Functional genetic diversity analysis and identification of associated SSRs and AFLPs markers to drought tolerance in Lentils landraces

Omar Idrissi, Sripada M. Udupa, Ellen De Keyser, Patrick Van Damme and Jan De Riek

Abstract

Genetic diversity of 70 Mediterranean lentil landraces was assessed using simple sequence repeat (SSR) and amplified fragment length polymorphisms (AFLP). Their variation for root and shoot traits and drought tolerance was evaluated using relative water content, water losing rate and wilting score. High level of genetic variation and clear differentiation of landraces from Morocco from those from northern Mediterranean originating from Italy, Turkey and Greece were found according to both SSR and AFLP techniques. High genetic variation for root and shoot traits as well as for drought tolerance was obtained. No correlation of drought response with landraces origin. Landraces with higher dry root biomass, chlorophyll content and root-shoot ratio were drought tolerant with higher relative water content and lower water losing rate and wilting severity. Kruskal-Wallis non-parametric test (K-W) was used to find associated SSRs and AFLPs to RWC, WLR and WS. Regression analysis showed six SSRs and AFLPs alleles explaining the highest phenotypic variation of RWC, WLR and WS. Functional genetic diversity based on drought response of landraces as estimated by RWC, WLR and WS was shown using SSRs and AFLPs alleles linked to these parameters according to K-W using canonical discriminant analysis. This highlighted the feasibility of association mapping studies aiming to find associated DNA markers with drought tolerance in larger number of lentil landraces.

Introduction

Lentil (Lens culinaris ssp. culinaris Medicus) is an annual grain legume wildly cultivated in North Middle East, North Africa, the Indian subcontinent, North America and Australia for its protein, minerals (Fe, Zn,…) and vitamins-rich seeds and valued straw for animal feed (Bhatty, 1988; Erskine et al. 1990; Grusak, 2009; Erskine et al. 2011). Lentil has other agronomic benefits thanks to its ability to fix atmospheric nitrogen in soils and as an important rotation component mainly in cereal based cropping systems, thus enhancing soil fertility and sustainability in these farming systems. Lentil annual average global production is 4.55 million tons (Mt) from 4.2 million hectares (FAOSTAT 2012).

Domestication event is thought to have taken place back to around 7000 BC in the foothills of the mountains between Turkey and Syria in the Eastern Mediterranean (Ladizinsky 1979; Ladizinsky 1987). Lentil was then spread to Greece, Central Europe, Egypt, Central Asia, India and South America. Lentil reached North Africa as well as Spain and the Italian islands of Sardinia and Sicily likely from either Central Europe or from the Levant (Sonnante and Pignone 2001, Faratini et al. 2011). Lentil have been introduced in the New world (North America and Australia) more recently (Ferguson[12] and W. Erskine, 2001).

Drought is one of the most challenging abiotic stresses causing important yield loses mainly in arid and semi-arid areas limiting the benefits of farmers. Breeding for this traits is considered as a major objective in these areas. With the global warming in the context of climate change, drought episodes are expected to worsen and to be more frequent. Thus, improving plant tolerance and adaptation to water limited availability to maintain growth and yield is a strategic research focus for breeders. Landraces selected over centuries are valuable genetic resources for adapted genotypes to different abiotic stresses especially drought.

Several screening methods based on parameters reflecting the water statue 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. 2010; Jain and Chattopadhyay 2010; Mullan and Pietragalla 2012; Singh et al. 2013; Khazaei 2013; Ammar et al. 2015; Idrissi et al. 2015). Well-developed roots, vigorous shoots at early seedling stage, root-shoot ration and chlorophyll content (SPAD value) were reported to play an important role for 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; Idrissi et al. 2015).

Association of molecular markers with trait of interest including drought tolerance is being studied using mapping populations based on quantitative trait loci approach and unrelated genetic resources like landraces based on association mapping approach taking advantage of historic linkage between phenotypic and genetic variations during the process of selection and adaptation. Based on genetic diversity analysis, Singh et al. 2013 reported SSR markers associated with Fusarium wilt (Fusarium udum) resistance in cultivated pigeonpea (Cajanuscajan), Razavi et al. 2010 identified associated AFLP and EST candidate gene markers to water deficit response in Fragaria and Mondal et al. 2010 reported association of SSR markers to rust and late leaf spot resistance in cultivated groundnut (Arachis hypogaea L.).

The Mediterranean region is expected to enclose high genetic diversity of lentils thanks to the rich history of domestication and cultivation as well as for the frequency of biotic and biotic stresses. In Mediterranean environments lentil as well as other crops experience intermittent drought during vegetative growth and terminal drought associated with increasing temperatures during reproduction stages (Slim et al. 1993; Materne and Siddique, 2009). This offer opportunities of identification of biotic and abiotic stress resistant landraces. Although the genetic diversity and relationship of lentil landraces from a number of Mediterranean countries have been reported 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; Idrissi et al. 2015), no published studies, as far as we know, reported functional genetic diversity in association with drought tolerance.

Thus, the objectives of our study were analysis of genetic diversity of 70 landraces from different Mediterranean countries (Morocco, Italy, Turkey and Greece) using Simple Sequence Repeat (SSRs) and Amplified Fragment Length (AFLP) DNA markers (1), root and shoot characterization and evaluation of their drought tolerance using physiological measures (2) and analysis of their functional genetic diversity in association with drought tolerance as first and preliminary step of testing association mapping studies (3).

Material and Methods

Plant material

Seventy landraces originating from four Mediterranean countries (Morocco, Italy, Turkey and Greece; Table 1) were evaluated for genetic diversity using SSRs and AFLPs DNA markers and drought tolerance under greenhouse using relative water content (Barrs and Weatherley 1962; Verslues et al. 2006), water losing rate (Suprunova et al. 2004) and wilting score (Singh et al. 2013).

Table 1 : list of lentil landraces analyzed and their respective origins.

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

DNA extraction

All landraces were planted in the greenhouse and young leaves were collected from 2 to 3-week-old plantlets and lyophilized. For each landrace, genomic DNA was isolated from five single plants according to the NucleoSpin® Plant (MACHEREY-NAGEL, MN; Duren, Germany) kit protocol. 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 2014.

SSRs analysis

SSR analysis was carried out as described in Idrissi et al. (2015). Thirty microsatellite markers developed by Hamwieh et al. (2005) were evaluated in this study. 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 selected and used in this study (Table 2).

Table 2: Primer sequences and PCR conditions used for the amplification of the microsatellites in the landarces

Locus name	Primer sequences (5'-3-)		Ta (°C)	Alleles size range (bp)	No of cycles	PCR multiplex set	Fluorescent label
	Forward	Reverse					
SSR113	CCGTAAGAATTAGGTGTC	GGAAAATAGGGTGGAAAG	53	211-245	25	1	NED
SSR154	GGAATTTATCACACTATCTC	GACTCCCAACTTGTATG	53	261-381	25	1	FAM
SSR199	GTGTGCATGGTGTGTG	CCATCCCCCTCTATC	53	180-211	25	2	FAM
SSR124	GTATGTGACTGTATGCTTC	GCATTGCATTTCACAAACC	56	174-177	25	3	NED
SSR233	CTTGGAGCTGTTGGTC	GCCGCCTACATTATGG	56	126-159	25	3	HEX
SSR80	CCATGCATACGTGACTGC	GTTGACTGTTGGTGTAAGTG	60	129-157	25	4	FAM
SSR184	GTGTGTACCTAAAGCCTTG	GTAAGTTGATCAAACGCCC	60	216-271	25	5	FAM
SSR48	CATGGTGGAATAGTGATGGC	CTCCATACACCACTCATTCAC	60	163-195	25	5	HEX
SSR19	GACTCATACTTTGTTCTTAGCAG	GAACGGAGCGGTCACATTAG	60	255-276	25	6	HEX
SSR99	GGGAATTTGTGGAGGGAAG	CCTCAGAATGTCCCTGTC	60	153-164	25	6	FAM
SSR302	CAAGCCACCCATACACC	GGGCATTAAGTGTGCTGG	60	231-276	30	7	FAM
SSR309-2	GTATGTCGTTAACTGTCGTG	GAGGAAGGAAGTATTCGTC	50	171-193	25	8	FAM
SSR204	CACGACTATCCCACTTG	CTTACTTTCTTAGTGCTATTAC	56	177-195	30	9	HEX
SSR336	GTGTAACCCAACTGTTCC	GGCCGAGGTTGTAACAC	56	235-270	30	9	FAM
SSR119	GAACTCAGTTTCTCATTG	GAACATATCCAATTATCATC	50	263-297	30	10	HEX
SSR212-1	GACTCATTGTTGTACCC	GCGAGAAGAATGGTTG	50	159-207	30	10	NED
SSR215	CATTAATATTTCTTTGGTGC	CTTTTCTTCTCTTCCCC	50	361-441	30	10	FAM
SSR130	CCACGTATGTGACTGTATG	GAAAGAGAGGCTGAAACTTG	56	195-198	30	11	NED
SSR33	CAAGCATGACGCCTATGAAG	CTTTCACTCACTCAACTCTC	56	250-321	30	11	HEX

PCR analysis was performed as described in De Keyser et al. (2010) according to the Qiagen Multiplex PCR kit protocol with a final volume of 10 µl per reaction. To 15 ng of DNA, 2 µM of each primer and 19 Qiagen MultiPlex Mastermix (Multiplex PCR Kit; Qiagen; Manchester, United Kingdom) were added. PCR was conducted in a GeneAmp 9700 Dual thermocycler. The Hot StarTaq enzyme was activated with a heating step of 15 min at 95°C, followed by 25 or 30 cycles (Table 2) of 30 s at 94°C, 90 s at Ta (Table 2) and 60 s at 72°C with a final extension step of 30 min at 60°C. Of the final PCR product, 1µl 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. Different multiplex sets, with similar reaction conditions, were composed. Forward primers were labelled fluorescently (FAM, HEX and NED, Table 2).

AFLPs analysis

The standard AFLP protocol (Vos et al. 1995) was followed according to De Riek et al. (2001), with minor modifications. The preamplification step was performed in 50 µl reaction mix containing 1xFlexi PCR buffer (Promega; Madison, Wisconsin, USA), 1.5 mM MgCl2, 0.5 µM EcoRI+A, 0.5 µM MseI+C, 1.25U Flexi Taq-polymerase (Promega) and 5 µl of the digest from the restriction–adaptor ligation reaction. 7 primer combinations were usde: 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). Fragments were separated, sized and visualized as described above for SSRs.

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 x D 24 cm) filled with fine perlite in order to extract intact roots without damage. The standard nutrition solution EEG MESTSTOF 19-8-16 (4) [NO3 11 %, NH4 8 %, P 2O5 8 %, K2O 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 the plant emergence. Water supply was then sopped in order to expose plants to progressive drought stress. The initial moisture in all the pots was 70 % of field capacity, it decreased to about 20 % at the 8^{th} week after sowing.

Response of landraces to drought stress was assed based on three fast and resources-effective phenotyping methods largely used in plant breeding programs, wilting score (WS), leaf relative water content (RWC) and leaf water losing rate (WLR). WS estimated 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 measured the plant water status in plant tissues estimating the 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); then leaves were soaked for 4 h in distilled water at room temperature under a constant light to determine the turgid weight (TW). Total 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)] x 100.**

WLR estimated the rate of water loss of leaves exposed to dehydration, it was determined on another set of young fully expanded leaves. Weight after 4 h drying on filter paper (W4) (at room temperature under a constant light) was recorded and total dry weight (DW) was recorded after oven-drying at 72 °C for 48 h. Leaf WLR was calculated according to Suprunova et al. (2004): **WLR (g h^{-1} g^{-1} DW) = [(FW - W4)]/[DW x 4].**

RWC and WLR were measured twice for each landrace and each replication during the 6^{th} week after sowing based on separated sets of leaves. The wilting score was estimated one day before harvest. At 60 days after sowing, plants were carefully extracted without damage to the roots, then shoots and roots were separated into plastic bags after root washing.

Chlorophyll content was estimated according to the SPAD values measured at 48 days after sowing using a SPAD-502Plus chlorophyll meter (Konica Minolta, Japan), four measures were taken in fully expanded leaves per plant. Shoot length was measured as the 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 the dry root weight by the 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; 5 = excellent. All the variable measures were recorded as the mean value based on the four plants per individual genotype in each pot.

Data analysis

For both SSR and AFLP analysis, allele pattern profiles corresponding to amplification products were visualized, sized and automatically scored using the GENEMAPPER 4.0 software (Applied Biosystems). Unique SSR pattern profile correspond to homozygote individual, while two different correspond to heterozygote. 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) using MS Access and MS Excel. Considering all genotypes (five single plants represents each landrace), genetic diversity parameters were estimated for SSRs 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). The probability of identity (PI) between all genotypes for SSR markers was calculated using the IDENTITY 1.0 programme (Wagner and Sefc 1999). Polymorphic information content (PIC) was calculated for AFLP using $\mathbf{PIC = 1 - \Sigma P_i^2}$, where Pi is the fragment frequency of the i^{th} allele (Smith et al. 1997).

Genetic distance matrix between all pairwise genotypes based on the Nei genetic distance (Nei 1973) using binary matrix for SSR and AFLP as well as the Mantel test (Mantel 1967) were computed on NTSYS-PC 2.1 (Rohlf 2004) program to construct neighbor joining clusters to show the associations between the studied landraces. The Bootstrap analysis of the neighbor joining dendrograms was performed using TREECON software (Van de Peer and De Wachter 1993) to test confidence and faithfulness of the obtained groupings.

SPSS Statistics 22 was used for variance analysis, correlation, principal component analyses of root and shoots traits, drought parameters and the genetic data from SSR and AFLP markers. It was also used to perform the non-parametric Kruskal-Wallis analysis to test the associations between SSR and AFLP markers with drought parameters as measured by WS, RWC and WLR. In order to test functional grouping according drought response of landraces, canonical discriminant analyses based on the linked SSR and AFLP markers to the three drought parameters were performed using prior information on landraces' response to drought as fellow. The five classes according to the wilting scores (Singh et al. 2013) were used as grouping variable. 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). Regression analysis based on linked SSRs and AFLPs markers to the three drought measures was performed to confirm association revealed by the K-W test and to identify the makers explaining the highest phenotypic variation. Canonical discriminant and regression analyses were performed using SPSS Statistics 22.

Results

Genetic diversity parameters

For all landraces' genotypes, 19 SSRs produced a total of 261 alleles with an average of 13.73 alleles per locus and 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 smallest number of alleles. The 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 the 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 10^{-24} (Table 3).

Table 3: Simple sequence repeat (SSR) polymorphism parameters in the 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 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 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 the total fragment obtained, 449 (64.24 %) 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 : Amplified fragment length polymorphism (AFLP) Primer combinations polymorphism parameters in the 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	51.77-479.90	0.4497
EcoRI-ACA+ *MseI*-CTG (PC2)	127	68.75	21.97	54.13	50.32-499.54	0.3387
EcoRI-ACA+ *MseI*-CTT (PC3)	162	91.42	16.25	56.43	50.16-469.48	0.3588
EcoRI-ACG+ *MseI*-CAA (PC4)	96	43.87	19.83	45.70	50.08-486.57	0.3195
EcoRI-AGC+ *MseI*-CAA (PC5)	104	53.28	17.39	51.23	51.06-492.58	0.3259
EcoRI-AGC+ *MseI*-CAG (PC6)	92	48.39	18.16	52.60	51.63-491.12	0.3393
EcoRI-AGC+ *MseI*-CTG (PC7)	83	42.77	9.25	51.54	50.48-498.72	0.3249
Total	812	449		54.28		0.3509
Average	116	64.24				

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

Genetic relationship among landraces was assessed for both microsatellites and AFLP markers taken separately using Neighbor Joining method (NJ) and the combined data sets using Principal Component Analysis (PCA).

Based on SSR markers the NJ dendrogram generated five groups. Landraces from northern Mediterranean from Italy, Turkey and Greece were grouped together in group 4 separately from those of Morocco, except six landraces (M29, M30, M39, M49, M26 and M8). The four other groups were from Morocco (Figure 1).

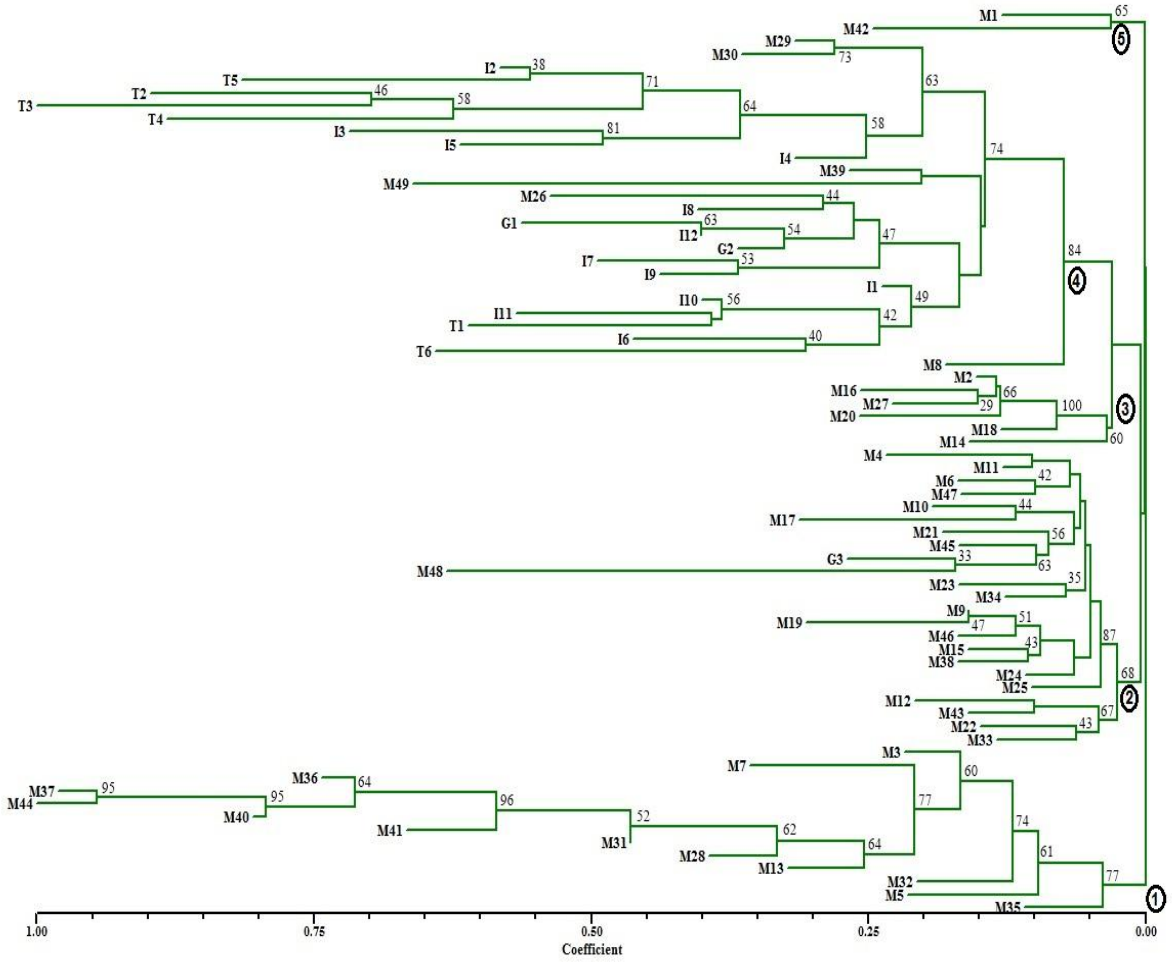

Figure 1 : Neighbor Joining dendrogram (NJ) generated based on Nei genetic distance from SSRs markers. Bootstrap values are given at the nodes.

NJ grouping based on AFLP markers (Figure 2) discriminated between landraces from Morocco and those from north Mediterranean. Landraces from Italy, Turkey and Greece were clustered in group 1. Landraces from Morocco could be separated in four groups, one large group containing 36 landraces, two groups containing 7 and 5 landraces respectively and one single landrace M7 separated from the rest.

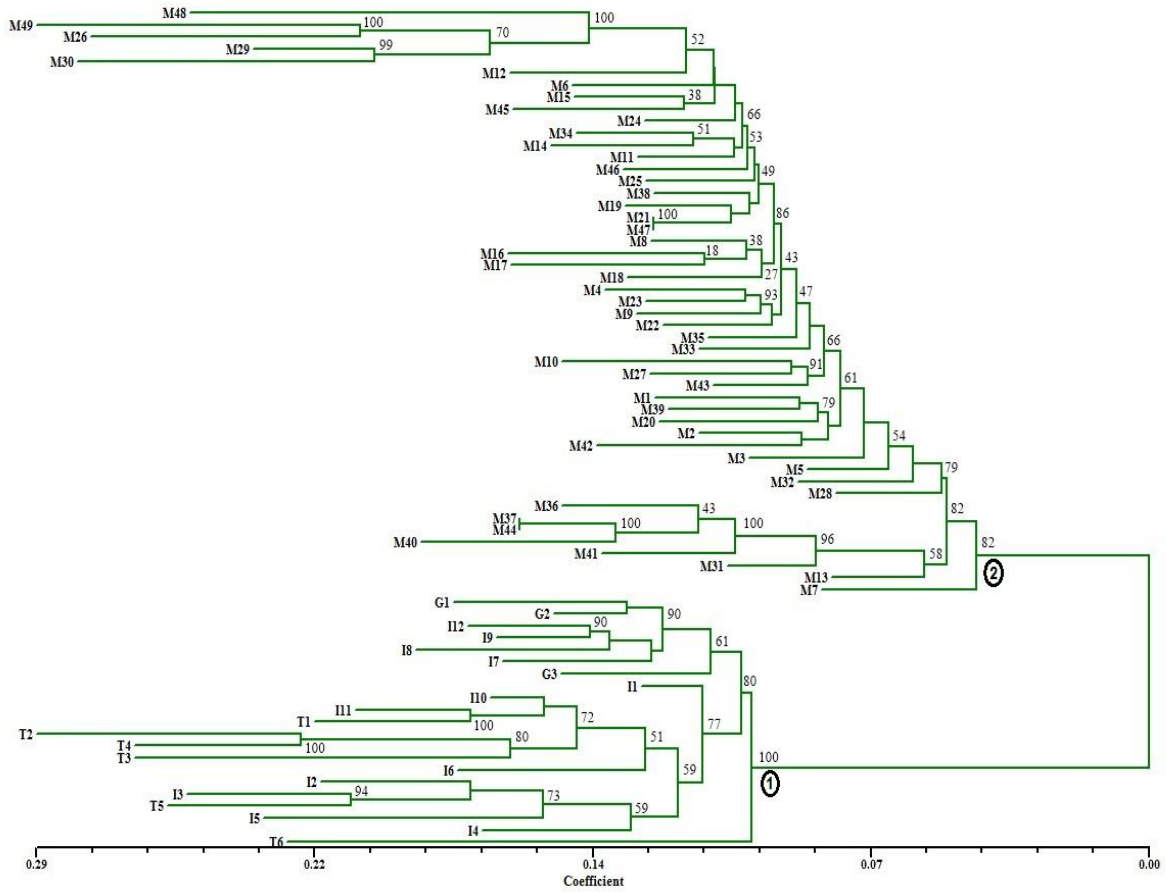

Figure 2: Neighbor Joining dendrogram (NJ) generated based on Nei genetic distance from AFLPs markers. Bootstrap values are given at the nodes.

Genetic similarity matrices between lentil landraces from the two data sets (SSR and AFLP) were compared using the Mantel test. A significant correlation between the two matrices was found with r = 0.6485 and Mantel t = 5.7477 ($P < 0.001$). Same patterns of groupings as shown is figures 2 and 3 were obtained based on all the 350 genotypes (five genotype per landrace) analyzed for both DNA markers (data not shown). Combined data sets from the SSR and AFLP analyses were used to construct a consensus grouping of the landraces by performing principal component analysis. The first and second axes of PCA explained 37.69% and 25.40 % of the total variance and separated lentil landraces into two main groups discriminating Moroccan landraces from those of Italy, Turkey and Greece. Landraces from both groups from northern Mediterranean region as well as from Morocco still enclose high genetic diversity (Figure 3).

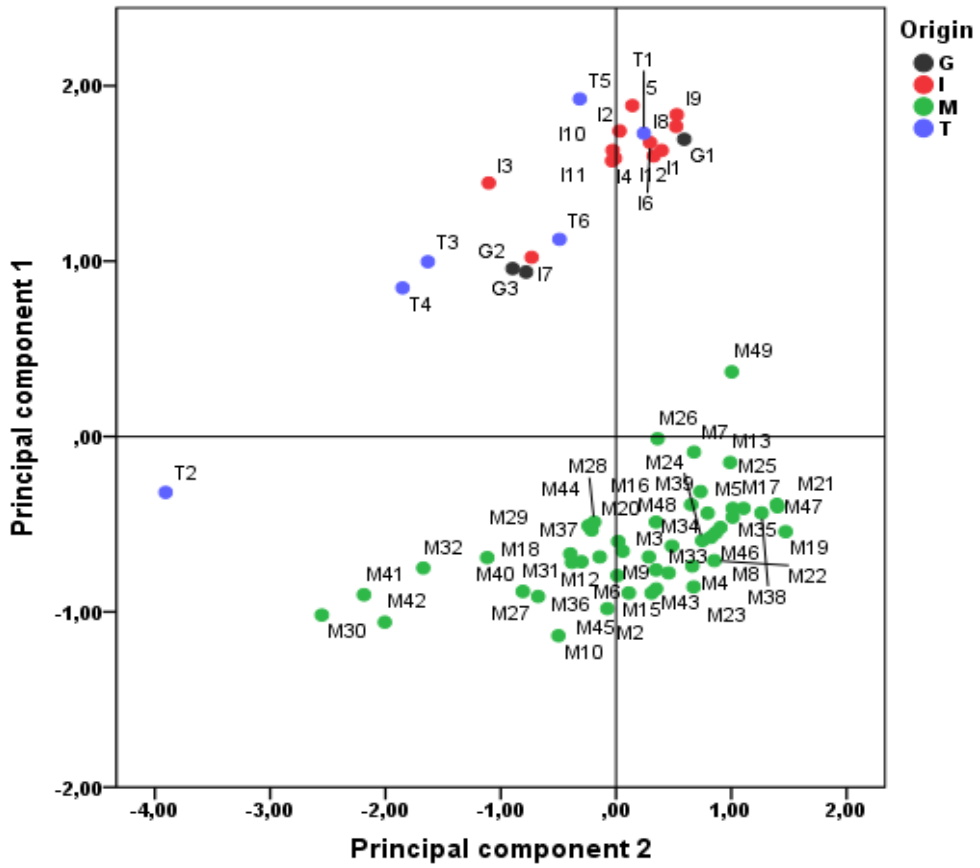

Figure 3: Principal component analysis (PCA) scatter plot based on SSRs and AFLPs of landraces sorted by country of origin. G: Greece; I: Italy; M: Morocco and T: Turkey.

Root and shoot characterization and drought tolerance evaluation

Analysis of variance showed a significant high variation for all traits among landraces (Table 5): shoot lengths at 12 and days after sowing, seedling vigor, dry shoot weight, chlorophyll content as estimated by the SPAD values, 100 seeds weight, dry shoot weight, root-shoot ratio, relative water content, water losing rate and wilting score (Table 5).

Table 5: Variation among root and shoot traits and drought parameters.

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

Important significant correlations were shown between (Table 6): seedling vigor and wilting score (0.252); SPAD and leaf relative water content (0.335), water losing rate (-0.325) and wilting score (-0.538); dry root weight and dry shoot weight (0.460), SPAD (0.573), relative water content (0.482), water losing rate (-0.288) and wilting score (-0..411); root-shoot ratio and relative water content (0.362), water losing rate (-0.256) and wilting score (-0.374). The three drought parameters were significantly correlated to each other. Water losing rate and wilting score were positively correlated (0.571), while relative water content was negatively correlated to both parameters with -0.577 and -0.610, respectively.

Table 6 : Correlations among root and shoot traits and drought parameters.

	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.

Principal component analysis based on all the variables among the landraces was performed. The first and the second axes explained 34.16 % and 24.59 % of the total variation, respectively (Figure 4). Principal component 1 was positively correlated with root-shoot ratio (0.766), leaf relative water content (0.609), dry root weight (0.529) and chlorophyll content (0.503); and negatively correlated with wilting score (-0.789), water losing rate (-0.603), shoot lengths after 12 and 22 DAS (-0.511; -0.643), seedling vigor (-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), seedling vigor (0.612), chlorophyll content (0.585), shoot lengths at 12 and 22 days after sowing (0.431; 0.569) and leaf relative water content (0.408). Weak but still significant negative correlations were observed with water losing rate (-0.303) and wilting score (-0.244).

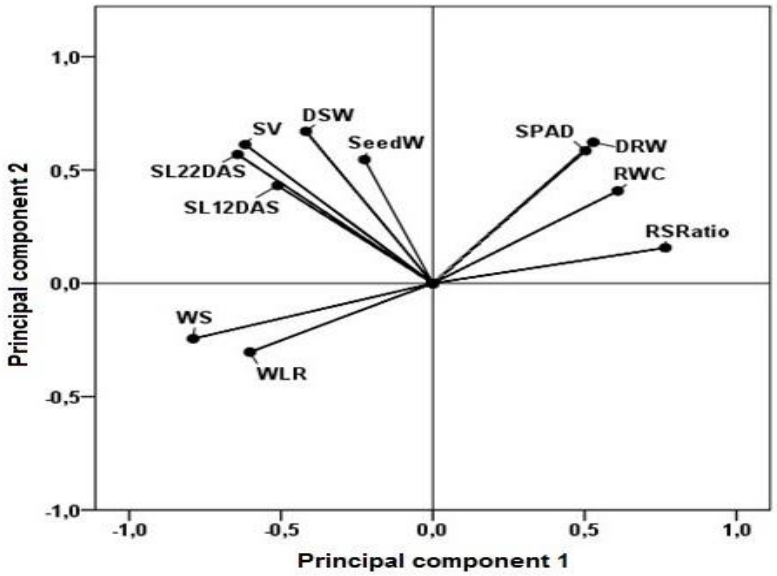

Figure 4: Principal component analysis based on all traits measured on the landraces.

Drought tolerance reaction as measured by RWC, WLR and WS score 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 5). No correlation between landraces' origin and drought response was observed.

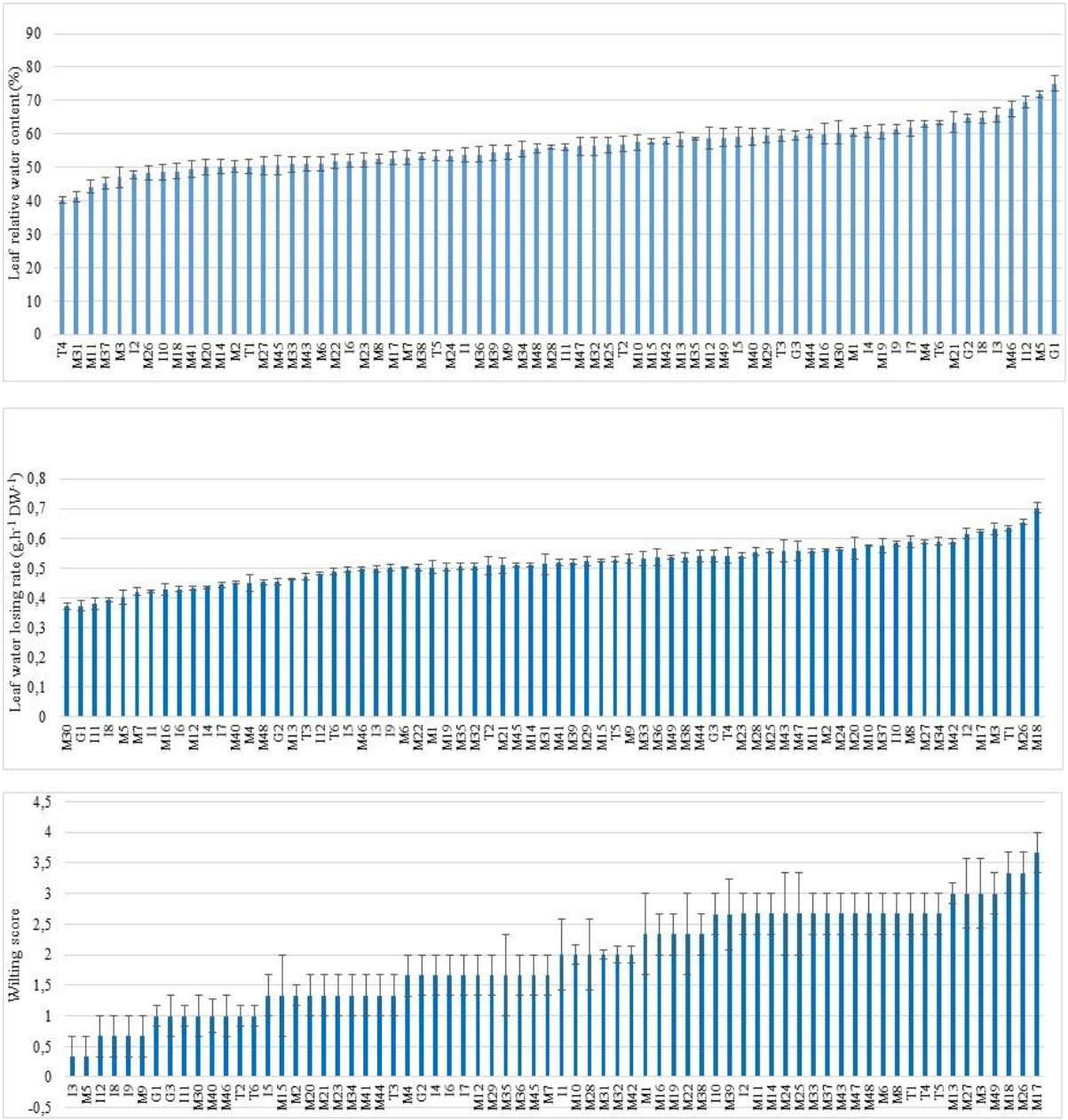

Figure 5: Variation of leaf relative water content (a), leaf water losing rate (b) and wilting score (c) among the landraces.

Principal component analysis was performed based on the three parameters used to estimate drought reaction (leaf relative water content, leaf water losing rate and wilting score) in order to sort the landraces according to a consensus classification in response to drought stress (Figure 6). Principal components 1 and 2 explained 65.25 % and 18.57 % of the total variation, respectively. The first axis was highly correlated with the three parameters : -0.826 with leaf relative water content, 0.807 with water losing rate and 0.791 with wilting score. Higher values of this axis indicated sensitive landraces, while lower values indicated tolerant landraces.

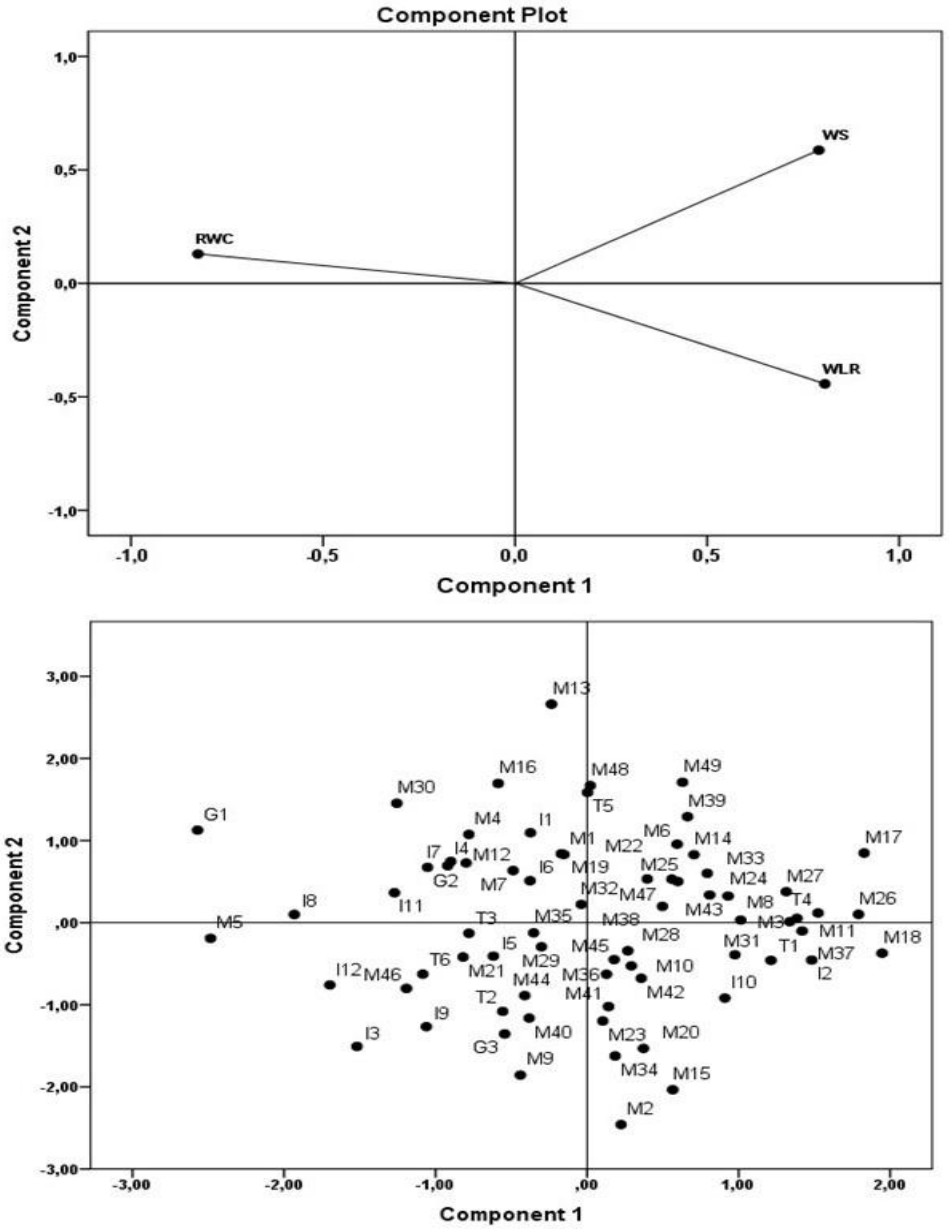

Figure 6: PCA based on leaf relative water content, leaf water losing rate and wilting score. Upper part sorts the 3 variables as associated to the two principal components (PC) while the lower part shows landraces according to the two PCs.

Associated SSRs and AFLPs candidates markers to drought tolerance

In order to determine SSRs and AFLPs markers linked to the individual measured physiological traits, Kruskal–Wallis analysis was applied. The test is based on the ranking of landraces according to leaf relative water content, water losing rate and wilting score separately and testing the association to the markers one by one as grouping variable. Six, four and five SSRs were identified as associated with leaf relative water content, leaf water losing rate and wilting score, respectively (Table 7). 91, 105 and 51 AFLPs, were found to be associated with leaf relative water content, water losing rate and wilting score, respectively (Tables 8, 9, 10).

Table 7: Linked SSRs to drought parameters according to Kruskal-Wallis H Test.

Linked SSRs to drought parameters	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance
Leaf relative water content				
SSR113_5	221	15.32	6	0.018
SSR184_17	263	8.36	3	0.039
SSR19_4	262	7.30	2	0.02
SSR233_13	155	11.5	5	0.04
SSR48_3	165	3.9	1	0.04
SSR80_12	153	18.1	7	0.01
Leaf water losing rate				
SSR215_9	388	6.07	2	0.04
SSR154_4	361	6.95	2	0.04
SSR184_17	263	8.86	3	0.04
SSR336_22	279	10.7	4	0.04
Wilting score				
SSR119_5	271.50	4.8	1	0.02
SSR154_12	379	3.96	1	0.04
SSR19_7	270.50	14.45	6	0.02
SSR204_1	177	5.64	1	0.01
SSR48_3	165.50	4.8	1	0.03

Table 8: Linked AFLPs to RWC according to Kruskal-Wallis H Test.

Linked AFLPs to RWC	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance	Linked AFLPs to RWC	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance
PC1_111	111	11.57	3	0.009	PC4_152	152	11.28	3	0.01
PC1_114	114	11.92	4	0.036	PC4_179	179	14.19	4	0.014
PC1_127	127	13.71	4	0.018	PC4_196	196	13.49	4	0.019
PC1_145	145	10.97	4	0.027	PC4_270	270	12.64	4	0.027
PC1_152	152	10.82	4	0.029	PC4_300	300	15.74	4	0.008
PC1_171	171	12.97	4	0.024	PC4_302	302	13.02	4	0.023
PC1_217	217	12.47	4	0.029	PC4_303	303	11.73	4	0.039
PC1_218	218	10.69	4	0.030	PC4_377	377	11.78	4	0.038
PC1_219	219	12.23	4	0.032	PC4_380	380	13.70	4	0.018
PC1_234	234	10.85	4	0.028	PC4_444	444	13.16	4	0.011
PC1_236	236	15.84	4	0.007	PC4_81	81	16.10	4	0.007
PC1_238	238	17.30	4	0.016	PC4_89	89	11.67	4	0.020
PC1_240	240	11.64	4	0.020	PC4_93	93	14.06	4	0.015
PC1_290	290	14.77	4	0.011	PC5_104	104	15.93	4	0.007
PC1_291	291	13.18	4	0.022	PC5_134	134	12.49	4	0.029
PC1_299	299	13.79	4	0.017	PC5_193	193	12.02	4	0.034
PC1_314	314	14.87	4	0.011	PC5_248	248	13.88	4	0.016
PC1_319	319	11.78	4	0.038	PC5_283	283	14.66	4	0.012
PC1_323	323	13.02	4	0.023	PC5_350	350	18.27	4	0.032
PC1_327	327	15.32	4	0.009	PC5_435	435	12.33	4	0.015
PC1_329	329	12.63	4	0.027	PC5_436	436	11.92	4	0.036
PC1_355	355	17.37	4	0.004	PC6_121	121	18.75	4	0.002
PC1_400	400	13.63	4	0.018	PC6_123	123	11.80	4	0.038
PC1_419	419	12.43	4	0.029	PC6_150	150	10.26	4	0.036
PC1_422	422	14.99	4	0.010	PC6_321	321	11.98	4	0.035
PC1_447	447	16.13	4	0.006	PC6_478	478	12.54	4	0.028
PC1_456	456	19.36	4	0.002	PC6_484	484	12.18	4	0.032
PC1_458	458	13.01	4	0.023	PC6_68	68	11.79	4	0.038
PC1_53	53	12.38	4	0.030	PC6_74	74	13.06	4	0.023
PC1_75	75	14.18	4	0.014	PC7_126	126	17.63	4	0.001
PC1_98	98	13.50	4	0.019	PC7_234	234	15.72	4	0.008
PC2_108	108	12.47	4	0.029	PC7_253	253	15.14	4	0.032
PC2_120	120	13.43	4	0.020	PC7_360	360	11.86	4	0.037
PC2_166	166	14.78	4	0.011	PC7_479	479	12.67	4	0.027
PC2_250	250	15.14	4	0.010	PC7_63	63	20.043	4	0.001
PC2_352	352	15.03	4	0.010	PC7_92	92	10.06	4	0.039
PC2_64	64	11.27	4	0.046					
PC2_98	98	17.09	4	0.004					
PC3_113	113	12.28	4	0.031					
PC3_140	140	15.72	4	0.008					
PC3_184	184	12.39	4	0.030					
PC3_185	185	13.78	4	0.017					
PC3_261	261	15.85	4	0.008					
PC3_305	305	14.52	4	0.024					
PC3_311	311	12.27	4	0.031					
PC3_333	333	11.85	4	0.037					
PC3_384	384	17.93	4	0.003					
PC3_471	471	7.87	3	0.049					
PC3_59	59	11.94	4	0.036					
PC3_64	64	7.92	3	0.048					
PC3_69	69	13.49	4	0.009					
PC3_88	88	14.53	4	0.006					
PC3_91	91	12.55	4	0.028					
PC3_93	93	13.04	4	0.023					
PC3_97	97	18.63	4	0.002					

Table 9: Linked AFLPs to WLR according to Kruskal-Wallis H Test.

Linked AFLPs to WLR	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance	Linked AFLPs to WLR	Allele size (bp)	Chi-square	Degree of freedom	Asymptotic significance
PC1_111	111	14.08	3	0.003	PC3_471	471	11.26	3	0.01
PC1_114	114	11.26	4	0.046	PC3_59	59	15.38	4	0.009
PC1_117	117	17.71	4	0.003	PC3_69	69	17.26	4	0.002
PC1_127	127	12.48	4	0.029	PC3_82	82	13.05	4	0.023
PC1_140	140	12.66	4	0.027	PC3_88	88	12.36	4	0.015
PC1_143	143	12.73	4	0.026	PC3_97	97	13.17	4	0.022
PC1_164	164	10.06	4	0.039	PC4_136	136	10.77	4	0.029
PC1_175	175	14.36	4	0.013	PC4_181	181	15.81	4	0.007
PC1_178	178	12.11	3	0.007	PC4_184	184	13.13	4	0.022
PC1_213	213	12.54	4	0.028	PC4_190	190	13.94	4	0.016
PC1_234	234	9.65	4	0.047	PC4_216	216	14.70	4	0.012
PC1_238	238	20.40	4	0.005	PC4_235	235	14.89	4	0.011
PC1_254	254	9.32	3	0.025	PC4_239	239	13.98	4	0.016
PC1_255	255	12.31	4	0.015	PC4_300	300	17.05	4	0.004
PC1_258	258	15.77	4	0.008	PC4_380	380	11.34	4	0.045
PC1_288	288	14.72	4	0.012	PC4_484	484	17.036	4	0.004
PC1_290	290	14.60	4	0.012	PC4_84	84	12.68	4	0.025
PC1_291	291	12.96	4	0.024	PC4_90	90	14.79	4	0.011
PC1_299	299	18.30	4	0.003	PC5_131	131	12.26	4	0.031
PC1_306	306	11.58	4	0.041	PC5_147	147	11.70	4	0.020
PC1_329	329	13.48	4	0.019	PC5_183	183	12.91	4	0.024
PC1_333	333	11.58	4	0.041	PC5_187	187	14.47	4	0.011
PC1_343	343	11.76	4	0.038	PC5_192	192	12.85	4	0.025
PC1_399	399	12.04	4	0.034	PC5_193	193	13.68	4	0.018
PC1_400	400	11.99	4	0.035	PC5_213	213	12.15	4	0.033
PC1_458	458	13.80	4	0.017	PC5_350	350	17.41	4	0.043
PC1_97	97	12.08	4	0.034	PC5_436	436	11.45	4	0.043
PC1_98	98	12.95	4	0.024	PC5_59	59	11.96	4	0.035
PC2_104	104	13.80	4	0.017	PC5_70	70	17.12	4	0.004
PC2_108	108	15.80	4	0.007	PC6_123	123	11.49	4	0.035
PC2_134	134	11.87	4	0.037	PC6_136	136	11.79	4	0.038
PC2_143	143	13.65	4	0.018	PC6_150	150	10.88	4	0.028
PC2_186	186	15.14	4	0.010	PC6_163	163	13.47	4	0.019
PC2_192	192	11.58	4	0.041	PC6_185	185	15.08	4	0.010
PC2_220	220	11.78	4	0.038	PC6_263	263	12.17	4	0.033
PC2_309	309	13.04	4	0.011	PC6_271	271	16.92	4	0.005
PC2_423	423	12.25	4	0.032	PC6_318	318	16.68	4	0.005
PC2_466	466	12.25	4	0.031	PC6_321	321	12.03	4	0.034
PC2_64	64	12.77	4	0.026	PC6_323	323	11.98	4	0.035
PC2_65	65	11.32	4	0.023	PC6_391	391	16.22	4	0.003
PC3_105	105	12.04	3	0.007	PC6_475	475	12.87	4	0.025
PC3_111	111	12.32	4	0.031	PC6_484	484	12.86	4	0.025
PC3_113	113	11.76	4	0.038	PC7_126	126	11.27	4	0.024
PC3_125	125	14.40	4	0.013	PC7_187	187	10.35	4	0.035
PC3_128	128	15.06	4	0.010	PC7_253	253	12.20	4	0.032
PC3_151	151	12.56	4	0.028	PC7_397	397	14.07	4	0.015
PC3_172	172	14.03	4	0.015	PC7_465	465	11.80	4	0.038
PC3_184	184	22.90	4	0.000					
PC3_185	185	13.62	4	0.018					
PC3_225	225	11.68	4	0.039					
PC3_237	237	15.28	4	0.009					
PC3_245	245	13.77	4	0.017					
PC3_305	305	15.47	4	0.017					
PC3_306	306	12.29	4	0.031					
PC3_308	308	11.82	4	0.037					
PC3_323	323	11.86	4	0.037					
PC3_350	350	12.91	4	0.024					
PC3_424	424	13.87	4	0.016					

Table 10: Linked AFLPs to WS according to Kruskal-Wallis H Test.

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

In order to test the genetic differentiation of landraces according to their drought reaction as measured by the three parameters, prior information related to their grouping based on relative water content, water losing rate and wilting score, canonical discriminant analyses were performed using pairwise genetic distances between landraces generated from linked SSRs and AFLPs markers to the respective parameters. The analyses highly discriminated landraces according to their drought reaction into the pre-defined groups based on relative water content, water losing rate and wilting score for both linked SSRs and AFLPs to these parameters (Figures 7, 8). First discriminant functions explained 96.9 %, 84.5 % and 93.7% of the total variation with canonical correlations of 0.883, 0.683 and 0.975 and Eigenvalues of 3.53, 0.876 and 19.57 for linked SSRs to relative water content, water losing rate and wilting score, respectively. For linked AFLPs to relative water content, water losing rate and wilting score, first discriminant functions explained 62.3 %, 58 % and 73.5 % of the 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 the 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 linked AFLPs to relative water content, water losing rate and wilting score.

Regression analysis based on linked SSRs alleles to relative water content, water losing rate and wilting score 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. SSR19_7 and SSR204_1 explained the highest phenotypic variation of WS with 33 % and 21 %, respectively.

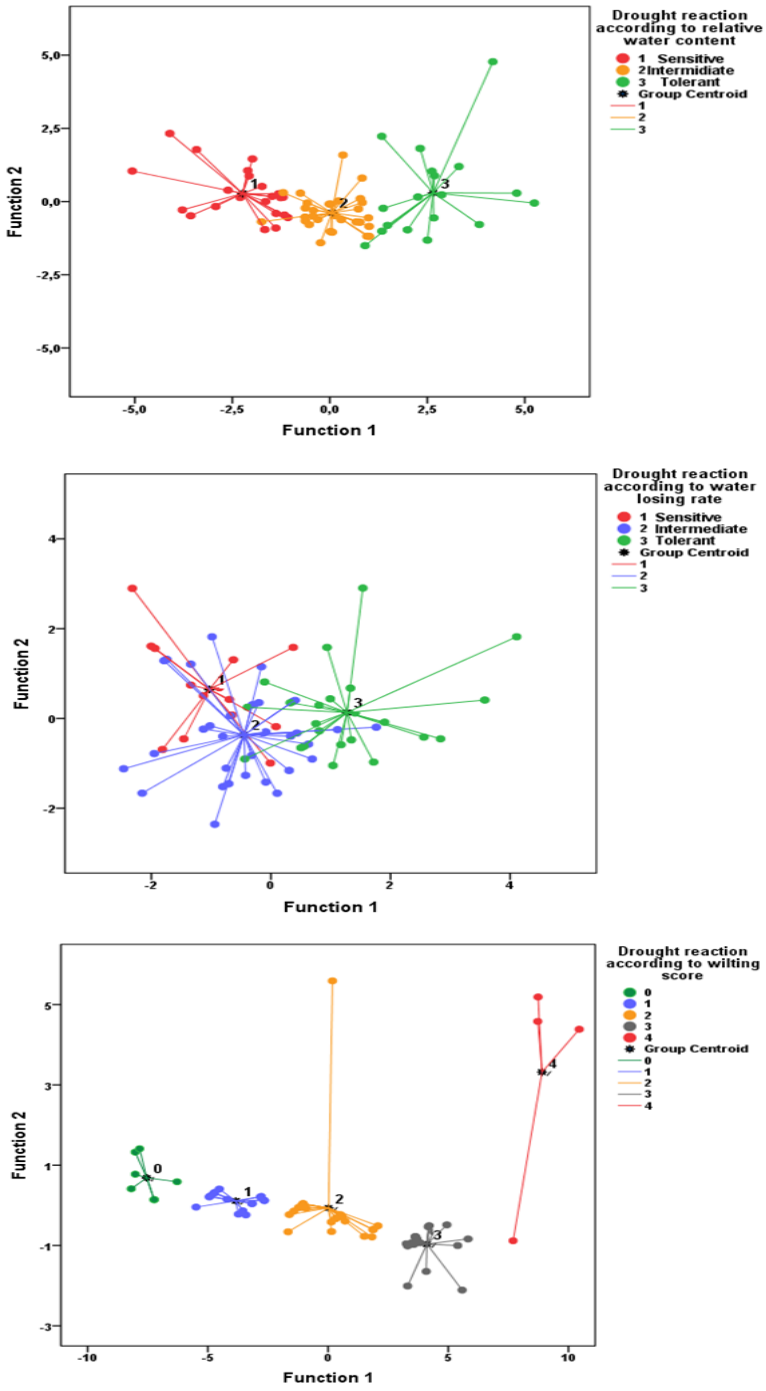

Figure 7 : Discriminant analysis based on SSRs linked to relative water content (a), water losing rate (b) and wilting score (c). 0 to 4 correspond to the following 0–4 score scale as described by Singh et al. (2013): 0 = healthy plants with no visible symptoms of drought stress; 1 = green plants with 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).

Regression analysis based on linked AFLPs alleles to relative water content, water losing rate and wilting score showed high associations with R^2=0.753, R^2=0.912 and R^2=0.832 respectively for the three drought measures as dependent variables. PC1_400 and PC7_92 explained the highest phenotypic variation of RWC with 32 % and 14 %, respectively. PC4_480 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, respectively.

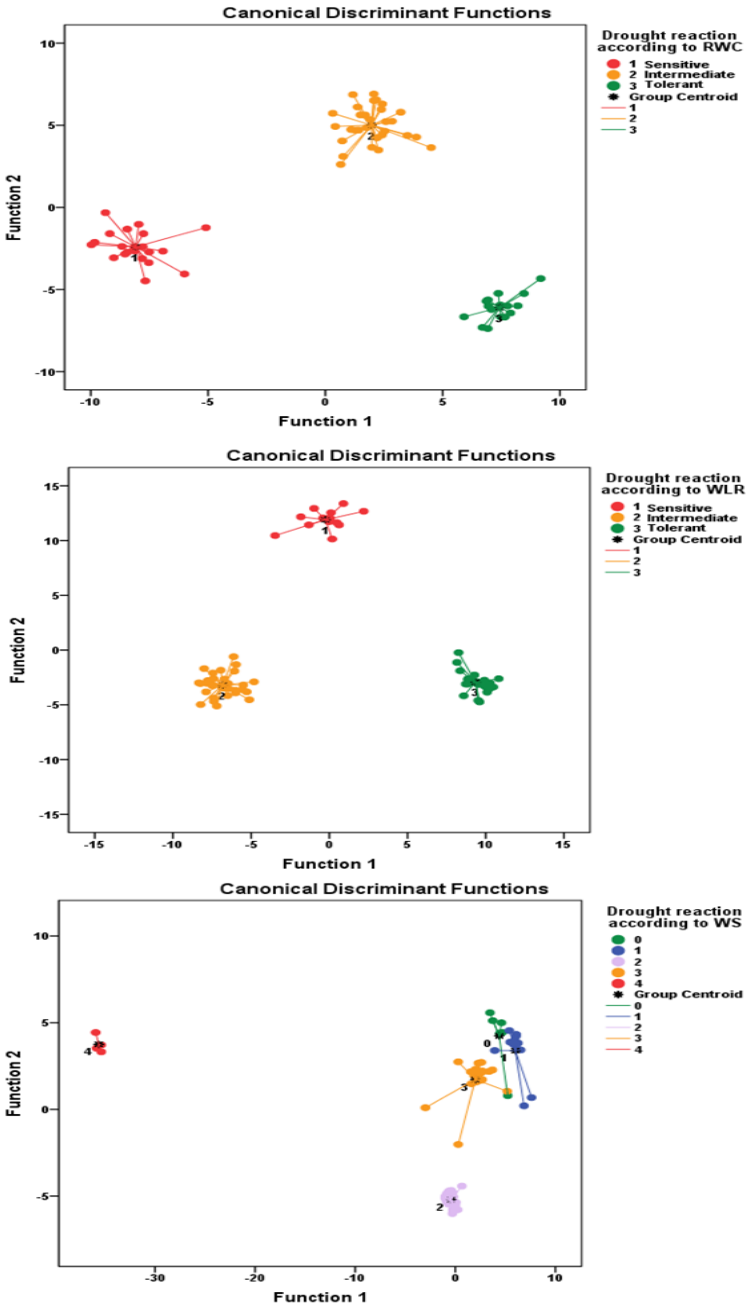

Figure 8 : Discriminant analysis based on AFLPs linked to relative water content (a), water losing rate (b) and wilting score (c). 0 to 4 correspond to the following 0–4 score scale as described by Singh et al. (2013): 0 = healthy plants with no visible symptoms of drought stress; 1 = green plants with 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).

Discussion

High genetic variation was shown in Mediterranean landraces from Morocco, Italy, Turkey and Greece by both SSRs and AFLPs DNA markers. 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 number of observed alleles from 2 to 22 at 16 loci for Italian landraces. Idrissi et al. (2015 b) reported 213 alleles at same 19 loci using Moroccan landraces. For AFLPs, a total of 812 fragments were yielded with 64.24 % were polymorphic and average PIC of 0.3509 over the seven primer combinations. Idrissi et al. (2015 b) reported 766 fragments with 54.78 % were polymorphic using same primer combination in Moroccan landraces and Torricelli et al. (2011) reported 698 fragments with 57.09 % were polymorphic using eight primer combinations in Italian landraces. Toklu et al. (2009) reported 212 fragments were 56.1 % were polymorphic and an average PIC of 0.579 using six primer combinations in Turkish landraces.

Based on NJ dendrogram and PCA using SSRs and AFLPs DNA markers separately and the combined data sets, landraces from northern Mediterranean namely from Italy, Turkey and Greece were clearly differentiated from those originating from southern Mediterranean form Morocco. Landraces from Italy, Turkey and Greece were different from each other as well. This highlight the high genetic diversity enclosed in the Mediterranean region for lentil landraces. Our results are in agreement with those of Lombardi et al. (2014) reporting very high level of genetic diversity of lentil landraces form the Mediterranean region using single nucleotide polymorphism markers.

The rich history of Mediterranean region regarding lentil domestication and cultivation overs centuries as well as the frequency and diversity of biotic and abiotic stresses make this region as an important source for well-adapted genotypes. Laghetti et al. (2008), Toklu et al. (2009) and Idrissi et al. (2015 b) reported the importance and genetic differentiation of lentil genetic resources for adaptive traits of some landraces from Italy, Morocco and Turkey.

High genetic variation for root and shoot traits as well as for drought response as estimated by leaf relative water content, water losing rate and wilting score were observed among the Mediterranean landraces included in our study. Sarker et al. 2005; Kashiwagi et al. 2005; Vadez et al. 2008; Gaur et al. 2008; Aswaf and Blair 2012; Kumar et al. 2012; Idrissi et al. 2015b reported the associations of these traits with drought tolerance in lentil and other crops. Under water limited stress, first plants response is to maintain water content as close as possible to non-stressed situation by stomatal control to limit water lose and by faster root growth and increased root-shoot ratio to improve water uptake. Increased root growth and the capacity to maintain higher water content are important for plants production under drought stress (Verslues et al. 2006) compared to other mechanisms with negative effect on yield.

Significant correlations were obtained between dry root biomass and dry shoot biomass and SPAD highlighting the possibility of indirect selection for these underground traits 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 root-shoot ratio were the most drought tolerant showing the highest leaf relative water content and the lowest water losing rate and wilting score. Thus, selection based on these three traits under water limited availability would result in improved drought tolerance. No correlation between drought tolerance and geographic origin of the landraces was observed, thus selection have to be made based on the individual response de each genotype.

Significant associations of SSRs and AFLPs DNA markers with leaf relative water content, water losing rate and wilting score based on Kruskal–Wallis Test were shown. Six, four and five SSRs as well as 91, 105 and 51 AFLPs were identified as linked to the three drought parameters, respectively. SSRs and AFLPs linked allele-markers highly discriminated landraces according to their drought reaction highlighting their genetic differentiation according to their drought tolerance level. Landraces with higher relative water content, lower water losing rate and wilting score were clearly separated from those with lower relative water content and higher water losing rate and wilting score. 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. These markers could be considered as functional markers to be used in functional genetic diversity analysis related to adaptative traits to drought tolerance. This finding suggest the reliability of association mapping studies for drought tolerance on a large number of landraces in lentil. Joshi-Saha and Reddy (2015) identified three SSRs alleles associated with drought tolerance using K-W test in 60 genotypes of chickpea. Using same method Razavi et al. (2011) reported five and 13 EST; 47 and 85 AFLPs markers linked to leaf relative water content and water losing rate in 23 fragaria cultivars, respectively.

Conclusion

High genetic variation of Mediterranean lentil landraces included here was shown suggestion the richness of germplasm from this region and potential interest for breeding programs. High variability for root and shoot traits and physiological parameters estimating drought tolerance and no correlation of drought response with geographic origin were observed. Association of some aboveground traits with underground traits (root) was demonstrated suggesting the reliability of indirect selection. Higher dry root biomass, chlorophyll content and root-shoot ratio were associated with drought tolerant.

A number of DNA markers were identified as associate to drought tolerance and phenotypic classes according to drought response better corresponded to groupings made on these correlated markers. Although plant response to drought stress is complex trait involving many aspect, this study showed evidences of genetic differentiation according to drought response. Thus further studies involving larger landraces and unrelated genotypes in associations mapping studies and quantitative trait studies based on mapping populations from contrasted parents using more efficient and effective DNA markers like Single Nucleotide Polymorphism markers, will allow better understanding of the genetic basis of drought tolerance.

References (to be finished)

M. H. Ammar1, F. Anwar1, E. H. El-Harty1, H. M. Migdadi1, S. M. Abdel-Khalik2, S. A. Al-Faifi1, M. Farooq1,3,4 & S. S. Alghamdi . Physiological and Yield Responses of Faba bean (Vicia faba L.) to Drought Stress in Managed and Open Field Environments. Journal of Agronomy and Crop Science. 01/2015; DOI: 10.1111/jac.12112.

Erskine et al. 2011

Bhatty, R.S. (1988) Composition and quality of lentil (Lens culinaris Medik.): a review. Canadian Institute of Food Science and Technology 21, 144–160.

Erskine, W., Rihawe, S. and Capper, B.S. (1990) Variation in lentil straw quality. Animal Feed Science and Technology 28, 61–69.

Grusak, M. A., 2009: Nutritional and health-beneficial quality. In: W. Erskine, F. J. Muehlbauer, A. Sarker, and B. Sharma (eds), The Lentil: Botany, Production and Uses, 368–390. CAB International, Oxfordshire.

Levitt 1980. Responses of Plants to Environmental Stresses. New York: Academic press

FAOSTAT 2012

Joshi-Saha and Reddy (2015). Repeat length variation in the 5'UTR of myo-inositol

monophosphatase gene is related to phytic acid content and contributes to drought tolerance in chickpea (Cicer arietinum L.). Journal of Experimental Botany. doi:10.1093/jxb/erv156

Ladizinsky, G., 1979: The genetics of several morphological traits in lentil. J. Heredity 70, 135—137.
Ladizinsky, G., 1987: Pulse domestication before cultivation. Econ. Bot. 41, 60—65.

Singh DH, Dikshit HK, Singh RA (2013) A new phenotyping techniqueforscreeningfordroughttoleranceinlentil (Lens culinaris Medik.). Plant Breed 132:185–190. doi:10.1111/ pbr.12033

Verslues et al. 2005 Methods and concepts……

Razavi et al. 2010

Khazaei H, Street K, Bari A, Mackay M, Stoddard FL (2013) The FIGS (Focused Identification of Germplasm Strategy) Approach Identifies Traits Related to Drought Adaptation in Vicia faba Genetic Resources. PLoS ONE 8(5): e63107. doi:10.1371/journal.pone.0063107

Sonnante and Pignone 2001, Faratini et al. 2011.

Jain and Chattopadhyay 2010. Analysis of gene expression in response to water deficit of chickpea (Cicer arietinum L.) varieties differing in drought tolerance. BMC Plant Biology 2010, 10:24.

Frederick J. Muehlbauer1,∗, Seungho Cho3, Ashutosh Sarker4, Kevin E. McPhee1,
Clarice J. Coyne2, P.N. Rajesh1 & Rebecca Ford5. Application of biotechnology in breeding lentil for resistance to biotic and abiotic stress. Euphytica (2006) 147: 149–165
DOI: 10.1007/s10681-006-7108-0

Idrissi et al. 2015

Singh et al. 2013

IBPGR and ICARDA (1985)

S. MONDAL and A. M. BADIGANNAVAR. Molecular diversity and association of SSR markers to rust and late leaf spot resistance in cultivated groundnut (Arachis hypogaea L.). 2010. Plant Breeding 129, 68—71 (2010).

Silim, S.N., Saxena, M.C. and Erskine, W. (1993) Adaptation of lentil to Mediterranean environment. I. Factors affecting yield under drought conditions. Experimental Agriculture 29, 9–19.

Mantel, N. A. 1967. The detection of disease clustering and a generalized regression approach. Cancer Res., 27:209-220.

M. Materne1 and K.H.M. Siddique2. Agroecology and Crop Adaptation. In: W. Erskine, F. J. Muehlbauer, A. Sarker, and B. Sharma (eds), The Lentil: Botany, Production and Uses, 368–390. CAB International, Oxfordshire.

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;

Idrissi et al. 2015

Suprunova et al. (2004

Barr and Weatherley (1962)

Mullan and Pietragalla (2012)

Van de Peer, Y., De Wachter, R. (1993) TREECON: a software package for the construction and drawing of evolutionary trees. *Comput. Applic. Biosci.* 9, 177-182.