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Effect of Water Stress on Rapeseed Cultivars Using Morpho-Physiological Traits and Their Relations with ISSR Markers

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Abstract
To study the effect of water stress in rapeseed cultivars at the seedling stage, 10 rapeseed cultivars were evaluated at three irrigation levels [normal irrigation (control) and irrigation after depletion of 60 and 85% of available soil water]. Analysis of variance showed considerable variation among cultivars. Water stress reduced all of the studied morphological characteristics, especially shoot and root dry weight, root volume and increased chlorophyll content and chlorophyll fluorescence. Cluster analysis at three levels of irrigation regime, assigned cultivars in different groups. Cultivars Licord, Opera and SLM043 were grouped together and showed higher average for all traits compared with other cultivars at all of the irrigation conditions. ISSR analysis using 11 primers produced 54 polymorphic bands in the studied cultivars. Mean PIC and MI of all primers were 0.21 and 1.03, respectively. Cluster analysis based on molecular data using Nei's genetic distance assigned the cultivars into three clusters. Associations between molecular markers and morpho-physiological traits, were assessed by stepwise multiple regression analysis at different stress levels. The highest amount of variation contributed by ISSR markers belonged to relative leaf water content (78%) at non-stress condition, to root/shoot index (66%) at moderate stress condition and to root length (53%) at severe stress condition.

Keywords: Genetic variation, ISSR markers, Rapeseed, Water stress

Introduction
Oil seeds are the second source of food after cereals. Rapeseed (*Brassica napus* L.) is an important agricultural crop grown primarily for its edible oil. The meal that remains after oil extraction has value as a source of protein for the livestock feed industry (Jensen *et al*. 1996). According to FAO (2009), rapeseed is the third most important source of oil seeds crop in the world after soybean and palm oil. Rapeseed contains about 40-44 % oil and is one of the major oilseed crops that grown profitably in rotation with wheat (Carmody 2001). Because of high water use efficiency, drought tolerance and also moderate tolerance to saline soil conditions, rapeseed has a special position for production in arid regions (Nielson 1997; Albarrak 2006).

Water shortage is the most significant factor restricting plant growth and crop productivity in majority of the agricultural fields of world (Tas and Tas 2007). The production of the rapeseed plant is limited by soil salinity and water shortage. Therefore, development of varieties with increased salinity and drought tolerance is important for growing this economical plant in regions where water is limited. Germination may occur in soils with low water content (Anastasi *et al.* 2003). However, this may cause delayed and

reduced germination and seedling growth, with negative effects on crop establishment, crop-weed competition and final grain yield (Willenborg *et al*. 2004; Andalibi *et al.* 2005).

DNA markers have been valuable in crop breeding, especially in studies on genetic diversity and gene mapping. The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs) or microsatellites (Staub *et al*., 1996; Gupta and Varshney 2000). The major limitations of these methods are low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism. ISSR-PCR (inter simple sequence repeat) is a technique that overcomes most of these limitations (Meyer *et al*. 1993; Wu *et al*. 1994; Gupta *et al*. 1994; Zietkiewicz *et al.* 1994). The technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility, possibly due to the use of longer primers (16–25 mers) as compared to RAPD primers (10-mers) which permits the subsequent use of high annealing temperature (45–60ºC) leading to higher stringency. ISSRs have been proposed as a new source of genetic markers, which overcome the technical limitation of restriction fragment length polymorphisms (RFLP) (Ibrahim *et al.* 2011). ISSRs have been successfully used in the study of genetic diversity at inter and intra specific level in a wide range of crop species including rice (Joshi *et al.* 2000), cotton (Liu and Wendel 2001; Sharaf *et al*. 2009), rapeseed (Wakui *et al*. 2009). Molecular markers associated with quantitative trait loci (QTL) for drought adaptive traits could greatly enhance progress in breeding for drought tolerance. Molecular markers improve the efficiency of breeding by allowing manipulation of the genome through marker-assisted selection (Ibrahim *et al*. 2011). Therefore, in this investigation, some morpho-physiological traits and their relations with ISSR markers in 10 rapeseed cultivars were studied under water stress conditions.

Material and Methods

In this study, 10 cultivars of rapeseed (*Brassica napus* L.) (Opera, Adder, SLM043, SLM046, Elvis, Okapi, Elite, Ebonit, Orient and Licord) were grown in a factorial experiment based on randomized complete block design with three replications in a controlled greenhouse (20±3°C temperature of day, 16±3°C temperature of night, 16/8h day/night photoperiod) under different irrigation conditions. Irrigation levels were full irrigation (control) and irrigation after depletion of 60 and 85% of available soil water.

Before planting, 100 cm^3 volumes of undisturbed soil samples were taken from four pots. Samples were oven dried at 105°C and bulk density was calculated from cylinder volume and dry soil mass (Jacob and Clark 2002).

Field capacity of a soil is the approximate water content at which the internal drainage of water through the soil profile due to gravity becomes negligible. The method for determination of water content at field capacity (q_{FC}) consisted of heavy watering of pots and monitoring the soil

moisture variation over time until the moisture of soil clearly tended to converge to a certain value taken as q_{FC} (Cavazza *et al*. 2007). During the monitoring of soil moisture, the top surface was covered with black plastic to prevent evaporation.

Particle size distribution was measured by using hydrometric method (Jacob and Clark 2002). Wilting point was estimated using the ROSETTA software (Schaap *et al*. 2001). Crop coefficients were obtained from FAO-56 tables (Allen *et al*. 1998). Relative humidity in the greenhouse and the amount of evaporation from class A pan (Epan) was recorded daily and reference evapo-transpiration (ET_O) was obtained using pan coefficient. Available water (AW), crop water requirement (ET_C) and irrigation frequency (I) were calculated based on Allen *et al*. (1998).

After depletion of 60 and 85% of available soil water, pots were irrigated to the field capacity level regarding the root depth at each growth stage.

In the control treatment, plants were irrigated based on their need from planting to harvest.

Two weeks after water stress treatment, chlorophyll and fluorescence indices were measured with Chlorophyll Meter SPAD-502 (Konica Minolta) and Chlorophyll Fluorometer OSI-30 (ADC Bioscietific), respectively. Root area was calculated by the following formula:

$$\text{Root area}=2*\{(\text{Root volume})*3/14*(\text{Root length})\}^{0/5}$$

Relative water content (RWC) was obtained by floating the leaf discs (five discs from each leaf with a 50 mm diameter) on distilled water for 24 hours at 4°C under dim light. Then, the turgid weight (TW) was determined after floating and the dry weight (DW) was obtained after the samples were dried for 24 hours at 70°C. Fresh weight (FW), TW and DW were used to calculate RWC as follows (Barrs and Weatherly 1962):

$$RWC= (FW-DW)/(TW-DW)$$

The DNA of leaf samples from 6 plants of each cultivar were extracted separately using the CTAB procedure according to Saghai-Maroof *et a*l. (1984). The quality and quantity of DNA samples were assessed using spectrophotometer and 0.8 percent agarose gel electrophoresis. All of the DNA samples were diluted to 25ng/µl and used in PCR reactions. Thirty four ISSR primers [from Bioneer Company (South Korea)] were used to study polymorphism in the plants and polymorphic primers were used for genotyping.

PCR reaction for ISSR primers was performed in a volume of 18µl containing 25 ng of DNA template, 2.22 mM $MgCl_2$, 0.11 mM each dNTPs, 0.44 µM primer, 1U *Taq* DNA polymerase and 1x PCR buffer. Amplification was programed for 5-min initial denaturation step at 94^{o}C followed by 35 cycles of denaturing at 94^{o}C for one min, annealing at 49-60^{o}C for one min, extension step at 72^{o}C for one min and a final extension step at 72^{o}C for 5 min. Amplification products were separated by 1.5% (w/v) agarose gel electrophoresis and were stained with ethidium bromide. ISSR bands were scored manually as present (1) or absent (0). Data were analyzed using SPSS 16, GenAelex 6.4, NTSYS 2.2 and PopGen32 software.

Results and Discussion

Physical properties of the soil were as follows: soil texture was clay loam; bulk density ($g.cm^{-3}$) was 0.9; water content at field capacity ($g.g^{-1}$) was 0.39 and wilting point moisture ($g.g^{-1}$) was 0.14.

The results showed that water stress had significant effect on all of the traits ($P \leq 0.01$). Significant differences were also observed among cultivars (Table 1). Water stress decreased shoot and root dry weight, root length, volume and area, number of leaves, root/shoot index and relative water content but increased SPAD and Fv/Fm (Table 2). It seemed that increased chlorophyll content caused decrease in light repression and increase in Fv/Fm. The Fv/Fm could be used as a physiological index for selecting osmotic stress tolerant cultivars (Paul Parkhill *et al.* 2001).

Licord and SLM043 cultivars had the highest shoot dry weight. Licord was superior for most of the traits (Table 3). Opera showed the highest root dry weight, root length and chlorophyll content. However, its shoot dry weight, number of leaves, root/shoot index and chlorophyll fluorescence were less than those of Licord. Among the studied cultivars, Licord and Ebonit showed the highest and the lowest number of leaves, respectively. Although water stress reduced the shoot dry weight, number of leaves and RWC in different cultivars, it was observed that Opera and Licord had relatively better performance under water stress conditions, because, they had higher root dry weight, root length, chlorophyll content and chlorophyll fluorescence.

Grouping the studied cultivars by UPGMA cluster algorithm using Euclidean distance is shown in Figure 1. In the normal condition, the first group consisted of Elite, Ebonit, Orient, Opera and SLM046. Elvis, Okapi and SLM043 were placed in another cluster. The mean of these clusters showed the lowest and the highest deviations from the total mean, respectively.

Three distinct clusters at 60% AW (irrigation after depletion of 60% of available soil water) were obtained. The third and first clusters had the lowest and the highest deviations from the total mean, for most of the studied traits, respectively. Opera, SLM043, SLM046 and Licord were located in the second cluster (Figure 1).

Two clusters were identified at 85% AW (irrigation after depletion of 85% of available soil water). Group 1 showed the highest deviation from the total mean, for most of the studied characters. SLM043, Opera, Elvis and Licord were located in the first cluster. Elite, Ebonit and Orient were located in the groups with low averages at the control and stress conditions. Therefore, these cultivars did not show better performance in this experiment. Adder was located in the group with higher means at the normal condition but located in the group with lower averages at the water stress conditions. This variety was not tolerant to the drought stress. Elvis was placed in groups with lower averages under the control and moderate stress conditions, but located in the group with higher averages at the severe water stress regime. Opera was located in the group with lower trait averages at the normal condition while at the water stress environments it was placed in the group with highest average values. This means that Opera was tolerant to water stress. Licord and SLM043 were located in the group that had higher trait

Table 1. Analysis of variance for studied traits of rapeseed cultivars at normal and water stress conditions

Source of variation	df	Mean squares									
		Shoot dry weight	Root dry weight	Root length	Root volume	Root area	Number of leaves	Root/ Shoot	Chlorophyll content	Relative water content	Chlorophyll fluorescence
Replication	2	2.17^{ns}	0.14^{**}	1.57^{ns}	225.81^{**}	1029.64^{**}	0.7^{ns}	0.002^{ns}	5.72^{ns}	58.38^{ns}	0.0001^{ns}
Water (W)	2	757.6^{**}	10.34^{**}	603.18^{**}	15340.54^{**}	105062.7^{**}	204.4^{**}	0.048^{**}	416.56^{**}	8179.28^{**}	0.033^{**}
Variety (V)	9	1.77^{*}	0.04^{*}	82.13^{**}	21.35^{ns}	218.62^{ns}	1.75^{**}	0.009^{**}	39.54^{**}	42.11^{ns}	0.0007^{*}
W*V	18	0.7^{ns}	0.016^{ns}	41.31^{ns}	7.95^{ns}	161.96^{ns}	0.61^{ns}	0.002^{ns}	12.27^{ns}	65.8^{ns}	0.0002^{ns}
Error	58	44.55	1.19	29.64	33.92	178.56	0.63	0.002	10.51	36.79	0.0003
C.V		15.91	13.1	14.53	30	15.64	10.93	19.37	6.03	9.69	2.16

ns, * and ** are non-significant and significant at 0.05 and 0.01 p values, respectively.

Table 2. Means of irrigation levels for the studied rapeseed traits

Water condition	Shoot dry weight (gr)	Root dry weight (gr)	Root length (cm)	Root volume (cm^3)	Root area (cm^2)	Number of leaves	Root/ Shoot	Chlorophyll content (SPAD)	Relative water content (%)	Chlorophyll fluorescence (ms)
Normal	11.17^{a}	1.76^{a}	42.61^{a}	45.43^{a}	153.18^{a}	10^{a}	0.27^{a}	51.03^{b}	7.32^{a}	0.77^{c}
60% AW	3.78^{b}	0.87^{b}	34.23^{b}	8.26^{b}	59.03^{b}	7^{b}	0.21^{b}	58^{a}	63.92^{b}	0.82^{b}
85% AW	1.59^{c}	0.68^{c}	35.23^{c}	4.53^{c}	44^{c}	4.8^{c}	0.28^{a}	52.23^{b}	45.38^{c}	0.83^{a}

Means with the same letter in each column are not significantly different at 0.05 probability level using Duncan's multiple test

Table 3. Means of measured traits in the rapeseed cultivars under study

Varieties	Shoot dry weight (gr)	Root dry weight (gr)	Root length (cm)	Number of leaves	Root/Shoot	Chlorophyll content (SPAD)	Chlorophyll fluorescence (ms)
Opera	5.33	1.15	40.77	7.33	0.29	56.62	0.816
Adder	5.64	0.97	39.11	7.66	0.21	52.22	0.817
SLM043	6.13	1.1	38.77	7.33	0.23	53.82	0.81
SLM046	5.29	1.14	36.66	6.88	0.27	54.35	0.8
Elvis	5.24	1.14	37.05	7.77	0.28	54.23	0.823
Okapi	5.2	1.09	35.27	7.11	0.26	52.72	0.814
Elite	5.42	1.01	34.66	7.11	0.22	55.61	0.8
Ebonit	5.24	1.11	33.05	6.66	0.28	50.62	0.8
Orient	5.1	1.02	36.05	6.77	0.25	51.04	0.821
Licord	6.44	1.17	43.11	8	0.3	56.34	0.821
LSD (5%)	0.82	0.13	5.13	0.74	0.047	3.06	0.016

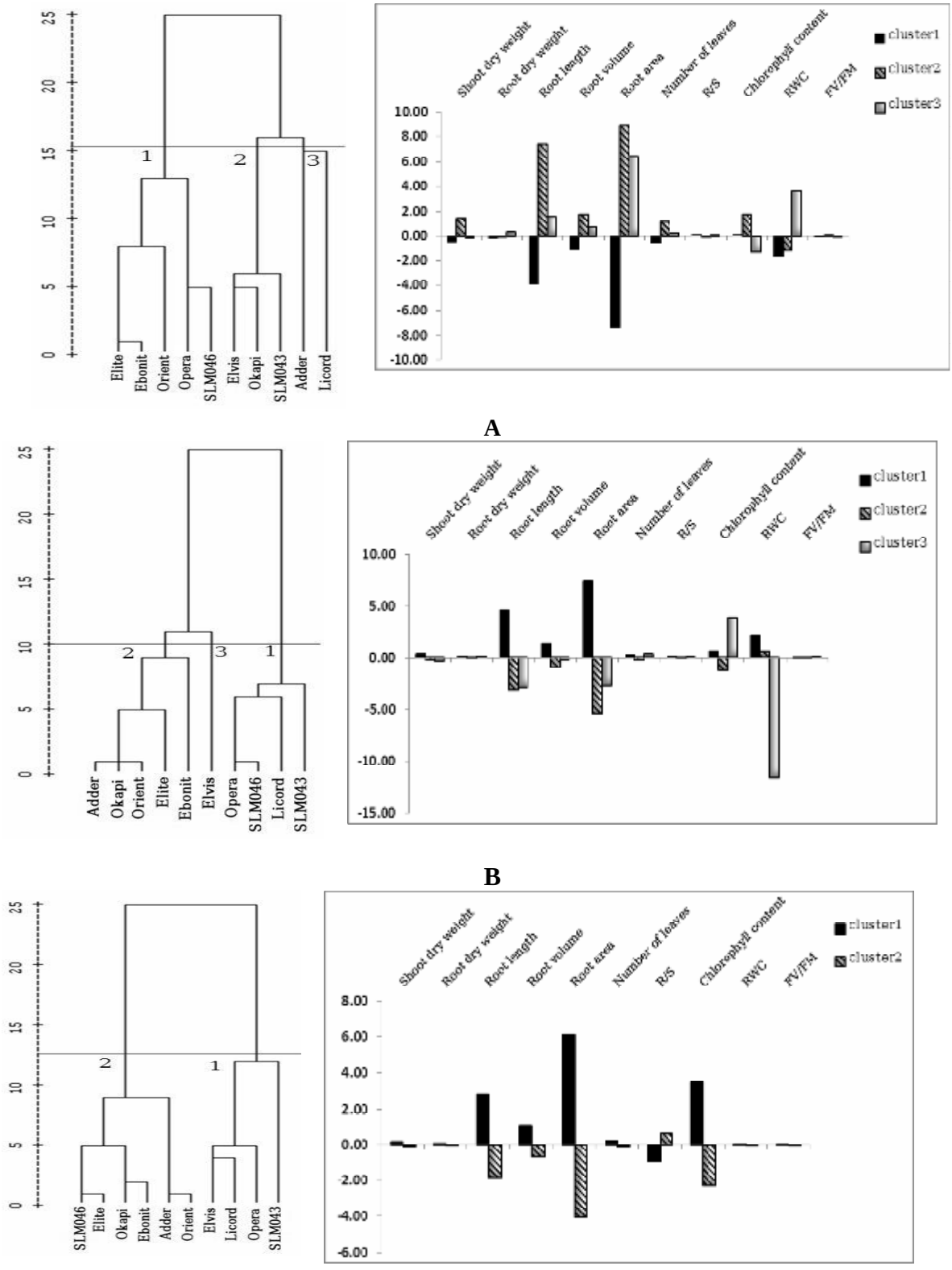

Figure 1. Grouping of 10 rapeseed cultivars using cluster analysis based on UPGMA method with the average deviation of group means from the grand mean of the traits under study. A (Normal), B (60% AW) and C (85% AW).

averages than the other groups at both water stress and non-stress conditions. These cultivars had, also, the largest distance from Elite, Ebonit, and Orient cultivars for evaluated traits. These cultivars could be crossed to produce the base populations for genetic studies and selection programs.

From 34 ISSR primers used in this study, 11 primers showed polymorphisms and produced 64 bands in total. Of these, 54 and 10 were polymorphic and monomorphic, respectively (Table 4). Figure 2 shows P8 primer banding pattern in the studied cultivars. P5, P11 and P13 primers had the highest polymorphism (100%). Charters *et al*. (1996) using three 5′ anchored primers together could distinguish 20 cultivars of *Brassica napus*.

P5 and P22 revealed the highest PIC (0.22) and MI (1.72), respectively (Table 4). PIC provides the value of a marker for detecting polymorphism introduced by Botstein *et al*. (1980). Marker index shows the potential of each primer in the production of more bands (Anderson *et al.* 1993; Powell *et al.* 1996). Chadha and Gopalakrishna (2007) reported PIC values of 0.1 to 0.5 and MI index of 1.54 in the study of genetic diversity using ISSR markers in rice.

The highest (0.4) and the lowest (0.21) amounts of mean genetic diversity within cultivars, based on Nei's gene diversity (Nei 1978), were found in Orient and Adder, respectively (Table 6). The total (H_T = 0.341), within (H_S = 0.221) and the average gene differentiation among the varieties over all loci (G_{ST}=0.34) indicated that there was a good genetic variation within and between the studied rapeseed varieties.

Figure 2 shows the grouping of the rapeseed cultivars based on molecular data using UPGMA cluster analysis method and Nei's genetic distance. The cluster analysis separated the rapeseed cultivars into three clusters. The first cluster consisted of five cultivars: Opera, Adder, SLM043, SLM046 and Elite. Four cultivars, Okapi, Ebonit, Orient and Licord were clusterd together and Elvis was located alone in the third cluster. These results had some similarities with the grouping based on morpho-physiological data.

Based on regression analysis, in total 23 and 30 ISSR markers were associated with the measured traits at the three irrigation levels (Tables 6 and 8). There was only one marker related to shoot dry weight, root area, chlorophyll content, RWC and R/S at the normal irrigation level, whereas three markers were associated with the root dry weight and chlorophyll fluorescence. Positive markers related to RWC could explain 78% of total variance of this trait. R/S related markers could explain 21% of the total variance (Table 5). At 60% AW three markers were associated with root length, number of leaves and RWC, and four markers were associated with shoot dry weight and root area. Markers in association with R/S explained 66% of the variation, whereas amount of explained variance by positive markers for chlorophyll content was 23%. At 60% AW condition, P11M2 marker was the most effective marker associated with studied traits (Table 6). At 85% AW, four markers were associated with chlorophyll fluorescence. Markers in association with root length explained 53% of

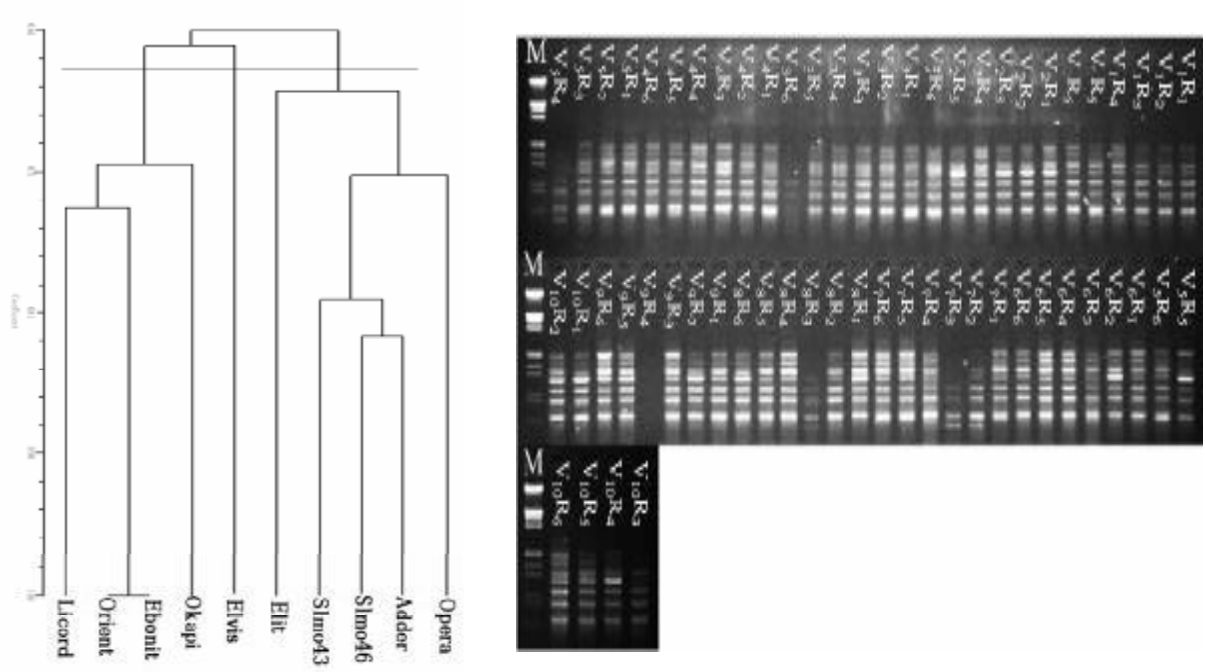

Figure 2. Grouping of the cultivars using molecular data and UPGMA cluster analysis method based on Nei's genetic distance (left) and banding pattern of ISSR-8 primer (right). (V: variety and R: repeat).

Table 4. Primers sequence, total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism, as revealed by ISSR analysis

Primer	Sequence	Total Amp	Mono Amp	Poly Amp	Polymorphism %	PIC	MI
P1	5' AGAC AGACGC 3'	6	2	4	66.6	0.222	0.88
P3	5' AGAGAGAGAGAGAGAGC 3'	6	1	5	83.3	0.203	1.01
P5	5' AACAACAACGC 3'	6	0	6	100	0.225	1.35
P8	5' GACGACGACGACG 3'	8	2	6	75	0.203	1.21
P11	5' GTGGTGGTGGC 3'	3	0	3	100	0.192	0.57
P12	5' TTGTTGTTGTTGTTGC 3'	5	1	4	80	0.197	0.98
P13	5' ACACACACACACACACYG 3'	7	0	7	100	0.215	1.5
P15	5' ACGACGACGACGAAC 3'	4	1	3	75	0.214	0.64
P16	5' CACACACACACAAG 3'	4	1	3	75	0.203	0.61
P22	5' ATGATGATGATGATGATG 3'	9	1	8	88.8	0.215	1.72
P32	5' AGAGAGAGAGAGAGAC 3'	3	1	2	66.6	0.218	0.87
	Total	61	10	51			
	Average	5	0.9	4.6	82.7	0.21	1.03

Mono= Monomorphic Poly= Polymorphic Amp= Amplicons

Table 5. Mean genetic diversity within cultivars based on Nie's (1978) gene diversity coefficient

Cultivar	Genetic diversity	Cultivar	Genetic diversity
Opera	0.25	Okapi	0.33
Adder	0.21	Elite	0.38
SLM043	0.24	Ebonit	0.36
SLM046	0.29	Orient	0.4
Elvis	0.36	Licord	0.37

Table 6. Regression coefficients and adjusted R^2 for the multiple regression of the morpho-physiological traits with ISSR marker at the normal condition

	Shoot dry weight	Root dry weight	Root length	Root volume	Root area	Number of leaves	Root/Shoot	Chlorophyll content	Relative water content	Chlorophyll fluorescence
Intercept	28.5	17.5	53	76.5	220.69	14.38	0.51	52.46	44.52	0.77
P1M1					0.313					
P3M1		-0.342		-0.322						
P8M4				-0.375						
P8M5						0.639		0.533		
P11M2			-0.304							
P11M3			-0.608							
P13M1						-0.339				
P13M6		-0.372								
P16M1	-0.354									0.674
P16M2										-0.352
P22M1									-0.642	
P22M8		0.406					-0.352			
P32M3										-0.357
R^2		0.399	0.54	0.591	0.23	0.417	0.211	0.698	0.787	0.405

the variation whereas amount of explained variance for number of leaves was 33%. At 85% AW, P13M6 marker was the most effective marker associated with the traits under study (Table 7).

DNA molecular markers are important tools which can be incorporated in these kinds of analyses. There is not enough marker data in rapeseed to screen rapeseed genotypes tolerant to water stress.

Conclusion

The plant performance of rapeseed cultivars reduced significantly under water stress, but some traits such as root/shoot ratio, chlorophyll content and chlorophyll fluorescence increased under this condition. These traits may be used for indirect

selection of high yielding rapeseed genotypes under water deficit condition. Licord, SLM043 and Opera were more tolerant to water deficit stress and had, also, the largest distance from Elite, Ebonit and Orient. These cultivars could be crossed to produce the base populations for genetic studies, selection programs and producing mapping populations. Furthermore, several ISSR markers were related with some rapeseed traits that may be used in QTL mapping programs. In addition, ISSR markers revealed relatively high genetic diversity for the rapeseed cultivars under study which can be utilized in the breeding programs.

Table 7. Regression coefficients and adjusted R^2 for the multiple regression of the morpho-physiological traits with ISSR markers at the 60% AW stress condition

	Shoot dry weight	Root dry weight	Root length	Root volume	Root area	Number of leaves	Root/ Shoot	Chlorophyll content	Relative water content	Chlorophyll fluorescence
Intercept	7.29	2.49	34.17	21.19	97.58	9.6	0.245	60.34	30.15	0.83
P1M1	-0.433			-0.38	-0.31					
P3M1			0.331							
P5M2			0.397							-0.343
P8M3									0.455	
P8M5		0.353					0.469		-0.781	
P11M2			0.471		0.426		0.426		-0.371	
P11M3					0.352					
P13M1	-0.338									
P13M5										0.423
P13M6					-0.436					
P15M1						-0.355				
P15M3	0.375							0.392		
P22M1	0.457					0.41				
P22M5						0.34				
R^2	0.391	0.434	0.49	0/3	0.544	0.392	0.661	0.238	0.649	0.431

Table 8. Regression coefficients and adjusted R^2 for the multiple regression of the morpho-physiological traits with ISSR markers at the 85% AW stress condition

	Shoot dry weight	Root dry weight	Root length	Root volume	Root area	Number of leaves	Root/ Shoot	Chlorophyll content	Relative water content	Chlorophyll fluorescence
Intercept	5.03	1.67	34.55	18.4	87.66	9.59	0.4	61.81	28.69	0.84
P3M2									-0.346	
P5M4			0.477							
P8M3			0.658							0.454
P8M4										-0.426
P8M5	-0.331				-0.327					
P11M2		0.689				-0.419				
P11M3				-0.402		-0.444	-0.452			
P12M3						-0.445			-0.445	
P13M1								-0.34	0.331	
P13M6	-0.438	-0.532		0.322					-0.356	
P16M1										0.412
P16M2										-0.523
P22M1		0.387		0.524						
P22M6			0.308							
P22M7								0/337		
R^2	0.533	0.588	0.591	0.356	0.404	0.335	0.438	0.424	0.553	0.449

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