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Increasing Efficiency of Tomato (*Lycopersicon esculentum* Mill.) Resistance to Drought Stress with Conventional and Molecular Genetic Methods

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Reliable and sustainable production of food crops is highly related to genetic diversity and continuous germplasm improvement, particularly, when the desirable traits show high heritability. Drought tolerance of tomato (*Lycopersicon esculentum* Mill.) is a trait to be urgently improved due to recent climate changes and limited water availability. Therefore, a greenhouse screening experiment was carried out at King Abdulaziz University (KAU, Jeddah, Saudi Arabia) in 2014 and 2015 under three levels of drought stress (600, 400 and 200 mL water on 3 days per week) to identify tomato cultivars having improved drought tolerant . Several sensitivity and tolerance indices were determined based on morphological markers. Aiming at establishing a correlation to these markers, a total of 16 Inter-Simple Sequence Repeat (ISSR) primers was used, additionally elucidating the genetic diversity among cultivars and clustering the cultivars into groups based on their molecular profiles. The results indicated that selection indices such as geometric mean productivity (GMP), mean productivity (MP), tolerance index (TOL), and stress tolerance index (STI) represented suitable indices for screening the drought tolerance of tomato cultivars. The cultivars C9 followed by C15 and C11 were identified as the most drought tolerant genotypes, while cultivars C1, C2, C6, C7 and C13 were classified as cultivars being sensitive to drought stress. An interesting correlation of the ISSR analyses to these morphological findings was established according to 83 detectable fragments derived from 10 primers. Among these 83 fragments, 35 were polymorphic across the cultivars. Specific fragments were proposed to be used for future drought tolerance screenings of larger cultivar germplasm. The highest value of the effective multiplex ratio (EMR) and marker index (MI) was detected for primer INC7 followed by INC1. Genetic relationships among the cultivars were evaluated by generating a similarity matrix based on the Jaccard's coefficient and the Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendrogram. Based on Jaccard's similarity coefficients, the genetic distance of the genotypes varied from 0.702 to 0.942 with a mean value of 0.882. The results showed a clear-cut separation of the 15 tomato cultivars due to their genetic variability as compared to local tomato accessions, making them a valuable genetic source for their incorporation into potential breeding programs. Molecular data were in good agreement with the results considering selection indices, and both of them will be useful tools for the future preservation and improvement of the tomato germplasm.

Key words: Tomato (*Lycopersicon esculentum* Mill.), drought stress, drought tolerant/sensitivity indices, genetic diversity, ISSR markers, polymorphic information, cluster analysis.

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Recent climate change scenarios including an increasingly limited water availability will severely affect tomato production in numerous producing countries worldwide. Most commercial cultivars of *L. esculentum* are sensitive to abiotic stresses, particularly to drought stress, during all stages of plant development (Kibreab *et al*., 2013; Allestrofa, 2014). Besides counteracting climatic changes, adaptation to drought stress is of high relevance to expand tomato cultivation to a wider range of environments, allow longer growing seasons, and increase quantitative and qualitative yield stability. In order to deduce an effective breeding strategy to achieve these goals, detailed knowledge of the nature and magnitude of genetic variability present in germplasm and the degree of transmission of the economic traits is a prerequisite of selecting suitable and promising parents (Reddy *et al*., 2012). Moreover, knowledge about the available genetic diversity is of utmost importance for: (i) classification of germplasm and analyses of genetic relationships among breeding material (Mohammadi and Prasanna, 2003), (ii) identification of possible loss of genetic diversity (Zubair *et al*., 2007), (iii) understanding the development of genotypic variations (Beyene *et al*., 2005), and (iv) selection of high-priority genotypes for conservation (Thormann *et al*., 1994).

Owing to recent developments, plant breeders can now complement phenotypic assessments of potential parents with a genotypic assessment of molecular diversity markers (Beyene *et al*., 2005). Various previous studies reported the genetic diversity among different accessions, including varieties and populations, which were selected based on morphological and agronomic traits (Pandey *et al*., 2009; 2011 and Sif *et al*., 2013) or physiological behavior (Avola *et al*., 2008). However, the applied model systems of identification were often restricted by a number of limitations, including low polymorphism, low heritability, and late expression. Moreover, variations in environmental factors and variable stages of plant development hampered the elucidation of real genetic variations, because of interactions of environment-dependent genetic control of polygenic morphological and agronomic traits (Smith and Smith, 1992; Fabio *et al*., 2010). Due to such disadvantages, breeders have recently focused on the use of DNA-based genetic markers, and integrated it into several plant systems.

The use of molecular markers has been proposed for breeding programs, where marker assisted selection (MAS) aims at the replacement or complementation of the conventional phenotypic selection (El-Nahas *et al*., 2011). MAS is highly useful for the development of new plant varieties, particularly when different pre-existing elite varieties are crossed to start a new cycle of selection (Lande and Thompson, 1990). By these means, a robust estimate of genetic similarity is derived, being otherwise inaccessible when using morphological data alone. Therefore, MAS represents a most valuable tool in breeding for stress tolerance (Abdel-Tawab *et al*., 2003a; Witcombe *et al*., 2008). Among the most promising and widely used markers, inter-simple sequence repeats (ISSR) markers have been successfully used to map plant genomes, identify stress tolerant cultivars, assess genetic diversity, and study interspecific and intraspecific relationships in different crops such as potato plant breeding (Gorji, 2011). For the determination of ISSRs, repeat-anchored primers are used to amplify DNA sequences between two inverted SSRs (Reddy *et al*., 2009). Particularly, AG or GA or $(GATA)_n$ repeats have been demonstrated to be highly informative and cost-effective in revealing genetic relationships among diverse tomato accessions (Rao *et al*., 2006). In addition, they are probably linked to genomic DNA sequences with significant effects on the abiotic stress tolerance (Kaushik *et al*., 2012). The presented study was conducted to compare the usefulness of morpho-agronomic and ISSR markers in order to decipher the extent of genetic variation, genetic relationship, and diversity among 15 tomato cultivars. Furthermore, correlations between distance estimates based on morpho-agronomic traits and DNA molecular marker should be investigated.

MATERIALS AND METHODS

15 tomato (*Lycopersicon esculentum* Mill.) cultivars were kindly provided and identified by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany. Their wide diversity of geographical origins is shown in

Table 1. Accession number, commercial name, serial code, botanical name, and origin of 15 tomato genotypes used for drought resistance evaluation

Accession no.	Commercial name	Co.	Botanical name	Origin
LYC3912	Dedication	C1	*Lycopersicon esculentum* Mill.	Russia
LYC4112	Anna Aasa	C2	*Lycopersicon esculentum* Mill. convar. *infiniens* Lehm. var. *flammatum*	Russia
LYC2019	Gelbfruechtig	C3	*Lycopersicon esculentum* Mill. convar. *infiniens* Lehm. var. *cordiforme*	Germany
LYC192	Australische Frühe	C4	*Lycopersicon esculentum* Mill. convar. *infiniens* var. *commune* L.H.Bailey	Australia
LYC3152	Australische Rosen	C5	*Lycopersicon esculentum* Mill.	Australia
LYC2431	Vencal	C6	*Lycopersicon esculentum* Mill. convar. *fruticosum* Lehm. var. *speciosum* Lehm	Netherlands
LYC2432	Zevat	C7	*Lycopersicon esculentum* Mill. convar. *fruticosum* Lehm. var. *speciosum* Lehm	Netherlands
LYC4242	Petomech	C8	*Lycopersicon esculentum* Mill. convar. *fruticosum* Lehm. var. *speciosum* Lehm	Italy
LYC4079	Sankt Ignatius	C9	*Lycopersicon esculentum* Mill. convar. *infiniens* Lehm. var. *commune*	Italy
LYC1346	Sintesti	C10	*Lycopersicon esculentum* Mill. convar. esculentum var. *esculentum*	Romania
LYC359	Tiganesti	C11	*Lycopersicon esculentum* Mill. convar. *infiniens* Lehm. var. *flammatum* Lehm	Romania
LYC2937	Florida MH-1	C12	*Lycopersicon esculentum* Mill. convar. *fruticosum* Lehm. var. *finiens* Lehm	USA
LYC2493	Sandpoint	C13	*Lycopersicon esculentum* Mill. convar. *fruticosum* Lehm. var. *pygmaeum* Lehm.	USA
LYC2987	California	C14	*Lycopersicon esculentum* Mill.	USA
LYC4113	California Red Cherry	C15	*Lycopersicon esculentum* Mill. convar. *parvibaccatum* Lehm. var. *cerasiforme* (Dunal) Alef.	USA

Table 1. The experiments were conducted during the period from September 2014 to March 2015 in greenhouses of the Biological Science Department, Faculty of Science, King Abdulaziz University, Jeddah, KSA in cooperation with the Institute of Food Science and Biotechnology, Plant Foodstuff Technology and Analysis, University of Hohenheim, Stuttgart, Germany.

Tomato plants (27 plants/cultivar) were grown in pots (30 cm diameter, volume of 1.1 L), containing a mixture of peat moss and quartz sand at a ratio of 1:3. Split plot combination of the treatments was laid out in a randomized complete block design (RCBD) with three replicates, setting up the pots in rows. Three levels of drought treatments were applied to the main plots, and tomato cultivars were assigned to the subplots. Each subplot consisted of one pot with three plants. Plants supplied with 600 mL of water three times a week were considered as control treatment (T_0), while two levels of reduced irrigation of 200 and 400 mL (two times a week) mimicking mild drought stress (T_1 and T_2, respectively). The plants developed at 22/16°C (day/night) and under a relative humidity of 60% for the entire growth period. They were fertilized twice, end of October and in December, using liquid fertilizer (A 15-10-5 fertilizer contains 15% nitrogen, 10% phosphorus, and 5% potassium). After 4 months from transplanting root length (RL), shoot length (SL), root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW), shoot/root length (S / R L), root/shoot dry weight (R / S D W), number of leaves (NL), leaf fresh weight (LFW), leaf dry weight (LDW), number of branches (NB), number of inflorescences (NI), number of fruits (NF), fruit fresh weight (FFW), and yield (Y) were determined. Analyses of variance and mean comparison of variables were performed for all traits recorded by MStat-C, version 2.10 (Michigan State University, USA) using Duncan's multiple range test (Duncan, 1955).

Drought tolerance/sensitivity indices

Drought tolerance and stress sensitivity indices were calculated for each genotype based on shoot fresh weight **(Table 2).**

Molecular Marker

Extraction and Purification of Genomic DNA

DNA was extracted from 0.2 g of randomly taken fresh young leaf tissue of plants, using the

Table 2. Drought tolerance/sensitivity indices and their equations.

Drought Tolerance/sensitivity indices	Equation	References
Stress Sensitivity Index (SSI)	$SSI= [(1- (Y_{si}/Y_{pi})/ SI]$	Fischer and Maurer (1978)
Stress Tolerance Index (STI)	$STI= [Y_{pi} \times Y_{si}] / (Y_p)^2$	Fernandez (1992)
Tolerance Index (TOL)	$TOL = Y_{pi} – Y_{si}$	Hossain et al. (1990)
Geometric Mean Productivity (GMP)	$GMP = (Y_{pi} \times Y_{si})^{0.5}$	Fernandez (1992)
Mean Productivity (MP)	$MP = (Y_{pi} + Y_{si}) / 2$	Hossain et al. (1990)
Yield Index (YI)	$YI = Y_{si} / Y_s$	Gavuzzi et al. (1997)
Yield Stability Index (YSI)	$YSI = Y_{si} / Y_{pi}$	Bouslama and Schapaugh (1984)

Y_{pi} and Y_{si} are the shoot fresh weight of a genotype after normal and stressed regeneration, respectively. SI is the stress intensity as calculated by $SI = 1-(Y_s/Y_p)$; Y_s and Y_p are the mean shoot fresh weights of all genotypes under stress and normal conditions, respectively

Qiagen DNeasy kit (Qiagen, Santa Clara, CA, USA). DNA concentration was determined after diluting the DNA 1:5 in dH_2O. Extracted DNA samples were electrophoresed in 0.7% agarose gel against 10 ìg of a DNA size marker (Lambda DNA digested with *HindIII* and Phi x 174 DNA digested with *HaeIII).* This marker covered a range of DNA fragment sizes from 310 to 23130 base pairs (bp) within a concentration range of 11 and 95 ng. DNA concentrations in a given sample were estimated by comparing the intensity of fluorescence of the unknown DNA band with that of bands of the DNA size marker.

Inter-Simple Sequence Repeat Analysis

PCR was performed in 25 µL reaction volume containing the 2X ready mix (Emerald Amp Max PCR master mix) by Takara Clontech (Madison, CA, USA), 25 pM oligonucleotide primer, and 50 ng genomic DNA. A set of 16 ISSR primers synthesized by Bioron (Ludwigshafen, Germany) were used in this study, although we only show results of 10 primers **(Table 3).** DNA amplification was performed applying 35 cycles using PerkinElmer (Akron, OH, USA). Cetus 480 DNT Thermal Cycler (Perkin Elmer Ltd, Norwalk, CA, USA) as follows: an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation step at 94°C for 1 minute, annealing temperature (Ta) for 1 minute, and an extension step at 72°C for 1 minutes, and final extension step at 72°C for 10 minutes. Amplification products were separated by horizontal gel electrophoresis using 1.5 % (w/v) agarose gel on 0.5×TBE buffers (50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.3) under a constant voltage of 80 V for 2 h, stained with 1 ìg mL^{-1} ethidium bromide. 1Kb DNA Ladder (250 to 1000 bp), supplied by Thermo Fisher Scientific (Watham, MA, USA), was used as DNA marker, and applied in the first column of the gel. The samples were arranged on the gel from left to right in numeric order.

Bands were visualized in a UV transilluminator (Sigma-Aldrich, St. Louis, MO, USA at 300 nm and photographed using gel documentation equipment (Bio Rad, Hercules, CA, USA). Amplified products were scored as 1 or 0 depending on its presence or absence, respectively. The ABI Gene Scan software (Applied Biosystems, Riyad, KSA) assigned non-integer base-pair size values to detected fragments. Individual fragments were assigned to alleles of the appropriate microsatellite loci. Allele binning was carried out as follows: (1) fragments were arranged by descending size; (2) fragments being separated by less than 2 bp were binned together, maintaining standard deviations below 2 bp; and (3) the obtained mean size was determined and rounded off to the nearest whole base pair integer to yield the molecular weight of the allele. Number of total loci (NTL) and number of polymorphism loci (NPL) were calculated for each primer. Polymorphic ratio (P%) was calculated based on the ratio of NPL/NTL. Each locus contained a maximum of two alleles (presence and absence) instead of multiple alleles (>2), where different sizes of amplified bands are assumed to be alleles of the same locus. The polymorphism information content (PIC) of a marker was calculated according to a simplified version of Anderson *et al*., (1993).

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

Table 3. Code name of primers, repeat motif and sequence of the primers used in ISSR detection

Marker	Repeat Motif	Sequence of primers	Marker	Repeat Motif	Sequence of primers
INC1	$(AG)_8$YC	5'-AGAGAGAGAGAGAGAGYC-3'	INC9	$(GATA)_4$GC	5'-GATAGATAGATAGATAGC-3'
INC2	$(AG)_8$YG	5'-AGAGAGAGAGAGAGAGYG-3'	INC10	$(GACA)_4$AT	5'-GACAGACAGACAGACAGACAAT-3'
INC3	$(AC)_8$YT	5'-ACACACACACACACACYT-3'	INC11	$(AC)_8$YA	5'-ACACACACACACACACYA-3'
INC4	$(AC)_8$YG	5'-ACACACACACACACACYG-3'	INC12	$(AC)_8$YC	5'-ACACACACACACACACYC-3'
INC5	$(GT)_8$YG	5'-GTGTGTGTGTGTGTGTYG-3'	INC13	$(AG)_8$YT	5'-AGAGAGAGAGAGAGAGYT-3'
INC6	CGC$(GATA)_4$	5'-CGCGATAGATAGATAGATA-3'	INC14	$(CTC)_4$TT	5'-CTCCTCCTCCTCCTCTT-3'
INC7	GAC$(GATA)_4$	5'-GACGATAGATAGATAGATA-3'	INC15	$(CT)_8$RG	5'-CTCTCTCTCTCTCTCTRG-3'
INC8	$(AGAC)_4$GC	5'-AGACAGACAGACAGACGC-3'	INC16	$(TC)_8$A	5'-TCTCTCTCTCTCTCTCA-3'

Y (C,T) and R (A,G)

Where *Pi* is the frequency of the *j*th allele of the *i*th marker, and n is the number of band positions analyzed in the set of accessions. Furthermore, average number of alleles, total heterozygosity and average PIC values were calculated. Resolving power (RP) for individual marker systems was calculated according to **RP = ΣIb,** where Ib (informativeness) takes the value of **1-[2 x |0.5-p|],** and p is the proportion of the tested accessions that contained the allele (Prevost and Wilkinson 1999). Effective multiplex ratio (EMR) is the product of the fraction of polymorphic bands and the number of polymorphic bands (Joshi and Nguyen, 1993). Marker index (MI) was determined according to (Powell *et al*., 1996) as the product of PIC and EMR. The presence or absence of alleles for each ISSR was recorded for all cultivars and converted into a genetic matrix. Employing the computer package NTSYS.pc (Rohlf, 2000), Jaccard's similarity coefficients were calculated and used to identify genetic relationships among the genotypes based on the unweighted pair group method of arithmetic averages (UPGMA) and sequential agglomerative hierarchical nested (SAHN) clustering for both the morpho-agronomic traits and molecular markers.

Statistical analysis

Analyses of variance and comparison of means of variables were performed by MStat-C, version 2.10 (Software, MSU, USA). Correlation analyses were performed regarding different selection indices and traits measured for each drought level using Microsoft Excel 2007. Ward's minimum variance clustering method was used to classify genotypes into discrete clusters (Romersburg, 1988).

RESULTS AND DISCUSSIONS

Drought tolerance and sensitivity indices

The analysis of variance revealed highly significant differences among tomato cultivars for all traits investigated under the same drought conditions **(Table 4)**, suggesting a high degree of phenotypic diversity among the cultivars. Particularly, fruit yield, shoot fresh weight, and leaf fresh weight showed a wide range of genotype-dependent variation, while root/shoot dry weight, number of inflorescences, number of branches and root dry weight exhibited a narrow or small

Table 4. Analysis of variance (ANOVA) for effect of cultivars and drought levels on different root, shoot, leaves, and fruits parameters of tomato (*Lycopersicon esculentum.*)

SOV	df	Root length(RL)	Shoot length(SL)	Root fresh weight(RFW)	Shoot fresh weight(SFW)	Root dry weight(RDW)	Shoot dry weight(SDR)	Shoot / Root Length (S/R L)	Root/Shoot dry weight (R/SDW)
Genotypes	14	166.2*	956.2*	282.5*	24325*	243.08*	818.1*	0.974*	0.262*
Treatments	2	591.2*	3315.1*	7807.9*	138274*	406.8*	4953.5*	0.4075*	0.0115ns
G. X T.	28	108.8*	114.73*	931.4*	2560*	61.28*	128.4*	0.1346*	0.055*
Error	88	41.62	26.47	60.99	302.7	10.91	18.54	0.078	0.020
LSD (0.05)	6.08	4.85	7.36	16.41	3.114	4.06	0.264	0.1354	
Genotypes	2.72	2.16	3.29	7.35	1.39	1.81	0.118	0.060	
Treatments G. x T.	10.53	8.40	12.75	28.4	5.39	7.03	0.457	0.234	

variance (data not shown). Most traits were negatively affected when plants were exposed to drought stress, in particular, when applying high stress treatment (T2). This effect was differently pronounced among the cultivars as shown by the exemplary photographs displayed in **Figure 1**. Improvement of these traits with a small value of variation might be limited if not impossible by simple selection of genotypes from the germplasm used in this study according to Ajmal *et al.* (2013).

To identify tomato cultivars having a superior resistance to drought stress, different sensitivity and tolerance indices were determined based on shoot fresh weight. Stress sensitivity indices (SSI) indicated a higher degree of drought susceptibility for cultivars C8, C9, C10, C11, and C13, while a lower sensitivity was found for cultivars C3 and C14. Clarke *et al*. (1992) and Amini *et al*., (2012) concluded that the identification of drought tolerant cultivars on the sole basis of SSI index may also include those that have low total yields. Therefore, the stress tolerance index (STI) and the geometric mean productivity (GMP) were further considered. The cultivars C1, C5, C15, C11 were ranked among those with highest STI and GMP, indicating their drought tolerance. While cultivars C6, C2, C7, C1, and C13 displayed the lowest values of STI and GMP and, thus, were classified as poorly drought tolerant, all other cultivars were characterized as semi-tolerant to drought stress. Accordingly, similar rankings for the tomato cultivars were observed when considering mean productivity (MP) and tolerance index (TOL) indices as well as STI and GMP, which suggested that these indices might be equally suitable for screening of drought tolerant genotypes. Based on these findings, STI, MP, TOL and GMP were proposed to be the most suitable indices for screening drought tolerance of tomato cultivars. Similar results have been reported by Mevlut and Sait (2011), Sharafi *et al.* (2011), Manal *et al*., (2013), Farshadfar *et al*., (2013) and Bradar-Jakanovic *et al*., (2014) for Turkish oat, barley, maize, and wheat and tomato respectively.

Beyond the mentioned parameters, the highest yield stability index (YSI) was obtained for C13 followed by C3, C2, C7 and C6, while lowest YSI values were determined for C10, C8 and C9. Noteworthy, substantial variations were observed among the populations for all the indices, and the

Fig. 1. The effect of different levels of drought levels on plant growth and fruit development of tomato cultivars C1 and C8. T0=600 mL, T1=400 mL, T2=200 mL. C1-C15; Cultivar code according to Table 1.

ranking was not the same for all indices, which may notably complicate the selection of the most drought tolerant cultivar. This different and partly contradictory relationships among the indices and parameters related to drought tolerance have been previously reported by other authors (Anwar *et al*., 2011; Iiker *et al*., 2011), even for the same genotypes tested in different seasons (Farshadfar *et al*., 2012).

Under drought conditions (T1 and T2), cluster analysis based on the indices shown in Table 5 revealed the existence of three groups, namely, drought tolerant, semi-tolerant and sensitive cultivars **(Fig. 2).** The characterization of each cluster group, i.e., the clustered means of selection indices, is summarized in **Table 6** and illustrated in Figure 2. In this analysis, the drought tolerant group, cluster III, contained only one cultivar (C9, Sankt Langatius), being the most drought tolerant cultivar according to its highest STI, GMP, and MP values. The semi-tolerant group (cluster I) contained a total of 9 cultivars, while the third group (cluster II) comprised the 5 cultivars having the lowest drought tolerance according to their sensitivity and yield indices. Thus, these cultivars were susceptible to drought being only suitable for cultivation under irrigated conditions.

Inter-Simple Sequence Repeat analysis

For molecular profiling and analysis of the genetic relationships among all 15 tomato cultivars listed in Table 1, a total of 16 ISSR primers were used for screening of suitable markers. Selection of ISSR primers was based on the number of amplicons recovered through PCR, and reproducibility of the patterns. Consequently, regarding all 16 ISSR primers used, 6 of the primers failed to amplify any fragment, while the rest of the primers (10 primers) was successfully applied to generate and reproducibly amplify specific DNA fragments. The size of the detected alleles ranged from 256 bp to 2300 bp (**Table 7**). These wide average size-range were probably refer to the

Table 5. Yield, tolerance, and productivity indices of 15 tomato (*Lycopersicon esculentum* Mill.) cultivars grown under two different levels of drought (T1 and T2), and control (T0).

Cultivars	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
Ypi	115.5	153	156	192	196	102.5	95	149	283.5	112	195.5	135	87	180.1	187
Ysi	108.75	125.5	155	149	196.5	84.75	97.25	156.25	279.5	166.7	192.75	148.5	74	168	199.25
SSI	1.1	0.619	0.53	0.92	0.87	0.96	1.102	1.19	1.14	1.81	1.14	1.17	0.293	0.58	0.984
STI	0.325	0.464	0.605	0.699	1.01	0.216	0.238	0.625	2.08	0.575	0.989	0.544	0.151	0.75	0.993
TOL	62	45.5	35.5	86.7	81.5	49.25	31.5	94.75	169.7	155.7	116	84	13	47.7	90.5
MP	121.5	142.3	161.2	177.6	210.3	98.9	101.6	169.6	309.1	174.6	213	158	80.5	180.2	210.25
GMP	116.32	139	160.11	168.08	205.6	93.94	100.35	161.87	294.6	155.8	203.2	151.8	79.9	177.9	204.9
YSI	0.593	0.724	0.802	0.607	0.675	0.601	0.732	0.565	0.569	0.384	0.572	0.58	0.85	0.666	0.646
YI	0.695	0.916	1.15	0.995	1.336	0.556	0.685	0.951	1.726	0.76	1.194	0.913	0.343	1.232	1.307

GMP, geometric mean productivity; SSI, stress sensitivity index; STI, stress tolerance index; TOL, tolerance index; YI, yield index; Mean Productivity (MP); Yield Stability Index (YSI); Y_{pi} and Y_{si} are the shoot fresh weight of a genotype after normal and stressed regeneration

enough number of cultivars were evaluated, and may also be due to the particular set of loci tested (El-Malky *et al.* 2007).

The level of polymorphism among the cultivars was evaluated by calculating allele numbers. The marker attributes for the ISSR primers were summarized as PIC, RP, EMR and MI values for each of the 10 primers evaluated **(Table 7).** The number of total amplified loci (NTL) was 83, of which 35 loci were polymorphic with an average polymorphic ratio (P %) of 42.16 %. This low ratio of polymorphisc loci may be probably due to an inherently narrow genetic base .

The number of total loci (NTL) ranged from 6 for primer (INC5) to 11 (INC7) with an average of 8.3 loci per marker. The number of polymorphic loci (NPL) ranged from 1 (INC10) to 7 (INC7) with an average of 3.5 loci. Three unique loci specific to the cultivars C2 and C3 were detected by the primers INC2, INC6 and INC8; these may be converted into specific-specific probes to quickly identify these genotypes or interspecific hybrids of interest during early stages of tomato selection programs. This was in concordance with previous studies (Gyana and Subhashree 2009; Razmjoo *et al*., 2015), where primers based on a GA/AG or GT/TG dinucleotide core as well as those with dinucleotide motifs $(GA)_n$, $(CT)_n$ or $(AG)_n$ repeats generated good banding profiles with a high level of polymorphism. These results were explained by Carvalho *et al*. (2009), who reported that dinucleotide primers were more suitable for amplifying ISSRs, since $(GA)_n$ dinucleotide repeats are the most abundant in plant species.

Moreover, the PIC values, reflecting allele frequency and information content among the cultivars, were estimated. The INC6 primer was the most informative showing the highest PIC value (0.658), whereas INC4 gave the lowest PIC (0.281). The overall average PIC was 0.3958. This moderate PIC value for the ISSR primers used could be attributed to the narrow genetic base of the tomato cultivars and/or highly informative ISSR markers used in this study (Razmjoo *et al*. 2015). EMR is the product of the fraction of polymorphic bands and the number of polymorphic bands. Consequently, primers with higher polymorphism had higher EMR values. The value of EMR varied from 1.001 (INC10) to 6.996 (INC7) with an overall mean of 3.498. MI is the product of PIC and EMR,

Table 6. Comparison profile of the tomato (*Lycopersicon esculentum* Mill.) cultivars group classified by Ward's minimum variance clustering method based on selection indices shown in Table 5.

Cluster groups	Drought levels (T1+T2)								
	$Y_p i$	Ysi	SSI	STI	TOL	MP	GMP	YSI	YI
Cluster I (9)	166.9	170.3	1.1	0.75	88.1	183.9	176.6	0.61	1.09
Cluster II (5)	110.9	98.05	0.82	0.28	40.25	108.9	105.9	0.7	0.64
Cluster III (1)	283.5	279.5	1.15	2.08	169.7	309.1	294.6	0.57	1.73

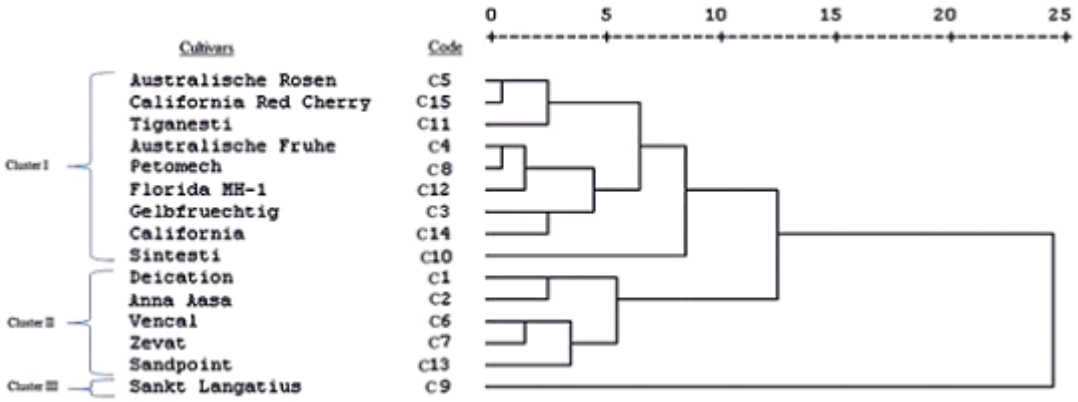

Fig. 2. Dendrogram using Ward's method for clustering tomato cultivars according to their drought tolerance indices shown in Table 5.

ranging from 0.391 to 3.07. The highest MI value (3.07) was observed for INC10, while the lowest MI (0.391) was obtained with INC10. In addition, INC7 showed the highest RP (14), while INC10 exhibited the lowest value (2) with an average RP of 0.7 **(Table 7).** Also, Three of the ISSR primers (INC1, INC4, and INC8) possessed high RP values (10) and, therefore, these three primers addition to INC7 seem to be most informative primers for distinguishing the tomato cultivars. Prevost and Wilkinosin (1999) stated the RP index to provide a moderately accurate estimate of the number of genotypes being distinguishable by a primer. However, RP does not provide accurate information on the ability of primer to reflect genetic or taxonomic relationships among a set of cultivars under investigation. Nevertheless, ISSR markers are expected to be more informative than random decamer primers used in random amplified polymorphism DNA (RAPD), since they allow the amplification DNA segments present at an amplifiable distance in between two identical microsatellite regions oriented in opposite directions. Moreover, ISSR markers are highly reproducible due to the use of longer primers (16-25 nucleotides), resulting in higher stringency as compared to that of random decamer primers (Reddy *et al*., 2002; Anil *et al*. 2015). Furthermore, Razmjoo *et al*. (2015) proposed the parameters MI and RP to be recommended for selecting informative primers. Previously, Kim *et al*. (2013) had employed GD (genetic diversity), PIC, EMR and MI to identify the most suitable primer for ISSR-marker based classification of germplasms, observing a highly significant positive correlation between them.

Among the detected polymorphism bands, a total of 9 bands was found to be useful as positive or negative markers of drought stress **(Fig 3).** These 9 bands were generated by all primers except the primers INC4 and INC10. INC7 and INC8 yielded cultivar-specific amplification fragments at 1393 (C7) and 1180 bp (C3 only) as shown in **Figure 3.** Moreover, INC1 and INC2 produced one amplified DNA fragment of 1950 (C6 and C7 only) and 950 bp (C2 only), which might be specific for these drought sensitive cultivars.

Regarding the ISSR profiles generated by

primers INC3 and INC5, bands with molecular weight 1113 and 662 bp were absent only in the drought sensitive cultivars C5 and C6 (1113 bp) as well as C2, C5, and C6 (662 bp), respectively. Using primer INC6, an amplified fragment of 653 bp was produced only in the drought sensitive cultivar C2, while an INC6-specific band at 285 bp was absent in the drought sensitive cultivars C2, C5 and C6. Polymorphic bands generated by primers INC9 ranged between 632-1847 bp. The smallest respective band (632 bp) was recorded solely in drought sensitive cultivars C1, C2, C5, C6, C7 and C13 with a size around 632 bp, which therefore may be considered a negative marker of drought tolerance. While primers INC2, INC3, INC5, INC8 and INC9 also contributed to generate negative markers by specfic bands (**Fig. 3**), positive markers of drought tolerance were generated by the primers INC4, INC7 and INC8. These positive markers generated specific and exclusive bands in the drought tolerant cultivar C9, and, eventually, also in the moderately drought tolerant cultivars, such as C3, C4, C8, C10, C11, C12, C14 and C15. According to the field trials and morphological parameters, these cultivars showed an acceptable drought tolerance. The correlation to our ISSR results may be useful to accelerate genetic advancement in tomato by using these cultivars as promising parent lines for future breeding. The proposed genetic markers may be more effective and less cost-intense than evaluations based on phenotypic traits, additionally eliminating environmental factors. Our results were in agreement with previous studies (Reddy *et al*., 2009; El-Nahas *et al*., 2011; Rasha K, 2013), which demonstrated the effectiveness of ISSR-PCR to enhance the identification of drought tolerant genotypes regarding different crops. The reliabilty on ISSR data may be improved by using a higher number of primers and cultivars in the future. As described below, ISSR analyses may also be used in detecting possible genetic relationships among cultivars with unknown ancestors (AL-Kordy *et al*., 2013).

Based on simple matching coefficients among the genetic attributes of the 15 tomato cultivars, a cluster analysis was carried out, and a dendrogram was generated The coefficients of genetic similarity obtained in the present study were characterized by a narrow range (0.702 to 0.942), i.e., that the genetic diversity among the 15 cultivars was comparably low (Table 8). Cultivars C7 and C12 revealed the maximum similarity of 0.942 followed by C5 and C10 (0.930), while cultivars C5 and C10 exhibited the least genetic similarity of 0.702 followed by C5 and C13 (0.706) and C2 and C10 (0.736), indicating that these cultivars were not closely related to each other, which was reflected by their highly distinct response to drought stress. Therefore, these cultivars may be considered as diverse genotypes for breeding programs, especially for improving resistance to abiotic stress.

Table 7. Attributes of markers produced by 10 ISSR primers

No.	ISSR Primer	Allele size (bp)		NTL	NML	NUL	NPL	P (%)	PIC	EMR	MI	RP
		Min	**Max**									
1	INC1	490	1950	8	3	0	5	62.5	0.352	5.00	1.76	10
2	INC2	550	2300	9	5	1	4	44.4	0.347	3.996	1.386	8
3	INC3	675	1926	9	7	0	2	22.2	0.365	1.998	0.729	4
4	INC4	524	1562	9	4	0	5	55.5	0.281	4.995	1.403	10
5	INC5	662	1794	6	4	0	2	33.3	0.320	1.998	0.639	4
6	INC6	285	1626	9	7	0	2	22.2	0.658	1.998	1.314	4
7	INC7	256	2633	11	4	0	7	63.6	0.439	6.996	3.07	14
8	INC8	295	1180	7	2	1	5	71.4	0.449	4.998	2.244	10
9	INC9	632	1847	8	6	0	2	25.0	0.356	2.00	0.712	4
10	INC10	464	1453	7	6	0	1	14.3	0.391	1.001	0.391	2
Total			83	48	2	35	414.4	3.958	34.98	13.648	70	
Average			8.3	4.8	0.2	3.5	41.44	0.3958	3.498	1.3648	0.7	

NTL, number of total loci; NML, number of monomorphic loci; NUL, number of unique loci; NPL, number of polymorphic loci; PIC, polymorphic information content; RP, resolving power; P (%), polymorphic ratio; EMR, effective multiplex ratio; MI, marker index.

Genetic cluster analysis was conducted using the unweight pair group method. Subsequently, we constructed a genetic relationship dendrogram according to the ISSR analysis. As illustrated by Fig. 4, the 15 tomato cultivars may be grouped into two major clusters. The first cluster (A) solely including the cultivar C9. As described above, this cultivar may be considered to be more drought tolerant according to its morphological traits. This correlation of results indicates that the ISSR analysis might be a useful tool for investigating drought tolerance of tomato based on genetic markers. In contrast to C9, all other cultivars were predominantly grouped in the second cluster (B), which may be divided into two subclusters (B1 and B2). Subcluster B1 consisted of C14, while subcluster B2 was again subdivided into two groups (B2a and B2b). The cultivars C8 and C5 were included in the group B2a, while the remaining cultivars were included in group B2b (Figure 4). Again, the latter group B2b had been divided into two subgroups (I and II). The first subgroup (I) included most of the moderately drought tolerant cultivars C3, C4, C10,

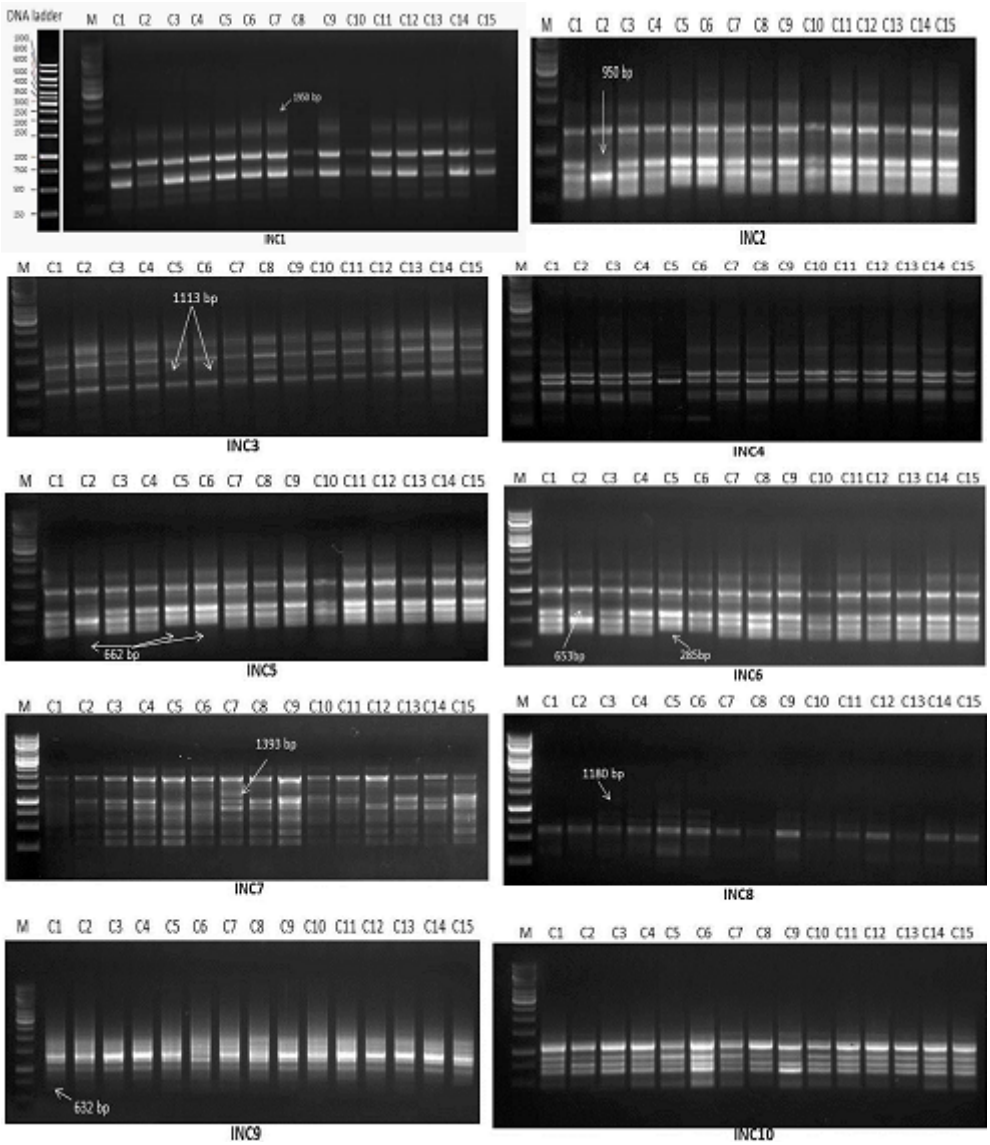

Fig. 3. PCR amplification profile generated from genomic DNA of 15 tomato cultivars with ISSR using 10 primers (INC1-INC10). (M: marker, C1-C15 :tomato cultivars)

Table 8. Similarity matrix for 15 tomato cultivars based on 10 ISSR markers

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
C1	1.000														
C2	0.840	1.000													
C3	0.810	0.776	1.000												
C4	0.845	0.760	0.930	1.000											
C5	0.780	0.746	0.769	0.776	1.000										
C6	0.786	0.753	0.820	0.828	0.916	1.000									
C7	0.871	0.808	0.805	0.837	0.800	0.828	1.000								
C8	0.823	0.736	0.808	0.816	0.753	0.736	0.890	1.000							
C9	0.797	0.786	0.855	0.840	0.826	0.831	0.842	0.794	1.000						
C10	0.820	0.732	0.805	0.867	0.702	0.733	0.814	0.904	0.767	1.000					
C11	0.855	0.816	0.837	0.847	0.783	0.789	0.873	0.826	0.901	0.823	1.000				
C12	0.869	0.805	0.826	0.835	0.797	0.826	0.942	0.840	0.888	0.811	0.898	1.000			
C13	0.850	0.838	0.859	0.842	0.706	0.714	0.842	0.848	0.845	0.818	0.880	0.867	1.000		
C14	0.884	0.819	0.840	0.849	0.786	0.792	0.875	0.828	0.826	0.800	0.859	0.873	0.882	1.000	
C15	0.855	0.791	0.813	0.847	0.760	0.789	0.873	0.852	0.800	0.878	0.830	0.845	0.826	0.8857	1.000

C11, C12, and C15, while all previously identified drought sensitive cultivars C1, C2, C6, C7, and C13 were grouped together in second subgroup (II), which again revealed the good agreement of our genetic ISSR data with the field evaluation data. The grouping of the moderately drought tolerant cultivars C3, C4, C10, C11, C12, and C15 in the same subgroup (I) confirmed their greater genetic similarity. Taking into account that cultivars aggregated together in the same cluster, this indicated a possible common origin of these genotypes (Saleh, 2013). Due to their higher genetic similarity, only low positive heterotic effects may be expected when generating hybrids from these cultivars and, thus, they may be less useful in transgressive breeding than those having less genetic similarity (C5 and C10, C5 and C13). Since C9 was found to be the most promising drought tolerant cultivar, the genetically most dissimilar genotype (C2, similarity index 0.786) may represent a promising mating partner for future breeding to increase drought tolerance. Although being less relevant for increasing drought tolerance, further cultivars of other clusters or subgroup may be combined with the cultivars in subgroup (I) to allow a general improvement of tomato germplasm diversity. Based on these findings, ISSR molecular markers may be used to identify further genotypes characterized by specific stress tolerances, e.g., salt stress tolerance. Most importantly, a sufficiently high number of genotypes with different genetic background must be tested to obtain relevant results. This observation was also supported by Caliskan *et al*. (2012), who concluded that, duplication in the present collection of germplasm should be avoided.

CONCLUSION

A total of 15 different tomato cultivars was grown under two different levels of drought stress, and the obtained morphological data and indices were compared to our ISSR analyses. By these means, a drought tolerant cultivar and several moderately drought tolerant cultivar were identified. Specific ISSR markers were proposed to facilitate future screening for drought tolerant cultivars among a larger germplasm database. The high correlation of our morphological and genetic

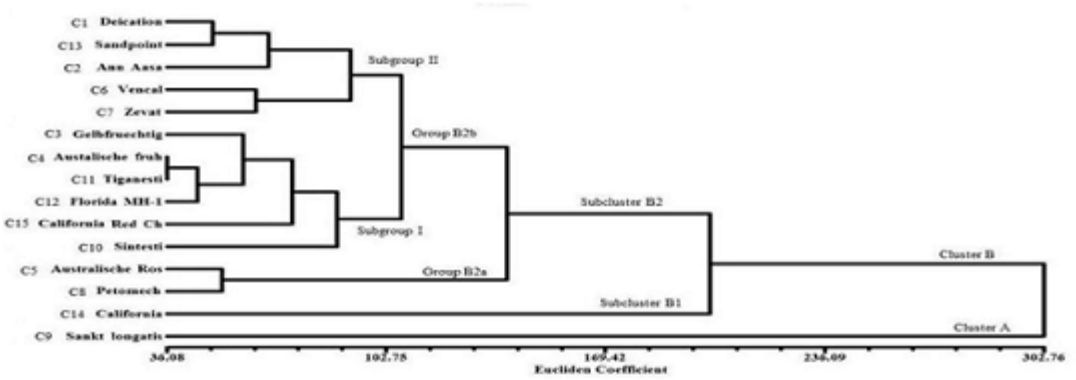

Fig. 4. Dendrogram derived from UPGMA cluster analysis of 15 tomato cultivars based on Nei and Li (1979) similarity coefficient using 10 ISSR markers

markers should encourage further investigators to seek for cultivars of other crops, which possess a tolerance against drought or other types of stress, such as salinity or light stress.

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