicker after treatment with 10 μM $Cd(NO_3)_2$. Cadmium-induced inhibition of root elongation could be related to the depolymerization of cell cytoskeleton microtubules and the production of

chromosome abnormalities, which cause meristematic cells to have decreased mitotic activity (Seth et al., 2008). García de la Torre et al. (2022) exposed *M. truncatula* cv. Parabinga seedlings to different Cd concentrations (0–40 µM $CdCl_2$) for 48, 72, and 96 h. They identified Cd-tolerant and -sensitive cultivars by testing 258 accessions of *M. truncatula* at the seedling stage, using the relative root growth (RRG) as an indicator of Cd tolerance. Clear differences in plant root growth were reported between the Cd-sensitive (CdS) and Cd-tolerant (CdT) accessions after Cd stress respectively, but in CdS the inhibition was stronger. Cd contents were analyzed, but no correlations with the tolerance trait were reported. Their results showed that the CdT accessions displayed a higher tolerance than CdS in all the studied stages. Cd-treated CdS roots were shown to lose plasma membrane integrity along the whole root. In contrast, Cd-treated CdT roots displayed minimal loss of plasma membrane integrity along the root surface and the root apex was not affected (around 4 mm). It has been suggested that oxidative damage produced by Cd was less in the roots of the tolerant cultivar. Cadmium stress causes increased root diameter as a result of larger parenchyma cells and enlarged cortical tissues, both of which play a role in increasing plant resistance to radial water and solute flows (Maksimovi et al., 2007). Root absorption capacity is proportional to total and specific root lengths, so decreases in root length, surface area, specific root length, and number of root tips, as well as increases in root diameter, indicate that plants under Cd stress have reduced resource acquisition capacity (water, nutrients, etc.) (He et al., 2017). Gratão et. al. (2009) reported a decrease in root diameter after Cd application to tomato (*Lycopersicon esculentum*). Moreover, the effects of Cd on root morphology might differ between hyperaccumulators and non-hyperaccumulators. In *Sedum alfredii*, a known hyperaccumulator, positive relationships were found between Cd concentrations and root morphological characteristics (root length, area, or volume), whereas such connections were dramatically reduced in non-hyperaccumulators (Li et al., 2009). Toxic symptoms of Cd stress in plant foliage include stunting, chlorosis, necrosis, and desiccation (Solís-Domínguez et al., 2007). In order to study the response of different accessions of *M. truncatula* to Cd toxicity, various seedling growth parameters were assessed by Saeidi et. al. (2012). According to their findings, Cd stress had a substantial effect on all growth indices except shoot fresh weight, root water content, dry shoot/root ratio, and fresh biomass. Compared with non-stress conditions, Cd-stress considerably reduced seedling root growth, root fresh weight, root and shoot dry weight, and dry biomass, but increased shoot water content, fresh shoot/root ratio, and root diameter. In our study, we did not observe significant effects of cadmium on root diameter and root surface, which is probably due to the lower concentration used. Root length analysis by ANOVA showed a significant interaction

between genotype × condition, which proved that root growth at the seedling stage is a good indicator of cadmium tolerance for genetic studies. Roots were stunted and brown, especially after six days. Susceptible genotypes showed faster and stronger reactions to cadmium treatment, whereas root growth difference between control and treatment conditions was less in tolerant genotypes. Nutrient leakages from Cd-treated roots have been reported, in agreement with described Cd deleterious effects on lipoperoxidation of membrane lipids (Rahoui et al., 2010, 2015; Sfaxi-Bousbih et al., 2010). Analysis of variance of obtained values for FEW, FLW, and DLW in our study showed a significant effect of genotype for all traits. The effect of the condition was significant for FRW and FLW, but the interaction effect of genotype and condition was not significant for all the traits. The results of DRW did not show any significant effects of genotype or condition, whereas Saeidi et. al. (2012) reported that *Medicago truncatula* root growth, root fresh weight, root and shoot dry weight significantly decreased but shoot water content and root diameter increased at treatment with 100 µM cadmium (i.e., 6.5 times as high as in our experiment). We did not find the significant effects of cadmium on root diameter, FLW, FRW, DLW and DRW that they reported. It may be assumed that these effects are greatly influenced by genotype and growth conditions such as cadmium concentration. The response of ten elite cultivars of rice under different levels of CdCl2 (0, 50 and 100 µM) was reported by Afzal et. al. (2019). The fresh and dry mass of rice seedlings was considerably reduced in all cultivars when compared to the control. This reduction was reported more pronounced at 100µM Cd than at 50 µM. At 50 µM, reduction in fresh weight varied from 22 to 49%. at 100 µM, fresh weight in some cultivars decreased to 50% or even more. The Cd treatments also reduced the dry weight by 14 to 40% and 19 to 50% respectively at 50 and 100 µM Cd concentration. According to Sager et. al. (2020) Cd treatment (0, 100, 200, and 400 µM Cd concentration) resulted in reduced growth of two genotypes (AG-10 and AP-3) of *Pisum sativum*. The length, fresh weight, and dry weight of roots and shoots in both genotypes were reduced by cadmium stress. At 400 µM, length, fresh weight, and dry weight of cultivar AG-10 decreased to 13.48, 37.11, and 30.94% (shoot) and 12.28, 35.79, and 9.7% (root), respectively. The AP-3 cultivar was shown to be more tolerant with a decrease of length, fresh weight, and dry weight of around 31.43, 37.11, 43.33% (shoot) and 20.53, 62.03, and 43.37% (root), respectively.

1.4. Genetic control of cadmium tolerance in plants

The first step to studying the genetic control of plant tolerance to Cd stress is selecting appropriate specific traits. According to the observed substantial root length phenotypic variation under Cd exposure in the *M. truncatula* LR4 population, root length data was selected as a main factor for detecting QTLs associated with cadmium stress. In addition, due to the highly significant effects of genotype and conditions for FLW data, the existence of QTLs associated with this response also was checked in our study.

Paape et al.(2022) studied 236 *M. truncatula* genotypes under 10 µM $CdCl_2$ after 48 h of Cd exposure in a Genome-Wide Association Study (GWAS) on the genetic control of cadmium tolerance. Relative root growth (RRG) and Cd leaf accumulation were considered as appropriate indices for Cd stress. Their results revealed SNPs with high association on almost all chromosomes. They found a large region (~1.3 Mb) of interest associated with Cd RRG on chromosome 2. Another cluster of low p-values associated with Cd RRG was found on chromosome 5 with SNPs in high LD. On chromosome 8 one candidate gene (Medtr8g015980) related to transmembrane ATPase activity was reported for RRG. 50 µM of Cd was chosen as an appropriate concentration for QTL studies in wheat (Ci et al., 2012), *Arabidopsis* (Courbot et al., 2007), and rice (Shilin et al., 2021). However, our aim was to check loci that are sensitive to low cadmium concentrations found in officially non-contaminated soils. Li et. al. (1995) reported that a considerable phenotypic difference between the parents in the objective quantitative traits is not necessarily required for successful QTL mapping. They successfully mapped two QTLs in rice with strong phenotypic effects using RIL populations of two parents (Lemont/Teqing) showing only a slight difference in plant height (only 6 cm). They argued that because the parents had so many distinct alleles, a tiny change in a trait between them could indicate transgressive segregation in the progeny. Similarly, Xue et. al. (2009) used a doubled haploid (DH) population to investigate the genetics of Cd tolerance and accumulation in rice. While the parents showed a small difference in the examined traits, QTLs were perfectly detected with 100µM Cd concentration. Shoot height (SH), root length (RL), shoot dry weight (SDW), root dry weight (RDW), total dry weight (TDW), and chlorophyll content (CC) were reported that have all been linked to 22 QTLs. and 10 and 12 QTLs were identified under the control and Cd stress conditions, respectively. Six QTLs were found on chromosomes 1, 3, 5, 8, and 10 for the Cd tolerant coefficient (CTC). On chromosomes 6 and 7, three QTLs influencing root and shoot Cd concentrations were discovered under Cd stress. The

transgressive segregation of all measured traits suggests that Cd tolerance and concentration are both quantitatively inherited properties that are influenced by a number of genes and the QTLs discovered could be beneficial for marker-assisted selection (MAS). Their findings revealed that the main effect of the QTLs linked to Cd tolerance was minor, as evidenced by low LOD scores and variation. Another study by Shilin et. al. (2021), identified QTLs for Cd toxicity tolerance in rice at the seedling stage, with 118 recombinant inbred lines (RILs) derived from a cross between parents 93-11 and PA64s. To determine the suitable Cd concentration for treatment, a gradient of Cd concentrations (0, 10, 20, 30, 40, 50, 100, and 150 μM) was applied to the two parents. finally, 50 μM Cd concentration was selected. A total of 36 putative QTLs were detected on chromosomes 1, 2, 3, 4, 5, 7, 9, 10 and 11. 15 QTLs were discovered under the control condition, accounting for 7.8% -18.1 % of phenotypic variation, 15 QTLs were discovered under the Cd stress condition, accounting for 0.9 %- 12.8% of phenotypic variation, and 6 QTLs for Cd tolerance coefficient were also discovered on chromosomes 1, 3, 7, and 9, accounting for 6.2 % - 15.4% of phenotypic variation. They mentioned that population size, threshold value, marker number, heritability, genetic background, and experimental conditions, all influence the discovery of QTLs. Ma et al. (2019) reported seven QTLs related to Cd tolerance on chromosomes 2, 4, 6, and 8 of *Oryza nivara*. There was one QTL for each of the following traits: difference in root dry weight (DRW), relative root dry weight (RRW), difference in shoot dry weight (DSW), and relative total plant dry weight (RTW); these explained 9.00–22.07 percent of phenotypic variation. Two QTLs for total plant dry weight (DTW) difference were discovered, accounting for 11.4 %and 11.8 % of the phenotypic variation, respectively.

In our study root length and fresh leaf weight was exploited as indexes to search for QTLs related to cadmium tolerance in the LR4 population of *M. truncatula* at 15 μM Cd. One single QTL for root length under Cd stress was detected on LG8 linked to MTE58 marker. It seems that at this concentration of cadmium and for the incubation time, only *loci* involved in a rapid growth response to cadmium stress could be identified, which appear to be located in the LG 8 region. Although no other significant QTLs were recognized for root length trait, the results indicated potential candidate QTL regions at lower LOD levels on LG1, LG3, LG5 and LG7. We propose the hypothesis that they would have appeared significantly with higher cadmium concentrations. Our results also showed one QTL for FLW trait on LG7, linked to the mtic509 marker. In the study of Arraouadi et. al. (2011), the *M. truncatula* LR5 RILs population was tested to identify QTLs related to salt tolerance. Fourteen quantitative traits associated with aerial and root growth were detected and most of them were on chromosome 8 with minor

effect. One QTL was detected for root length trait in LG8 which was linked to the MTE54 marker. It can be suggested that this region harbors several loci related to stress on roots.

V.2. Gene expression related to cadmium stress

2.1. Genes involved in oxidative stress and their expression in response to Cd treatment

2.1.1. *Peroxidase* (*PRX*) and *Superoxide dismutase* (*SOD*)

Abiotic stresses are among the factors that have a significant impact on the growth and development of plants and thereby cause economic damage. One of the responses is the overproduction of ROS like singlet oxygen(O), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical. This causes oxidative damage and leads to oxidation of proteins, peroxidation of lipids, inhibition of enzymes, and damage of DNA and RNA. ROS are produced in both unstressed and stressed cells. The cell must strike a balance between the concentrations of radicals required to elicit an appropriate response to a changing condition and the low levels of reactive oxygen species required to prevent large-scale cellular damage. Plants have highly developed defense systems against ROS, which include reducing ROS generation as well as removing it. Under unstressed conditions, the formation and removal of O_2 radicals are in balance and they occur mostly at low levels in organelles like chloroplasts, mitochondria, peroxisomes, plasma membranes, and apoplastic spaces (Alscher et al., 2002). However, the defense system, when presented with increased ROS generation under stress conditions, can be overwhelmed. Antioxidants are important in preventing oxidative stress produced by abiotic stressors. To deal with oxidative stress, plants have evolved two defensive mechanisms: enzymatic and non-enzymatic detoxification. The antioxidant enzymes include *SOD, CAT, GPX, APX, PRX*, and *GR*, and the non-enzymatic antioxidants include low molecular mass compounds such as GSH, AsA, a-tocopherol, carotenoids, and phenolic compounds. Non-enzymatic hydrophilic antioxidants (such as ascorbic acid and glutathione) are active in the aqueous phase, whereas lipophilic antioxidants (such as α -tocopherol and β -carotene) are active in the membrane environment. The increase in antioxidant production is a common response to abiotic stress. These antioxidants on the one hand work as redox buffers that interact with ROS and on the other hand as a metabolic interface that controls the optimal induction of acclimation responses (Waśkiewicz et al., 2014). While O_2 can be produced in any

compartment of the cell, chloroplasts, mitochondria, and peroxisomes are regarded to be the most important ROS producers. The first line of defense appears to be increased *SOD* activity (Kumar et al., 2008) that catalyzes the formation of H_2O_2 and dioxygen from two molecules of singlet oxygen ($1O_2$) (Noctor et al., 1998). H_2O_2 has to be rapidly metabolized to guarantee effective protection, otherwise, its accumulation can be harmful (Michiels et al., 1994). As a result, *PRX* is the next line of defense. This enzyme employs H_2O_2 to oxidize a wide range of substrates. *SODs* occur at any place within a cell where an electron transport chain is present, therefore O2 activation can happen in different parts of the cell including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxisomes, apoplasts, and the cytosol (Alscher et al., 2002). Phospholipid membranes have been shown to be impermeable to charged O_2^- molecules. As a result, the presence of *SOD*s in the compartments where O_2^- radicals are produced is critical for their local elimination. *SODs* are divided into three classes based on the metal co-factor employed by the enzyme: iron *SOD* (Fe SOD), manganese *SOD* (Mn-SOD), and copper-zinc *SOD* (Cu-Zn SOD), and these classes are found in distinct parts of the cell. Despite the fact that all three isoforms are encoded by nuclear DNA, they each have a signal peptide that directs their transport to different subcellular compartments. Fe SODs are found in the chloroplast, Mn SODs in the mitochondrion and the peroxisome, and Cu-Zn SODs in the chloroplast, the cytosol, and possibly the extracellular space (Mishra & Sharma, 2019). Increased SOD activity in the response to Cd has been reported (Deng et al., 2010; Guo et al., 2019; Luo et al., 2011). When Populus plants were exposed to 50 µM of Cd in the study of Zhang et. al. (2014), a significant change was observed in *SOD*, *CAT*, *GPX*, and *APX* activities. It was reported that SOD gene expression demonstrated a 107.7% and 138.5% increase compared to control, with a maximum after 24h. In another study on chickpeas treated with 200 µM Cd, it was observed that SOD expression was different depending on the isoforms as well as on plant organs. The Mn-SOD activity was increased by 56%, Cu/Zn-SOD activity was reduced by 85%, and Fe-SOD activity was maintained at the control level (Sakouhi et al., 2021). In the present study, *SOD* and *PRX* genes showed a significant upregulation on the first day in the susceptible pool which is in agreement with a coordinated action of the two enzymes to counteract ROS and oxidative burst. To ensure that *SOD*s provide effective protection, H_2O_2 created by *SOD*s has to be quickly oxidized by peroxidases, otherwise, H_2O_2 accumulation could be hazardous (Michiels et al., 1994). However, in the tolerant pool, we observed only a weak response in *PRX* gene expression at 96h and none at all in *SOD* gene expression. The Peroxidase superfamily plays a role as an electron receiver and catalyzes the oxidation reaction in living organisms (Vicuna, 2005). The work of Shah et. al. (2001) with rice showed that

increasing Cd concentrations were linked to a marked elevation in *SOD* and *PRX* activities and that the increase in *PRX* activity was greater than in *SOD* activity. These results are in agreement with our results. We observed that *PRX* gene upregulation in both susceptible and tolerant pools was stronger than that of *SOD*. García de la Torre et al.(2022) also investigated the differences in the antioxidant defense response to Cd stress between CdT and CdS *M. truncatula* accessions. The expression of genes that codify for enzymes involved in the antioxidant machinery and PC biosynthesis was studied in seedling roots exposed to 0 or 50 µM CdCl2 for 12 h. Their results showed that SOD and GR expression in roots of the susceptible line increased after 12h but were unaffected the tolerant line. This result is in agreement with our results. In the investigations of Zhang et. al. (2009) on *Vicia faba* submitted to Cd stress PRX activity in roots increased with increasing Cd concentration (from 10 to 50µM) during 6 days and then dropped, whereas no effect on *SOD* activities was reported. Peroxidases are encoded by a gene family that provides a wide range of enzyme specificities. As a result, peroxidases can play a role in a variety of physiological and biochemical processes, including plant growth control and lignification. The *PRX* superfamily is divided into heme and non-heme *PRX*s. Heme-containing *PRX*s, commonly known as heme *PRX*s, are split into two categories: animal and non-animal. Non-animal heme *PRX*s are divided into three groups: Class I, II, and III, all of which have a comparable three-dimensional structure and a heme group made up of protoporphyrin IX and Fe (III). However, they have different amino acid sequences that allow to perform unique reaction mechanism and functions with different subcellular localization. Class III peroxidases are heme oxidoreductase enzymes found in higher plants. They are also known as secretory *PRX*s because they are secreted into the extracellular space or the vacuole. By oxidizing phenolic compounds, Class III *PRX*s lower the H_2O_2 level inside the cell and eventually generate phenolic polymers. As a result, their ability to lower ROS levels makes them an efficient member of the antioxidant system against biotic and abiotic stressors, whereas the formation and deposition of phenolic polymer strengthens the cell wall and inhibits the entry of heavy metal and pathogens. They are also engaged in stages of plant development such as seed germination, cell wall metabolism, suberization, fruit growth and development, pollination, wound healing, auxin and anthocyanin catabolism, and lignification. Lignification in plants gets triggered by biotic and abiotic stresses (Kidwai et al., 2020). Another mechanism believed to be involved in the response to Cd stress is sensing by the reduced glutathione-oxidized glutathione ratio (GSH/ GSSG). In various plant species, a drop in the GSH/GSSG ratio has been reported during Cd stress, resulting in the activation of response genes (Dalcorso et al., 2010). GSH and GSH-related enzymes such as *GR*, *GPX*, and

GSTs participate in controlling the ROS concentrations and redox balance by protecting plants against oxidative stress induced by heavy metals (Asgher, Per, Anjum, Masood, et al., 2017). Reduced glutathione (GSH), a peptide containing three amino acids (Glu-Cys-Gly), plays a key role in plants' antioxidative system by scavenging ROS (ZHANG & GE, 2008). Besides its antioxidant role this tripeptide is also important in the detoxification of heavy metals through the synthesis of phytochelatins (PCs). In fact, Glutathione is a precursor of phytochelatins which bind Cd with their thiol group and thus reduce Cd toxicity by forming a complex and directing it into the vacuole. It is generally considered that GSH content positively correlates with metal stress. PCs are a class of peptides with different repetitions of the -Glu-Cys motif followed by a terminal Gly (-Glu- Cys)n-Gly), and have been found in a wide range of plant species. The enzyme phytochelatin synthase synthesies PCs from GSH, and it is assumed that PCs-metal complexes are delivered to the vacuole in the final step of metal detoxification (Sobrino-Plata et al., 2014).

2.2. Genes involved in the Glutathione pathway

To address Cd's impact on the glutathione pathway, we assessed the expression of *GR* and *GST* genes. As mentioned before the GSH/GSSG ratio decreases in plants under stress conditions, and GR has the potential to increase this GSH/GSSG ratio (Romero-Puertas et al., 2007).

2.2.1. *Glutathione reductase* (GR)

GR is involved in oxidative stress defence, and under stress helps in the maintenance of a reduced glutathione pool, which contributes to the strengthening of antioxidative mechanisms in plants. *GR* is a flavoenzyme and a substrate-specific antioxidant enzyme found in all species, from prokaryotes to eukaryotes.*GR* in plants is primarily found in chloroplasts (70–80%), however, small quantities occur also in mitochondria and the cytosol. Chloroplast *GR* is known to play a role in photosynthetic protection against oxidative stress. In plants, the molecular mass of *GR* varies from 60 to 190 kDa. *GR* conducts redox interconversions depending on the availability of substrate (Gill & Tuteja, 2010). It catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) via the AsA-GSH cycle, which is mediated by NADPH

oxidation. The AsA-GSH cycle is responsible for ROS detoxification and the reduced form of oxiglutathione (GSSG) and GSH/GSSG ratio (~300:1) in the cell has been regulated by *GR*. GSH/GSSG balance, protection against chloroplast and mitochondrial cytotoxicity, and enhancement of cell viability and stability against inter-intracellular stressors are all reported to be significant functions of *GR* (Gill et al., 2013; Trivedi et al., 2013). In *Brassica napus* leaves GR activity increased at low concentrations of Cd, but decreased at higher concentrations; in *Brassica juncea* leaves however, *GR* activity remains unchanged at the Cd concentrations tested (Nouairi et al., 2009). Mobin et. al. (2007) reported that two cultivars of *Brassica juncea* L. (Varuna and RH-30) treated with 25, 50, and 100 mg kg^{-1} soil showed 122.0%, 144.0% and 136.0% increases in *GR* activity for Varuna and 124.0%, 132.0% and 79.0% for RH-30, in comparison to the control.

2.2.2. *Glutathione S-transferase* (*GST*)

Glutathione S-transferases, a group of well-known dimeric, multifunctional enzymes, catalyze the conjugation of GSH with xenobiotic compounds for detoxification. Many plant *GST*s are induced by abiotic stress such as herbicides, heat, ozone, heavy metals, and plant hormones. It has been suggested that a common effect of all these abiotic stress processes is the generation of active oxygen species (AOS) produced during oxidative stress and that the GSTs induced response to oxidative stress to protect cellular components from damage (Marrs, 1996; ZHANG & GE, 2008). Most *GST*s described to date are dimers composed of 22–30 kDa subunits, and each subunit has a GSH binding site (G-site) and an adjacent electrophilic substrate binding site (H-site). These enzymes are mainly found in the cytosol and may be divided into several classes such as phi, tau, theta, zeta, lambda, and dehydroascorbate reductase, among which phi and tau classes are specific in plants (Banerjee & Goswami, 2010; Edwards et al., 2000). It has been found that GST is involved in GSH-Cd formation, decreasing Cd uptake in *Saccharomyces cerevisiae* (Adamis et al., 2004) due to the sulfhydryl group in cysteine, enabling GSH to chelate metals and participate in redox cycling (Jozefczak et al., 2012). Similarly, Hu et al. (2009) demonstrated that less Cd was translocated to rice shoots when the thiol content and GST activity in the root were higher. GST also functions as glutathione peroxidase (GPOX), using GSH as a reductant to scavenge reactive oxygen species produced by Cd stress. As a result, the total and specific activities of GST were greater in the Cd-treated sample than in the control sample (ZHANG & GE, 2008). Rice GSTs are organized into four main phylogenetic classes. Based on protein sequence alignments, 40 of the genes

were assigned to the phylogenetic class Tau, 16 to Phi, 3 to Zeta, and 2 to Theta. These GSTs occur in different organs and tissues of rice, and the expression of specific GSTs varies differently during plant development (Soranzo et al., 2004). As an important enzyme in Cd detoxification, the response of *GST* activity to Cd stress differed greatly between shoots and roots in rice according to ZHANG & GE (2008). Their results showed that enzyme activity and Cd level have a significant positive correlation. *GST* activity in roots was dramatically higher than that in shoots. *GST* activity increased by roughly two-fold in shoots when exposed to 40 M Cd compared to the control, while its activity, has shown a decreasing trend against the increase of cadmium concentration from 0 to 40 M in the root. There have been some reports on the effect of Cd stress on *GST* activity in peas (Dixit et al., 2001), maize (Marrs, 1996), wheat (Mauch & Dudler, 1993), and Phragmites (Iannelli et al., 2002). Except for the study of Marrs (1996), all others revealed that Cd stress increased *GST* activity. ZHANG & GE (2008) reported that Cd level and GSH content, as well as *GST* activity, were found to have significant connections, suggesting that these two anti-oxidant defenses could be employed as biomarkers of Cd-induced stress. Zhang et. al. (2013) reported that rice *GST* occurs as many isozymes, some of which are expressed constitutively while others are triggered by Cd exposure as evidenced by enzyme activity and gene expression. In conclusion, upon Cd exposure, GSH is consumed in the synthesis of phytochelatin, which forms complexes with Cd and subsequently sequestrates into the vacuole. Then, *GST*s that also possess glutathione peroxidase activities can use GSH as a reductant for scavenging the reactive oxygen species formed upon Cd stress. Thus, the total and specific activities of *GST* were higher in the Cd-treated sample than those in the control. In our study of *M. truncatula* response to cadmium, *GR* and *GST* gene expression was upregulated with a similar pattern, but which was different in susceptible and tolerant plants with earlier upregulation in susceptible plants. The expression and activity of *GR* and *GST* genes in tomato leaves when exposed to heavy metals such as cadmium, lead, and copper were investigated by Kısa (2017). reporting that both genes demonstrated the highest expression at the same concentration of 20 ppm (around 177 μM) of cadmium. Transgenic tobacco constitutively expressing a *GST* gene of *Pyrus pyrifolia* was found to be more tolerant against abiotic stresses such as drought, NaCl, and Cd (Liu et al., 2013). Previous gene expression analysis on 6 *M. truncatula* lines by Rahoui et. al. (2014) showed that *GST* gene expression was significantly upregulated after 24 hours, which corresponds to our results for plants from the susceptible pool. *GR* gene was reported to be overexpressed in all lines, with 4 lines showing overexpression at 24 and 96 hours and 2 lines showing upregulation at 24 hours.

2.3. A gene that plays a role in monosaccharide transportation

2.3.1. Monosaccharide transporter 1 (*MST1*)

Sugar is a vital source of energy and carbon for the growth and development of plants. In addition to serving as an energy source, sugars also function as a signaling molecule, regulating plant growth, development, storage, and stress responses (Eveland & Jackson, 2012; Sami et al., 2016). Sugar is exclusively biosynthesized in photosynthetic organs; hence a sophisticated transport system is required to efficiently move sugar from source tissues to heterotrophic sinks. When sucrose reaches the sink tissue, it is discharged and taken up by sink cells both symplasmically and apoplasmically (Monfared et al., 2020; Slewinski, 2011). Several sugar transporters have been identified as components regulating carbohydrate partitioning as well as in signal transduction involved in the sensing of biotic and abiotic stressors. Sugar transporters such as sugars will eventually be exported transported transporter (sweets), sucrose transporters (SUTs), and monosaccharide transporters (*MSTs*) are involved in sugar loading and unloading as well as long-distance transport (source to sink) besides orchestrating oxidative and osmotic stress tolerance (Saddhe et al., 2021). Plasma membrane sucrose transporters (SUTs) and *monosaccharide transporters (MSTs)* are the principal types of transporters that facilitate the transport of soluble photo-assimilate through hydrophobic membranes in apoplasmic transport. In higher plants, there is a large family of monosaccharide transporter genes. It is worth noting that, based on their tissue-specific expression and differential expression in response to distinct developmental stages and environmental stimuli, the majority of the encoded proteins appear to play a specialized role. Monosaccharide transporters are membrane proteins with 12 transmembrane-spanning domains that work together to form a central pore that transports soluble monosaccharides across hydrophobic membranes. The monosaccharide transporter (- like) gene superfamily consists of 53 members in *A. thaliana* and even more in rice (Wang et al., 2008). Drought, salinity, and cold are all abiotic stressors that cause considerable sugar buildup in plant tissues. As a result, it is not surprising that many *MSTs* (-like) exhibit an increase in transcript abundance when plants are subjected to such conditions. The accumulation of sugar and altered subcellular partitioning of soluble carbohydrates were suggested to protect cells by stabilizing membranes and providing osmotic adjustment. Furthermore, significant changes in soluble carbohydrate concentrations

may play a role in redox and sugar signaling events required to elicit downstream stress-tolerance responses (Monfared et al., 2020; Slewinski, 2011). Using transgenic *A. thaliana* overexpressing the rice monosaccharide transporter gene OsMST6, showed that these plants had improved drought and salt tolerance, as seen by lower water loss, higher relative water content, and increased cell membrane stability. The overexpression of the OsMST6 gene in *A. thaliana* can increase soluble sugar content in cells and improve osmotic adjustment capability which leads to a significant increase in the tolerance of transgenic plants to various abiotic stresses (Monfared et al., 2020). OsMST6 is thought to have a role in rice response to water stress by maintaining the water status of plant cells. The rapid accumulation of OsMST6 transcript in response to salt stress has also been described by Wang et. al. (2008). In our study, Mt*MST1* gene expression increased in the susceptible plants after 24 hours, whereas we did not observe any change in the tolerant plants. Rahoui et. al. (2015) reported that expression of the Mt*MST1* gene was upregulated following Cd treatment in 5 *M. truncatula* lines except for TN1.11 at 24h, which is consistent with our results in the susceptible pool. TN1.11 has been shown to downregulate Mt*MST1* and had high glucose levels in the Cd-treated condition. It was proposed that glucose buildup in embryonic tissues was due to transporter blockage under Cd treatment, and that tolerance to Cd poisoning is related to the ability to metabolize glucose under Cd stress. Taken together, all genes investigated here displayed an unusual pattern compared to the literature, in that the susceptible plants showed a stronger induction of the gene. It should be noted, however, that our investigation was conducted at a relatively low cadmium concentration. We propose that the gene expression response in susceptible plants is an overreaction that, rather than protecting them from the stress, makes them weaker. A parallel could be made to a study on the response of tomato plants to the root pathogen *Verticillium dahliae* by Robb *et al*. (2012) which reported that a susceptible isoline produced more defense proteins, among them *peroxidase*s, than the resistant isoline when inoculated with the pathogen. The authors proposed that increased defense protein production may be the cause of symptoms rather than the cure. This might also apply to the *M. truncatula* LR4 population that was under mild cadmium stress. Repeating our findings at higher cadmium concentrations would be informative. If our theory is correct, this should result in a different pattern of gene induction and might even help find more QTLs.

V.3. Cadmium response of Iranian *Medicago*

As mentioned in previous parts, root growth inhibition has been identified as one of the earliest and most identifiable indicators of Cd toxicity (Lux et al., 2011). Iranian accessions of *M. truncatula* and *M. scutellata* also showed the same reaction against cadmium treatment at 15μM concentration. The genotypes that had a higher growth rate in control conditions were more affected by cadmium. The difference in root growth in control and treatment conditions with cadmium was considered as the tolerance index. Genotype 7321. B has the lowest difference in root length growth in control and cadmium treatment conditions with an average of about 1.2 cm (The most resistant line) and SCU. A genotype has the largest difference with an average of about 6.3 cm (The most sensitive line) and LR4 parents have a difference of about 2.4 and 2.7 cm for DZA315.16 and A17, respectively. We also observed significant effects of cadmium on the root surface. Root surface trait analysis showed inhibition against cadmium treatment. The response of the genotypes was similar to the response observed in the root length trait. Genotype 7321. B has the lowest root surface difference in control and cadmium treatment conditions with an average of 0.1 cm (the most resistant line) and SCU. A genotype has the highest difference with an average of 0.48 cm (the most sensitive line) and LR4 parents with a difference of 0.22 and 0.26 cm for DZA315.16 and A17, respectively. Analysis of variance of both root length and surface showed highly significant effects of genotype and condition (±15 μM Cd) as well as a highly significant interaction of genotype and condition, indicating genetic variation in response to Cd among lines. In other words, the difference in root growth and surface between control conditions and treatment with cadmium was less in tolerant genotypes. These results were consistent with other research (Rahoui et al., 2010, 2015; Sfaxi-Bousbih et al., 2010). Shorter but thicker primary and lateral roots were frequently observed in plant species grown in soils or culture medium containing Cd (Kubo et al., 2011; Lu et al., 2013). Our results were in agreement with Wang et. al. (2016) who reported that compared with the control, the total root length, root surface area, and root volume of five soybeans (*Glycine max* L. Merr) cultivars were decreased significantly at four Cd levels. also, it was reported that the root surface area of the five cultivars showed the same trends as the root total lengths.in peanut (*Arachis hypogaea* L.) cultivars Cd considerably has decreased the root lengths, surface area, and number of root tips, but has increased the root diameters (Lu et al., 2013). Our results of variance analysis of root diameter data showed a significant effect of

genotype and condition but their interaction was not significant. The reaction of the root diameter trait in the genotypes did not follow a constant trend. Saeidi et. al. (2012) observed that Cd-stress considerably reduced seedling root growth, root fresh weight, root and shoot dry weight and dry biomass, but increased shoot water content, fresh shoot/root ratio, and root diameter. In Our study, fresh and dry weight Analysis of variance showed significant genotypic effects for leaf fresh weight (FLW), root fresh weight (FRW), leaf dry weight (DLW), and root dry weight (DRW). The effect of condition was significant for FRW, but the interaction effect of genotype and condition was not significant. The response of ten selected rice cultivars under different levels of $CdCl_2$ (0, 50 and 100 µM) was investigated in the study of Afzal et. al. (2019). With the application of 50 and 100 µM cadmium treatments, the fresh and dry weight of rice seedlings in all cultivars decreased significantly compared to the control. Also, changes in the dry mass weight of cultivars have been reported. At the concentration of 50 µM cadmium, the dry weight decreased by 14 to 50%. According to Sager et. al. (2020), cadmium treatment (0, 100, 200 and 400 µM Cd concentration) has led to a decrease the growth of pea plants (*Pisum sativum*). Cadmium stress decreased the length, fresh weight and dry weight of roots and shoots The length, fresh weight, and dry weight of roots and shoots were lower when Cd treatment was applied. Hence the results obtained with this small sample of accessions showed that interesting genes for cadmium tolerance can be found in the Iranian gene pool.

VI - Conclusion and Perspectives

VI.1.English Conclusion and Perspective

Presently, various pollutants especially heavy metals have been mostly created by human activities such as industry, agriculture, transportation, and natural variabilities. In general, the first human exposure to heavy metals is through the diet (water and food). Access to healthy and sufficient food, drinking water, and clean air is one of the most obvious rights of all human beings, and the production and provision of these needs for citizens is the inherent duty of all governments. On the other hand, environmental protection is very important along with agricultural and industrial production activities. The complications of exposure to heavy metals depend on the concentration and duration of exposure to these metals, *i.e*., acute or chronic. They can quickly contaminate the environment, accumulate, be dispersed, and move among compartments. Consequently, it is necessary to study their transport and behaviors in air-water-soil-biota.

The use of tolerant plants with a mechanism to avoid the entry of cadmium, resistant plants with a detoxification mechanism, hyperaccumulating plants for Phytoremediation purposes, and identification of resistance genes for modern plant breeding purposes are very worthy and safe ways to control and struggle with negative effects of heavy metals on the growth and development of living organisms. Another important additive component of the plant defense system is the symbiotic association with *Arbuscular mycorrhizal* (AM) fungi. AM can effectively immobilize heavy metals and reduce their uptake by host plants via binding metal ions to hyphal cell walls and excreting several extracellular biomolecules (Pakniat et al., 2010) Additionally, AM fungi can enhance the activities of the antioxidant defense machinery of plants. It was reported that AM symbiosis had a protective effect on *M. truncatula* plants grown in Cd-contaminated substrate with regard to plant biomass and phytotoxicity symptoms. Because mycorrhized *M. truncatula* plants also were found to accumulate Cd in their shoots, it was chosen as a model to investigate the mechanisms by which legume shoots tolerate Cd toxicity upon AM symbiosis (Aloui et al., 2011).

With this scenario as background, the tolerance of *Medicago truncatula* toward Cd stress was studied in the early stages after germination, using the recombinant inbred lines (RILs) LR4 population and several growth traits to identify QTLs involved in Cd tolerance. In addition, the expression of genes involved in responses to Cd stress was studied in tolerant and susceptible RILs. Cadmium at low concentrations (15 µM) caused inhibition in root growth that varied among the 147 LR4 RILs population of *M. truncatula*. Implying that different adaptation

methods exist in different accessions. Two QTLs associated with root length and fresh leaf weight (FLW) were detected on LG8 and LG7, respectively, but were too large to search for candidate genes. It can be assumed that if the cadmium concentration gradually increases, other responsive gene regions can be detected at each concentration level.

The *GR*, *GST*, *SOD*, *PRX*, and *MtMST1* gene expression study revealed an interesting and unexpected discovery regarding the response to cadmium stress. The susceptible accessions presented a short-term response towards the cadmium contamination, in contrast to the tolerant ones that presented a long-term response. In addition, the response in susceptible and tolerant pools was entirely different from each other, with a downward trend in susceptible and an upward trend in the tolerant pools. It will be interesting to continue the gene expression study in susceptible and tolerant accessions to know more about the expression pattern of these genes during longer exposure (more than 96 hours). This might be helpful to have a more precise idea about the roles of these genes in cadmium tolerance. The reaction of Iranian lines against cadmium stress was similar to that of the LR4 population. Root growth inhibition was one of the earliest and most identifiable indicators of Cd toxicity. We also observed significant effects of cadmium on the root surface. Analysis of variance (ANOVA) of both root length and surface showed highly significant effects of genotype and condition (±15 µM Cd) as well as a highly significant interaction of genotype and condition, indicating genetic variation in response to Cd among the Iranian accessions.

1.1.1. Further study of the genetic control of resistance response towards Cd stress using a Genome-wide association study

In the study conducted by Ben et. al. (2013) on resistance to *Verticillium alfalfae* fungal pathogen, they reported some QTLs for resistance to this soil-born pathogen. Subsequent supplementary studies were conducted through a Genome-wide association study (GWAS). Using a large number of HapMap accessions led to the discovery of 2 SNPs in previously reported QTL regions and to the identification of candidate genes involved in resistance to the pathogen (Mazurier, 2018). We propose to study the resistance to cadmium through the GWAS approach with the collection of about 250 *M. truncatula* HapMap accessions. This should lead to the identification of more loci involved in tolerance to cadmium, present in various genotypes. It would be interesting to compare such a study with the results of Garcia de la

Torre et al. (2022), to see if the identified loci are robust under varying conditions. It is also possible that some SNPs involved in resistance to Cadmium may be located within the QTLs detected during this thesis. Like in the case of *Verticillium* resistance it can be expected that the *loci* will be narrow and allow the identification of candidate genes for cadmium tolerance.

1.1.2. Test new morphological and physiological traits in selected tolerant and susceptible lines of the LR4 population treated with a gradient of cadmium concentration

The present study led to the selection of 10 lines as susceptible and 10 lines as tolerant. It is suggested conduct another study examining more physiological traits, for example, levels of proline, glycine betaine, soluble proteins, sugar content (Sager et al., 2020), the level of Mitogen-activated protein kinase, Auxin transporter, protein kinases, (Chen et al., 2019), the lengths of shoots and roots (Chen et al., 2021), in plants treated with a cadmium concentration gradient. Furthermore, Cd accumulation in leaves, shoots, roots, and grain, Root-to-shoot Cd translocation, should be investigated at different time points notably the ones used in the present study.

1.1.3. Iranian accessions deserve more research in the field of stress tolerance

Considering the response of Iranian *M. truncatula* and *M. scutellata* lines to cadmium stress, it is recommended to create a recombinant inbred line population using contrasting lines (7321 and 1724) as parents for further studies, such as QTL detection. In this case, constructing a genetic map will be a prerequisite. Additionally, for future studies, we suggest performing full genome sequencing of Iranian lines to include them in the HapMap collection. Furthermore, the response of these Iranian accessions to other abiotic and biotic stresses should be investigated. This will help expand the pool of genetic resources and contribute to international efforts in understanding and breeding resistance to environmental constraints.

VI.2.Conclusion et perspectives (Français)

Des polluants divers, en particulier les métaux lourds, sont créés principalement par les activités humaines telles que l'industrie, l'agriculture, les transports et les variabilités naturelles. En général, la première exposition de l'homme aux métaux lourds se fait par l'alimentation (eau et aliments). L'accès à une alimentation saine et suffisante, à de l'eau potable et à un air pur sont des droits les plus évidents de tous les êtres humains. Ainsi, la protection de l'environnement en face des activités de production agricole et industrielle, est d'importance primordiale. Les conséquences d'une exposition aux métaux lourds dépendent de la concentration et de la durée de l'exposition, c'est-à-dire aiguë ou chronique. Ils peuvent contaminer l'environnement plus ou moins rapidement, s'accumuler, se disperser et se déplacer entre les compartiments. Par conséquent, il est nécessaire d'étudier leur transport et leurs comportements dans les compartiments air-eau-sol-biote. L'utilisation de plantes tolérantes évitant l'entrée de cadmium, de plantes résistantes avec un mécanisme de détoxification, de plantes hyperaccumulatrices à des fins de phytoremédiation, et l'identification de gènes de résistance à des fins de sélection végétale moderne sont des moyens pour contrôler et lutter contre les effets négatifs des métaux lourds sur la croissance et le développement des organismes vivants. Un autre aspect important du système de défense des plantes est l'association symbiotique avec les champignons *Mycorhiziens arbusculaires* (AM), capables d'immobiliser efficacement les métaux lourds (Pakniat et al., 2010). De plus, les champignons AM peuvent améliorer les activités antioxydantes des plantes. Il a été rapporté que la symbiose AM avait un effet protecteur sur les plantes de *Medicago truncatula* cultivées dans un substrat contaminé au Cd en ce qui concerne la biomasse végétale et les symptômes de phytotoxicité. Étant donné que les plantes mycorhizées de *M. truncatula* accumulaient également du Cd dans leurs parties aériennes, elles ont été choisies comme modèle pour étudier les mécanismes par lesquels les légumineuses tolèrent la toxicité du Cd grâce à la symbiose AM (Aloui et al., 2011). Avec ce scénario en arrière-plan, la tolérance de *M. truncatula* au Cd a été étudiée dans les premiers stades après la germination, en utilisant la population LR4 des lignées recombinantes (RIL) et plusieurs traits de croissance pour identifier les QTLs impliqués dans se contrôle génétique. De plus, l'expression de gènes impliqués dans les réponses au stress lié au Cd a été étudiée chez les RILs tolérants et sensibles. Le cadmium à de faibles concentrations (15 µM) a provoqué une inhibition de la croissance des racines qui variait au sein de la population des 147 RILs, ce qui implique que différentes méthodes d'adaptation existent dans différentes

accessions. Deux QTL associés à la longueur des racines et au poids des feuilles fraîches (FLW) ont été détectés respectivement sur les chromosomes 8 et 7, mais ils étaient trop larges pour pouvoir rechercher des gènes candidats. On peut supposer que si la concentration de cadmium augmente progressivement, d'autres loci pourraient être détectées. L'étude de l'expression des gènes *GR*, *GST*, *SOD*, *PRX* et *MtMST1* a révélé une découverte intéressante et inattendue concernant la réponse au stress cadmium. Les accessions sensibles ont présenté une réponse rapide et transitoire à la contamination par le cadmium, contrairement aux accessions tolérantes qui ont présenté une réponse à long terme. De plus, la réponse dans les plantes sensibles et tolérants était entièrement différente l'une de l'autre, avec une tendance à la baisse dans les plantes sensibles et une tendance à la hausse dans les plantes tolérantes. Il sera intéressant de poursuivre l'étude de l'expression des gènes dans les accessions sensibles et tolérantes pour en savoir plus sur le schéma d'expression de ces gènes lors d'expositions plus longues (plus de 96 heures). Cela pourrait être utile pour avoir une idée plus précise du rôle de ces gènes dans la tolérance au cadmium. La réaction des lignées iraniennes face au stress cadmium était similaire à celle de la population LR4. L'inhibition de la croissance des racines était l'un des indicateurs les plus précoces et les plus identifiables de la toxicité du Cd. Nous avons également observé des effets significatifs du cadmium sur la surface des racines. L'analyse de la variance (ANOVA) de la longueur et de la surface des racines a montré des effets très significatifs du génotype et de la condition (± 15 µM de Cd) ainsi qu'une interaction très significative entre génotype et condition, indiquant une variabilité génétique concernant la réponse au Cd parmi ces accessions.

Une poursuite de cette étude pourrait être envisagée par l'approche de génétique d'association (GWAS). Environ 300 accessions de *M. truncatula* ont été séquencées dans le projet international *Medicago* Hapmap et sont disponibles pour la communauté scientifique. Cette approche a été utilisée avec succès au laboratoire pour identifier des gènes potentiellement impliqués dans la résistance au champignon pathogène *Verticillium alfalfae*, à des températures différentes et avec deux isolats fongiques différents (Fartash et al., 2023; Mazurier, 2018).

Comparé à une étude antérieure, cette approche a permis de délimiter la zone des loci identifiés par QTL mapping (Ben et al., 2013) et on peut supposer qu'une étude GWAS de la réponse au Cd mènera également à l'identification de gènes candidats impliqués dans la tolérance à ce polluant.

Une étude de paramètres physiologies supplémentaires tels que : niveaux de proline, glycine bétaine, protéines solubles, et sucres solubles (Sager et al., 2020), accumulation de Cd dans les différents organes de la plante et les niveaux d'activités d'enzymes comme les transporteurs

d'auxine et protéine kinases (Chen et al., 2019), donnera également plus d'information sur les mécanismes de tolérance. L'accumulation de Cd dans les racines et parties aériennes devrait être déterminée après différents temps d'exposition. Ceci était prévu dans le cadre de cette thèse mais n'a pas pu être réalisé à cause de problèmes sanitaires et techniques.

Finalement, les accessions iraniennes semblent être une bonne ressource génétique pour étudier la tolérance au Cd, puisqu'elles ont montré une variabilité génétique dans leur réponse à ce polluant. Pour une étude de QTL on devra faire des croisements entre une accession sensible et une tolérante (par exemple 7321 et 1724). Il sera également intéressant de séquencer leurs génomes pour les inclure dans la collection *Medicago* HapMap. La réponse de ces lignées à d'autres stress abiotique et biotiques devrait aussi être étudiée. Ainsi elles vont augmenter le nombre de ressources génétiques et pourront contribuer aux efforts internationaux pour comprendre et améliorer la tolérance des légumineuses aux contraintes environnementales.

VII - Annex

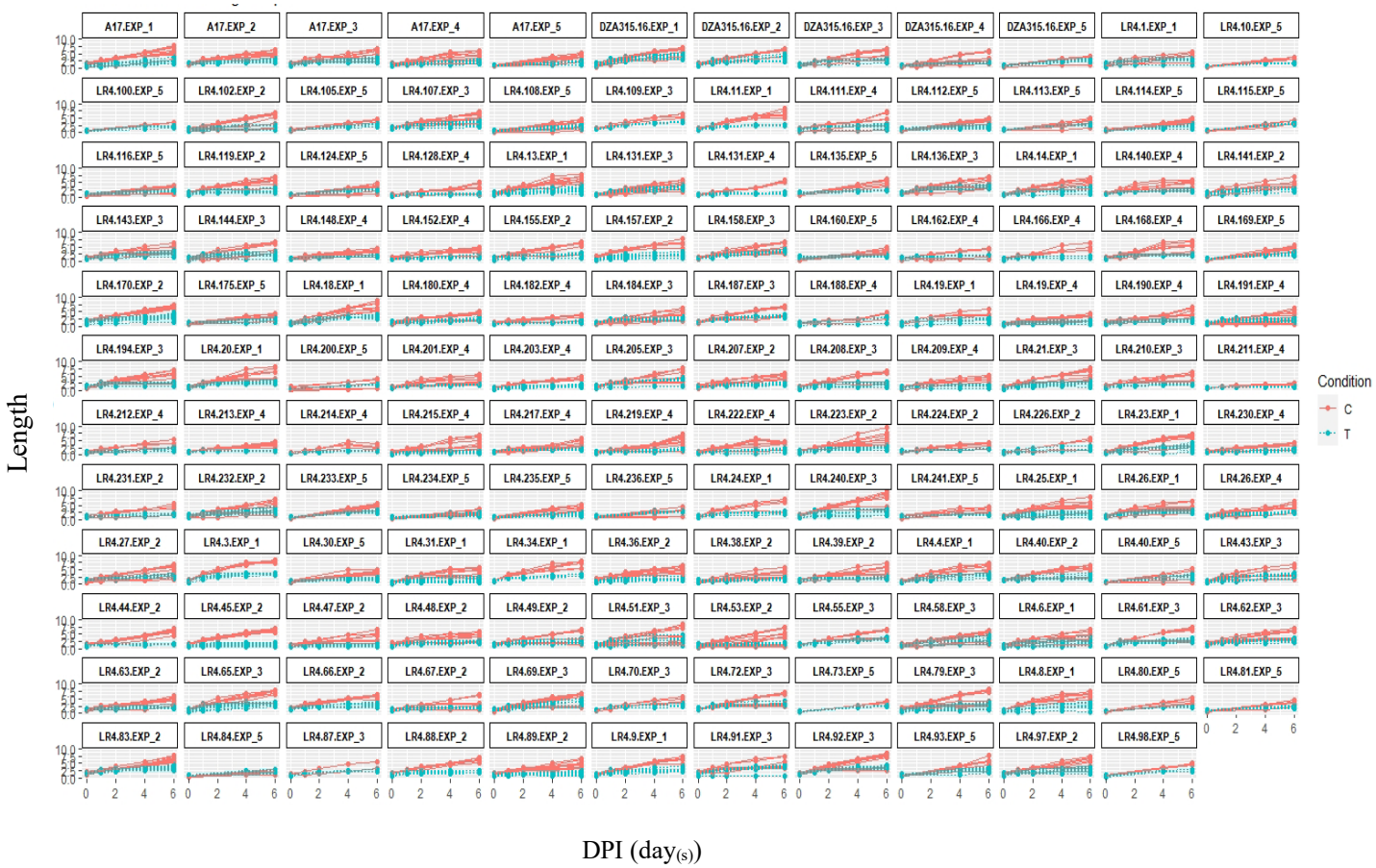


Figure A1- Root growth curve of *Medicago truncatula* LR4 genotypes during 6 days in control (C) and Cd-treatment (T) conditions, repeat 1.

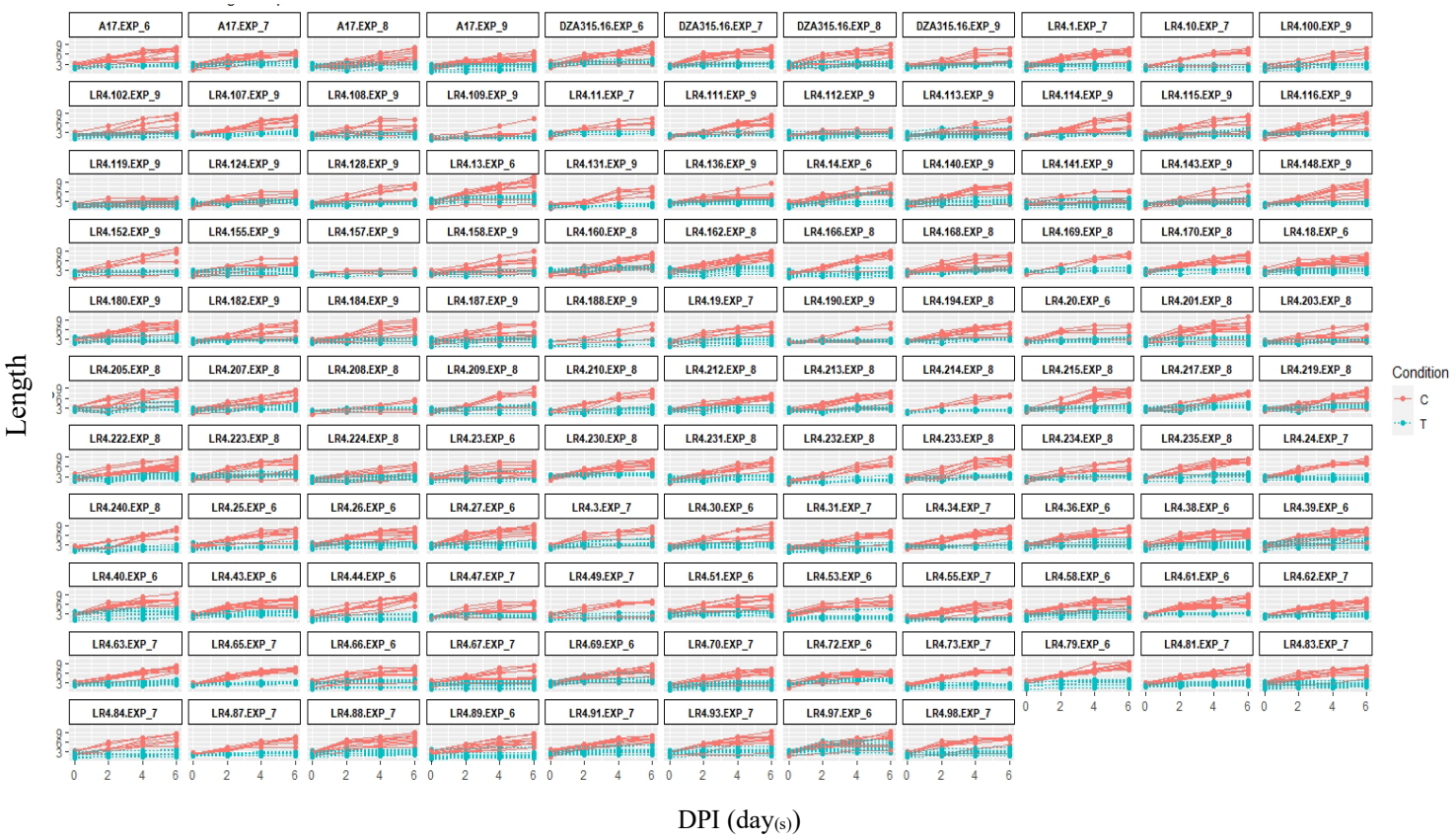


Figure A2- Root growth curve of *Medicago truncatula* LR4 genotypes during 6 days in control (C) and Cd-treatment (T) conditions, repeat 2.

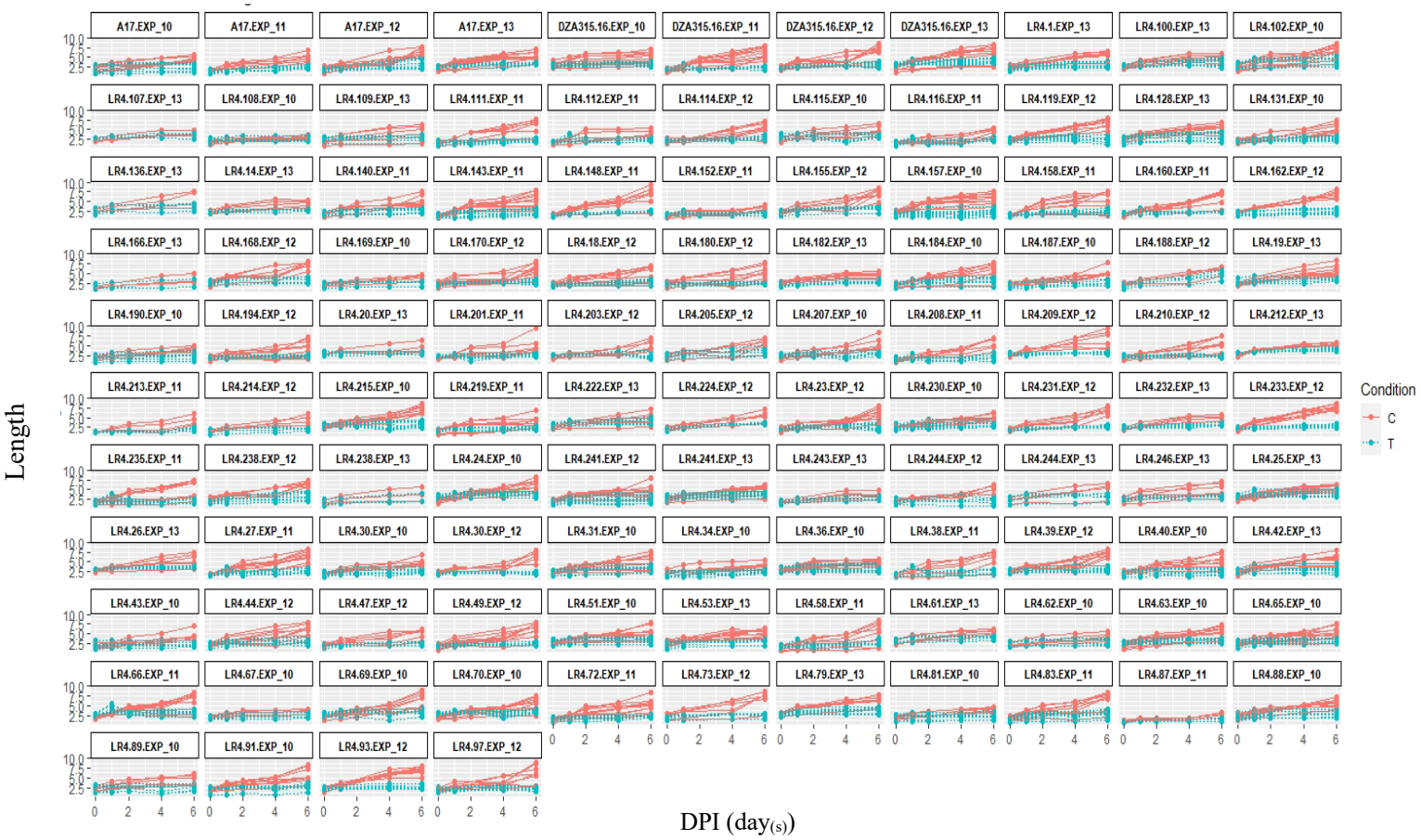


Figure A3- Root growth curve of *Medicago truncatula* genotypes during 6 days in control (C) and Cd-treatment (T) conditions, repeat 3.

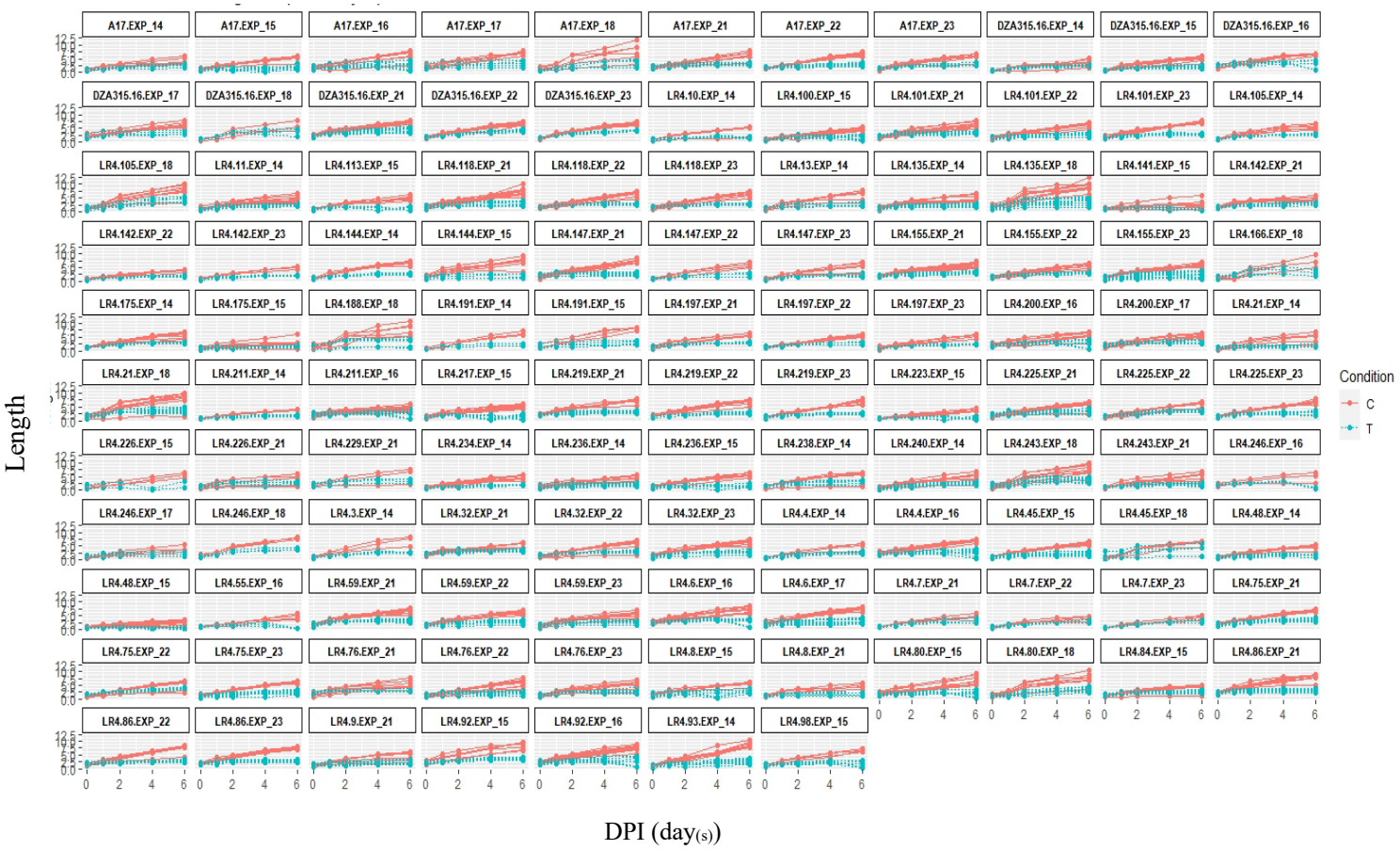


Figure A4- Root growth curve of *Medicago truncatula* LR4 genotypes during 6 days in control (C) and Cd-treatment (T) conditions, complementary repeat.

VIII - Bibliography

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