**Promoters:**

Prof. dr. ir. Patrick Van Damme

Ghent University, Faculty of Bioscience Engineering, Department of Plant Production

Coupure links 653, 9000 Ghent, Belgium

Dr. ir. Jan De Riek

Institute for Agricultural and Fisheries Research (Instituut voor Landbouw- en Visserijonderzoek; ILVO), Plant Sciences Unit, Applied Genetics and Breeding

Caritasstraat 39, Melle, Belgium

**Dean:**

Prof. dr. ir. Marc Van Meirvenne

**Rector:**

Prof. dr. Anne De Paepe

**Ir. Omar Idrissi**

# Molecular diversity and quantitative trait loci related to drought tolerance in lentil (*Lens culinaris* Medik., Fabaceae)

Thesis submitted in fulfilment of the requirement for the degree of Doctor (PhD) in Applied Biological Sciences: Agricultural Sciences

**Dutch translation of the title:** Moleculaire diversiteit en “quantitative trait loci” gecorreleerd met droogtetolerantie in linzen (*Lens culinaris* Medik., Fabaceae)

**Citation:**
Idrissi, O. (2016). Molecular diversity and quantitative trait loci related to drought tolerance in lentil (*Lens culinaris* Medik., Fabaceae). PhD thesis, Ghent University, Ghent, Belgium, p. 238

**ISBN: 978-90-5989-898-1**

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To all those who supported me, no achievements would have been accomplished without your encouragements,

*Hanane*, my beloved wife, thank you for your very generous and unconditional support!
*Yasmine* and *Nour*, our lovely daughters, You are making our lives very pleasant !
My dearest parents: My father *Youssef Idrissi* and my mother *Hougga Doramane* with my wishes of healthy and long life.

My brothers and sisters, *Lhaj Mohamed*, *Hammou*, *Mustapha*, *Latifa*, *Malika* and *Ghita*.

My uncle *Moulay Ali Idrissi*

My whole family *Idrissi*,

Families: *Salhi*, *Lahmouddi*, *El Messadi*, *Antari*, *Toudghi*, *EL Moudni* and *Bouhriz*

My friends: *Mohamed Gadi*, *Toufik Chedadi* and *Mohamed El Idrissi*.

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## Acknowledgments

This thesis was directed by Professor Patrick Van Damme and co-promoted by dr. Jan De Riek. Prof. dr. ir. Patrick Van Damme is full professor at the Department of Plant Production, Faculty of Bioscience Engineering, Ghent University. Dr. ir. Jan De Riek is senior researcher at the Institute for Agricultural and Fisheries Research (Instituut voor Landbouw- en Visserijonderzoek; ILVO), Plant Sciences Unit, Applied Genetics and Breeding Group.

I sincerely express my profound gratitude to Prof. Patrick Van Damme and dr. Jan De Riek for their determinant roles during the different steps of my PhD study. I have learned a lot from their rich experience. They also helped me to largely enhance my knowledge and acquire skillful scientific thinking. Besides this, their support and orientations always paved the ways for more optimal solutions making my work pleasant.

I also would like to thank dr. ir. Ellen De Keyser, senior researcher at ILVO, for her kind help and suggestions. I am very grateful to Veerle Buysens and Laurence Desmet for their assistance during the experimental work in the laboratory. Likewise to all the laboratory, technical, research and administrative staff of ILVO, Caritasstraat 39, Melle, Belgium and of Faculty of Bioscience Engineering, 653, Coupure links, Ghent, Belgium.

To my colleagues, PhD students: we shared wonderful moments. I wish you good luck and all success during your careers!

My special thanks go to Isabel Vandriessche, dr. ir. Wouter Vanhove and ir. Kaat Verzelen from the Laboratory of Tropical and Subtropical Agronomy and Ethnobotany, they were always willing to help.

Thanks to dr. Sripada M. Udupa from ICARDA and Prof. Fred Muehlbauer from WSU for their advice and remarks during the realization of my research project. My thanks go to drs. Rebecca McGee and Clarice Coyne from WSU for their fruitful collaboration.

I would like to thank my home institute, INRA-Morocco, and the IDB Merit Scholarship program for High Technology for the support and funding of my thesis.

All my thanks go to the jury members for kindly accepting to review my research work.

## Summary

Drought is one of the major abiotic stresses limiting lentil (*Lens culinaris* Medik.) productivity especially in rainfed agricultural systems. Rooting patterns are often associated with promising drought avoidance mechanisms targeted in breeding programs. Their molecular mapping aims to develop tools for efficient selection. Molecular genetic diversity analysis of landraces is a key step for enhanced use of these important genetic resources in developing adapted cultivars as well as for their better valorization for the benefits of local farmers. Lentil contributes to sustainable farming by biological fixation of nitrogen in soils and enhances nutrition thanks to its proteins and micronutrients-rich grains. Improvement of drought tolerance and targeted use of genetic resources will contribute to enhance lentil production to face increasing demand for staple food in the world.

In the absence of earlier molecular characterization, we assessed the genetic diversity of 51 Moroccan lentil landraces using simple sequence repeats (SSR) and amplified fragment length polymorphisms (AFLP). Nineteen SSRs yielded 213 alleles, whereas seven AFLP primer combinations gave 766 fragments of which 422 were polymorphic. Overall, we observed moderate to high genetic variation. We also differentiated several groups of landraces. Interestingly, one of these groups contained short-cycle landraces with high rapid vegetative growth. Landraces in that group were from the dryland location of Abda in west-central Morocco, where they were likely selected for adaptation to drought and heat stress over centuries. Another group contained landraces from highland areas that may have been selected for specific adaptation to cold stress. A third group contained one landrace from the Zear region, in north-western Morocco, known for its seed quality (especially short cooking time) and has been proposed in the national catalog of local products for a protected designation of origin (PDO) quality mark. Both molecular techniques evidenced that the latter landrace developed on its own some specific characteristics supporting the idea of PDO attribution. Genetic differentiation according to agro-environmental origins, cycle duration and early vegetative vigour was observed when combining genetic and agronomic information. Landraces from dry areas were differentiated from those of more favourable climatic condition areas and higher locations. Specific adaptation of these landraces to their respective agro-environments may be the reason of their genetic differentiation.

Additionally, we assessed the genetic diversity of a collection of 70 Mediterranean lentil landraces using the same 19 SSRs and seven AFLP primer combinations. These landraces were assessed for variation in root and shoot traits as well as for drought tolerance as estimated by

relative water content (RWC), water losing rate (WLR) and wilting score (WS). We found clear genetic diversity and netted differentiation of Moroccan landraces from those from northern Mediterranean regions (Italy, Turkey and Greece). Population structure analysis of Mediterranean lentil germplasm revealed two major gene pools and a possibility one minor gene pool. The latter contained mainly landraces from the dryland location of Abda in west-central Morocco. The two major gene pools contained the southern (Morocco) and the northern Mediterranean landraces, respectively. High genetic variation in root and shoot traits and drought tolerance level was also observed. However, no relationship was found between drought tolerance of landraces and their geographic origin. Landraces with higher dry root biomass, chlorophyll content and root–shoot ratio were shown to be more drought tolerant as evidenced by their higher RWC, and lower WLR and wilting severity. Kruskal–Wallis non-parametric test (KW) was used to find SSRs and AFLPs associated with RWC, WLR and WS. Regression analysis showed six SSR and AFLP alleles explaining the highest percentage of phenotypic RWC, WLR and WS variation (ranging from 21 to 50 % for SSRs and from 14 to 33 % for AFLPs). Functional genetic diversity analysis showed statistically significant relationships between drought response of landraces and linked SSR and AFLP alleles. We used canonical discriminant analysis based on genetic distance between landraces and their response to drought to show that drought-tolerant landraces were differentiated from sensitive and intermediate ones. Our results confirm the feasibility of using association mapping to find DNA markers associated with drought tolerance in larger numbers of lentil landraces.

Only limited information is available about genetic control of shoot and root traits in association with drought tolerance. We studied the latter issue in lentil using a mapping population of 133 $F_{6\text{-}8}$ recombinant inbred lines (RIL) from the ILL6002 x ILL5888 cross. We found important variation between genotypes and also high variation in heritability values for root and shoot traits at 38 days after sowing under both well-watered and drought-stressed treatments in the greenhouse during two consecutive seasons. Higher heritability values were obtained under the drought-stressed treatment suggesting that selection in water-limited environments would be more effective in achieving higher genetic gains. Drought had reduced root and shoot growth compared to well-watered conditions. However, root–shoot ratio was likely to be enhanced under drought-stressed treatment underlying the importance of this trait for drought tolerance. Quantitative and continuous distributions of variation in root and shoot traits shown here are evidence of their polygenic control in the perspective of identification and mapping associated quantitative trait loci (QTL). Statistically significant associations between root and shoot traits

such as dry shoot biomass and chlorophyll content were noted, highlighting the reliability of indirect selection for underground traits (root) based on these aboveground traits in breeding programs. Significant correlations and regressions were demonstrated between dry root biomass, lateral root number, root surface area, dry shoot biomass, root–shoot ratio, chlorophyll content and drought tolerance as estimated by wilting severity at limited water supply. This shows the importance of a well-developed root system and early biomass development for conveying drought tolerance. Identification and mapping of QTLs related to the traits studied in this population would be a first step for starting marker-assisted selection in lentil.

The thus phenotyped RIL population (ILL6002 x ILL5888) was genotyped using SNP, SSR, AFLP, SRAP and RAPD DNA markers. In all, 252 co-dominant and dominant markers were used for genetic linkage map construction. QTL analysis based on greenhouse experiments for root and shoot traits during two seasons under progressive drought-stressed conditions was performed using the thus developed map. A genetic map of nine linkage groups (LG) spanning a total distance of 2022.8 cM was constructed. Eighteen QTLs controlling a total of 14 root and shoot traits were identified. A QTL-hotspot genomic region related to a number of root and shoot characteristics associated with drought tolerance such as dry root biomass, root surface area, lateral root number, dry shoot biomass and shoot length was identified on LG VII at position 21-22 cM. Interestingly, a QTL related to root-shoot ratio, an important trait for drought avoidance, was detected for both seasons explaining the highest phenotypic variance of 27.6 % and 28.9 %, respectively. This QTL was close to the co-dominant Single Nucleotide Polymorphism (SNP) TP6337 marker located at 2.30 cM on LG IX and also flanked by the two SNPs TP518 and TP1280, located respectively at 0 and 2.9 cM on LG IX. These markers could be used for marker-assisted selection. An important QTL related to lateral root number was identified in LG III at 98.64 cM position close to TP3371 and flanked by TP5093 and TP6072 SNP markers. Also, a QTL associated with specific root length was identified on LG IV at 61.63 cM position close to TP1873 SNP marker and flanked by F7XEM6b SRAP marker and TP1035 SNP marker. These two QTLs were detected in both seasons. Our results could be used for marker-assisted selection in lentil breeding programs targeting root and shoot characteristics conferring drought avoidance as an efficient alternative to slow and labour-intensive conventional breeding methods.

**Keywords**: genetic differentiation, agro-environmental origins, ecophysiology, drought tolerance, breeding, marker-trait association, QTL, marker-assisted selection.

## Samenvatting

Droogte is een van de belangrijke abiotische factoren die de productiviteit van linzen (*Lens culinaris* Medik.) aantast. Dat is vooral het geval in niet-geïrrigeerde landbouwsystemen. Het wortelpatroon is vaak geassocieerd met droogte-vermijdende plantmechanismen die in de plantenveredeling worden beoogd. De (moleculaire) mechanismen van deze wortelpatronen vastleggen in een genetische kaart stelt de veredelaars dan ook in staat de linzenplanten op een meer efficiënte wijze te selecteren. Moleculaire genetische diversiteitsanalyse van landrassen van linzen is dus belangrijk bij de ontwikkeling van aangepaste variëteiten. Linzen dragen bij tot een meer duurzame landbouw door biologische fixering van stikstof in de bodem. Bovendien verhogen ze de voedselzekerheid door hun hoge proteïne- en micronutriëntengehaltes. De verhoging van de droogtetolerantie door een meer doelgericht gebruik van de genetische diversiteit bij linzen, draagt bijgevolg bij tot een verhoogd productiepotentieel, en biedt een antwoord op de steeds stijgende wereldwijde vraag naar voedingsproducten.

Omdat een moleculaire karakterisering van linzen nooit eerder werd uitgevoerd, werd de genetische diversiteit bij 51 Marokkaanse landrassen in kaart gebracht met behulp van "S*imple Sequence Repeats*" (SSR) en "*Amplified Fragment Length Polymorphism*" (AFLP). Negentien SSRs resulteerden in 213 allelen. Zeven AFLP primer combinaties leverden 766 fragmenten op, waaronder 422 polymorfe. In het algemeen werd een matige tot hoge diversiteit geobserveerd, maar konden niettemin verschillende groepen landrassen worden onderscheiden. Opmerkelijk was dat een van deze groepen landrassen met een korte teeltcyclus en een snelle vegetatieve groei bevatte. Deze landrassen zijn afkomstig uit de droge gebieden in Abda in West-Centraal Marokko, waar ze de voorbije eeuwen vermoedelijk werden geselecteerd op basis van hun droogte- en hittetolerantie. Een andere groep bevatte landrassen uit hogere gebieden die mogelijks geselecteerd werden op basis van koudetolerantie. Een derde groep bevatte 1 enkel landras uit de Zear-regio, in het noordwesten van Marokko, dat gekend is voor zijn hoge kwaliteit (vooral een korte kooktijd). Dat ras werd voorgedragen voor een geografische oorsprongsbescherming in de nationale catalogus voor Marokkaanse producten. De twee gehanteerde moleculaire technieken toonden aan dat het landras specifieke kenmerken heeft, die het idee voor een oorsprongscertificaat ondersteunen. Door de genetische met landbouwkundige informatie te combineren, kan genetische differentiatie naargelang de agro-ecologische oorsprong, de duur van de teeltcyclus en de vroege vegetatieve groei worden aangetoond. Landrassen uit de droge gebieden werden onderscheiden van die uit gebieden met

een gunstiger klimaat en ook van die uit hoger gelegen gebieden. De genetische differentiatie kan verklaard worden door aanpassingen aan de specifieke ecologische omstandigheden die deze landrassen hebben doorgemaakt.
Aanvullend werd ook de genetische diversiteit van een collectie van 70 mediterrane linzenlandrassen bestudeerd met behulp van dezelfde 19 SSRs en de 7 AFLP primer combinaties. De landrassen werden ook fenotypisch gekarakteriseerd waarbij de variatie in wortel- en stengelkenmerken in kaart werden gebracht, evenals de droogtetolerantie. Dat laatste gebeurde aan de hand van gemeten parameters zoals het relatief watergehalte (RWC), de snelheid waarmee de plant water verliest (WLR) en de verwelkingsscores (WS). De resultaten onthulden een duidelijke differentiatie tussen de linzen-genotypen uit Marokko en die uit de noordelijke mediterrane regio (Italië, Turkije en Griekenland). Analyse van de populatiestructuur van de mediterrane linzendiversiteit onthulde twee grote "*genepool*" en daarnaast mogelijks een kleinere "*genepool*" die vooral landrassen uit de droge gebieden uit Abda in West-Centraal Marokko bevatte. De twee grote "*genepools*" bevatten de landrassen uit respectievelijk de zuidelijke (Marokko) en de noordelijke mediterrane gebieden. Er werd een grote genetische variatie geobserveerd in wortel- en stengelkenmerken en in de mate van droogtetolerantie. Niettemin kon geen verband worden aangetoond tussen de droogtetolerantie bij landrassen en hun geografische oorsprong. Landrassen met een hogere droge wortel biomassa, chlorofylgehalte en wortel-stengelverhouding waren meer droogtetolerant zoals aangetoond door hun hogere RWC, lager WLR en WS. De niet-parametrische Kruskal-Wallistest werd gebruikt om SSRs en AFLPs te vinden die geassocieerd konden worden met RWC, WLR en WS. Een regressieanalyse toonde aan dat 6 SSR en AFLP allelen het hoogste percentage van de fenotypische (RWC, WLR en WS) variatie verklaarden (21 % tot 50 % voor SSRs en 14 % tot 33 % voor AFLPs). Functionele genetische diversiteitsanalyse toonde significante verbanden aan tussen droogtetolerantiekenmerken en daaraan gekoppelde SSR- en AFLP-allelen. Aan de hand van discriminantanalyse op basis van de genetische afstand tussen de landrassen en hun droogtetolerantiekenmerken konden droogtegevoelige en intermediair tolerante landrassen worden onderscheiden. De resultaten bevestigen de haalbaarheid om DNA merkers te vinden die gelinkt zijn met droogtetolerantie in een populatie landrassen van linzen.

Er is momenteel slechts beperkte informatie beschikbaar over de genetische controle van wortel- en stengelkenmerken die gelinkt zijn aan droogtolerantie bij linzen. Daarom werd een studie verricht op een "*mapping population*" van 133 $F_{6-8}$ recombinante inteeltlijnen (RIL) van de kruising tussen de genotypen ILL6002 x ILL5888. Er werd een grote variatie tussen de

genotypen gevonden, maar ook in de overerfbaarheidswaarden voor wortel- en stengelkenmerken, 38 dagen na het uitzaaien, zowel onder goed geïrrigeerde als onder droogtestress-omstandigheden, gedurende twee opeenvolgende seizoenen. Hogere overerfbaarheidswaarden werden verkregen onder de droogtestress-omstandigheden wat erop wijst dat selectie voor veredeling in droge omgevingen grotere genetische winst kan opleveren met betrekking tot droogtetolerantie. De droge omstandigheden in onze proef hadden zowel de wortel- als de stengelgroei ingeperkt in vergelijking met de goed geïrrigeerde omstandigheden. Niettemin verhoogde de droogtebehandeling de verhouding tussen de biomassa van wortel en stengel bij de bestudeerde RILs, wat aantoont dat deze verhouding een belangrijk kenmerk is bij droogtetolerantie. De kwantitatieve en continue verdeling van de variatie in wortel- en stengelkenmerken toonden aan dat ze polygeen worden gecontroleerd. Dit is belangrijk met het oog op de identificatie en het in kaart brengen van de met deze kenmerken geassocieerde "*quantitative trait loci*" (QTL). Er werden statistisch significante verbanden gevonden tussen wortel- en stengelkenmerken zoals droge stengel biomassa en chlorofylgehalte, wat de betrouwbaarheid aantoont van indirecte selectie van ondergrondse (wortel) kenmerken op basis van bovengrondse kenmerken in veredelingsprogramma's. Er werden significante correlaties en regressies aangetoond tussen de droge wortel biomassa, het aantal laterale wortels, de totale worteloppervlakte, droge stengel biomassa, wortel-stengel verhouding, chlorofylgehalte enerzijds, en droogtetolerantie (benaderd door de WS bij de droogtebehandeling) anderzijds. Dit illustreert het belang van een goed ontwikkeld wortelsysteem en snelle vegetatieve groei bij droogtetolerantie. De identificatie van en het in kaart brengen van QTLs die gelinkt zijn aan de bestudeerde kenmerken, maken een "*marker-assisted selection*" (MAS) van droogtetolerante linzen mogelijk.

De fenotypisch gekarakteriseerde RIL populatie (ILL6002 x ILL5888) werd ook genotypisch gekarakteriseerd met behulp van SNP, SSR, AFLP, SRAP en RAPD DNA merkers. Alles samen werden 252 co-dominante en dominante merkers gebruikt in de constructie van een genetische "*linkage map*". Met behulp van deze "*linkage map*" werd op basis van serre-experimenten - waarin het effect van verschillende droogtestressbehandelingen op wortel en stengelkenmerken werd gemeten - een QTL-analyse verricht. Op die manier werd een genetische kaart met 9 "*linkage groups*" (LG) geconstrueerd, die een totale genetische afstand van 2022,8 cM overbrugden. Er werden 18 QTLs die in totaal 14 wortel- en stengelkenmerken controleerden geïdentificeerd. Er werd ook een QTL-"*hotspot*" regio geïdentificeerd in het genoom in LG VII op positie 21-22 cM, die is gerelateerd aan kenmerken zoals droge wortel

biomassa, totale worteloppervlakte, aantal laterale wortels, droge stengel biomassa en de lengte van de stengel. Bovendien werd ook een QTL gelinkt aan de wortel-stengel verhouding gevonden die de hoogste fenotypische variantie kon verklaren in beide seizoenen (respectievelijk 27,6 % en 28,9 %). Deze QTL bevond zich dicht bij de co-dominante SNP TP6337 merker, op positie 2,30 cM in LG IX en werd geflankeerd door de SNPs TP518 en TP1280, die zich op respectievelijk posities 0 en 2,9 cM bevonden in LG IX. Deze merkers zijn dus bruikbaar bij MAS. Een belangrijke QTL, gerelateerd aan het aantal laterale wortels kon worden geïdentificeerd in LG III op positie 98,64 cM, dichtbij TP3371 en geflankeerd door de SNP merkers TP5093 en TP6072. Tenslotte werd een QTL, geassocieerd met de specifieke wortellengte geïdentificeerd in LG IV op positie 61.63 cM dicht bij SNP merker TP1873 en geflankeerd door SRAP merker F7XEM6b en SNP merker TP1035. Deze twee QTLs werden in beide seizoenen gedetecteerd. De resultaten van deze doctoraatstudie kunnen worden aangewend in MAS in veredelingsprogramma's voor linzen, waarin verbeterde wortel- en stengelkenmerken bijdragen aan een verhoogde droogtetolerantie. MAS vormt daarbij een efficiënt alternatief voor de relatief tragere en meer arbeidsintensieve conventionele veredelingsmethodes.

**Key words**: genetische differentiatie, agro-ecologische origine, eco-fysiologie, droogtetolerantie, veredeling, merker-kenmerk associatie, QTL, "*marker-assisted selection*"

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## List of abbreviations

**A**: Adenine

**AFLP**: Amplified Fragment Length Polymorphism

**bp**: base pair

**C**: Cytosine

**CIM**: Composite Interval Mapping

**cM**: centiMorgan

**DNA**: Deoxyribonucleic Acid

**DMN**: Direction de la Météorologie Nationale du Maroc

**DR**: Drought-stressed treatment (watering regime)

**DRW**: Dry Root Weight

**DSW**: Dry Shoot Weight

**FAO**: Food and Agriculture Organization

**G**: Guanine

**GR**: Growth rate

**$H_0$**: Observed Heterozygosity

**He**: Expected Heterozygosity

**I**: Shannon Information Index

**IBPGR**: International Board for Plant Genetic Resources

**ICARDA**: International Center for Agriculture Research in the Dry Areas

**ILVO:** Instituut voor Landbouw- en Visserijonderzoek (Institute for Agricultural and Fisheries Research)

**INRA**: Institut National de la Recherche Agronomique

**LOD**: Logarithm of odds

**LRN**: Lateral Root Number

**MGB**: Moroccan Gene Bank

**MQM**: Multiple-QTL Models mapping

**ne**: Number of expected alleles

**NJ**: neighbor-joining

**no**: Number of observed alleles

**PDO**: Protected Designation of origin quality mark

**PCA**: Principal Component Analysis

**PCO**: Principal Coordinate Analysis

**PCR**: Polymerase Chain Reaction

**PI**: Probability of Identity

**PIC**: Polymorphism Information Content

**QTL**: Quantitative Trait Loci

**RAPD**: Randomly Amplified Polymorphic DNA

**RIL:** Recombinant Inbred Line

**RSA**: Root Surface Area

**RS ratio**: Root-shoot ratio

**RWC**: Relative Water Content

**SCL**: Strongest Cross Link

**SIM**: Simple Interval Mapping

**SL12DAS**: Shoot Length at 12 Days After Sowing

**SL22DAS**: Shoot Length at 22 Days After Sowing

**SNP**: Single-Nucleotide Polymorphism

**SPAD**: Soil Plant Analysis Development

**SRAP**: Sequence Related Amplified Polymorphism

**SRL**: Specific Root Length

**SRSA**: Specific Root Surface Area

**SSR**: Simple Sequence Repeat

**SV**: Seedling Vigour (Early vegetative vigour)

**T**: Thymine

$T_a$: Annealing temperature

**TRD**: Average Taproot Diameter

**TRL**: Taproot Length

**UPGMA**: Unweighted Pair Group Method with Arithmetic Mean

**WLR**: Water Losing Rate

**WS**: Wilting Score

**WSU**: Washington State University

**WW**: Well-watered treatment (watering regime)

# Chapter 1. General introduction

# Chapter 1. General introduction

## 1.1. Justification

Worldwide population is continuously increasing especially in developing countries. As a result, the demand for food is also expected to dramatically increase during the next years. Moreover, climate change and the frequency of drought stress periods that can cause substantial yield losses for plant crops, add to the challenge of achieving food security. Food legumes including lentils, consumed as staple foods, are expected to play an important role in future food security. Thus, enhancing their productivity through innovative approaches such as modern breeding methods and better use and conservation of genetic resources would contribute to ensure food supply in the world.

The 68$^{th}$ United Nations General Assembly declared 2016 the International Year of Pulses (lentil, chickpea, bean, and other dry grain food legumes) in order to increase public awareness of the nutritional benefits of food legumes. The latter come from the rich grains they produce, whereas the plants also form part of sustainable farming and food production aiming at increased food security and nutrition (FAO 2015).

Lentil (*Lens culinaris* Medik.) is one of the most important food legumes worldwide. It contributes to reduce hunger and malnutrition especially with low-income people. Its grains are largely consumed as staple food especially in developing countries, providing an inexpensive source of proteins, vitamins and some important micronutrients like iron and zinc (Carbonaro *et al.* 2015; Grusak 2009; Grusak and Coyne 2009; Thavarajah *et al.* 2011). Lentil grains are also used to prepare various dishes including vegetarian meals and salads in many parts of the word. Furthermore, the crop provides a number of additional agronomic, environmental and economic benefits. As a leguminous crop, lentil is able to enhance soil fertility and thus contributes to farming sustainability by fixing atmospheric nitrogen thanks to the symbiotic association of its roots with *Rhizobium leguminosarum* bacterium. This offers an opportunity to significantly save on input costs due to lower needs for chemical nitrogen fertilizer applications. The productivity of cereal-based cropping systems, in which lentil is often included as a rotational crop, can thus be improved with less nitrogen fertilizers (McNeil and Materne 2007). The latter considerations and the fact that lentil is a low-input crop, highlights the environmental importance of lentil as a component in sustainable farming systems. Of late, lentil growers incomes have been significantly increasing with rising worldwide demand (case of exporting countries like Canada), whereas similar trends can be seen at local demand level (case of Morocco, India,…). The crop is widely cultivated in the Middle East, North Africa,

Ethiopia, the Indian subcontinent, North America and Australia for its nutritionally rich seeds and also for its straw which is valued as animal feed (Bhatty 1988; Erskine *et al.* 1990; Muehlbauer and Tullu 1997; Ferguson and Erskine 2001; Sarker *et al.* 2002, Yadav *et al.* 2007; Coyne and McGee 2013).

Evaluation and knowledge of diversity of genetic resources such as landraces are important for defining appropriate strategies for their management and use helping a better valorization and protection of the benefit of local farmers, limiting the risk of their permanent loss and helping an efficient utilization in breeding programs. Introgression of useful alleles from landraces and related wild species will greatly determine future genetic gains in specific target crops (Blum 2011; Coyne and McGee 2013). Landraces locally selected over centuries by farmers for specific adaptations to different types of stress offer a valuable genetic resource for developing genotypes adapted to different abiotic stresses, especially drought. Selection pressure of their respective agro-environments over time is a major factor for their genetic differentiation and the accumulation of favourable alleles in their germplasm. In fact, one of the farmers' strategies is to select seeds for the next sowing season from plants performing better under abiotic and biotic stress that frequently occurs in their specific regions. Natural selection and evolution of genetic resources over time under specific conditions, such as drought stress, may result in adapted genotypes that could be important for plant improvement.

The Mediterranean basin is known for its species richness with "diversity hot spots" for various food legumes diversity (Davis *et al.* 1994; Akeroyd 1999; Maxted and Bennett 2001). It is the center of diversity of important crop species such as cereals, legumes and olives among others, it has one of the richest floras of the world containing some 25 000 plant species (Maxted and Bennett 2001). The Mediterranean region has a rich history of domestication and cultivation of lentil where local farmers have repeatedly selected landraces and local cultivars for adaptation to biotic and abiotic stress conditions over a long period of time. In this region, biotic stress and abiotic stress such as intermittent drought during vegetative growth and end-cycle drought associated with increasing temperatures during lentil flowering and maturity stages frequently occur (Silim *et al.* 1993; Materne and Siddique 2009). Also, a wide diversity of agro-environments (highlands, drylands, more favourable areas,…) is known to occur thanks to the diversity of climatic and edaphic conditions. Lentil collected from these different regions most probably have high molecular diversity and different adaptations to abiotic and biotic stress as a result of reproductive isolation and evolutionary difference of populations (Heywood 1995; Akeroyd 1999). We therefore focused to Mediterranean lentil landraces in our study.

In addition, we studied a lentil recombinant inbred lines (RIL) population to investigate genetic variability, genetic control and molecular markers linked to genes controlling root and shoot traits and their association with drought tolerance. RIL populations are $F_2$-derived lines by single seed descent and self-pollinating for several generations resulting in useful genetic variations and associations for molecular mapping studies. They provide valuable genetic material for identification and mapping of DNA markers associated with phenotypic traits and related quantitative trait loci (QTL). The numerous meiosis recombination events that occurred during the development of the RIL population allow such associations and their detection (Broman 2005).

On a global scale, drought is a major constraint for crop production, especially in arid and semi-arid areas. As lentil is often cultivated in rainfed regions, its productivity is frequently limited by irregular rainfall and thus drought (Shrestha *et al.* 2009). Moreover, with global warming in the context of climate change becoming more and more important, drought episodes are expected to worsen and become more frequent. As a result, improving crop tolerance and adaptation to this challenging abiotic stress is a strategic research focus. Lentil has been reported to be less affected by drought than other food legumes experiencing lower yield reduction under drought conditions (Daryanto *et al.* 2015). Thus, lentil would offer an adequate alternative for cropping systems in areas suffering from water deficiency.

Enhancing productivity under drought-stressed environments using improvement of agro-morphological, phenological and physiological characteristics of plant shoots has known little success so far. Thus, exploring the hidden plant parts (root systems) directly involved in water uptake is of potential interest to identify relevant drought-tolerance related traits. These traits can be used in developing drought-tolerant cultivars. Furthermore, change in root systems is an efficient drought avoidance mechanism that does not negatively impact plant functions under drought stress, thus allowing higher production compared to other mechanisms such as stomatal closure (Verslues *et al.* 2006).

Having a well-developed and prolific root system combined with shoot characteristics such as early vegetative vigour are of major importance for conveying drought tolerance to crops (Verslues *et al.* 2006; Blum 2011; Comas *et al.* 2013). In low-moisture soils, water and nutrient uptake is increased by deeper and well-developed roots as they allow plants to explore higher volumes and deeper layers of soils, thus enhancing plant survival and yield under limited water conditions. Breeding for superior root traits is consequently important for the development of drought-tolerant cultivars. Compared with aboveground plant characteristics, studies dealing with root systems including genetic aspects are limited in numbers. However, recently research

and studies focusing on root characteristics gained more interest (Aswaf and Blair 2012; Comas *et al.* 2013; Varshney *et al.* 2014; Manavalan *et al.* 2015).

Exploring the genetic variation in root and shoot traits and their association with drought tolerance as well as understanding the genetic control behind them are important elements in designing better-targeted breeding strategies focusing on these important traits.

Plant tolerance to drought and related root and shoot characteristics are complex traits governed by different mechanisms involving genetic, physiological, and environmental factors (Verslues *et al.* 2006; Blum 2011; Comas *et al.* 2013). The latter authors reported that these traits are under quantitative and polygenic control where several quantitative trait loci (QTL) contribute to the average genetic effect. QTL are genomic regions that contain genes associated with a specific continuous variable that affect with additive values when present in an individual plant (Collar *et al*. 2005). Thus, QTL analysis aiming to statistically determine the relationship between phenotypic (from trait measurement) and genotypic (from molecular markers) variations is important for better understanding the genetic basis of variation of these features (Falconer and Mackay 1996). Hence, studying DNA marker/trait association and QTL related to root and shoot traits as well as to drought tolerance will allow the identification of DNA markers linked to genes controlling these traits. This is important for developing marker-assisted selection in breeding programs. Also, indirect selection for prolific root systems and drought tolerance based on correlated shoot traits, which are more easy to measure, has been shown to be a time- and resource-effective breeding strategy (Sarker *et al.* 2005; Kumar *et al.* 2012). Ultimately, these are alternative strategies for programs targeting root traits as phenotypic selection is a slow and labour-intensive work.

Overall, the biological hypothesis behind this thesis is that understanding genetic variation related to root and shoot traits conferring drought tolerance and adaptation of genetic resources to specific environments, especially drought stressed ones, is a key step in defining strategies for improving lentil productivity under water-limited availability. Hence, studying related molecular and physiological characteristics is critical for developing new breeding technologies and for identification, valorization and conservation of valuable genetic resources. Therefore, this would contribute to efficiently enhance knowledge concerning plant improvement and biodiversity conservation and use.

## 1.2. Objectives

The main objective of this doctoral research project is to investigate by molecular analysis the functional diversity in relation with agro-environmental origin, drought tolerance and associated root and shoot traits, as well as to identify molecular markers linked to genes controlling these traits in lentil. A set of landraces from the Mediterranean region (Morocco, Italy, Greece and Turkey) as well as a mapping population composed of recombinant inbred lines (RIL) were used in this study. This $F_{6-8}$ RIL mapping population was developed by Prof. Fred J. Muehlbauer (Washington State University, United State of America) from the cross of two lentil parents contrasting for targeted traits (root and shoot characteristics and drought tolerance), i.e. ILL6002 and ILL5888.

The specific objectives of our study are:

1. analysis of the extent of genetic diversity and population structure of Mediterranean lentil landraces using Simple Sequence Repeat (SSR) and Amplified Fragment Length Polymorphism (AFLP) DNA markers using non-structured statistical analysis techniques like UPGMA clustering and bootstrapping;
2. investigation of genetic differentiation of landraces according to agro-environmental origins, some adaptive traits and drought tolerance as a first and preliminary step in association mapping studies using a structured statistical analysis technique like discriminant analysis;
3. characterization of root and shoot traits' genetic variability and evaluation of their association with drought tolerance using a number of physiological parameters in order to understand the genetic control behind these traits;
4. identification of DNA markers linked to drought tolerance and QTLs associated with root and shoot characteristics in the perspective of developing a marker-assisted breeding protocol for these traits.

Previous studies that used molecular markers for studying lentil diversity were limited to the description of genetic diversity and classification. We aimed to improve knowledge about the relationship between genetic diversity and phenotypic traits. Therefore, we analysed molecular variation in association with some functional traits, especially those linked to drought tolerance, allowing specific adaptation among landraces. Furthermore, in the absence of previous reports on genetic control of drought tolerance-related root and shoot traits in lentil, we analysed genetic variability aiming to identify related QTLs using a recombinant inbred line population (RIL).

## 1.3. Organization of the thesis

As a first part of this thesis, the general introduction in chapter 1 presents the research problem and justification of the topic of the research project, as well as the objectives that will be considered.

The literature review is presented in chapter 2 to introduce the axes and concepts studied and to discuss the state-of-the-art of the research questions addressed in this doctoral research.

Four experimental chapters present our main results. For each chapter, an introduction about the specific topic, followed by materials and methods used, results and discussion as well as conclusions are included.
Chapters 3 and 4 are about the molecular analysis of functional diversity of landraces, but in chapter 3 the focus is on differentiation of lentil landraces according to their agro-environmental origins and some adaptive traits related to drought tolerance. While in chapter 4 we focus more on population structure, identification of DNA markers linked to drought tolerance and differentiation of lentil landraces according to their drought responses.

In chapter 5, genetic variability of root and shoot characteristics and their association with drought tolerance is presented. The extent of genetic variability, genetic control and heritability of these traits and practical considerations for use in breeding programs are presented and discussed.

Chapter 6 presents QTL analysis of root and shoot traits and identification of the linked DNA markers in the RIL population (ILL6002 x ILL5888).

Each experimental chapter has been adapted from articles published in international pre-reviewed journals and from other documents highlighting our participation in international research conferences.

The thesis concludes by chapter 7 that presents the general conclusions from the results from the four experimental chapters. We also present perspectives and suggestions for future research in this chapter.

# Chapter 2. Literature review

# Chapter 2. Literature review

## 2.1. Origin, distribution and nutritional benefits of lentil

Lentil (*Lens culinaris* Medikus subsp. *culinaris*) is an annual food legume from the Fabaceae family, subtribe of Papilionaceae and tribe of Vicieae. The plant is generally 15 to 75 cm high (Duke 1981; Muehlbauer *et al.* 1985) with many ramifications carrying two to four flowers in the axils of the leaves producing pods with two seeds each (Sandhu and Sarvjeet Singh 2007) (Figure 2.1). It is a herbaceous plant with indeterminate growth exhibiting high variation in growth habits as it displays single-stem, erect, semi-erect, compact growth or much-branched low bushy forms. The species root system is characterized by a slender taproot with a mass of fibrous lateral roots that may be shallow, intermediate or deep (Saxena 2009).

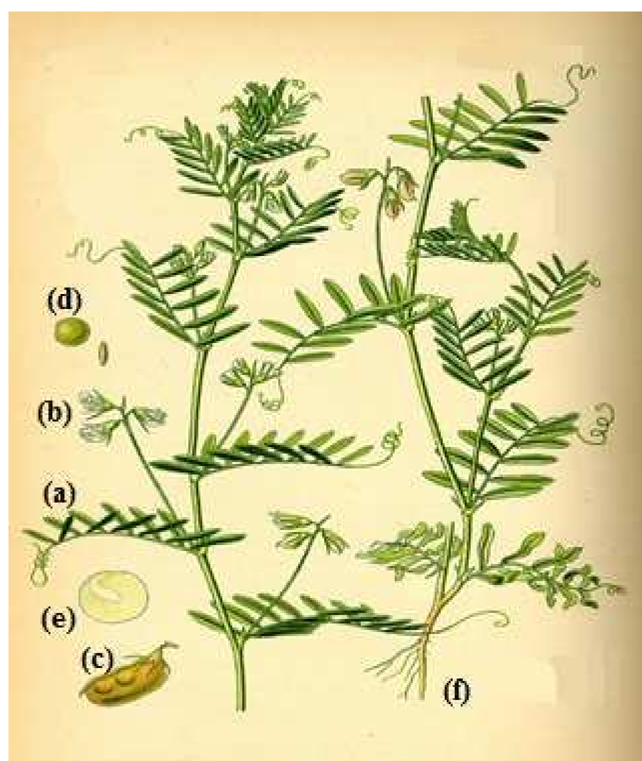


**Figure 2.1.** Lentil plant: branch with leaves carrying several leaflets ending by a tendril (a); flowers (b); 2-seeded pod (c); mature seeds (d); germinating seed (e); and part of superficial roots (f) (adapted from Thomé 1885).

Lentil is one of the oldest crops that man has domesticated. It has been used since the beginning of the first agricultural activities in the "the cradle of agriculture" (Harlan 1992; Sandhu and Sarvjeet Singh 2007; Sonnante *et al.* 2009) close to the area where other important crops like wheat were domesticated in the Fertile Crescent (Lev-Yadun *et al.* 2000). The Near East in the foothills of the mountains between southern Turkey and northern Syria is thought to be the center of origin and domestication of lentil (Ladizinsky 1979; Sandhu and Singh 2007; Cubero *et al.* 2009; Faratini *et al.* 2011). It was domesticated from its wild progenitor *Lens orientalis*

(Boiss.) Hand.-Mazz. [synonym of *L. culinaris* Medik. subsp. *orientalis* (Boiss.) Ponert] (Ladizinsky 1979). The oldest archaeological remains of lentil seeds start from the Neolithic (Sonnante *et al.* 2009) back to 13000 years BC (Sandhu and Singh 2007). Ladizinsky (1987) in his well-known work on pulse domestication suggested that lentil cultivation might have started before completed domestication. Seed dormancy and pod dehiscence were the main traits targeted during the domestication process by selecting seeds with higher germination rate and plants with pods retaining seeds at maturity (Ladizinsky 1987; Sonnante *et al.* 2009).

Lentil was diffused from the Near East together with wheat, barley, pea, faba bean and chickpea to Europe, North Africa, Central Asia and India later. Lentil first diffused to Cyprus then through the Danube river to south-eastern Europe and Central Europe. Lentil further reached Ethiopia either via the Nile river or from the Arabian coast (Cubero *et al.* 2009; Sonnante *et al.* 2009). Lentil spread later to Georgia and reached Russia likely from the west coast of the Black Sea or from the Danube valley. The crop later reached Pakistan and India (Sonnante *et al.* 2009). Lentil reached Morocco, Spain and the Italian islands of Sardinia and Sicily likely from either Central Europe or the route of isles. Lentil was introduced into North and South America, and Australia more recently (Ferguson and Erskine 2001).

Annual average global production of lentil is 4.55 million tons (t) harvested from 4.2 million hectares (ha) (FAOSTAT 2013). It is the world's fifth most important pulse crop produced in over 70 countries. Canada, India, Turkey, Australia and USA are the major producers of lentil worldwide (Table 2.1). They are also the five top-exporting countries. Syria, Nepal and China export significant quantities as well (Erskine 2009). In Africa, Ethiopia and Morocco are the two major producers.

**Table 2.1.** Major producers of lentil (mean of the period 2009-2013) (FAOSTAT, 2013)

| Region | Country | Harvested area (1000 ha) | Annual production (1000 t) | Average yield (kg/ha) |
|---|---|---|---|---|
| Africa | Ethiopia | 104.90 | 122.81 | 1173.2 |
| | Morocco | 50.91 | 32.41 | 681.4 |
| Asia | Bangladesh | 81.45 | 77.04 | 941 |
| | China | 63.70 | 138 | 2164.4 |
| | India | 1621.54 | 1024.28 | 633.8 |
| | Iran | 144.76 | 82.8 | 508.2 |
| | Nepal | 198.60 | 188.3 | 941.2 |
| | Pakistan | 24.57 | 11.87 | 485 |
| | Syria | 124.41 | 110.37 | 881.6 |
| | Turkey | 235.63 | 402.10 | 1712.2 |
| Europe | Spain | 33.32 | 25.20 | 754.2 |
| North America | Canada | 1051.04 | 1681.52 | 1637.4 |
| | USA | 181.94 | 268.11 | 1474.8 |
| Australia | Australia | 163.33 | 289.95 | 1713.4 |

The largest demand and consumption of lentil seeds are situated in the developing world, mainly in Asia and Africa. Some lentil producing countries where it is historically grown in larger areas are also the largest consumers. Some of those countries are not self-sufficient in lentil production and import lentils to meet the local demand or to complete trading contracts. Some European countries import lentil to meet the rising demand for vegetarian food (Erskine 2009).

Lentil seeds have many nutritional and health benefits as they are an important source of proteins, energy, essential minerals and vitamins (Table 2.2). Lentil seeds contain several essential amino acids present generally in proportions recommended for human consumption by the World Health Organization. Proteins of lentil seeds have high lysine and tryptophan content but they lack the sulfur containing amino acids (methionine and cysteine). When combined with wheat or rice, lentil provides a balanced diet for essential amino acids for human nutrition (Shewry and Halford 2002; Grusak 2009).

**Table 2.2.** Nutritional composition of lentil seeds (adapted from Grusak *et al.* 2009 and references within)

| **Components** (per 100 g dry matter) | **Range of published values** |
|---|---|
| Protein (g) | 15.9-31.4 |
| Energy (kJ) | 1418-2010 |
| Carbohydrates (g) | 43.4-74.9 |
| Fat (g) | 0.3-3.5 |
| Total fibre (g) | 5.1-26.6 |
| Calcium (Ca) (mg) | 42-165 |
| Magnesium (Mg) (mg) | 13-167 |
| Phosphorus (P) (mg) | 240-1287 |
| Potassium (K) (mg) | 38-1360 |
| Iron (Fe) (mg) | 3.1-13.3 |
| Zinc (Zn) (mg) | 2.3-10.2 |
| Manganese (Mn) (mg) | 0.6-1.0 |
| Copper (Cu) (mg) | 0.4-9.9 |
| Sodium (Na) (mg) | 0.4-79 |
| Chromium (Cr) (mg) | 0.03 |
| Selenium (Se) (mg) | 0.009-0.012 |
| Molybdenum (Mo) (mg) | 0.08-0.22 |

## 2.2. Genetic resources, diversity and adaptation

The taxonomy of the genus *Lens* has been discussed by several authors using morphological, molecular markers and hybridization studies (Cubero 1981; Ladizinsky 1979, 1986, 1997; Ladizinsky *et al.* 1984; Van Oss *et al.* 1997; Ferguson *et al.* 1999; Singh *et al.* 2014; Wong *et al.* 2015). Seven species were described: *L. ervoides*, *L. nigricans*, *L. lamottei*, *L. odemensis*, *L. tomentosus*, *L. orientalis* and *L. culinaris*. *L. orientalis* is commonly reported as the progenitor of the cultivated species *L. culinaris*, while *L. nigricans* is considered as the most distant wild relative. Recently Wong *et al.* (2015) reported four gene pools, based on phylogenetic tree and genetic structure analysis using Single Nucleotide Polymorphism (SNPs) DNA markers, corresponding respectively to primary: *L. culinaris*/*L. orientalis*/*L. tomentosus*, secondary: *L. lamottei*/*L. odemensis*, tertiary: *L. ervoides*, and quaternary: *L. nigricans*.

Lentil genetic resources including landraces, local cultivars, breeding lines and wild accessions are conserved in several collections worldwide. The largest world collection of *Lens* (10800) accessions is conserved *ex-situ* as seeds by the International Center of Agricultural Research in the Dry Areas (ICARDA) which includes 8860 cultivated *L. culinaris* accessions originating from more than 70 countries, 583 accessions of the six wild species from 24 country and 1373 breeding lines. This collection was recently secured in the Svalbard Global Seed Vault, Norway. Some 5250 accessions of *Lens* are conserved in the Australian Temperate Field Crops Collection, hosted by the Department of Primary Industries, Victoria, Australia. United States Department of Agriculture, Agricultural Research Service, maintains 2797 accessions of lentils, whereas the N.I. Vavilov All-Russian Research Institute of Plant Industry preserves 2396 accessions of *Lens*. The National Bureau of Plant Genetic Resources, India, maintains 2212 accessions (Furman *et al*. 2009 and references within). A number of other countries conserve a number of local accessions and landraces in their national gene banks (Coyne and McGee 2013). Some 293 lentil landraces are maintained in the Moroccan Gene bank (MGB) at the National Institute of Agricultural Research (INRA), Settat (Ouabbou and Quariouh 2015, personal communication).

Genetic variability and its magnitude are key points for plant breeding in order to achieve significant genetic gains.

Although lentil has experienced a number of bottleneck events during its domestication process reducing its genetic base (Erskine *et al.* 1998), high genetic diversity was reported in several studies for different traits in cultivated lentil: morphological, phenological, nutritive, and biotic and abiotic stresses resistance. Also, a wide molecular genetic diversity was reported using different kinds of DNA markers including RAPD, AFLP, SSR, ISSR and SNP (Abo-Elwafa *et al.* 1995; Ferguson *et al.* 1998; Sonnante and Pignone 2001; Sonnante *et al.* 2003; Duran and Perez de la Vega 2004; Hamwieh *et al.* 2005; Sultana and Ghafoor 2008; Liu *et al.* 2008; Babayeva *et al.* 2009; Toklu *et al.* 2009; Bacchi *et al.* 2010; Reddy *et al.* 2010; Datta *et al.* 2011; Alo *et al.* 2011; Zaccardelli *et al.* 2011; Lombardi *et al.* 2014; Singh *et al.* 2014).

High variation for plant height was reported with cultivars as short as 15 cm and as tall as 75 cm (Duke 1981; Muehlbauer *et al.* 1985). High variation for growth habit and ramification was observed from single stem and erect to plants with many ramifications and prostrate (Figure 2.2) (Saxena and Hawtin 1981). Leaflet size was reported to range from small (8–15 × 2–5 mm) to large (15–27 × 4–10 mm) (IBPGR and ICARDA 1985; Cubero *et al.* 2009). Variation for early vegetative vigour and root systems such as evidenced by lateral root numbers and root length was also reported (Sarker *et al.* 2005). Also, variation for seed type and diameter exists

with two major types: macrosperma (large seeds, seed diameter: 6–9 mm) and microsperma (small seeds, seed diameter: 2–6 mm). Seed colour ranges from black, gray, brown, pink to green (Erskine and Witcombe 1984). Seed testa pattern was reported to be absent, dotted, spotted, marbled or complex (IBPGR and ICARDA 1985).

Lentil also exhibits a wide level of diversity regarding its phenology such as time to flowering and maturity, although the concrete environment also affects these characters. Periods to reach maturity of 75–100, 120–160 and over 180 days after sowing were reported (Saxena and Hawtin 1981; Saxena 2009).

Genetic diversity for nutritive composition of seeds was reported in several studies. High variation for seed iron, zinc and selenium concentrations as well as in protein content was observed (Grusak 2009; Thavarajah *et al.* 2009; Thavarajah *et al.* 2011; Kumar *et al.* 2015). Lentil harbours a wide variability in resistance against rust, *stemphylium blight*, *fusarium wilt* and *ascochyta* diseases. Genes responsible for conferring resistance for these diseases were studied by Bayaa *et al.* (1994), Saha *et al.* (2010) and Sari (2014) among others. For abiotic stresses, a wide range in variation in responses for drought, heat and cold stresses was reported (Kahraman *et al.* 2004; Sarker *et al.* 2005; Kumar *et al.* 2013).

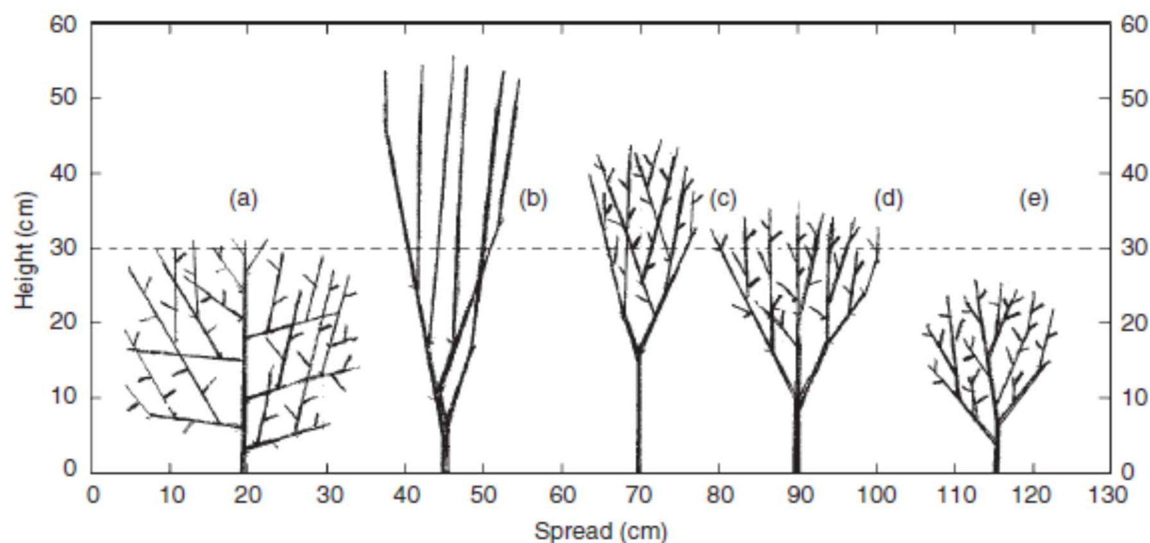


**Figure 2.2.** Schematic representation of lentil plant habit. (a) highly branched, bushy; (b) sparsely branched, tall erect; (c) moderately branched, semi-tall erect; (d) moderate to highly branched, semi-tall, subcompact; (e) moderate to highly branched, short, subcompact (Saxena 2009).

Lentil has been grown in three major different climatic regions in the world (Materne and Siddique 2009):

**-** north Africa, West Asia and Australia: as a winter crop receiving annual rainfall <450 mm, with vegetative growth being low during winter and rapid during spring. Maturity occurs under low rainfall and high temperatures. Longer period for vegetative and reproductive growth, rapid canopy development and greater absorption of photosynthetically active radiation of early sown cultivars result in more water use, greater dry matter production, seed yield and water use efficiency in Mediterranean-type environments (Siddique *et al*. 1998),

**-** subtropical regions (India, Nepal, Bangladesh and Pakistan): as a winter crop grown on residual soil moisture and in higher temperature, and

- high altitude and/or latitude (Turkey, Europe, USA and Canada): as a spring crop grown under stored soil moisture and rainfall during spring and warm long days during summer.

Lentil adaptation to specific environments depend mainly on temperature, and rainfall distribution and quantity affecting the selection pressure imposed by biotic and abiotic constraints (Materne and Siddique 2009). Morphological traits such as plant habit, rate of early vegetative growth, ramifications and plant height as well as phenological traits such as flowering and maturity time are important adaptive traits for specific agro-environments (Silim *et al.* 1993; Materne and Siddique 2009). Earliness with short vegetative and reproductive periods, early vegetative vigour and faster seed growth are traits well-adapted to dry areas where water availability decreases during the end of the crop's development stage (Silim *et al.* 1993; Sarker *et al.* 2005; Materne and Siddique 2009). This is typically the case in many Mediterranean regions, while late flowering and maturing lentils with less additional vegetative growth during the reproductive period are well-adapted to high-altitude areas and areas where water is more available. Macrosperma types were found to be more readily adapted to cooler seasons (Erskine 1996).

## 2.3. Lentil genetics and genomics

### 2.3.1. Molecular markers for genetic diversity and mapping analyses

DNA markers can be simply defined as fragments or sequences of DNA corresponding to variations that could be used to detect polymorphism between genotypes of a population. They are widely used for germplasm characterization, determining seed purity, systematic sampling of germplasm, phylogenetic and population structure analysis and mapping traits of interest in breeding and genetic resources conservation programs (Varshney *et al*. 2009). The development of molecular marker and genome sequencing techniques resulted in a clear understanding of

the polymorphism at DNA level for many plant species. Examples of these molecular markers are:

- RFLP (*Restriction Fragment Length Polymorphism*; Feinberg and Vogelstein 1983; Saiki *et al.* 1985);
- RAPD (*Random Amplification of Polymorphic DNA*; Williams *et al.* 1990; Karp *et al.* 1997);
- AFLP (*Amplified Fragment Length Polymorphism*; Vos *et al.* 1995);
- SRAP (*Sequence-Related Amplified Polymorphism*; Li and Quiros 2001);
- ISSR (*Inter-Simple Sequence Repeat*; Reddy *et al.* 2002);
- SSR (*Simple Sequence Repeat*; Tautz and Rentz 1984; Powell *et al.* 1996);
- CAPS (*Cleaved Amplified Polymorphic Sequence*; Glazebrook *et al.* 1998);
- SCAR (*Sequence-Characterized Amplified Region*; Joshi and Chavan 2012); and
- SNP (*Single Nucleotide Polymorphism*; Schafer and Hawkins 1998; Rafalski 2002, Sharpe *et al.* 2013).

These molecular markers could be divided into three classes according to the detection method: hybridization-based, polymerase chain reaction- (PCR-) based, and DNA sequence-based (Collard *et al.* 2005). Nowadays, hybridization-based markers such as RFLPs are outdated; PCR-based markers such as AFLPs are still valuable but they are being replaced by sequence-based markers, especially SNPs. Although RFLPs are reproducible and codominant, their detection is expensive, labor- and time-consuming process, making these markers eventually obsolete. With the invention of PCR technology and its application for the rapid detection of polymorphisms, a new generation of PCR-based markers emerged: RAPD, AFLP, and SSR markers. The anonymous character and very low reproducibility of RAPDs limited their success. Although AFLPs are anonymous too, the level of their reproducibility and sensitivity is high. Thus, AFLP markers are still popular in molecular diversity research in crops lacking genome sequence information. However, application in molecular breeding of AFLP markers is limited. AFLPs are among PCR-based dominant markers used when other more efficient marker types such as SNPs and SSRs are lacking (Kumar *et al.* 2015; Yu *et al.* 2015). AFLP technique has the advantage over RAPDs and RFLPs to generate higher number of polymorphic bands distributed throughout the genome with higher reproducibility, thus they are considered as more efficient for genetic diversity analysis (Mba and Tohme 2005; Koopman *et al.* 2008). Likewise, Powell *et al.* (1996) reported congruence between SSRs, AFLPs and RFLPs as evidenced by high correlations between their respective genetic similarity matrices. While they found lower correlations with RAPDs. Also, Sharma *et al.* (1996) compared RAPD and AFLP markers in a study of diversity and phylogeny of *Lens* and concluded that AFLPs detected

higher level of polymorphism. As AFLPs, SRAP markers targeting the open reading frames of the genome are of potential interest when SNP and SSR markers are not available in sufficient number. They are highly variable and are less technically demanding compared to AFLPs (Robarts and Wolfe 2014). After the discovery of SSR markers able to overcome above-mentioned limits of previous technologies, they were declared as "markers of choice" (Powell *et al.* 1996). But, with the discovery of SNPs known to be the most abundant forms of genetic variation among individuals of the same species (Schafer and Hawkins 1998), the latter surpassed SSRs.

SSR and SNP markers have several advantages over other markers especially for gene mapping and subsequent practical use in marker-assisted selection thanks to their high genomic abundance and coverage, co-dominance, high reproducibility and suitability for automation. SSRs have some limitations such as PCR and electrophoresis artefacts that may cause errors in allele sizing, unequal allele amplification due to PCR competition and possibility of null alleles caused by mutations in primer region flanking the marker (Jones *et al.* 2007). Also, the development of related primers requires substantial resources and time. Thus SNPs are considered as efficient alternatives.

SNP markers are expected to be used more largely in the future because their costs keep on decreasing and because of their ability for multiplexing for high-throughput genotyping and automated detection. Also, SNPs from transcribed regions of the genome offer the possibility of establishment of a direct association between polymorphism and functional variations (Andersen and Lübberstedt 2003). Furthermore, reference genome sequences are more and more available for several plant species (Jiang 2013). However, their application in some laboratories can still be rather expensive.

Overall, ideal markers should have the following characteristics: high level of polymorphism, even distribution across the whole genome, co-dominant inheritance, clear distinct allelic features, single copy and no pleiotropic effect, cost-efficiency, easy assay/detection and automation, high reproducibility, high availability and suitability for duplication/multiplexing. However, appropriate choice of markers depends on the objectives, marker availability and allocated resources. Different kinds of markers have been used in studying genetic diversity, linkage map development and QTL mapping (Jiang 2013). However, codominant markers are generally more informative than the dominant markers.SNPs are the most appropriate markers for studying diversity, for QTL mapping and genome-wide association study approach in plant species. They have many advantages over other markers especially for QTL/gene discovery

and practical use in plant breeding (Mammadov *et al.* 2012). In situations when SSR primers were developed and SNPs are not available, SSRs could be used for genetic diversity analysis and mapping.

Important advances in Next Generation Sequencing technologies have resulted in the development of a powerful and promising technique: Genotyping-By-Sequencing (GBS). GBS is a sequencing and genotyping technique aiming to reduce the complexity of the genome by sequencing the ends of restriction fragments obtained from digested DNA by restriction enzymes (Elshire *et al.* 2011; Poland *et al.* 2012; Appendices A.1, A.2). This technique allows simultaneous sequencing and marker discovery resulting in the detection of large numbers of both SNP and SSR markers (Elshire *et al.* 2011).

### 2.3.2. Lentil genetics and genomic resources

Lentil is a self-pollinating, diploid crop with $2n=2X=14$ chromosomes (Sharma *et al.* 1996) and a relatively large nuclear genome of 4 Gbp (Arumuganathan and Earle 1991). Recently, significant advances in genetic marker development and their use for lentil genetics and breeding have been achieved, although slow compared to other crops like cereals and soybean. RAPD, RFLP, ISSR and AFLP markers have been used for the assessment of genetic diversity as well as for construction of first linkage maps in lentil (Havey and Muehlbauer 1989; Eujayl *et al.* 1998; Ferguson *et al.* 1998; Sonnante and Pignone 2001; Rubeena *et al.* 2003).

The development of microsatellite or SSR markers in lentil (Hamwieh *et al.* 2005, 2009; Saha *et al.* 2010) resulted in an enhanced understanding of inter- and intra-specific genetic relationships as well as improved gene mapping. The 670 SSRs developed by Hamwieh *et al.* (2005, 2009) and Saha *et al.* (2010) were used for several genetic diversity analysis and QTL mapping studies. Another set of 122 functional SSRs was developed and tested for exploring genetic variability within lentil and across related legumes by Verma *et al.* (2014). However, lentil SSR markers are still not sufficient for a genome-wide coverage (Kumar *et al.* 2015).

Efficient use of markers for marker-assisted selection (MAS) in lentil improvement programs is limited by the large size of the genome, narrow genetic base, lack of candidate genes and the difficulty in identifying beneficial alleles (Kumar *et al.* 2015). However, the development of SNP markers and their use for mapping important traits offer an opportunity for breeding programs targeting genes linked to these markers and for better understanding of genetic diversity. GBS was used recently to identify SNPs in lentil to classify and characterize different species within the genus *lens* to resolve phylogenetic relationship and genetic diversity (Wong *et al.* 2015). The latter authors developed an automated GBS pipeline. Efforts are being deployed to use GBS for mapping QTLs associated with economically important traits in lentil

mainly in Saskatchewan University (Canada) and Washington State University (USA). Furthermore, an ambitious and promising project is under way for lentil genome sequencing: *LenGen* (http://knowpulse2.usask.ca/portal/project/Lentil-genome-sequencing-%28LenGen%29%3A-establishing-a-comprehensive-platform-for-molecular-breeding). A first version of genome v1.0 has been made publicly available.

In addition to the GBS, other techniques of Next Generation Sequencing technology such as Illumina Golden Gate and competitive allele-specific PCR (KASPar) markers were used for identifying SNPs in lentil (Sharpe *et al.* 2013; Kumar *et al.* 2015; Temel *et al.* 2014; Lombardi *et al.* 2014). These techniques have resulted in a large number of SNPs in lentil covering over half the genome (2.7 Gb) that have been used by the latter authors for linkage map construction and diversity analysis.

Functional genomic and transcriptomic approaches lead to the development of Expressed Sequence Tag (ESTs) markers. ESTs correspond to short DNA sequences (150-400 bp) from a complementary DNA (cDNA) of a particular Messenger RNA (mRNA). Vijayan *et al.* (2009) published the first ESTs library for lentil. Kaur *et al.* (2011) developed $1.38 \times 10^6$ lentil ESTs. Another set of 10163 lentil ESTs was recently published by Saskatchewan University (Kumar *et al.* 2015).

After the first genetic linkage maps based on morphological and isozyme markers had been developed (Zamir and Ladizinsky 1984), Hevey and Muehlbauer (1989) were the first to construct a lentil genetic map of 333 cM based on DNA markers using 20 RFLPs in addition to 8 isozyme and 6 morphological markers. Later, Eujayl *et al.* (1998) developed a map of 1073 cM with more DNA markers i.e.: 177 RAPD, AFLP, RFLP and morphological markers. Both maps were interspecific between *L. culinaris* and *L. orientalis*. The first intraspecific lentil map of 784.1 cM was published by Rubeena *et al.* (2003) with 114 RAPD, inter-simple sequence repeat (ISSR) and resistance gene analog (RGA) markers. SSR markers were first included in maps developed by Duran *et al.* (2004) and Hamwieh *et al.* (2005). Other authors reported several maps using different kinds of markers mainly PCR-based ones, thus improving genome coverage and marker density. Gupta *et al.* (2012) constructed a map of 3847 cM with 199 markers: 28 SSRs, 9 ISSRs and 162 RAPDs. More recently, Sharpe *et al.* (2013) developed a map of 834.7 cM comprising seven linkage groups likely representing the seven chromosomes using six SSRs and 537 SNPs. Kaur *et al.* (2014) constructed a genetic map with 10 linkage groups having 57 SSRs and 267 SNPs markers (Table 2.3).

Although SNP markers are now widely available, their extended use is still limited for genetic diversity and mapping studies in lentil because related genotyping methods require expensive

and sophisticated platforms (Kumar *et al.* 2015). Thus, integration of other markers such as SSRs and AFLPs for studying genetic diversity and in genetic map development would result in a better genome coverage and enhance marker density as well as filling gaps in linkage groups. AFLPs and SSRs are sometimes combined with SNPs for gene map construction and gene mapping in lentil and several other species such as *Populus nigra* L. and *Cichorium intybus* L. (Gaudet *et al.* 2008; Muys *et al.* 2014; Ting *et al.* 2014; Sharpe *et al.* 2013; Kaur *et al.* 2014).

**Table 2.3.** Examples of lentil genetic maps developed

| **Populations used for map construction** | **Number of loci** | **Type of markers** | **Genetic map length (cM)** | **Number of linkage groups** | **References** |
|---|---|---|---|---|---|
| $F_2$ (*Lens culinaris* ssp. *culinaris* X *L.* c. ssp. *orientalis*) | 34 | 20 RFLPs, 8 Isozymes, 6 Morphological | 333 | 9 | Havey and Muehlbauer (1989) |
| RIL (ILL5588 X L692-16-1) | 177 | RAPD, AFLP, RFLP | 1073 | 7 | Eujayl *et al.* (1998) |
| $F_2$ (ILL5588 X ILL7537) | 114 | RAPD, ISSR | 784 | 9 | Rubeena *et al.* (2003) |
| *Lens culinaris* ssp. *culinaris* X L. c. ssp. *orientalis* | 161 | 71 RAPD, 39 ISSR, 83 AFLP, 2 SSR | 2172 | 10 | Duran *et al.* (2004) |
| RIL (ILL5588 X L692-16-1) | 283 | 41 SSR, 45 AFLP | 751 | 14 | Hamwieh *et al.* (2005) |
| RIL (ILL6002 X ILL5888) | 139 | 21 SSR, 27 RAPD, 89 SRAP, 2 Morphological | 1565.2 | 14 | Saha *et al.* (2010) |
| $F_2$ (L830 X ILWL77) | 199 | 28 SSRs, 9 ISSRs and 162 RAPD | 3843.4 | 11 | Gupta *et al.* (2012a) |
| RIL (ILL5588 X ILL5722) | 196 | RAPD, ISSR, 15 MtEST-SSR and SSR | 1156.4 | 11 | Gupta *et al.* (2012b) |
| RIL (CDC Robin X 964a-46) | 543 | 6 SSRs and 537 SNPs | 834.7 | 7 | Sharpe *et al.* (2013) |
| RIL (Cassab X IIL2024) | 318 | 57 SSRs and 267 SNPs | 1178 | 10 | Kaur *et al.* (2014) |
| RIL (Precoz X L830) | 216 | 216 SSRs | 1183.7 | 7 | Verma *et al.* (2015) |

## 2.4. Breeding for drought tolerance

### 2.4.1. Plant drought responses

Water availability is a *sine qua non* condition for any living organism and thus also plant growth, development and reproduction. Water scarcity can cause total destruction of crop production and can lead to serious social problems and economic losses (Osakabe *et al.* 2014; Fang and Xiong 2015). It can also cause ecological damages, desertification and soil erosion. Under the continuously growing world demand for food and agricultural products, looking for potential solutions using scientific approaches to enhance crop production under water-limited availability is utmost importance (Fita *et al.* 2015).

Drought is obviously the major abiotic stress constraint for crop production worldwide causing substantial yield losses. For example, about 67 % of crop losses over the last 50 years were due to drought stress in the United State of America (Comas *et al.* 2013). Although, water shortages are frequent in some environments like the Mediterranean region and other dryland farming systems in arid/semi-arid regions, they are expected to worsen in the coming decades as a result of global warming and climate change (Chaves *et al.* 2002; Dilley *et al.* 2005; Dai 2013). Drought is also one of the major abiotic stresses severely limiting yield of cool-season food legumes such as chickpea (*Cicer arieticum*), faba bean (*Vicia faba*), lentil and pea (*Pisum sativum*) (Stoddard *et al.* 2006). Heavy production losses due to drought stress in cool-season food legumes were reported by several authors: Saxena (1993), Singh *et al.* (1994), Subbarao *et al.* (1995) and Siddique *et al.* (1999). Lentil is relatively more drought tolerant than other food legumes, and yield losses can range between 6 and 54 %. Pea is more sensitive to drought and in that species yield losses range from 21 to 54 %. Yield losses in chickpea due to terminal drought can vary between 30 and 60 %, while faba bean is considered to be very sensitive to drought and losses in potential yield can be as high as 70 % (Siddique *et al.* 1999).

In lentil, drought stress has several effects on growth and yield (Shrestha *et al.* 2009). Water deficit reduces dry matter production at maturity by up to 32–61 % by reducing plant height, leaf area and the number of leaves, nodes and reproductive structures (Ashraf *et al.* 1992; Turay *et al.* 1992; Shrestha *et al.* 2006b). The total number of flowers produced under drought stress is reduced by up to 45% and the flowering duration by 12 days compared with well-watered conditions (Shrestha *et al.* 2009; Idrissi *et al.* 2013). End-cycle drought reduces the total numbers of filled pods and final seeds produced (Shrestha, 2005). The latter author also reported a reduction of up to 60–70 % of final pod and seed numbers with an increase of seed abortion of 55–75 % under sever water deficit occurring during pod development stage. Under such

conditions new flowers and pods production was reported to be completely stopped. Lentil seed yield is reduced by terminal drought by reducing pod and seed numbers (Shrestha *et al.*, 2006a). $N_2$ fixation is affected by water deficit through reduced carbon supply to the nodules, reduced flow of carbon into nodules via the phloem, or due to a direct effect on the activity of the nodules (Shrestha *et al.* 2009).

Drought is caused by a decrease in water availability in soils in natural agricultural systems or other growing mediums resulting in decreased water potential. In the field, it is a period of below normal and sufficient precipitation limiting plant productivity (Kramer and Boyer 1995; Verslues *et al.* 2006). Decrease in soil water potential leads to disturbing the soil/plant/air water flux system, based on water potential gradient under normal conditions, thus causing difficulties for the plant to uptake water.

Plants respond to decreased water availability by several morphological and physiological mechanisms involving damaging and/or adaptive changes that are affecting often productivity especially when the drought period is severe and long. Several kinds of drought tolerance mechanisms exist. In addition to drought escape mechanisms based on completing growth cycle during water availability period, two major plant responses and mechanisms after reduction in water availability: low water potential stress avoidance and low water potential stress tolerance (dehydration avoidance and dehydration tolerance) were reported by Levitt (1980).

- **Low water potential escape (drought escape):**

Drought escape is defined as the mechanism that consists of natural or artificial changes in the plant growth period, life cycle, planting and harvesting time to prevent the growing season from encountering local seasonal or climatic drought (Boyer 1996). Escape mechanisms allow the plant to complete its life cycle before water deficit severity reaches critical levels that could affect crop development and yield. These include: rapid germination, early vegetative vigour, early flowering and maturity and seed set acceleration (Turner *et al.* 2001). Differences in phenological development involved in this mechanism explain 45–60 % of the variation in seed yield (Silim *et al.* 1993; Turner *et al.* 2001). Early cultivars are potentially more productive in arid/semi-arid environments where end-cycle drought and high temperature episodes at the beginning of flowering and maturity stages frequently occur.

- **Low water potential stress avoidance (drought avoidance):**

Plant productivity is optimum when tissue water potential is maintained at normal values as for situations where water availability is not limited. Thus, under water deficit stress the plant first develops mechanisms to avoid low water potential by balancing water uptake and water loss. This drought avoidance is defined as the ability of plants to maintain normal physiological

functions under mild or moderate water deficit conditions by changes in certain morphological or growth rate traits to avoid the negative effects caused by limited water availability (Hall and Schulze 1980; Blum 2005; Osakabe *et al.* 2014). This mechanism principally tries to maintain a high plant water potential.

The plant's first reaction is stomatal closure and leaf rolling to limit water loss by transpiration to maintain cell turgor whereby plant functions remain as close as possible to those of unstressed conditions (Turner *et al.* 2001; Osakabe *et al.* 2014). Increasing root-shoot ratio, tissue water storage capacity and water permeability are drought avoidance strategies occurring later in situations of longer-term water deficit (Verslues *et al.* 2006). Deep rooting and high root density allow plants to extract water from greater depth thus enhancing avoidance capacity. Shrestha (2005) reported 14-100 % increase in root-shoot dry matter ratio in lentil under drought occurring at the reproductive stage compared to well-watered conditions. Other morphological modifications such as reduction in leaf size, shape and numbers and change in leaf angle contribute to drought avoidance (Turner *et al.* 2001; Shrestha *et al.* 2009).

Low water potential stress avoidance mechanisms are important to maintain plant production and performance under limited drought duration (Kramer and Boyer 1995). Decreased stomatal conductance and increased root system development are potentially important to increase crop productivity under drought. However, a prolonged drought stress limits the benefits of these mechanisms, as $CO_2$ uptake during photosynthesis will decrease and development of roots may be at the expense of vegetative and reproductive tissue development (Verslues *et al.* 2006).

- **Low water potential stress tolerance (drought tolerance):**

When the plant experiences low water potential over a relatively longer period and water deficit stress becomes more severe, low water potential stress avoidance mechanisms are not enough as water uptake and loss cannot be balanced. Thus, the plant will respond by using other mechanisms to maintain continued growth and survival. Drought tolerance is defined as the ability of plants to sustain a certain level of physiological activities under severe drought stress conditions and to reduce or repair the resulting stress damage (Passioura *et al.* 1997). In this case, two situations corresponding to two different mechanisms are possible (Levitt 1980):

**Dehydration avoidance:** In order to maintain an adequate water flux, the plant tissue water potential is lowered as a result of decreased soil water potential. This is possible either by water loss or solute accumulation, and cell wall hardening which can help to achieve low tissue water potential ($\Psi_w$) and avoid water loss. Solute accumulation is known as osmotic adjustment which allows maintenance of water relations (turgor) and corresponds to the active accumulation of additional solutes (such as sugars, polyols, mannitol, proline, glycine, betaine, trehalose,

fructan, inositol, amino acids, alkaloids and inorganic ions) to reduce osmotic potential ($\Psi_s$), increase pressure potential ($\Psi_p$; $\Psi_w = \Psi_s + \Psi_p$; Kramer and Boyer 1995; Verslues *et al.* 2006) and thus improve cell water retention in response to low water potential caused by water deficit stress (Morgan 1984). In addition to the maintenance of turgor, these changes contribute to enhance stomatal conductance, photosynthesis and plant growth at progressively lower leaf water potentials (Subbarao *et al.* 1995). In lentil, osmotic adjustment was reported to range from 0.0 to 1.8 MPa under different soil water potentials (Ashraf *et al.* 1992; Turner *et al.* 2001; Shrestha 2005). Although osmotic adjustment can be energy-intensive for the plant and of limited effect when soil water content is very low, it is considered as an important factor for inducing drought tolerance in the field (Kramer and Boyer 1995; Morgan 1984). Also, cell wall hardening (rigid cell wall) allows little change in cell volume and rapid decrease of turgor and water potential as a result of a small loss of water thus avoiding further water loss (Verslues *et al.* 2006). The rapid decrease in turgor when water is lost from cells allows the achievement of lower cell water potential compared to its surroundings thus restoring water potential gradient. It is physiologically achieved by the accumulation of substances like lignin and cellulose in cell membranes (Blum 2011).

**Dehydration tolerance:** When the already low water potential becomes lower as a result of severe drought stress, mechanisms for dehydration avoidance are not sufficient and the plant responds using other mechanisms to allow it to cope with dehydration and avoid cellular damage caused by water loss. In this case, protective proteins such as dehydrins and late-embryogenesis abundant proteins start accumulating to protect membrane structures (Turner *et al.* 2001; Bravo *et al.* 2003; Verslues *et al.* 2006). Other changes result from dehydration mechanisms like metabolic change and reactive oxygen species (ROS) detoxification (Verslues *et al.* 2006). However, there are dehydration critical levels which are lethal for most crop plants (Turner *et al.* 2001).

Plant responses to drought stress are summarized in Figure 2.3. Interactions between avoidance and tolerance mechanisms occur maybe as a consequence of an integrated plant response to drought stress. In fact, plant responses to low water availability do not exclude one or another of avoidance/tolerance strategies. For example, under dehydration avoidance, osmotic adjustment in roots could result in a deeper root system which can enhance water uptake which is one of the mechanisms of low water potential avoidance (Verslues *et al.* 2005). Also, dehydrin proteins involved in dehydration tolerance by their role in protecting cellular structures may play a role in dehydration avoidance by acting to bind and retain water (Close 1997).

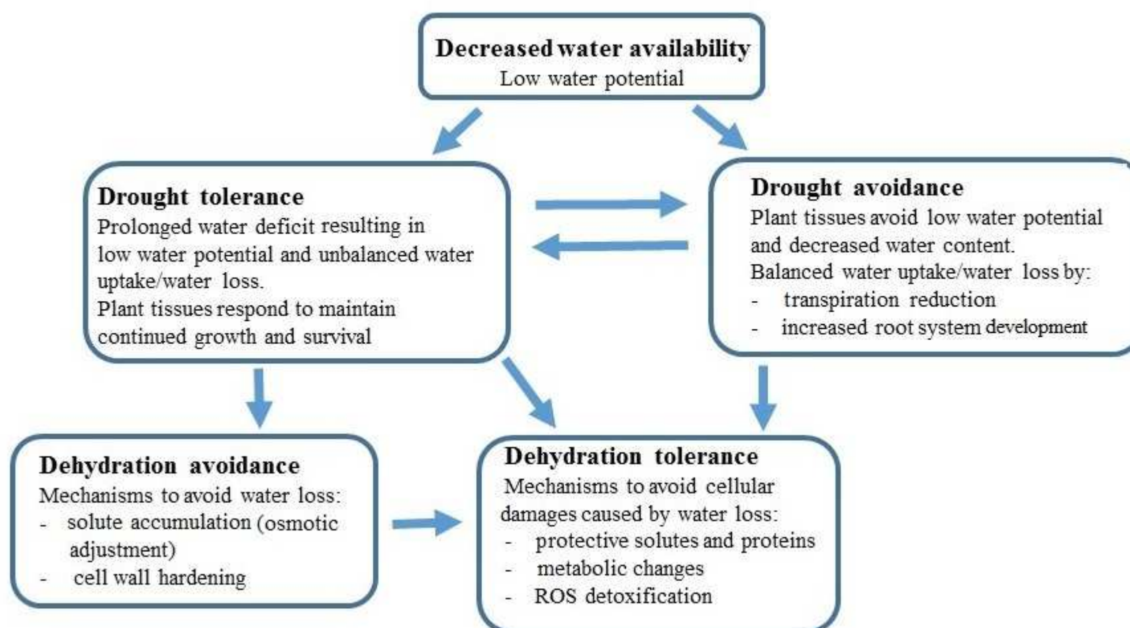


**Figure 2.3.** Avoidance/tolerance plant responses to drought stress. Blue arrows show interactions and time overlap between different mechanisms (adapted from Verslues *et al.* 2006).

### 2.4.2. Breeding for drought tolerance

Drought tolerance is an important trait targeted in breeding programs worldwide, but mainly in and for arid and semi-arid areas. Significant advances have been made in developing adequate methods and approaches as well as molecular characterization studies of this abiotic stress (Ur Rehman 2009; Khazaei 2014). However, drought tolerance is still a difficult and complex trait for breeders. As drought occurrence and severity are unpredictable in field experiments, breeding and screening for this challenging abiotic stress are mainly based on controlled-environment experiments as an alternative, that may not always reflect reality (Stoddard *et al.* 2006). Also, performing multi-environmental and multi-annual trials is costly and slow. Physiological selection criteria and genomic approaches offer efficient options in breeding programs targeting drought tolerance for water-limited environments. However, synchronization with plant breeding concept, theory and methods determines the potential of these approaches (Blum 2011).

Among the plant responses to water deficit stress, both dehydration avoidance and low water potential avoidance related to the maintenance of a high tissue water potential and controlled by plant constitutive and adaptive traits are the most effective mechanisms of drought resistance (Kramer and Boyer 1995; Blum 2011). Drought escape is effective when the growing season matches the periods of soil moisture availability, especially in areas where terminal drought

stress predominates (Turner 1986). In these areas, breeding for short-season varieties is of major importance. Dehydration tolerance allowing plant tissues to function in a dehydrated state is rare but can sometimes be important (Blum 2011).

Ancient farmers are considered as the first pre-scientific era plant breeders who achieved significant progress in conveying drought resistance to their crops (Blum 2011). By selecting seeds for next season sowing in their environment, they were indirectly developing drought resistant landraces of sorghum, pearl millet, wheat, barley and other species (Blum and Sullivan 1986; Blum *et al.* 1989 and Ceccarelli *et al.* 1998). Nowadays, plant breeders in addition to select short cultivars that can escape terminal drought, use different measures and methods to identify drought tolerant genotypes.

Different screening techniques to elucidate drought tolerance/avoidance are being used in plant breeding programs. Effectiveness, reliability and acceptance of any screening system depend on simple selection criteria, rapid and accurate screening of large numbers of genotypes, non-destructive ways, reproducibility, and relationship to field performance (Wery *et al.* 1994; Serraj *et al.* 2003; Verslues *et al.* 2006). Several evaluation and selection criteria were used in drought tolerance studies in food legumes (Table 2.4).

**Table 2.4.** Evaluation and selection criteria used in food legume drought tolerance studies

| Species | Evaluation and selection criteria | References |
|---|---|---|
| Lentil | Relative water content | Salam and Islam (1994), Shrestha *et al.* (2006) |
| | Stomatal conductance | Talukdar (2013) |
| | Wilting score | Singh *et al.* (2013) |
| | Leaf metabolic content (proline,..) | Muscolo *et al.* (2015) |
| | Root and shoot characteristics | Sarker *et al.* (2005), Kumar *et al.* (2012) |
| | Osmotic regulation | Ashraf *et al.* (1992), Stoddard *et al.* (2006) |
| Pea | Relative water content | Iglesias-García *et al.* (2015), Alexieva *et al.* |
| | Osmotic adjustment | (2001) |
| | Abscisic acid accumulation | Rodrigues-Maribona *et al.* (1992) |
| Chickpea | Osmotic adjustment | Upreti and Murti (1999) |
| | relative water content | Morgan *et al.* (1991) |
| | | Lecoeur *et al.* (1992), Turner *et al.* (2006), Jain and Chattopadhyay (2010), Kumar Patel *et al.* (2011) |
| | Wilting score | Ur Rehman (2009) |
| | Metabolic content (proline, …) | Ur Rehman (2009) |
| | Stomatal conductance | Kumar Patel *et al.* (2011) |
| | Chlorophyll fluorescence | Ur Rehman (2009) |
| | Root and shoot characteristics | Ur Rehman (2009), Serraj *et al.* (2004) |
| Faba | Relative water content | Kashiwagi *et al.* (2014) |
| bean | Canopy temperature | Khazaei *et al.* (2013), Khan *et al.* (2007) |
| | Osmotic potential, stomatal | Khazaei *et al.* (2013) |
| | Conductance | Khan *et al.* (2007) |

Breeding for drought tolerance in crops could be simply defined as developing cultivars able to maintain sufficient water balance to achieve better yield under water-limited conditions compared to a normal situation. Genetically, drought tolerance has been found to be a complex quantitative trait controlled by a large number of minor genes (Ur Rehman 2009; Fleury *et al.* 2010; Ravi *et al.* 2011; Khazaei *et al.* 2013; Fang and Xiong 2015). Identification of these genes and their associated agronomically important alleles controlling plant responses to drought using genomic approaches should allow an effective improvement of drought tolerance and yield under water deficit environments (Tuberosa and Salvi 2006).

Many morphological, physiological and phenological parameters related to escape/avoidance/tolerance plant reactions were developed as phenotyping and selection criteria in breeding programs (Verslues *et al.* 2006; Blum 2011; Fang and Xiong 2015):

- **Selection criteria based on dehydration avoidance**

  **Early vigour:** fast vigorous seedling growth may limit the loss of water due to direct evaporation by soil surface shading and reduction of inhibition of stomatal conductance occurrence; thus optimizing water use efficiency of annual crops (Blum 2011; Tuberosa 2012). It is therefore targeted in breeding programs. It has been reported to improve water use efficiency and yield in wheat (Asseng *et al.* 2003; Richards 2006; Rebetzke *et al*. 2007). Tuberosa (2012) reported that there is an optimal degree of vigour that should be targeted, depending on the environmental conditions, to avoid causing early reduction of soil moisture.

  **Relative leaf water content** (Barrs and Weatherley 1962): is a parameter expressing plant water status and the effect of osmotic adjustment on leaf water content. It estimates the volumetric water content of the leaf at any moment relative to its water content at full turgor. Leaf relative water content is one of the major components of dehydration avoidance allowing cell turgor maintenance (Blum 2011). Under drought stress, plants with higher relative leaf water content are drought tolerant (Ashraf *et al.* 1992; Jain and Chattopadhyay 2010; Talukdar 2013; Khazaei 2013; Iglesias-García *et al.* 2015). Ashraf *et al.* (1992) found that lentil drought-tolerant accessions produced greater biomass and had higher capacity to maintain high relative water content. Rodriguez-Maribona *et al.* (1992) and Morgan (1995) reported a positive correlation of relative leaf water content with yield in pea and wheat, respectively.

  **Stomatal conductance:** transpiration decreases as a result of stomatal closure in response to lower water potential in soils, thus lowering $CO_2$ uptake and photosynthesis. This drought avoidance strategy is not effective for maintaining plant productivity under drought. Indeed, high stomatal conductance under drought stress was shown to be important to maintain high yields in wheat, rice, cotton, and chickpea (Blum *et al.* 1982; Sanguineti *et al.* 1999; Izanloo *et al.* 2008; Ur Rehman 2009). Delayed stomatal closure allows continuous supply of water and maintaining cell turgidity, thus improving dehydration avoidance (Verslues *et al.* 2006). In lentil, stomatal conductance variation ranged from 169 to 400 mmol/m$^2$/s for well-watered conditions and decreased to only

19–100 mmol/m$^2$/s under water deficit (leaf water potential below –2.5 MPa) (Shrestha, 2005).

**Canopy temperature:** stomatal closure causes leaf temperature increase indicating decreasing transpiration in a plant subjected to water deficit. Thus, lower canopy temperature under drought stress testifies increased transpiration, and optimal water status and photosynthesis, key factors for guaranteeing better yield. Stomatal conductance is strongly associated with canopy temperature and could be indirectly estimated using an infrared thermometer (Ur Rehman 2009). The latter also reported that lower canopy temperature was associated with higher stomatal conductance and strong positive associations with yield in chickpea. Also, correlations ranging from 0.6-0.8 with grain yield were reported for wheat by Reynolds and Pfeiffer (2000). Blum *et al.* (1982) and Pinter *et al.* (1990) reported that monitoring this parameter is an effective technique for drought tolerance screening.

**Leaf pubescence:** under drought stress, leaf spectral reflectance is higher thanks to leaf pubescence lowering net radiation and leaf temperatures. This characteristic may be considered for drought tolerance screening by selecting cultivars carrying leaves with hairs (Blum 2011).

**Osmotic adjustment:** is an important component of dehydration avoidance allowing to maintain cellular hydration, turgor and high relative leaf water content (Blum 2011). It has a role also in cellular membrane stability maintenance (Bohnert and Shen 1999). Several studies reported osmotic adjustment as an effective criterion for drought tolerance (Morgan 1984, 1991; Ahsraf *et al.* 1992; Blum 1988; Kramer and Boyer 1995; Babu *et al.* 1999; Verslues *et al.* 2006). Furthermore, it is effective for both plant productivity and survival (Blum *et al.* 2011). Solute accumulation leads to lower osmotic potential of the plant cells in both shoots and roots, thus attracting water into the cells and maintaining their turgor. Higher accumulation of osmotic adjustment solutes initiated in response to water deficit stress, allows continuous stomatal and photosynthetic activities resulting in continuous growth of the plant. A significant increase in yield with higher osmotic adjustment genotypes was reported to occur in chickpea (Morgan *et al.* 1991; Moinuddin and Khanna-Chopra 2004).

**Root characteristics:** under drought stress, changes in root growth are one of the earliest plant responses. Increased root formation allows to explore more soil moisture in deeper layers, thus improving water uptake and balancing plant water status. Increased root growth has the potential to maintain or even to increase crop productivity

under water-limited conditions (Verslues *et al.* 2006). A well-developed root system resulting in enhanced dehydration avoidance is one of the morphological characteristics that plant breeders may target for the development of drought tolerant cultivars. Higher root-shoot ratio is to be considered as a key trait for screening drought tolerant cultivars. Root characteristics, their genetic variability, their association with drought tolerance and specific root traits important for breeding will be further discussed (chapter 2.5 below).

**Water losing rate:** is a typical measure of decrease in fresh weight over time of detached leaves. This measure allows to estimate gradual decline in leaf water content and dehydration as described by Clarke and McCaig (1982), Suprunova *et al.* (2004) and Verslues *et al.* (2006). This criterion stands for a short-term dehydration avoidance, whereby the rate of water loss is largely determined by stomatal conductance (Verslues *et al.* 2006). Furthermore, it could be also an indicator of altered accumulation of or sensitivity to abscisic acid, because the latter is largely involved in the control of stomatal conductance. Water losing rate was reported to be negatively correlated with relative water content (Verslues *et al.* 2006; Ravazi *et al.* 2011). Thus, lower values are associated with tolerant cultivars. This parameter has been used for drought avoidance evaluation in maize, barley, and strawberry (Ristic and Jensk 2002; Suprunova *et al.* 2004; Ravazi *et al.* 2011).

**Stay-Green:** this parameter is related to a delay in the loss of leaf greenness, which is a normal process in leaf senescence (Blum 2011). Delayed senescence (longer greenness) has a positive effect on chlorophyll content maintenance and high leaf water status, thus playing an important role as dehydration avoidance component. A parameter that is negatively correlated to this criterion, and largely used in breeding and genetic studies related to drought, is the wilting or drought tolerance score, visually accessed using a 0-4 or a 1-9 scale (Singh *et al.* 1997; Ur Rehman 2009; Singh *et al.* 2013).

- **Selection criteria based on drought escape mechanisms**

  Drought escape is the ability of plant to achieve its life cycle during water availability period. This mechanism is of interest mainly in dryland areas where terminal drought occur frequently reducing yield by affecting seed quality and causing poor grain filling or even complete failure of grain or fruit set (Blum 2011). Plant breeders select short-growth duration cultivars with optimum yield adapted to environments where water availability decreases by the end of season such as in the Mediterranean region.

- **Selection criteria based on dehydration tolerance mechanisms**

  **Plant survival:** allows continuous water extraction under severe drought to delay plant death potentially resulting in subsequent recovery after water supply resumes (Volaire and Lelievre 2001; Blum 2011). Although plant survival occurring under severe drought is most likely to have only limited benefits on yield, it is of great importance for smallholder farmers (Blum 2011). The latter author reported the importance of plant seedling survival capacity for some farming systems for arid and semi-arid agriculture like in Mediterranean regions. In these areas, winter rain germinates seeds but frequently following episodes of drought stress may occur leading to plant wilting as a result of water deficit. After rain, plants with higher survival capacity will regenerate and could produce green biomass and grains, valuables resources for smallholder farmers. Thus, using this selection criterion in breeding programs targeting such environments to develop cultivars with higher recovery after re-watering would result in significant interest.

  **Cell membrane stability:** drought and other abiotic stresses can cause increased cell wall permeability and thus leakage of ions as a result of water loss associated with these structural changes (Blum 2011). Cell membrane stability is estimated by measuring the electro-conductivity of aqueous media containing leaf discs after applying an in vitro physiological drought stress to leaflet and leaf discs using polyethylene glycol. In vitro stress causes destruction of cell wall membrane, thus resulting in efflux of electrolytes. Thus it is used as screening technique for drought tolerance (Blum and Ebercon 1979; Singh *et al.* 2008).

  **Reactive oxygen species (ROS):** ROS plays a complex role in plants (Apel and Hirt 2004; Miller *et al.* 2010). Biotic and abiotic stresses cause ROS concentrations in plants to increase (Blum 2011). Some studies have reported their role for conveying drought resistance, but there still exist doubts about their possible use in breeding for drought resistance (Lascano *et al.* 2001; Mungur *et al.* 2006; Rodrigues *et al.* 2006). Blum (2011) considered that ROS as well as other antioxidants need more investigation before serious use in plant breeding for drought resistance could be considered.

## 2.5. Root traits and their association with drought tolerance

### 2.5.1. Roles of root systems

Basically, roots are the morphological part of the plant that ensure its physical anchorage in the soil (or any other growth medium) and its needs for water and mineral elements. Roots are involved in the synthesis of some important growth regulators such as abscisic acid, cytokinins and gibberellins. Roots of leguminous species are also active in atmospheric nitrogen fixation in symbiotic association with *Rhizobium* bacteria.

Different root systems exist. Comas *et al.* (2013) described root systems for woody and herbaceous plants. Woody plants root systems consist of two major parts: coarse woody roots corresponding to aboveground stems and ensuring permanent fixation, carbohydrate and nutrient storage, and transport of water and nutrients to shoot parts; and fine roots (first and second branches) ensuring water and nutrients uptake. For herbaceous plants, there are also two major parts: tap and lateral roots (tap root system), or seminal/nodal and lateral roots (fibrous root system) (Fitter 2002). Tap and seminal roots play roles of anchorage, establishment of the whole root architecture and control of rooting depth (Henry *et al.* 2011). Lateral roots are considered to be the most active root parts and compose the majority of surface area and total root length, whereas they also ensure water and nutrients uptake (Rewald *et al.* 2011).

### 2.5.2. Root growth under drought stress conditions

Drought stress results in an increase of root-shoot dry matter ratio in many crop plants (Verslues 2006; Blum 2011). This is one of the first mechanisms that plants use to avoid low water potential by relatively rapid root growth to explore more soil surface and enhance water uptake to maintain high water content. In fact, this shift in allometry (biomass partitioning between shoots and roots) allows continuous production of new root tips increasing root capacity for water supply for existing shoots and leaves (Comas *et al.* 2013). Root length density, the length of roots per unit volume of soil, increases under drought compared to what happen in unstressed conditions (Kashiwagi *et al.* 2005; Blum 2011; Aswaf and Blair 2012). In certain cases, increase in root growth under water deficiency is due to greater osmotic adjustment in roots compared to leaves (Ober and Sharp 2007), and accumulation of carbohydrates in roots as a result of reduced (or arrested) leaf expansion (Blum 2011); abscisic acid synthesis inhibits shoot growth but conversely promotes root growth (Ober and Sharp 2007); cell wall expansion enhances root growth and soil moisture determines the interaction between root and shoot growth thus enhancing root development under drying soils (Blum 2011). However, root growth may be limited under severe drought (Comas *et al.* 2005). Increased branching of roots and increased

total root length under drought conditions were reported (Franco *et al.* 2008). Other root characteristics may also be affected by drought: root length, fresh weight, dry weight, diameter and surface area, deep rooting and cortex thickness (Franco 2011).

### 2.5.3. Root-based breeding for drought tolerance

Root system size, proprieties and distribution are key factors in defining whole-plant access to water. Hence, improvement of root characteristics increasing water uptake and maintaining plant productivity (yield) under drought stress has received increased interest during recent years (Comas *et al.* 2013). The underground character of roots may be one of the reasons to be less investigated by researchers compared to other plant traits. Breeding for specific root traits requires efficient screening methods and deeper knowledge of their specific functions under water-limited environments (Vadez *et al.* 2008). Identification of DNA markers linked to these desired root characteristics using genomic approaches will facilitate the adoption of marker-assisted selection and ultimately, efficient development of drought tolerant cultivars with a well-developed root system. In fact, breeding methods based on genetic control of these traits is feasible (Varshney *et al.* 2011; Comas *et al.* 2013).

Kashiwagi *et al.* (2005) and Varshney *et al.* (2011) considered root biomass, root depth and root proliferation as the most promising traits to be targeted in chickpea (*Cicer arietinum*) for drought avoidance and end-cycle drought tolerance. Later, Varshney *et al.* (2014) reported the importance of targeting root length density, root surface area and the ratio root dry weight/total plant dry weight in the same species. In common beans (*Phaseolus vulgaris* L), Aswaf and Blair (2012) reported cumulative importance of deep rooting, longer root length, thicker roots, increasing root length distribution with depth, root volume and root biomass. In wheat (*Triticum* spp.), increased total root biomass and length, number of roots, seminal root number and angle, and deep root growth were reported to be associated with a higher capacity for water uptake (Sharma *et al.* 2011; Hamada *et al.* 2012; Christopher *et al.* 2013). Comas *et al.* (2013) summarized, in a review, root traits contributing to maintain plant productivity under drought as: small fine root diameter, long specific root length and high root length density. The same authors reported that small xylem diameters of seminal roots contribute to save soil water in deep profiles to be used during plant maturation thus contributing to enhance yield under late season water deficit. Larger xylem diameters and deeper root growth may improve water absorption when the latter is available at greater depths. Higher root-shoot ratio under drought was reported to enhance plant hydraulic conductance and productivity under water deficit conditions (Addington *et al.* 2006; Maseda and Fernandez 2006; Verslues *et al.* 2006). Plant

productivity under drought is also enhanced following root distribution plasticity and the ability of plants to produce roots depending on soil water availability in the different soil layers (Comas *et al.* 2013).

In areas where drought is episodic, root response to re-watering is important to ensure significant yield. For example, drought-adapted wheat genotypes rapidly produce "rain roots" after a rainfall succeeding a drought episode (Sadras and Rodriguez 2007).

### 2.5.4. Genetic control of root characteristics

Four main areas of research could be defined under root genomics (Costa de Oliveira and Varshney 2011): root growth and development, functional analyses of abiotic stress responses, functional analyses of biotic stress responses, and QTL analysis and molecular breeding. The latter deals with developing efficient breeding strategies using molecular tools required for better understanding of the genetic basis of root characteristics involved in drought tolerance. Root characteristics are highly influenced by the environment and mostly controlled by many genes with small effects each (Costa de Oliveira and Varshney 2011; Gruber *et al.* 2011). Thus, QTL analysis is widely used to investigate genetic control of root systems. For instance, several studies about root genetics have been reported for a number of crop species using mapping pupulations (Manavalan *et al.* 2015; Lou *et al.* 2014; Varshney *et al.* 2014; Bai *et al.* 2013; Brensha *et al.* 2012; Aswaf and Blair 2012; Sayed 2011; Ur Rehman 2009; Ruta 2008; Serraj *et al.* 2004; Sarker *et al.* 2005; Kashiwagi *et al.* 2005). These authors reported high genetic variability, evidence of quantitative polygenic control and high heritability, and identified valuable QTLs for root traits related to drought tolerance. For example, among food legumes, Varshney *et al.* (2014) identified in chickpea (*Cicer arietinum*) 9 QTLs clusters with high potential to enhance drought tolerance of which 3 main effects-QTLs for root length density, root surface area and root dry weight/total plant dry weight ratio with heritability values of 0.61, 0.46 and 0.56, and explaining 10.9 %, 10.26 % and 16.67 % of the phenotypic variation, respectively. For common bean (*Phaseolus vulgaris*), Aswaf and Blair (2012) reported heritability values ranging from 0.31 to 0.57 and 9 putative QTLs for seven rooting pattern traits explaining a phenotypic variation ranging from 17.4 % to 40 %.

### 2.5.5. Lentil root system

The lentil root system is characterized by a slender taproot system with a mass of fibrous lateral roots (Saxena 2009). The upper layer of taproot and lateral roots carries small round or elongated nodules which are a major source of fixed nitrogen for the crops, resulting from their association with *Rhizobium leguminosarum*. Figure 2.4 shows the three main architectural

structures of lentil root systems differing mainly in relative depth of the taproot and the proliferation of lateral roots.

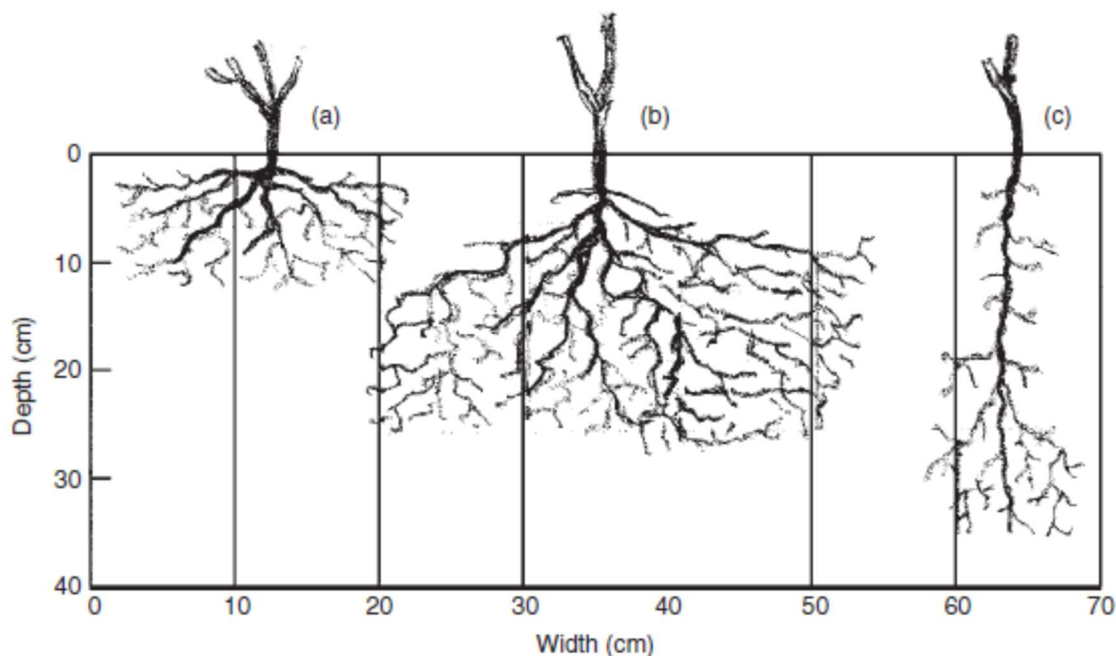


**Figure 2.4.** Lentil root system: (a) shallow (b) intermediate and (c) deep (adapted from Nezamuddin 1970 and Saxena 2009). X axis reports the width of root systems and Y axis reports the depth.

High genetic variation has been reported for lentil germplasm from different origins for both root and shoot traits such as stem length, and weight, taproot length, lateral root number, total root length, root growth rate and total root weight (Mia *et al.* 1996; Sarker *et al.* 2005; Gahoonia *et al.* 2005, 2006; Kumar *et al.* 2012, 2013) but also for other grain legumes (Serraj *et al.* 2004; Kashiwagi *et al.* 2005; Vadez *et al.* 2008; Aswaf and Blair 2012). Gahoonia *et al.* (2005) reported that differences in root morphologies may increase micronutrient uptake from the soil and account for 10 to 20 % yield increases in lentil.

Little information is available about lentil shoot and root traits in association with drought tolerance. Lentil is relatively deep-rooting compared to other food legumes. Lentil is more drought tolerant compared to other grain legumes like chickpea (*Cicer arietinum*), pea (*Pisum sativum* L.) and faba bean (*Vicia faba*) (McKenzie and Hill 2004). Also, Daryanto *et al.* (2015) reported lower drought-induced yield reduction for lentil compared to other legumes such as cowpea (*Vigna unguiculata*) and green gram (*Vigna radiate*) under similar drought conditions. Shrestha *et al.* (2005) reported a 60-65 cm root depth to be normal in lentil. Lentil can extract water from soil layers as deep as 90 cm (Sharma and Prasad 1984; McKenzie 1987). Sarker *et al.* (2005) identified lentil breeding line ILL6002 as having a prolific and well-developed root system.

## 2.6. Marker-trait association and quantitative trait loci analysis

Since the rediscovery of Mendel's laws on genetics and inheritance by Hugo de Vries, Carl Correns and Erich von Tschermak-Seysenegg in 1900 (Acquaah 2007), a significantly better understanding of the genetic bases of qualitative plant traits has been achieved. Fisher (1918) was the first to develop a quantitative genetics theory combining Mendelian genetics, biometric and statistic approaches. In brief, he stated that the genetic variance of quantitative traits in a population is due to a large number of Mendelian factors, each making a small additive contribution to a particular phenotype (Nelson *et al.* 2013). Quantitative genetics is a branch of genetics where individual genotypes are not clearly identified as for qualitative traits and the traits of individuals are quantitatively measured (Acquaah 2007). This is because several genes contribute with different effects to the overall phenotypic expression of a quantitative trait (Falconer 1989). Quantitative genetics focus on the degree of difference between individuals rather than difference between classes of individuals with similar characteristic (Falconer 1989). Quantitative traits in plants are more affected by the environment than qualitative traits, thus environmental and genetic variations due to simultaneous segregation of polygenes can result in continuous variation (Poehlman 1987; Acquaah 2007). Polygenic control of a character is characterized by the involvement of several segregating genes lacking dominance and having additive gene effects (Poehlman 1987; Acquaah 2007). This makes breeding successes difficult to obtain for quantitative traits compared to qualitative ones. In fact, a qualitative trait is usually controlled by a few genes with major effects, while for a quantitative trait, the average gene effect depends on several individual genes contributing each with small effects (Poehlman 1987, Acquaah 2007). Also, breeding progress depends on heritability, and quantitative traits tend to have lower heritability than qualitative ones (Acquaah 2007).

Continuous developments in informatics and statistics now allow scientists to construct genetic (linkage) maps using molecular markers. Genetic linkage maps are composed of several linkage groups corresponding to sets of markers linked to each other and that are tend to be inherited together (Acquaah 2007). Genetic linkage is based on the basic principle of recombination frequency explained by crossing-over events during gamete formation (meiosis). Crossing-over is the exchange of chromosomal parts of homologous chromosomes during meiosis occurring in anthers and ovaries, the reproductive organs of flowering plants (Acquaah 2007). This exchange results in new individual genotypes in the offspring population that are different from both parents. The proportion of these new (recombinant) genotypes is called recombination frequency (Griffiths *et al*. 2000). Thomas Hunt Morgan and Alfred Henry Sturtevant, around 1913, were the first to describe the chromosome theory of inheritance that uses genetic

recombination for gene mapping (Griffiths *et al.* 2000). Informatics and statistics packages allowed to identify linked genes to several economically important quantitative and qualitative traits for many crops. More details about genetic linkage analysis and some computer programs methods used are provided in the sections below.

Marker-trait association analysis is a useful statistical tool for detecting DNA variants associated with specific agronomical crop traits (Wang and Sheffield 2005). Finding DNA markers linked to certain phenotypic traits is based on two different methods: linkage analysis and association mapping analysis.

### 2.6.1. Linkage analysis for quantitative trait loci mapping

Linkage maps present positions of molecular (and/or other kinds of) markers in the chromosomes based on the relative genetic distance between them (Collard *et al.* 2005). The basic principle is that during meiosis, recombination events (crossing-overs) represented by exchange events of chromosomal parts between chromosomes results in co-segregation of markers that are situated close to each other. Thus, they tend to be inherited together and transmitted from the parents to the respective progeny (Paterson 1996a). The closer the markers to each other, the less frequently crossing-over will occur indicating that they are more likely located in the same chromosome. In segregating populations ($F_2$, $F_1$-derived hybrids, backcross, recombinant inbred lines), the frequency of recombinant genotypes (individuals with new characteristics different from both parents as results of crossing-overs) can be used to calculate recombination fractions, used to infer the genetic distance between markers (Griffiths *et al.* 2000). Recombination frequencies are converted to genetic distances expressed in centiMorgans (cM) using mapping functions (Collard *et al.* 2005). Two types of mapping functions are commonly used for linkage mapping (Hartl and Jones 2001; Kearsey and Pooni 1996):

- the Kosambi mapping function, assuming interaction between recombination events, whereby one crossing-over influences the occurrence of other adjacent crossing-over events; and
- the Haldane mapping function, which assumes no interference between adjacent crossing-over events.

As the number of markers used for genetic map construction is generally large, manual calculations are not possible. Thus, a number of computer programs were developed and are being used for genetic linkage map construction (JoinMap, Stam 1993; MapMaker, Lander *et al.*1987; MapManager QTX, Manly *et al.* 2001; Carthagene, de Givry *et al.* 2005). Genetic maps consist of several linkage groups of linked markers based on the calculation of odd ratios

from recombination frequencies between each pair of markers (the ratio of linkage versus no linkage). The logarithm (base 10) of the ratio (LOD: logarithmic of odds; Morton 1955) of likelihoods under the null and alternative hypotheses is usually estimated using the following formula:

$$\boldsymbol{LOD\ score} = \boldsymbol{log_{10}} \frac{(1-\theta)^{NR} x\ \theta^{R}}{0.5^{(NR+R)}} = \boldsymbol{log_{10}} \frac{L(\theta)}{L(0.5)}$$ , (Morton 1955),

where $\theta$ is the recombinant fraction, L($\theta$) is the probability that two loci are linked under a given recombination fraction of $\theta$, L(0.5) is the probability that two loci are not linked, R is the number of offspring recombinant individuals in a mapping population and NR is the number of offspring non-recombinant individuals in a mapping population.

For instance, a LOD score of 3 indicates that evidence that two markers are linked is 1000 times more likely than the probability they are not linked. Thus, LOD scores of 3 and higher are commonly used as thresholds in linkage analysis studies (Mauricio 2001; Collard *et al.* 2005). Computer programs, such as JoinMap, used for linkage map construction are based on the two following major algorithms: regression mapping algorithm (Stam 1993; Van Ooijen 2006) and maximum likelihood mapping algorithm (Jansen *et al*. 2001; Van Ooijen 2006).

For the **regression mapping algorithm**, first all pairwise recombination frequencies and LOD scores are calculated in order to form groups. Then, markers with recombination frequencies smaller than a predefined threshold and LOD scores larger than a predefined threshold are mapped together. For each linkage group, mapping starts by the most informative pairwise: i.e. the lowest recombination frequency and the higher LOD score. Markers are then added one by one by estimating the distances and searching for the best position vis-a-vis the first mapped pair of markers. Determining the optimal map order with the best fitting position of each added marker without disturbing the order of earlier mapped markers is performed in an action called a *ripple*. A *ripple* is based on the calculation of the normalized difference in goodness-of-fit chi-square measures before and after adding a given marker locus testing all permutations within each three adjacent markers. This difference is known as a *jump*. For each pair of markers:

$$\boldsymbol{The\ goodness - of - fit} = \frac{\boldsymbol{direct\ distance\ based\ on\ recombination\ frequencies\ from\ genotypic\ data}}{\boldsymbol{map{-}derived\ distance\ based\ on\ reverse\ map\ function}}$$

When adding a given marker results in too large a *jump* or negative distance compared to the predefined threshold (commonly 3 to 5), the marker fitting position is considered as poor and the marker is removed. Regression mapping calculation algorithm is based on linear regression using weighted least squares (Stam 1993).

Regression mapping algorithm implemented in JoinMap program (Stam 1993) is hampered by typing errors, laboratory errors and missing observations that may lead to less consistent results and substantially large amounts of computer time (Jansen *et al.* 2001). To overcome these kinds of situations, the latter authors developed the maximum likelihood mapping algorithm.

The **maximum likelihood mapping algorithm** is based on three main techniques that help to optimize mapping order, reduce the influence of missing data and genotyping errors and construct dense linkage maps (Jansen *et al*. 2001): *Gibbs sampling*, *simulated annealing* and *spatial sampling*. *Gibbs sampling* is a Monte Carlo Expectation Maximization algorithm estimating multipoint recombination frequencies used to calculate likelihoods. *Simulated annealing* searches the marker order that has the maximum likelihood to be used in mapping. *Spatial sampling* based on gradual map construction is used to find global optimum order of markers. In *simulated annealing* the optimization criterion used is the sum of recombination frequencies in adjacent map segments by finding the order with the highest likelihood (or lower total number of recombination events: lower sum of adjacent recombination frequencies; Jansen *et al.* 2001). It is based on performing repeated iterations of random replacement of a random locus using an acceptance probability value for steps leading to improvement. Each *chain* (1000 iterations), the acceptance probability is reduced (*cooling*). When no improvement is achieved, the system stops and stores the current map order.

*Gibbs sampling* is employed to obtain maximum likelihood multipoint recombination frequency estimates given the current map order; it is based on Monte Carlo Expectation Maximization (MCEM) cycles. In each cycle, all genotypes (including those with missing data and dominant scores) are sampled and pairwise values of recombination frequencies over all loci are recalculated. This iteration is repeated and at the end, the average recombination frequencies of the set of sampled genotypes is calculated. In the next MCEM cycle, these averages are used as new map distances according to which other set of genotypes are sampled. After 3 to 5 cycles, multipoint recombination frequency estimates are stabilized resulting in new and improved recombination frequencies. Then, a new round of *simulated annealing* optimization is performed resulting in an improved map order for which new multipoint estimates of recombination frequencies is required. Usually 3 rounds of *simulated annealing* followed by *Gibbs sampling* are performed. More rounds can be added if changes still occur. *Gibbs sampling* and *simulated annealing* are particularly important to deal with missing genotyping data (Jansen *et al.* 2001).

The *spatial sampling* approach that could be set as calculation option in situations with many unknown genotypes or genotyping errors allows gradual building of the map. At a given

recombination frequency threshold, loci are arranged in a list. For the first locus, recombination frequencies (with all other loci) are compared with this threshold; if recombination frequency is lower than the threshold, then the locus is excluded from the list. The next locus is dealt with and so on. This process ends with a list of loci with recombination frequencies above the threshold called a *spatial sample*. Using the above procedure (*simulated annealing* followed by *Gibbs sampling*), the map is estimated for each *spatial sample* according to the next sampling thresholds (less stringent, lower LOD score and higher recombination frequency).

### 2.6.2. Recombinant inbred lines as mapping population

One of the commonly used mapping populations for linkage map construction and QTL analysis is the Recombinant Inbred Lines (RIL) (Jansen 2003; Pollard 2012) (Figure 2.5). RILs are developed from a cross of phenotypically and genetically contrasting parents. The obtained $F_1$ progeny is self-pollinated to obtain $F_2$ generation, then repeated single seed descent is used in next generations until homozygosity is achieved (Burr and Burr 1991; Broman 2005; Pollard 2012). After 6 to 10 generations, each recombinant inbred line is fixed for a different combination of linked blocks of parental alleles (Burr and Burr 1991). For diploid species, and when the parents are both homozygote, then for each locus two alleles can segregate and alleles of two linked loci from the same parents ($F_{6-10}$) will remain associated more frequently in the progeny than if they were distributed randomly (Burr and Burr 1991). This fact is important for linkage mapping studies.

The genome of recombinant inbred lines is a mosaic of the two parental genomes with a mixture of genotypes of parental types and new recombinant types (Figure 2.5; Broman 2005).

Although developing a RIL mapping population requires substantial time and resources, it is considered as a powerful tool for genetic mapping that can be efficiently used for genetic studies and for the location of QTLs whereas it has several other advantages (Burr and Burr 1991; Alonso-Blanco *et al.* 1998; Broman 2005): once developed, $F_{6-10}$ recombinant inbred lines are fixed and stable, thus easily and homogeneously conserved by seed increasing; recombinant inbred lines ($F_{6-10}$) may be propagated continuously without further segregation and can be used for mapping by different researchers sharing genotyping data; and the greater probability of recombination during multiple meioses from generation to generation offers better chances for identification of linked markers or genes. In fact, Haldane and Waddington (1931) reported that the amount of observed recombination between very closely linked markers is twice higher for recombinant inbred lines than for populations derived from a single meiosis.

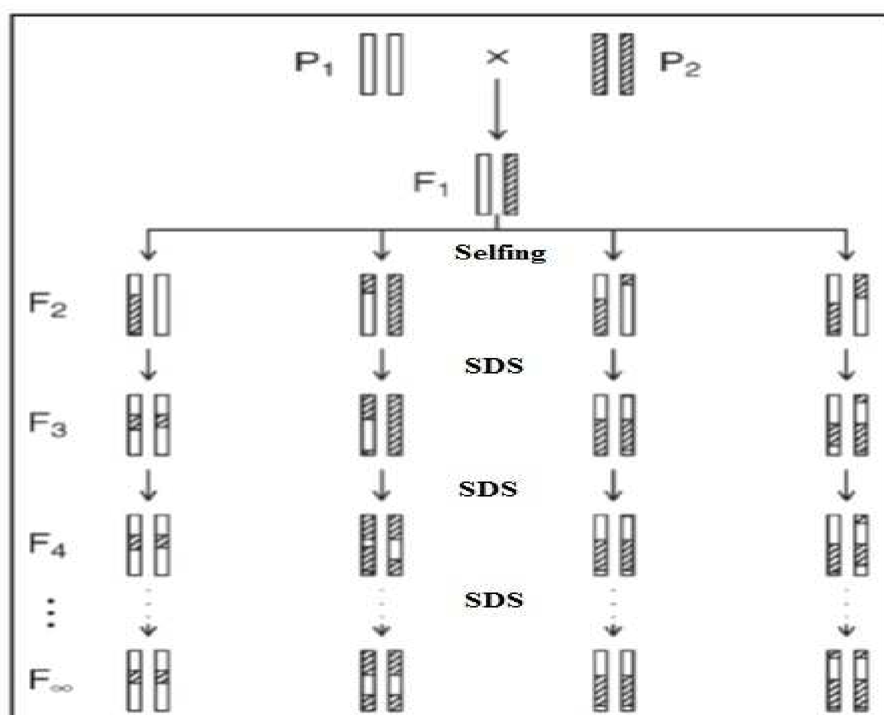


**Figure 2.5.** Mosaic structure of chromosomes of recombinant inbred lines used for linkage analysis (adapted from Broman 2005). [$P_1$ and $P_2$: parents 1 and 2, $F_1$: hybrid (generation 1), $F_n$: generation n, SDS: Single Seed Decent].

### 2.6.3. Family-based quantitative trait loci mapping methods

Quantitative trait loci (QTL) are associated with the domain of quantitative genetics and are genomic regions containing genes controlling particular quantitative traits (Collard *et al.* 2005). Identification of QTLs through statistical analysis of relationships between phenotypic variations of a quantitative trait and genotypic classes constructed based on allelic variations of a particular DNA marker, is known as QTL mapping (Paterson 1996b; Mauricio 2001). This method leads to identification of valuable alleles linked to a QTL to be ultimately used in marker-assisted selection based on the presence/absence of these alleles at specific markers as an efficient, resource- and time-effective substitute for (or to assist in) phenotypic selection in plant breeding programs.

The basic principle of QTL mapping is illustrated in Figure 2.6. After partitioning a mapping population into genotypic classes based on the presence/absence of different alleles at a given locus, significant differences between these classes according to the phenotypic variation of the quantitative trait are tested. If the difference is significant the marker is linked to a QTL, and conversely, if there is no significant difference between the two genotypic classes the marker is not linked to a QTL (Young 1996; Mauricio 2001).

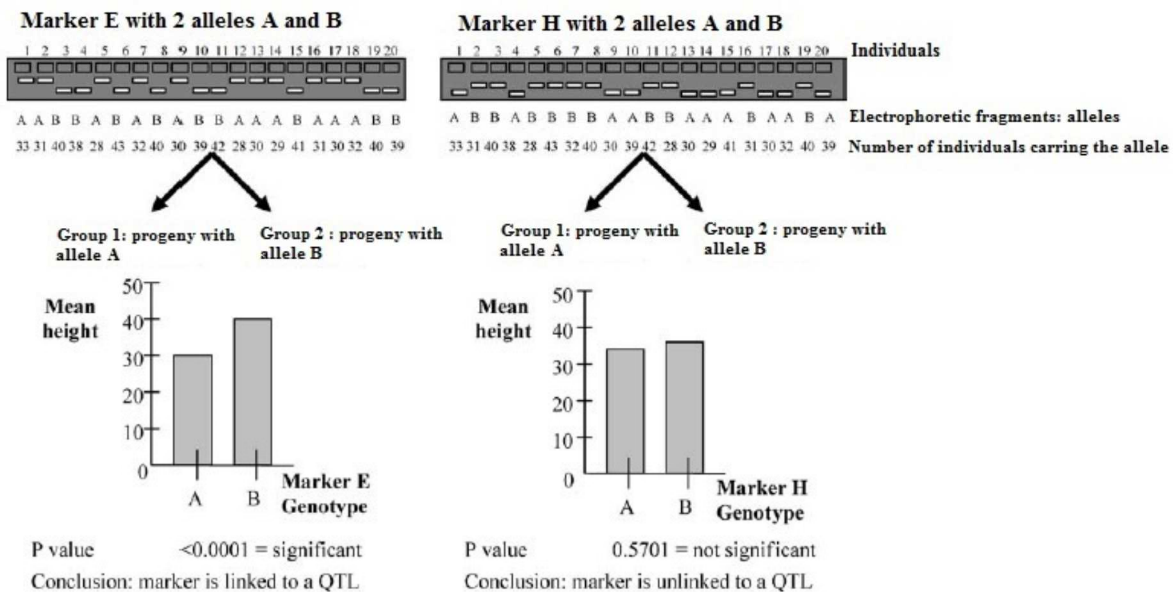


**Figure 2.6.** Basic principle of QTL mapping (adapted from Collard *et al.* 2005).

Different methods exist for identification of QTLs and estimation of their effects: *single-marker analysis (SM)*, *simple interval mapping (SIM)* and *composite interval mapping (CIM)*. Single-marker analysis is the simplest method and does not require any prior genetic maps. It can be based on t-tests, ANOVA and linear regression analysis. The coefficient of determination $R^2$ explains the phenotypic variation from the QTL linked to the marker. However, recombination may occur between the marker and the QTL. Thus, the weakness of this method is that detection of a QTL decreases when the closest marker is located further from the QTL. Also, the effect of the QTL could be underestimated (Tanksley 1993; Mauricio 2001). Kruskal-Wallis test (Lehmann 1975) is one of the commonly used single-marker analysis methods. It is based on a locus-by-locus analysis for QTL mapping. It is a non-parametric, one-way analysis of variance (Van Ooijen 2004). For each marker, genotypic classes are created and individuals are ranked according to the quantitative trait under study. Significant differences in average ranks between genotypic classes indicate the presence of a QTL linked to the marker under consideration. The higher the numbers of individuals and genotypic classes, the more powerful the test is.

Simple interval mapping is more powerful than single-marker mapping (Lander and Botstein 1989). It is based on searching within a chromosomal interval of a genetic map for QTLs between two adjacent markers, thus limiting the effect of possible recombination between QTLs and markers. Probabilities of the presence of a QTL that affects the trait studied between each pair of two markers are calculated and a likelihood-ratio test statistic is applied to estimate the corresponding LOD score. Above a given LOD score threshold (usually ≥3 or determined using a permutation test), a likelihood-ratio is considered to be significant. A QTL is then declared to

be located between the flanking markers. The LOD score is calculated using the expectation maximization algorithm based on the following formula:

$$\boldsymbol{LOD\ score} = \mathbf{log_{10}} \frac{\boldsymbol{Probability\ that\ a\ QTL\ is\ present}}{\boldsymbol{Probability\ that\ a\ QTL\ is\ absent}} = \mathbf{log_{10}} \frac{\boldsymbol{L_1}}{\boldsymbol{L_0}}$$ (Morton 1955; Van Ooijen 2004).

Where, $\boldsymbol{L_1} = \prod_{n=1}^{N} \sum_{q=1}^{Q} \boldsymbol{\pi_q f_q(x_n)}$ and $\boldsymbol{L_0} = \prod_{n=1}^{N} \sum_{q=1}^{1} \boldsymbol{\pi_1 f_q(x_n)}$. $L_1$ and $L_2$ are the likelihoods under the hypothesizes that a QTL is present and absent, respectively. *N* is the population size, *Q* is the number of QTL genotype determining the number of component distributions (depends on population type, 3 for recombinant inbred lines), $f_q(x_n)$ is the component densities and $\pi_q$is their probabilities. When there is no QTL, there is only one component distribution (*Q=1,* no QTL is assumed to be segregating) (Van Ooijen 2004).

However, this method (*SIM*) can lead to false positive QTLs if other QTLs are linked to the interval of adjacent markers being considered (Mauricio 2001).

Composite interval mapping, also called *multiple-QTL models (MQM)*, is a powerful method based on a multiple linear regression model of a quantitative phenotype on genotype (putative QTLs and markers) (Jansen and Stam 1994). It combines the two methods described here above: simple interval mapping and linear regression. In addition to the two adjacent linked markers used for simple interval mapping, additional markers are included (Zeng 1994; Jansen and Stam 1994). Probability of presence of a QTL between two markers is tested using the LOD score as described above, considering other markers outside the interval that are significantly associated with the trait (from *SIM*) as cofactors reducing the residual variation and enhancing the power of QTL detection. Subsequent analysis is performed after adding cofactors and those for which the model performs better (more QTL detected, higher LOD scores and percentage of explained variance) are kept and will serve as covariates during mapping. Because when taking several segregating QTLs into account while testing for a QTL at a given position, their respective variances will be considered thus reducing the residual variance. Curves of LOD scores obtained for each linkage group are used to identify the most likely position of a QTL corresponding to the position where the highest LOD value is obtained (Figure 2.7). *CIM* (or *MQM*) method offers also the possibility of the completion of missing genotypic observations using genetic information from markers surrounding the assumed QTL map position (Jansen and Stam 1994; Van Ooijen 2004).

Permutation tests are used to identify appropriate LOD score thresholds (Van Ooijen 2004). This permutation test is a resampling approach without replacement and interval mapping is performed on the obtained data giving the frequency distributions of the maximum LOD score under the null-hypothesis (no QTL) after a large number of iterations (commonly 1000). Over

each iteration, the marker data remain fixed while the quantitative trait data are permuted over all individuals, thus testing any possible association with the respective markers. Subsequently, the LOD score significance threshold is determined based on a given P-value by the interval mapping method. For instance, for a P-value of 0.05, the relative cumulative count of the permutation test results above 0.95 is taken to find the corresponding LOD score, which is the significance value.

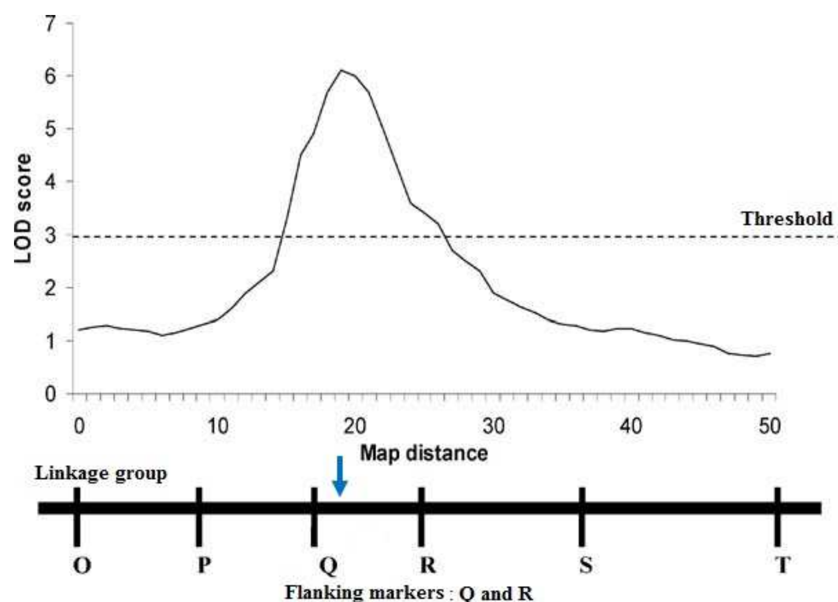


**Figure 2.7.** Illustration of the LOD profile from a composite interval mapping exercise showing the most probable position of a QTL (blue arrow) and the two flanking markers in a linkage group (adapted from Collard *et al.* 2005).

### 2.6.4. Linkage disequilibrium-based quantitative trait loci analysis and association mapping

Genetic dissection of complex traits such as quantitative characteristics is based on two major tools: linkage analysis (as described above in section 2.6.3) and association mapping (Zhu *et al.* 2008). Marker-trait association mapping and QTL detection methods based on linkage disequilibrium in non-related accessions are characterized by greater precision and higher resolution than family-based linkage analysis methods (Figure 2.8) (Mackay and Powell 2007). The latter authors defined linkage disequilibrium as a non-random association of alleles at separate loci located on the same chromosome. Association mapping evidences the significant association of a molecular marker with a phenotypic trait (Gupta *et al.* 2005). Natural diversity of genetically diverse genotypes in plants, evolution history and domestication events result in marker-trait associations. Thus, association mapping makes use of the degree of linkage disequilibrium between DNA markers and functional characteristics in a set of landraces, local

accessions or elite lines to find and locate genes and QTLs related to these traits by identifying closely linked markers (Zhu *et al.* 2008; Sorkheh *et al.* 2008).

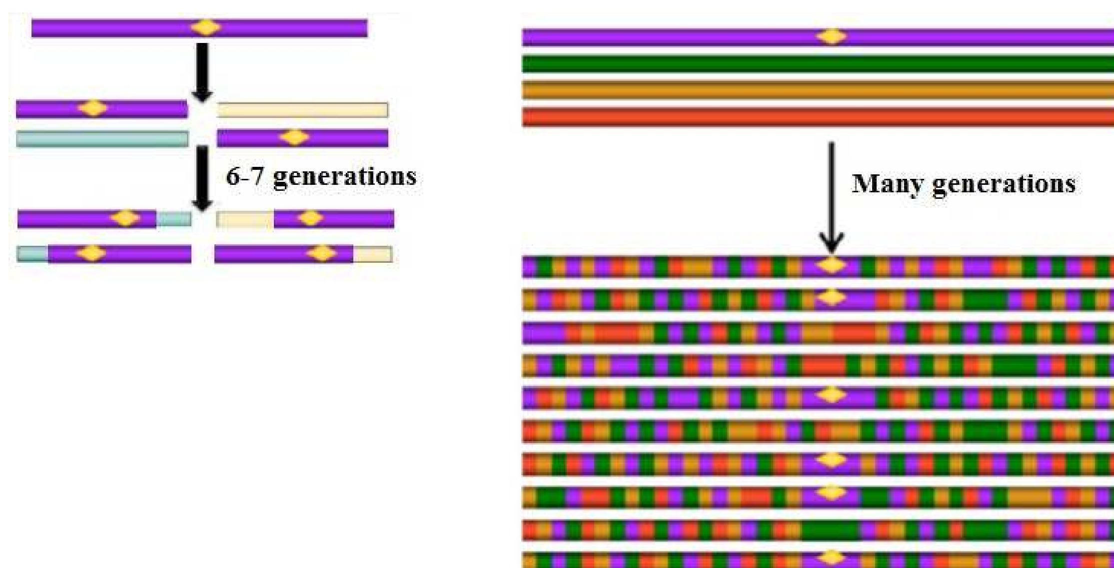


**Figure 2.8.** Association mapping based on linkage disequilibrium from unrelated accessions (right) compared to linkage mapping based on segregating populations (left) (adapted from Cardon and John 2001; Zhu *et al.* 2008). [yellow diamond: functional locus or marker closely linked to the genomic region of interest].

Two approaches for association mapping studies exist: genome-wide association mapping where a large number of DNA markers are tested for association with different traits, and candidate gene association mapping where prior known information about candidate genes related to the trait studied from previous linkage, pathway, biochemical, or physiological analyses are used (Zhu *et al.* 2008).

Recent advances in pulse molecular biology offer opportunities of association studies for identification of DNA markers linked to targeted traits in breeding programs (Bohra *et al.* 2014). We report here some examples. Fedoruk *et al.* (2013) using SNPs and SSRs markers on an association panel of lentil reported significant marker/trait associations related to seed quality and flowering time that could be used for marker-assisted selection and further candidate gene analysis. Thudi *et al.* (2014) used genome-wide and candidate gene-based association mapping approaches for genetic dissection of drought and heat tolerance in chickpea. They found 312 significant marker/trait associations related to root traits, heat tolerance, yield and its components that can be used, after validation, in chickpea breeding programs. Also in chickpea, Diapari *et al.* (2014) identified SNP alleles associated with seed iron and zinc concentrations using 94 different accessions. Ahmad *et al.* (2015) identified SSR markers linked to lipid content in pea by analyzing genetic diversity, population structure and

association mapping. Many putative QTLs for 13 morphological and physiological traits related to frost tolerance in faba bean were identified by Sallam and Martsch (2015) based on association mapping using a general linear model and mixed linear model.

### 2.6.5. Use of quantitative trait loci for breeding purposes

Many economically important traits in plants such as yield and drought tolerance are quantitatively inherited thus are under polygenic control. Phenotypic selection used in conventional plant breeding for the evaluation of large populations requires substantial time and resources. Furthermore, phenotypic expression of a specific quantitative trait is affected by the environment. As a result, QTL analysis aiming to find and map genomic regions involved in such trait variation is important for breeding purposes. QTL mapping permits to link observable DNA markers to desirable phenotypic characteristics that could be used for screening in a markers-assisted selection (MAS) approach (Kumar *et al.* 2015). Large plant populations could be tested using these markers at seedling stage to select potential individuals for final evaluation under field conditions (Collard and Mackill 2008). These could lead to accelerated variety development and more efficient use of resources. Examples of the use of MAS for breeding purposes were reported by Barloy *et al.* (2007) for cereal cyst nematode resistance genes and Varshney *et al.* (2014b) to introgress QTLs conferring resistance to *fusarium wilt* race 1 and *ascochyta blight* in an elite cultivar of chickpea. Also, QTLs associated with virus resistance and plant architecture were applied to MAS in pea (Smýkal *et al.* 2012). In rice, a model cereal crop, MAS benefited from advances in genomics thus it is being widely used in breeding programs targeting QTLs of agronomic traits, cooking and nutritional quality as well as several biotic stresses (Mackill 2007).

For lentil, several QTLs were identified for a number of important traits: winter hardiness (Kahraman *et al.* 2004), height of the first ramification (Duran et al. 2004), *Fusarium wilt* resistance (Hamwieh et al. 2005), *Ascochyta blight* (Taylor *et al.* 2006; Gupta *et al.* 2012b; Sari 2014), earliness and plant height (Tullu *et al.* 2008), *Stemphylium blight* (Saha *et al.* 2010), days to 50 % flowering, plant height, 100-seed weight and seed diameter (Saha *et al.* 2013), seed size, seed plumpness and thickness (Fedoruk *et al.* 2013), boron tolerance (Kaur *et al.* 2014). Among these QTLs, those linked with *fusarium wilt* resistance and boron tolerance were found to be closed to SSR and SNP markers, respectively, thus they could be useful for MAS (Kumar *et al.* 2015). The recent advances in lentil genomics and genome sequencing resulting in more efficient markers could help to use MAS in breeding programs in coming years.

# Chapter 3. Genetic diversity analysis of Moroccan lentil (*Lens culinaris* Medik.) landraces using simple sequence repeat and amplified fragment length polymorphisms reveals functional adaptation towards agro-environmental origins.

Based on:

Idrissi, O.[1,2], Udupa, S.M.[3], Houasli, C.[2], De Keyser, E.[4], Van Damme, P.[1,5], De Riek, J.[4] (2015). Genetic diversity analysis of Moroccan lentil (*Lens culinaris* Medik.) landraces using simple sequence repeat and amplified fragment length polymorphisms reveals functional adaptation towards agro-environmental origins. Plant Breeding, 134, 322-332. Doi:10.1111/pbr.12261.

1 Department of Plant Production, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.
2 Institut National de la Recherche Agronomique du Maroc (INRA), Centre Régional de Settat, P.O. Box 589, Settat, Morocco.
3 ICARDA-INRA Cooperative Research Project, International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 6299, Rabat, Morocco.
4 Institute for Agricultural and Fisheries Research (ILVO) Plant Sciences Unit, Applied Genetics and Breeding, Caritasstraat 21, 9090 Melle, Belgium.
5 Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Kamycka 129, 165 21 Prague 6- Suchdol, Czech Republic.

# Chapter 3. Genetic diversity analysis of Moroccan lentil (*Lens culinaris* Medik.) landraces using Simple Sequence Repeat and Amplified Fragment Length Polymorphisms reveals functional adaptation towards agro-environmental origins

## 3.1. Introduction

Lentil (*Lens culinaris* Medik.) is a self-pollinated diploid annual food legume domesticated in the foothills of the mountains between Turkey and Syria in the Eastern Mediterranean (Ladizinsky 1979, 1987). It is believed that lentil is one of the earliest domesticated crops, with oldest remains dating back to 13 000 years BC (Sandhu and Singh 2007) probably belonging to wild lentil as suggested by Ladizinsky (1987) and later by Sonnante *et al.* (2009) in a review about the origin and domestication of lentil. Among grain legumes, it could be the oldest domesticated crop (Bahl *et al.* 1993). Ladizinsky (1987) in his famous work about pulse domestication suggested that cultivation may have started before complete domestication. Seed dormancy and pod indehiscence were the main targeted traits during the domestication process (Ladizinsky 1987). Selection for specific adaptation to different environments and for seed traits and other characteristics occurred later (Sonnante *et al.* 2009). After domestication, lentil spread to Greece, Central Europe, Egypt, Central Asia and India. Lentil probably reached North Africa, Spain and the Italian islands of Sardinia and Sicily eventually either from Central Europe or the Levant (Sonnante and Pignone 2001; Faratini *et al.* 2011). After the discovery of the New World, lentil was introduced into North and South America and more recently to Australia (Ladizinsky 1979; Cubero 1981; Ferguson and Erskine 2001).

In Morocco, lentil is currently grown as a rain fed crop in rotation with cereals occupying around 14 % of the area yearly cultivated by food legumes in the country. Within the legume group, it ranks third, after faba bean (42 %) and chickpea (20 %) contributing to the sustainability of the cereal-based cropping systems (Ministère de l'Agriculture et de la Pêche Maritime 2012). Morocco is one of the countries where lentil has been traditionally grown for several centuries. Cultivation is based on local germplasm and landraces selected by farmers over many years for their specific adaptation under different environments (climate, soil type, dryland farming, more favourable regions and highlands) and for their seed characteristics (mainly cooking time, seed colour and seed size) in relatively small areas.

Morocco is geographically located in the northwest corner of Africa in a meeting area of very distinct natural blocks: the Mediterranean Sea in the north, the Atlantic Ocean in the west and northwest and the desert in the southeast. This explains the high range of bio-climates occurring

in the country: humid, sub-humid, Saharan, arid and semi-arid and highland climate in the Rif, the middle and high Atlas mountains (Saidi *et al.* 2007). The diversity of climatic conditions in Morocco resulted in a rich diversity in agro-environmental zones where crops experience different biotic and abiotic stresses like drought, high temperatures and cold (Direction Nationale de la Météorologie du Maroc 2014). Hence, high spatial heterogeneity of total rainfall and temperature in Morocco determines the classification and diversity of vegetation (Augustin 1921; Balaghi *et al.* 2013). Morocco is one of the hotspots countries for crop diversity of 35 food crops, including lentil, listed in the international treaty of plant genetic resources for food and agriculture (Bellon *et al.* 2014; Figure A.5, appendix). However, the genetic diversity of the local material and landraces has not been studied and is still not well known. There are a limited number of reports on morphological characterization of Moroccan lentil germplasm, but thus far, molecular characterization has not been reported. Molecular markers including simple sequence repeat (SSR, microsatellite) and amplified fragment length polymorphism (AFLP) were applied successfully and were reported as efficient methods for studying the genetic diversity of lentil landraces from diverse origins (Abo-Elwafa *et al.* 1995; Ferguson *et al.* 1998; Sonnante and Pignone 2001; Sonnante *et al.* 2003; Duran and Perez de la Vega 2004; Hamwieh *et al.* 2005; Sultana and Ghafoor 2008; Liu *et al.* 2008; Babayeva *et al.* 2009, Toklu *et al.* 2009, Bacchi *et al.* 2010, Reddy *et al.* 2010, Datta *et al.* 2011; Alo *et al.* 2011; Zaccardelli *et al.* 2011).

This study investigates the genetic diversity among 51 lentil landraces collected from diverse regions in Morocco and maintained in the Moroccan gene bank located at the National Institute of Agricultural Research of Settat (INRA, Morocco) using SSR and AFLP markers. The two earliest selected local cultivars L24 and L56 of the INRA Morocco breeding program were included in the study in order to determine their relationship to other landraces. This study also aims to analyze functional genetic differentiation among the landraces according to their agro-environmental origins and to a number of adaptive traits. The overall goal is to provide information for defining targeted germplasm conservation strategies and valorization and also provide information for use in breeding programs.

## 3.2. Materials and methods

### 3.2.1. Plant material

Fifty-one landraces from five different regions of Morocco known for their long-standing growing tradition were evaluated for genetic diversity: Abda, western-central; Chaouia, north-central; Sais Meknes, northern; Zaer, north-western and Middle Atlas mountains, central-

Morocco (Figure A.3, appendix). Two local cultivars (L24 and L56), representing the first lentil cultivars registered in Morocco, were also included. All accessions are maintained by the Moroccan gene bank at INRA Settat, Morocco. We also included four Moroccan landraces of unknown origin that had been repatriated to Moroccan gene bank from the International Center for Agricultural Research in Dry Areas (ICARDA) (Table 3.1). Three contrasted agro-environments represent the origins of the landraces (Table 3.2; Figures 3.1 and A.4, appendix).

### 3.2.2. DNA extraction

All seeds were planted in the greenhouse, and young leaves were collected from 2 to 3-week-old plantlets and lyophilized. As landraces could be composed of a mixture of different genotypes and as this study aims to estimate genetic diversity among landraces rather than within each landrace, DNA was isolated from five single plants from each landrace.

Genomic DNA was isolated according to the NucleoSpin Plant (MACHEREY-NAGEL, MN; Duren, Germany) kit protocol as follows. Tissue Lyser (Qiagen; Manchester, United Kingdom) was used to homogeinize 20 mg of dry weight (lyophilized) plant material. Then 450 µl of PL2 lysis buffer was added to the resulting powder allowing to solubilize the cell membrane and therefore release DNA. Tubes were then mixed thoroughly and 15 µl of RNase A was added to remove RNA before incubating the mixture for 30 min at 65 °C. After adding 112.5 µl of PL3 buffer and mixing, tubes were incubated for 5 min on ice in order to precipitate SDS completely followed by 5 min of 14000 rpm centrifugation step. The obtained crude lysate was loaded onto the column of NucleoSpin® Filter and centrifuged for 2 min at 11000 rpm to collect the clear flow-through. For adjusting DNA binding conditions, 675 µl of PC buffer was added and tubes were mixed thoroughly. After that, a maximum of 700 µl of each sample was loaded to a new collection of tubes using the NucleoSpin® Plant II Column followed by a centrifugation step for 1 min at 11000 rpm. Wash buffers PW1 (2 washs of 400 µl and 700 µl followed by a centrifugation of 1 min at 11000 rpm) and PW2 (200 µl, 2 min at 11000 rpm centrifugation) were used to wash away contaminants and dry the silica membrane. Finally, genomic DNA was eluted with low salt elution buffer PE (65°C) with a twice repeated step of adding 50 µl, incubation at 65°C for 5 min and a centrifugation of 1 min at 11000 rpm.

Concentration and quality of DNA were verified using a NanoDrop Spectrophotometer ND-1000 (Isogen; De Meern, The Netherlands). Isolated DNA was diluted to 15 ng/µl and stored at -20 °C. The experiments were carried out at ILVO-Melle, Belgium during 2013.

**Table 3.1.** List of lentil plant material analyzed, their respective origins, days to flowering, days to maturity, early vegetative vigour, 100-seed weight and seed type

| Accession Code | Locality | Days to 50 % flowering | Days to maturity | Early vegetative vigour * | 100-seed weight (g) | Seed type ** |
|---|---|---|---|---|---|---|
| MGB1000 | Chaouia (I) | 113 | 150 | 3 | 4.08 | m |
| MGB1013 | Chaouia (I) | 115 | 149 | 4 | 4.25 | m |
| MGB1015 | Chaouia (I) | 110 | 152 | 4 | 4.38 | m |
| MGB1016 | Chaouia (I) | 109 | 155 | 4 | 4.72 | M |
| MGB1017 | Chaouia (I) | 112 | 156 | 3 | 4.04 | m |
| MGB1019 | Chaouia (I) | 113 | 149 | 4 | 4.60 | M |
| MGB1020 | Chaouia (I) | 109 | 148 | 2 | 4.32 | m |
| MGB1022 | Chaouia (I) | 114 | 150 | 4 | 4.56 | M |
| MGB1023 | Chaouia (I) | 115 | 145 | 3 | 4.43 | m |
| MGB1024 | Chaouia (I) | 113 | 154 | 4 | 4.03 | m |
| MGB1025 | Chaouia (I) | 115 | 151 | 3 | 4.44 | m |
| MGB1029 | Chaouia (I) | 111 | 154 | 4 | 3.47 | m |
| MGB1030 | Chaouia (I) | 114 | 154 | 4 | 3.59 | m |
| MGB1031 | Chaouia (I) | 116 | 156 | 3 | 4.48 | m |
| MGB1032 | Chaouia (I) | 114 | 156 | 4 | 3.18 | m |
| MGB1034 | Chaouia (I) | 115 | 161 | 4 | 3.17 | m |
| MGB1035 | Chaouia (I) | 111 | 158 | 4 | 4.32 | m |
| MGB1036 | Chaouia (I) | 114 | 156 | 3 | 4.35 | m |
| MGB1045 | Chaouia (I) | 117 | 152 | 4 | 4.42 | m |
| MGB1049 | Chaouia (I) | 114 | 154 | 4 | 4.28 | m |
| MGB1050 | Chaouia (I) | 108 | 150 | 3 | 3.83 | m |
| MGB1051 | Zaer (II) | 113 | 166 | 2 | 3.32 | m |
| MGB1052 | Zaer (II) | 118 | 162 | 1 | 4.46 | m |
| MGB1053 | Zaer (II) | 115 | 177 | 2 | 3.34 | m |
| MGB1054 | Zaer (II) | 116 | 175 | 2 | 4.44 | m |
| MGB1055 | Zaer (II) | 118 | 170 | 3 | 5.16 | M |
| MGB1056 | Zaer (II) | 120 | 175 | 2 | 4.22 | m |
| MGB1058 | Zaer (II) | 117 | 173 | 3 | 4.24 | m |
| MGB1008 | Middle Atlas mountains (III) | 113 | 163 | 3 | 5.03 | M |
| MGB1010 | Middle Atlas mountains (III) | 120 | 160 | 3 | 4.87 | M |
| MGB1043 | Middle Atlas mountains (III) | 109 | 158 | 4 | 4.29 | m |
| MGB1044 | Middle Atlas mountains (III) | 114 | 165 | 3 | 4.49 | m |
| MGB996 | Middle Atlas mountains (III) | 116 | 163 | 4 | 4.03 | m |
| MGB997 | Middle Atlas mountains (III) | 114 | 161 | 3 | 2.97 | m |
| MGB999 | Middle Atlas mountains (III) | 116 | 160 | 3 | 4.10 | m |
| MGB1026 | Abda (IV) | 95 | 131 | 4 | 2.58 | m |
| MGB1027 | Abda (IV) | 101 | 135 | 5 | 3.05 | m |
| MGB1037 | Abda (IV) | 97 | 131 | 3 | 3.31 | m |
| MGB1038 | Abda (IV) | 100 | 129 | 5 | 4 | m |
| MGB1039 | Abda (IV) | 95 | 126 | 4 | 3.75 | m |
| MGB1040 | Abda (IV) | 96 | 124 | 5 | 3.97 | m |
| MGB1041 | Abda (IV) | 102 | 130 | 4 | 4.32 | m |
| MGB1042 | Abda (IV) | 105 | 131 | 4 | 4.60 | M |
| MGB1047 | Abda (IV) | 94 | 123 | 4 | 4.58 | M |
| MGB1060 | Sais-Meknès (V) | 114 | 177 | 1 | 3.62 | m |
| MGB1061 | Sais-Meknès (V) | 118 | 172 | 2 | 4.36 | m |
| MGB1062 | Sais-Meknès (V) | 114 | 173 | 3 | 3.53 | m |
| L24 | Local cultivar (VI) | 128 | 170 | 3 | 3.58 | m |
| L56 | Local cultivar (VI) | 125 | 165 | 3 | 4.72 | M |
| MGB7377 | Unknown *** (VII) | 118 | 160 | 3 | 4.36 | m |
| MGB7386 | Unknown *** (VII) | 118 | 166 | 3 | 4.11 | m |
| MGB7389 | Unknown *** (VII) | 116 | 165 | 3 | 3.25 | m |
| MGB7457 | Unknown *** (VII) | 114 | 158 | 3 | 3.45 | m |

* Early vegetative vigour: 1=very poor, 2=poor, 3=average, 4=good, 5=very good (Kumar *et al.* 2012); **: *m* refers to microsperma (smaller seeds with 100-seed weight less than 4.5g) and *M* to macrosperma (larger seeds with 100-seed weight more than 4.5g); *** Unknown origin: Moroccan landraces repatriated from ICARDA. I to VII refer to collection localities. MGB refers to Moroccan Gene Bank.

**Table 3.2.** Ago-environmental origins of the Moroccan lentil landraces according to their geographical origins

| | Agro-environments | | | | |
|---|---|---|---|---|---|
| | Dry areas | | Favourable areas | | Highlands |
| **Localities** | **Chaouia** | **Abda** | **Zaer** | **Sais-Meknes** | **Middle Atlas mountains** |
| **Average annual precipitation (mm) * and main characteristics** | 200-300<br>Frequent drought and heat stress | | 300-500<br>Favourable climatic conditions | | >500<br>Altitude of more than 1300 m<br>Frequent cold stress |

*(Direction Nationale de la Météorologie du Maroc, 2014)

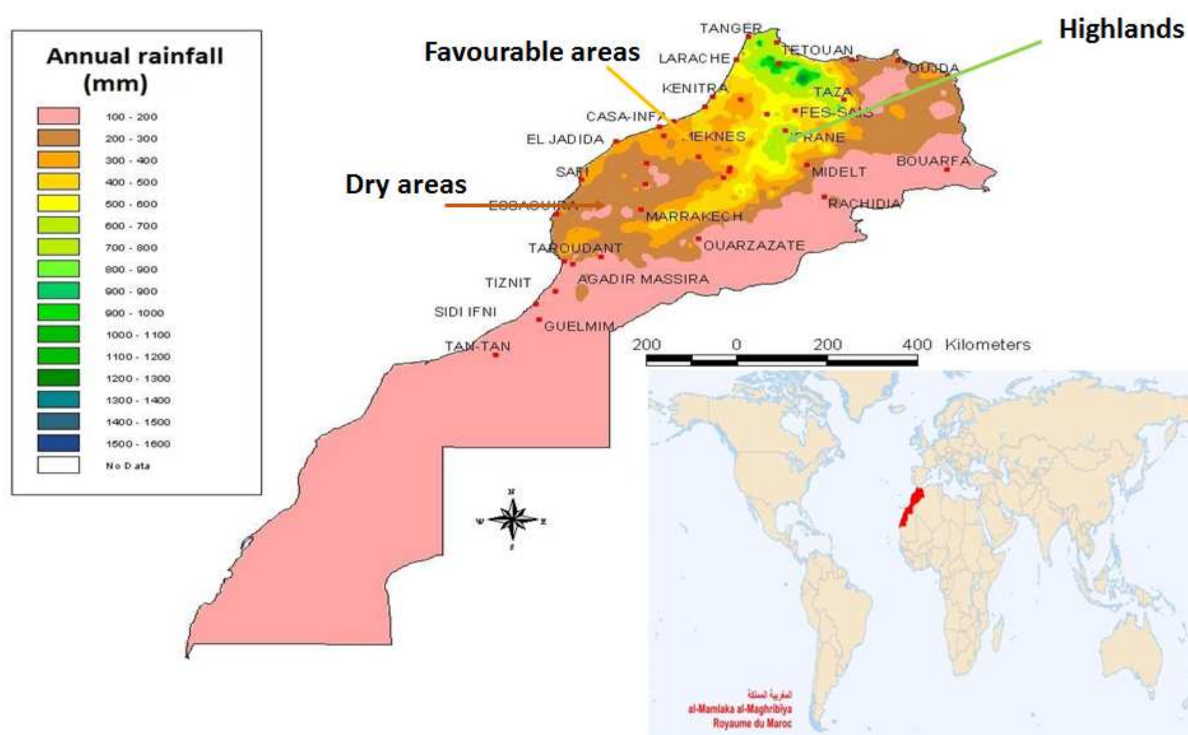


**Figure 3.1.** Agro-climatic zones of Morocco (adapted from Direction de la météorologie nationale du Maroc; http://www.marocmeteo.ma/).

### 3.2.3. SSR analysis

Thirty microsatellite markers developed by Hamwieh *et al.* (2005) were evaluated in this study. All SSRs were first tested for amplification and polymorphism on a subset of 16 DNA samples. Based on the published polymerase chain reaction (PCR) conditions (Hamwieh *et al.* 2005), annealing temperature (*Ta*) and number of PCR cycles were optimized for each marker to produce clear and reproducible microsatellite profiles. Of the 30 tested SSRs, 19 were polymorphic and as such selected for further use in this study (Table 3.3). PCR analysis was performed according to the Qiagen Multiplex PCR kit protocol with a final volume of 10 µl per reaction. Each reaction mix contained 5 µl of 2x Qiagen MultiPlex Mastermix (Multiplex PCR

Kit; Qiagen; Manchester, United Kingdom), 0.2 µl of each primer (10 µM), RNase-free water and 1 µl of DNA (15 ng/µl). Different multiplex sets, with similar reaction conditions, were composed containing two or three microsatellites. Forward primers were labelled fluorescently (FAM, HEX and NED, Table 3.3).

PCR was conducted in a GeneAmp 9700 Dual thermocycler. The Hot StarTaq DNA polymerase enzyme was activated with a heating step of 15 min at 95 °C, followed by 25 or 30 cycles (Table 3.3) of 30 s at 94 °C (denaturation), 90 s at *Ta* (annealing, table 3.3) and 60 s at 72 °C (extension) with a final extension step of 30 min at 60 °C. Samples were then stored at -20°C and protected against light. Of the final PCR product, 1 µl was mixed with 13.5 µl Hi-DiTM Formamide (Applied Biosystems; Carlsbad, California, USA) and 0.5 µl of the GeneScanTM-500 Rox Size Standard (Applied Biosystems; Carlsbad, California, USA). Products were denatured by heating for 3 min at 90 °C. Capillary electrophoresis and fragment detection were performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). GENEMAPPER 4.0 software (Applied Biosystems) was used for scoring the alleles.

### 3.2.4. AFLP analysis

The AFLP protocol (Vos *et al.* 1995) was followed according to De Riek *et al.* (2001), with minor modifications. The analysis was performed in three steps according to the Applied Biosystems kit protocol: restriction-adaptor ligation, pre-amplification and selective amplification. The restriction-adaptor ligation step was carried out using 5µl of 5x reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM MgAc, 250 mM Kac), 0.25 µl MseI (10 U/µl), 0.17 µl EcoRI (5 U/µl), 0.33 $H_2O$, 19.25 µl of diluted DNA (15 ng/µl) and 24 µl adaptor-ligation solution (19.96 µl $H_2O$, 0.24 µl 1M MgAc, 0.60 µl 2M KAc, 0.24 µl 1M Tris-HCl pH 7.5, 0.96 µl 10 mM ATP, 1 µl MseI-adaptor 50 pmol/µl (MesI-ad 14 : TACTCAGGACTCAT and MseI-ad 16: GACGATGAGTCCTGAG), 1 µl EcoRI-adaptor 5pmol/µl (EcoRI-ad 17: CTCGTAGACTGCGTACC and EcoRI-ad 18: AATTGGTACGCAGTCTAC) and 1 µl T4-DNA-ligase (1 U/µl). The pre-amplification step was performed in 50 µl reaction mix containing 10 µl of 5xFlexi PCR buffer (Promega; Madison, Wisconsin, USA), 3 µl of 1.5 mM $MgCl_2$, 29 µl $H_2O$, 0.5 µl of EcoRI+A (50 ng/µl), 0.5 µl of MseI+C (50 ng/µl), 1.75 µl of 5 mM d'NTP's, 0.25 µl of Flexi Taq-polymerase (5 U/µl) (Promega) and 5 µl of the digest from the restriction–adaptor ligation reaction. The selective amplification step was carried out in 20 µl total volume using 2 µl of 10xPCR buffer, 0.20 µl of 20mM d'NTP's, 12.33 µl of $H_2O$, 1.35 µl of 1 µM EcoRI-primer labeled with fluorescent dye (FAM, HEX), 1µl of 5 uM MseI-primer, 0.12 µl of Taq-polymerase (5 U/µl) and 3 µl of pre-amplification product.

The quality of the restriction-adaptor ligation and pre-amplification were checked by loading, respectively, 2 µl and 5 µl of the reaction of each sample on a 1.5 % agarose gel in 1x Tris Acetate EDTA (TAE) along with λPst for 1 h. After staining with ethidium bromide (100 µl of 10 mg/ml in 2 l $H_2O$), gels were visualized under UV-light and corresponding images were stored. The quality of migration of bands (DNA fragments) were examined before proceeding with next steps. The pre-amplification PCR settings program were: 25 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min. While the selective amplification PCR settings program were: 1 cycle at 94°C for 2 min, 65°C for 30 s, 72°C for 2 min; followed by 8 cycles of 94 °C for 1 s, 64°C to 56°C for 30 s (annealing temperature was decreased by 1°C/cycle), 72°C for 2 min, and a final step of 23 cycles of 94°C for 1 s, 56°C for 30 s, 72°C for 2 min. Samples were then stored at -20°C and protected against light.

A total of 12 primer combinations were tested: *EcoRI-ACA* + *MseI-CAG, EcoRI-ACA* + *MseI-CTG, EcoRI-ACA* + *MseI-CTT, EcoRI-ACG* + *MseI-CAA, EcoRI-AGC* + *MseI-CAA, EcoRI-AGC* + *MseI-CAG, EcoRI-AGC* + *MseI-CTG, EcoRI-ACT* + *MseI-CAA, EcoRI-ACT* + *MseI-CTT, EcoRI-AAG* + *MseI-CAT, EcoRI-AAT* + *MseI-CGG* and *EcoRI-ACT* + *MseI-CAG*. Fragments were separated, sized and visualized as described above for SSRs.

### 3.2.5. Agronomic characterization

The phenotypic characteristics (Table 3.1) used in this study were recorded in a field trial during 2010 at *Sidi El Aidi* INRA Morocco research station (a dry site with an annual average rainfall of 250 mm and Vertic Calcixeroll soil; altitude 230 m, 33.17°N, 7.40°W). The trial was performed under rainfed conditions and the rainfall during this season was 393 mm. Each landrace was sown in two lines of 4 m length and 0.35 m between lines in a completely randomized block design with three replications. Early vegetative vigour (Kumar *et al.* 2012), days to flowering and to maturity and seed type were recorded as shown in table 3.1.

**Table 3.3.** Primer sequences and PCR conditions used for the amplification of the microsatellites in the Moroccan lentil landraces and local cultivars

| Locus name | Primer sequences (5'-3-) | | Repeat unit | *Ta* (°C) | Alleles size range (bp) | No of cycles | PCR multiplex set | Fluorescent label |
|---|---|---|---|---|---|---|---|---|
| | Forward | Reverse | | | | | | |
| SSR113 | CCGTAAGAATTAGGTGTC | GGAAAATAGGGTGGAAAG | (AC)17(AT)13 | 53 | 211-245 | 25 | 1 | NED |
| SSR154 | GGAATTTATCACACTATCTC | GACTCCCAACTTGTATG | (AC)3ATAG(AC)7(AT)2 | 53 | 261-381 | 25 | 1 | FAM |
| SSR199 | GTGTGCATGGTGTGTG | CCATCCCCCTCTATC | (GT)4GC(GT)8GC(GT)3 | 53 | 180-211 | 25 | 2 | FAM |
| SSR124 | GTATGTGACTGTATGCTTC | GCATTGCATTTCACAAACC | (TGC)3+(GT)9TA(TG)2 | 56 | 174-177 | 25 | 3 | NED |
| SSR233 | CTTGGAGCTGTTGGTC | GCCGCCTACATTATGG | (GT)9 | 56 | 126-159 | 25 | 3 | HEX |
| SSR80 | CCATGCATACGTGACTGC | GTTGACTGTTGGTGTAAGTG | (TC)14(AC)12(AT)2 | 60 | 129-157 | 25 | 4 | FAM |
| SSR184 | GTGTGTACCTAAAGCCTTG | GTAAGTTGATCAAACGCCC | (GT)10(AT)15(GT)19 | 60 | 216-271 | 25 | 5 | FAM |
| SSR48 | CATGGTGGAATAGTGATGGC | CTCCATACACCACTCATTCAC | (TG)13 | 60 | 163-195 | 25 | 5 | HEX |
| SSR19 | GACTCATACTTTGTTCTTAGCAG | GAACGGAGCGGTCACATTAG | (TG)14 | 60 | 255-276 | 25 | 6 | HEX |
| SSR99 | GGGAATTTGTGGAGGGAAG | CCTCAGAATGTCCCTGTC | (TG)8TC(TG)2 | 60 | 153-164 | 25 | 6 | FAM |
| SSR302 | CAAGCCACCCATACACC | GGGCATTAAGTGTGCTGG | (TA)15(CA)11 | 60 | 231-276 | 30 | 7 | FAM |
| SSR309-2 | GTATGTCGTTAACTGTCGTG | GAGGAAGGAAGTATTCGTC | (AT)3GT(TA)3T(TAT)6 | 50 | 171-193 | 25 | 8 | FAM |
| SSR204 | CACGACTATCCCACTTG | CTTACTTTCTTAGTGCTATTAC | (TG)4+(AC)7 b | 56 | 177-195 | 30 | 9 | HEX |
| SSR336 | GTGTAACCCAACTGTTCC | GGCCGAGGTTGTAACAC | (TAA)6AGA(TAA)4 | 56 | 235-270 | 30 | 9 | FAM |
| SSR119 | GAACTCAGTTTCTCATTG | GAACATATCCAATTATCATC | (TA)4TT(TA)11(TG)19 | 50 | 263-297 | 30 | 10 | HEX |
| SSR212-1 | GACTCATTGTTGTACCC | GCGAGAAGAATGGTTG | (AT)2(TC)26(AC)8 | 50 | 159-207 | 30 | 10 | NED |
| SSR215 | CATTAATATTTCTTTGGTGC | CTTTTCTTCTCTTCCCC | (CA)15(TA)25 | 50 | 361-441 | 30 | 10 | FAM |
| SSR130 | CCACGTATGTGACTGTATG | GAAAGAGAGGCTGAAACTTG | (GT)9 | 56 | 195-198 | 30 | 11 | NED |
| SSR33 | CAAGCATGACGCCTATGAAG | CTTTCACTCACTCAACTCTC | (CA)21(GA)25 | 56 | 250-321 | 30 | 11 | HEX |

b (TG)4CTTAAGCCTAGGTAGGAGGCTTATCTCTCAAGTAAAACACCCATAACCTAACAAT(AC)7

### 3.2.6. Data analysis

Allele pattern profiles corresponding to amplification products of all SSR loci, as well as AFLP fragments, were visualized, sized and automatically scored using the GENEMAPPER 4.0 software (Applied Biosystems) combined with both MS Access and MS Excel. For all genotypes (single plants), binary matrices were constructed based on scoring presence of amplification products of all SSR loci and AFLP fragments of all primer combinations as (1) and absence as (0). Unique SSR amplification products correspond to homozygous individual, while two different correspond to heterozygous. Genetic diversity parameters were estimated for microsatellites taking into consideration whether the individual is homozygote or heterozygote at each given locus (observed number of alleles, *na*; expected number of alleles, *ne*; Shannon's information index, *I*; Nei's genetic distances (Nei 1973); observed heterozygosity, *Ho*; and expected heterozygosity, *He*); and for AFLP (number of fragments, percentage of polymorphic fragments), using POPGENE 1.31 (Yeh *et al.* 1999). Polymorphic information content (*PIC*) was calculated for AFLP using $PIC = 1\text{-}\sum P_i^2$, where $P_i$ is the frequency of the i$^{th}$ allele (fragment) (Smith *et al.* 1997). A binary matrix was used to construct a genetic distance matrix between all pairwise genotypes based on the Nei's genetic distance and the chi-square measure for SSRs or the Jaccard similarity index for AFLP. The probability of identity (*PI*) between genotypes for SSR markers was computed using the IDENTITY 1.0 program (Wagner and Sefc 1999). Assignment of genotypes to their origin (collection sites or cultivar) was computed using the assignment test combined with canonical discriminant analysis (De Riek *et al.* 2001, 2013) using SPSS Statistics 22 to display genetic variation coming from the origin of the landraces. The assignment test (De Riek *et al.* 2001, 2007, 2013) was carried out by first ranking all individual genotypes (single plants) to each other based on chi-square distances for the SSRs analysis and on Jaccard's similarity index for AFLP. For SSRs, a ranking of the most resembling genotypes (single plants) per individual was made, and pairs of genotypes with chi-square distance above 7 were excluded. For AFLP, a ranking of the most resembling single plants per individual was made whereby pairs of genotypes with Jaccard's similarity index below 0.45 were excluded. This allowed producing assignment tables showing for each origin (geographic location of landraces or cultivars) the most-related single plants. Assignment tables were then used as input files for discriminant function analysis (De Riek *et al.* 2007, 2013) in order to classify the genotypes according to their geographical origins. NTSYS-PC 2.1 (Rohlf 2004) was used for genetic distance analysis using Nei's genetic distance (Nei 1973) for both SSRs and AFLP, for the construction of a cluster based on unweighted pair group method with arithmetic means (UPGMA) and principal coordinate analysis (*PCo*) scatter

plot to show the associations between the landraces studied. UPGMA cluster analysis based on the combined data sets was carried out using the Jaccard similarity index. The Bootstrap analysis of the clustering methods was performed by the WINBOOT software (Yap and Nelson 1996) to test confidence and faithfulness of the obtained groupings. Using SPSS Statistics 22, discriminant function analysis was performed based on combined data sets of genetic data (SSR and AFLP markers) and the agronomic data used as grouping factors (agro-environment, early vegetative vigour, flowering and maturity time and seed type) in order to sort landraces according to most discriminating agronomic factors that may correspond to functional grouping of the landraces. Agronomic data were used as dependent variables while genetic data were used as predictor variables. The genetic data used were based on the distance matrix from chi-square measures and Jaccard's similarity index between all pairs of individual plants of each accession (calculated using SPSS Statistics 22) for SSRs and AFLPs, respectively. Factor analysis based on agronomic data was performed to extract principal components and then used to present the landraces in a biplot graph.

## 3.3. Results

### 3.3.1. SSR genetic diversity and allelic variation

Nineteen SSRs produced a total of 213 alleles for all landraces with an average of 11.21 alleles per locus (Table 3.4). The number of alleles per locus ranged from 2 to 26. The largest number of observed alleles (*no*) was produced at the SSR215 locus, while the smallest number of alleles was produced at SSR99, SSR124 and SSR130 loci. The Shannon's information index (*I*) ranged from 0.03 (SSR130) to 2.59 (SSR215) with an average over all loci for all genotypes of 1.55. The expected heterozygosity (*He*), expressing the level of genetic diversity as the probability at a given locus of two alleles taken at random from the population to be different of each other, ranged from 0.0124 (SSR130) to 0.9098 (SSR212-1) with an average over all loci for all landraces of 0.6531. Total *PI* between two randomly chosen genotypes of the landraces and the two cultivars over all loci was very low with a value of $1.62 \times 10^{-19}$, highlighting the high level of genetic diversity of the material studied. The lowest *PI* of 0.013 was found for SSR212-1 locus. The highest probabilities of identity were found for the three loci with lowest polymorphism with only two alleles (SSR99, SSR124 and SSR130). The Nei's genetic distance between all pairwise genotypes ranged from 0 to 1.58.

**Table 3.4.** SSR polymorphism parameters in the Moroccan lentil landraces and local cultivars

| Locus Name | Number of observed alleles (*no*) | Number of expected alleles (*ne*) | Shannon Information Index (*I*) | Observed heterozygosity (*Ho*) | Expected heterozygosity (*He*) | Probability of Identity (*PI*) |
|---|---|---|---|---|---|---|
| SSR19 | 8 | 4.86 | 1.71 | 0.0602 | 0.7964 | 0.073 |
| SSR33 | 21 | 3.77 | 1.84 | 0.2983 | 0.7370 | 0.091 |
| SSR48 | 10 | 4.17 | 1.69 | 0.0462 | 0.7623 | 0.090 |
| SSR80 | 12 | 5.08 | 1.86 | 0.0561 | 0.8054 | 0.064 |
| SSR99 | 2 | 1.10 | 0.19 | 0.0000 | 0.0917 | 0.829 |
| SSR113 | 14 | 7.28 | 2.21 | 0.0506 | 0.8645 | 0.032 |
| SSR119 | 17 | 9.16 | 2.41 | 0.0932 | 0.8927 | 0.021 |
| SSR124 | 2 | 1.02 | 0.06 | 0.0045 | 0.0223 | 0.951 |
| SSR130 | 2 | 1.01 | 0.03 | 0.0042 | 0.0124 | 0.970 |
| SSR154 | 13 | 5.54 | 2.02 | 0.9342 | 0.8215 | 0.05 |
| SSR184 | 18 | 2.90 | 1.64 | 0.0881 | 0.6572 | 0.122 |
| SSR199 | 3 | 2.24 | 0.94 | 0.3571 | 0.5550 | 0.259 |
| SSR204 | 7 | 3.66 | 1.43 | 0.0905 | 0.7287 | 0.100 |
| SSR212-1 | 18 | 10.85 | 2.55 | 0.1172 | 0.9098 | 0.013 |
| SSR215 | 26 | 7.50 | 2.59 | 0.8305 | 0.8685 | 0.023 |
| SSR233 | 11 | 3.21 | 1.54 | 0.5325 | 0.6900 | 0.123 |
| SSR302 | 14 | 2.87 | 1.55 | 0.2922 | 0.6540 | 0.157 |
| SSR309-2 | 8 | 4.09 | 1.61 | 0.9831 | 0.7575 | 0.090 |
| SSR336 | 7 | 4.56 | 1.65 | 0.3433 | 0.7825 | 0.077 |
| **Total** | 213 | | | | | $1.62 \times 10^{-19}$ |
| **Average** | 11.21 | 4.47 | 1.55 | 0.2727 | 0.6531 | |
| **Standard deviation** | 6.82 | 2.68 | 0.76 | 0.3222 | 0.2861 | |

### 3.3.2. AFLP genetic diversity parameters

Of the 12 primer combinations tested, seven produced clear polymorphic fragments (Table 3.5). The scoring of the seven primer combinations yielded a total of 766 fragments ranging from 50.42 to 499.54 bp over all landraces with an average of about 109 fragments per primer combination. The highest number of fragments was produced by primer combination *EcoRI-ACA + MseI-CTT* with 144 fragments, whereas the lowest number was produced by primer combination *EcoRI-ACG + MseI-CAA* with 80 fragments. Of the 766 fragments, 422 (54.78 %) were polymorphic with polymorphic band percentages ranging from 49.61 (*ACG-CAA*) to 64.40 % (*ACA-CAG*). The *PIC* for the seven primer combinations ranged from 0.3122 to 0.4160. The Jaccard similarity index calculated between all pairwise genotypes ranged from 0.13 to 0.86.

**Table 3.5.** AFLP Primer combinations polymorphism parameters in the Moroccan lentil landraces and local cultivars

| Primer combinations | Number of fragments | Polymorphic fragments | | | Fragment size range (bp) | *PIC* |
|---|---|---|---|---|---|---|
| | | Number | Standard deviation | Percentage | | |
| *EcoRI*-ACA+ *MseI*-CAG | 122 | 78.58 | 22.08 | 64.40 | 51.69-479.90 | 0,4160 |
| *EcoRI*-ACA+ *MseI*-CTG | 114 | 60.83 | 16.88 | 53.35 | 50.70-499.54 | 0,3247 |
| *EcoRI*-ACA+ *MseI*-CTT | 144 | 77.17 | 19.62 | 53.59 | 50.42-496.48 | 0,3293 |
| *EcoRI*-ACG+ *MseI*-CAA | 80 | 39.69 | 10.93 | 49.61 | 50.92-492.47 | 0,3152 |
| *EcoRI*-AGC+ *MseI*-CAA | 118 | 62.20 | 15.78 | 52.71 | 50.59-492.58 | 0,3170 |
| *EcoRI*-AGC+ *MseI*-CAG | 97 | 57 | 13.84 | 58.76 | 51.63-487.87 | 0,3593 |
| *EcoRI*-AGC+ *MseI*-CTG | 91 | 46.50 | 13.38 | 51.09 | 50.48-498.72 | 0,3122 |
| **Total** | 766 | 422 | | - | - | - |
| **Average** | 109.42 | 60.28 | | 54.78 | - | 0.3391 |

### 3.3.3. Genetic relationship between landraces

Cluster analysis based on UPGMA was used for studying the genetic relationship among landraces for both microsatellites and AFLP markers taken separately and the combined data sets. The UPGMA dendrogram based on SSR markers discriminated between seven clusters with contrasting sizes: one large group, two small groups and four single groups. The large group contained 42 landraces, while the small ones contained five landraces (MGB1026, MGB1027, MGB1047, MGB1039 and MGB1040) and two landraces (MGB1008 and MGB1010) for the other. The four remaining clusters contained either one landrace or one cultivar: L24, L56, landrace MGB7389 and landrace MGB1055 (Figure 3.2).

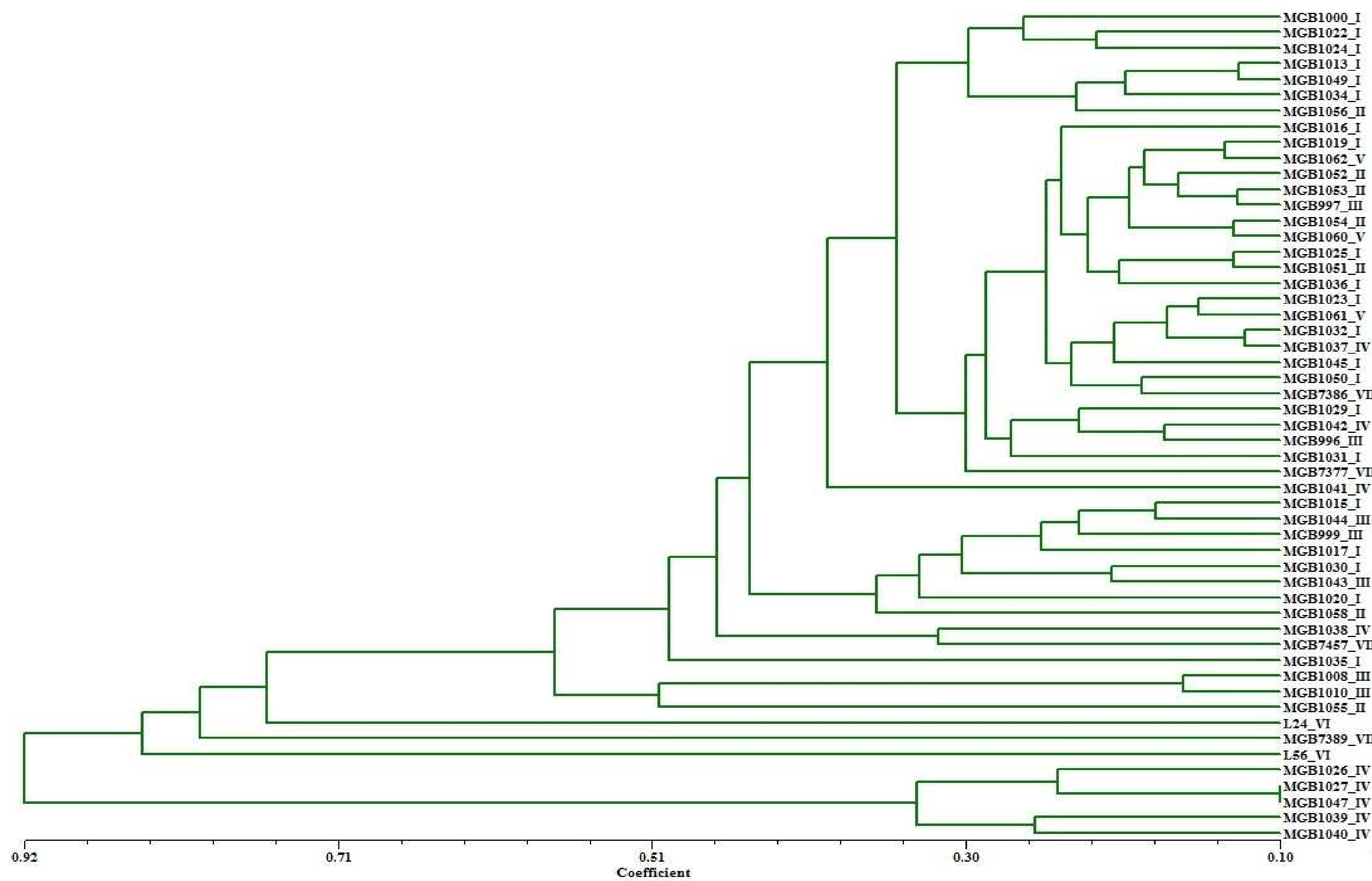


**Figure 3.2.** UPGMA of the Moroccan local lentil accessions based on Nei's genetic distance, as revealed by SSRs data. Accession's origin codes are listed right of the accession codes. Groups are indicated by continuous lines right of the figure.

UPGMA clustering based on AFLP data (Figure 3.3) divided the landraces into eight groups: one large group with 41 landraces, two small groups with, respectively, five (MGB1040, MGB1047, MGB1027, MGB1039 and MGB1026) and two (MGB1008 and MGB1010) landraces and five single groups. The latter contained each: L24, L56, MGB1055, MGB1024 and MGB7389.

Genetic similarity matrices between lentil landraces and cultivars obtained from the two data sets (SSR and AFLP) were compared using the Mantel test. A highly significant correlation between the two matrices was found with $r^2 = 0.8472$ and Mantel $t = 8.8457$ ($P < 0.001$). Consensus clustering was performed using the combined data sets from the SSR and AFLP analyses to build UPGMA dendrogram (Figure 3.4) based on the Jaccard similarity index. The local material could be divided into closely similar clusters displayed by the two UPGMA ordinations related to each separate marker data set. Five groups could be shown at bootstrap values of 89%, 93%, 94%, 100% and 100%, respectively. The only difference regarding groups is that landrace MGB7389 is grouped with the five landraces from Abda region (MGB1040, MGB1047, MGB1027, MGB1039 and MGB1026). Sub-groups could not be defined within the largest group due to low bootstrap values (<50%).

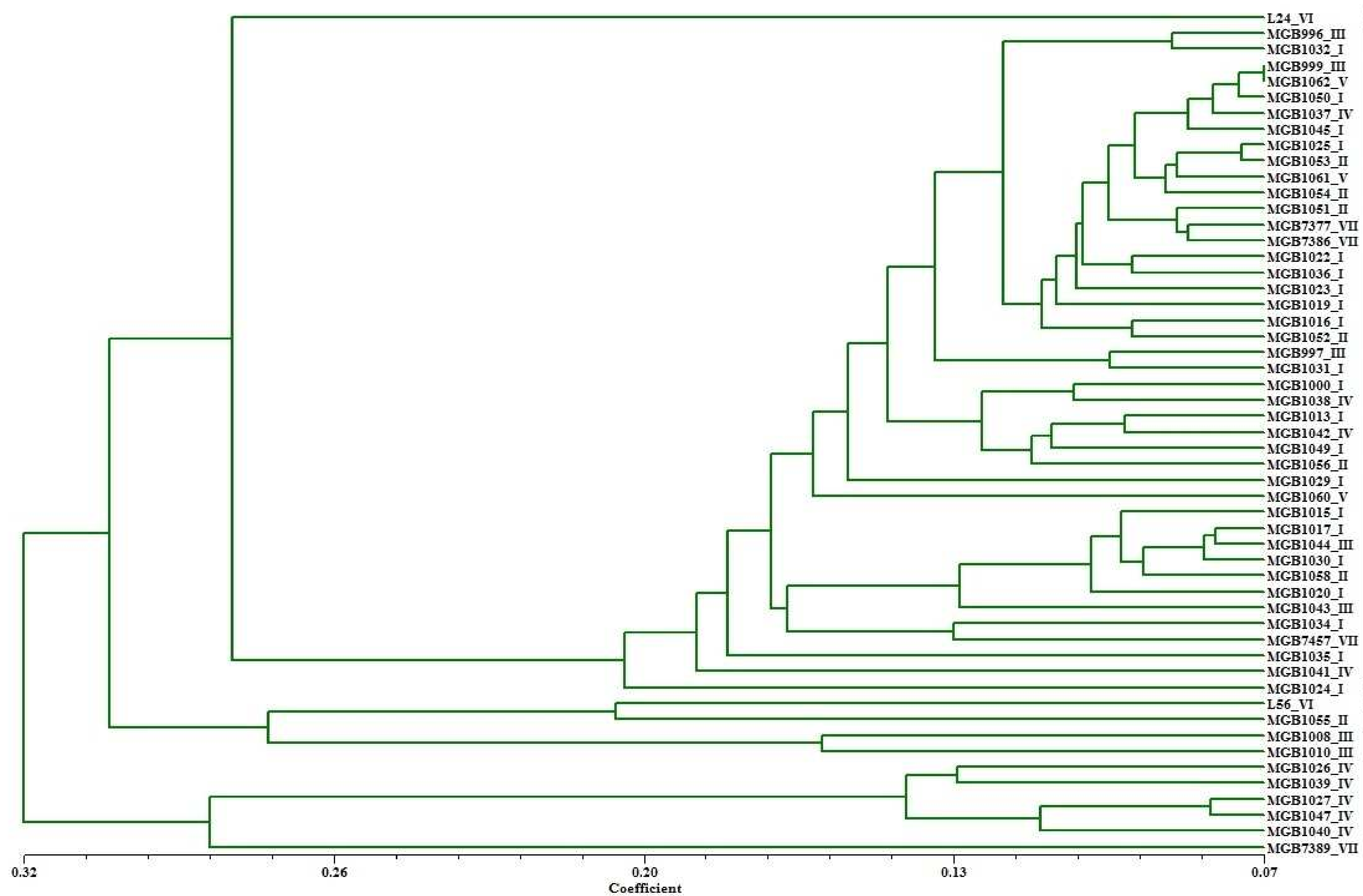


**Figure 3.3.** UPGMA of the Moroccan local lentil accessions based on Nei's genetic distance, as revealed by AFLP data. Accession's origin codes are listed right of the accession codes. Groups are indicated by continuous lines right of the figure.

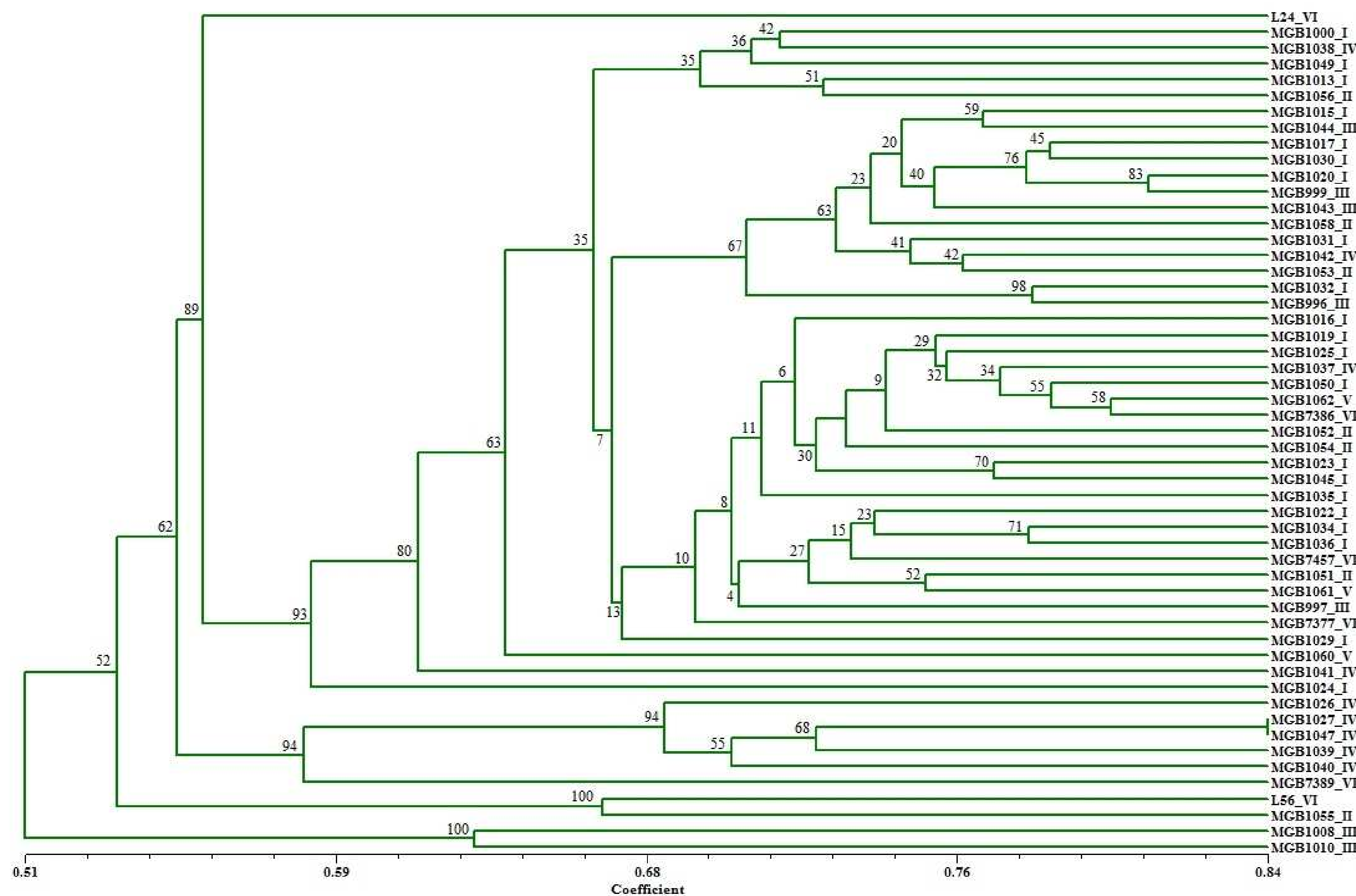


**Figure 3.4** Association between the Moroccan local lentil accessions as revealed by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis with Jaccard's similarity index calculated based on the combined data sets from SSR and AFLP markers. Bootstrap values are given at the nodes. Accession's origin codes are listed right of the accession codes. Groups are indicated by continuous lines right of the figure.

Principal coordinate analysis of SSR data indicates that the first three coordinates accounted for 89.92 % of total observed variation (Figure 3.5 a). The percentages of variation displayed by the first three coordinates were 62.59 %, 16.09 % and 11.23 %, respectively. The landraces and cultivars separated from the main cluster shown by UPGMA for SSR data were very closely mirrored by the 3-dimensional scatter plot of the PCo. The only difference is that here, landrace MGB7389 is not clearly separated from the main group as in the UPGMA.

Based on AFLP data, the PCo is reported in Figure 3.5 b. Total variation explained by the first three coordinates was 73.94 %, and 44.03 %, 18.5 % and 11.41 % for the individual coordinates, respectively. As for the SSR data, the results of the PCo for AFLP markers were close to those displayed by UPGMA clustering except for cultivar L24 which is not separated from the main group. Landrace MGB1043 is also detached from the main group.

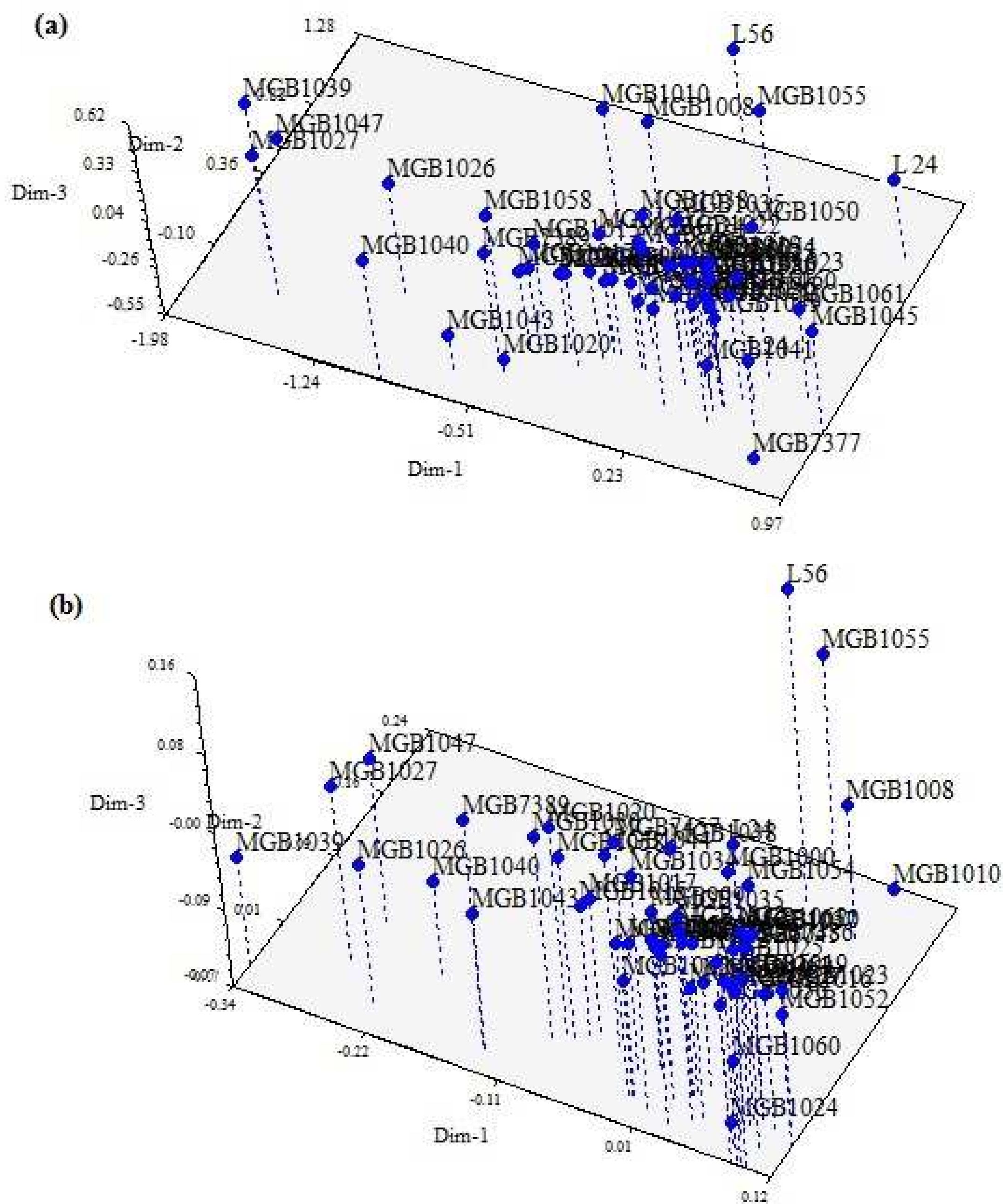


**Figure 3.5.** Relationship between the Moroccan local lentil accessions as revealed by principal coordinate analyses based on SSR markers (a) and AFLP markers (b).

### 3.3.4. Genetic differentiation among landraces according to agro-environmental origins and agronomic characteristics

Discriminant analysis based on SSR and AFLP was performed using prior information related to geographical origin of the landraces. Eigenvalues were as low as 1.98 for the first function (55.1 % of the total variation and canonical correlation of 0.78) and 0.7 for the second (24.6 % of the total variation and canonical correlation of 0.64), respectively, for SSRs. Slightly higher eigenvalues were observed for AFLP with 2.26 and 0.8 for the first (52 % of the total variation and canonical correlation of 0.81) and second functions (28.3 % of the total variation and canonical correlation of 0.69), respectively. Although no clear separation of landraces according to their origins was possible as reported by the canonical discriminant functions analysis, both cultivars and relatively small sets of landraces could be separated from the larger set of landraces in concordance with the results shown by the UPGMA and PCo. The detached landraces were mainly composed of genotypes from Abda, Middle Atlas mountains and Zaer regions as well as single landraces from other origins. The two local cultivars are clearly separated from other groups and from each other.

The first two axes of principal component analysis based on agronomic data (agro-environment, days to flowering, days to maturity, early vegetative vigour and seed type) explained 76.77 % of the total variation, with 57.71 % and 19.05 % from the first and second components, respectively. The biplot of the landraces sorted according to their origin (agro-environment or cultivar) based on these two axes is presented in Figure 3.6. A number of landraces were clearly separated: MGB1041, MGB1037, MGB1027, MGB1026, MGB1039, MGB1038, MGB1040, MGB1042, MGB1047, MGB1022, MGB1016, MGB1019, MGB1008, MGB1010 and MGB1055 as well as the two local cultivars.

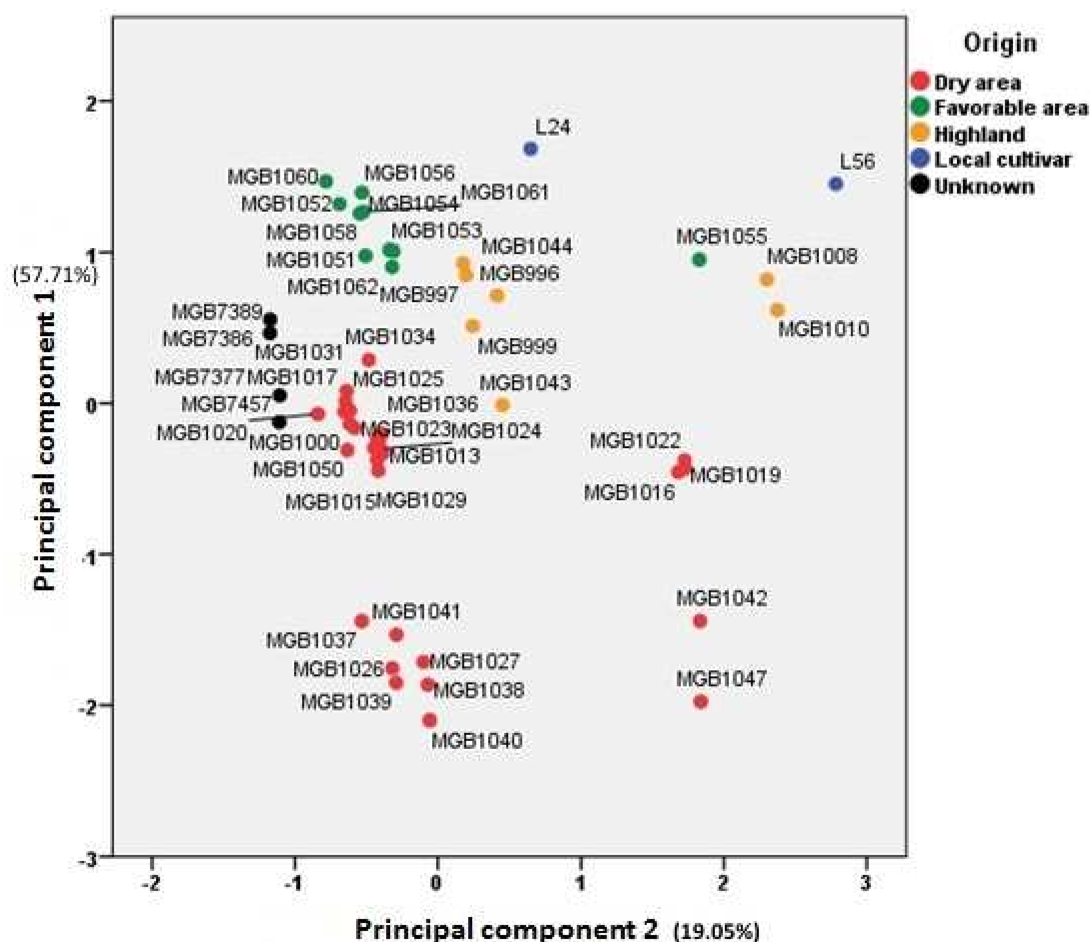


**Figure 3.6.** Scatter plot of the Moroccan local lentil accessions based on the two principal component axes of the agronomic data.

Discriminant analysis based on combined data sets (genetic and agronomic) showed discrimination based on agro-environmental origin of the landraces. The two first functions explained 72.8 % of the total variation, with 55.1 % for the first function and 17.7 % for the second and canonical correlations of 0.98 and 0.94, respectively (Figure 3.7 a). The eigenvalues were as high as 26.08 and 8.39 for the first and second functions, respectively. The two local cultivars L24 and L56 were the most differentiated. Some overlapping was observed between the highland origin and the unknown landraces' origin. When discriminant analysis was performed based only on the three agro-environments, excluding local cultivars and unknown origin, landraces from dry areas were clearly separated from those of both favourable and highland environments (Figure 3.7 b). The first function accounted for 54.6 % of the total variation and the second for 45.4 % with canonical correlations of 0.95 and 0.94 and eigenvalues of 9.3 and 7.7, respectively. Clear discrimination of landraces into three groups according to cycle duration (early flowering and maturity, medium and late) was observed as shown in Figure 3.7 c. The first function explained 53 % of the total variation and the second

47 %, with eigenvalues of 6.51 and 5.77 and canonical correlations of 0.93 and 0.92, respectively. When grouping landraces according to early vegetative vigour, functional grouping according to this trait was observed. Landraces with very poor vegetative vigour are clearly differentiated compared to others. The first function explained 37.8 % of the total variation, and the second explained 24.2 %, with eigenvalues of 9.2 and 5.9 and canonical correlations of 0.95 and 0.92, respectively. Landraces with average and good early vigour were close to each other's (Figure 3.7 d). Only poor differentiation of landraces was observed according to geographical origins (eigenvalues of the first two axes of 9.05 and 8.78, respectively) and seed type (macrosperma and microsperma). The highest eigenvalues of the two first functions of discriminant analyses were obtained for agro-environmental origin.

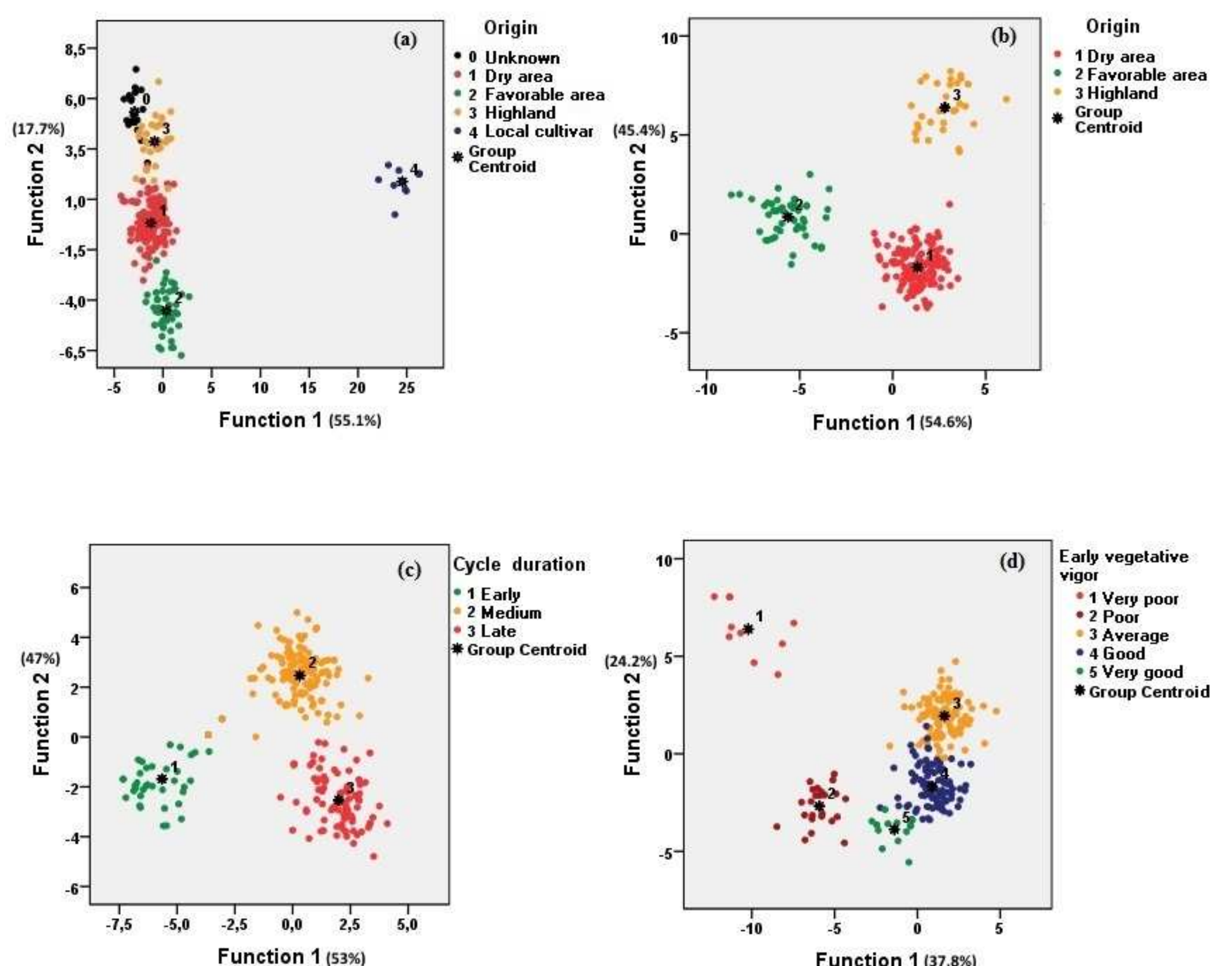


**Figure 3.7.** Discriminant analysis scatter plots of the Moroccan local lentil accessions based on landraces origins (a) agro-environmental origins (b), cycle duration (c) and early vegetative vigour (d) using combined data sets from SSR, AFLP and agronomic data. For cycle duration, early refers to days after sowing to maturity <140; medium refers to ≥140 and ≤160; long refers to >160.

## 3.4. Discussion

### 3.4.1. Genetic diversity among landraces

Both SSR primers and AFLP primer pairs revealed a moderate to high level of polymorphism among Moroccan lentil landraces and local cultivars included in this study. For SSRs, 213 alleles with an average genetic diversity of 0.6531 were obtained. Large variation among SSRs was found for gene diversity and number of alleles. Three loci (SSR99, SSR124 and SSR130) showed only two alleles. Compared to the results of Sonnante *et al.* (2007) where the number of observed alleles ranged from 1 to 22 at 16 loci, our results indicated a slightly higher number of alleles and ranged from 2 to 26 at 19 loci. For AFLP, a total of 766 fragments with 422 polymorphic fragments were recorded. Average PIC over the seven AFLP primer combinations was 0.3391. The highest diversity was obtained from the *EcoRI-ACA* + *MseI-CAG* primer combination with 64.40 % polymorphic bands and 0.4160 as PIC which was greater than that reported by Torricelli *et al.* (2011) using eight primer combinations on Italian landraces and revealing 404 polymorphic fragments (57 %).

Mean genetic diversity between all landrace genotypes analyzed in this study was 0.1307. Less diversity was obtained by Sharma *et al.* (1995) and by Ferguson *et al.* (1998) who reported values of 0.037 and 0.049, respectively, using RAPD markers on lentil accessions from different origins. Moderately higher values were found by Toklu *et al.* (2009) who studied 38 Turkish landraces using inter simple sequence repeats (ISSR) and AFLP markers that indicated mean genetic diversity of 0.180. Similarly, a mean genetic diversity of 0.175 was reported by Fikiru *et al.* (2007) using 70 genotypes representing seven Ethiopian landraces and ISSR markers.

### 3.4.2. Genetic differentiation towards agro-environmental origins and agronomic characteristics

Based on different statistical analyses and ordinations using both molecular data (UPGMA, PCo, DA), a small set of landraces namely MGB1026, MGB1027, MGB1039, MGB1040, MGB1047, MGB1043, MGB1010, MGB1008, MGB1055 and MGB7389 as well as the two local cultivars (L24 and L56) could be separated from the main group containing all other landraces. These genotypes are likely to have different genetic characteristics from the rest. This was supported by agronomic data. In fact, all these accessions, except MGB1043 and MGB7389, were also differentiated from other landraces according to growth cycle duration, early vegetative vigour, seed type and agro-environmental origins. Other landraces differentiated by phenotypic data were not shown as such by both molecular techniques. Interestingly, the first five of these landraces were from Abda region in west-central Morocco

(mainly from small communities near *Jemaat Shaim* village), which is the driest area where lentil has been grown ancestrally by farmers (Sakr 2005; Idrissi *et al.* 2012). This area is characterized by the lowest annual precipitation and the highest temperatures during the lentil cultivation period especially during flowering and pod set stages compared to others areas. The drought frequencies in this area may have had a significant effect on the genetic differentiation by the selection of well-adapted material for this agro-environmental area. These five landraces from Abda region are the earliest to flower and to mature among the landraces studied with <105 days to flowering and <135 days to maturity compared to other landraces showing clearly late flowering and maturity (Table 3.1). This adaptation to dry areas appears to have been an escape mechanism from late season drought and heat. They also have high early vegetative growth vigour (Table 3.1) conferring drought tolerance for lentil as reported by Sarker *et al.* (2005).

Two landraces, MGB1008 and MGB1010, originated from the highland region of Morocco where cold tolerance is one of the most important characteristic targeted by farmers in their selection. Low temperatures, reaching sometimes below zero values, are frequent in these areas during December, January and February when lentil is at seedling stage (Balaghi *et al.* 2013; DNM 2014). These two landraces are macrosperma type having large seeds with 100-seed weight of about 5.03 and 4.87 g, respectively. They are also late flowering and late maturing, a typically useful trait for lentil genetic material adapted to highlands farming as the reproductive stage begins when the temperature increases. Erskine (1996) reported higher cold tolerance of large seeded genotypes compared to small seeded ones.

MGB1055 collected in *Ain Sbit* in *Zaer* region, north-western of Morocco, was different from the other landraces according to UPGMA clustering and PCo using both SSRs and AFLPs. Interestingly, the lentil maintained and cultivated by local communities in this small area is specifically known in Morocco for its excellent seed quality and specific adaptation while being produced ancestrally. As a consequence, it was proposed for a protected geographic denomination (*lentil of Ain Sbit*) as it would have the characteristics of a protected designation of origin quality mark (‘*produit de terroir*’) (Benbrahim *et al.* 2011; Ministère de l’Agriculture et de la Pêche Maritime 2011). This would offer the perspective of better valorization of the landrace maintained by local communities of this area with a perspective of enhancing farmers’ incomes as this quality label would result in higher unit prices. Moreover, molecular characterization may help to avoid frauds, thus protecting farmer’s interest. The annual average cultivated area and production of this landrace are 1100 ha and 2200 tonnes, respectively (Ministère de l’Agriculture et de la Pêche Maritime, Direction de Développement des Filières

de Production 2011). Benbrahim *et al.* (2011) reported high seed protein content of this landrace of 31 %, which is 6 % higher than the next protein-rich landrace among 35 local populations analyzed. The latter authors also reported high iron concentration in the seeds (65 ppm), which was 12 % higher than the next highest concentration in the landraces analyzed. MGB1055 is a macrosperma type and has large seeds with a 100-seed weight of about 5.16 g (Table 3.1). The average cooking time of the seeds of the *Ain Sbit* lentil was 44 min, the shortest among the material analyzed, and the texture remains good after cooking (Benbrahim *et al.* 2011).

The differentiated landraces are in agreement with results of Erskine *et al.* (1981, 1989, 1990), where they reported great differences between landraces that have undergone genetic adaptation in response to extremes of temperature, photoperiod and cold. Thus, specific adaptation and possible evolution into distinct ecotypes of these landraces to their respective ecological environments may be indicated by their genetic differentiation using SSRs and AFLPs compared to other landraces.

Although the two local cultivars included in this study were selected from the local germplasm, there is clearly variation compared to other landraces as well as to each other. Cultivar L24 was clearly distant from all the landraces as shown by the results of the UPGMA clustering based on both SSRs and AFLPs as well as on combined data sets. While cultivar L56 was separated from all the landraces by SSR markers, the results related to AFLP data and to the combined data sets showed that this cultivar was grouped with few landraces and was closely related to MGB1055. It might be speculated that it was selected from this landrace or similar germplasm collected from the same region.

UPGMA cluster analyses based on all genotypes (252) using Nei's genetic distance for SSR and Jaccard's similarity index for AFLP were carried out to determine genetic associations between all individuals (resulting trees not shown as they were unreadable because of the large number of individuals). For both types of markers, the same patterns as shown for the accessions were observed, with a separation of one large group and a number of small groups. Interestingly, one of the smallest clusters contained genotypes from two regions Abda (IV) and Chaouia (I) with the proportions of total genotypes of 75 % for SSR data and 79.40 % for AFLP data. These two regions are located in dry area which differ them from other more favourable regions (Zear, Sais-Meknes and highland). Hence, these two regions may share closely related genetic material selected over time for specific adaptation to drought and heat stress typical of these dry areas.

Despite the cited exceptions, most landraces would not be clearly sorted by geographical origin. For instance, geographically close landraces that were genetically distant were different,

whereas by contrast, others that were geographically distant were genetically similar. The migration and contamination of genetic material between regions as well as the selection for specific adaptations to local conditions (mainly environmental) by farmers are likely to be the reasons for these results. Sonnante and Pignone (2007) and Toklu *et al.* (2009) reported similar results for Italian and Turkish lentil landraces, respectively.

Taking into consideration the genetic data with the agronomic data, cycle duration, early vegetative vigour and agro-environmental origin discriminated landraces into different functional groups. Short-cycle landraces were clearly separated from late flowering and maturity ones. Most of these landraces were from dry areas and had high early vegetative vigour which discriminated the landraces. Agro-environmental origin of landraces namely dry areas, favourable and highlands was the most discriminant factor among landraces as shown by the highest eigenvalues obtained for the two discriminant functions. This highlights the important role of specific adaptation to agro-environment in the genetic differentiation of landraces.

## 3.5. Conclusions

Preservation, characterization and use of local germplasm in breeding programs can become more efficient based on the knowledge of the genetic variation related to the material. Based on our results using SSRs and AFLPs combined with the available phenotypic information, the landraces had a moderate to high degree of genetic variation. Both SSR and AFLP techniques provided nearly the same results strengthening our conclusions. Although no clear differentiation towards geographical origins was observed, indications for differentiation according to agro-environmental origins and agronomic traits were shown. In fact, the differentiation of a number of landraces from the dry areas (mainly from Abda region) indicated that these areas may contain useful genotypes with well-adapted characteristics potentially conferring drought and heat tolerance traits that can be incorporated into breeding programs. This is also true for the two distinct landraces from the highland areas that may have been selected for cold tolerance in the middle Atlas mountains. Additional landraces collected from these areas in relatively distant localities could provide more information about the genetic variation of the gene pool of these regions compared to that of others. Furthermore, our results provide evidence for better valorization and protection of lentil landrace of *Ain Sbit* as a ‘protected designation of origin’. Phenotypic characterization considering more morphologic, agronomic and seed quality traits of these landraces would provide additional information for better understanding of the differentiated landraces.

# Chapter 4. Functional genetic diversity analysis and identification of associated Simple Sequence Repeats and Amplified Fragment Length Polymorphism markers with drought tolerance in Mediterranean lentil (*Lens culinaris* Medik.) landraces

Based on:

Idrissi, O.[1,2], Udupa, S.M.[3], De Keyser, E.[4], Van Damme, P.[1,5], De Riek, J.[4] (2016). Functional genetic diversity analysis and identification of associated simple sequence repeats and amplified fragment length polymorphism markers to drought tolerance in lentil (*Lens culinaris* ssp. *culinaris* Medicus) Landraces. Plant Molecular Biology Reporter, 34(3), 659-680. Doi: 10.1007/s11105-015-0940-4.

1 Department of Plant Production, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.
2 Institut National de la Recherche Agronomique du Maroc (INRA), Centre Régional de Settat, P.O. Box 589, Settat, Morocco.
3 ICARDA-INRA Cooperative Research Project, International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 6299, Rabat, Morocco.
4 Institute for Agricultural and Fisheries Research (ILVO) Plant Sciences Unit, Applied Genetics and Breeding, Caritasstraat 21, 9090 Melle, Belgium.
5 Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Kamycka 129, 165 21 Prague 6- Suchdol, Czech Republic.

# Chapter 4. Functional genetic diversity analysis and identification of associated Simple Sequence Repeats and Amplified Fragment Length Polymorphism markers with drought tolerance in Mediterranean lentil (*Lens culinaris* Medik.) landraces

## 4.1. Introduction

Globally, drought is one of the most challenging abiotic stresses causing yield losses limiting benefits to farmers. With increasing global warming in the context of climate change becoming more and more important, drought episodes are expected to worsen and become more frequent. Thus, improving plant tolerance and adaptation to water-limited conditions to maintain growth and yield is an important strategic research focus for breeders. Breeding for drought tolerance is a major objective in arid and semi-arid areas. Landraces selected over centuries are valuable genetic resources for developing genotypes adapted to different abiotic stresses, particularly drought (Grando and Ceccarelli, 1995; Peleg *et al.*, 2007, 2008; Tuberosa 2012). They provide valuable opportunities in conferring resistance to drought by higher expression of secondary traits such as root mass and osmotic adjustment. Well-developed roots, vigorous shoots at early seedling stage, high root–shoot ratio and chlorophyll content (Soil Plant Analysis Development (SPAD) value) have all been reported to be important indicators in promoting drought avoidance in lentil and other food legumes (Sarker *et al.* 2005; Kashiwagi *et al.* 2005; Vadez *et al.* 2008; Gaur *et al.* 2008; Aswaf and Blair 2012).

Screening methods that use parameters reflecting water status in plants, such as relative water content, water losing rate and wilting score, have been reported as suitable and effective for genetic studies (Levitt 1980; Verslues *et al.* 2006; Shrestha *et al.* 2006; Razavi *et al.* 2011; Jain and Chattopadhyay 2010; Mullan and Pietragalla 2012; Singh *et al.*2013; Khazaei 2013; Talukdar 2013; Ammar *et al.* 2015; Iglesias-García *et al.* 2015; Esmaeilpour *et al.* 2015).

Association of molecular markers with such traits of interest as those linked to drought tolerance is being studied using mapping populations to identify quantitative trait loci; in addition, unrelated genetic resources such as landraces are being used in association mapping to take advantage of the historic linkage between phenotypic and genetic variations during the process of selection and adaptation. Based on genetic diversity analysis, Singh *et al.*(2013) reported Simple Sequence Repeats (SSR) markers associated with fusarium wilt (*Fusarium udum*) resistance in cultivated pigeon pea (*Cajanus cajan*), Razavi *et al.* (2011) identified Amplified Fragment Length Polymorphism (AFLP) and Expressed Sequence Tag (EST) candidate gene markers associated with water deficit response in *Fragaria*, whereas Mondal *et al.* (2010)

reported association of SSR markers with genes for rust and late leaf spot resistance in cultivated groundnut (*Arachis hypogaea* L.).

The Mediterranean region is expected to harbor high genetic diversity in lentil thanks to the rich history of domestication and cultivation as well as because of the frequency of biotic and abiotic stresses as selection pressure. In Mediterranean environments, lentil, as well as other crops, experiences intermittent drought during vegetative growth and end-cycle drought associated with increasing temperatures during reproduction stage (Silim *et al.* 1993; Materne and Siddique 2009). This offers opportunities for the identification of biotic and abiotic stress-resistant landraces. Although genetic diversity and relationships between lentil landraces have been reported from a number of Mediterranean countries using different molecular markers (Ferguson *et al.* 1998; Sonnante and Pignone 2001; Sonnante *et al.* 2003; Duran and Perez de la Vega 2004; Toklu *et al.* 2009; Bacchi *et al.*2010; Zaccardelli *et al.* 2011; Lombardi *et al.* 2014), to our knowledge, no studies have reported on lentil genetic diversity in association with drought tolerance.

Thus, the objectives of our study were to (1) analyze genetic diversity of 70 landraces from different Mediterranean countries (Morocco, Italy, Turkey and Greece) using SSR and AFLP DNA markers, (2) characterize their root and shoot traits and to evaluate their drought tolerance using physiological parameters and (3) analyze their functional genetic diversity in association with drought tolerance as a first and preliminary step of testing association mapping studies in lentil.

## 4.2. Materials and methods

### 4.2.1. Plant materials

Seventy landraces collected in four different Mediterranean countries (Morocco, Italy, Turkey and Greece; Table 4.1) were evaluated for their genetic diversity using SSR and AFLP DNA markers and for their drought tolerance under greenhouse conditions using relative water content (Barr and Weatherley 1962; Verslues *et al.* 2006), water losing rate (Suprunova *et al.* 2004) and wilting score (Singh *et al.* 2013) as drought characterization parameters. Landraces were kindly provided by the Moroccan National Gene Bank, INRA-Settat, Morocco, the Italian National Council of Research, Institute of Biosciences and Bioresources, Bari, Italy and by the National Plant Germplasm System, United States Department of Agriculture, USA (for landraces from Turkey and Greece).

**Table 4.1.** List of the 70 Mediterranean lentil landraces analyzed and their respective origins.

| Name | Code | Origin | Name | Code | Origin* |
|---|---|---|---|---|---|
| ALTAMURA | I1 | Italy | MGB1032 | M15 | Morocco |
| TIPO ASSTELLUCCIO | I2 | Italy | MGB1034 | M16 | Morocco |
| MOUNTAIN LENTIL | I3 | Italy | MGB1035 | M17 | Morocco |
| TIPO TURCHE NO2 | I4 | Italy | MGB1036 | M18 | Morocco |
| MG110288 | I5 | Italy | MGB1045 | M19 | Morocco |
| MG110438 | I6 | Italy | MGB1049 | M20 | Morocco |
| MG106892 | I7 | Italy | MGB1050 | M21 | Morocco |
| MG110287 | I8 | Italy | MGB1051 | M22 | Morocco |
| MG111854 | I9 | Italy | MGB1052 | M23 | Morocco |
| MG111863 | I10 | Italy | MGB1053 | M24 | Morocco |
| MG106899 | I11 | Italy | MGB1054 | M25 | Morocco |
| MG111849 | I12 | Italy | MGB1055 | M26 | Morocco |
| AKCA MUCIMEGI | T1 | Turkey | MGB1056 | M27 | Morocco |
| YERLI1 | T2 | Turkey | MGB1058 | M28 | Morocco |
| ADI | T3 | Turkey | MGB1008 | M29 | Morocco |
| YERLI2 | T4 | Turkey | MGB1010 | M30 | Morocco |
| ILL183 | T5 | Turkey | MGB1043 | M31 | Morocco |
| ILL171 | T6 | Turkey | MGB1044 | M32 | Morocco |
| ILL306 | G1 | Greece | MGB996 | M33 | Morocco |
| ILL312 | G2 | Greece | MGB997 | M34 | Morocco |
| ILL298 | G3 | Greece | MGB999 | M35 | Morocco |
| MGB1000 | M1 | Morocco | MGB1026 | M36 | Morocco |
| MGB1013 | M2 | Morocco | MGB1027 | M37 | Morocco |
| MGB1015 | M3 | Morocco | MGB1037 | M38 | Morocco |
| MGB1016 | M4 | Morocco | MGB1038 | M39 | Morocco |
| MGB1017 | M5 | Morocco | MGB1039 | M40 | Morocco |
| MGB1019 | M6 | Morocco | MGB1040 | M41 | Morocco |
| MGB1020 | M7 | Morocco | MGB1041 | M42 | Morocco |
| MGB1022 | M8 | Morocco | MGB1042 | M43 | Morocco |
| MGB1023 | M9 | Morocco | MGB1047 | M44 | Morocco |
| MGB1024 | M10 | Morocco | MGB1060 | M45 | Morocco |
| MGB1025 | M11 | Morocco | MGB1061 | M46 | Morocco |
| MGB1029 | M12 | Morocco | MGB1062 | M47 | Morocco |
| MGB1030 | M13 | Morocco | L24 (local cultivar) | M48 | Morocco |
| MGB1031 | M14 | Morocco | L56 (local cultivar) | M49 | Morocco |

### 4.2.2. DNA Extraction

DNA was extracted as described above in section 3.2.2 of chapter 3.

### 4.2.3. SSR Analysis

SSR analysis was carried out as described above in section 3.2.3 of chapter 3.

### 4.2.4. AFLP Analysis

AFLP analysis was carried out as described above in section 3.2.4 of chapter 3.

### 4.2.5. Root and shoot characterization and drought tolerance evaluation

Landraces were evaluated for drought tolerance in a plastic pot experiment in a greenhouse arranged in a completely randomized block design with three replications. Four uniformly germinated seeds were planted in plastic pots (H 35 cm × D 24 cm) filled with fine perlite

(diameter ≤ 2 mm) in order to be able to extract intact roots without damage (Day 1991; Anon 2002; Rabah Nasser 2009). Standard nutrition solution EEG MESTSTOF 19-8-16 (4) [$NO_3$ 11 %, $NH_4$ 8 %, $P_2O_5$ 8 %, $K_2O$ 16 %, MgO 4 %, B 0.02 %, Cu EDTA 0.03 %, Fe EDTA 0.038 %, Mn EDTA 0.05 %, Mo EDTA 0.02 %, Zn EDTA 0.01 %] was supplied only during the first week after plant emergence. Water supply was then stopped in order to expose plants to progressive drought stress. Initial moisture in all pots was 70 % of field capacity and decreased to about 20 % at the eighth week after sowing. In the greenhouse, temperature ranged from 8 to 15 °C with 20 to 35 % relative humidity. The photoperiod was 11/13-h light/dark with light intensity of 240 W $m^{-2}$. The experiment was carried out at Ghent University greenhouse, Melle, Belgium, during November and December 2014.

Response of landraces to drought stress was assessed based on three fast and resource-effective phenotyping methods widely used in plant breeding programs: wilting score (WS), leaf relative water content (RWC) and leaf water losing rate (WLR). WS estimates visual symptoms of tissue damages under drought stress as the degree of wilting severity using the following 0–4 score scale as described by Singh *et al.* (2013): 0 = healthy plants with no visible symptoms of drought stress, 1 = green plants with slight wilting, 2 = leaves turning yellowish green with moderate wilting, 3 = leaves yellow–brown with severe wilting and 4 = completely dried leaves and/or stems. RWC measures the plant water status in plant tissues estimating dehydration avoidance under drought stress. Fresh weight (FW) was recorded on fully expanded excised leaves after 4-h drying on filter paper (at room temperature under a constant light) (Razavi *et al.* 2011); then, leaves were soaked for 4 h in distilled water at room temperature under constant light to determine turgid weight (TW). Total leaf dry weight (DW) was recorded after oven-drying at 72 °C for 48 h. RWC was calculated according to Barr and Weatherley (1962):

$$RWC\,(\%) = [(FW - DW)/(TW - DW)] \times 100.$$

WLR estimates rate of water loss of leaves exposed to dehydration and was determined on a separate set of young fully expanded leaves. Fresh leaf weight (FW) was measured immediately after excision. Weight after 4-h drying on filter paper (W4) (at room temperature under constant light) was recorded, and total leaf DW was recorded after oven-drying at 72 °C for 48h. Leaf WLR was calculated according to Suprunova *et al.* (2004) and Verslues *et al.* (2006):

$$WLR\,(g\,h^{-1}\,g^{-1}\,DW) = [(FW - W4)]/[DW \times 4].$$

RWC and WLR were measured twice for each landrace and each replication at week 6 after sowing using separate sets of leaves. WS was estimated 1 day before harvest. At 60 days after sowing, plants were carefully extracted, the roots were washed without damage, and then, shoots and roots were put into separate plastic bags.

Chlorophyll content was estimated via SPAD values measured at 48 days after sowing using a SPAD-502 Plus chlorophyll meter (Konica Minolta, Japan) and four measures were performed on fully expanded leaves per plant. Shoot length was measured as stem length (cm) at 12 and 22 days after sowing. Dry root and shoot biomass (DRW, DSW; mg plant$^{-1}$) were measured after oven-drying at 72 °C for 48 h. Root–shoot ratio (RS ratio) was calculated by dividing dry root weight by dry shoot weight. Seedling vigour (SV) was recorded following the 1–5 IBPGR and ICARDA (1985) scale: 1 = very poor, 2 = poor, 3 = average, 4 = good, and 5 =excellent. All variables were analyzed as mean values based on four plants per pot and per genotype.

**4.2.6. Data analysis**

Molecular genetic diversity analysis from SSR and AFLP markers was performed as described above in the section 3.2.6 of chapter 3. Genetic distance matrices between all pairwise genotypes based on Nei's genetic distance (Nei 1973) using binary matrices for SSR and AFLP as well as Mantel test (Mantel 1967) were computed and performed on NTSYSPC 2.1 (Rohlf 2004) program to construct neighbor-joining clusters to show the associations between the studied landraces. Bootstrap analysis of neighbor-joining dendrograms was performed using TREECON software (Van de Peer and De Wachter 1993) to test confidence and faithfulness of the obtained groupings.

Structure 2.3.4 software (Pritchard *et al.* 2005, 2010) was used to investigate the population structure of the lentil Mediterranean germplasm studied including all genotypes (5 for each landrace) using SSR multilocus genotype data. The admixture model was assumed to perform ten runs for $K=1$ to K=10 with the "length of burning period" and the "number of Markov chain Monte Carlo" repeats of 100000 both. In the admixture model both possibilities of correlated allele frequencies and independent allele frequencies were tested. The output of the ten runs were used to estimate the most likely number of gene pools (*k*) according to the method described by Evanno *et al.* (2005) using the *ad hoc* statistic *Delta k* ($\Delta K$). This method allows the identification of genetically homogeneous groups of individuals using a Bayesian algorithm. It is based on the rate of change in the log-probability (computed from posterior likelihoods) of data generated by successive *K* values, whereby the highest $\Delta K$ corresponds to the true number of gene pools (*K*). *Delta K* was estimated following the formula of Evanno *et al.* (2005):

$Delta\ K = [L''(K)]/S$, where *S* is the standard deviation of the estimated probability values [$L(K)$] from the ten runs and $L''(K)$ is the absolute value of the second order rate of change of the likelihood distribution.

SPSS Statistics 22 was used for normality test, variance, correlation, and principal component analyses of root and shoots traits, drought parameters, and genetic data from SSR and AFLP markers. It was also used to perform the nonparametric Kruskal–Wallis analysis to test the associations between individual SSR and AFLP markers and drought parameters as measured by WS, RWC and WLR. In order to test functional groupings according to drought responses of landraces, canonical discriminant analyses based on genetic distance between landraces from SSR (chi-square distance dissimilarity measure) and AFLP (Jaccard similarity index) markers linked to the three drought parameters were performed using prior information on landraces' response to drought as follows. The five classes obtained according to WS (Singh *et al.* 2013) as described above were used as grouping variable (dependent variables). Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate (52.5≤RWC<60) and tolerant (RWC ≥60). Similarly, three classes were defined for WLR: sensitive (WLR ≥0.56), intermediate (0.56<WLR≤0.50) and tolerant (WLR <0.50). As for WS, these classes were used as grouping variables with genetic data from SSR and AFLP as predictor variables for canonical discriminant analysis.

Regression analysis based on SSR and AFLP markers linked to the three drought measures was performed to confirm association revealed by the K-W test and to identify the markers explaining the highest phenotypic variation. Canonical discriminant and regression analyses were performed using SPSS Statistics 22.

## 4.3. Results

### 4.3.1. Genetic diversity

For all landraces' genotypes, 19 SSRs produced a total of 261 alleles with an average of 13.73 alleles per locus whereby the number of alleles per locus ranged from 2 to 26. SSR215 locus produced the largest number of observed alleles (*no*) while SSR124, SSR99 and SSR130 loci produced the lowest number of alleles. Average Shannon information index was 1.73, ranging from 0.15 for SSR99 to 2.80 for SSR215. The level of genetic diversity as estimated by expected heterozygosity (*He*), expressing the probability at a given locus of two alleles taken at random from the population to be different of each other, ranged from 0.0694 (SSR99) to 0.9253 (SSR212-1) with an average over all loci for all landraces of 0.6775. Total probability of identity (PI) between two randomly chosen genotypes of the landraces over all loci was as low as $4.89 \times 10^{-24}$ (Table 4.2).

**Table 4.2.** SSR polymorphism parameters in the Mediterranean lentil landraces

| Locus Name | Number of observed alleles (*no*) | Number of expected alleles (*ne*) | Shannon Information Index (*I*) | Observed heterozygosity (*Ho*) | Expected heterozygosity (*He*) | Probability of Identity (*PI*) |
|---|---|---|---|---|---|---|
| SSR113 | 19 | 10.11 | 2.52 | 0.0403 | 0.9024 | 0.0088 |
| SSR154 | 12 | 2.50 | 1.47 | 0.7708 | 0.6018 | 0.0224 |
| SSR199 | 5 | 2.20 | 1.06 | 0.3311 | 0.5480 | 0.1069 |
| SSR124 | 2 | 1.12 | 0.24 | 0.0095 | 0.1115 | 0.7283 |
| SSR233 | 13 | 2.98 | 1.59 | 0.5545 | 0.6661 | 0.0698 |
| SSR80 | 14 | 7.95 | 2.28 | 0.0476 | 0.8757 | 0.0118 |
| SSR184 | 22 | 4.34 | 2.11 | 0.1516 | 0.7713 | 0.0572 |
| SSR48 | 17 | 6.87 | 2.22 | 0.0526 | 0.8557 | 0.0217 |
| SSR19 | 10 | 5.43 | 1.84 | 0.0466 | 0.8174 | 0.0519 |
| SSR99 | 2 | 1.07 | 0.15 | 0.0000 | 0.0694 | 0.5161 |
| SSR302 | 16 | 3.29 | 1.75 | 0.2322 | 0.6974 | 0.0873 |
| SSR309_2 | 8 | 3.88 | 1.57 | 0.8899 | 0.7439 | 0.0591 |
| SSR204 | 7 | 3.46 | 1.40 | 0.0521 | 0.7127 | 0.0642 |
| SSR336 | 15 | 7.09 | 2.11 | 0.4509 | 0.8604 | 0.0255 |
| SSR119 | 24 | 10.13 | 2.60 | 0.0000 | 0.9027 | 0.0095 |
| SSR212_1 | 22 | 13.14 | 2.77 | 0.0947 | 0.9253 | 0.0080 |
| SSR215 | 26 | 10.32 | 2.80 | 0.7273 | 0.9046 | 0.0272 |
| SSR130 | 2 | 1.13 | 0.26 | 0.0116 | 0.1207 | 0.7671 |
| SSR33 | 25 | 4.61 | 2.09 | 0.3567 | 0.7845 | 0.0217 |
| **Total** | 261 | | | | | $4.89 \times 10^{-24}$ |
| **Average** | 13.73 | 5.35 | 1.73 | 0.2537 | 0.6775 | |
| **Standard deviation** | 7.72 | 3.59 | 0.82 | 0.2923 | 0.2776 | |

Seven AFLP primer combinations yielded a total of 812 fragments ranging from 50.08 to 499.54 bp over all landraces, with an average of about 116 fragments per primer combination. The highest number of fragments was produced by primer combination *EcoRI-ACA* + *MseI-CTT* (PC3) with 162 fragments, while the lowest number was produced by primer combination *EcoRI-AGC* + *MseI-CTG* (PC7) with 83 fragments. Of all fragments obtained, 449 (54.28 %) were polymorphic. Polymorphic band percentages ranged from 45.70 (*EcoRI-ACG* + *MseI-CAA* (PC4)) to 68.33 % (*EcoRI-ACA* + *MseI-CAG* (PC1)). Polymorphic information content (*PIC*) ranged from 0.3195 (*EcoRI-ACG* + *MseI-CAA* (PC4)) to 0.4497 (*EcoRI-ACA* + *MseI-CAG* (PC1)), with an average over the seven primer combinations of 0.3509 (Table 4.3).

**Table 4.3.** AFLP primer combinations polymorphism parameters in the Mediterranean lentil landraces

| Primer combinations | Number of fragments | Polymorphic fragments | | | Fragment size range (bp) | *PIC* |
|---|---|---|---|---|---|---|
| | | Number | Standard deviation | Percentage | | |
| *EcoRI*-ACA+ *MseI*-CAG (PC1) | 148 | 101.2 | 25.4 | 68.33 | 52-480 | 0.4497 |
| *EcoRI*-ACA+ *MseI*-CTG (PC2) | 127 | 68.75 | 21.97 | 54.13 | 50-499 | 0.3387 |
| *EcoRI*-ACA+ *MseI*-CTT (PC3) | 162 | 91.42 | 16.25 | 56.43 | 50-469 | 0.3588 |
| *EcoRI*-ACG+ *MseI*-CAA (PC4) | 96 | 43.87 | 19.83 | 45.70 | 50-486 | 0.3195 |
| *EcoRI*-AGC+ *MseI*-CAA (PC5) | 104 | 53.28 | 17.39 | 51.23 | 51-493 | 0.3259 |
| *EcoRI*-AGC+ *MseI*-CAG (PC6) | 92 | 48.39 | 18.16 | 52.60 | 52-491 | 0.3393 |
| *EcoRI*-AGC+ *MseI*-CTG (PC7) | 83 | 42.77 | 9.25 | 51.54 | 50-499 | 0.3249 |
| **Total** | 812 | 449 | | - | - | - |
| **Average** | 116 | 64.24 | | 54.28 | - | 0.3509 |

### 4.3.2. Genetic relationship between landraces as revealed by SSR and AFLP DNA markers

Genetic relationship among landraces was assessed for both microsatellite and AFLP markers taken separately using neighbor-joining (NJ) method and the combined data sets using principle component analysis (PCA). Based on SSR markers, the NJ dendrogram generated five groups. Landraces from the northern Mediterranean (Italy, Turkey and Greece) were grouped together in group 4 with a bootstrap value of 84 % separately from those of Morocco, except for six landraces (M29, M30, M39, M49, M26 and M8). The four other groups were from Morocco (Figure 4.1).

NJ grouping based on AFLP markers (Figure 4.2) discriminated between landraces from Morocco and those from northern Mediterranean. Landraces from Italy, Turkey and Greece were clustered in group 1 with a bootstrap value of 100 %. Landraces from Morocco could be separated into seven groups, one large group containing 36 landraces, two groups containing 7 and 5 landraces, respectively, and three single landrace (M7, M12 and M13) separated from the rest.

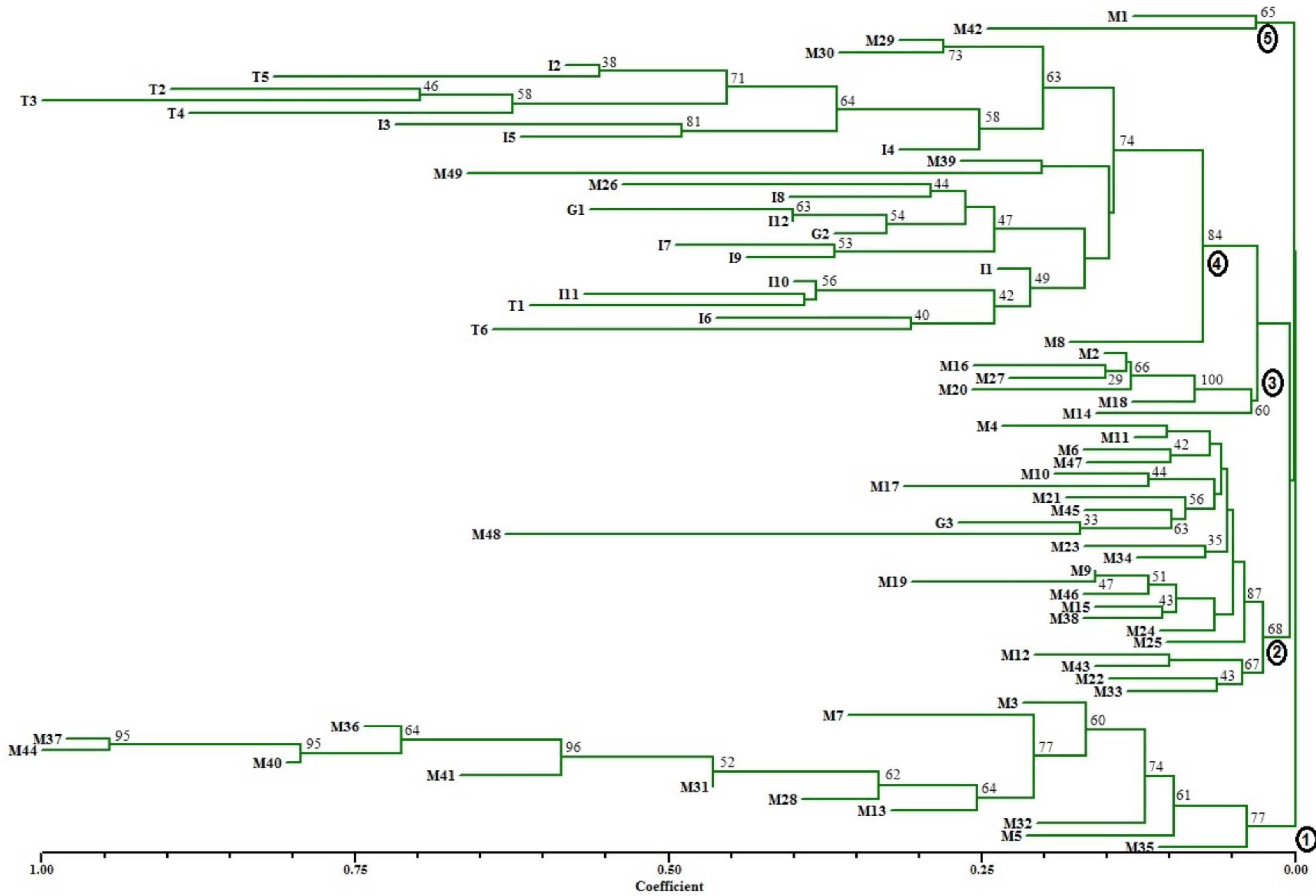


**Figure 4.1.** Neighbor-joining (NJ) dendrogram of the Mediterranean lentil landraces obtained via Nei's genetic distance from SSR markers. Bootstrap values are given at the nodes.

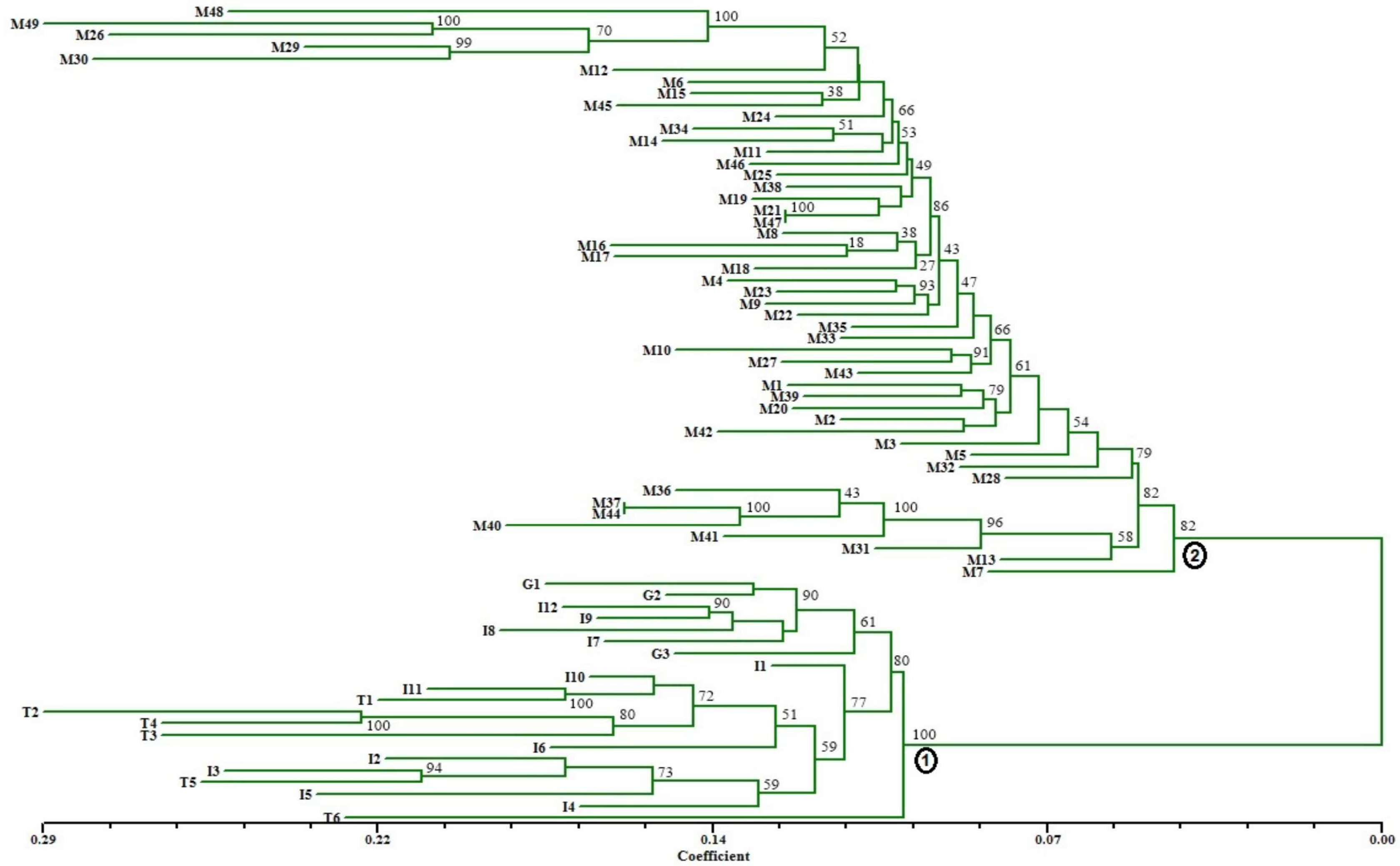


**Figure 4.2.** Neighbor-joining (NJ) dendrogram of the Mediterranean lentil landraces obtained via Nei's genetic distance from AFLP markers. Bootstrap values are given at the nodes.

Genetic similarity matrices between lentil landraces from the two data sets (SSRs and AFLPs) were compared using the Mantel test. A significant correlation between the two matrices was found with $r^2=0.6485$ and Mantel $t=5.7477$ ($P<0.001$). Same grouping patterns as shown in Figures 4.1 and 4.2 were obtained based on all 350 genotypes (five genotypes per landrace) analyzed for both DNA markers (data not shown).

Combined data sets from SSR and AFLP analyses were used to construct a consensus grouping of landraces by performing PCA based on allele frequencies. The first and second axes of PCA explained respectively 37.69 % and 25.40 % of total variance and separated lentil landraces into two main groups discriminating Moroccan landraces from those of Italy, Turkey and Greece. Landraces from both the northern Mediterranean region as well as from Morocco enclose high genetic diversity (Figure 4.3).

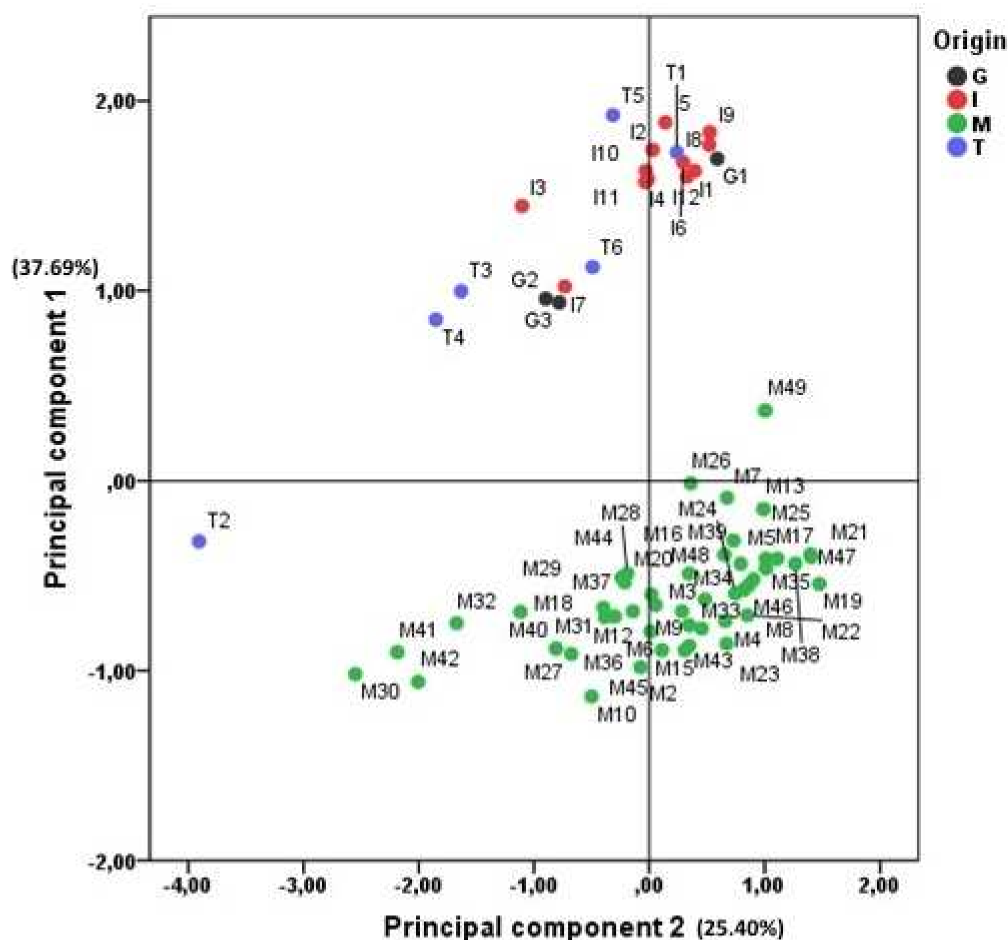


**Figure 4.3.** Principal component analysis (PCA) scatter plot based on allele frequencies from combined SSR and AFLP data sets of the Mediterranean lentil landraces sorted by country of origin. G: Greece, I: Italy, M: Morocco, T: Turkey.

### 4.3.3. Population genetic structure

Results obtained from STRUCTURE program using SSRs markers for all genotypes are reported in figure 4.5. According to the method suggested by Evanno *et al.* (2005) for the estimation of the most likely number of gene pools (*k*) in a population based on the *ad hoc* statistic *Delta K* ($\Delta K$), the Mediterranean lentil germplasm used in this study could be divided into two or three gene pools (Figure 4.4).

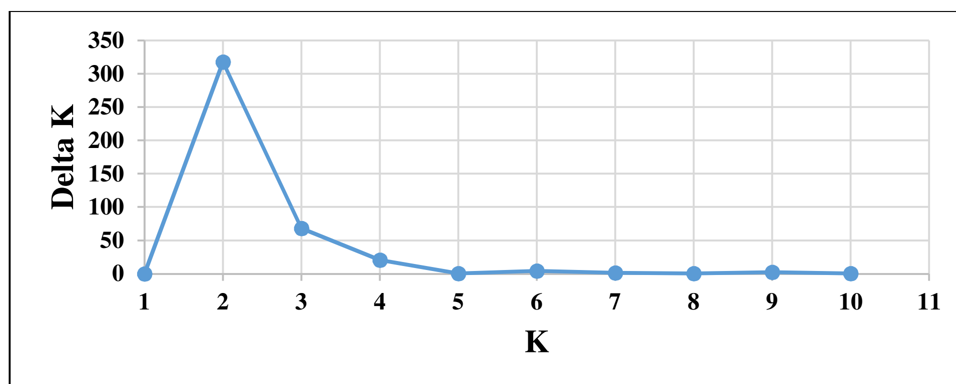


**Figure 4.4.** Variation of Evanno *et al.* (2005) $\Delta K$ for each $K$ calculated for 350 genotypes of the Mediterranean lentil landraces based on SSR markers. Admixture model with correlated allele frequencies were assumed.

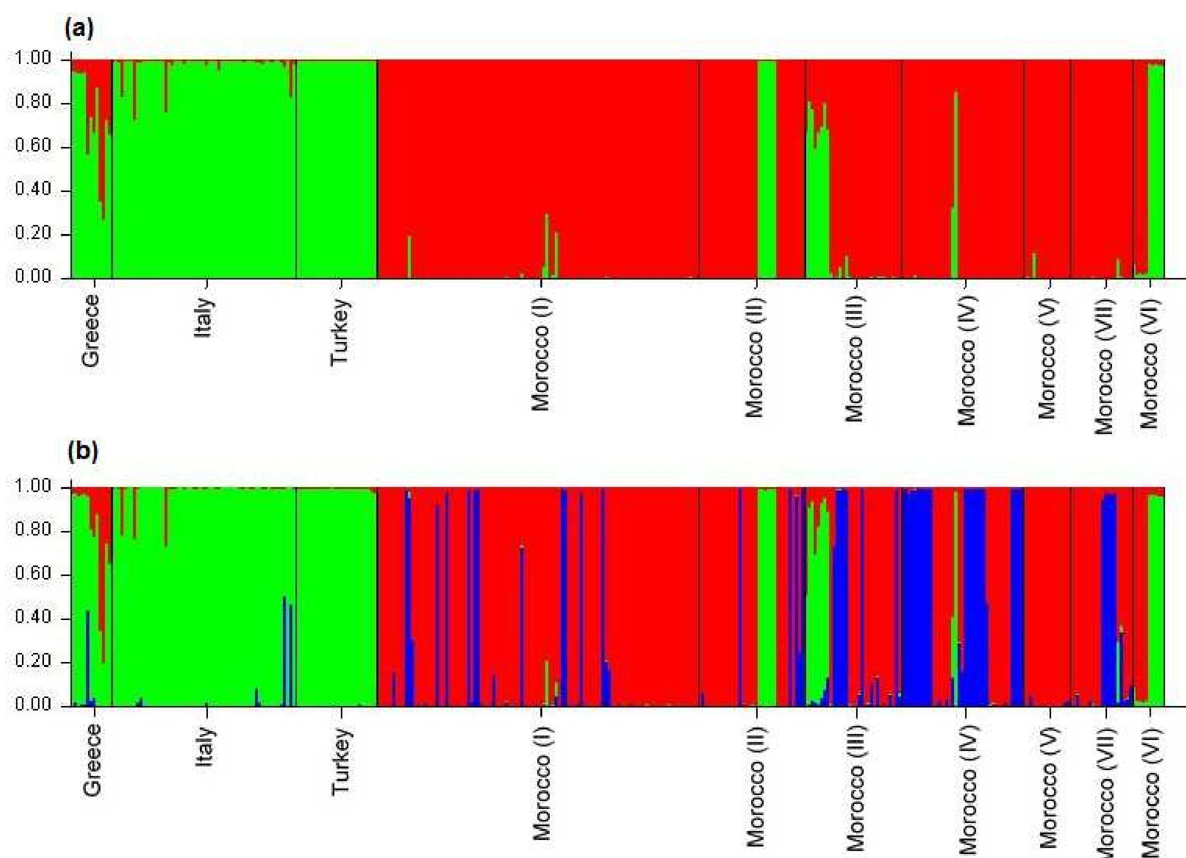


**Figure 4.5.** Inferred population genetic structure for $K$=2 (a) and $K$=3 (b) for 350 genotypes of the Mediterranean lentil landraces based on SSR markers. Each individual genotype is presented by a vertical line divided into $K$ coloured segments corresponding to the estimated fractions belonging to each gene pool shown in the vertical axis. An admixture model with correlated allele frequencies were assumed.

The best population genetic structure model is likely to be at *K*=2 which displays the clear highest value of *ΔK* (317.61). For *K* =3, a fairly higher value of *ΔK* (68.25) compared to other values of ΔK suggests also the possibility of three gene pools (Figure 4.4).

For *K=2*, landraces from the northern Mediterranean countries were clustered together in one gene pool (green cluster, figure 4.5 a) with high membership proportions of assignation of genotypes of 73.7 %, 97.8 % and 99.9 % for landraces from Greece, Italy and Turkey, respectively. The second gene pool (red cluster, figure 4.5 a) contained predominantly landraces from Morocco with high proportions of membership of each sub-population sorted by geographic origin of 99 %, 82.3 %, 80.6 %, 96.7 %, 98.9 %, 49.5 % and 99.2 % for Chaouia (I), Zear (II), middle Atlas mountains (III), Abda (IV), Sais Meknès, local cultivars (VI) and unknown origin (VII), respectively. The two gene pools shared only small proportions of landraces from northern and southern Mediterranean region, expect for the two Moroccan local cultivars that were shown to be assigned to the two different gene pools. Genetic diversity in same cluster estimated by expected heterozygosity was as high as 0.59 and 0.72 for the gene pool containing Moroccan landraces and for the once containing landraces from northern Mediterranean, respectively.

For *K=3*, landraces from the northern Mediterranean countries were shown to belong to the same gene pool (green cluster, figure 4.5 b) as found for *K=2* with closely similar proportions of membership (74.2 %, 96.5 % and 99.6 % respectively for landraces from Greece, Italy and Turkey). Moroccan landraces were assigned to the three different gene pools with different proportions. But, they were mainly clustered together in the same gene pool as shown for *K=2* (red cluster, figure 4.5 b). Proportions of membership for the latter gene pool (87.2 %, 69.7 %, 55.3 %, 99.2 %, 50 % and 71 % respectively for Chaouia, Zaer, middle Atlas mountains, Sais Meknès, local cultivars and unknown origin) were the highest compared to the two other gene pools except for landraces from Abda region. The third gene pool (blue cluster, figure 4.5 b) contained 56.7 % of landraces from Abda region, which is the highest proportions of membership for this gene pool. Expected heterozygosity in same cluster was 0.58, 0.69 and 0.56 for the red cluster, the green cluster and the blue cluster, respectively.

For both cases (*K=2* and *K=3*), genomes of some landraces include segments from different gene pools with proportions over all genotypes of 10.28 % and 15.42 %, respectively.

### 4.3.4. Root and shoot characterization and drought tolerance evaluation

All variables were normally distributed. A slight deviation from normal distribution was observed for WS, RS ratio, and shoot lengths at 12 and 22 days after sowing. Analysis of variance showed a significantly high variation for all traits measured (Table 4.4; Figure A.8, appendix): shoot lengths at 12 and 22 days after sowing, SV, dry shoot weight, chlorophyll content as estimated by the SPAD values, 100-seed weight, dry root weight, RS ratio, RWC, WLR and WS (Table 4.4). Also, variations were significant within each geographical origin.

**Table 4.4.** Variation among root and shoot traits and drought parameters in the Mediterranean lentil landraces

| Traits | Mean±sd | Min | Max | CV (%) |
|---|---|---|---|---|
| Shoot length at 12 days after sowing (SL12DAS, cm) | 6.82±1.42 | 3.53 | 10.13 | 20.82 |
| Shoot length at 22 days after sowing (SL22DAS, cm) | 17.17±3.46 | 10.53 | 21.15 | 20.15 |
| Seedling vigour (SV) | 3.38±0.93 | 1.66 | 4.66 | 27.51 |
| Dry shoot weight (DSW, $g.plant^{-1}$) | 0.8490±0.19 | 0.4763 | 1.2220 | 22.37 |
| Chlorophyll content (SPAD) | 38.23±3.18 | 31.10 | 46.6 | 8.31 |
| 100-seeds weight (SeedW, g) | 4.13±1.38 | 2.02 | 5.16 | 33.41 |
| Dry root weight (DRW, $g.plant^{-1}$) | 0.6578±0.1912 | 0.3177 | 1.1823 | 29.06 |
| Root-shoot ratio (RSRatio) | 0.7906±0.2188 | 0.3125 | 1.5501 | 27.67 |
| Leaf relative water content (RWC, %) | 56.03±9.98 | 40.12 | 75.13 | 17.81 |
| Leaf water losing rate (WLR, $g.h^{-1}.g^{-1}$ DW ) | 0.5158±0.1221 | 0.3717 | 0.7027 | 23.67 |
| Wilting score (WS) | 1.92±0.8128 | 0.33 | 3.66 | 42.33 |

sd: standard deviation, Min: minimum, Max: maximum, CV: coefficient of variation

Significant correlations were shown between the following: SV and WS (0.252); SPAD and leaf RWC (0.335), WLR (−0.325), and WS (−0.538); dry root weight and dry shoot weight (0.460), SPAD (0.573), RWC (0.482), WLR (−0.288), and WS (−0.411); and RS ratio and RWC (0.362), WLR (−0.256) and WS(−0.374) (Table 4.5). The three drought parameters were also significantly correlated to each other. WLR and WS were positively correlated (0.571), while RWC was negatively correlated to both parameters with values of −0.577 and −0.610, respectively.

**Table 4.5.** Correlations between root and shoot traits and drought parameters in the Mediterranean lentil landraces

| | **SL12DAS** | **SL22DAS** | **SV** | **DSW** | **SPAD** | **SeedW** | **DRW** | **RSRatio** | **RWC** | **WLR** | **WS** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **SL12DAS** | 1 | 0.577** | 0.578** | 0.320** | 0.059 | 0.050 | -0.015 | -0.222 | -0.062 | 0.013 | 0.167 |
| **SL22DAS** | 0.577** | 1 | 0.761** | 0.533** | -0.050 | 0.524** | 0.040 | -0.372** | -0.098 | 0.214 | 0.267* |
| **SV** | 0.578** | 0.761** | 1 | 0.571** | 0.005 | -0.177 | 0.127 | -0.259* | -0.077 | 0.095 | 0.252* |
| **DSW** | 0.320** | 0.533** | 0.571** | 1 | 0.105 | 0.235 | 0.460** | -0.453** | 0.052 | 0.072 | 0.126 |
| **SPAD** | 0.059 | -0.050 | 0.005 | 0.105 | 1 | -0.177 | 0.573** | 0.298* | 0.335** | -0.325** | -0.538** |
| **SeedW** | 0.050 | 0.524** | -0.177 | 0.235 | -0.177 | 1 | -0.153 | -0.313** | -0.232 | 0.310* | 0.319* |
| **DRW** | -0.015 | 0.040 | 0.127 | 0.460** | 0.573** | -0.153 | 1 | 0.737** | 0.482** | -0.288* | -0.411** |
| **RSRatio** | -0.222 | -0.372** | -0.259* | -0.453** | 0.298* | -0.313** | 0.737** | 1 | 0.362** | -0.256* | -0.374* |
| **RWC** | -0.062 | -0.098 | -0.077 | 0.052 | 0.335** | -0.232 | 0.482** | 0.362** | 1 | -0.577** | -0.610** |
| **WLR** | 0.013 | 0.214 | 0.095 | 0.072 | -0.325** | 0.310* | -0.288* | -0.256* | -0.577** | 1 | 0.571** |
| **WS** | 0.167 | 0.267* | 0.252* | 0.126 | -0.538** | 0.319* | -0.411** | -0.374** | -0.610** | 0.571** | 1 |

** Significant at 0.01 level; * Significant at 0.05 level.
Shoot length at 12 days after sowing (SL12DAS, cm), Shoot length at 22 days after sowing (SL22DAS, cm), Seedling vigour (SV), Dry shoot weight (DSW, $g.plant^{-1}$), Chlorophyll content (SPAD), 100-seeds weight (SeedW, g), Dry root weight (DRW, $g.plant^{-1}$), Root-shoot ratio (RSRatio), Leaf relative water content (RWC, %), Leaf water losing rate (WLR, $g.h^{-1}.g^{-1}$ DW ), Wilting score (WS)

We also performed PCA based on all phenotypic variables among landraces. The first and second axes explained 34.16 % and 24.59 % of total variation, respectively (Figure 4.6).

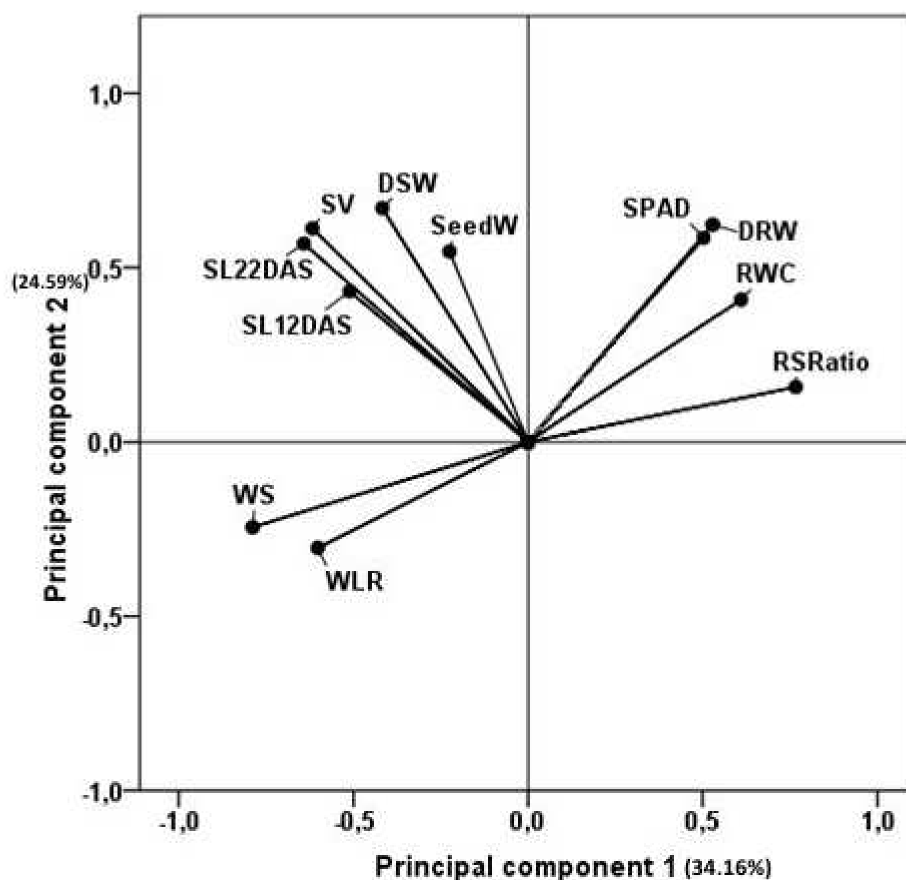


**Figure 4.6.** Principal component analysis (PCA) scatter plot based on all phenotypic traits measured on the 70 Mediterranean lentil landraces tested (shoot length at 12 days after sowing (SL12DAS), shoot length at 22 days after sowing (SL22DAS), seedling vigour (SV), dry shoot weight (DSW), chlorophyll content (SPAD), 100-seed weight (SeedW), dry root weight (DRW), root–shoot ratio (RSRatio), leaf relative water content (RWC), leaf water losing rate (WLR) and wilting score (WS)).

Principal component 1 was positively correlated with RS ratio (0.766), leaf RWC (0.609), dry root weight (0.529) and chlorophyll content (0.503), and negatively correlated with WS (−0.789), WLR (−0.603), shoot lengths at 12 and 22 DAS (−0.511; −0.643), SV (−0.618) and dry shoot weight (–0.418). Principal component 2 was positively correlated with dry shoot weight (0.670), dry root weight (0.623), SV (0.612), chlorophyll content (0.585), shoot lengths at 12 and 22 days after sowing (0.431; 0.569) and leaf RWC (0.408). Weak but still significant negative correlations of principal component 2 were observed with WLR (–0.303) and WS (–0.244).

Weak but significant differentiation (low eigenvalues of discriminant analysis) according to geographical origin was observed based on phenotypic data, and landraces from Morocco and

Greece had slightly higher shoot length, biomass, and seedling early vigour compared to those from Italy and Turkey. Turkish landraces had the lowest biomass (Figures 4.7 and 4.8). When discriminant analysis was performed based on the northern Mediterranean versus Moroccan origin as grouping variable, differentiation is more evident (Figure 4.7).

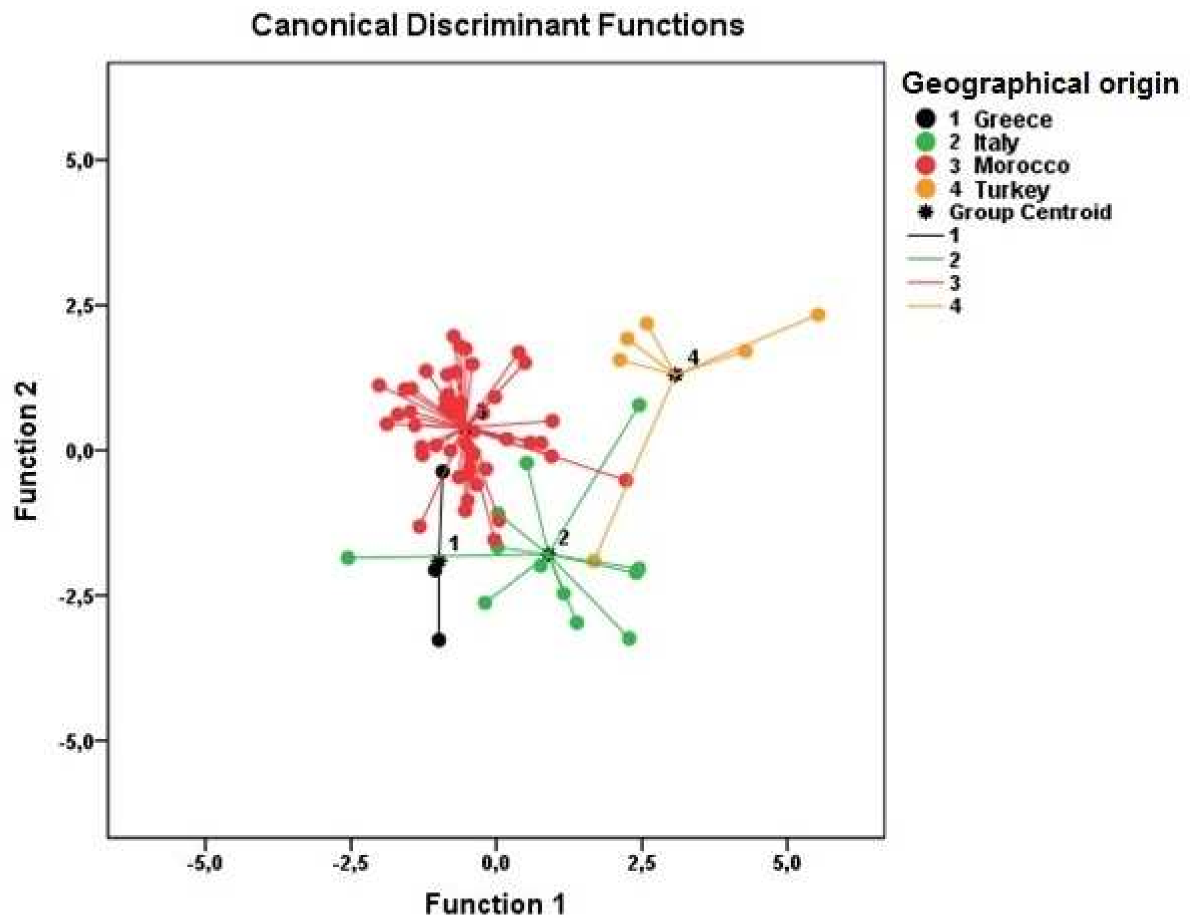


**Figure 4.7.** Discriminant analysis based on phenotypic data according to country of origin of the Mediterranean lentil landraces.

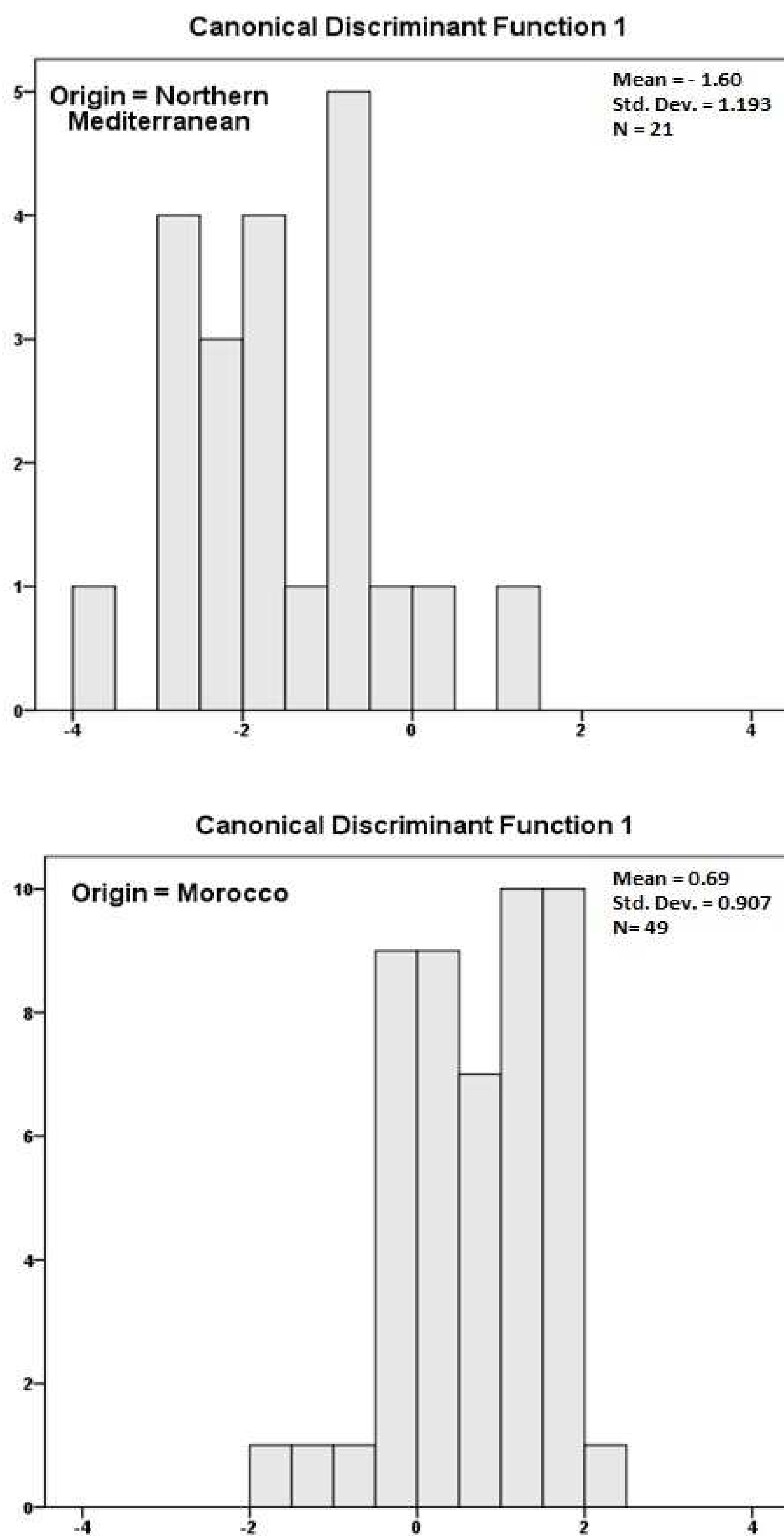


**Figure 4.8.** Discriminant analysis based on phenotypic data according to geographical origin of the Mediterranean lentil landraces.

Drought tolerance level as evaluated by RWC, WLR and WS showed high genotypic variations among landraces. RWC ranged from 40.12 % in T4 to 75.13 % in G1; WLR ranged from 0.3717 in M30 to 0.7027 in M18; WS ranged from 0.33 in I3 to 3.66 in M17 (Figure 4.9). No correlation between landrace' origin and drought response was observed.

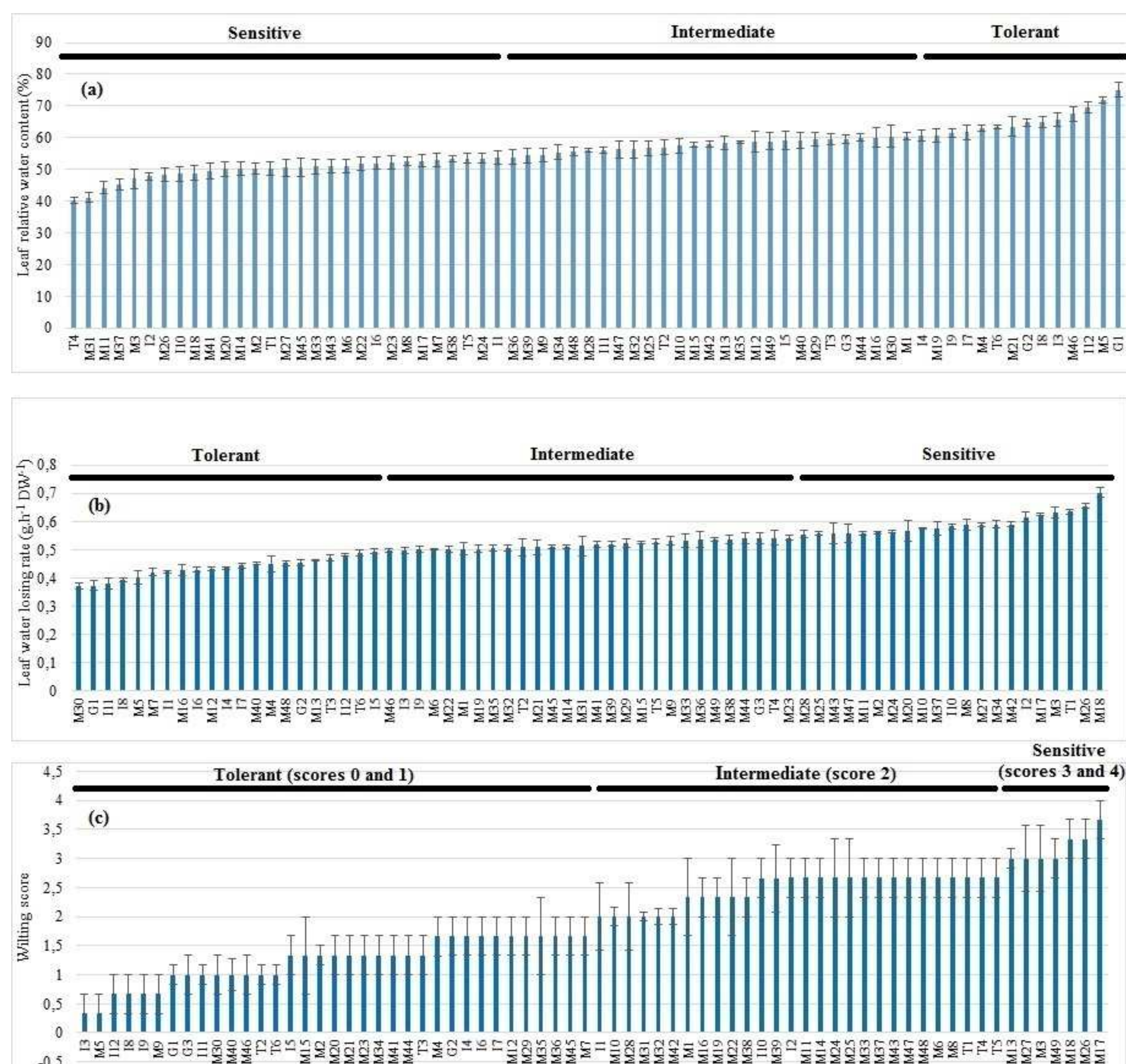


**Figure 4.9.** Variation of relative water content (a), leaf water losing rate (b) and wilting score (c) among the Mediterranean lentil landraces tested. Wilting score: 0 to 4 corresponds to the following 0–4 score scale as described by Singh *et al.* (2013): 0 = healthy plants with no visible symptoms of drought stress, 1 = green plants with slight wilting, 2 = leaves turning yellowish-green with moderate wilting, 3 = leaves yellow–brown with severe wilting and 4 = completely dried leaves and/or stems. Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate (52.5≤RWC<60) and tolerant (RWC ≥60). Similarly, three classes were defined for WLR: sensitive (WLR ≥0.56), intermediate (0.56<WLR≤0.50) and tolerant (WLR <0.50).

PCA was performed with the three parameters used to estimate drought tolerance (leaf RWC, leaf WLR and WS) in order to sort the landraces according to a consensus classification in response to drought stress (Figure 4.10). Principal components 1 and 2 explained 65.25 % and

18.57 % of total variation, respectively. The first axis was highly correlated with the three parameters: –0.826 with leaf RWC, 0.807 with WLR and 0.791 with WS. Higher values of this axis indicated sensitive landraces, while lower values indicated tolerant landraces.

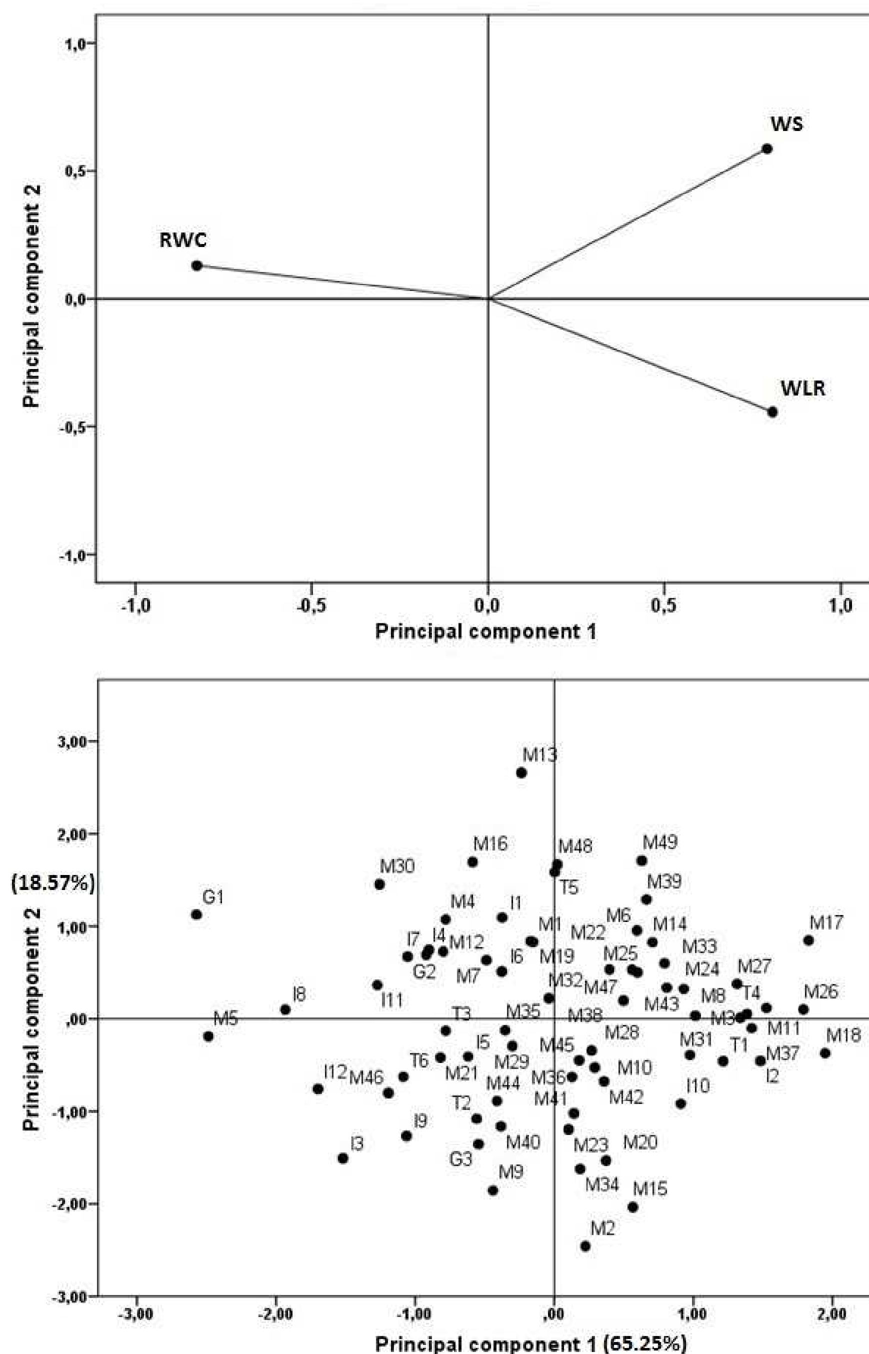


**Figure 4.10.** PCA of the Mediterranean lentil landraces based on leaf relative water content (RWC), leaf water losing rate (WLR) and wilting score (WS). The first upper figure sorts the three variables as associated with the two principal components (PC) whereas the lower part shows landraces according to the two PCs.

### 4.3.5. SSR and AFLP markers associated with drought tolerance

In order to determine SSR and AFLP markers that are linked to the individually measured physiological traits, a Kruskal– Wallis analysis was applied. The test was based on the ranking of landraces according to leaf RWC, WLR, and WS separately and testing the association to the markers one by one as grouping variable. Six, four and five alleles from SSRs loci were identified to be associated with leaf RWC, leaf WLR and WS, respectively (Table 4.6). On the other hand, 91, 105 and 51 AFLP markers were found to be associated with leaf RWC, WLR and WS, respectively (Tables 4.7, 4.8 and 4.9).

**Table 4.6.** SSR markers linked to drought parameters according to Kruskal-Wallis H test

| SSRs linked to drought parameters | Allele size (bp) | Chi-square | Degree of freedom | Asymptotic significance* | Correlation* |
|---|---|---|---|---|---|
| **Relative water content (RWC)** | | | | | |
| **SSR113_5** | 221 | 15.32 | 6 | 0.018 | -0.24* |
| **SSR184_17** | 263 | 8.36 | 3 | 0.039 | 0.42** |
| **SSR19_7** | 262 | 7.30 | 2 | 0.02 | 0.32** |
| **SSR233_13** | 155 | 11.5 | 5 | 0.04 | 0.26* |
| **SSR48_3** | 165 | 3.9 | 1 | 0.04 | 0.25* |
| **SSR80_12** | 153 | 18.1 | 7 | 0.01 | 0.27* |
| **Leaf water losing rate (WLR)** | | | | | |
| **SSR215_9** | 388 | 6.07 | 2 | 0.04 | -0.33** |
| **SSR154_4** | 361 | 6.95 | 2 | 0.04 | 0.27* |
| **SSR184_17** | 263 | 8.86 | 3 | 0.04 | -0.28* |
| **SSR336_22** | 279 | 10.7 | 4 | 0.04 | -0.28* |
| **Wilting score (WS)** | | | | | |
| **SSR119_5** | 271.50 | 4.8 | 1 | 0.02 | 0.25* |
| **SSR154_12** | 379 | 3.96 | 1 | 0.04 | 0.24* |
| **SSR19_7** | 270.50 | 14.45 | 6 | 0.02 | 0.25* |
| **SSR204_1** | 177 | 5.64 | 1 | 0.01 | 0.36** |
| **SSR48_3** | 165.50 | 4.8 | 1 | 0.03 | -0.32** |

*Significant at p<0.05, **Significant at p<0.01

**Table 4.7.** AFLP markers linked to relative water content (RWC) according to Kruskal-Wallis H test

| AFLPs Linked to RWC | Allele size (bp) | Chi-square | Degree of freedom | Asymptotic significance | Correlation |
|---|---|---|---|---|---|
| PC1_111 | 111 | 11.57 | 3 | 0.009 | 0.30* |
| PC1_114 | 114 | 11.92 | 4 | 0.036 | 0.33** |
| PC1_127 | 127 | 13.71 | 4 | 0.018 | -0.20* |
| PC1_145 | 145 | 10.97 | 4 | 0.027 | -0.20* |
| PC1_152 | 152 | 10.82 | 4 | 0.029 | 0.36** |
| PC1_171 | 171 | 12.97 | 4 | 0.024 | 0.27* |
| PC1_217 | 217 | 12.47 | 4 | 0.029 | 0.19* |
| PC1_218 | 218 | 10.69 | 4 | 0.030 | 0.26* |
| PC1_219 | 219 | 12.23 | 4 | 0.032 | -0.27* |
| PC1_234 | 234 | 10.85 | 4 | 0.028 | 0.32** |
| PC1_236 | 236 | 15.84 | 4 | 0.007 | 0.33** |
| PC1_238 | 238 | 17.30 | 4 | 0.016 | -0.30* |
| PC1_240 | 240 | 11.64 | 4 | 0.020 | 0.31** |
| PC1_290 | 290 | 14.77 | 4 | 0.011 | 0.19* |
| PC1_291 | 291 | 13.18 | 4 | 0.022 | 0.35** |
| PC1_299 | 299 | 13.79 | 4 | 0.017 | -0.27* |
| PC1_314 | 314 | 14.87 | 4 | 0.011 | 0.42** |
| PC1_319 | 319 | 11.78 | 4 | 0.038 | 0.19* |
| PC1_323 | 323 | 13.02 | 4 | 0.023 | 0.35** |
| PC1_327 | 327 | 15.32 | 4 | 0.009 | 0.49** |
| PC1_329 | 329 | 12.63 | 4 | 0.027 | 0.37** |
| PC1_355 | 355 | 17.37 | 4 | 0.004 | 0.44** |
| PC1_400 | 400 | 13.63 | 4 | 0.018 | 0.33** |
| PC1_419 | 419 | 12.43 | 4 | 0.029 | 0.33** |
| PC1_422 | 422 | 14.99 | 4 | 0.010 | 0.41** |
| PC1_447 | 447 | 16.13 | 4 | 0.006 | 0.45** |
| PC1_456 | 456 | 19.36 | 4 | 0.002 | 0.46** |
| PC1_458 | 458 | 13.01 | 4 | 0.023 | 0.21* |
| PC1_53 | 53 | 12.38 | 4 | 0.030 | 0.36** |
| PC1_75 | 75 | 14.18 | 4 | 0.014 | 0.33** |
| PC1_98 | 98 | 13.50 | 4 | 0.019 | 0.41** |
| PC2_108 | 108 | 12.47 | 4 | 0.029 | 0.22* |
| PC2_120 | 120 | 13.43 | 4 | 0.020 | 0.33** |
| PC2_166 | 166 | 14.78 | 4 | 0.011 | 0.36** |
| PC2_250 | 250 | 15.14 | 4 | 0.010 | 0.33** |
| PC2_352 | 352 | 15.03 | 4 | 0.010 | 0.32** |
| PC2_64 | 64 | 11.27 | 4 | 0.046 | 0.32** |
| PC2_98 | 98 | 17.09 | 4 | 0.004 | 0.44** |
| PC3_113 | 113 | 12.28 | 4 | 0.031 | 0.35** |
| PC3_140 | 140 | 15.72 | 4 | 0.008 | -0.27* |
| PC3_184 | 184 | 12.39 | 4 | 0.030 | 0.25* |
| PC3_185 | 185 | 13.78 | 4 | 0.017 | 0.29* |
| PC3_261 | 261 | 15.85 | 4 | 0.008 | 0.30* |
| PC3_305 | 305 | 14.52 | 4 | 0.024 | -0.26* |
| PC3_311 | 311 | 12.27 | 4 | 0.031 | 0.28* |
| PC3_471 | 471 | 7.87 | 3 | 0.049 | 0.30* |

**Table 4.7. Continued**

| AFLPs Linked to RWC | Allele size (bp) | Chi-square | Degree of freedom | Asymptotic significance* | Correlation* |
|---|---|---|---|---|---|
| PC3_59 | 59 | 11.94 | 4 | 0.036 | 0.25* |
| PC3_64 | 64 | 7.92 | 3 | 0.048 | 0.24* |
| PC3_69 | 69 | 13.49 | 4 | 0.009 | 0.26* |
| PC3_88 | 88 | 14.53 | 4 | 0.006 | 0.23* |
| PC3_91 | 91 | 12.55 | 4 | 0.028 | 0.25* |
| PC3_93 | 93 | 13.04 | 4 | 0.023 | 0.26* |
| PC3_97 | 97 | 18.63 | 4 | 0.002 | 0.27* |
| PC3_333 | 333 | 11.85 | 4 | 0.037 | 0.25* |
| PC3_384 | 384 | 17.93 | 4 | 0.003 | 0.26* |
| PC4_152 | 152 | 11.28 | 3 | 0.01 | 0.25* |
| PC4_179 | 179 | 14.19 | 4 | 0.014 | 0.36** |
| PC4_196 | 196 | 13.49 | 4 | 0.019 | -0.37** |
| PC4_270 | 270 | 12.64 | 4 | 0.027 | 0.28* |
| PC4_300 | 300 | 15.74 | 4 | 0.008 | 0.35** |
| PC4_302 | 302 | 13.02 | 4 | 0.023 | 0.40** |
| PC4_303 | 303 | 11.73 | 4 | 0.039 | 0.41** |
| PC4_377 | 377 | 11.78 | 4 | 0.038 | 0.26* |
| PC4_380 | 380 | 13.70 | 4 | 0.018 | 0.24* |
| PC4_444 | 444 | 13.16 | 4 | 0.011 | 0.30* |
| PC4_81 | 81 | 16.10 | 4 | 0.007 | 0.32** |
| PC4_89 | 89 | 11.67 | 4 | 0.020 | -0.25* |
| PC4_93 | 93 | 14.06 | 4 | 0.015 | 0.48** |
| PC5_104 | 104 | 15.93 | 4 | 0.007 | 0.29* |
| PC5_134 | 134 | 12.49 | 4 | 0.029 | 0.22* |
| PC5_193 | 193 | 12.02 | 4 | 0.034 | 0.37** |
| PC5_248 | 248 | 13.88 | 4 | 0.016 | 0.24* |
| PC5_283 | 283 | 14.66 | 4 | 0.012 | 0.38** |
| PC5_350 | 350 | 18.27 | 4 | 0.032 | 0.30* |
| PC5_435 | 435 | 12.33 | 4 | 0.015 | 0.40** |
| PC5_436 | 436 | 11.92 | 4 | 0.036 | 0.23* |
| PC6_121 | 121 | 18.75 | 4 | 0.002 | 0.29* |
| PC6_123 | 123 | 11.80 | 4 | 0.038 | 0.36** |
| PC6_150 | 150 | 10.26 | 4 | 0.036 | 0.26* |
| PC6_321 | 321 | 11.98 | 4 | 0.035 | -0.27* |
| PC6_478 | 478 | 12.54 | 4 | 0.028 | 0.24* |
| PC6_484 | 484 | 12.18 | 4 | 0.032 | 0.23* |
| PC6_68 | 68 | 11.79 | 4 | 0.038 | 0.45** |
| PC6_74 | 74 | 13.06 | 4 | 0.023 | -0.24* |
| PC7_126 | 126 | 17.63 | 4 | 0.001 | 0.31** |
| PC7_234 | 234 | 15.72 | 4 | 0.008 | -0.24* |
| PC7_253 | 253 | 15.14 | 4 | 0.032 | 0.37** |
| PC7_360 | 360 | 11.86 | 4 | 0.037 | 0.26* |
| PC7_479 | 479 | 12.67 | 4 | 0.027 | 0.24* |
| PC7_63 | 63 | 20.043 | 4 | 0.001 | -0.37** |
| PC7_92 | 92 | 10.06 | 4 | 0.039 | 0.24* |

*Significant at $p<0.05$, **Significant at $p<0.01$

**Table 4.8.** AFLP markers linked to water losing rate (WLR) according to Kruskal-Wallis H test

| AFLPs linked to WLR | Allele size (bp) | Chi-square | Degree of freedom | Asymptotic significance* | Correlation* |
|---|---|---|---|---|---|
| PC1_111 | 111 | 14.08 | 3 | 0.003 | -0.24* |
| PC1_114 | 114 | 11.26 | 4 | 0.046 | -0.25* |
| PC1_117 | 117 | 17.71 | 4 | 0.003 | -0.34** |
| PC1_127 | 127 | 12.48 | 4 | 0.029 | -0.24* |
| PC1_140 | 140 | 12.66 | 4 | 0.027 | -0.24* |
| PC1_143 | 143 | 12.73 | 4 | 0.026 | 0.24* |
| PC1_164 | 164 | 10.06 | 4 | 0.039 | -0.26* |
| PC1_175 | 175 | 14.36 | 4 | 0.013 | -0.39** |
| PC1_178 | 178 | 12.11 | 3 | 0.007 | -0.24* |
| PC1_213 | 213 | 12.54 | 4 | 0.028 | -0.27* |
| PC1_234 | 234 | 9.65 | 4 | 0.047 | -0.27* |
| PC1_238 | 238 | 20.40 | 4 | 0.005 | 0.41** |
| PC1_254 | 254 | 9.32 | 3 | 0.025 | -0.23* |
| PC1_255 | 255 | 12.31 | 4 | 0.015 | -0.24* |
| PC1_258 | 258 | 15.77 | 4 | 0.008 | -0.25* |
| PC1_288 | 288 | 14.72 | 4 | 0.012 | -0.30* |
| PC1_290 | 290 | 14.60 | 4 | 0.012 | -0.24* |
| PC1_291 | 291 | 12.96 | 4 | 0.024 | -0.31** |
| PC1_299 | 299 | 18.30 | 4 | 0.003 | 0.30* |
| PC1_306 | 306 | 11.58 | 4 | 0.041 | 0.26* |
| PC1_329 | 329 | 13.48 | 4 | 0.019 | -0.24* |
| PC1_333 | 333 | 11.58 | 4 | 0.041 | -0.24* |
| PC1_343 | 343 | 11.76 | 4 | 0.038 | -0.28* |
| PC1_399 | 399 | 12.04 | 4 | 0.034 | -0.30* |
| PC1_400 | 400 | 11.99 | 4 | 0.035 | -0.30* |
| PC1_458 | 458 | 13.80 | 4 | 0.017 | -0.25* |
| PC1_97 | 97 | 12.08 | 4 | 0.034 | -0.24* |
| PC1_98 | 98 | 12.95 | 4 | 0.024 | -0.36** |
| PC2_104 | 104 | 13.80 | 4 | 0.017 | -0.35** |
| PC2_108 | 108 | 15.80 | 4 | 0.007 | -0.24* |
| PC2_134 | 134 | 11.87 | 4 | 0.037 | 0.25* |
| PC2_143 | 143 | 13.65 | 4 | 0.018 | 0.24* |
| PC2_186 | 186 | 15.14 | 4 | 0.010 | 0.32** |
| PC2_192 | 192 | 11.58 | 4 | 0.041 | -0.24* |
| PC2_220 | 220 | 11.78 | 4 | 0.038 | 0.24* |
| PC2_309 | 309 | 13.04 | 4 | 0.011 | -0.39** |
| PC2_423 | 423 | 12.25 | 4 | 0.032 | -0.32** |
| PC2_466 | 466 | 12.25 | 4 | 0.031 | -0.30** |
| PC2_64 | 64 | 12.77 | 4 | 0.026 | -0.36** |
| PC2_65 | 65 | 11.32 | 4 | 0.023 | -0.32** |
| PC3_105 | 105 | 12.04 | 3 | 0.007 | 0.25* |
| PC3_111 | 111 | 12.32 | 4 | 0.031 | 0.27* |
| PC3_113 | 113 | 11.76 | 4 | 0.038 | -0.28* |
| PC3_125 | 125 | 14.40 | 4 | 0.013 | 0.25* |
| PC3_128 | 128 | 15.06 | 4 | 0.010 | 0.26* |
| PC3_151 | 151 | 12.56 | 4 | 0.028 | 0.29* |
| PC3_172 | 172 | 14.03 | 4 | 0.015 | 0.30* |
| PC3_184 | 184 | 22.90 | 4 | 0.000 | -0.26* |
| PC3_185 | 185 | 13.62 | 4 | 0.018 | -0.27* |
| PC3_225 | 225 | 11.68 | 4 | 0.039 | -0.24* |
| PC3_237 | 237 | 15.28 | 4 | 0.009 | 0.24* |
| PC3_245 | 245 | 13.77 | 4 | 0.017 | 0.24* |
| PC3_305 | 305 | 15.47 | 4 | 0.017 | 0.29* |

**Table 4.8. Continued**

| Linked AFLPs to WLR | Allele size (bp) | Chi-square | Degree of freedom | Asymptotic significance* | Correlation* |
|---|---|---|---|---|---|
| PC3_306 | 306 | 12.29 | 4 | 0.031 | 0.26* |
| PC3_308 | 308 | 11.82 | 4 | 0.037 | 0.25* |
| PC3_323 | 323 | 11.86 | 4 | 0.037 | -0.27* |
| PC3_350 | 350 | 12.91 | 4 | 0.024 | 0.28* |
| PC3_424 | 424 | 13.87 | 4 | 0.016 | 0.24* |
| PC3_471 | 471 | 11.26 | 3 | 0.01 | -0.29* |
| PC3_59 | 59 | 15.38 | 4 | 0.009 | -0.27* |
| PC3_69 | 69 | 17.26 | 4 | 0.002 | -0.29* |
| PC3_82 | 82 | 13.05 | 4 | 0.023 | 0.26* |
| PC3_88 | 88 | 12.36 | 4 | 0.015 | -0.29* |
| PC3_97 | 97 | 13.17 | 4 | 0.022 | -0.30* |
| PC4_136 | 136 | 10.77 | 4 | 0.029 | -0.30* |
| PC4_181 | 181 | 15.81 | 4 | 0.007 | -0.39** |
| PC4_184 | 184 | 13.13 | 4 | 0.022 | -0.27* |
| PC4_190 | 190 | 13.94 | 4 | 0.016 | 0.35** |
| PC4_216 | 216 | 14.70 | 4 | 0.012 | -0.33** |
| PC4_235 | 235 | 14.89 | 4 | 0.011 | -0.38** |
| PC4_239 | 239 | 13.98 | 4 | 0.016 | -0.24* |
| PC4_300 | 300 | 17.05 | 4 | 0.004 | -0.33** |
| PC4_380 | 380 | 11.34 | 4 | 0.045 | -0.31** |
| PC4_484 | 484 | 17.036 | 4 | 0.004 | -0.29* |
| PC4_84 | 84 | 12.68 | 4 | 0.025 | -0.32** |
| PC4_90 | 90 | 14.79 | 4 | 0.011 | 0.35** |
| PC5_131 | 131 | 12.26 | 4 | 0.031 | -0.24* |
| PC5_147 | 147 | 11.70 | 4 | 0.020 | -0.24* |
| PC5_183 | 183 | 12.91 | 4 | 0.024 | -0.32** |
| PC5_187 | 187 | 14.47 | 4 | 0.011 | -0.39** |
| PC5_192 | 192 | 12.85 | 4 | 0.025 | 0.27* |
| PC5_193 | 193 | 13.68 | 4 | 0.018 | -0.26* |
| PC5_213 | 213 | 12.15 | 4 | 0.033 | -0.24* |
| PC5_350 | 350 | 17.41 | 4 | 0.043 | -0.24* |
| PC5_436 | 436 | 11.45 | 4 | 0.043 | -0.33** |
| PC5_59 | 59 | 11.96 | 4 | 0.035 | -0.26* |
| PC5_70 | 70 | 17.12 | 4 | 0.004 | -0.27* |
| PC6_123 | 123 | 11.49 | 4 | 0.035 | -0.29* |
| PC6_136 | 136 | 11.79 | 4 | 0.038 | -0.35** |
| PC6_150 | 150 | 10.88 | 4 | 0.028 | -0.26* |
| PC6_163 | 163 | 13.47 | 4 | 0.019 | -0.24* |
| PC6_185 | 185 | 15.08 | 4 | 0.010 | -0.27* |
| PC6_263 | 263 | 12.17 | 4 | 0.033 | -0.33** |
| PC6_271 | 271 | 16.92 | 4 | 0.005 | 0.25* |
| PC6_318 | 318 | 16.68 | 4 | 0.005 | -0.38** |
| PC6_321 | 321 | 12.03 | 4 | 0.034 | 0.24* |
| PC6_323 | 323 | 11.98 | 4 | 0.035 | 0.24* |
| PC6_391 | 391 | 16.22 | 4 | 0.003 | -0.44** |
| PC6_475 | 475 | 12.87 | 4 | 0.025 | -0.30* |
| PC6_484 | 484 | 12.86 | 4 | 0.025 | -0.30* |
| PC7_126 | 126 | 11.27 | 4 | 0.024 | -0.30* |
| PC7_187 | 187 | 10.35 | 4 | 0.035 | -0.32** |
| PC7_253 | 253 | 12.20 | 4 | 0.032 | -0.27* |
| PC7_397 | 397 | 14.07 | 4 | 0.015 | 0.25* |
| PC7_465 | 465 | 11.80 | 4 | 0.038 | -0.27* |

*Significant at p<0.05, **Significant at p<0.01

**Table 4.9.** AFLP markers linked to wilting score (WS) according to Kruskal-Wallis H test

| AFLPs linked to WS | Allele size (bp) | Chi-square | Degree of freedom | Asymptotic significance* | Correlation* |
|---|---|---|---|---|---|
| PC1_114 | 114 | 11.94 | 4 | 0.036 | -0.27* |
| PC1_143 | 143 | 13.84 | 4 | 0.017 | 0.35** |
| PC1_217 | 217 | 12.97 | 4 | 0.024 | -0.24* |
| PC1_314 | 314 | 13.47 | 4 | 0.019 | -0.24* |
| PC1_333 | 333 | 13.57 | 4 | 0.019 | -0.25* |
| PC1_355 | 355 | 16.86 | 4 | 0.005 | -0.26* |
| PC1_399 | 399 | 13.94 | 4 | 0.016 | -0.36** |
| PC1_468 | 468 | 15.99 | 4 | 0.007 | -0.39** |
| PC1_73 | 73 | 13.29 | 4 | 0.021 | -0.28* |
| PC1_75 | 75 | 16.35 | 4 | 0.006 | -0.28* |
| PC1_92 | 92 | 10.82 | 4 | 0.029 | -0.35** |
| PC2_104 | 104 | 11.21 | 4 | 0.047 | -0.35** |
| PC2_166 | 166 | 12.95 | 4 | 0.024 | -0.25* |
| PC2_250 | 250 | 13.40 | 4 | 0.020 | -0.28* |
| PC3_113 | 113 | 12.48 | 4 | 0.029 | -0.30* |
| PC3_131 | 131 | 9.71 | 3 | 0.021 | 0.26* |
| PC3_137 | 137 | 16.66 | 4 | 0.005 | 0.27* |
| PC3_184 | 184 | 16.59 | 4 | 0.005 | -0.29* |
| PC3_211 | 211 | 11.87 | 4 | 0.036 | 0.24* |
| PC3_213 | 213 | 10.08 | 4 | 0.039 | 0.25* |
| PC3_274 | 274 | 14.42 | 4 | 0.013 | 0.26* |
| PC3_305 | 305 | 15.47 | 4 | 0.017 | 0.30* |
| PC3_360 | 360 | 11.92 | 4 | 0.036 | 0.26* |
| PC3_64 | 64 | 10.63 | 3 | 0.014 | -0.27* |
| PC3_69 | 69 | 9.66 | 4 | 0.047 | -0.30* |
| PC3_87 | 87 | 14.04 | 4 | 0.015 | 0.26* |
| PC3_88 | 88 | 11.58 | 4 | 0.006 | -0.31** |
| PC4_117 | 117 | 10.23 | 4 | 0.037 | -0.35** |
| PC4_136 | 136 | 9.86 | 4 | 0.043 | -0.34** |
| PC4_152 | 152 | 12.58 | 3 | 0.006 | -0.24* |
| PC4_179 | 179 | 12.94 | 4 | 0.024 | -0.33** |
| PC4_184 | 184 | 11.89 | 4 | 0.036 | -0.37** |
| PC4_219 | 219 | 8.16 | 3 | 0.043 | -0.25* |
| PC4_235 | 235 | 11.47 | 4 | 0.043 | -0.39** |
| PC4_239 | 239 | 12.28 | 4 | 0.031 | 0.25* |
| PC4_300 | 300 | 14.93 | 4 | 0.011 | -0.31** |
| PC4_380 | 380 | 13.41 | 4 | 0.020 | -0.28* |
| PC4_66 | 66 | 12.59 | 4 | 0.027 | -0.28* |
| PC4_75 | 75 | 12.55 | 4 | 0.028 | 0.24* |
| PC5_104 | 104 | 16.27 | 4 | 0.006 | -0.28* |
| PC5_126 | 126 | 15.89 | 4 | 0.007 | 0.34** |
| PC5_192 | 192 | 11.87 | 4 | 0.036 | 0.37** |
| PC5_248 | 248 | 12.37 | 4 | 0.030 | -0.24* |
| PC5_88 | 88 | 13.56 | 4 | 0.019 | 0.43** |
| PC6_121 | 121 | 12.46 | 4 | 0.029 | -0.30* |
| PC6_271 | 271 | 12.55 | 4 | 0.028 | 0.42** |
| PC6_323 | 323 | 12.69 | 4 | 0.026 | 0.24* |
| PC6_391 | 391 | 12.014 | 4 | 0.017 | -0.41* |
| PC6_97 | 97 | 11.42 | 4 | 0.044 | 0.38** |
| PC7_280 | 280 | 17.52 | 4 | 0.004 | 0.51** |
| PC7_400 | 400 | 12.81 | 4 | 0.025 | -0.24* |

*Significant at $p<0.05$, **Significant at $p<0.01$

In order to test the genetic differentiation of landraces according to their drought reaction as measured by the three parameters, we used discriminant analysis. Prior information related to landraces' drought responses based on RWC, WLR, and WS, were used as grouping variable in canonical discriminant analyses using pairwise genetic distances between landraces generated from SSR and AFLP markers linked to the respective parameters. The analyses highly discriminated landraces according to their drought reaction into the predefined groups based on RWC, WLR, and WS for both SSRs and AFLPs linked to these parameters (Figures 4.11 and 4.12). First discriminant functions explained 96.9 %, 84.5 %, and 93.7 % of total variation with canonical correlations of 0.883, 0.683, and 0.975 and eigenvalues of 3.53, 0.876 and 19.57 for SSRs linked to RWC, WLR and WS, respectively. Although significant, second functions explained only a small amount of variation for SSRs linked to the three parameters. Some overlapping was observed for SSRs linked to WLR (eigenvalues <1), but the three groups still could be well differentiated.

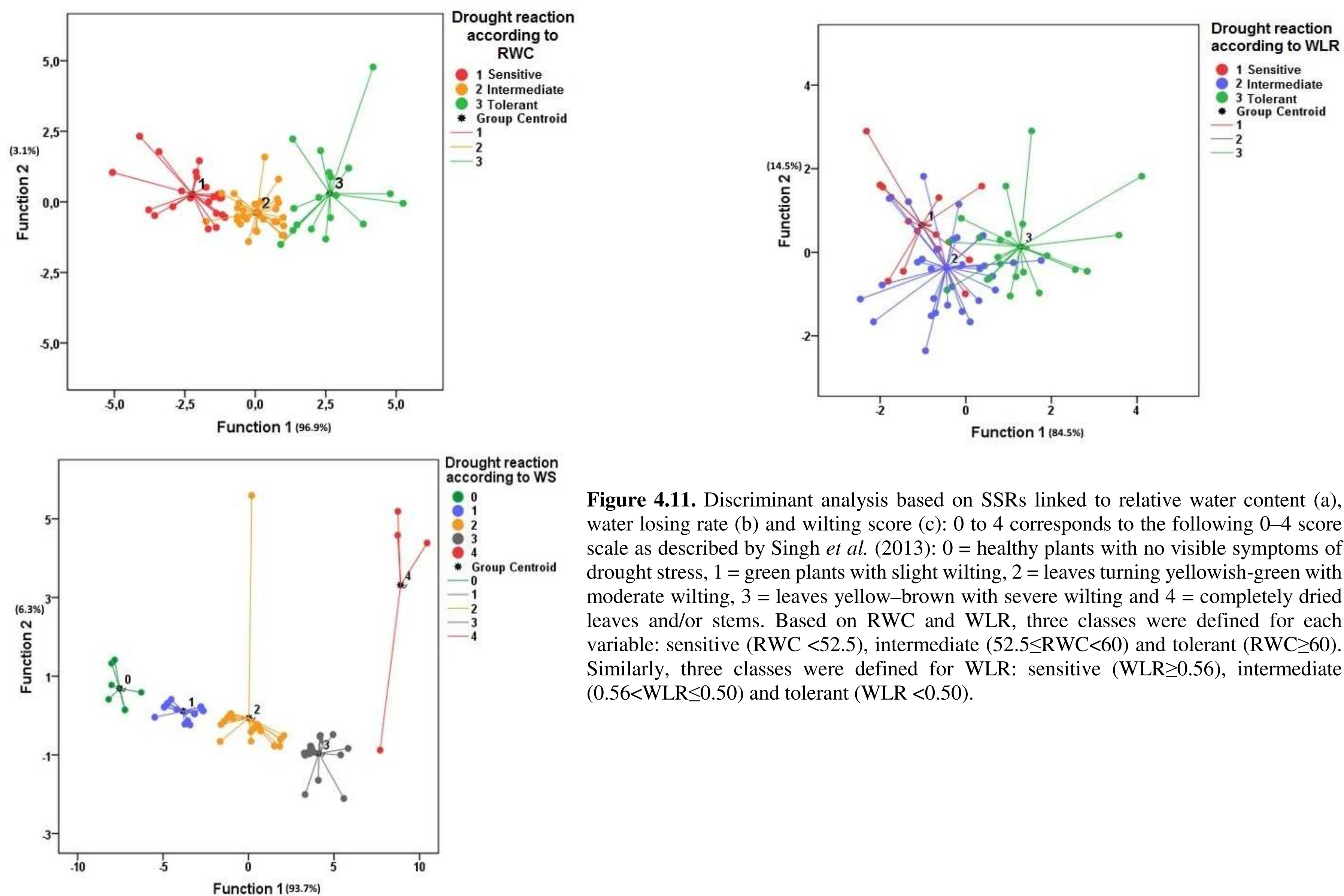


**Figure 4.11.** Discriminant analysis based on SSRs linked to relative water content (a), water losing rate (b) and wilting score (c): 0 to 4 corresponds to the following 0–4 score scale as described by Singh *et al.* (2013): 0 = healthy plants with no visible symptoms of drought stress, 1 = green plants with slight wilting, 2 = leaves turning yellowish-green with moderate wilting, 3 = leaves yellow–brown with severe wilting and 4 = completely dried leaves and/or stems. Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate (52.5≤RWC<60) and tolerant (RWC≥60). Similarly, three classes were defined for WLR: sensitive (WLR≥0.56), intermediate (0.56<WLR≤0.50) and tolerant (WLR <0.50).

For AFLPs linked to RWC, WLR and WS, first discriminant functions explained 62.3 %, 58 % and 73.5 % of total variation with canonical correlations of 0.987, 0.991 and 0.995 and eigenvalues of 37.49, 53.14 and 91.97, respectively. Second discriminant functions explained 37.7 %, 42 % and 13.3 % of total variation with canonical correlations of 0.979, 0.987 and 0.971 and eigenvalues of 22.66, 38.44 and 16.63, respectively, for AFLPs linked to RWC, WLR and WS.

Regression analysis based on SSR alleles linked to RWC, WLR and WS showed moderate associations with $R^2$=0.504, $R^2$=0.289 and $R^2$=0.363, respectively, for the three drought measures as dependent variables. SSR19_7 and SSR80_12 explained the highest phenotypic variation of RWC with 33 % and 30 %, respectively. SSR336_22 and SSR184_17 explained the highest phenotypic variation of WLR with 50 % and 41 %, respectively, whereas SSR19_7 and SSR204_1 explained the highest phenotypic variation of WS with 33 % and 21 %, respectively. Linked SSR alleles with major effects on the drought parameters are reported in Table 4.10.

**Table 4.10.** Effects of major SSR alleles on the drought parameters

| **SSRs linked to drought parameters** | | | | | |
|---|---|---|---|---|---|
| **RWC** | | **WLR** | | **WS** | |
| **SSRs allele names** | **Allele effect (%)** | **SSRs allele names** | **Allele effect (%)** | **SSRs allele names** | **Allele effect (%)** |
| SSR19_7 | 32.7 | SSR184_17 | 40.7 | SSR19_7 | 32.7 |
| SSR80_12 | - 30.5 | SSR336_22 | 50 | SSR204_1 | 21.3 |
| SSR184_17 | 17.7 | SSR154_4 | 10 | SSR154_12 | -17 |
| | | | | SSR48_3 | 13.5 |

Regression analysis based on AFLP alleles linked to RWC, WLR and WS showed high associations with $R^2$=0.753, $R^2$= 0.912 and $R^2$=0.832, respectively, for the three drought measures used as dependent variables. PC1_400 and PC7_92 explained the highest phenotypic variation of RWC with 32 % and 14 %, respectively. PC4_484 and PC4_239 explained the highest phenotypic variation of WLR with 28 % and 16 %, respectively.PC7_400 and PC1_314 explained the highest phenotypic variation of WS with 33 % and 17 %, respectively. Linked AFLP alleles with major effects on the drought parameters are reported in Table 4.11.

**Table 4.11.** Effects of major AFLP alleles on the drought parameters

| AFLPs linked to drought parameters | | | | | |
|---|---|---|---|---|---|
| **RWC** | | **WLR** | | **WS** | |
| **AFLPs allele names** | **Allele effect (%)** | **AFLPs allele names** | **Allele effect (%)** | **AFLPs allele names** | **Allele effect (%)** |
| PC1_400 | 32.2 | PC4_484 | -28.3 | PC7_400 | 33 |
| PC7_92 | 14 | PC4_239 | -16.1 | PC1_314 | 17 |
| PC4_89 | -12 | PC1_178 | -12 | PC1_399 | -10.2 |
| PC4_58 | 11 | PC1_127 | -7 | PC5_88 | 8 |
| PC1_419 | 10 | PC1_458 | -5 | PC5_126 | 7 |
| PC1_217 | 9 | PC1_114 | 5 | PC6_323 | -6.5 |
| PC6_121 | -6 | PC7_397 | -4 | PC4_75 | 5 |
| PC2_98 | 3 | PC4_380 | 3.5 | PC6_391 | 5 |
| PC1_329 | 3 | PC6_123 | 3.4 | PC1_73 | 4 |
| | | PC4-300 | 3.1 | PC5_248 | 2.3 |

Higher correlations were observed between matrices based on drought parameters (RWC, WLR, and WS) and similarity matrices based on the linked SSR and AFLP markers, compared to matrices based on total and randomly selected markers. This confirms the reliability of genetic differentiation according to drought response classes revealed by the markers linked to the traits. The latter clearly discriminated between groups of landraces corresponding to the drought response classes (sensitive, intermediate and tolerant). Also, closely similar patterns of clustering based on total markers as in figures 4.1 and 4.2 were obtained differentiating the two major groups of landraces (Moroccan versus Northern Mediterranean) when using the linked markers.

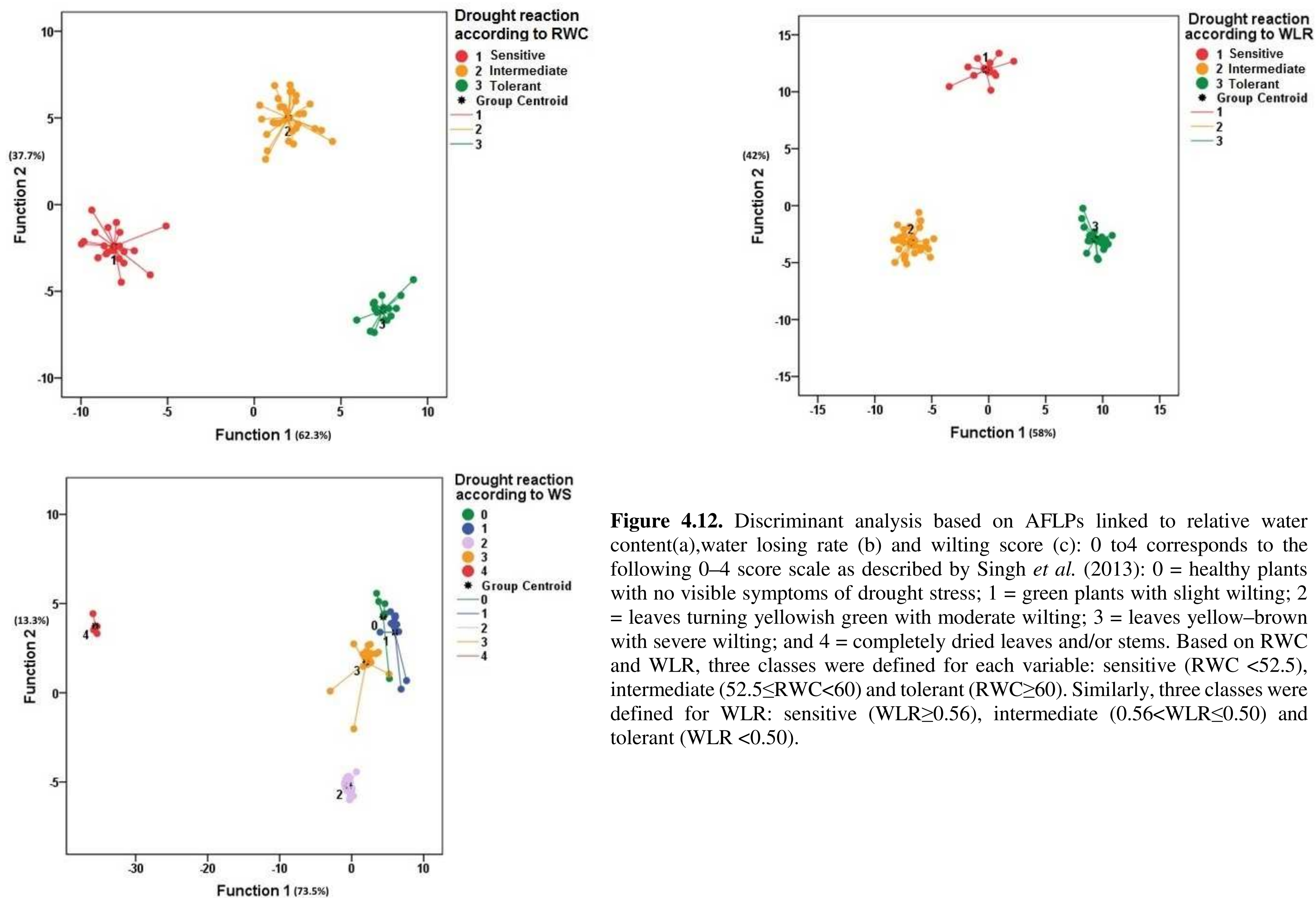


**Figure 4.12.** Discriminant analysis based on AFLPs linked to relative water content(a),water losing rate (b) and wilting score (c): 0 to4 corresponds to the following 0–4 score scale as described by Singh *et al.* (2013): 0 = healthy plants with no visible symptoms of drought stress; 1 = green plants with slight wilting; 2 = leaves turning yellowish green with moderate wilting; 3 = leaves yellow–brown with severe wilting; and 4 = completely dried leaves and/or stems. Based on RWC and WLR, three classes were defined for each variable: sensitive (RWC <52.5), intermediate (52.5≤RWC<60) and tolerant (RWC≥60). Similarly, three classes were defined for WLR: sensitive (WLR≥0.56), intermediate (0.56<WLR≤0.50) and tolerant (WLR <0.50).

## 4.4. Discussion

High genetic variation was shown to exist among Mediterranean landraces originating from Morocco, Italy, Turkey and Greece by using both SSR and AFLP DNA markers. Overall, 261 alleles with an average expected heterozygosity of 0.6775 and number of observed alleles ranging from 2 to 26 were reported at 19 loci, for SSRs. Sonnante *et al.* (2007) reported 170 alleles and between 2 and 22 alleles at 16 loci for Italian landraces. We obtained 213 alleles at the same 19 loci using Moroccan landraces as shown in chapter 3. For AFLPs, a total of 812 fragments were obtained whereby 54.28 % were polymorphic with an average *PIC* of 0.3509 over the seven primer combinations. We reported 766 fragments whereby 54.78 % were polymorphic using the same primer combinations in Moroccan landraces, whereas Torricelli *et al.* (2011) reported 698 fragments where 57.09 % were polymorphic using eight primer combinations on Italian lentil landraces. Toklu *et al.* (2009) reported 212 fragments whereby 56.1 % were polymorphic and with an average *PIC* of 0.579 using six primer combinations in Turkish landraces.

Based on NJ dendrogram and PCA using SSR and AFLP DNA markers separately, and the combined data sets, landraces from the northern Mediterranean, i.e., from Italy, Turkey and Greece, could clearly be differentiated from those originating from the southern Mediterranean, i.e., from Morocco. Landraces from Italy, Turkey and Greece also differed between them as well. This confirms the presence of high genetic diversity in the Mediterranean region for lentil landraces and the possibility of different gene pools. Our results are in agreement with those of Lombardi *et al.* (2014) who reported very high levels of genetic diversity among lentil landraces from the Mediterranean region using single-nucleotide polymorphism markers. Similar results of geographic differentiation have been reported for Mediterranean tetraploid wheat landraces by Oliveira *et al.* (2014) showing four groups: an eastern group (Cyprus, Croatia, Egypt, Iran, Iraq, Israel, Jordan, Lebanon and Turkey), a western group (Algeria, France, Morocco, Portugal, Spain and Tunisia), a second mainly eastern cluster (some accessions not only from Croatia and Turkey, but also from Greece and one Portuguese accession), and a fourth cluster (all Italian accessions and also accessions from Spain and Tunisia).

Population structure analysis confirmed the obtained results from principal component and neighbor-joining analyses which revealed two distinct gene pools: northern Mediterranean landraces (Greece, Italy and Turkey) versus southern Mediterranean landraces (Morocco). When assuming the possibility of three gene pools as shown following Evanno *et al.* (2005) method, landraces from Abda region of Morocco, the driest area of origin among those included in this study, were shown to be assigned to a third gene pool. These is in agreement with results

reported and discussed in the previous section of this chapter 3 evidencing the genetic differentiation of landraces from this area. Assuming either correlated allele frequencies or independent allele frequencies in the admixture model of Structure software leads to closely similar results about the detected population genetic structure (Porras-Hortado *et al*. 2013). High genetic diversity obtained was confirmed for each gene pool by high values of expected heterozygosity estimating average genetic distances between landraces obtained from population structure analysis.

The rich history of the Mediterranean region regarding lentil domestication and cultivation together with the frequency and diversity of biotic and abiotic stresses makes this region an important source for genotypes that have developed tolerance mechanisms. Laghetti *et al.* (2008) and Toklu *et al.* (2009) reported the importance and genetic differentiation of lentil genetic resources for adaptive traits of some landraces from Italy and Turkey, respectively. In chapter 3, we demonstrated functional adaptation of Moroccan landraces according to their agro-enviromental origins and traits conferring specific adaptation.

High genetic variation for root and shoot traits as well as for drought response as estimated by leaf RWC, WLR and WS was observed among the Mediterranean landraces included in our study. The association of these latter traits with drought tolerance in lentil and other crops has often been reported before (Sarker *et al.* 2005; Kashiwagi *et al.* 2005; Vadez *et al.* 2008; Gaur *et al.*2008; Aswaf and Blair 2012; Kumar *et al.* 2012). Under water-limited conditions, the first plant response is to maintain water content as close as possible to that of the non-stressed situation by stomatal control to limit water loss and by faster root growth and increased RS ratio to improve water uptake. Increased root growth and the capacity to maintain higher water content levels are important in order to maintain plant growth and production under drought stress conditions (Verslues *et al.* 2006) compared to other mechanisms which have a more negative effect on yield. Significant positive correlations were obtained between dry root biomass and dry shoot biomass and SPAD. This highlights the possibility of indirect selection for this underground trait using simple measures of chlorophyll content and aboveground biomass weight in breeding programs targeting vigorous root systems. Landraces with higher dry root weight, chlorophyll content and RSratio were the most drought tolerant as evidenced by their higher leaf RWC and lower WLR and WS. Thus, selection of accessions that score well on these parameters under water-limited conditions would result in developing improved cultivars with drought tolerance. No correlation between drought tolerance and geographical origin of landraces was observed. Thus, selection has to be based on the individual response of each genotype. Significant but rather weak grouping based on shoot and root traits was observed

showing landraces from Morocco with slightly higher shoot length, biomass, and seedling early vigour compared to those from northern Mediterranean. Additional phenotypic characterization including morphological and phenological traits is needed to understand the genetic differentiation shown by SSR and AFLP markers.

Significant marker–trait associations of SSR and AFLP DNA markers with leaf RWC, WLR and WS were evidenced based on Kruskal–Wallis test. Six, four and five SSRs and 91, 105 and 51 AFLPs were identified to be linked to the three drought parameters, respectively. SSR- and AFLP- linked allele markers highly discriminated landraces according to their drought reaction highlighting genetic differentiation according to their drought tolerance level (high eigenvalues of discriminant analyses). Landraces with higher RWC and lower WLR and WS could be clearly separated from those with lower RWC and higher WLR and WS. Among these markers, alleles SSR19_7 and SSR80_12, SSR336_22 and SSR184_17, and SSR19_7 and SSR204_1 explained the highest phenotypic variation of RWC, WLR and WS, respectively, as shown by the regression analysis (ranging from 21 to 50 %). These markers can thus be considered as associated markers and potential functional markers to be used in functional genetic diversity analysis related to finding adaptive traits to drought tolerance. The highest phenotypic variation explained by linked AFLPs ranged from 14 to 33 %. This finding suggests the reliability of association mapping studies for evidencing drought tolerance on a large number of landraces in lentil as an interesting approach for the identification of genes and quantitative trait loci (QTL) controlling traits of interest for marker-assisted selection (Kumar *et al.* 2015). Joshi-Saha and Reddy (2015) identified three SSR alleles associated with drought tolerance using K-W test in 60 genotypes of chickpea (*Cicer arietinum*). Using the same method, Razavi *et al.* (2011) reported five and 13 EST and 47 and 85 AFLP markers linked to leaf RWC and WLR in 23 *Fragaria* cultivars, respectively. Iglesias-García *et al.* (2015) reported four QTLs associated with drought adaptation as estimated by leaf RWC in pea (*Pisum sativum* L.).

## 4.5. Conclusions

Our study evidenced substantial genetic variation in Mediterranean lentil landraces for traits related to drought tolerance and for molecular diversity at several SSR and AFLP loci. Further phenotypic evaluation is needed to understand the genetic differentiation between landraces from Morocco and those from the northern Mediterranean. Germplasm included in this study has great potential for lentil breeding for developing drought-tolerant lentil varieties. High variability for root and shoot traits and physiological parameters related to drought tolerance observed in this study showed no correlation with geographical origin. Higher dry root biomass,

chlorophyll content and RS ratio were associated with higher drought tolerance. Association of certain aboveground traits with root biomass indicates the potential for reliable indirect selection for drought tolerance in lentil.

A number of DNA markers were identified to be associated with drought tolerance, and phenotypic classes according to drought response better corresponded to groupings based on these correlated markers. Although plant response to drought stress is a complex trait involving many aspects, this study showed evidences of genetic differentiation according to drought response. Thus, further studies involving larger numbers of landraces and unrelated genotypes in association mapping and quantitative trait studies based on mapping populations from contrasted parents using more efficient and effective DNA markers like single-nucleotide polymorphism markers would allow better understanding of the genetic basis of their drought tolerance.

# Chapter 5. Genetic variability for root and shoot traits in a lentil (*Lens culinaris* Medik.) recombinant inbred line population and their association with drought tolerance

Based on:

Idrissi, O.[1,2], Houasli, C.[2], Udupa, S.M.[3], De Keyser, E.[4], Van Damme, P.[1,5], De Riek, J.[4] (2015).
Genetic variability for root and shoot traits in a lentil (*Lens culinaris* Medik.) recombinant inbred line population and their association with drought tolerance. Euphytica, 204, 693–709.
Doi: 10. 1007/s10681-015-1373-8.

1 Department of Plant Production, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.
2 Institut National de la Recherche Agronomique du Maroc (INRA), Centre Régional de Settat, P.O. Box 589, Settat, Morocco.
3 ICARDA-INRA Cooperative Research Project, International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 6299, Rabat, Morocco.
4 Institute for Agricultural and Fisheries Research (ILVO) Plant Sciences Unit, Applied Genetics and Breeding, Caritasstraat 21, 9090 Melle, Belgium.
5 Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Kamycka 129, 165 21 Prague 6-Suchdol, Czech Republic.

# Chapter 5. Genetic variability for root and shoot traits in a lentil (*Lens culinaris* Medik.) recombinant inbred line population and their association with drought tolerance

## 5.1. Introduction

Drought is one of the major factors limiting lentil production in the world mainly in arid and semi-arid areas such as North Africa and Middle East (Malhotra *et al.* 2004; Stoddard *et al.* 2006; Sarker *et al.* 2009).

Among several physiological, morphological and phenological traits that have been reported to be involved in crop adaptation to drought stress, well-developed roots and vigorous shoots at early seedling stage have been proposed as the main drought avoidance traits to contribute to seed yield under drought environments (Turner *et al.* 2001; Sarker *et al.* 2005; Kashiwagi *et al.* 2005; Verslues *et al.* 2006; Vadez *et al.* 2008; Gaur *et al.* 2008; Aswaf and Blair 2012). In a low moisture soil under drought conditions, uptake of water and nutrients is increased by enhanced root growth and development (Sarker *et al.* 2005; Abdelhamid 2010; Costa de Oliveira and Varshney 2011; Wu and Cheng 2014). Sarker *et al.* (2005) reported high correlations between stem length, taproot length and lateral root number with lentil grain yield. High genetic variation has been reported for root and shoot traits such as stem length, stem weight, taproot length, lateral root number, total root length and total root weight for lentil germplasm from different origins (Mia *et al.* 1996; Sarker *et al.* 2005; Gahoonia *et al.* 2005, 2006; Kumar *et al.* 2012, 2013) as well as for other grain legumes (Serraj *et al.* 2004; Kashiwagi *et al.* 2005; Vadez *et al.* 2008; Aswaf and Blair 2012). The latter authors reported high heritability estimates in chickpea (*Cicer arietinum*) and common bean (*Phaseolus vulgaris*) suggesting evidence for feasibility of breeding for these traits and making use of this genetic variability for the development of cultivars combining these characters.

Identifying allelic variation for specific traits is important for providing proofs of their genetic control and thus evidences to be exploited for crop breeding (Pacheco-Villalobos and Hardtke 2012). In the same trend, Materne *et al.* 2007 reported that genetic variability for traits that are associated with water deficiency tolerance is necessary to breed lentil cultivars that are tolerant to drought. Also, the availability of sufficient genetic variability of a targeted trait determines the effectiveness of breeding programs (Tuberosa 2012). Therefore, understanding genotypic variation of root and shoot characteristics and identification of specific traits associated with drought tolerance are important for designing efficient breeding strategies in lentil. Also, estimation of the degree of genetic variability and heritability will help to estimate the expected

genetic gains for these traits from breeding (Acquaah 2007). Using recombinant inbred line population developed from a cross of contrasting parents will allow better understanding of the genetic control behind these traits in the perspective of quantitative trait loci (QTL) mapping. Root phenotyping for genetic research is commonly based on the use of controlled growing environments such as greenhouse pots or tubes, growth chambers, hydroponic systems, and agar gel (Comas *et al.* 2013). Different watering conditions are often used (Kashiwagi *et al.* 2005; Ruta 2008; Sayed 2011; Aswaf and Blair 2012; Kashiwagi *et al.* 2014). Interestingly, phenotypic and genotypic variation in controlled environments is more likely to be similar to variation under field conditions for traits with high heritability (Comas *et al.* 2013).

The objectives of this study were to (1) determine the genetic variability of root and shoot traits at an early growth stage (38 days after sowing) in a recombinant inbred line (RIL) population under well-watered and drought-stressed treatments and (2) relate their association to each other and to drought tolerance. It should be pointed out that, as far as we know, this is the first study on lentil roots using a mapping population derived from a cross involving contrasting parents.

## 5.2. Materials and methods

A population of 133 $F_{6–8}$ RILs derived from a cross between two contrasting parents ILL6002 and ILL5888 (obtained from Muehlbauer, F.J., USDA-ARS, Washington State University, Pullman, USA) was used in this study (Figures A.6 and A.7, appendix). The parent ILL6002, a pure line selection from the Argentinian early variety *Precoz*, was first reported by Sarker *et al.* (2005) as exhibiting significant superior root and shoot traits and grain yield compared to 40 lentil genotypes from different origins. Also, Kumar *et al.* (2013) used this line as rapid early growth vigour parent in a cross aimed to study genetics of early vegetative growth in lentil. Singh *et al.* (2013) reported similar observations and, furthermore, showed this line to be drought tolerant among 80 lentil genotypes from different origins. The parent ILL5888 is a pure line selection from a landrace from Bangladesh. It is short cultivar with prostrate growth habit (Saha *et al.* 2013). The two parents also differ in diseases resistance (ILL6002 is drought tolerant and resistant to *Stamphylium blight* while ILL5888 is susceptible to both stresses), flowering and maturity time, seed diameter and 100-seed weight (Saha *et al.* 2010, 2013).

The two parents and RIL were phenotyped for root and shoot traits at an early growth stage (38 days after sowing) for two consecutive growing seasons (during February and March 2013 and 2014) at ILVO-Melle, Belgium under two contrasting watering regimes i.e. well-watered and progressively drought-stressed (three replications each) in a plastic pot experiment in a greenhouse arranged in a completely randomized block design. Four uniformly germinated

seeds were planted in plastic pots (H 35 cm × D 24 cm) filled with fine perlite in order to extract intact roots without damage. Fine perlite (diameter ≤ 2 mm), a physically stable and chemically inert material with enhanced holding capacity of readily available water, is an optimum growth medium allowing root extraction with minimal damage compared to sand and sand/compost mixt (Day 1991; Anon 2002; Rabah Nasser 2009). The standard nutrition solution EEG MESTSTOF 19-8-16 (4) [$NO_3$ 11 %, $NH_4$ 8 %, $P_2O_5$ 8 %, $K_2O$ 16 %, MgO 4 %, B 0.02 %, Cu EDTA 0.03 %, Fe EDTA 0.038 %, Mn EDTA 0.05 %, Mo EDTA 0.02 %, Zn EDTA 0.01 %] was supplied as needed twice a week for the well-watered treatment during the experiment and just once in the beginning for the progressively drought-stressed treatment. The two watering regimes differed in terms of field capacity. The initial moisture in all the pots was 75 % of field capacity, it decreased to about 22 % for the drought-stressed regime, while it was maintained at 75 % for the well-watered treatment (Figure 5.1). Dry weight of filled pots as well as water-saturated weight were recorded before watering in order to estimate the field capacity. During the experiment pots weights were recorded every 2 days in order to keep the field capacity of well-watered treatment at 75 % and follow up the moisture decrease in drought-stressed treatment. Temperature ranged from 8 to 14 °C. The photoperiod was11/13 h light/dark with light intensity of 240 Watt $m^{-2}$.

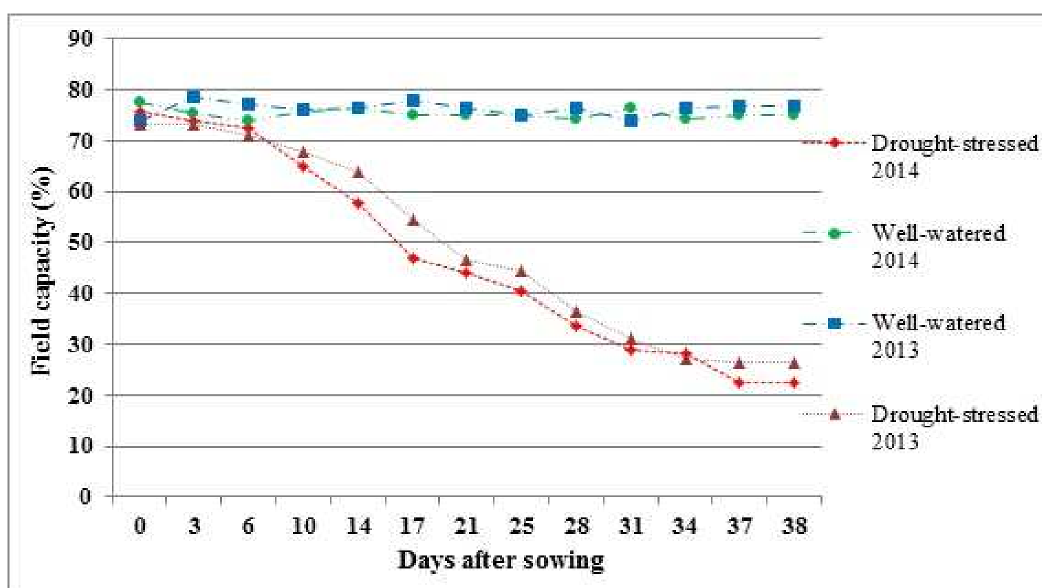


**Figure 5.1.** Changes in field capacity under the two watering regimes during the two seasons.

At 38 days after sowing, plants were carefully extracted without damage to the roots, then shoots and roots were separated into plastic bags. Washed roots were preserved in a refrigerator (4 °C, 90 % relative humidity) to avoid drying before being scanned as images using EPSON Scan scanner. The images were then analyzed using Image J software (Abramoff *et al.* 2004)

combined with Smart Roots software (Lobet *et al.* 2011). From the scanned images, taproot length (TRL; cm $plant^{-1}$), average taproot diameter (TRD; mm $plant^{-1}$), root surface area (RSA; $cm^2$ $plant^{-1}$) and lateral root number (LRN) were measured. Dry root and shoot biomass (DRW, DSW; mg $plant^{-1}$) were measured after oven-drying at 72 °C for 48 h. Chlorophyll content was estimated according to the SPAD values measured at 32 days after sowing using a SPAD-502Plus chlorophyll meter (Konica Minolta, Japan), four measures were taken in fully expanded leaves per plant.

Drought tolerance was estimated by the wilting score (WS) as the degree of wilting severity using the following 0–4 score scale as described by Singh *et al.* (2013): 0 = healthy plants with no visible symptoms of drought stress; 1 = green plants with slight wilting; 2 = leaves turning yellowish green with moderate wilting; 3 = leaves yellow–brown with severe wilting; and 4 = completely dried leaves and/or stems. Seedling vigour (SV) was recorded following the 1–5 IBPGR and ICARDA (1985) scale: 1 = very poor; 2 = poor; 3 = average; 4 = good; 5 = excellent. Root–shoot ratio (RS ratio) was calculated by dividing the dry root weight by the dry shoot weight. Growth rate (GR; cm) was estimated as the gain of length between 12 (SL12DAS; cm) and 22 days after sowing (SL22DAS; cm) (GR = SL22DAS–SL12DAS; cm). Shoot length was measured as the stem length (cm) at 12 and 22 days after sowing. All the variable measures were recorded as the mean value based on the four plants per individual genotype in each pot.

The analysis of variance was used to assess variability of root and shoot traits under both watering regimes. Variability was partitioned into variability between genotypes (RILs), variability due to watering regimes and variability due to the interaction of RIL with watering regimes. The phenotypic correlations among the variables in both drought and well-watered treatments were calculated as Pearson's correlations between the RIL combined mean values of the two seasons 2013 and 2014. Principal component analysis (PCA) and linear regression analyses were performed based on the combined data sets of the two seasons for the drought-stressed treatment in order to assess the relationship between the variables, especially the association of root and shoot traits with drought tolerance.

Analysis of variance, correlation analysis, PCA, regression analysis and normality test were computed using SPSS Statistics 21. The analysis of variance on each variable was computed based on the following statistical model:

$$\boldsymbol{Y = \mu + G + WR + R + G * WR + E}$$

where μ is the general mean, G the genotype effect, WR the watering regime effect, R the replication effect within each watering regime, G*WR the genotype watering regime interaction effect and E the residual error effect. The generalized heritability based on variances was

computed as the percentage of total variance explained by genotypic variance (between the RIL) using Genstat 12.1 based on the formula:

$\boldsymbol{h^2 = 1 - \left(\frac{EV}{2\sigma_g{}^2}\right)}$, as described by Cullis *et al.* (2006) and Oakey *et al.* (2006); $\boldsymbol{\sigma_g{}^2}$ corresponds to genotypic variance whereas EV corresponds to the predicted error variance.

## 5.3. Results

### 5.3.1. Effect of drought stress and genetic variation on root and shoot traits of parents and derived RIL

The analysis of variance showed a significant genotype effect for all the traits in both seasons regardless of watering regime (WR). The effect of watering regime was significant for all traits except for root surface area in both seasons and shoot length at 22 days after sowing and seedling vigour for 2014. The interaction of the two factors (genotype and watering regime) was significant except for shoot length at 22 days after sowing for 2013 and for growth rate for both seasons (Table 5.1).

The two parents contrasted for root and shoot traits in both well-watered and drought-stressed treatments except for taproot length and RS ratio under well-watered condition for the two seasons and average taproot diameter under the 2014 drought condition (Tables 5.2, 5.3). The ILL6002 parent is a vigorous line with a well-developed root system showing drought tolerance (score 0), while the parent ILL5888 has a less-developed root system and vegetative biomass showing drought sensitivity (score 3).

Differences among the RIL derived from the two parents were significant for all traits under both treatments and for the two seasons. Drought stress significantly reduced root and shoot characteristics compared to well-watered conditions except for RS ratio that was increased (RIL mean from 1.25 to 2.12 and 1.24 to 2.05, respectively, for 2014 and 2013). Early seedling vigour, shoot length at 12 days after sowing and taproot length remained stable under the two watering regimes (Tables 5.2, 5.3).

**Table 5.1.** Mean squares and significance levels of genotypic, watering regime and their interaction effects on root and shoot traits according to ANOVA analysis

| Source of variation | Df | DRW | LRN | TRL | TRD | RSA | DSW | SL12DAS | SL22DAS | GR | SV | SPAD | RSratio | WS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2013 | | | | | | | | | | | | | |
| **RIL** | 132 | 1010.4*** | 305*** | 71.3** | 0.06** | 68*** | 766.1*** | 8.3*** | 24.8*** | 7.4*** | 2.1*** | 137*** | 0.4*** | 1.5*** |
| **WR** | 1 | 878.7** | 2896.6*** | 520.1* | 0.23** | 35.3ns | 112147.8*** | 5.8** | 45.5*** | 18.7** | 0.4*** | 19160.6*** | 109.5*** | 221*** |
| **RIL*WR** | 124 | 364.7*** | 176.9*** | 49.3* | 0.02** | 28.3*** | 168.4** | 1.7*** | 2.42ns | 1.8ns | 1.2*** | 47.9*** | 0.1*** | 1.2*** |
| **Error** | 444 | 178.6 | 94.5 | 38.1 | 0.01 | 15.6 | 115.6 | 0.5 | 1.9 | 1.7 | 0.04 | 9.9 | 0.05 | 0.23 |
| | 2014 | | | | | | | | | | | | | |
| **RIL** | 132 | 813.1*** | 286.3*** | 69.1*** | 0.04*** | 54.8*** | 623*** | 5.8*** | 15.2*** | 4.09*** | 1.9*** | 115.2*** | 0.45*** | 1.47*** |
| **WR** | 1 | 41.7** | 461.3* | 536* | 0.2** | 1.52ns | 86953*** | 12*** | 0.4ns | 9.04** | 0.07ns | 16560.3*** | 116.7*** | 149*** |
| **RIL*WR** | 124 | 286.6*** | 149.4* | 57.1* | 0.03*** | 23.71*** | 132.9* | 1.2*** | 1.5* | 1.12ns | 0.8*** | 41.1*** | 0.14** | 1.1*** |
| **Error** | 444 | 142.2 | 86.7 | 40.9 | 0.02 | 12.56 | 90.6 | 0.24 | 1.14 | 1.02 | 0.12 | 8.34 | 0.05 | 0.18 |

Significance level: ns: P>0.05; *: P<0.05; **: P<0.01; ***: P<0.001; Df: degree of freedom;

**Table 5.2.** Phenotypic variation, generalized heritability and coefficient of variation of root and shoot traits for the 2013 well-watered and drought-stressed treatments

| Traits 2013 | Drought-stressed | | | | | | | Well-watered | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Parents means±SE | | RIL means±SD | Min | Max | CV (%) | h² | Parents means±SE | | RIL means±SD | Min | Max | CV (%) | h² |
| | ILL6002 | ILL5888 | | | | | | ILL6002 | ILL5888 | | | | | |
| **DRW** | 96.85±3.82 | 54.85±4.08 | 73.76±19.74 | 9.03 | 145.52 | 26.77 | 61.06 | 124.95±17.75 | 57.94±2.75 | 81.76±19.63 | 20.2 | 166.74 | 25.91 | 43.22 |
| **LRN** | 55.67±3.92 | 30±1.73 | 39.14±11.15 | 12 | 70 | 28.51 | 71.98 | 50.67±11.68 | 42±4.08 | 47.81±13.03 | 12 | 81 | 29.75 | 10.95 |
| **TRL** | 33.09±1.82 | 23.6±0.81 | 23.9±4.1 | 4.61 | 32.11 | 27.31 | 56.32 | 25.01±1.01 | 21.06±2.1 | 25.9±9.9 | 5.1 | 34.11 | 42.8 | 39.1 |
| **TRD** | 0.84±0.02 | 0.51±0.003 | 0.78±0.1 | 0.3 | 1.16 | 21.3 | 52.19 | 0.97±0.08 | 0.71±0.021 | 0.81±0.21 | 0.33 | 1.66 | 27.6 | 13.9 |
| **RSA** | 23.11±0.78 | 14.13±0.52 | 17.12±5.6 | 7.2 | 35.2 | 35.6 | 62.7 | 27.1±1.82 | 15.2±2.01 | 23.21±6.02 | 4.11 | 37.6 | 37.2 | 55.3 |
| **DSW** | 68.15±4.53 | 27.43±2.29 | 37.75±12.16 | 15.25 | 76.07 | 32.23 | 72.66 | 102.64±14.62 | 44.08±7.72 | 63.24±19.75 | 20.05 | 131.33 | 31.23 | 48.71 |
| **SL12DAS** | 9.62±0.6 | 4.61±0.09 | 6.72±1.48 | 2.75 | 10.8 | 22.06 | 97.80 | 9.65±0.50 | 4.93±0.29 | 6.91±1.58 | 2.4 | 12.46 | 22.88 | 69.73 |
| **SL22DAS** | 15.5±1.23 | 6.71±0.74 | 9.82±2.64 | 5.18 | 18.63 | 24.89 | 76.89 | 15.62±0.78 | 8.19±0.47 | 12.14±2.58 | 5.08 | 18.92 | 23.19 | 66.65 |
| **GR** | 5.88±0.65 | 2.09±0.68 | 3.88±1.72 | 0.03 | 9.02 | 44.38 | 49.95 | 5.97±0.30 | 3.26±0.71 | 4.2±1.76 | 0.09 | 12.09 | 42.03 | 31.36 |
| **SV** | 4±0.00 | 1.67±0.33 | 3.05±0.79 | 1 | 5 | 26.01 | 90.47 | 5±0.00 | 2±0.00 | 3.01±0.83 | 1 | 5 | 27.58 | 96.82 |
| **SPAD** | 48.36±2.79 | 40.74±2.15 | 35.97±7.46 | 10.75 | 53.05 | 20.74 | 68.73 | 54.1±0.65 | 47.36±0.75 | 46.9±5.24 | 22.63 | 61.22 | 13.18 | 98.21 |
| **RSratio** | 1.43±0.13 | 2.03±0.28 | 2.05±0.51 | 0.18 | 3.81 | 25.13 | 63.03 | 1.2±0.009 | 1.4±0.25 | 1.24±0.28 | 0.54 | 2.37 | 22.71 | 49.00 |
| **WS** | 0±0.00 | 2.33±0.33 | 1.22±1.2 | 0 | 4 | 88.12 | 63.26 | | | | | | | |

SE: mean standard error; SD: standard deviation; min: minimum; max: maximum; CV: coefficient of variation; h²: generalized heritability; DRW: dry root weight (mg plant$^{-1}$); LRN: lateral root number; TRL: taproot length (cm plant$^{-1}$); TRD: average taproot diameter (mm plant$^{-1}$); RSA: root surface area (cm² plant$^{-1}$); DSW: dry shoot weight (mg plant$^{-1}$); SL12DAS: shoot length at 12 days after sowing (cm plant$^{-1}$); SL22DAS: shoot length at 22 days after sowing (cm plant$^{-1}$); GR: growth rate (cm plant$^{-1}$); SV: seedling vigour; SPAD: chlorophyll content; RSratio: root-shoot ratio; WS: wilting score

As expected, both belowground and aboveground plant biomass values were higher for the well-watered treatment compared to the drought-stressed treatment. Dry root weight ranged from 18.2 to 136 and from 9.09 to 145.52 mg plant$^{-1}$ for drought-stressed treatment for 2014 and 2013, respectively. For the well-watered treatment, it ranged from 21.3 to 158.8 and from 20.2 to 166.74 mg plant$^{-1}$ for 2014 and 2013, respectively. Dry shoot weight ranged from 13.5 to 71.1 and from 15.25 to 76.07 mg plant$^{-1}$ for drought-stressed treatment for 2014 and 2013, respectively. For the well-watered treatment, it ranged from 18.9 to 114.2 and from 20.05 to 131.33 mg plant$^{-1}$ for 2014 and 2013, respectively.

High genetic variability was observed among RIL for dry root weight, lateral root number, taproot length, average taproot diameter, root surface area, dry shoot weight, shoot length, growth rate, seedling vigour, SPAD value, root-shoot ratio and wilting score (drought score) under the two watering regimes and during the two seasons as shown by the range of variation of minimum–maximum as well as coefficient of variation values (CV; Tables 5.2, 5.3). Under drought treatment, coefficient of variation ranged from 18.9 to 108.8 and from to 20.74 to 88.12 %, respectively for 2014 and 2013. It ranged from 10.95 to 43.93 and from 13.18 to 42.03 %, respectively for 2014 and 2013, under well-watered treatment. Lateral root number and taproot length ranged under drought treatment respectively from 11 to 67 and from 1.44 to 34.91 cm for 2014. The wide variation observed for the wilting score (CV of 108.8 and 88.12 %, for 2014 and 2013, respectively) highlighted the considerable genetic variation in this population for drought tolerance.

**Table 5.3.** Phenotypic variation, generalized heritability and coefficient of variation of root and shoot traits for the 2014 well-watered and drought-stressed treatments

| Traits 2014 | Drought-stressed | | | | | | | Well-watered | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Parents means±SE | | RIL means±SD | Min | Max | CV (%) | h² | Parents means±SE | | RIL means±SD | Min | Max | CV (%) | h² |
| | ILL6002 | ILL5888 | | | | | | ILL6002 | ILL5888 | | | | | |
| **DRW** | 91.93±3.32 | 51.43±3.21 | 69.64±18.21 | 18.2 | 136 | 26.15 | 64.58 | 114±14.11 | 53.46±2.07 | 75.56±17.98 | 21.3 | 158.8 | 25.85 | 41.33 |
| **LRN** | 50.66±3.38 | 30±1 | 37.56±10.86 | 11 | 67 | 29.10 | 74.62 | 63.33±3.52 | 34.66±2.02 | 46.22±12.9 | 6 | 75 | 32.90 | 17.49 |
| **TRL** | 32.89±1.07 | 21.48±0.64 | 22.31±5.6 | 1.44 | 34.91 | 25.26 | 53.27 | 26.32±0.72 | 22.80±2.55 | 23.68±8.3 | 1.23 | 36.5 | 35.05 | 36.14 |
| **TRD** | 0.90±0.05 | 0.59±0.005 | 0.74±0.14 | 0.34 | 1.24 | 18.9 | 54.23 | 1.05±0.09 | 0.74±0.025 | 0.79±0.19 | 0.37 | 1.71 | 24.82 | 10.81 |
| **RSA** | 25.11±0.66 | 12.21±0.65 | 16.56±4.75 | 5.50 | 36.69 | 28.68 | 58.97 | 25.79± 2.64 | 12.63±2.10 | 21.39±5.46 | 3.85 | 32.99 | 33.31 | 33.31 |
| **DSW** | 61.90±5.37 | 28.50±4.36 | 34.36±11.13 | 13.5 | 71.10 | 32.41 | 74.92 | 96.40±7.9 | 42.63±3.62 | 57.40±17.48 | 18.9 | 114.2 | 30.46 | 48.64 |
| **SL12DAS** | 8.21±0.34 | 4.32±0.11 | 5.96±1.22 | 3.2 | 9.18 | 20.50 | 79.80 | 7.7±0.43 | 4.2±0.15 | 5.69±1.27 | 2 | 9.6 | 22.44 | 72.52 |
| **SL22DAS** | 13.33±0.49 | 5.50±0.50 | 8.02±2.07 | 4.40 | 15.10 | 23.75 | 76.55 | 12.56±0.84 | 6.5±0.28 | 10.71±1.33 | 4 | 14.9 | 23.20 | 76.63 |
| **GR** | 4.91±0.36 | 1.17±0.49 | 2.76±1.35 | 0.21 | 7.46 | 48.87 | 70.12 | 4.85±0.6 | 2.30±0.35 | 3.03±1.35 | 0.1 | 9.3 | 43.93 | 68.2 |
| **SV** | 4±0.33 | 2±0.33 | 3.13±0.79 | 1 | 5 | 25.50 | 55.52 | 3.66±0.00 | 1.66±0.00 | 3.12±0.79 | 1 | 5 | 25.22 | 77.94 |
| **SPAD** | 45.32±2.17 | 37.47±2.77 | 33.64±7.04 | 9.96 | 50.70 | 20.92 | 69.52 | 50.55±0.31 | 44.26±0.36 | 44.07±4.82 | 21.33 | 56.35 | 10.95 | 68.23 |
| **RSratio** | 1.51±0.15 | 1.92±0.38 | 2.12±0.51 | 0.75 | 3.88 | 24.4 | 64.44 | 1.14±0.009 | 1.26±0.07 | 1.25±0.28 | 0.60 | 2.31 | 22.31 | 46.47 |
| **WS** | 0±0.00 | 3±0.00 | 1.07±1.16 | 0 | 4 | 108.8 | 65.80 | | | | | | | |

SE: mean standard error; SD: standard deviation; min: minimum; max: maximum; CV: coefficient of variation; h²: generalized heritability

**Table 5.4.** Correlations among root and shoot traits (correlation given above diagonal are for drought-stressed treatment and below diagonal are for well-watered treatment)

| | DRW | LRN | RSA | TRL | TRD | DSW | SL12DAS | SL22DAS | SV | GR | SPAD | RSratio | WS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **DRW** | 1 | 0.699** | 0.859** | 0.25** | 0.162 | 0.653** | 0.384** | 0.488** | 0.243** | 0.483** | 0.464** | 0.191* | -0.684** |
| **LRN** | 0.630** | 1 | 0.701** | 0.537** | 0.133 | 0.602** | 0.28** | 0.425** | 0.111 | 0.419** | 0.266** | -0.030 | -0.583** |
| **RSA** | 0.872** | 0.598** | 1 | 0.316** | 0.328** | 0569** | 0.248** | 0.39** | 0.206* | 0.398** | 0.407** | 0.139 | -0.527** |
| **TRL** | 0.206** | 0.557** | 0.143 | 1 | -0.147 | 0.315** | 0.161 | 0.19* | 0.017 | 0.145 | 0.094 | -0.097 | -0.136 |
| **TRD** | 0.037 | -0.186* | 0.289** | -0.479** | 1 | 0.162 | 0.052 | 0.084 | 0.016 | 0.10 | 0.042 | 0.033 | 0.013 |
| **DSW** | 0.734** | 0.677** | 0.685** | 0.298** | 0.064 | 1 | 0.468** | 0.671** | 0.366** | 0.652** | 0.196* | -0.583** | -0.428** |
| **SL12DAS** | 0.438** | 0.342** | 0.308** | 0.162 | -0.135 | 0.484** | 1 | 0.841** | 0.633** | 0.389** | -0.145 | -0.334** | -0.066 |
| **SL22DAS** | 0.551** | 0.450** | 0.471** | 0.19** | -0.018 | 0.637** | 0.846** | 1 | 0.539** | 0.825** | -0.11 | -0.455** | -0.039 |
| **SV** | 0.177* | 0.138 | 0.125 | 0.127 | 0.069 | 0.257** | 0.509** | 0.533** | 1 | 0.289** | -0.074 | -0.242** | -0.072 |
| **GR** | 0.516** | 0.426** | 0.510** | 0.174* | 0.110 | 0.607** | 0.432** | 0.838** | 0.388** | 1 | -0.03 | -0.434** | -0.003 |
| **SPAD** | 0.255** | 0.164 | 0.205* | 0.204* | -0.134 | 0.155 | -0.053 | -0.09 | -0.079 | -0.074 | 1 | 0.298** | -0.484** |
| **RSratio** | 0.133 | -0.212* | 0.051 | -0.149 | -0.069 | -0.545** | -0.214* | -0.304** | -0.214 | -0.289** | 0.109 | 1 | -0.307** |
| **WS** | | | | | | | | | | | | | 1 |

** Significant at 0.01 level; * Significant at 0.05 level;

DRW: dry root weight (mg $plant^{-1}$); LRN: lateral root number; TRL: taproot length (cm $plant^{-1}$); TRD: average taproot diameter (mm $plant^{-1}$); RSA: root surface area ($cm^2$ $plant^{-1}$); DSW: dry shoot weight (mg $plant^{-1}$); SL12DAS: shoot length at 12 days after sowing (cm $plant^{-1}$); SL22DAS: shoot length at 22 days after sowing (cm $plant^{-1}$); GR: growth rate (cm $plant^{-1}$); SV: seedling vigour; SPAD: chlorophyll content; RSratio: root-shoot ratio; WS: wilting score

Moderate to high generalized heritability values were noted in both watering regimes for the two experimental seasons. Under drought-stressed treatment, the heritability estimates ranged from 53.27 to 79.8 % for 2014 and from 49.95 to 97.8 % for 2013. It was low for average taproot diameter (10.81 % for 2014) and lateral root number (17.49 % for 2014 and 10.95 % for 2013) under the well-watered treatment. Compared to the well-watered treatment, the heritability estimates were fairly higher under the drought treatment except for seedling vigour. The frequency distribution and normality test of root and shoot traits were performed based on pooled data of the two seasons. Figure 5.2 shows the frequency distributions of the traits in the RIL population under drought-stressed treatment. The population distributions were quantitative, continuous and normally distributed for dry root weight, lateral root number, shoot lengths at 12 and 22 days after sowing, average taproot diameter, RS ratio (Shapiro–Wilk P values of respectively 0.59, 0.51, 0.57, 0.13, 0.52 and 0.87). A slight deviation from normal distribution was observed for other traits.

Figure 5.3 shows the frequency distributions of the traits under the well-watered treatment. The population distributions were quantitative, continuous and normally distributed for all variables (Shapiro–Wilk P values of 0.11, 0.37, 0.29, 0.42, 0.63, 0.37, 0.64, 0.28, 0.28 and 0.25 for dry root weight, lateral root number, root surface area, taproot length, average taproot diameter, dry shoot weight, shoot lengths at 12 and 22 days after sowing, growth rate, RS ratio, respectively) except for seedling vigour and SPAD value for which slight deviation was observed. Positive and negative transgressive segregant RIL were observed under drought-stressed as well as under well-watered treatments.

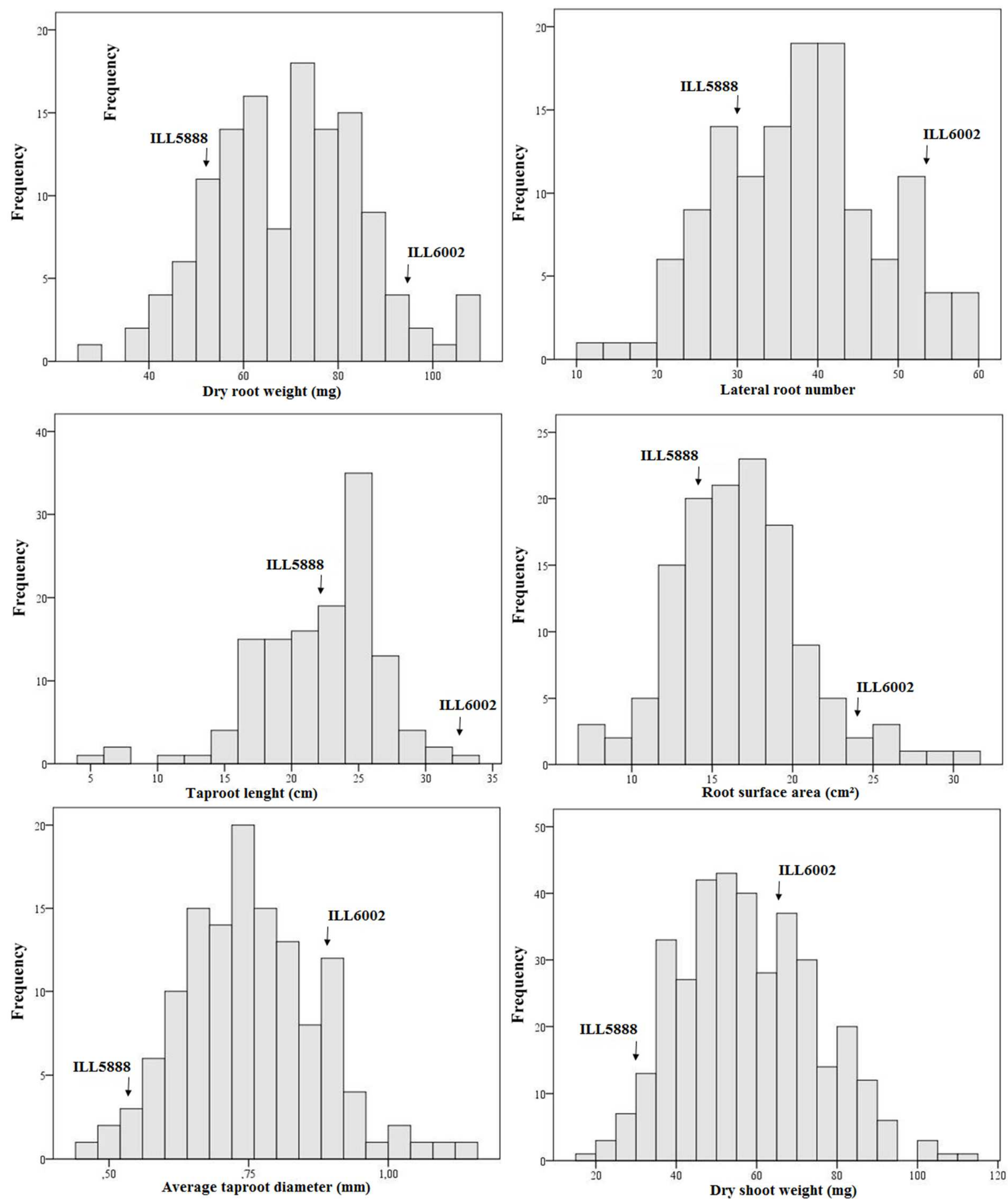


**Figure 5.2.** Frequency distributions of root and shoot traits under the drought-stressed treatment on the RIL population; Y axis reports the number of RIL, while X axis reports traits.

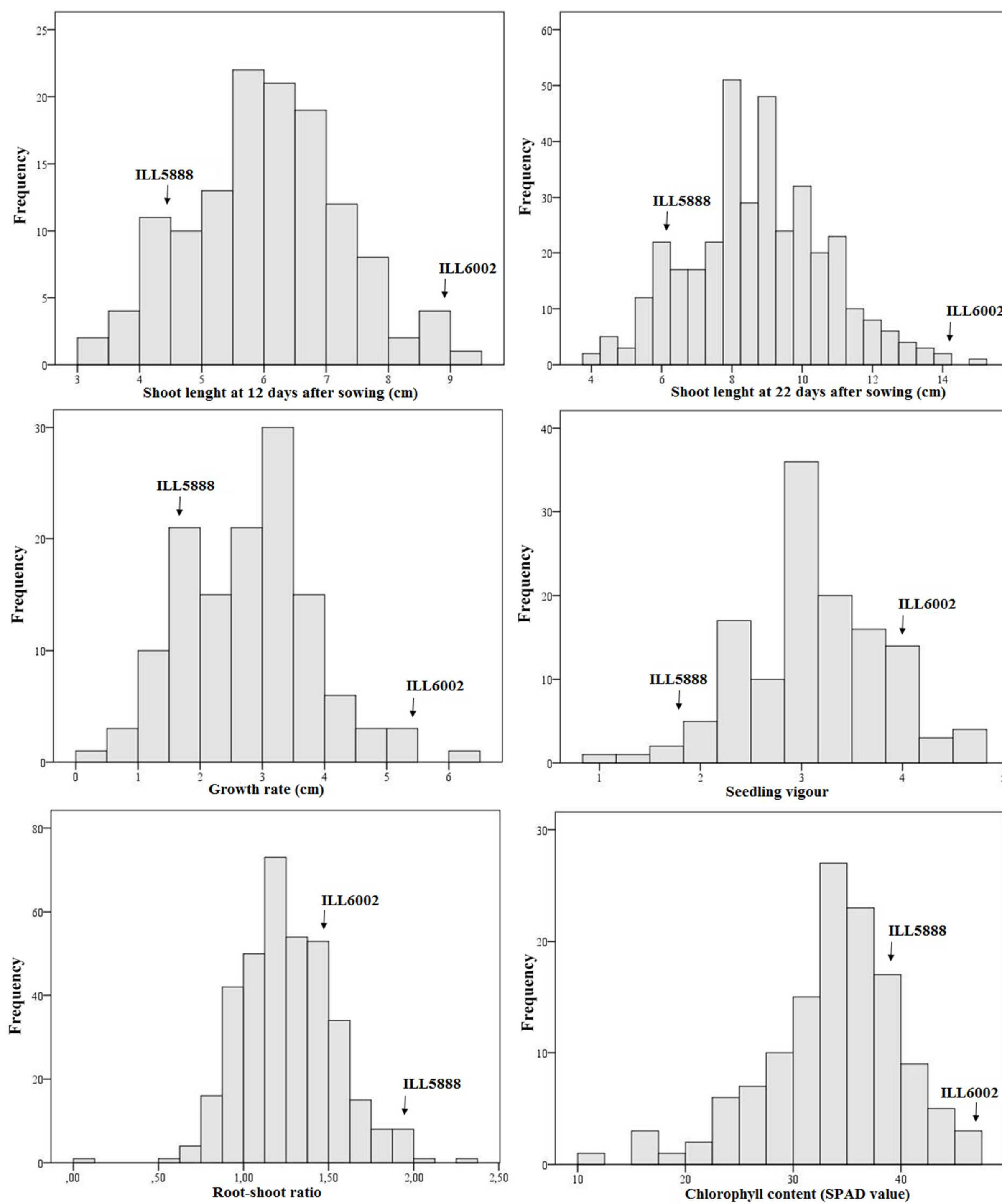


**Figure 5.2.** Continued.

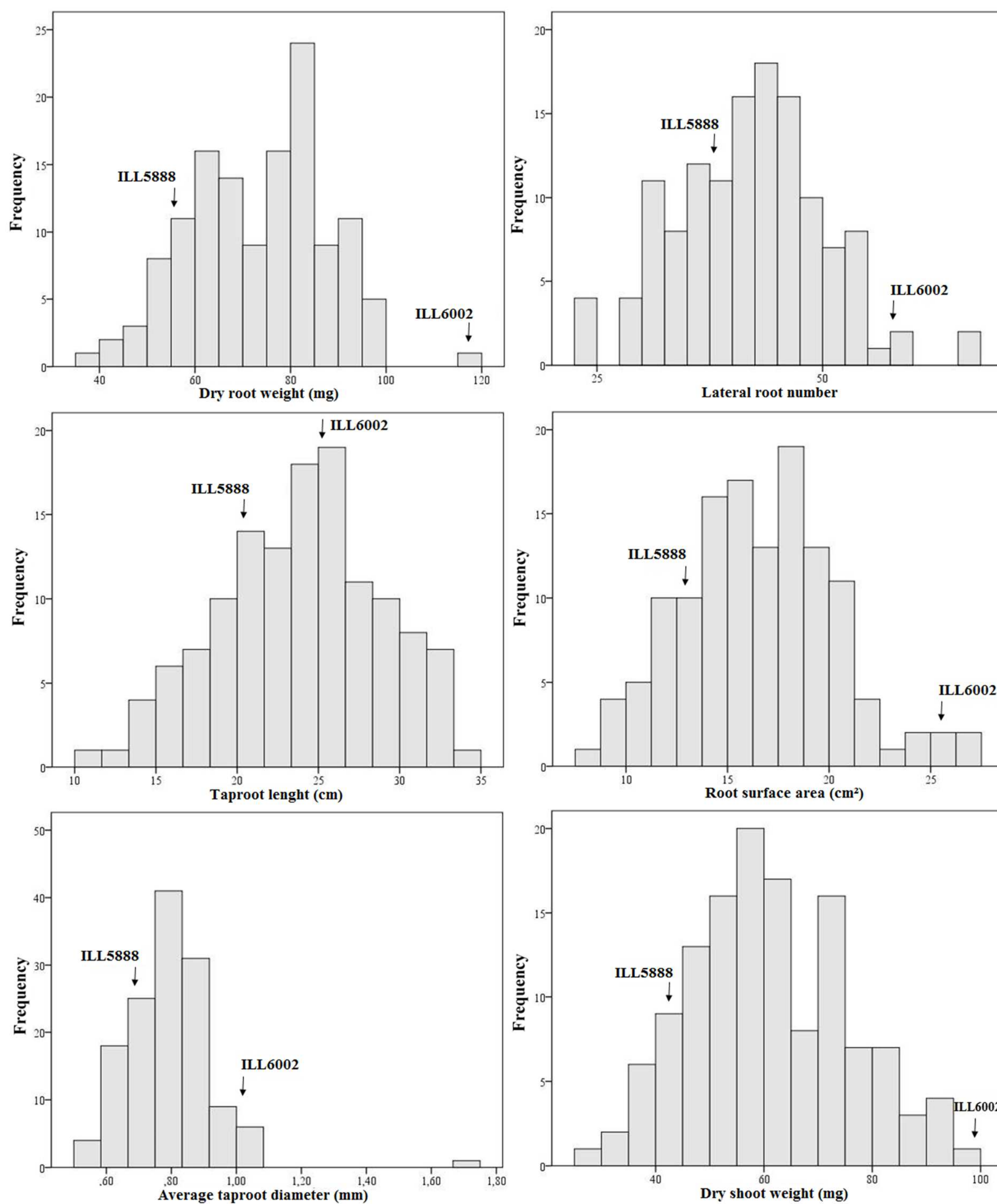


**Figure 5.3.** Frequency distributions of root and shoot traits under the well-watered treatment on the RIL population; Y axis reports the number of RIL, while X axis reports traits.

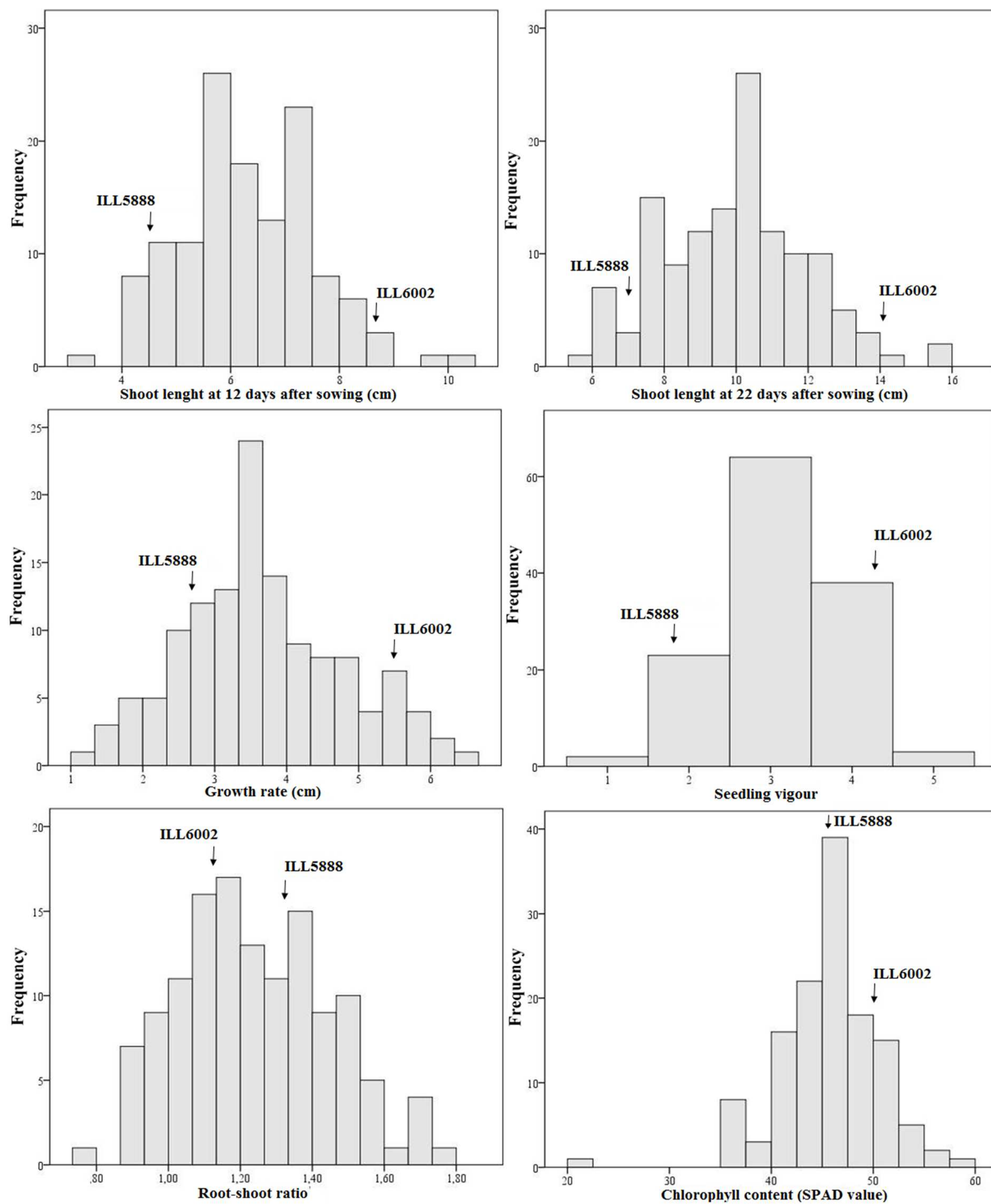


**Figure 5.3.** Continued.

### 5.3.2. Relationship between root and shoot traits and their association with drought tolerance

Correlations between root and shoot characteristics for the two watering treatments were computed based on the mean values of the pooled data of the two seasons (Table 5.4). Significant correlations were noted among root traits and among shoot traits in both treatments. Under the well-watered treatment, dry root weight and lateral root number were significantly and positively correlated with dry shoot weight (0.73 and 0.68, respectively), shoot length at 22 days after sowing (0.55 and 0.45, respectively) and growth rate (0.52 and 0.43, respectively). Under drought treatment, positive significant correlations were observed between dry root weight and dry shoot weight (0.653), shoot length at 22 days after sowing (0.488), growth rate (0.48) and SPAD value (0.46). Similarly, lateral root number was significantly and positively correlated with dry shoot weight (0.60), shoot length at 22 days after sowing (0.43), growth rate (0.42) and SPAD value (0.27). Interestingly, significant high to moderate negative correlations were observed between wilting score inversely estimating drought tolerance and dry root weight (-0.68), lateral root number (-0.58), root surface area (-0.53), dry shoot weight (-0.43), SPAD value (-0.48) and RS ratio (-0.31) for the drought-stressed treatment.

Principal component analysis was performed based on mean values of all variables for drought treatment of the two seasons. The two first components explained 71.79 % for the first axis and 10.75 % for the second corresponding to a total variation covered of 82.54 % (Figure 5.4 a, b). The first principal component (vertical) was highly and positively correlated with RS ratio (0.75), SPAD value (0.64) and negatively with wilting score (-0.61). The second principal component (horizontal) was highly and positively correlated with dry shoot weight (0.84), dry root weight (0.83), root surface area (0.79), lateral root number (0.75), shoot length at 22 days after sowing (0.74) and growth rate (0.68). Positions of the RIL and the two parents are reported in Figure 5.4 b together with the variables.

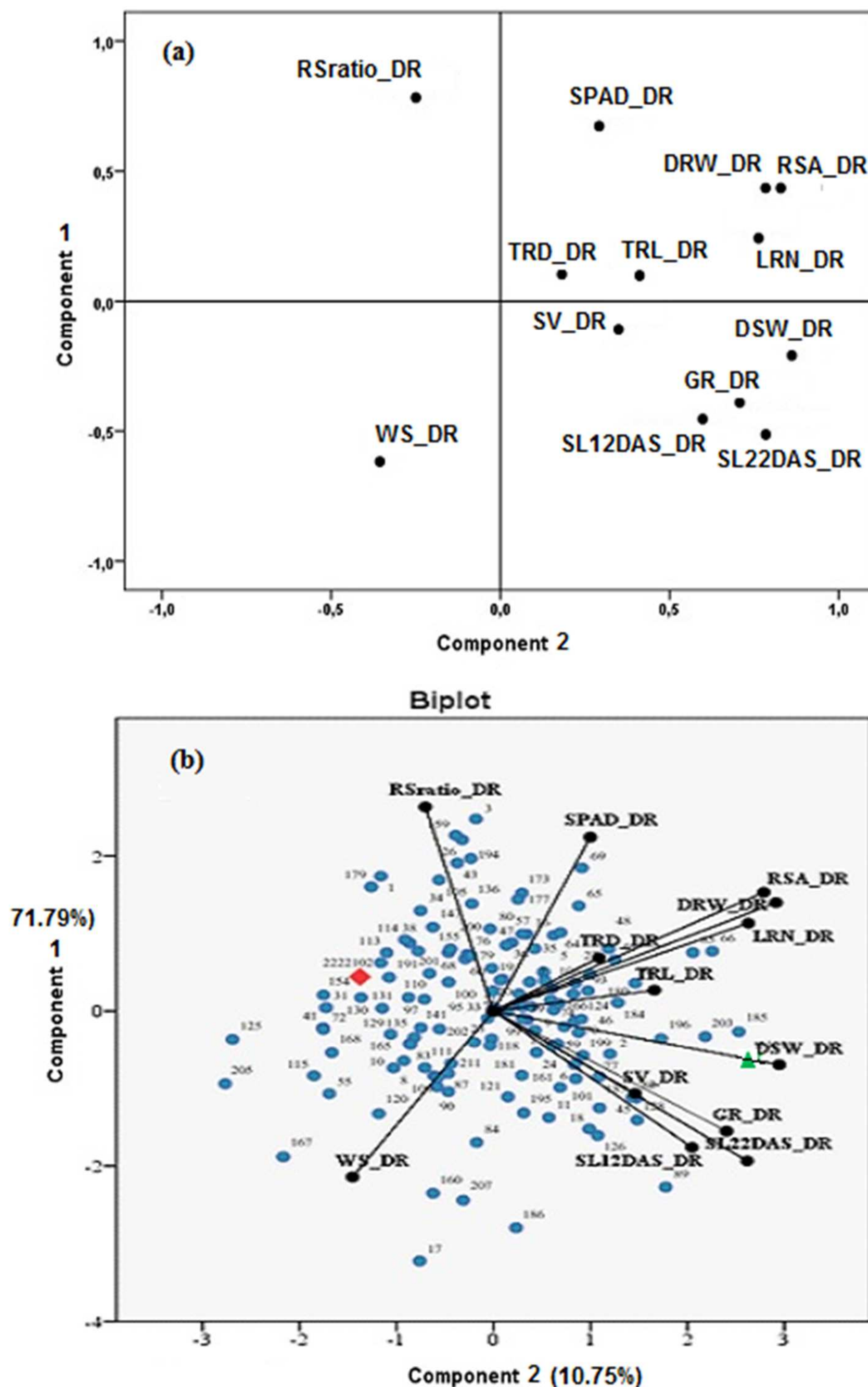


**Figure 5.4.** Principal component analysis of root and shoot traits under drought treatment showing variables (a) and variables together with the RIL and both parents (b). RIL are indicated by blue dots, parent ILL6002 is indicated with a green triangle and parent ILL5888 is indicated by a red square. DRW_DR dry root weight (mg plant$^{-1}$), LRN_DR lateral root number, TRL_DR taproot length (cm plant$^{-1}$), TRD_DR average taproot diameter (mm plant$^{-1}$), RSA_DR root surface area (cm$^{2}$ plant$^{-1}$), DSW_DR dry shoot weight (mg plant$^{-1}$), SL12DAS_DR shoot length at 12 days after sowing (cm plant$^{-1}$), SL22DAS_DR shoot length at 22 days after sowing (cm plant$^{-1}$), GR_DR growth rate (cm plant$^{-1}$), SV_DR seedling vigour, SPAD_DR chlorophyll content, RS ratio_DR root–shoot ratio, WS wilting score.

Regression analysis was performed between root characteristics and shoot traits in order to show relationship among traits. Significant and positive associations were observed between both dry root weight and lateral root number with dry shoot weight, with coefficients of determination of 0.488 and 0.394, respectively (Figure 5.5 a, b). Dry root weight was also significantly associated to SPAD value, and shoot length at 22 days after sowing with determination coefficients of $R^2 = 0.245$ and $R^2 = 0.185$, respectively.

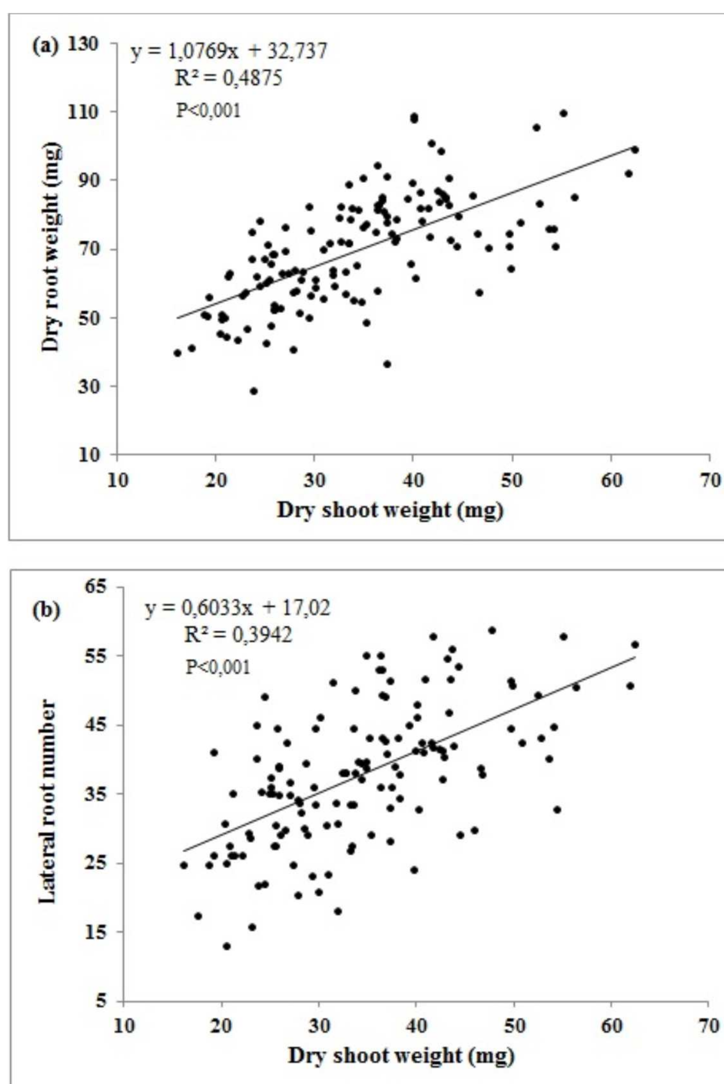


**Figure 5.5.** Relationship of dry root weight (a) and lateral root number (b) with the dry shoot weight under drought treatment.

In order to show the relationship between root traits and drought tolerance as estimated by the wilting score, a regression analysis was performed. Dry root weight was significantly and negatively associated with wilting score ($R^2 = 0.37$). The relationship of root and shoot traits with the wilting score was fairly enhanced ($R^2 = 0.48$) by using the second component of the principal component analysis as independent variable in the regression analysis (Figure 5.6 a,

b). As shown above, the second component is highly and positively correlated with root and shoot traits (dry root weight, root surface area, lateral root number, shoot length at 22 days after sowing, growth rate and dry shoot weight).

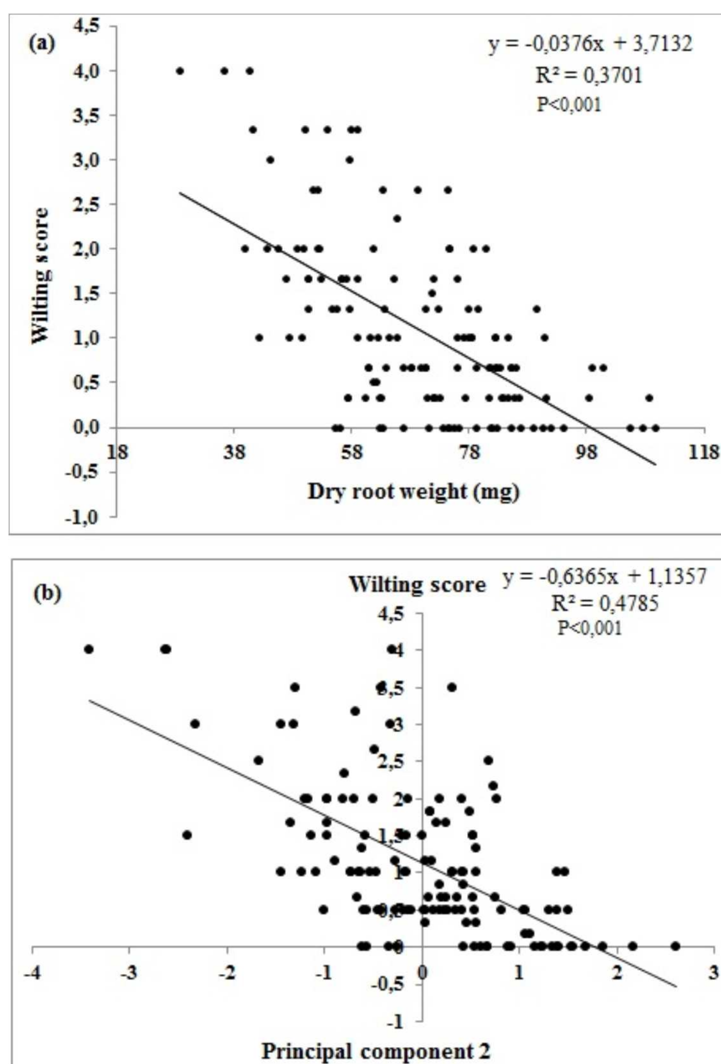


**Figure 5.6.** Relationship of dry root weight (a) and second principal component of the principal component analysis (b) with the wilting score under drought treatment.

## 5.4. Discussion

Studies on root and shoot traits on lentil have been limited to germplasm from different origins (Gahoonia *et al.* 2005, 2006; Sarker *et al.* 2005; Kumar *et al.* 2012). Hence, our study aimed to investigate root and shoot traits at early stage (38 days after sowing) on a lentil mapping population including 133 $F_8$ RILs developed from a cross between two contrasting lines, ILL6002 and ILL5888. ILL6002 has been recommended by Sarker *et al.* (2005) as having characteristics of developed root system and high early biomass conferring drought tolerance that could be used in breeding programs targeting root and shoot traits.

Considerable genetic variation was noted between the parents and the RIL used in this study for dry root biomass, lateral root number, taproot length, root surface area, mean taproot diameter, dry shoot biomass, shoot length, early seedling vigour, chlorophyll content, RS ratio and drought tolerance as estimated by the wilting score. This was true under the well-watered conditions as well as under the drought-stressed treatment during the two seasons. This was evidenced by the wide range of minimum–maximum values and the high coefficients of variation.

In agreement with our results, Sarker *et al.* (2005) and Kumar *et al.* (2012) reported high genetic diversity in lentil accessions from diverse countries for stem length, stem weight, taproot length, lateral root number, total root length and total root weight. Similarly, significant genetic variation was observed in RIL populations of chickpea (*Cicer arietinum* L.) (Serraj *et al.* 2004), common bean (*Phaseolus vulgaris* L.) (Aswaf and Blair 2012) and soybean (*Glycine max* (L.) Merr.) (Brensha *et al.* 2012).

Our study showed that drought stress significantly reduced root and shoot biomass development in both parents and derived RIL indicating that these traits were negatively affected by drought stress as plant growth and development in general. However, RS ratio increased under drought stress in response to less-favourable conditions for aboveground biomass development and probably for more belowground (root) biomass development to explore more space for more water and nutrient uptake. In contrast, under favourable conditions (well-watered treatment) aboveground biomass development increased, thus reducing RS ratio. This is in agreement with the findings of Aswaf and Blair (2012) on common bean. High RS ratio under water-limited conditions may be important for selection of drought tolerant lines (Comas *et al.* 2013).

The frequency distributions of the root and shoot traits among the RIL under both watering regimes showing continuous and quantitative variation as well as normal distribution suggests polygenic control behind these traits. Thus, many QTLs contributing with variable effects are probably involved in the genetic control of the studied traits. Similar observations have been reported for maize (Ruta 2008), common bean (Cichy *et al.* 2009; Aswaf and Blair 2012), barley (Sayed 2011), soybean (Brensha *et al.* 2012) and chickpea (Kashiwagi *et al.* 2014) for which the QTLs related were mapped.

Positive and negative transgressive recombinant lines with trait values higher and lower than those recorded for the two parents were observed for all root and shoot studied traits. This suggests that probably both QTLs of minor effects and major effects are involved. Also positive and negative alleles may come from both parents.

High heritability values were observed for all root and shoot traits included in this study under the drought treatment and during the two seasons. They were also high under well-watered conditions except for lateral root number for both seasons and mean taproot diameter for 2014. The high heritability values exhibited suggest the feasibility and reliability for effective selection and breeding of root and shoot traits at an early growth stage. For both seasons, except for seedling vigour and SPAD value, heritability estimates were higher under drought than under well-watered conditions. This suggests that selection for lentil root and shoot characteristics for drought tolerance would be more effective to achieve genetic gain under water-limited environments than in favourable ones. Similarly to our findings, Sarker *et al.* (2005) reported high heritability values in the lentil germplasm studied for stem length, taproot length and lateral root number. Kumar *et al.* (2012) reported high heritability for shoot length but, in contrast, they obtained low values for root length and dry root weight for the studied lentil accessions. Also, higher heritability values under progressive drought-stressed were reported by Aswaf and Blair (2012) in common bean.

In this study, we observed high association between shoot traits, mainly dry shoot biomass, shoot length and chlorophyll content and root traits in both well-watered and drought-stressed treatments. This is important for practical use in breeding programs as aboveground traits, easy to measure, could be used for indirect selection for underground traits (root) that are more difficult and labour-intensive to measure especially for large numbers of genotypes. High and positive correlations as well as significant regressions were highlighted in agreement with the results of Sarker *et al.* (2005), Kumar *et al.* (2012) and Singh *et al.* (2013). Interestingly, on the other hand, high correlations and significant regressions between drought tolerance (as estimated by the wilting score) and dry root biomass, lateral root number, root surface area, dry shoot biomass, RS ratio and SPAD value were noted indicating the important role of well-developed root systems, early biomass development and high chlorophyll content for drought tolerance in lentil. Drought tolerance related to root development could be actually qualified as avoidance by contributing to maintenance of high water content in plant tissues through improved water uptake by an extensive and prolific root system.

## 5.5. Conclusions

Taking in consideration the fact that drought tolerance is a complex trait depending on diverse aspects such as morphology, phenology, agronomy and physiology, our findings in this study, especially, the relationship between traits and their association with drought tolerance may be used for selection and breeding purposes. Mapping of QTLs related to studied traits in this

population would be a first step for starting marker-assisted selection in breeding programs targeting drought tolerance.

# Chapter 6. Identification of quantitative trait loci controlling root and shoot traits conferring drought tolerance in a lentil (*Lens culinaris* Medik.) recombinant inbred line population

Based on:

Idrissi, O.[1,2], Udupa, S.M.[3], De Keyser, E.[4], McGee, R.J.[5], Coyne, C.J.[6], Saha, G.C.[7], Muehlbauer, F.J.[6], Van Damme, P.[1,8], De Riek, J.[4] (2016). Identification of quantitative trait loci controlling root and shoot traits conferring drought tolerance in a lentil (*Lens culinaris* Medik.) recombinant inbred line population. Submitted to a journal included in the Web of Science Journals list, under review.

1 Department of Plant Production, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium.
2 Institut National de la Recherche Agronomique du Maroc (INRA), Centre Régional de Settat, P.O. Box 589, Settat, Morocco.
3 ICARDA-INRA Cooperative Research Project, International Center for Agricultural Research in the Dry Areas (ICARDA), P.O. Box 6299, Rabat, Morocco.
4 Institute for Agricultural and Fisheries Research (ILVO) Plant Sciences Unit, Applied Genetics and Breeding, Caritasstraat 39, 9090 Melle, Belgium.
5 USDA-ARS Grain Legume Genetics and Physiology Research, 305 Johnson Hall, Pullman, WA 99164-6434, USA.
6 USDA-ARS Western Regional Plant Introduction, Washington State University, 59 Johnson Hall, Pullman, WA 99164-6402, USA.
7 Brotherton Seed Company, 451S Milwaukee Ave, Moses Lake, Washington, WA 98837, USA.
8 Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Kamycka 129, 165 21 Prague 6 – Suchdol, Czech Republic.

# Chapter 6. Identification of quantitative trait loci controlling root and shoot traits conferring drought tolerance in a lentil (*Lens culinaris* Medik.) recombinant inbred line population

## 6.1. Introduction

In the arid and semi-arid areas and also in the context of climate change and global warming, drought is one of the major constraints that can limit lentil production and cause substantial yield losses (Malhotra *et al.* 2004; Stoddard *et al.* 2006; Sarker *et al.* 2009). Developing cultivars with enhanced drought tolerance by conventional breeding often has limited success due to the complexity of this trait and the difficulties with finding reliable and suitable phenotyping methods. For example, well-developed root systems have been shown to be linked to drought tolerance as an avoidance mechanism guaranteeing plant productivity under water-limited conditions (Sarker *et al.* 2005; Kashiwagi *et al.* 2005; Verslues *et al.* 2006; Vadez *et al.* 2008; Gaur *et al.* 2008; Aswaf and Blair 2012; Comas *et al.* 2013). However, it is difficult to screen large numbers of accessions for these root traits using conventional methods. Thus, applying a marker-assisted selection for these traits would offer an interesting alternative in breeding programs targeting drought tolerance. As such, identifying and mapping DNA markers linked to genes controlling rooting patterns associated with drought tolerance will assist in reliable and efficient identification and development of tolerant cultivars. Several studies have shown that root traits are polygenically controlled, whereas they also identified related quantitative trait loci (QTL) for different species such as maize (Ruta 2008), common bean (Cichy *et al.* 2009; Aswaf and Blair 2012), barley (Sayed 2011), soybean (Brensha *et al.* 2012) and chickpea (Kashiwagi *et al.* 2014).

Lentil has a genome size of about 4 Gbp (Arumuganathan and Earle 1991); several kinds of DNA markers have been developed and mapped, including RAPDs, ISSRs, AFLPs, SRAPs, SSRs and SNPs (Eujayl *et al.* 1998; Rubeena *et al.* 2003; Hamwieh *et al.* 2005; Saha *et al.* 2010; Sharpe *et al.* 2013). In chapter 4, we confirmed evidence of high genetic variability, high heritability and polygenic control of root and shoot characteristics. To our knowledge, no QTL related to root traits have been reported for lentil to date. Thus, the objective of this study was to identify and map QTLs related to root and shoot traits associated with drought tolerance in a lentil recombinant inbred line population (RIL) as a promising step for initiating marker-assisted selection approach. It also aimed to investigate the stability of detected QTLs by performing the analysis on two consecutive seasons.

## 6.2. Materials and methods

### 6.2.1. Plant materials

A recombinant inbred line (RIL) population developed from a cross between two contrasting parents, ILL6002 and ILL5888 (Saha *et al.* 2010), obtained from Fred J. Muehlbauer, USD AARS, Washington State University, Pullman, USA, was used in this study. The RIL population consisted of the two parents and 132 $F_{6\text{-}8}$ lines. The lines were advanced to the $F_{6\text{-}8}$ generation from individual $F_2$ using single seed descent. The ILL6002 parent is a vigorous line reported as drought tolerant and with a well-developed root system (Sarker *et al.* 2005; Singh *et al.* 2013). On the other hand, ILL5888 is a drought sensitive line and has a less-developed root system and vegetative biomass. The two parents also differ in resistance to *Stemphylium blight*, flowering and maturity time, seed diameter, 100-seed weight, growth habit and plant height (Saha *et al.* 2010, 2013).

### 6.2.2. RIL root and shoot traits phenotyping and drought tolerance evaluation

The population was evaluated under greenhouse conditions for root and shoot traits associated with drought tolerance under two contrasting watering regimes (well-watered and progressive drought-stressed) for two consecutive seasons (2013 and 2014) as previously described in chapter 5. Dry root weight (DRW), lateral root number (LRN), taproot length (TRL), average taproot diameter (TRD), root surface area (RSA), dry shoot weight (DSW), shoot lengths at 12 (SL12DAS) and 22 (SL22DAS) days after sowing, growth rate (GR), seedling vigour (SV), leaf chlorophyll content (Soil Plant Analysis Development: SPAD), root-shoot ratio (RSratio) and wilting score (WS) were measured.

### 6.2.3. RIL genotyping

A total of 220 polymorphic Single Nucleotide Polymorphism (SNP) markers developed using Genotyping By Sequencing (GBS) technique and 180 polymorphic Amplified Fragment Length Polymorphism (AFLP) markers from seven primer combinations were used to enhance the previously developed linkage map (Saha *et al.* 2010) that was created based on the same RIL population used in this study.

### 6.2.4. Simple Sequence Repeat (SSR), Sequence Related Amplified Polymorphism (SRAP) and Randomly Amplified Polymorphic DNA (RAPD) genotyping

Data related to 23 SSR, 108 SRAP and 30 RAPD markers used in this study were obtained from the previous work of Saha *et al.* (2010, 2013) on linkage map development, inheritance and mapping of genes and QTLs controlling *Stemphylium blight*, and agromorphological traits in

lentil. The latter authors developed and mapped these markers for the same population used in our study (ILL6002 x ILL5888) (Tables A.1 and A.2, appendix). Experimental details regarding marker development and genotyping, are provided in Saha *et al.* (2010, 2013).

### 6.2.5. Genotyping-by-sequencing for SNP identification

SNP data obtained from 92 RILs using Genotyping-By-Sequencing (GBS) were used. The GBS procedure of Poland *et al.* (2012) was used, including their 48 bar-coded adapters with a *Pst* I overhang; genomic DNA was digested with the enzymes *Pst I* and *Msp I*. The ligation reaction was completed using bar-coded Adapter 1 and the common Y-adapter in a master mix of buffer, ATP and $T_4$-ligase. Ligated samples were pooled and PCR-amplified in a single tube, producing libraries of 48 samples each. The libraries were sequenced on two lanes of Illumina HiSeq2000 (University of California Berkeley V.C. Genomic Sequencing Lab). The sequencing data was processed to remove low quality data using in-house scripts and analyzed using Stacks software (Catchen *et al.* 2011, 2013). 220 SNPs that proved to be polymorphic between both parents of the RIL population ILL6002 x ILL5888 (Wong *et al.* 2015) were analysed (Table A.3, appendix). GBS for SNP identification was carried out at Washington State University, USA (Appendices A.1, A.2).

### 6.2.6. AFLP genotyping

DNA extraction and AFLP analysis for genotyping the RIL population (plus the two parents) were performed following experimental details provided in sections 3.2.2. and 3.2.4. of chapter 3, respectively. Out of 12 primer combinations tested, seven (*EcoRI-ACA* + *MseI-CAG*, *EcoRI-ACA* + *MseI-CTG*, *EcoRI-ACA* + *MseI-CTT*, *EcoRI-ACG* + *MseI-CAA*, *EcoRI-AGC* + *MseI-CAA*, *EcoRI-AGC* + *MseI-CAG*, *EcoRI-AGC* + *MseI-CTG*) were selected and used for genotyping all the RIL population.

### 6.2.7. Linkage analysis and map construction

A presence-absence matrix for the alleles from a total of 561 molecular markers on 132 RILs was used for linkage analysis and construction of genetic maps using JoinMap®4 program (Van Ooijen 2006) (Table 6.1). SNP markers were available for 92 RILs. First, segregation according to Mendelian expectation ratio of 1:1 was tested using the chi-square test at a significance level of 0.05, and markers with distorted segregation were removed before further analysis. The grouping tree of the JoinMap® program was calculated using independent *LOD* (Logarithm of odds) as grouping parameter with threshold ranges of 6 for start and 30 for end, and 1 for step. Stable sets of markers at higher LOD values were selected. After initial creation of groups, the *Strongest Cross Link* (*SCL*) information from the output results was used for inspecting

assignment of markers to groups, those with *SCL-values* larger than 5, indicating that they have strong linkage outside their respective groups, were assigned to the corresponding groups. This was repeated until all markers of each group had *SCL-values* smaller than 5. Linkage groups were calculated using the maximum likelihood mapping algorithm with default values as in the software. Map order in each linkage group was verified using the regression mapping algorithm with the following parameters: *LOD* threshold larger than 4, recombination frequency smaller than 0.25, Kosambi function as mapping function for genetic distance calculation and the second round map of the algorithm. Also, monitoring parameters such as *the nearest neighbour fit* and *plausible positions* of markers from the output results of maximum likelihood mapping algorithm were used to inspect and validate the obtained marker order for each linkage group. *The nearest neighbour fit* for each marker should be arrond 1 cM and *plausible positions* of markers should show regular patterns arrond the diagonal of the table (best position) according to Van Ooijen (2006). The final linkage map was generated using MapChart© 2.3 program (Voorrips 2002).

**Table 6.1.** Marker types used for linkage map development

| Marker types | Number of polymorphic markers | Final number of mapped markers |
|---|---|---|
| SSR | 23 | 13 |
| SRAP | 108 | 56 |
| RAPD | 30 | 17 |
| SNP | 220 | 106 |
| AFLP | 180 | 60 |
| **Total number of markers** | 561 | 252 |

### 6.2.8. QTL analysis

QTL analysis was performed for each season for drought-stressed treatments separately in order to check the stability of detected QTLs using MapQTL® 5 program (Van Ooijen 2004). First, Kruskal-Wallis test was performed to determine set of linked markers to each quantitative trait. Simple Interval Mapping was performed to identify linkage groups and positions with significant LOD scores. For each trait, LOD score threshold was determined based on a permutation test using 1000 iterations at a P value of 0.05; LOD scores above these values were considered as significant. Cofactor selection was performed based on automatic cofactor selection implemented in the software for each linkage group and on manual selection of individual markers with significant LOD scores from Simple Interval Mapping output before applying Multiple-QTL Models (MQM) mapping (also called Composite Interval Mapping). Performing MQM mapping with markers close to significant LOD score positions as cofactors

allows reduction of residual variance, thus enhancing the power of QTL detection. For each quantitative trait, cofactor selection and MQM mapping were repeated until no further enhancement was obtained (no more QTLs detected, no increase in LOD scores and explained variances). From MQM mapping output, closest marker, flanking markers, additive effect and percentage of explained variance for each detected QTL and for each quantitative trait were determined for both seasons. Final results, with significant LOD scores and intervals, for each detected QTL per linkage group, were generated using MapChart© 2.3 program (Voorrips 2002).

## 6.3. Results

### 6.3.1. GBS for SNP identification

Selection of the genotyping-by-sequencing two enzyme method of Poland *et al* (2012) and the enzymes *MspI* and *PstI* was based on the results of Wong et *al* (2015) lentil SNP discovery across the lentil species. Using GBS, 220 polymorphic SNPs were deemed high quality for mapping, after satisfying quality control filtering based on deleting low quality and redundant SNPs using haplotype information for read depth (3), lack of redundancy and segregation in the parents. Genome coverage was reasonable, but incomplete, across six linkage groups (LG I, LG II, LG III, LG IV, LG VI and LG IX; Figure 6.1).

### 6.3.2. Linkage analysis and map construction

Marker distortion tested by Chi-square test ($P<0.05$) revealed that 35.4 % of SNPs, 43 % of SSRs, 18 % of SRAPs, 52.7 % of AFLPs and 20 % of RAPDs did not segregate according to the expected 1:1 ratio and were removed from the analysis. Out of 17 stable groups selected from the grouping tree, a total of 252 out of the 561 polymorphic markers were finally mapped in nine linkage groups spanning a total length of 2022.8 cM (Tables 6.1, 6.2). Final linkage groups were established using the *SCL* information. Linkage group length ranged from 71.7 cM to 531.1 cM whereas average distance between two markers ranged from 5.12 (LG V) to 9.8 cM (LG II) (Table 6.2; Figure 6.1). Seven linkage groups had a length of more than 100 cM (LG I, LG II, LG III, LG IV, LG VI, LG VII and LG IX). Both co-dominant (SNP and SSR) and dominant (SRAP, AFLP and RAPD) markers were present in six linkage groups, while dominant markers composed three linkage groups (LG V, LG VII and LG VIII).

**Table 6.2.** Linkage groups of the developed lentil linkage map and marker distribution

| Linkage groups | Number of mapped markers | Length (cM) | Average distance between markers (cM) |
|---|---|---|---|
| LGI | 71 | 531.1 | 7.4 |
| LGII | 48 | 473.4 | 9.8 |
| LGIII | 35 | 256.1 | 7.3 |
| LGIV | 17 | 141.6 | 8.3 |
| LGV | 14 | 71.7 | 5.12 |
| LGVI | 22 | 180.5 | 8.2 |
| LGVII | 12 | 118.3 | 9.8 |
| LGVIII | 16 | 96.1 | 6.0 |
| LGIX | 17 | 154.0 | 9.0 |
| **Total** | 252 | 2022.8 | 8.0 |

### 6.3.3. RIL root and shoot traits phenotyping and drought tolerance evaluation

High genetic variability, quantitative, continuous and normally distributed variation as well as high heritability estimate values of: dry root weight (DRW), lateral root number (LRN), taproot length (TRL), average taproot diameter (TRD), root surface area (RSA), dry shoot weight (DSW), shoot lengths at 12 (SL12DAS) and 22 (SL22DAS) days after sowing, growth rate (GR), seedling vigour (SV), leaf chlorophyll content (SPAD), root-shoot ratio (RS ratio) and wilting score (WS) were reported in chapter 5. Phenotyping evaluation was carried out under two watering regimes during two consecutive seasons. Evidence for polygenic control of these traits and the possibility of mapping the quantitative trait loci in the RIL population derived from ILL6002 x ILL5888 cross were reported in chapter 5. Also, significant associations of dry root biomass, lateral root number, root surface area, dry shoot biomass, root-shoot ratio and chlorophyll content with drought tolerance as estimated by the wilting score were demonstrated (chapter 5).

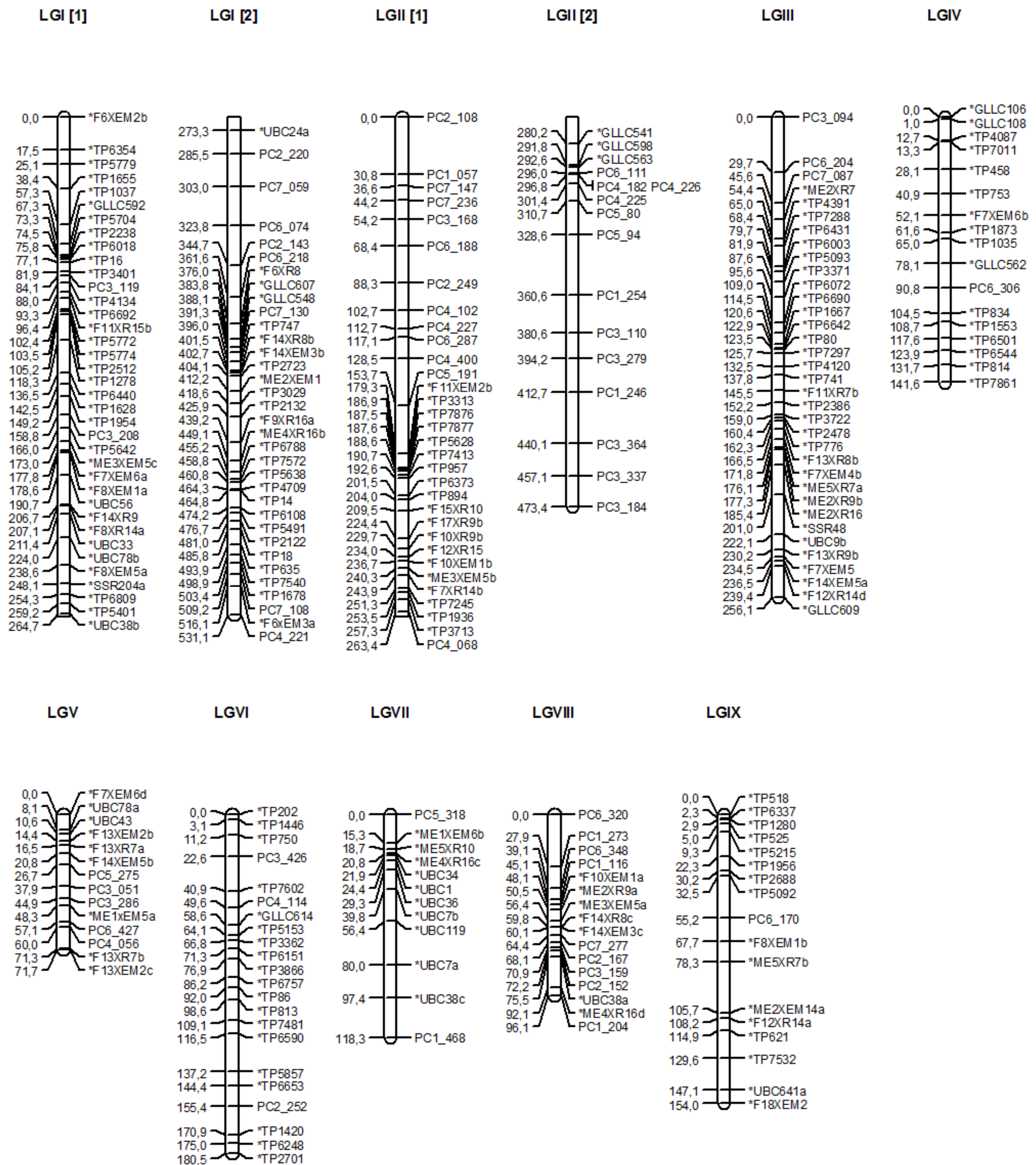


**Figure 6.1.** Genetic linkage map of lentil developed at LOD score ≥ 6 using maximum likelihood mapping algorithm of JoinMap® 4 program and drawn using MapChart© program. LGI-LGIX correspond to linkage groups, marker names are presented right to the linkage groups and the genetic positions in centiMorgans (cM) left. SNP markers are denoted by *TP_, SSR by *GLLC_, SRAP starting by *M_ or F_, RAPD by *UBC_ and AFLP by PC_.

### 6.3.4. QTL identification

A total number of 18 QTLs associated with 14 root and shoot traits were detected under drought-stressed conditions during the two seasons 2013 and 2014 (Table 6.3; Figures 6.2, 6.3). LOD score, percentage of explained phenotypic variance and additive effect of detected QTLs ranged from 2.75 (TRL) to 8.14 (DSW), from 4.3 ($QRSrati_{IX\text{-}77.72}$) to 28.9 % ($QRSratio_{IX\text{-}2.30}$) and from -5.17 (LRN) to 8.10 (DRW), respectively. Individual QTLs with higher explained phenotypic variance tend to have higher LOD scores than QTLs explaining less phenotypic variance. But this was not the case for some linked multiple QTLs. Although the parent ILL 6002 has predominantly contributed with positive alleles in the RIL population, the parent ILL 5888 also affected some traits as shown by the obtained positive and negative values of the additive affect (Table 6.3). This results in transgressive segregation with some recombinant inbred lines showing trait values higher than those of both parents as observed and reported in chapter 5.

Seven of the detected QTLs were co-located on LG VII at position 21-22 cM, with UBC34 as the closest marker and ME5XR10 - UBC1 as the two flanking markers: $QDRW_{VII\text{-}21.94}$, $QLRN_{VII\text{-}21.94}$, $QRSA_{VII\text{-}21.94}$, $QDSW_{VII\text{-}21.94}$, $QSL12_{VII\text{-}20.75}$, $QSL22_{VII\text{-}21.75}$ and $QGR_{VII\text{-}21.94}$ (Table 6.3).

Among the 18 detected QTLs, 12 were evidenced for the drought-stressed treatment for both seasons: $QDRW_{VII\text{-}21.93}$, $QRSA_{VII\text{-}21.94}$, $QDSW_{VII\text{-}22.94}$, $QRSratio_{IX\text{-}2.30}$, $QSL12_{IV\text{-}103.83}$, $QSL12_{VI\text{-}170.87}$, $QSL12_{VII\text{-}19.71}$, $QSL22_{VII\text{-}21.94}$, $QLRN_{III\text{-}98.64}$, $QLRN_{VII\text{-}21.94}$, $QSRL_{IV\text{-}61.63}$ and $QSPAD_{VIII\text{-}72.15}$. Interestingly, among these stable QTLs, $QRSratio_{IX\text{-}2.30}$, located at 2.30 cM on LG IX, is associated with a high root-shoot ratio and had LOD scores of 6.20 and 5.11 for 2013 and 2014 seasons, respectively. The explained phenotypic variance of this QTL was the highest with 27.6 % and 28.9 % and an additive effect of 1.23 and 1.84 for 2103 and 2014 seasons, respectively. The closest marker to this QTL is SNP marker TP6337 located at 2.3 cM whereby the two flanking markers are TP518 and TP1280, located respectively at 0 and 2.9 cM.

Two QTLs were identified for dry root biomass, $QDRW_{VII\text{-}21.93}$ accounted for 22.2 % (with a LOD score of 7.21) and 21.3 % (with a LOD score of 6.88) of the phenotypic variance with additive effects of 8.10 and 7.47 for 2013 and 2014 seasons, respectively.

Among the three QTLs detected for lateral root number, $QLRN_{III\text{-}98.64}$, was located at 98.64 cM position on LG III close to TP3371 SNP marker and flanked by the two SNP markers TP5093 - TP6072. The LOD scores, percentage of explained phenotypic variances and additive effects were 2.94, 23.5 % and -5.17, and 3.31, 24 % and -5.15 for 2013 and 2014 seasons, respectively. An important QTL was also identified for specific root length namely $QSRL_{IV\text{-}61.63}$ that was

detected for both seasons with LOD scores, percentage of explained phenotypic variances and additive effects of 3.84, 16.8 % and 0.83 and 3.63, 16.2 % and 0.32, respectively for 2013 and 2014.

Three QTLs were identified to be linked to chlorophyll content in which one was common for both seasons. The latter is the QTL $QSPAD_{VIII-72.15}$, which was detected with LOD scores, percentage of explained phenotypic variances and additive effects of respectively 3.98, 10.7 % and -2.20 for 2013 season and 4.25, 13.1 % and -2.20 for 2014 season.

Also, a QTL related to early vegetative vigour estimated by seedling vigour was detected for the 2013 experiment. This QTL, $QSV_{VII-4}$, was located on LG VII at position 4 cM, had a LOD score of 3.46, an additive effect of 0.29 and explained 14.9 % of total phenotypic variance.

A QTL $QWS_{I-22.53}$, related to drought tolerance as estimated by wilting score, is located at 22.53 cM position on LG I with a LOD score of 3.08 and 18.8 % as percentage of explained phenotypic variance.

**Table 6.3.** Characteristics of quantitative trait loci (QTL) identified under progressive drought stress in the RIL population (ILL6002 x ILL5888) for the 2013 and 2014 seasons

| **Drought-stressed treatment (2013)** | | | | | | |
|---|---|---|---|---|---|---|
| **Trait *** | **QTL**** | **LOD Score ***** | **PVE % ****** | **Additive effect ******* | **Closest marker** | **Flanking markers** |
| DRW | **QDRW$_{VII-21.94}$** | 7.21 | 22.2 | 8.10 | UBC34 | ME5XR10 - UBC1 |
| LRN | **QLRN$_{III-98.64}$** | 2.94 | 23.5 | -5.17 | TP3371 | TP5093 - TP6072 |
| | **QLRN$_{VII-21.94}$** | 2.91 | 5.3 | 2.78 | UBC34 | PC5_318 - UBC36 |
| RSA | **QRSA$_{VII-21.94}$** | 4.36 | 14.1 | 1.68 | UBC34 | ME5XR10 - UBC1 |
| DSW | **QDSW $_{VII-21.94}$** | 8.14 | 25.7 | 5.18 | UBC34 | ME5XR10 - UBC1 |
| RS ratio | **QRSratio$_{IX-2.30}$** | 6.20 | 27.6 | 1.23 | TP6337 | TP518 - TP1280 |
| SRL | **QSRL$_{IV-61.63}$** | 3.84 | 16.8 | 0.83 | TP1873 | F7XEM6b - TP1035 |
| | QSRL$_{VII-31.25}$ | 2.83 | 10.2 | -0.03 | UBC36 | ME4XR16c - UBC7b |
| SL12DAS | **QSL12$_{IV-102.83}$** | 4.02 | 16.5 | -0.54 | TP834 | PC6_306 - TP1553 |
| | **QSL12$_{VI-170.87}$** | 3.58 | 15.9 | 0.50 | TP1420 | PC2_252 - TP6248 |
| | **QSL12$_{VII-20.75}$** | 2.90 | 8.1 | 0.38 | ME4XR16c | ME5XR10 - UBC34 |
| SL22DAS | **QSL22$_{VII-21.75}$** | 4.55 | 12.2 | 0.78 | UBC34 | ME5XR10 - UBC1 |
| | QSL22$_{IV-102.83}$ | 4.22 | 19.2 | -0.94 | TP834 | PC6_306 - TP1553 |
| GR | QGR$_{VII-21.94}$ | 2.82 | 9.4 | 0.41 | UBC34 | ME5XR10 - UBC1 |
| SV | QSV$_{VII-4}$ | 3.46 | 14.9 | 0.29 | PC5_318 | PC5_318 - ME1XEM6b |
| SPAD | **QSPAD$_{VIII-72.15}$** | 3.98 | 10.7 | -2.20 | PC2_152 | PC3_159 - UBC38a |
| | QSPAD$_{I-158.76}$ | 3.41 | 9.2 | 2.14 | PC3_208 | TP1954 - TP5642 |
| WS | QWS$_{I-22.53}$ | 3.08 | 18.8 | 0.46 | TP5779 | TP6354 - TP1655 |

* DRW: dry root weight (mg plant$^{-1}$); LRN: lateral root number; TRL: taproot length (cm plant$^{-1}$); SRL: specific root length (root length/dry root weight; cm mg$^{-1}$ plant$^{-1}$); TRD: average taproot diameter (mm plant$^{-1}$); RSA: root surface area (cm$^2$ plant$^{-1}$); DSW: dry shoot weight (mg plant$^{-1}$); SL12DAS: shoot length at 12 days after sowing (cm plant$^{-1}$); SL22DAS: shoot length at 22 days after sowing (cm plant$^{-1}$); GR: growth rate (cm plant$^{-1}$); SV: seedling vigour; SPAD: chlorophyll content; RS ratio: root-shoot ratio; WS: wilting score.

** Bolded and underlined QTLs were identified for both seasons.

*** The presence of QTL was declared when the LOD score is above the threshold value obtained by a permutation test for each quantitative trait.

**** PVE: percentage of variance explained.

*****Positive values of additive effect mean that positive allele comes from the ILL6002 parent, while negative values mean that positive allele comes from the ILL5888 parent.

**Table 6.3. Continued**

| **Drought-stressed treatment (2014)** | | | | | | |
|---|---|---|---|---|---|---|
| **Trait *** | **QTL** | **LOD Score *** ** | **PVE % ******** | **Additive effect ***** ** | **Closest marker** | **Flanking markers** |
| DRW | **QDRW$_{VII-21.93}$** | 6.88 | 21.3 | 7.44 | UBC34 | ME5XR10 - UBC1 |
| LRN | **QLRN$_{III-98.64}$** | 3.31 | 24 | -5.15 | TP3371 | TP5093 - TP6072 |
| | **QLRN$_{VII-21.94}$** | 2.89 | 10 | 6.98 | UBC34 | ME5XR10 - UBC1 |
| RSA | **QRSA$_{VII-21.94}$** | 4.24 | 13.8 | 1.54 | UBC34 | ME5XR10 - UBC36 |
| DSW | **QDSW$_{VII-22.94}$** | 6.96 | 20.7 | 4.40 | UBC34 | ME5XR10 - UBC1 |
| | QDSW$_{IX-73.72}$ | 2.90 | 9 | -2.89 | ME5XR7b | F8XEM1b - ME2XEM14a |
| RS ratio | **QRSratio$_{IX-2.30}$** | 5.11 | 28.9 | 1.84 | TP6337 | TP518 - TP1280 |
| | QRSrati$_{IX-77.72}$ | 3.45 | 4.3 | 0.14 | ME5XR7b | F8XEM1b - ME2XEM14a |
| | QRSratio$_{III-49.62}$ | 2.95 | 14.7 | -0.14 | PC7_087 | PC6_204 - ME2XR7 |
| TRL | QTRL$_{IV-52.11}$ | 2.75 | 9.4 | 1.44 | F7XEM6b | TP753 - TP1873 |
| SRL | **QSRL$_{IV-61.63}$** | 3.63 | 16.2 | 0.32 | TP1873 | F7XEM6b - TP1035 |
| TRD | QTRD$_{IX-111.15}$ | 3.39 | 12.9 | -0.04 | F12XR14a | ME2XEM14a - TP621 |
| SL12DAS | **QSL12$_{VI-170.87}$** | 3.64 | 15.8 | -0.48 | TP6248 | TP1420 - TP2701 |
| | **QSL12$_{IV-103.83}$** | 3.55 | 13.5 | -0.45 | TP834 | PC6_306 - TP1553 |
| | **QSL12$_{VII-19.71}$** | 2.94 | 8.1 | 0.35 | ME5XR10 | ME1XEM6b - ME4XR16c |
| SL22DAS | **QSL22$_{VII-21.94}$** | 4.28 | 12.1 | 0.65 | UBC34 | ME5XR10 - UBC1 |
| SPAD | **QSPAD$_{VIII-72.15}$** | 4.25 | 13.1 | -2.20 | PC2_152 | PC3_159 - UBC38a |

* DRW: dry root weight (mg plant$^{-1}$); LRN: lateral root number; TRL: taproot length (cm plant$^{-1}$); SRL: specific root length (root length/dry root weight; cm mg$^{-1}$ plant$^{-1}$); TRD: average taproot diameter (mm plant$^{-1}$); RSA: root surface area (cm² plant$^{-1}$); DSW: dry shoot weight (mg plant$^{-1}$); SL12DAS: shoot length at 12 days after sowing (cm plant$^{-1}$); SL22DAS: shoot length at 22 days after sowing (cm plant$^{-1}$); GR: growth rate (cm plant$^{-1}$); SV: seedling vigour; SPAD: chlorophyll content; RS ratio: root-shoot ratio; WS: wilting score.

** Bolded and underlined QTLs were identified for both seasons.

*** The presence of QTL was declared when the LOD score is above the threshold value obtained by a permutation test for each quantitative trait.

**** PVE: percentage of variance explained.

*****Positive values of additive effect mean that positive allele comes from the ILL6002 parent, while negative values mean that positive allele comes from the ILL5888 parent.

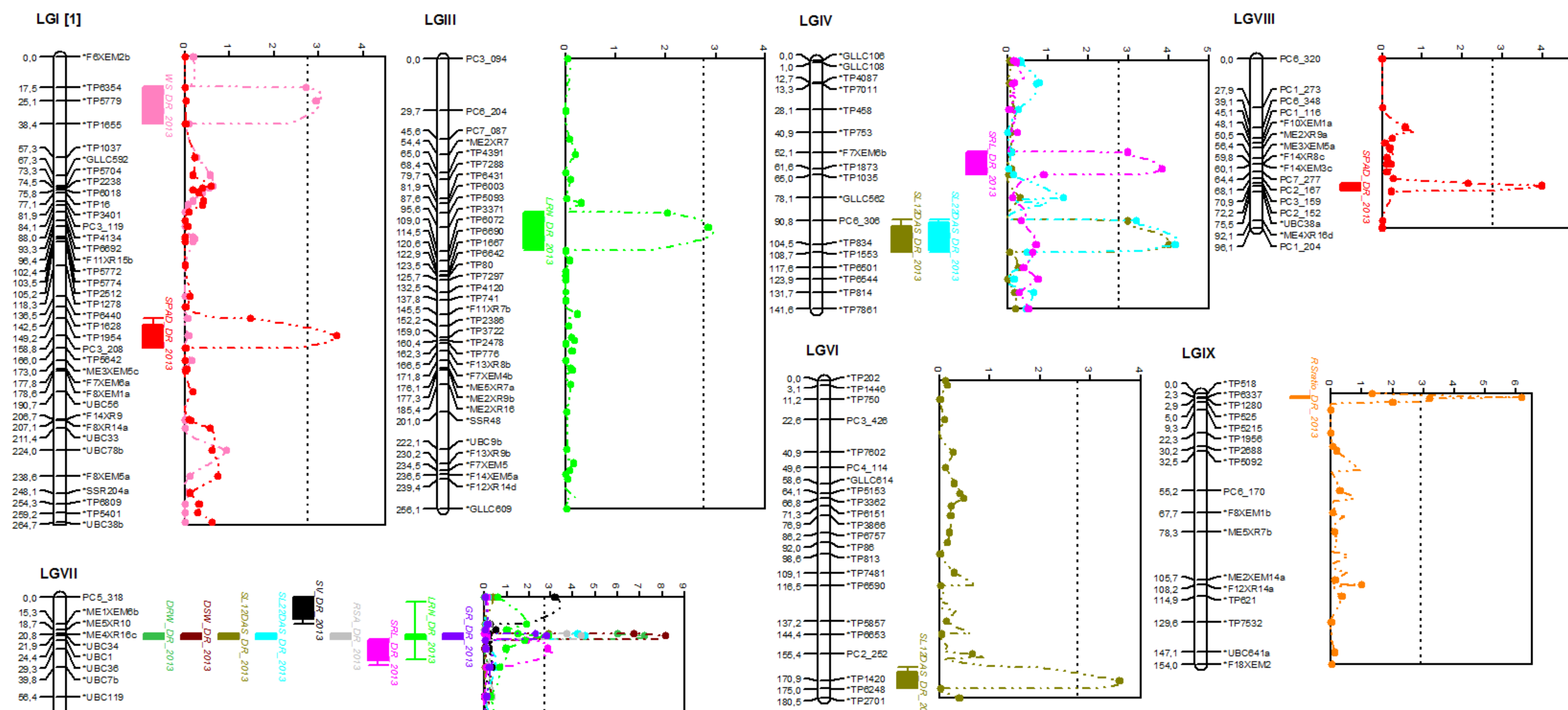


**Figure 6.2.** Linkage groups with identified QTLs related to root and shoot traits under progressive drought stress for the 2013 season. LOD score curves are presented right to linkage groups and significant thresholds are presented by dotted lines (when more than one QTL for different quantitative traits are detected in same linkage group, the dotted line correspond to the smallest threshold value). WS_DR_2013: wilting score; SPAD_DR_2013: Chlorophyll content as estimated by SPAD value; LRN_DR_2013: lateral root number; SL12DAS_DR_2013: shoot length at 12 days after sowing; SL22DAS_DR_2013: shoot length at 22 days after sowing; SRL_DR_2013: specific root length; DRW_DR_2013: dry root weight; DSW_DR_2013: dry shoot weight; SV_DR_2013: seedling vigour; RSA_DR_2013: root surface area; GR_DR_2013: growth rate; RSratio_DR_2013: root-shoot ratio.

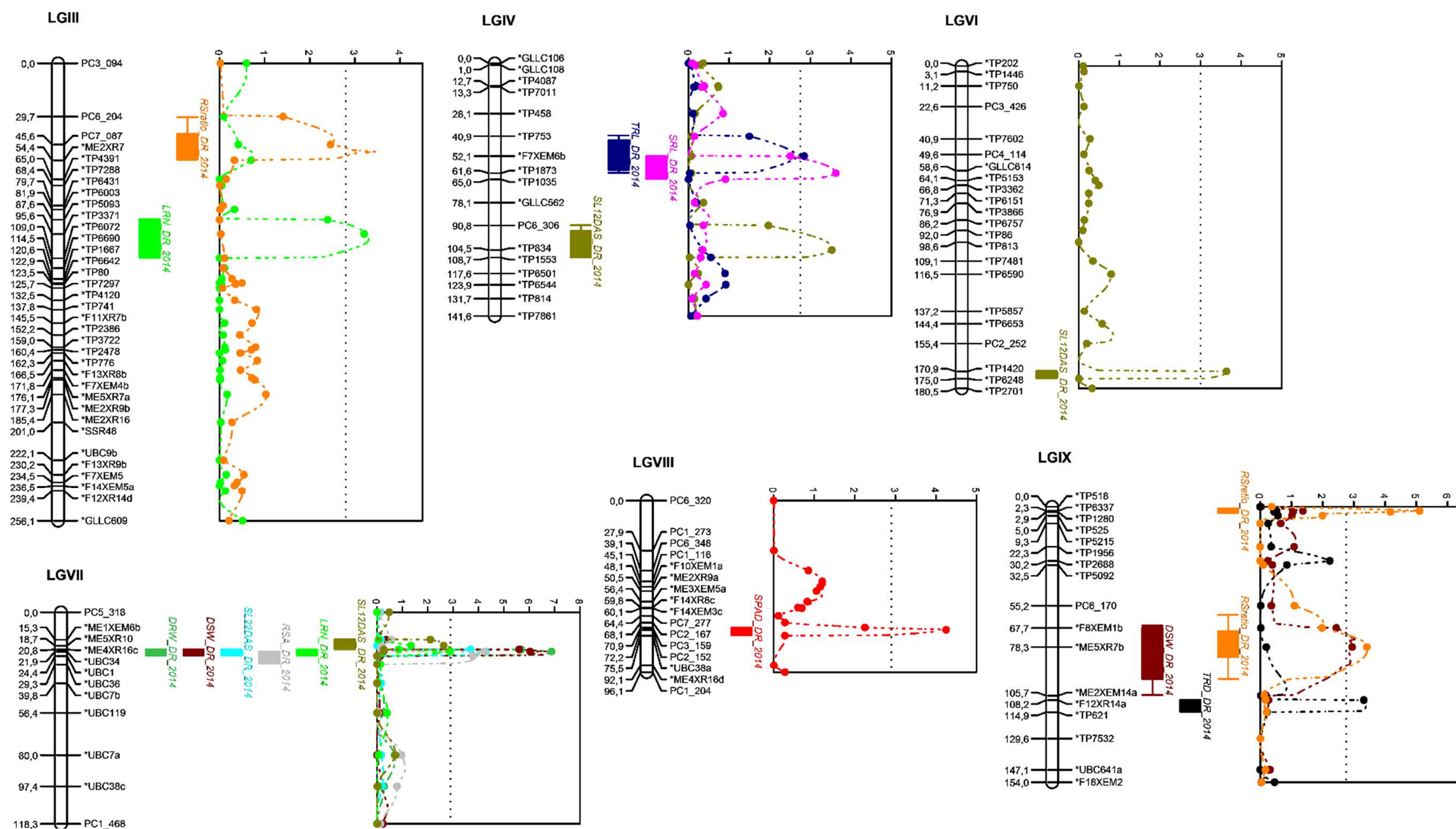


**Figure 6.3.** Linkage groups with identified QTLs related to root and shoot traits under progressive drought stress for the 2014 season. LOD score curves are presented right to linkage groups and significant thresholds are presented by dotted lines (when more than one QTL for different quantitative traits are detected in same linkage group, the dotted line correspond to the smallest threshold value). RSratio_DR_2014: root-shoot ratio; LRN_DR_2014: lateral root number; SRL_DR_2014: specific root length; TRL_DR_2014: taproot length; SL12DAS_DR_2014: shoot length at 12 days after sowing; DRW_DR_2014: dry root weight; DSW_DR_2014: dry shoot weight; SL22DAS_DR_2014: shoot length at 22 days after sowing; RSA_DR_2014: root surface area; SPAD_DR_2014: Chlorophyll content as estimated by SPAD value; RSratio_DR_2014: root-shoot ratio; TRD_DR_2014: average taproot diameter.

## 6.4. Discussion

The genetic linkage map of lentil initially developed by Saha *et al.* (2010) using a ILL6002 x ILL5888 RIL population containing 139 markers and 14 linkage groups was enhanced by adding SNP co-dominant markers and AFLP dominant markers, thereby increasing marker density and total spanned length. The number of linkage groups was reduced to nine with a total number of 252 mapped markers covering 2022.8 cM compared to 1565.2 cM in the previous genetic map. Average distance between markers was reduced from 11.3 to 8 cM. Sharpe *et al.* (2013) reported a lentil map with seven linkage groups using SNP and SSR markers. 62.77 % of markers from the Saha *et al.* (2010) linkage map were also mapped in the genetic map developed in our study. Several sets of markers from the previous genetic map were confirmed to be linked to each other in our map. For instance, all markers from LG 1 from the map of Saha *et al.* (2010) were also mapped in LG I of our map. Thirteen markers out of a total of nineteen mapped in LG 2 were mapped in LG III and four in LG IV of our map. Nine markers from LG 3 were mapped in LG V of our map and all those from LG 4 except for two that ended up in LG VII of our enhanced map. All markers from LG 11 of the previous map (except two) were mapped in LG II. All markers of LG 13 and LG 14 were mapped in LG IX and LG VIII of our map, respectively. Our linkage groups could not be assigned per Sharpe *et al.* (2013), the best lentil linkage map with seven linkage groups likely corresponding to the seven chromosomes of the genome developed to date, due to lack of common markers. We used a combination of dominant and co-dominant markers to develop a linkage map with reduced gaps. Since SNP data were not available for the whole population, we added also dominant AFLP markers for map construction. In other studies, dominant markers were also used together with co-dominant ones for the development of linkage maps and QTL analysis to overcome different limits such as genetic marker availability and large gaps in linkage groups when the number of mapped SSRs and SNPs is not sufficient (Gaudet *et al.* 2007; De Keyser *et al.* 2010; Muys *et al.* 2014; Ting *et al.* 2014; Kaur *et al.* 2014). Although maximum likelihood mapping algorithm often results in increased map length, it is considered to be more robust with missing data, genotyping errors and the use of markers with low information content (Lincoln and Lander 1992; Van Ooijen 2006; Cartwright *et al.* 2007; De Keyser *et al.* 2010). This algorithm uses multipoint analysis to approximate missing genotypes using nearby markers (De Keyser *et al.* 2010). Genetic linkage maps based on this approach giving the most likely marker order (De Keyser *et al.* 2010) were reported to be suitable for QTL mapping (Kim 2007; De Keyser *et al.* 2010). Thus, we adopted this approach as the main objective of our study was to identify QTLs related to root and shoot traits. Furthermore, although we used dominant markers such as AFLPs

known to result in longer maps, our linkage groups did not have extreme lengths and the total map length of 2022.8 cM is among common reported values in similar studies on lentil. Duran *et al.* (2004) reported a genetic linkage map of 2172 cM length using SSR, AFLP, ISSR and RAPD markers. More recently, Gupta *et al.* (2012) used SSR, ISSR and RAPD markers to construct a map of 3843.4 cM length. Also, Kaur *et al.* (2014) used SSR and SNP markers to develop a map of 1178 cM length. Using predominantly SNP markers and few SSRs, Sharpe *et al.* (2013) constructed a shorter map of 834.7 cM length. More recently, Ates *et al.* (2016) developed a map spanning a total length of 4060.6 cM and composed of seven linkage groups using SSR and SNP markers to identify QTLs controlling genes for Selenium uptake in lentil.
High genetic variability, quantitative, continuous and normally distributed variation as well as high heritability estimate values of all studied traits were shown in chapter 5.
In all, 18 QTLs were identified for root and shoot traits for both seasons under progressive drought-stressed treatments in the lentil RIL population ILL6002 x ILL5888. Among these QTLs, 12 were evidenced for both seasons. Aswaf and Blair (2012) reported a total of 15 putative QTLs for seven rooting pattern traits and four shoot traits under drought-stressed treatments in common bean (*Phaseolus vulgaris* L.). Also, Varshney *et al.* (2014) reported drought tolerance-related root trait QTLs in chickpea (*Cicer arietinum* L.). In soybean (*Glycine max* L.), Manavalan *et al.* (2015) identified a QTL region controlling a number of root and shoot architectural traits. In lentil, to our knowledge, this is the first report on QTLs related to root and shoot traits associated with drought tolerance. Interestingly, QTL $QRSratio_{IX\text{-}2.30}$ related to root-shoot ratio, an important trait for drought avoidance (Verslues *et al.* 2006), was confirmed to be present on LG IX at 2.30 cM position during the two seasons. Among detected QTLs, this QTL explained the highest percentage of phenotypic variance and was close to the co-dominant SNP marker TP6337 (C/T) and furthermore was flanked by the two SNP markers TP518 (A/G) and TP1280 (G/T). These markers are potentially important for their practical use for marker-assisted selection in breeding programs targeting drought tolerance. It should be pointed out that the same SNP markers were confirmed as being linked to root-shoot ratio when using only SNP markers on 92 RILs for linkage map construction and QTL mapping (data not shown).
A QTL-‘hotspot’ genomic region was identified on LG VII close to UBC34 RAPD marker and ME4XR16c SRAP marker, and was identified to be linked to the genetic control of a number of root and shoot traits for both seasons: dry root weight, lateral root number, root surface area, dry shoot weight and shoot length at 12 and 22 days after sowing. These traits were demonstrated to be significantly correlated as shown in chapter 4. Similarly, a *QTL-‘hotspot’*

related to 12 root traits was reported by Varshney *et al.* (2014) in chickpea (*Cicer arietinum* L.). Although practical efficient use of the identified genomic region in the ILL6002 x ILL5888 lentil population for marker-assisted selection could be limited by the dominant character of the closest RAPD marker, SRAP markers identified close to this genomic region could be used for assisting in the selection for linked traits. SRAP markers targeting the coding regions of open-reading frames of the genome, considered as better than RAPDs and technically less challenging than AFLPs, are of potential interest for QTL mapping (Chen *et al.* 2007; Yuan *et al.* 2008; Zhang *et al.* 2009; Saha *et al.* 2010, 2013; Robarts and Wolfe 2014). Furthermore, up to 20 % of SRAP markers were found to be co-dominant (Li and Quiros 2001). Dry root weight, associated with drought tolerance in lentil as shown in chapter 4, and other root and shoot traits such as root surface area and dry shoot weight also associated with drought tolerance are linked to this 'hotspot' genomic region.

QTL $QLRN_{III\text{-}98.64}$, related to lateral root number located at 98.64 cM position on LG III, was identified during both seasons explained 23.5 % and 24 % variations for 2013 and 2014, respectively. This QTL was close to SNP marker TP3371 (C/T) whereas its significant interval is between TP5093 (C/T) and TP6072 (A/G) SNP markers. Thus, the efficient use of these markers in breeding programs is possible for screening for higher lateral root number. High lateral root number was previously reported to be associated with drought tolerance and yield in lentil under drought stress (Sarker *et al.* 2005). We also found lateral root number to be correlated with drought tolerance with a correlation of -0.58 with the wilting severity as reported in chapter 4. Similarly, QTL $QSRL_{IV\text{-}61.63}$, located at 61.63 cM on LG IV and related to specific root length, was detected in both seasons with fairly high LOD scores of 3.84 and 3.63 for 2013 and 2014 respectively. This QTL, explaining 16.8 % of phenotypic variance, was close to TP1873 (A/C) SNP marker (61.6 cM) and flanked by the two F7XEM6b SRAP (52.1 cM) and TP1035 (A/T) SNP markers (65 cM). Specific root length is considered an important root trait that can contribute to plant productivity under drought (Comas *et al.* 2013). Therefore, the use of these linked markers to screen lines with longer root length should be of potential interest. Three QTLs were identified for chlorophyll content as estimated by the SPAD value. Among them, $QSPAD_{I\text{-}158.76}$ is located at 158.76 cM on LG I close to AFLP marker PC3_208 and flanked by the two co-dominant SNP markers TP1954 (A/T) and TP5642 (A/T) that could be efficiently used in marker-assisted selection. In chapter 4, correlations of SPAD value of 0.46 and 0.48 with dry root biomass and drought tolerance, respectively, in the same mapping population used here were shown.

A QTL $QWS_{I-22.53}$ related to drought tolerance as estimated by the wilting score, located at 22.53 cM on LG I and explaining 18.8 % of total phenotypic variance, is close to TP5779 (A/T) and flanked by TP6354 (C/T) and TP1655 (C/T) SNP markers, was identified during the 2013 season. After validation, these markers could be used for screening for drought tolerance. Wilting severity due to drought stress was found to be correlated with relative water content in lentil landraces as shown in chapter 3 indicating the importance of this parameter for the identification of drought tolerant cultivars. QTLs for drought tolerance as estimated by relative water content were reported for pea (*Pisum sativum*) by Iglesias-García *et al.* (2015).

It should be pointed out that results of QTL analysis using the second round map of JoinMap®4 program (Van Ooijen 2006) obtained from regression mapping algorithm were closely similar to those obtained using maximum likelihood algorithm, although total lengths of the two maps were different (data not shown).

## 6.5. Conclusions

In this study, a total of 18 QTLs related to root and shoot traits associated with drought tolerance such as dry root biomass, lateral root number, root-shoot ratio and specific root length were identified under progressive drought-stressed treatment. Interestingly, 12 of these QTLs were detected for both seasons, confirming their potential importance in conveying drought tolerance. DNA markers linked to these QTLs could be used for marker-assisted selection, thus making subsequent breeding efforts more reliable and efficient as the respective phenotyping-based methods are slow and labour-intensive, and affected by environment. Although root characteristics are difficult to study as many environmental effects (especially soil characteristics) interact with genetic factors, our results provide significant information about QTLs related to root and shoot traits that could be used in marker-assisted breeding after validation. A drought tolerance breeding strategy could be first based on laboratory screening of large collections of genetic material for the presence of the identified markers. Then, lines carrying alleles linked to QTLs of targeted traits could be evaluated under field conditions to finally identify drought-tolerant individuals. More focus should be on QTLs related to root-shoot ratio, lateral root number, specific root length and wilting score shown to be flanked by SNPs markers

# Chapter 7. Conclusions and further research perspectives

# Chapter 7. Conclusions and further research perspectives

## 7.1. Introduction

Genetic diversity analysis and identification of valuable genotypes tolerant to drought stress offer an opportunity to enhance crop productivity in arid/semi-arid environments and thus local farmers' livelihoods. Identifying and characterizing local cultivars and landraces with specific characteristics, such as adaptation to dry areas or other quality mark distinction, would contribute to enhanced valorization of these genetic resources. Furthermore, high genetic diversity is of utmost importance in breeding, as it allows to achieve higher genetic gains by offering a large specter for selecting superior genotypes to introduce in breeding steps. In fact, genetic diversity represented by the standard deviation around the mean of a population is a key factor in the equation of genetic gain (Acquaah 2007).

Certain root and shoot characteristics convey drought tolerance in lentil like they would in other crop species. Studying the genetic control mechanisms governing these traits and identification of the DNA markers linked to them are promising steps for developing efficient molecular breeding tools.

Lentil contributes to sustainable farming by its ability to fix nitrogen in soils, reducing the need for chemical fertilizer use and enhancing the productivity of a number of cereal-based cropping systems where it is usually included in rotation with wheat and barley. Furthermore, lentil seeds are consumed as staple food. They thus constitute an important source of proteins and micronutrients for a large population especially in developing countries. Thus, efficient breeding methods and enhanced valorization of lentil genetic resources would contribute to improving both human nutrition and sustainable agriculture.

### 7.2. Genetic diversity analysis of lentil landraces

Both microsatellites (SSR) and AFLP markers evidenced a high level of genetic diversity in the lentil landraces from the Mediterranean region (Morocco, Italy, Turkey and Greece) that were covered by our study. Similar results were obtained through these two techniques, thus confirming and strengthening our findings. We also evidenced high genetic variability for root and shoot traits and drought response among these Mediterranean landraces. For instance, dry root biomass ranged from 0.31 to 1.18 $g.plant^{-1}$ and dry shoot biomass from 0.47 to 1.22 $g.plant^{-1}$ while relative water content ranged from 40 to 75 %, water losing rate from 0.37 to 0.70 $g.h^{-1}.g^{-1}$ DW and wilting score from 0.33 to 3.66. For each geographic origin, the level of genetic variation as revealed by SSR and AFLP markers as well as the concomitant drought responses

was high. Thus, high genetic gain could be expected when using genotypes selected among these landraces in breeding for drought tolerance.

Regarding the populations' genetic structure, two major gene pools were identified through both microsatellites and AFLP DNA markers: a southern Mediterranean genepool composed by Moroccan landraces and a northern Mediterranean genepool consisting of landraces from Italy, Greece and Turkey. This suggests that the introduction and/or evolution (once introduced) of lentil to these two areas probably followed different itineraries. Similar to our results, Lombardi *et al.* (2014) reported a very high level of genetic diversity for landraces from the Mediterranean region, especially those from Greece and Turkey, using SNP markers. Furthermore, Dilshit *et al.* (2015) concluded that Mediterranean landraces could be used for broadening the genetic base of lentil grown in South Asia. The latter authors attributed the low productivity of lentil in this region to a greater susceptibility to biotic and abiotic stresses due to its narrow genetic base.

## 7.3. Genetic differentiation of landraces according to agro-environmental origins and drought reactions

We demonstrated genetic differentiation of Moroccan landraces according to their agro-environmental origins. Landraces from dry areas, where drought and heat stresses are frequent, were genetically distinct from those originating from highlands, where cold stress is frequent, and more favourable climatic condition areas. Specific adaptation to the respective environments and possible evolution into distinct ecotypes, as a result of a continuous selection over time, probably explain this genetic differentiation. We identified some specific functional drought adaptation traits, such as early flowering and seed-maturing, and early vegetative vigour. Landraces originating from dry areas all have a shorter production growth cycle and can be associated with higher early vegetative vigour than the other landraces studied. Early maturity, early vigour and high ground coverage were found to be strongly associated with higher seed yield and biomass (straw) in lentil under Mediterranean environment during dry seasons (Silim *et al.* 1993). The latter authors reported that 49 % of variation in obtained seed yield from a dry season (<180 mm rainfall) was due to variation in flowering time. Mediterranean landraces showed high levels of variation in their response to drought stress. We evidenced genetic differentiation of these landraces according to their drought response based on genetic distances and physiological parameters quantifying drought tolerance. We were able to clearly discriminate drought-tolerant landraces from those showing moderate and sensitive reactions. This was done by analyzing genetic distances between landraces from microsatellites

and AFLP markers linked to drought tolerance together with relative water content, water losing rate and wilting score. Similar to our results, Erskine *et al.* (1989) reported lentil landraces from different geographical regions revealing specific adaptation to their respective ecological environments. Differentiation among lentil landraces could be explained by the adaptation for better phenological characteristics (Erskine *et al.* 1981), different responses for temperature and photoperiod (Erskine *et al.* 1990), climatic stresses such as cold (Erskine *et al.* 1981) and edaphic factors such as iron deficiency (Erskine *et al.* 1993). More recently, Singh *et al*. (2016) reported molecular clustering of *Lens* species including wild and cultivated forms with different adaptations to drought conditions using SSR markers.

Our results also evidenced one landrace, i.e. *lentil of Ain Sbit,* to be genetically distinct from the other landraces. Lentils from *Ain Sbit*, a small rural area in Morocco where lentil has been grown since times immemorial, are well-known for their adaptation to their environment and for their excellent seed quality (high protein content and short cooking time with intact seed texture after cooking). Our findings support the idea that this landrace should receive a Protected Designation of Origin (PDO) quality mark as a local product, "*produit de terroire*". This would allow better valorization and protection of this landrace from frauds for the benefits of local farmers. Sonnante and Pignone (2007) equally considered that molecular characterization of Italian lentil landraces can contribute to promote the economic value of elite landraces and protect producers from frauds. Molecular profiles from SSR and AFLP markers could be used to confirm the genetic identity of this landrace.

## 7.4. Identification of QTLs related to rooting patterns and shoot traits, and DNA markers associated with drought tolerance

### 7.4.1. Identification of SSR and AFLP markers associated with drought tolerance in Mediterranean lentil landraces

Single-marker analysis using a Kruskal-Wallis test was used to identify SSR and AFLP markers associated with drought tolerance in Mediterranean lentil landraces. Several microsatellites and AFLP DNA markers were identified to be linked to drought tolerance as estimated by relative water content, water losing rate and wilting score in a number of Mediterranean landraces. Among these markers, six potentially important main effect SSR and AFLP alleles explaining high phenotypic differences between accessions (ranging from 21 to 50 % for SSRs, and from 14 to 33 % for AFLPs) were identified. In fact, our results could be considered as first steps for identifying genes related to drought tolerance based on the degree of association between alleles and a measured trait (linkage disequilibrium) used in association mapping studies.

### 7.4.2. Genetic variability, heritability estimates and identification of QTLs related to root and shoot traits in a lentil recombinant inbred line population

Certain rooting patterns, early vegetative vigour, high root-shoot ratio and other shoot traits such as chlorophyll content can be associated with drought tolerance in lentil. Developing cultivars with prolific root systems is important for enhancing lentil productivity in water deficiency environments. However, techniques for field screening of root characteristics are slow and labour-intensive, especially when dealing with large numbers of lines, which is generally the case in breeding programs. Thus, the need for designing efficient alternative strategies for identifying genotypes with well-developed root systems is of utmost importance for breeding programs to be successful. As marker-assisted selection could be an effective solution for selecting genotypes with superior root systems, identifying and mapping DNA markers linked to QTLs controlling rooting patterns is a promising step to assist breeding for these traits. It should be pointed out that although there exist a limited number of reports on lentil root and shoot traits related to drought tolerance (Sarker *et al.* 2005; Kumar *et al.* 2012; Singh *et al.* 2013), we are the first to report on the use of a RIL mapping population aiming to understand genetic control of these characteristics.

We found high genetic variability for root and shoot traits associated with drought tolerance to occur in a recombinant inbred line population derived from the ILL6002 x ILL5888 cross. We obtained high coefficients of variation and wide ranges between minimum and maximum values within the population for dry root biomass, lateral root number, dry shoot biomass and other characteristics. We observed high variability for reaction to drought stress ranging from healthy plants with no visible symptoms to completely dried plants passing by several intermediate levels. This confirmed the results we obtained on root traits using landraces. We also demonstrated a normal distribution and evidence of the polygenic control of these traits. Comparable results were obtained by Varshney *et al.* (2014) for chickpea (*Cicer arietinum)* and by Aswaf and Blair for soybean (*Glycine max).* We found high heritability estimates, highlighting the potential of obtaining high genetic gains when using this germplasm for breeding for these parameters. Interestingly, higher values of heritability ranging from 50 % to 97.8 % were obtained under progressive drought stress indicating that breeding for root and shoot traits would be more effective under water-limited environments. Similar observations were reported by Aswaf and Blair (2012) for soybean. Drought stress reduced all root and shoot growth, whereas root-shoot ratio was increased suggesting that the latter could be an important selection criterion for drought tolerance in lentil. Under moderate drought stress, one of the first reactions of plants is to maintain a higher water content in their shoots by increasing root growth

to explore higher soil volumes thus increasing water uptake (Verslues *et al.* 2006; Comas *et al.* 2013). We obtained important correlation and regression values between root (underground, difficult to measure) and shoot traits (aboveground, more easy to measure) that could be used as proxy parameters. For instance, dry root biomass and lateral root number were positively associated with dry shoot biomass ($R^2 = 0.49$, $R^2 = 0.39$) as well as with chlorophyll content as estimated by SPAD values. Also, higher dry root biomass, lateral root number, root surface area, dry shoot biomass, chlorophyll content and root-shoot ratio were associated with drought tolerance. We obtained a similar trend of associations using landraces. Similarly, Sarker *et al*. (2005) reported that shoot length accounted for 85 % of variation in seed yield and Kumar *et al.* (2012) reported significantly positive correlations of SPAD with dry root weight and root length in lentil.

Compared to the previous map developed by Saha *et al*. (2010) which was based on the same population, an enhanced linkage map of ILL6002 x ILL5888 recombinant inbred line population was developed using both dominant and co-dominant markers. By adding SNP and AFLP markers, we reduced the number of linkage groups from 14 to 9, whereas marker density corresponding to the average distance between two markers went from 11.3 to 8 cM.

QTL analysis allowed to identify a total of eighteen QTLs controlling rooting patterns under progressive drought-stressed treatments for two consecutive seasons. Among these QTLs, twelve were common during the two seasons. A QTL-hotspot genomic region located on linkage group LGVII at 21-22 cM position was identified to be linked to several co-located QTLs related to dry root biomass, lateral root number, root surface area, shoot length and dry shoot biomass. Among these QTLs, $QDRW_{VII-21.94}$ linked to dry root weight, which was shown to be associated with drought tolerance, has the highest additive effect of 8.10 (with a LOD score of 7.21) for 2013 and 7.44 (with a LOD score of 6.88) for 2014. Although practical use of these QTLs is limited by the dominant character of the closest RAPD marker, UBC34, the flanking SRAP marker ME4XR16c could be used in marker-assisted selection. Similarly, Varshney *et al.* (2014) found a number of co-located QTLs in a genomic hotspot region related to drought tolerance for chickpea using two mapping populations. This region contained 12 out of 25 detected QTLs for: 100-seed weight, root length density, days to flowering, days to maturity, biomass, plant height, pods per plant, harvest index, root dry weight/total plant dry weight ratio, shoot dry weight, seeds per pod and yield.

One interesting QTL ($QRSratio_{IX-2.3}$) identified on linkage group LGIX at position 2.30 cM was shown to be related to a high root-shoot ratio and also to explain the highest percentage of phenotypic variation with 27.6 % and 28.9 % for 2013 and 2014 seasons respectively. This

QTL was detected with fairly high LOD scores and additive effects of 6.2 and 1.23 for 2013 and 5.11 and 1.84 for 2014, respectively. High root-shoot ratio is an important selection criterion for drought tolerance as it is based on an avoidance mechanism that allows higher plant productivity under water deficit conditions. Interestingly, the identified QTL was close to and flanked by co-dominant SNP markers TP6337 and TP518 - TP1280, respectively. These markers are efficient in successful use for marker-assisted selection (Rafalski 2002). Furthermore, this QTL and its related markers are likely to be stable as we demonstrated them to occur in both seasons. Similarly for chickpea, Varshney *et al.* (2014) confirmed several QTLs related to root traits conferring drought tolerance in two mapping populations based on multi-location and multi-annual experiments. We also identified other important QTLs close to and flanked by SNP markers for specific root length, lateral root number and wilting score. A QTL located at 61.63 cM on linkage group LGIV related to specific root length was detected in both seasons in the drought-stressed treatment. High specific root length (ratio of root length to root biomass, estimates root length increase achieved per unit root biomass invested) is important for better exploration of soil for water forage (Paula and Pausas 2011). It allows higher root-soil contact, therefore greater absorption potential by higher surface to volume ratio for the same carbone allocated (Larcher 1995). For lateral root number, a QTL located at 98.64 cM position of linkage group LGIII was identified in the drought-stressed treatment during both seasons. A QTL related to wilting score, that provides a good estimate of drought tolerance (Singh *et al.* 1997, 2013; Ur Rehman 2009; Blum 2011), located at 22.53 cM on linkage group LGI, close to TP5779 and flanked by TP6354 and TP1655 SNP markers was identified during the 2013 season. A number of QTLs for drought tolerance as estimated by relative water content, that we demonstrated to be correlated with wilting score in lentil, were reported for pea (*Pisum sativum*) by Iglesias-García *et al.* (2015).

### 7.5. Root phenotyping for QTL mapping

Rooting patterns are difficult to study as they are highly affected by their environment, especially edaphic conditions. Getting accurate measures for roots from experiments on field-grown plants is prevented by heterogeneity in the soil structure (Tuberosa 2012). Furthermore, available phenotyping methods so far do not allow non-destructive assessment in real-field conditions. However, a number of phenotyping methods were reported to yield reliable results for the estimation of root characteristics under controlled conditions (Tuberosa 2012; Comas *et al.* 2013). These methods are mainly based on experiments in simple plastic pots or in large and deep tubes under greenhouse conditions. Hydroponic culture was also reported to work well

and is widely used (Varshney *et al.* 2011; Aswaf and Blair 2012; Kumar *et al.* 2012; Singh *et al.* 2013). Growing plants in pots or columns filled with soil is considered as a compromise to avoid both the difficulties of studying roots in the field and the unnatural conditions of hydroponics (Tuberosa 2012). Nevertheless, it should be pointed out that hydroponics combined with some growing media like perlite, the method that we used here for phenotyping roots, offer the possibility of getting roots with limited damage (Rabah Nasser 2009; Day 1991). Rabah Nasser (2009) tested different growth media to extract lentil roots and concluded that perlite is optimum to get intact roots for further analysis. Likewise, we were able to extract and analyze roots without damage at seedling stage. Other techniques have been used to study bi- and tri-dimensional root architecture: magnetic resonance imaging, X-ray microtomography, soil-filled chambers, or soil sacs, pouches and paper rolls (Tuberosa 2012).

Although significant differences may exist between results obtained from pot experiments and those obtained in the field, phenotypic and genotypic variation in controlled environments is more likely to be similar to variation under field conditions for traits with high heritability (Comas *et al.* 2013). Interestingly, Kashiwagi *et al.* (2005) and Sayar *et al.* (2007) reported high heritability across different environments and associations with improvements in grain yield for root traits in chickpea and wheat, respectively. Furthermore, Watt *et al.* (2013) showed that controlled-environment seedling root screen for wheat correlates well with rooting depths at vegetative stages at two field sites. However, the same authors showed different results between controlled conditions and field conditions at mature developmental stages because of root sensibility to soil and climatic conditions.

## 7.6. Summarized outcomes and practical significance

In our PhD research project, we demonstrated high genetic variability in Mediterranean landraces as well as in the RIL population for root and shoot traits conferring drought tolerance. Also, high heritability values were shown for these traits. Thus, our findings are of potential interest for practical use in breeding programs, especially those aiming to develop drought tolerant cultivars. The expected genetic gain related to these traits would be high because it is highly dependent on large genetic variability and high heritability of targeted traits.

The evidenced genetic differentiation following drought tolerance and different agro-environmental origins (dry areas, highlands and favourable areas) could allow a well-targeted and oriented selection of genotypes to be included in breeding programs. Selecting landraces from dry areas would result in greater genetic gains in drought tolerance breeding, while landraces from highlands (example of middle Atlas mountains, Morocco) could yield better

results in cold tolerance breeding. Biodiversity conservation, *in situ* or *ex situ*, of such landraces is of critical importance for preserving these potential sources of biotic and abiotic stress tolerance/resistance.

Another useful importance of our results for breeding purposes is the identified molecular markers linked to drought tolerance and root traits that could be used in marker-assisted selection. Phenotypic evaluation alone is costly and requires substantial labor. Also, indirect selection shown by high correlations and regressions that we obtained with aboveground characteristics (easy to measure) could be used for screening belowground traits (difficult to measure).

Also, promising individuals from both populations (RILs and landraces) could be tested in field yield trials (multi-locations and multi-annuals) of a breeding program that may result in the identification of superior (well-adapted and high yielding) candidates for registration as a variety.

On the other hand, the disctinction between the two major gene pools from northern and southern Mediterranean that we found is important when defining suitable strategies aimed to improve germplasm conservation and utilization. Likewise for the obtained genetic diversity using SSR and AFLP markers. For instance, the evidenced classification could be used prior to the selection of core collections by sampling from both defined groups. Also, our results from SSR analysis of landraces provide preliminary information about the allelic richness that could be targeted for construction of such core sets aiming at maximization of genetic variability.

## 7.7. Perspectives

Plants are subjected to different kinds of biotic and abiotic stresses under different frequency and severity. Drought, heat, cold and other biotic stresses are limiting plant production in different parts of the world. Studying these stresses will lead to find better strategies to limit their impact and enhance yield. However, it is frequent that several stresses occur simultaneously (Atkinson and Urwin 2012; Ramegowda and Senthil-Kumar 2014). In fact, each environment is characterized by a combination of factors interacting with plants. Hence, testing plant response for each stress individually may not be adequate (Mittler and Blumwald 2010). For instance, drought stress is often combined with increased temperature, especially under Mediterranean conditions. Lentil is known to be sensitive to high temperatures during flowering and seed development growth stages (Materne and Siddique 2009). Studies about drought should also include heat stress to understand their combined effect, the interaction between these two abiotic constraints and to investigate the genetic control behind them. Lentil cultivars

combining drought and heat stress would result in better yield in areas where end-cycle drought and high temperatures occur frequently.

In this thesis we reported high genetic variability, high heritability and identified some associated QTLs and markers for root and shoot traits and drought tolerance in pot experiments under controlled conditions. Also, genetic differentiation according to some adaptive traits were demonstrated. Our results are of significant importance for better understanding of genetic adaptation and tolerance for drought stress that could be important for breeding and genetic resource conservation and valorization strategies in lentil. However, investigation of these issues under real field conditions using larger populations is of utmost importance for comparison and validation of these findings. This would require more powerful QTL detection and lower false positive discovery thus yielding more accurate results.

Further phenotypic evaluation of larger numbers of landraces from different agro-environments in the Mediterranean region using additional adaptation traits and drought response measures at physiological, metabolic and morphological levels would enhance the understanding of the genetic differentiation that took place during centuries of adaptation processes. Also, testing these landraces under contrasting field conditions in repeated experiments would allow estimation of the extent of genotype by environment interaction (landraces by agro-environments). Furthermore, recent progress in lentil genome sequencing, such as genotyping-by-sequencing, and increasing availability of efficient co-dominant SNPs markers (Sharpe *et al.* 2013; Wong *et al.* 2015), would result in better potential for analyzing the relationship between functional adaptation and genetic differentiation. The results we reported could be a first step towards genome-wide association studies in lentil to identify markers linked to QTLs conferring drought tolerance using sufficient numbers of landraces, allowing higher expression of linkage disequilibrium, and efficient DNA markers such as SNPs.

The methods we described on genetic differentiation of landraces according to agro-environmental origin and drought response could be used for other crop species. It offers an efficient tool to explore plant agro-biodiversity. Genetic resource collections originating from regions of different climatic conditions have undergone different selection pressures over time. Identifying groups of genotypes genetically different for their reaction to biotic and abiotic stresses, using the method we described, would result in efficient selection for specific objectives in breeding programs of economically important crops.

In this study, we reported on QTLs related to root traits in repeated greenhouse conditions under drought-stressed treatment at seedling stage (38 days after sowing). Although several

identified QTLs and associated DNA markers are likely to be stable, as they were detected for both seasons, validation using other phenotyping methods in larger populations will be necessary in order to compare the results obtained. For instance, we used *Image J* (Abramoff *et al.* 2004) and *Smart Roots* (Lobet *et al.* 2011) for root measurements from image analysis of scanned roots. These programs were largely used for phenotyping roots at seedling stage and have yielded accurate results. However, possible overlapping of lateral roots might limit their use when root systems are more developed. Therefore, using more specialized systems specifically designed for roots such as *WinRHIZO* (Arsenault *et al.* 1995) that combines image acquisition and analysis would enhance the quality of data and allow the study of roots at a more developed growth stage. Also, it would be of interest to measure root traits at maturity to investigate relationships with grain yield under drought stress and identify associated QTLs. Also, future studies should include the interaction between QTLs and different watering regimes. Further research studies using multiple mapping populations in multi-location and multi-annual experiments would result in the validation of the QTLs that we identified in this study. In the same vein, Varshney *et al.* (2014) reported a study based on up to 7 seasons at 5 locations to confirm a set of important QTLs and related DNA markers for their introgression into elite cultivars to enhance drought tolerance in chickpea. Also, further research under different environmental conditions would result in clearly identifying “constitutive” QTLs (consistently detected across most environments; Collins *et al.* 2008) and “adaptive” ones (detected only in specific environmental conditions or increases in expression with the level of an environmental factor; Collins *et al.* 2008) among the QTLs we detected in this study.

Molecular markers that we have used consisting of a combination of co-dominant and dominant markers may hamper practical use of our results. Genomic studies in lentil have recently achieved important advances that could be used to overcome this situation. Promising results are expected from the ongoing project (*LenGen*) about the whole genome sequencing of lentil. Yet, a recent first version v1.0 sequence of the lentil genome has been made available at the *knowPulses* web-site related to the lentil genome sequencing project of Saskatchewan University, Canada. The latter research group developed also the GBS protocol in lentil for high density SNPs identification (Wong et *al.* 2015). These tools should also be used for further investigation of drought tolerance in the genus *Lens* in association studies in the perspective of identification of valuable genotypes and of better characterization, valorization and preservation of lentil biodiversity. Screening for drought tolerance-related root traits in wild lentil-related species (such as *Lens orientalis*) and identification of related genes through

association mapping studies or in a mapping population from interspecific crosses should be considered in future studies. Singh *et al.* (2016) suggest that drought adaptation varies among wild and cultivated lentil. These authors found significant differences in physiological and morphological characters under drought stress among lentil clusters based on microsatellite markers.

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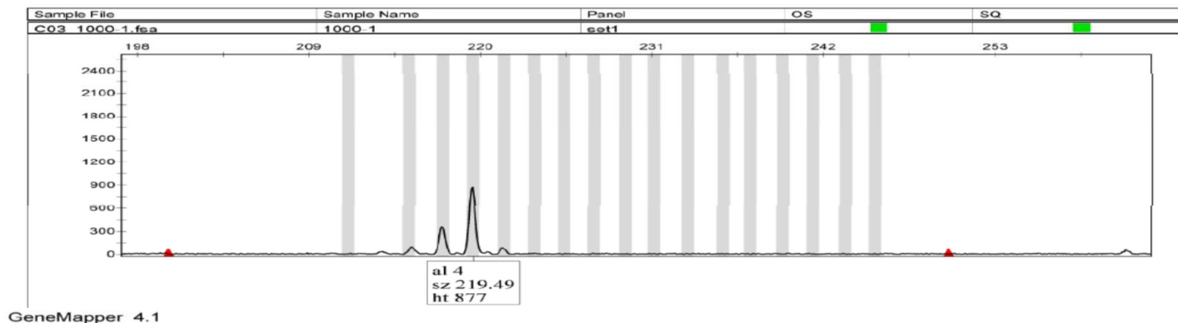
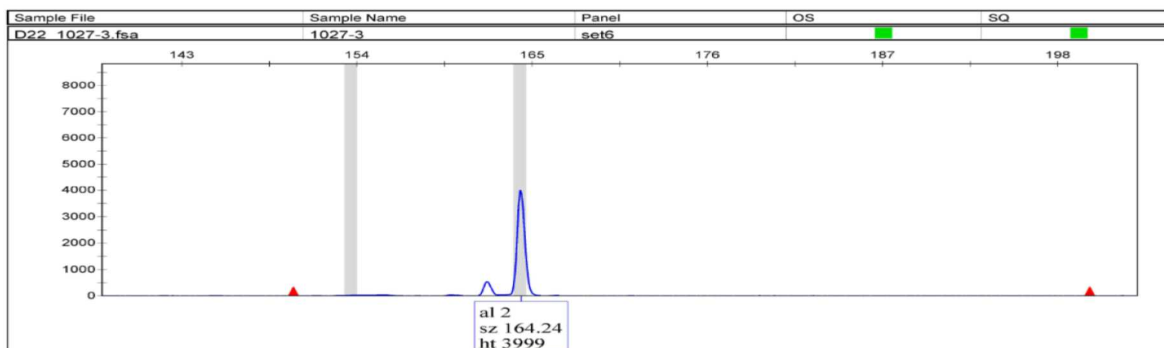
# Appendix

# Appendix



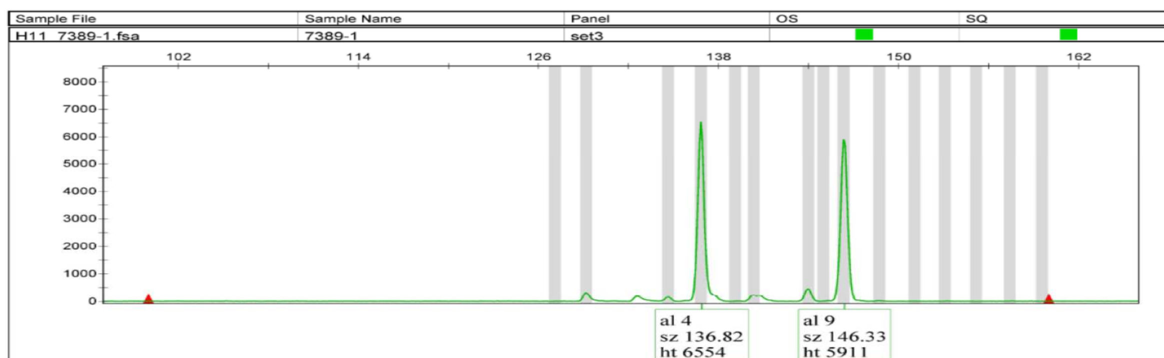




**Figure A.1.** Examples of SSR electrophoretogram profiles on lentil landraces used in chapter 3 (3 different samples at 3 different loci). The 2 upper profiles correspond to homozygous individuals at these two loci, while the lower profile corresponds to a heterozygous individual at this locus.

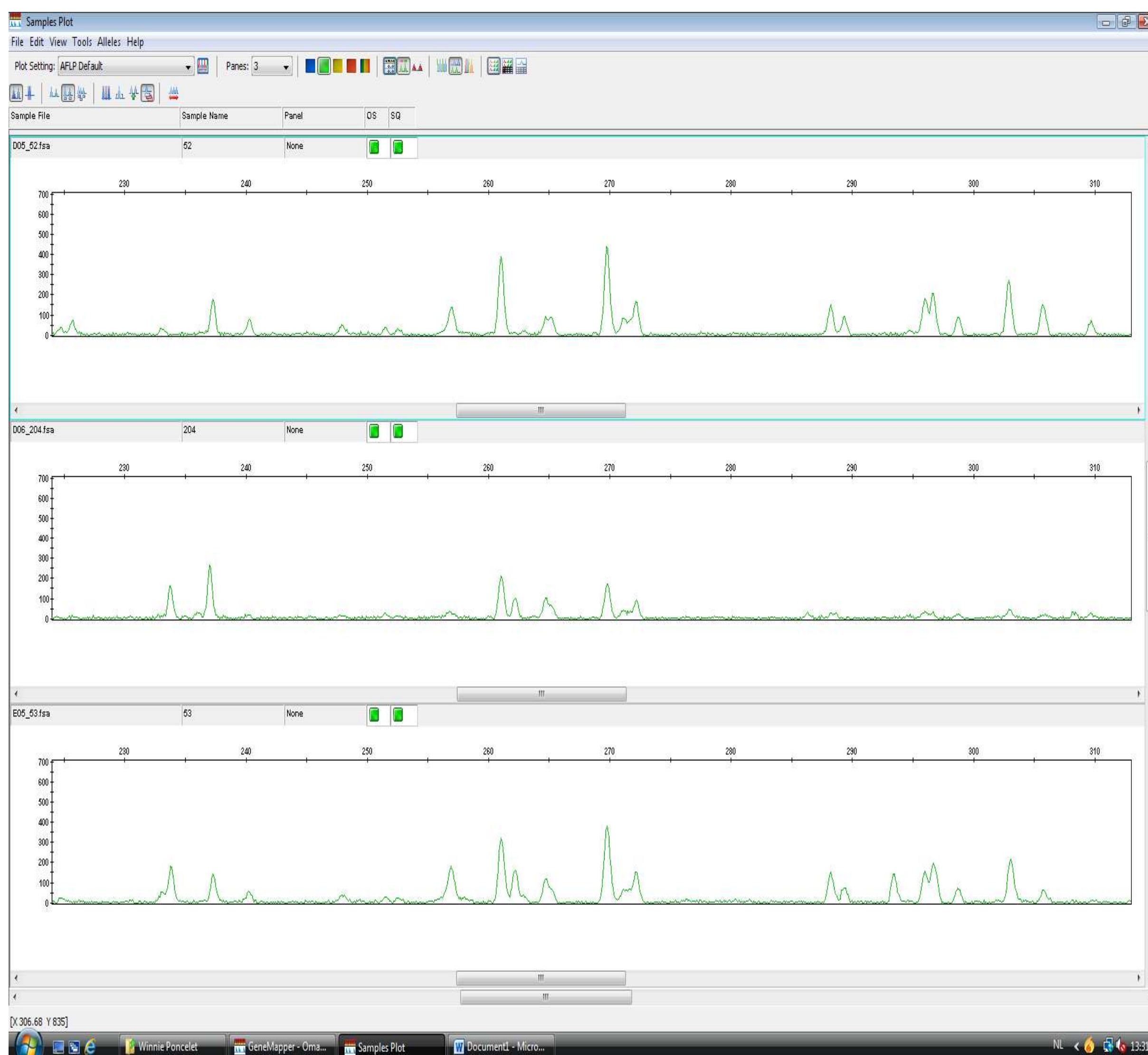



**Figure A.2.** Examples of AFLP electrophoretogram profiles on lentil landraces used in chapter 3 (3 different samples with a same primer combination).

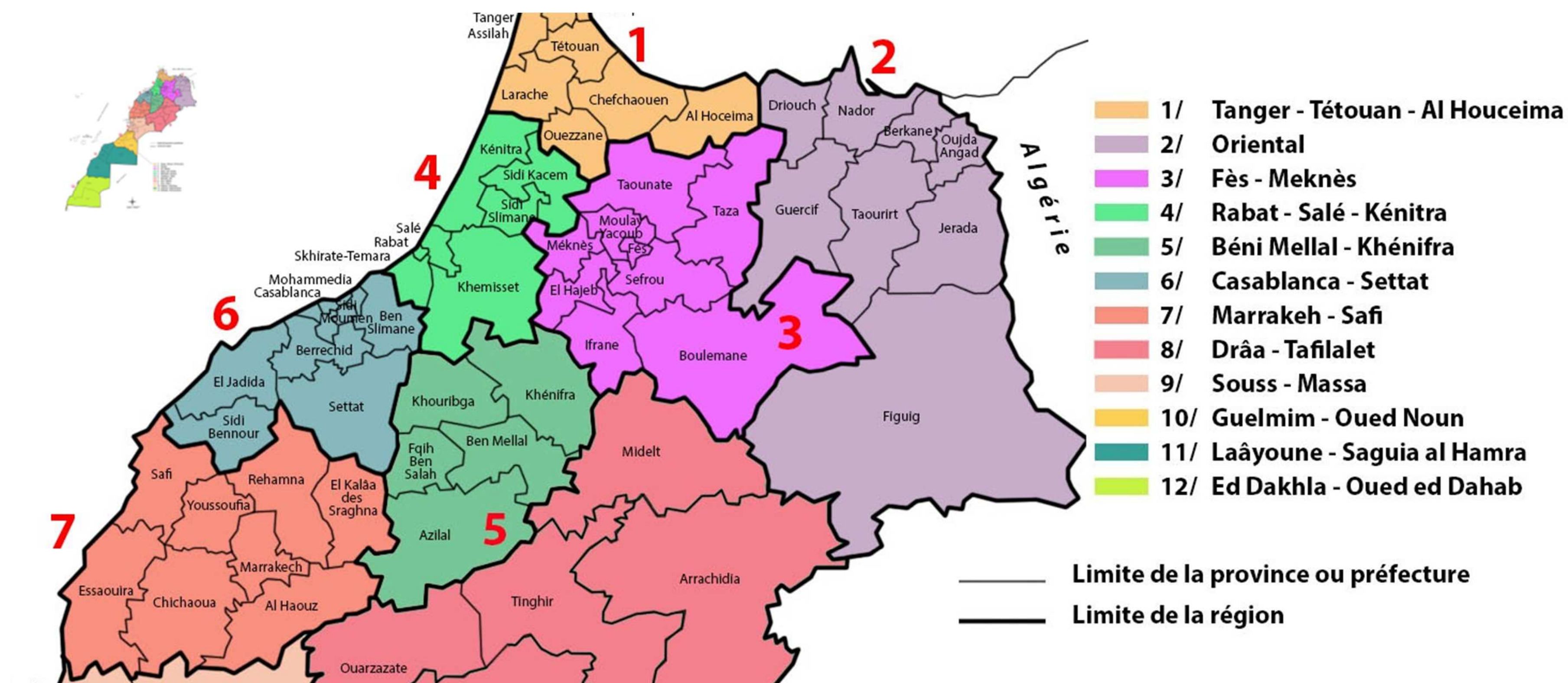



**Figure A.3.** Geographic origin of Moroccan lentil landraces used in chapter 3. Landraces from Chaouia (Region 6/ Casablanca-Settat) were from the provinces of Settat, Benslimane and Berrchid. Landraces from Abda (Region 7/ Marrakech-Safi) were from the provinces of Safi and Youssoufia. Landraces from Zaer (Region 4/ Rabat-Salé-Kénitra) were from the provinces of Khemisset and Sidi Slimane. Landraces from Saîs-Meknès (Region 3/ Fès-Meknès) were from the provinces of Meknès, Moulay Yacoub and Taounate. Landraces from highlands of middle Atlas mountains (Region 3/ Fès-Meknès) were from the provinces of Ifrane, Sefrou, Boulemane and Khénifra.

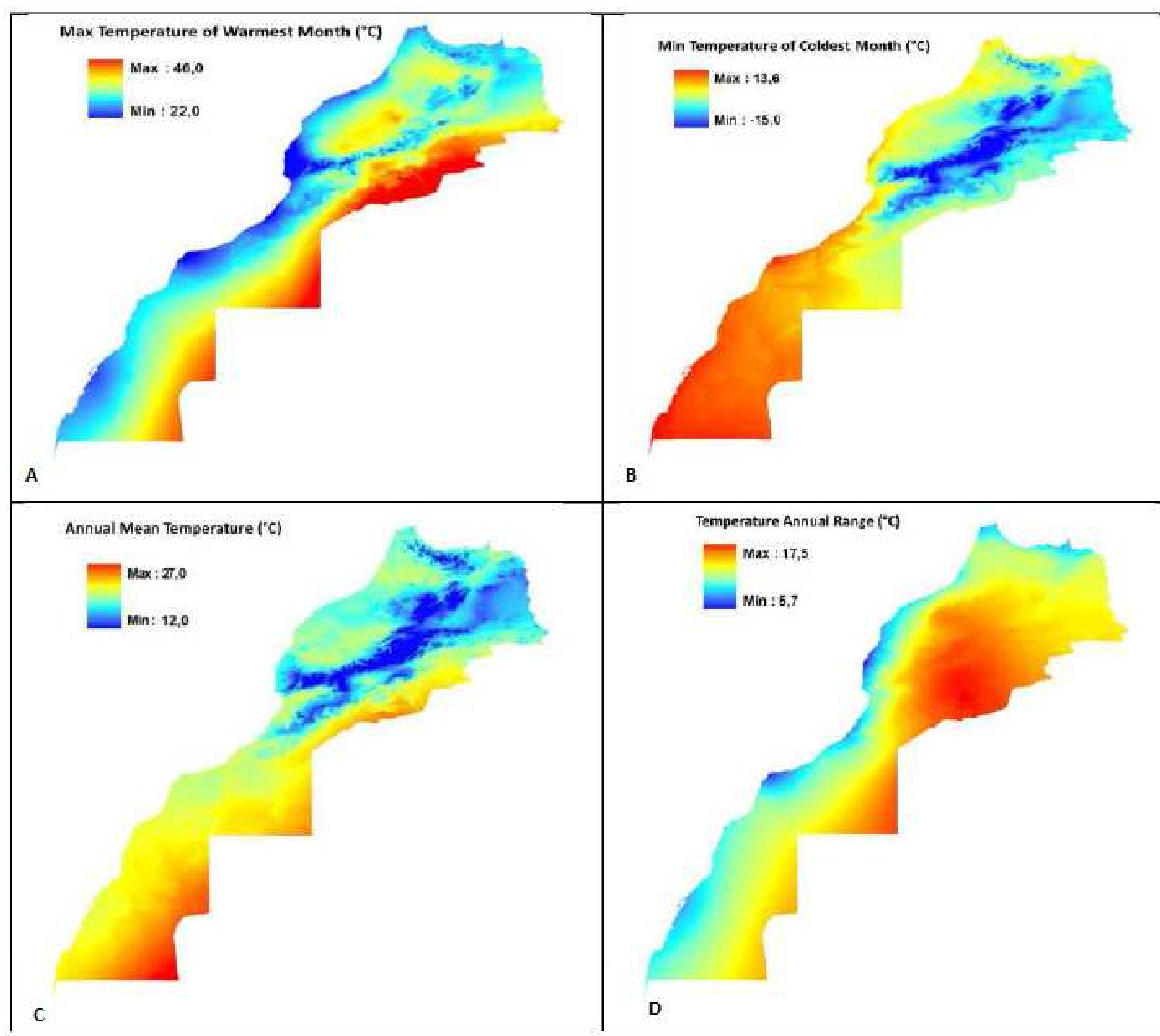



**Figure A.4.** Range of temperatures in Morocco showing high diversity for areas of origin of the lentil landraces included in chapter 3 (Balaghi *et al.* 2013 and related reference cited within).

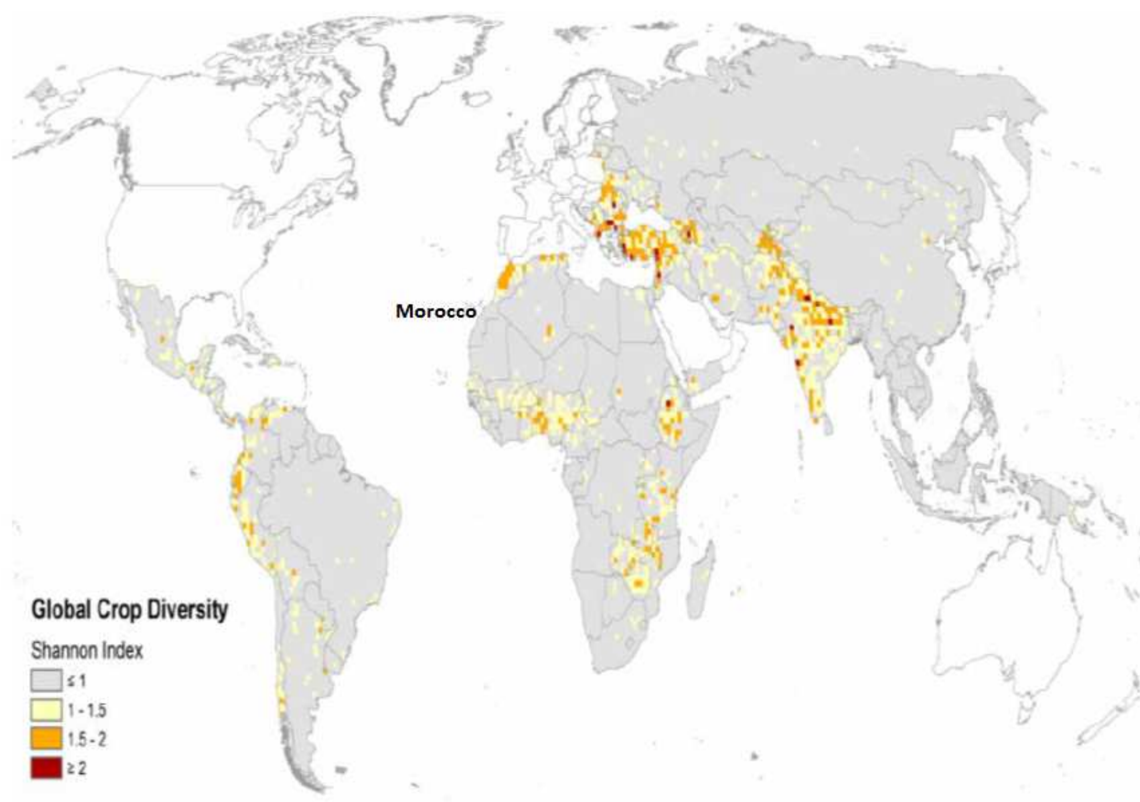



**Figure A.5.** Map of hotspots of crop diversity of the 35 food crops included in the International Treaty on Plant Genetic Resources for Food and Agriculture showing high level of diversity in the Mediterranean region (adapted from Bellon *et al.* 2014).

**Figure A.6.** Root systems at early seedling stage of ILL6002 (left) and ILL5888 (right) lentil lines, the parents of the RIL population used for root and shoot genetic diversity analysis and QTL mapping (chapters 4 and 5).

**Figure A.7.** Root systems at maturity of ILL6002 (left) and ILL5888 (right) lentil lines, the parents of the RIL population used for root and shoot genetic diversity analysis and QTL mapping (chapters 4 and 5).

**Figure A.8.** Roots and shoots of some lentil landraces at early seedling stage used chapter 3.

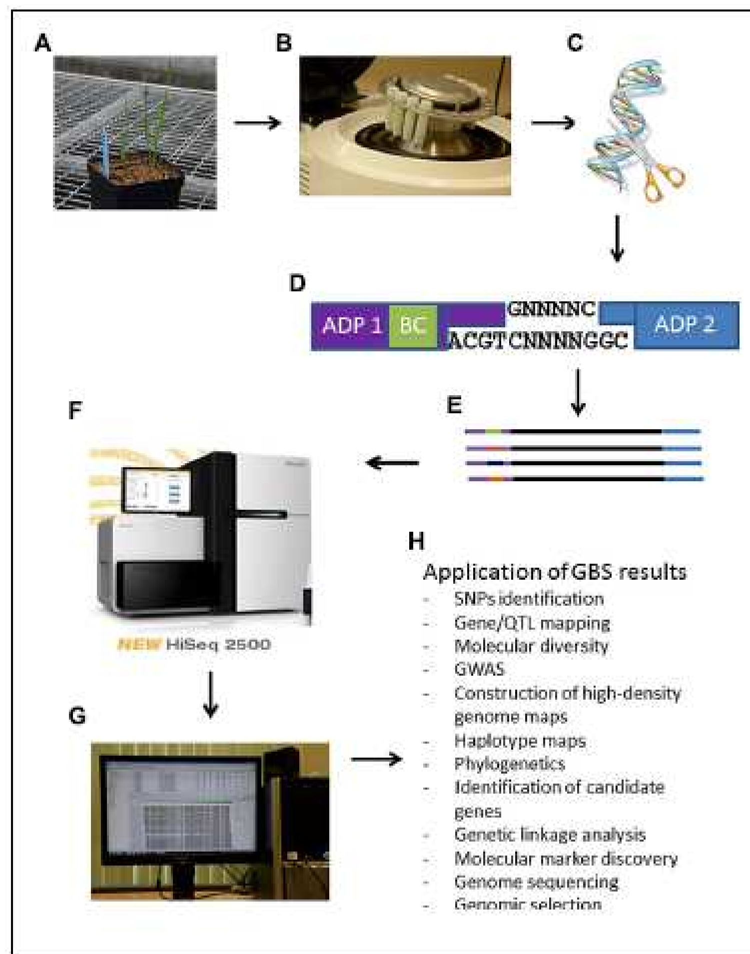
**Table A.1.** Forward and reverse primers of SSR markers used in developing genetic linkage map in chapter 5 (Saha *et al*. 2010)

| Marker names | Forward primer sequences | Reverse primer sequences |
|---|---|---|
| GLLC 106 | ACGACAATCCTCCACCTGAC | AACAAGGAAGGGGAGAGGAG |
| GLLC 108 | CGACAATCCTCCACCTGAC | ACAAGGAAGGGGAGAGGAAG |
| GLLC 511 | ATTGAGAGGAGGCGGAGAA | CGCGTGTCTCTCTCTCTCAC |
| GLLC 527 | GTGGGACGGTTTGAATTTGA | GAACATAAAATGGGAGTGTCACAA |
| GLLC 538 | AAGGGAAGGAAAAGGGAAGT | GCACGAAGAGGGTACGTAGG |
| GLLC 541 | TGGGCTCATTGAACCAAAAG | CCCCCTTTTAAGTGATTTTCC |
| GLLC 548 | CTGTTGTGGCCAAGAGGATT | CCAAAACCCCCACTACTTCA |
| GLLC 556 | TGCATTTGGATGAGAAAGGA | GGGTTACAAATAACCCGCTTG |
| GLLC 559 | CATGGATCCAAATGCAAAAA | GCTTCTTCAAGAGCACGTTTC |
| GLLC 562 | TGTGTAGGCACATCAACAAAA | GGTGGGCATGAGAGGTGTTA |
| GLLC 563 | ATGGGCTCATTGAACAAAAG | CCCCCTCTAAGAGATTTTCCTC |
| GLLC 587 | GTGGGACGGTTTGATTTTGA | GAACATAAAATGGGAGTGTCAAAA |
| GLLC 591 | TGTTTGATGTACCTCAGGCTTA | TGAACTGATGAGGAGGACGTT |
| GLLC 592 | GCGACATGGATCCTTACCC | TCATTCAAACAAAAACAAAACAAAA |
| GLLC 595 | TTGTCTGGTGGTGTTTTTGG | CACAAAGTTTCTCACCTCACG |
| GLLC 598 | TGGGCTCATTGAACCAAAAG | CCCCCTTCTAAGTGATTTTCC |
| GLLC 607 | AAGTTGTGGCCAAGAGGATT | CCAAAACCCCCACTACTTTA |
| GLLC 609 | GCGACATGGAATTGGATTTG | GCACAAAGTCGAGGAGCCTA |
| GLLC 614 | AACCCCAGCCAGATCTTACA | AAGGGTGGTTTTGGTCCTATG |

**Table A.2.** Forward and reverse primers of SRAP markers used in developing genetic linkage map in chapter 5 (Saha *et al*. 2010)

| Forward primer* | Forward primer sequences | Reverse primer* | Reverse primer sequences |
|---|---|---|---|
| ME1 | TGAGTCCAAACCGGATA | EM1 | GACTGCGTACGAATTAAT |
| ME2 | TGAGTCCAAACCGGAGC | EM2 | GACTGCGTACGAATTTGC |
| ME3 | TGAGTCCAAACCGGAAT | EM3 | GACTGCGTACGAATTGAC |
| ME4 | TGAGTCCAAACCGGACC | EM4 | GACTGCGTACGAATTTGA |
| ME5 | TGAGTCCAAACCGGAAG | EM5 | GACTGCGTACGAATTAAC |
| F6 | GTAGCACAAGCCGGATA | EM6 | GACTGCGTACGAATTGCA |
| F7 | FTAFCACAAGCCGGAGC | R7 | GACACCGTACGAATTTGC |
| F8 | GTAGCACAAGCCGGAAT | R8 | GACACCGTACGAATTGAC |
| F9 | GTAGCACAAGCCGGACC | R9 | GACACCGTACGAATTTGA |
| F10 | GTAGCACAAGCCGGAAA | R10 | GACACCGTACGAATTAAC |
| F11 | CGAATCTTAGCCAGATA | R12 | CCTTGCTACGCAATTGAC |
| F12 | CGAATCTTAGCCGGAGC | R13 | CCTTGCTACGCAATTAAC |
| F13 | CGAATCTTAGCCGGCAC | R14 | CGCACGTCCGTAATTAAC |
| F14 | GGAATCTTAGCCGGAAT | R15 | CGCACGTCCGTAATTCCA |
| F15 | GATCCAGTTACCGGATA | R16 | CGCACGTCCGTAATTTAC |
| F16 | GATCCAGTTACCGGAGC | | |
| F17 | GATCCAGTTACCGGCAC | | |
| F18 | GATCCAGTTACCGGAAT | | |

* All combinations of forward primers with reverse primers were used.




**Appendix A.1.** Main steps of Genotyping-By-Sequencing. (A): tissue is obtained from any plant species; (B): DNA extraction; (C): DNA digestion with restriction enzymes; (D): ligations of adaptors (ADP) including a bar coding (BC) region in adapter 1 in random *PstI-MseI* restricted DNA fragments; (E): representation of different amplified DNA fragments with different bar codes from different samples/lines. These fragments represent the GSB library; (F): analysis of sequences from library on a NGS sequencer; (G): bioinformatic analysis of NGS sequencing data; (H): Applications of GBS results. (source: He *et al.* 2014).

**Appendix A. 2. Description of GBS analysis steps followed for SNP genotyping (Poland *et al.* 2012):**

**1.Adapters:** 48 barcoded adapters with a *PstI* overhang were designed based on a custom script in Java (www.sourceforge.net/projects/tassel/). Three main criteria were considered: each barcode must be 2 or more bp different from all other barcodes, barcodes with a run of maximum 2 of the same nucleotide and barcodes cannot contain or recreate after ligation the *PstI* or *MspI* restriction site. Barcodes were designed to optimize the uniformity of each nucleotide at each position: barcodes had different lengths (4 bp to 9 bp) and balanced bases in the restriction site by selecting appropriate nucleotides. An example of a barcoded adapter pair (barcode sequence shown with capitol bases):

A01_AAGTGA_top

5′ – gatctacactctttccctacacgacgctcttccgatctAAGTGAtgca – 3′

A01_AAGTGA_bot

5′ – TCACTTagatcggaagagcgtcgtgtagggaaagagtgtagatc – 3′

The common adapter (Y-adapter) was designed in such way to prevent amplification of the more common *MspI*-*MspI* fragments and adapter-dimers formed by self-ligation of adapter fragments. During the PCR amplification the reverse primer is identical to the Y-tail of the common adapter and can only anneal if the complimentary strand has first been synthesized from the other end of the fragment containing Adapter 1 (Figure A.9).

Before use, the double-stranded adapter were formed by annealing in complimentary pairs standard oligos in a high-salt solution. PCR analysis for adapters annealing was performed first by heating at 95 °C for 1 minute followed by slowly cooling step of −1 °C/min to 30 °C for 65 cycles. After ligation, the adapters were quantified and uniform concentration of 0.1 uM of the adapters was used.

```
1) Ligation

   Forward Adapter    Barcode       Genomic DNA       Reverse Y-Adapter
5' CACGACGCTCTTCCGATCTXXXXXXTGCAGNNNN...NNNNCCGAGATCGGAAGAGCGGGGACTTTAAGC              3'
3' GTGCTGCGAGAAGGCTAGAYYYYYYTGCACNNNN...NNNNNNGGCTCTAGCCTTCTCGCCAAGTCGTCCTTACGGCTCTGGCTAG 5'


2) First PCR Cycle

    Forward Primer                                           PCR ⇒
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTXXXXXXACG
                                            3' GTGCTGCGAGAAGGCTAGAYYYYYYTGCACNNNN.....

                                       PCR ⇒
                     .....NNNNNCCGAGATCGGAA
                     .....NNNNNGGCTCTAGCCTTCTCGCCAAGTCGTCCTTACGGCTCTGGCTAG 5'


3) Second PCR Cycle

                  5' ....NNNNNCCGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATC   3'
                                            TCGAAGTCGTCCTTACGGCTCTGGCTAGAGCATACGGCAGAAGGACGAAC 5'
                                          ⇐ PCR                             Reverse Primer
```

**Figure A.9.** Adapter design, PCR amplification of fragments for GBS. 1- The ligation product of a genomic DNA fragment (black) with a *PstI* and a *MspI* restriction site. The forward adapter (blue) binds to a *PstI* generated overhang (corresponding 4–9 bp barcode in bold with ‘‘X’’). The *MspI* generated overhang corresponds to the reverse Y-adapter (green). The unpaired tail of the Y-adapter is underlined. 2- Annealing of only the forward primer (red) during the first round of PCR. PCR reaction of the complementary strand synthesizes the compliment of the Y-adapter tail in the end of the fragment. 3- Annealing of the reverse primer (orange) to the newly synthesized compliment of the Y-adapter tail during the second round of PCR. Then on the other end of the same fragment, this PCR synthesizes the compliment of the forward adapter/primer (Poland *et al.* 2012).

**2.Restriction digest:** 200 ng of genomic DNA of each sample was digested in 20 ul reaction volume of NEB Buffer 4 with 8 U of *HF-PstI* and 8 U of *MspI* at 37 °C for 2 h. Enzymes were then inactivated at 65 °C for 20 min.

**3.Ligation:** the ligation reaction was carried out at 22 °C for 2 h. 0.1 pmol of the respective Adapter 1 (0.1 pmol for 200 ng of genomic DNA) and 15 pmol of the common Y-adapter were added to the restriction digest samples. To each sample, a master mix of NEB Buffer 4 (1× final), ATP (1 mM final), and 200 U T4 ligase (NEB T4 DNA Ligase #M0202) was added. After ligation, the ligase was inactivated at 65 °C for 20 min.

**4.Multiplexing and amplification:** single library from 48 samples was obtained from ligated samples after PCR amplification (18 cycles of 95 °C for 30 s, 62 °C for 30 s and 68 °C for 30 s) in a single tube. The library was sequenced on a single lane of Illumina GAII or HiSeq2000.

**5.Sequencing:** to construct full libraries, the two parents and some RIL lines were replicated. The libraries were each sequenced on two lanes of Illumina HiSeq2000.

**6.Processing of Illumina raw data:** the barcode sequences were used to assign sequences to individuals from the Illumina data trimmed to 64 bp using a custom script in Java (www.maizegenetics.net, www.sourceforge.net/projects/tassel/). In order to check the quality of generated data, only sequences that had an exact match to a barcode followed by the expected

sequence of 5 nucleotides remaining from a *PstI* cut-site were kept. All reads were then examined for unique tags present in more than five different lines. A matrix of presence/absence for each sample was generated then from tags. This matrix was used for SNP calling.

**Table A.3.** SNP markers and their respective alleles obtained from GBS used for genetic map construction and QTL mapping (chapter 5)

| SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles |
|---|---|---|---|---|---|---|---|
| TP14 | C/G | TP372 | A/G | TP776 | A/C | TP1204 | C/G |
| TP16 | A/G | TP398 | C/T | TP790 | C/G | TP1227 | C/T |
| TP18 | G/T | TP401 | A/G | TP796 | A/C | TP1229 | C/T |
| TP31 | A/C | TP412 | A/G | TP804 | C/T | TP1241 | A/G |
| TP36 | A/G | TP421 | C/G | TP811 | A/C | TP1256 | C/T |
| TP37 | A/G | TP429 | A/C | TP813 | A/G | TP1278 | A/C |
| TP52 | A/G | TP430 | A/C | TP814 | A/G | TP1280 | G/T |
| TP70 | A/T | TP458 | C/G | TP816 | A/C | TP1313 | A/G |
| TP72 | C/T | TP478 | A/G | TP818 | C/T | TP1331 | A/T |
| TP80 | A/T | TP481 | C/T | TP828 | A/G | TP1336 | A/G |
| TP81 | A/G | TP498 | C/T | TP834 | C/T | TP1347 | A/T |
| TP84 | C/T | TP518 | A/G | TP870 | A/G | TP1357 | A/T |
| TP85 | A/G | TP525 | C/T | TP878 | A/G | TP1360 | A/G |
| TP86 | A/G | TP527 | C/T | TP894 | A/G | TP1361 | G/A |
| TP93 | T/C | TP540 | A/T | TP899 | A/T | TP1371 | A/G |
| TP100 | A/C | TP543 | A/G | TP913 | A/C | TP1373 | A/G |
| TP145 | G/A | TP579 | A/T | TP926 | C/T | TP1376 | A/C |
| TP163 | A/C | TP585 | A/T | TP953 | A/T | TP1386 | C/T |
| TP164 | A/C | TP607 | A/C | TP957 | A/T | TP1399 | A/G |
| TP177 | A/G | TP611 | A/C | TP984 | A/G | TP1407 | C/T |
| TP194 | C/G | TP621 | A/G | TP1004 | C/T | TP1410 | C/T |
| TP200 | A/T | TP635 | C/T | TP1013 | A/T | TP1415 | G/T |
| TP202 | A/G | TP639 | A/G | TP1034 | C/T | TP1416 | A/T |
| TP205 | C/T | TP640 | A/G | TP1035 | A/T | TP1419 | A/G |
| TP209 | C/T | TP657 | C/G | TP1037 | C/G | TP1420 | G/T |
| TP212 | A/G | TP663 | G/T | TP1038 | A/G | TP1426 | G/T |
| TP217 | C/T | TP682 | A/T | TP1045 | A/G | TP1427 | C/T |
| TP226 | C/T | TP699 | A/T | TP1057 | A/T | TP1431 | A/C |
| TP229 | C/T | TP701 | A/G | TP1058 | A/G | TP1446 | A/C |
| TP242 | C/T | TP709 | G/T | TP1069 | C/T | TP1473 | C/T |
| TP257 | A/G | TP716 | A/T | TP1094 | A/G | TP1480 | A/G |
| TP273 | A/G | TP725 | A/G | TP1099 | A/G | TP1484 | A/G |
| TP276 | C/T | TP727 | C/T | TP1100 | A/C | TP1501 | C/T |
| TP288 | A/G | TP741 | A/G | TP1107 | C/T | TP1508 | C/T |
| TP291 | A/G | TP743 | C/T | TP1119 | A/G | TP1519 | C/T |
| TP292 | C/T | TP747 | C/G | TP1123 | A/G | TP1545 | G/T |
| TP299 | C/T | TP750 | C/G | TP1142 | G/T | TP1548 | A/C |
| TP306 | A/G | TP752 | A/C | TP1152 | C/T | TP1553 | A/T |
| TP318 | C/T | TP753 | A/C | TP1160 | G/T | TP1558 | G/T |
| TP332 | G/T | TP760 | C/T | TP1168 | C/T | TP1561 | G/T |
| TP335 | C/T | TP762 | C/T | TP1170 | C/G | TP1576 | A/G |

**Table A.3.** Continued

| SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles |
|---|---|---|---|---|---|---|---|
| TP343 | A/C | TP772 | C/G | TP1180 | A/G | TP1598 | A/G |
| TP1625 | C/T | TP2078 | A/G | TP2556 | T/C | TP3160 | C/T |
| TP1628 | A/G | TP2086 | C/T | TP2561 | A/G | TP3162 | A/T |
| TP1633 | C/T | TP2119 | C/T | TP2563 | G/T | TP3170 | C/T |
| TP1649 | A/G | TP2121 | A/G | TP2580 | C/G | TP3209 | C/T |
| TP1655 | C/T | TP2122 | C/T | TP2581 | A/C | TP3238 | A/G |
| TP1656 | A/G | TP2132 | G/T | TP2586 | A/G | TP3266 | A/G |
| TP1665 | G/A | TP2151 | A/G | TP2589 | A/G | TP3270 | A/C |
| TP1667 | G/T | TP2155 | C/T | TP2604 | A/C | TP3272 | A/G |
| TP1668 | A/G | TP2161 | G/T | TP2642 | G/T | TP3310 | C/G |
| TP1678 | C/T | TP2190 | A/C | TP2646 | C/T | TP3313 | C/T |
| TP1683 | A/G | TP2193 | C/T | TP2649 | C/T | TP3321 | A/T |
| TP1687 | A/G | TP2217 | G/T | TP2665 | A/G | TP3323 | C/T |
| TP1690 | A/G | TP2227 | C/G | TP2676 | A/G | TP3339 | A/T |
| TP1715 | G/T | TP2232 | C/G | TP2680 | C/T | TP3362 | A/G |
| TP1722 | C/T | TP2238 | A/G | TP2688 | C/G | TP3364 | A/G |
| TP1763 | A/G | TP2241 | A/C | TP2701 | C/T | TP3369 | C/T |
| TP1772 | A/T | TP2255 | C/T | TP2703 | C/T | TP3371 | C/T |
| TP1786 | A/G | TP2276 | A/G | TP2723 | A/G | TP3380 | C/T |
| TP1791 | A/G | TP2293 | A/G | TP2728 | A/G | TP3392 | A/T |
| TP1804 | C/G | TP2309 | A/T | TP2767 | A/G | TP3401 | A/T |
| TP1807 | C/T | TP2312 | A/G | TP2768 | C/T | TP3448 | A/T |
| TP1811 | C/T | TP2340 | A/G | TP2785 | A/G | TP3494 | A/C |
| TP1836 | A/G | TP2344 | C/T | TP2799 | C/T | TP3496 | C/T |
| TP1863 | A/G | TP2386 | C/T | TP2804 | C/T | TP3518 | C/T |
| TP1873 | A/C | TP2390 | C/T | TP2870 | A/C | TP3536 | A/G |
| TP1878 | A/T | TP2403 | C/T | TP2871 | C/T | TP3538 | C/T |
| TP1883 | A/T | TP2414 | A/T | TP2905 | A/T | TP3541 | A/G |
| TP1891 | C/T | TP2415 | A/T | TP2951 | G/T | TP3585 | C/T |
| TP1894 | C/T | TP2445 | A/G | TP2982 | C/T | TP3598 | C/T |
| TP1927 | C/G | TP2467 | A/T | TP2986 | G/T | TP3601 | A/C |
| TP1936 | C/T | TP2470 | C/T | TP2991 | C/G | TP3611 | A/C |
| TP1951 | A/G | TP2478 | A/G | TP3028 | C/G | TP3645 | C/T |
| TP1954 | A/T | TP2484 | A/G | TP3029 | A/G | TP3648 | C/G |
| TP1956 | A/G | TP2492 | A/G | TP3034 | A/G | TP3649 | C/T |
| TP1957 | C/T | TP2496 | A/G | TP3050 | C/T | TP3675 | A/C |
| TP1979 | A/G | TP2501 | A/C | TP3092 | A/G | TP3677 | A/C |
| TP1992 | A/T | TP2502 | C/T | TP3113 | A/G | TP3681 | C/T |
| TP2001 | C/T | TP2503 | C/T | TP3121 | C/T | TP3690 | A/G |
| TP2002 | A/G | TP2505 | C/T | TP3127 | A/G | TP3692 | A/G |

**Table A.3.** Continued

| SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles |
|---|---|---|---|---|---|---|---|
| TP2023 | A/G | TP2512 | A/C | TP3139 | C/T | TP3697 | A/G |
| TP2029 | A/G | TP2524 | A/G | TP3142 | A/G | TP3704 | C/T |
| TP2033 | G/T | TP2526 | A/C | TP3147 | C/T | TP3709 | A/C |
| TP2070 | C/T | TP2534 | G/T | TP3153 | G/T | TP3710 | A/C |
| TP2076 | A/G | TP2550 | A/G | TP5093 | C/T | TP5614 | A/G |
| TP3713 | C/T | TP4560 | C/T | TP5099 | A/C | TP5628 | A/C |
| TP3719 | C/T | TP4563 | C/G | TP5116 | C/T | TP5638 | A/G |
| TP3722 | A/G | TP4567 | A/T | TP5119 | C/T | TP5642 | A/T |
| TP3757 | C/G | TP4571 | A/T | TP5139 | A/T | TP5644 | A/T |
| TP3779 | C/T | TP4579 | A/C | TP5153 | A/C | TP5656 | C/G |
| TP3791 | G/T | TP4658 | C/T | TP5161 | A/G | TP5661 | A/G |
| TP3805 | C/T | TP4660 | C/T | TP5176 | A/G | TP5687 | A/C |
| TP3815 | A/G | TP4683 | A/C | TP5189 | G/T | TP5704 | A/G |
| TP3825 | A/G | TP4705 | A/T | TP5199 | C/T | TP5713 | A/C |
| TP3866 | G/T | TP4709 | A/G | TP5215 | A/G | TP5719 | A/C |
| TP3875 | A/G | TP4712 | A/G | TP5227 | C/G | TP5772 | G/T |
| TP3976 | C/T | TP4714 | C/G | TP5265 | A/T | TP5773 | G/T |
| TP3985 | T/A | TP4742 | A/G | TP5268 | C/T | TP5774 | G/T |
| TP4051 | G/T | TP4756 | A/G | TP5269 | C/T | TP5779 | A/T |
| TP4055 | A/G | TP4761 | C/T | TP5272 | A/G | TP5812 | C/T |
| TP4087 | C/T | TP4763 | G/T | TP5343 | C/T | TP5852 | C/T |
| TP4098 | A/G | TP4778 | A/C | TP5366 | C/T | TP5856 | A/T |
| TP4104 | G/T | TP4797 | A/G | TP5368 | A/G | TP5857 | A/T |
| TP4120 | C/T | TP4811 | C/T | TP5384 | G/T | TP5871 | C/T |
| TP4123 | C/T | TP4845 | G/A | TP5391 | A/G | TP5887 | A/T |
| TP4128 | G/T | TP4846 | A/C | TP5401 | C/T | TP5899 | C/T |
| TP4134 | A/C | TP4850 | A/G | TP5406 | C/G | TP5900 | A/G |
| TP4142 | C/T | TP4853 | A/C | TP5407 | A/G | TP5903 | A/G |
| TP4146 | G/T | TP4872 | G/T | TP5420 | C/T | TP5939 | A/G |
| TP4165 | A/C | TP4873 | A/G | TP5421 | A/T | TP5943 | A/G |
| TP4186 | C/T | TP4878 | A/T | TP5431 | C/T | TP5949 | A/T |
| TP4194 | C/G | TP4883 | A/C | TP5434 | A/G | TP5953 | C/G |
| TP4247 | A/G | TP4890 | G/T | TP5439 | A/G | TP5957 | A/T |
| TP4268 | C/T | TP4912 | A/T | TP5442 | C/T | TP5962 | A/G |
| TP4276 | G/T | TP4941 | A/G | TP5443 | C/T | TP6003 | C/T |
| TP4293 | G/T | TP4967 | C/T | TP5449 | C/T | TP6018 | A/T |
| TP4295 | C/T | TP4968 | A/G | TP5481 | C/T | TP6033 | A/G |
| TP4311 | A/C | TP4975 | A/G | TP5491 | C/T | TP6050 | A/G |
| TP4321 | G/T | TP4976 | C/T | TP5502 | C/T | TP6063 | C/T |
| TP4336 | A/G | TP5004 | C/T | TP5523 | C/G | TP6072 | A/G |
| TP4344 | C/T | TP5005 | C/T | TP5546 | C/T | TP6088 | A/G |

**Table A.3.** Continued

| SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles |
|---|---|---|---|---|---|---|---|
| TP4368 | A/T | TP5006 | C/G | TP5554 | C/T | TP6096 | C/T |
| TP4391 | C/T | TP5053 | A/C | TP5555 | G/T | TP6108 | C/T |
| TP4402 | C/G | TP5071 | A/G | TP5575 | C/T | TP6121 | A/G |
| TP4463 | A/G | TP5084 | C/T | TP5585 | C/T | TP6151 | A/G |
| TP4495 | A/T | TP5085 | C/T | TP5607 | G/T | TP6181 | A/T |
| TP4517 | C/G | TP5089 | C/T | TP5613 | A/T | TP6183 | A/T |
| TP4556 | A/G | TP5092 | A/G | TP6985 | C/T | TP7423 | C/T |
| TP6185 | C/T | TP6603 | A/T | TP7006 | C/T | TP7428 | C/T |
| TP6194 | A/T | TP6607 | G/T | TP7011 | C/T | TP7439 | G/T |
| TP6197 | C/T | TP6611 | C/T | TP7012 | C/T | TP7460 | G/T |
| TP6212 | A/C | TP6624 | A/G | TP7015 | A/G | TP7481 | G/T |
| TP6216 | C/T | TP6631 | C/G | TP7020 | A/G | TP7484 | C/T |
| TP6248 | C/T | TP6638 | A/C | TP7028 | C/T | TP7487 | A/T |
| TP6265 | A/G | TP6642 | A/G | TP7032 | A/G | TP7488 | A/G |
| TP6270 | C/T | TP6646 | C/T | TP7053 | A/C | TP7497 | C/G |
| TP6290 | G/T | TP6653 | A/G | TP7057 | C/T | TP7505 | A/G |
| TP6311 | G/T | TP6665 | A/G | TP7068 | C/T | TP7507 | C/G |
| TP6315 | C/T | TP6673 | A/G | TP7077 | A/G | TP7509 | C/G |
| TP6326 | C/T | TP6674 | C/T | TP7086 | C/T | TP7522 | C/T |
| TP6335 | A/G | TP6690 | A/C | TP7116 | G/T | TP7532 | C/T |
| TP6337 | C/T | TP6691 | A/G | TP7154 | C/T | TP7540 | A/G |
| TP6340 | A/G | TP6692 | A/C | TP7170 | A/G | TP7546 | A/T |
| TP6354 | C/T | TP6694 | A/T | TP7196 | C/T | TP7555 | C/T |
| TP6373 | C/T | TP6703 | A/T | TP7203 | C/G | TP7570 | C/T |
| TP6379 | C/G | TP6714 | C/T | TP7210 | A/T | TP7571 | A/G |
| TP6381 | C/T | TP6718 | C/T | TP7211 | G/T | TP7572 | A/T |
| TP6384 | C/T | TP6755 | C/T | TP7236 | A/T | TP7602 | C/T |
| TP6390 | G/T | TP6757 | A/G | TP7239 | C/T | TP7630 | C/T |
| TP6430 | C/G | TP6777 | A/G | TP7242 | A/G | TP7669 | C/T |
| TP6431 | A/C | TP6782 | A/G | TP7245 | C/G | TP7688 | C/T |
| TP6440 | A/G | TP6783 | A/T | TP7248 | C/G | TP7694 | G/T |
| TP6445 | A/G | TP6788 | A/G | TP7252 | A/G | TP7737 | C/G |
| TP6469 | A/G | TP6789 | A/G | TP7268 | A/C | TP7738 | C/G |
| TP6483 | C/T | TP6790 | C/G | TP7272 | C/T | TP7741 | C/T |
| TP6484 | A/T | TP6809 | A/T | TP7283 | A/G | TP7747 | G/T |
| TP6490 | C/T | TP6823 | G/T | TP7288 | G/A | TP7835 | A/G |
| TP6501 | G/T | TP6834 | C/T | TP7293 | C/T | TP7840 | G/A |
| TP6544 | G/A | TP6850 | A/G | TP7297 | C/T | TP7853 | A/C |
| TP6545 | C/G | TP6854 | C/T | TP7306 | A/G | TP7861 | A/G |
| TP6546 | C/T | TP6864 | C/T | TP7328 | A/G | TP7873 | C/T |
| TP6551 | A/C | TP6878 | A/G | TP7331 | C/T | TP7875 | A/G |

**Table A.3.** Continued

| SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles | SNP markers | Alleles |
|---|---|---|---|---|---|---|---|
| TP6557 | G/T | TP6891 | A/G | TP7355 | C/T | TP7876 | A/C |
| TP6561 | C/T | TP6892 | C/T | TP7360 | G/T | TP7877 | A/C |
| TP6562 | C/T | TP6931 | A/G | TP7362 | A/C | TP7905 | C/T |
| TP6569 | C/T | TP6935 | A/G | TP7391 | A/G | TP7916 | C/T |
| TP6577 | C/T | TP6939 | C/T | TP7395 | A/T | TP7917 | C/T |
| TP6583 | A/G | TP6940 | C/T | TP7411 | C/T | TP7924 | A/G |
| TP6590 | C/T | TP6949 | C/G | TP7413 | A/C | TP6597 | C/G |
| TP6591 | A/T | TP6969 | A/G | TP7419 | A/T | TP6982 | C/T |

# Curriculum Vitae

## Personal information

First name: Omar

Family name: Idrissi

Nationality: Morocco

Date of birth: 22/06/1978

Email: Omar.Idrissi@UGent.be; o.idrissi@yahoo.fr

Marital status: Married, 2 children

## Education

- Lycée Zaid Ouhmad, Tinghir, Morocco:
  1997: Baccalauréat Sciences Expérimentales.
- Ecole Nationale d'Agriculture de Meknès, Morocco:
  2003: Ingénieur D'Etat en Agronomie, Département de production végétale et amélioration des plantes.
- Faculté Universitaire des Sciences Agronomique de Gembloux, Belgium:
  2004: Diplôme des Etudes Spécialisées en Génie Sanitaire.
- Ghent University, Faculty of Bioscience Engineering, Ghent, Belgium:
  2012-2016: PhD candidate.

## Professional Experience

2006-present: Researcher at Institut National de la Recherche Agronomique du Maroc, Centre Régional de Settat, Unité de Recherche d'Amélioration génétique et biotechnologies.

## Language Skills

- French: Fluency in speaking, reading and writing.
- English: Satisfactory in speaking, fluency in reading and writing.
- Arabic: Fluency in speaking, reading and writing.

## Conferences and Seminars attended

- 2016 International Conference on Pulses for Health, Nutrition and Sustainable Agriculture in Drylands, Marrakech, Morocco, April 18-20, 2016.
- 20$^{th}$ National Symposium of Applied Biological Sciences (NSABS-2015), Louvain-La-Neuve, Belgium, 30 January 2015.
- 6$^{th}$ International Food Legumes Research Conference (IFLRC VI) and 7$^{th}$ International Conference on Legume Genetics and Genomics (ICLGG VII), Saskatoon, Canada, 6-11 July 2014.
- 3$^{rd}$ International Ascochyta Workshop - Ascochyta 2012, Cordoba, Spain, 2012.
- 5$^{th}$ International Food Legumes Research Conference and 7$^{th}$ European Conference on Grain Legumes, 26-30April, 2010, Antalya, Turkey.

- $5^{èmes}$ journées de la Biodiversité, Faculté des Sciences et Techniques de Fès, Fès, Morocco, 18- 20 Décembre 2008.
- Congrès National pour l'Amélioration de la Production Agricole, Faculté des Sciences et Techniques de Settat & Centre Régional de la Recherche Agronomique de Settat, Settat, Morocco, 03-04 Avril 2007. Membre du comité d'organisation.

## Publications and communications

### A1 articles

- **Idrissi, O. et al. (2016, expected).** Identification and mapping of QTLs related to root and shoots traits conferring drought tolerance in lentil. **Submitted**, **under review.**
- **Idrissi, O., Udupa, S.M., De Keyser, E., Van Damme, P. and De Riek, J. (2015).** Functional genetic diversity analysis and identification of associated simple sequence repeats and amplified fragment length polymorphism markers to drought tolerance in lentil (*Lens culinaris* ssp. *culinaris* Medicus) landraces. **Plant Molecular Biology Reporter 10/2015; Doi: 10.1007/s11105-015-0940-4.**
- **Idrissi, O., Udupa, S.M., Houasli, Ch., De Keyser, E., Van Damme, P. and De Riek, J. (2015).** Genetic diversity analysis of Moroccan lentil (*Lens culinaris* Medik.) landraces using Simple Sequence Repeat and Amplified Fragment Length Polymorphisms reveals functional adaptation towards agro-environmental origins. **Plant Breeding, 134, 322-332. Doi: 10.1111/pbr.12261.**
- **Idrissi, O., Houasli, Ch., Udupa, S.M., De Keyser, E., Van Damme, P. and De Riek, J. (2015).** Genetic variability for root and shoot traits in a lentil (*Lens culinaris* Medik.) recombinant inbred line population and their association with drought tolerance. **Euphytica, 204 (3), 693-709. Doi: 10.1007/s10681-015-1373-8.**
- **Idrissi, O., Sakr, B., Dahan, R., Houasli, Ch., Nsarellah, N., Udupa, S.M., Sarker, A. (2012).** Registration of 'Chakkouf' lentil in Morocco. **Journal of Plant Registrations, 6, 268–272. Doi: 10.3198/jpr2011.07.0384crc.**
- **Idrissi, O., Houasli, Ch., Fatmi, Z.E.A., Krimi Benchekroun, S., Agrawal, S.K., Imtiaz, M., Maalouf, F. (2013).** Status and prospects of food legumes improvement in Morocco. **Phytopathologia Mediterranea, 52(1), 237.**
- **Thavarajah, D., Thavarajah, P., Sarker, A., Materne, M., Vandemark, G., Shrestha, R., Idrissi, O., Hacikamiloglu, O., Bucak, B., Vandenberg, A. (2011)**. A global survey of effects of genotype and environment on selenium concentration in lentils (*Lens culinaris* L.): Implications for nutritional fortification strategies. **Food Chemistry, 125(1), 72-76. Doi: 10.1016/j.foodchem. 2010.08.038.**

**Other articles and communications**

- **Omar Idrissi, Sripada M. Udupa, Ellen De Keyser, Patrick Van Damme and Jan De Riek. (2016).** Use of DNA markers in Moroccan lentil breeding program: molecular diversity and mapping towards enhanced valorization of genetic resources and marker-assisted selection for efficient cultivar development. **International Conference on Pulses for Health, Nutrition and Sustainable Agriculture in Drylands, 18-20 April 2016, Abstracts. p.112-112, Marrakech, Morocco.**
- **Omar Idrissi, Sripada M. Udupa, Ellen De Keyser, Patrick Van Damme and Jan De Riek. (2015).** Screening for drought tolerance and genetic diversity analysis in landraces of lentil. **Recent progress in drought tolerance: from genetics to modelling: conference handbook. p.118-118. Montpellier, France; 06/2015.**
- **Omar Idrissi, Ellen De Keyser, Patrick Van Damme and Jan De Riek. (2015).** Genetic structure of Moroccan lentil landraces from different origins as revealed by Short Sequence Repeat molecular markers using the software STRUCTURE. **Applied Biological Sciences, 20$^{th}$ National symposium, Abstracts. p.64-64, Louvain-la-Neuve, Belgium.**
- **Omar Idrissi, Chafika Houasli, Ellen De Keyser, Patrick Van Damme and Jan De Riek. (2014).** Genetic variation of root and shoot traits at early stage in a lentil (*Lens culinaris* Medik.) recombinant inbred lines (RILs) population under drought: towards Quantitative Trait Loci (QTL) mapping**. IFLRC VI & ICLGG VII, Abstracts, Saskatoon, Canada.**
- **Omar Idrissi, Chafika Houasli, Ellen De Keyser, Patrick Van Damme and Jan De Riek. (2014).** Assessment of genetic variation of lentil (*Lens culinaris* Medik.) landraces from Morocco. **IFLRC VI & ICLGG VII, Abstracts, Saskatoon, Canada.**
- **Omar Idrissi, Chafika Houasli and Nasserlhaq Nsarellah. (2013).** Comparaison de lignées avancées de lentille sous stress hydrique durant la phase de floraison et formation des gousses. **Nature & Technologie, 8, 53-61**.
- **Idrissi Omar, Houasli Chafika, Nsarellah Naserelhaq, Agrawal Shiv Kumar (2011).** Intégration de la sélection pour la qualité nutritive dans le programme d'amélioration génétique de la lentille : cas des concentrations en fer et en zinc. Poster, **Congrès International pour l'Amélioration de la Production Agricole, Faculté des Sciences et Techniques de Settat, Maroc. 17-18 Mars 2011.**
- **Houasli Chafika, Idrissi Omar, Nsarellah Naserelhaq (2011).** Evaluation et sélection de génotypes de pois chiche pour la tolérance à la sécheresse. Poster, **Congrès International pour l'Amélioration de la Production Agricole, Faculté des Sciences et Techniques de Settat, Maroc. 17-18 Mars 2011.**
- **Idrissi Omar, Nsarellah Nsarelhaq, Houasli Chafika, Sarker Ashutosh (2010).** Comparison of some lentil advanced genotypes under water deficit during flowering and pod development time. Poster, ***5$^{th}$* International Food Legumes Research Conference and 7$^{th}$ European Conference on Grain Legumes April 26 to 30, 2010, Turkey**.

- **Ashutosh Sarker, Renuka Shrestha, M.J Uddin, Rajib Podder, Omar Idrissi (2009).** Genetic variation and enhancement for nutritional quality in lentil. Poster, **International Annual Meetings in Sustainability through Plant and Soil Sciences. November 1-5, 2009, Pissburgh PA, USA.**
- **Omar Idrissi (2008)**. Classification de variétés et de lignées avancées de lentille selon leur adaptation au milieu semi-aride. Poster, ***5èmes*** **Journées Nationales de la Biodiversité au Maroc, FST de Fès, 18-20 Décembre 08.**
- **Omar Idrissi et Nsarelhaq Nsarellah (2007)**. Effet du déficit hydrique sous serre lors de la phase de reproduction sur des lignées avancées de lentille, comparaison et sélection. Poster, **Congrès National pour l'Amélioration de la Production Agricole, FST de Settat, 03-04 Avril 2007**.