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Selection for Drought Tolerance Genotypes in Bread Wheat (*Triticum Aestivuml.*) Under *in Vitro* Conditions Based on Molecular Approaches

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Abstract

Drought is one of a major environmental factors affecting growth and yield of wheat in arid and semi-arid areas of the world, response of four genotypes of bread wheat (Triticum aestivum L.) to mature embryo culture, callus production and in vitro drought tolerance was used in a Completely Randomized Design (CRD) with ten replications. The effect of water stress induced by polyethylene glycol (PEG 6000). Whereas formed calli on MS medium supplemented with 4.0 mg/l of 2, 4- dichlorophenoxyacetic acid (2, 4-D) and 0.5 mg/l kinetin were sub cultured on media containing different concentrations of PEG (0.0, 50, 100, 150 and 200 g/l). DNA-based molecular markers DNA (RAPD) were used in this study to examine the genetic differences among four Iraqi wheat genotypes. Results showed that all primers, except for OPA-15 and VBC3-13, produced different-sized fragments with DNA from one or more of the tested genotypes. RAPD analysis detected a total of 92 DNA fragments of which 86 (93.5%) were polymorphic. The genotype Iba99 produced the highest total number of fragments (47), whereas the Dijlah produced the lowest number (30). Primer B-18 produced the highest number of bands (16). while the least number of bands (8) was recorded for primer VBC3-14. The amplified DNA fragments normally ranged from150bp to1600bp.The maximum polymorphism (100%) was recorded for the primers VBC2-6, VBC3-8 and VBC3-14, whilst the minimum polymorphism was produced by the primer B-18.Maximum numbers of unique DNA fragments (11) were detected in Dijlah genotype, whereas the minimum (2) were detected in Furat genotype. Investigation of genetic distance using RAPD analysis showed that the lowest genetic distance (0.40) 40% was obtained between Iba99 and Furat genotypes, while the highest genetic distance (0.79) 79% was observed between Dijlah and Sham6 genotypes. Analysis of genetic relationship showed there were three groups, the first included Iba99 and Furat. The second contained the genotype Sham6, and the most distant genotype (Dijlah) in the dendrogram stands in the third group. In conclusion, Construction of genetic relatedness tree can done using RAPD molecular marker, the use of molecular markers will be good alternative to the agronomic selection, where it allow a quick selection and provides the breeder with the genetic marker for drought stress. The results of this study can be used as database for Iraqi wheat genotypes to be used in the future development of new varieties in breeding programs.

Keywords: *Bread wheat; Tissue Culture; PEG; Drought stress; RAPD*

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Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereals in the world and Iraq. However, Iraq still imports tons of wheat every year. Abiotic environmental factors are considered the main source for reduction of yield by 71% (Khan., *et al*. 2013). Any excess or deficit of the abiotic stress factors adversely affects the plant growth and productivity (Koyro., *et al*. 2012). Drought and salinity are widespread problems around the world, especially in the middle and south of Iraq. Water deficit stress, which can arise from many environmental conditions, including drought, salinity, and high temperature has adverse effects on the growth and yield of crops, Understanding the physiological, biochemical, and molecular mechanisms of drought tolerance is very important in development of selection and breeding strategies (Aliyev and Huseynova, 2014). In vitro culture of plant cells and tissues such as mature embryos and immature embryos has attracted considerable interest over recent years because it provides a means to study plant physiological and genetic processes and offers a potential to improve cultivars by increasing genetic variability and are considered to be an important complement to classical plant breeding methods (Sorkheh., *et al*. 2011).

In vitro selection technique has been used to improve abiotic environmental stresses such as cold hardiness, salt tolerance and drought tolerance (Zair., *et al*. 2003; Benderradji., *et al*. 2012; El Ameen, 2013). One of the screening techniques based on physiological traits is the use of various osmotica to induce stress in plant tissues. Germination in mannitol and polyethylene glycol (PEG) has been suggested for drought screening (Shabir, 2010; Geravandi., *et al*. 2011). PEG cannot cross embranes and cannot get into the cell to change its osmotic potential (Dragiiska., *et al*. 1996). It stimulates water deficit conditions in cultured cells in a manner similar to that observed in the cells of intact plants subjected to true drought conditions (Yao., *et al*. 2007). Callus culture are used as an *in vitro* technique for biochemical and physiological studies in response to stress at the cellular level (Liu., *et al*. 2006). Many researchers have used the in vitro culture of cells on media supplemented with PEG to study the mechanisms of drought tolerance and to utilize the somaclonal variation, as a source of variability to improve the drought tolerance (El-Shafey., *et al*. 2009; El Ameen, 2013; Hemaid., *et al*. 2013). The response of plants to different abiotic stresses in the field or in greenhouse conditions is often difficult to be analyzed, due to variable nature of these stresses that cannot be controlled, thus in vitro tissue culture-based tools have been allowed a deeper understanding of the physiology and biochemistry in comparison to plants cultured under adverse environmental conditions (Benderradji., *et al*. 2012). Mature embryos can be used as an efficient explants source in wheat tissue culture due to their high frequency for callus induction and plant regeneration capacity (Ozgen., *et al*. 1998).

One of the main phenomena occurring in the cell during water shortage is extensive modification of gene expression (Liu., *et al*. 2013; Savitri., *et al*. 2013). Therefore, through a variety of molecular and biochemical approaches, key genes responsible for drought resistance were studied (Wei., *et al*. 2009). Randomly amplified polymorphic DNA primers (RAPD) is one of the most widely used molecular techniques to detect polymorphism among accessions of different plant species (Shiran., *et al*. 2007). RAPD associated with drought tolerance was used initially to search genetic diversity in wheat plants (El Ameen, 2013). RAPD technology is an important tool for rapid identification of markers associated with drought resistance and is very effective in determining the genetic variation among wheat genotypes (Ali., *et al*. 2013). The RAPD technique has been widely applied in studies of wheat genetic diversity (Iqbal., *et al*. 2007). Soliman and Headway (2013) used RAPD-PCR with four primers to distinguish plantlets with regenerated from the PEG tolerant and control plantlets, four bread wheat genotypes and one wheat line were evaluated for molecular indicators of drought tolerance using RAPD-PCR, and found that RAPD analysis is a valuable diagnostic tool when different sets of RAPD primers were used to study the polymorphism at the molecular level (ElSayed and Rafudeen, 2012). The results of (El Ameen, 2013) indicate that tolerant wheat genotypes harbored seven positive RAPD markers, while sensitive genotypes had only one negative RAPD markers. Hence the objective of the present investigations to evaluate the ability of genotypes to induce callus using mature embryo culture and screening bread wheat genotypes for drought tolerance under in vitro condition using different concentrations of PEG and test RAPD markers associated with tolerant calli.

Materials and Method

Plant Materials

This study was carried out in the Tissue Culture Laboratory, Institute of Genetic Engineering and Biotechnology for postgraduate Studies, University of Baghdad, Iraq. Grains of four bread wheat (*Triticumaestivum* L.) genotypes (Sham-6, Dijlah, Furat and Iba 99) were obtained from Seed Testing and Certification Center - AbuGraib/Ministry of Agriculture, the genotype and their pedigree are presented in table 1. Mature embryos of these genotypes were cultured on callus induction medium to obtain calli which were used for DNA extraction.

Genotype	Pedigree
Dijla	F2 materials derived from a commercial wheat breeding program, USA→ (radiation)
Furat	F2 materials derived from a commercial wheat breeding program, USA→ (radiation)
Iba99	Ures/Bow s / 3/Jup/Biy s /ures
Sham6	W3918A/JUP

Table 1: *The genotypes and their pedigree used in this study.*

Callus Induction

After separation of the mature embryos from ripe grains in a laminar flow hood by using a sterilized metallic scalpel and forceps they were inoculated in the callus induction media (Murashige and Skoog, 1962) supplemented with 4.0 mg/l 2, 4-D, and one embryo per vial with 100 replication for all cultivars respectively. The pH of themedium was adjusted to 5.7 prior to autoclaving at 121°C for 20 min. The scutellums side were kept upward and maintained at 25 ± 1°C temperatures in complete darkness for four weeks for callus induction. During the entire period of inoculation the autoclaved scalpels, forceps, were dipped in 90% absolute alcohol contained in a glass beaker inside the cabinet and flaming the scalpel and forceps with a burner (Bunzen burner) and both the hands were rinsed with 70% ethyl alcohol. All measures were taken to obtain maximum free condition from contamination during the operation. Afterward, induced calli were shifted to callus multiplication media (MS2) supplemented with 4 mg/l 2, 4-D, and 0.5 mg/l kinetin for another period of four weeks. The culture media were refreshed every14 days.

In vitro Selection Procedure of Drought Tolerant Callus.

After four weeks of incubation on callus multiplication media, Healthy calli were separately sub cultured in MS2 medium supplemented with different concentrations of polyethylene glycol (PEG 6000) (0, 50, 100, 150, 200 g/l).

PEG-6000 solutions of (-0.50,-1.48, -2.95, -4.91 Bars was prepared according to the method of Michel and Kaufmann (1973) :

OP = (-1.18 × 10-2) × C - (1.18 × 10-4) × C+ (2.67 x 10-4) × CT +

(8.39 x l0-7) × C2T

Where OP = the osmotic pressure

C = PEG concentration

T = Temperature.

The concentration of the PEG 6000 and their osmotic pressure are illustrated in table 2.

PEG6000 g/kg	Bars of OP at 25°C
50	-0.50
100	-1.48
150	-2.95
200	-4.91

Table 2: *The osmotic pressure (OP) of the PEG 60000 concentration.*

Primer Selection

Ten random primers (decamer) were used in this study to detect genetic polymorphism at DNA level in four genotypes of bread wheat. The primers were supplied by Bioneer Company (Korea). The sequence of RAPD primers were shown in table 3.

Primer name	PCR RAPD primers sequences (5'–3')	References
OPA-15	TTCCGAACCC	Khaled., *etal*. (2015)
E - 10	CACCAGGTGA	El Ameen (2013)
B-18	CCACAGCAGT	ElSayed and Rafudeen (2012)
VBC 3-13	AAGCCTCGTC	Jerodieh., *et al*. (2015)
VBC 2-6	TGCTCTGCCC	
VBC 3-2	GTGAGGCGTC	
VBC 3-8	TGGACCGGTG	
VBC 3-9	CTCACCGTCC	
VBC 3-14	TGCGTGCTTG	
VBC 3-20	ACTTCGCCAC	

Table 3: *The sequence of the 10-base nucleotide primers used for rapd analysis.*

DNA extraction

In this study, DNA was extracted from callus tissues without the use of liquid nitrogen, the aim of using liquid nitrogen is to broken the cell wall. Since callus tissues are soft and freshly, the cell wall can be broken by crushing and grinding the sample into a fine paste using a sterile pestle and mortar, DNA was extracted by using genome DNA purification kit (Geneaid/korea). DNA concentration and purity were assessed by the use of nanodrop.

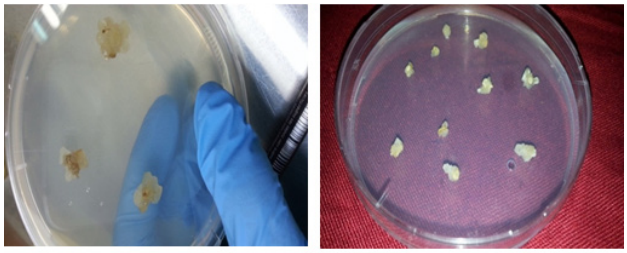
Polymerase Chain Reaction (PCR)

After checking the concentration of genomic DNA by agarose gel electrophoresis for all four wheat genotypes which will use to detect a marker related to drought tolerance. Out of the 10 random primers screened, only eight primers clear reproducible bands. The PCR mixture (25 µl total) consisted of 12 µl PCR pre Mix (Ready to use): Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffer (PH 8.5), 5 µl of primers,3 µl DNA template and 5 µl nuclease free water. Amplification was carried using a program that consisted of initial denaturation for 5 min. at 95°C, followed by 35 cycle of 1 min at 95°C, 45s at 37°, and 1 min at 72°C, and final extension for 7 min at 72°C.

Results and Discussion

Callus Induction and Growth

Mature embryos were cultured on MS medium supplemented with 4.0 mg/l 2, 4-D for four wheat genotypes and incubated in darkness at 25 ± 1°C for four weeks (Figure 1A). 2, 4-D was reported as the most widely used growth regulator for callus induction and maintenance in wheat). Good callus growth was observed on MS medium containing 4 mg/l 2, 4-D, and these results are consistent with the findings of Hemaid., *et al*. (2013). But differ from the results of other researchers (Yu., *et al*. 2008; Salama., *et al*. 2013) who reported that wheat callus induction frequency peaked with 2.0 mg/l 2, 4-D in the induction medium. Induced calli were shifted to callus multiplication (Callus proliferation) on MS medium supplemented with 4.0 mg/l 2, 4-D, 0.5 mg/l kinetin, for 4 weeks (Figure1B). Solid MS medium was optimum for mature embryo culture of wheat supplement with different combinations of plant growth regulators (Mendoza and Kaeppler, 2002).



(A) (B)

Figure 1(A): *Callus formation of wheat after 1 week from inoculation on induction medium which containing MS + 4.0 mg/l 2,4-D.*
(B): *callus at proliferation period after growing on multiplication medium containing 4.0 mg/l 2,4-D and 0.5 mg/l kinetin.*

The callus was growing on callus MS2 medium with different concentrations of PEG for four weeks (Figures 2, 3). The addition of PEG in the medium leads to a loss of cell turgor and hence a loss of growth. The growth of the calli is significantly restricted by increasing and continuous presence of PEG in the media (Dragiiska., *et al*. 1996). Levitt (1980) stated that at the cellular level, the effect of water stress on the retard of cell divisions and elongation by the loss of turgor, and loss of growth (Hemaid., *et al*. 2013).

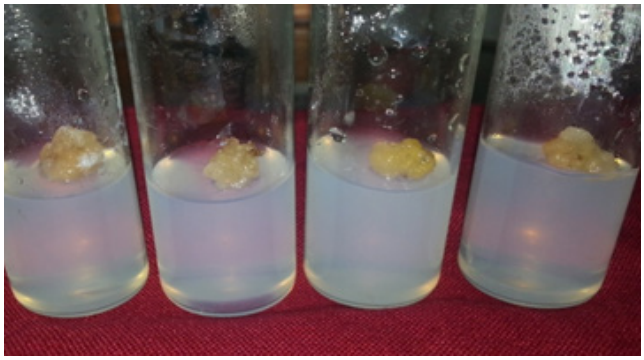


Figure 2: *Callus grown on MS medium solidified with agar at 50% PEG concentration.*

(A) (B)

Figure 3(A): *Callus grown on MS liquid medium with filter paper bridges as carrier for the 100, 150, 200 concentration of PEG.*
(B): *Callus without stress condition.*

Molecular Genetic Marker

In the four wheat genotypes 92 DNA fragments were detected giving an average of 23 alleles per genotype. The number of fragments generated for each primer and for each genotype ranged from 1 to 16. The genotype Iba99 produced the highest total number of fragments (47), whereas the Dijlah produced the lowest number (30). Primer B-18 produced the highest number of bands (16) followed by VBC3-2 (13), VBC3-8 andVBC3-2 which produced 12 bands each, E-10 and VBC3-9 which produced 11 bands each, VBC2-6(9). The least number of bands (8) was recorded for primer VBC3-14 (Figure 4-7). The amplified DNA fragments normally ranged from150bp to 1600bp. ElAmeen (2013) reported that E-10Marker could be considered as reliable marker for drought tolerant in wheat.

In this study RAPDs detected a high degree of polymorphism among studied genotypes. From the 92 fragments detected, with all primers, in the four wheat genotypes 86 (93.5%) were polymorphic with 10.8 polymorphic bands per primer on average. Different percentages of polymorphism in the range of 87.5-100% were obtained by the primers used in this study (Table 4). It is generally reported that polymorphism among genotypes can arise through nucleotide changes that prevent amplification by introducing a mismatch at one priming site; deletion of a priming site; insertions that render priming site too distant to support amplification and insertions or deletions that change the size of the amplified product (Powell., *et al*. 1996).

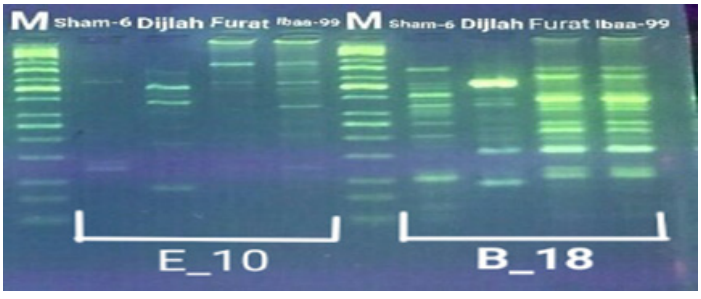


Figure 4: *RAPD_PCR fragments of tow primers (E_10) and (B_18).*

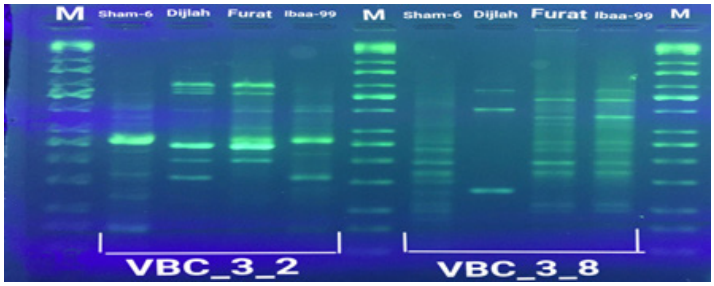


Figure 5: *RAPD_PCR fragments of tow primers (VBC_3_2) and (VBC_3_8).*

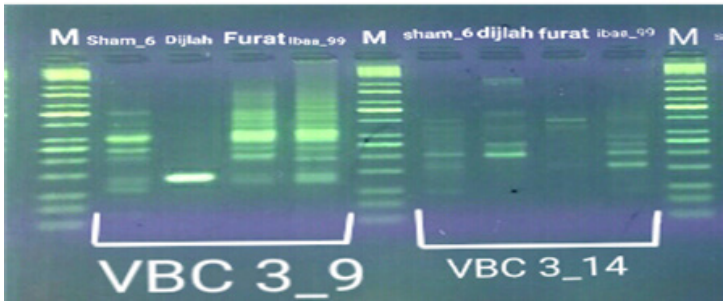


Figure 6: *RAPD_PCR fragments of tow primers (VBC_3_9) and (VBC_3_14).*

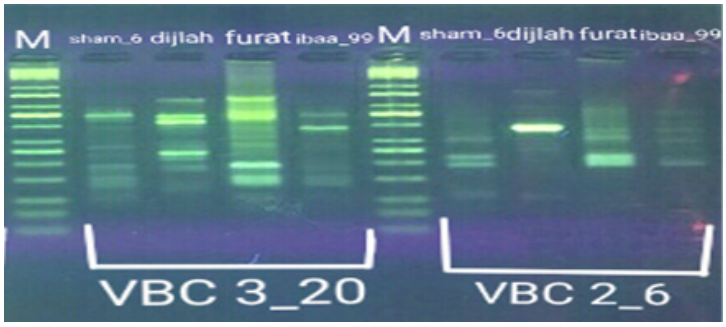


Figure 7: *RAPD_PCR fragments of tow primers (VBC_3_20) and (VBC_2_6).*

No.	Primers	Total number of main bands	Number of Polymorphic bands	Polymorphism (%)
1	E-10	11	10	90.9
2	B-18	16	14	87.5
3	VBC3-2	13	12	92.3
4	VBC3-8	12	12	100
5	VBC3-9	11	10	90.9
6	VBC3-14	8	8	100
7	VBC3-20	12	11	91.7
8	VBC2-6	9	9	100
Total		92	86	Mean = 93.5

Table 4: *Polymorphism rate for the four wheat genotypes using random primers*

The maximum polymorphism (100%) was recorded for the primers VBC2-6, VBC3-8 and VBC3-14, whilst the minimum polymorphism was produced by the primer B-18. The polymorphism obtained in this study (93.5%) were similar with the results of Jerodieh., *et al*. (2015) who used the same primers that used in this study and found 93.8% polymorphic. However, the polymorphism obtained in this study are comparatively high when compared with the results obtained by Nimbal., *et al*. (2009) who reported 80% polymorphism for bread wheat. Ahmed., *et al*. (2010) observed 61% polymorphism for 32 wheat genotypes using 15 RAPD primers. ElSayed and Rafudeen (2012) stated that a total of 72 alleles were amplified with six random primers out of which 61% were monomorphic and 38% were polymorphic. Tonk., *et al*. (2014) showed that of 145 bands70 (41.28%) were polymorphism. Shukre., *et al*. (2015) reported polymorphism were 12.5-80.76% for wheat genotypes. Sabbour., *et al*. (2015). The different primers revealed different levels of polymorphism among the four studied wheat genotypes. This corresponds to a level of polymorphism of 63.7%. The use of considerable and high polymorphic DNA markers eliminates the limitations associated with morphological and biochemical characterization, especially for closely related varieties (Schulman, 2007).

Unique DNA fragments with different sizes were detected in a particular genotype but not in the others (Table 5). In this respect, the 8 unique DNA fragment of 950 and 800 bp length (B-18), 340,250and 150 bp (VBC3-2), 300bp (VBC3-8)220bp (VBC3-9) and 500 bp (VBC3-20) were unique positive markers to the genotype Sham6. The 11 bands780, 500,370 and 160bp (E-10), 180bp (B-18), 1100bp (VBC3-2), 800 and 260bp (VBC3-8), 1100bp (VBC3-14) and900 and 450bp (VBC3-20) were positive markers for genotype Dijlah. A 2DNA fragment of 1600 bp (VBC3-9) and 600bp (VBC3-20) were a unique positive marker to genotype Furat. The 3DNA fragment having a size of 700 and400 bp, and 800bp which were amplified by the primers E-10 and VBC3-14, respectively, were unique to genotype Iba99. Results revealed that DNA markers E-10 and VBC3-14 exhibited no unique band for the genotypes Sham6 and Furat. DNA markers E-18 and VBC3-2 were exhibited no unique band for the genotypes Furat and Iba99. DNA marker VBC3-9 exhibited no unique band for the genotypes Dijlah and Iba99.whereas DNA marker VBC3-20 exhibited no unique band for the genotype Iba99. However no negative markers were recorded for all genotypes Figures (4-7). The results obtained demonstrated that the RAPD assay have a considerable potential for identification and recognition of different wheat cultivars. Such potentiality has also been highlighted by Tonk., *et al*. (2014).

Genotypes Names	Primers Names	unique bands (bp)	numbers of unique bands	Total
Sham6	B-18	950,800	4^{th}, 5^{th}	8
	VBC3-2	340,250,150	10^{th}, 12^{th}, 13^{th}	
	VBC3-8	300	9^{th}	
	VBC3-9	220	11^{th}	
	VBC3-20	500	7^{th}	
Dijlah	E-10	780,500,370,160	5^{th}, 7^{th}, 9^{th}, 11^{th}	11
	B-18	180	11^{th}	
	VBC3-2	1100	2^{nd}	
	VBC3-8	800,260	3^{rd}, 10^{th}	
	VBC3-14	1100	I^{st}	
	VBC3-20	900, 450	3^{rd}, 8^{th}	
Furat	VBC3-9	1600	I^{st}	2
	VBC3-20	600	5^{th}	
Iba99	E-10	700, 400	6^{th}, 8^{th}	3
	VBC3-14	800	4^{th}	

Table 5: *Wheat genotypes and primers that appeared unique bands of genotypes, unique bands (bp) and numbers of these bands.*

The genetic distance (RAPD-GD) of the 4 wheat genotypes, based on the data of 8 RAPD primers was calculated using genetic program (PAST program Ver.1.91 (Hammer., *et al*. 2001), and based on the similarity scale Hamming similarity index). Table 6 illustrates the values of genetic distance of wheat genotypes. The RAPD-GD value ranged from (0.40) to (0. 79). The lowest genetic distance (0.40) 40% was obtained between Iba99 and Furat genotypes indicating that these are relatively less variable, while the highest genetic distance (0.79) 79% was observed between Dijlah and Sham6 genotypes.

	Sham-6	Dijlah	Furat	Ibaa-99
Sham-6	0	0.79	0.51	0.58
Dijlah	0.79	0	0.68	0.75
Furat	0.51	0.68	0	0.4
Ibaa-99	0.58	0.75	0.4	0

Table 6: *Values of genetic distance between wheat genotypes based on RAPD data.*

Dendrogram was constructed the genetic distance using UPGMA cluster analysis and depicted genetic relationships among four wheat genotypes, as clear from in Figure 8, wheat genotypes are grouped into three clusters. Cluster a included genotypes Iba99 and Furat, cluster B included Sham6 and the most distant genotype (Dijlah) in the dendrogram stands in cluster c. The closest genotypes were Iba99 and Furat (60%), while genotype Dijlah was the most diverse and have a low similarity (21%) when compared with other genotypes. Shukre., *et al*. (2015(reported that the value of similarity coefficient ranged from 0.088 to 9999.00. Sabbour., *et al*. (2015) reported that genetic similarity matrices among the four wheat genotypes showed that the range of pair similarity coefficient between genotypes was from 62.65 to 91.95. Al-Kaab., *et al*. (2016) indicated that the Similarity coefficient among sixteen Iraqi varieties depending on twenty seven RAPD primers ranged from 0.339 to 0.983.

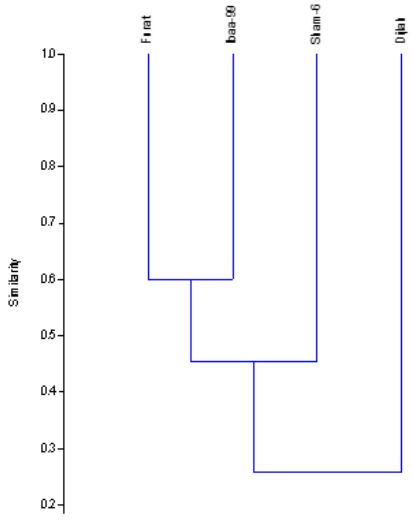


Figure 8: *Dendrogram of wheat genotypes based on data generated using eight RAPD primers.*

Conclusions

In vitro tissue culture could be an important means of improving crop tolerance through genetic transformation as well as by induced somaclonal variation. The results of this study indicated that effect of callus selection media comprising PEG-6000 induced drought stress on callus growth rate for screening drought tolerant callus lines of wheat genotypes. Also, the results showed that all wheat genotypes were not always identical in their DNA ability to be amplified. RAPD analysis showed good potentiality to determine phylogenetic relationships among wheat genotypes. Construction of genetic relatedness tree can done using RAPD molecular marker, the use of molecular markers will be good alternative to the agronomic selection, where it allow a quick selection and provides the breeder with the genetic marker for drought stress.

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