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Effect of Irrigation Water Type on WRKY33 Gene Expression in Wheat, *Triticum Aestivum* and Barley, *Hordeum Vulgare*

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ABSTRACT

An experiment was conducted in plastic pots in an open place in Salah al-Din/ Samarra during the winter season 2020/2021 on wheat plants Triticum aestivum L. (Sham 6) and barley Hordeum vulgare L. (White Arabic) to measure the expression of the WRKY33 gene in response to the salinity of the irrigation water. Two types of water were used to irrigate the plants: river water and well water. The study was conducted on the leaves in the flowering stage. The results showed that there was a significant increase in the gene expression in the wheat plant when irrigated with well water, compared to irrigation with river water. while there was a significant decrease in the gene expression in the barley plant when irrigated with well water, compared to irrigation with river water.

Introduction

Wheat *Triticum* ranks first among cereal crops in terms of economic importance and cultivated area globally (FAO, 2014). Wheat germ oil, gluten and wheat starch are used in food industry (Nagarajan, 2013). Barley *Hordium* is the fourth important cereal crop in the Poaceae family (Gramineae) that is widely cultivated in saline regions (IBSC, 2012). Barley grain flour is used alone or a mixture with wheat flour in the manufacture of various types of pastries, especially bread. Some people prefer barley bread over wheat bread (Fischbeck, 2002). The problem of salinity is one of the main problems that limit the cultivation of many agricultural crops (Marschner, 1995) and negatively affects the various stages of growth and unfolding such as germination, the beginning of the seedling stage, vegetative growth, and the date of flowering and fruiting (Anwar et al., 2011). To adapt and counteract the effects of such abiotic stresses, plants have evolved several molecular mechanisms involving signal transduction and gene expression (Yoon et al., 2020; Ma et al., 2019). Transcription factors regulate the expression of functional genes, which are essential for the stress response (Hennig, 2012). Several transcription factors related to stress tolerance have been identified, such as DREB, bZIP, NAC, and WRKY (Agarwal et al., 2006; Vinocur and Altman, 2005). The transcription factor WRKYs are of particular interest as they are involved in the response to diverse biotic and abiotic stresses as well as in physiological processes (Jiang et al., 2015). WRKY33 has been shown to confer

tolerance to salt stress (Jiang and Deyholos, 2009). The degree of salinity tolerance in plants depends on the degree of salinity tolerance of the gene, which will be reflected in the degree of its gene expression under suitable salinity conditions for the gene. Therefore, the characteristic of salinity tolerance in plants depends mainly on the amount of gene expression (Munns, 2005). Due to the importance of the salinity factor in plant life, this study aimed to demonstrate the effect of irrigation water salinity on the expression of the WRKY33 gene in wheat and barley plants.

Materials and methods

First. Implementation and design of the experiment:

This study was conducted in an open place in Samarra / Salah Al-Din Governorate during the winter season 2020/2021 on wheat plants *Triticum aestivum* L. (Chamber 6) and barley *Hordeum vulgare* L. (Arabic white variety), in plastic pots, the length of the pot is 25 cm and the diameter of its end is 26 cm.The seeds were obtained from the General Authority for Seed Examination and Certification in Salah Al-Din / Tikrit / Al-Zuhur Street. The pots were prepared with soil taken from one of the fields in the city of Samarra, and urea fertilizer and triple superphosphate were added by mixing it with the soil and according to the recommendations. As 15 seeds were planted in each pot, two types of water were used to irrigate the plants: river water and well water, and their chemical characteristics were analyzed, Table 1. After 24 days of cultivation, the plants were thinned to seven plants per pot.

The experiment included two factors, river water and well water, which were applied to wheat and barley plants, Thus, the total number of treatments becomes 4, and each treatment contained 5 replicates. When the plant reached the flowering stage, gene expression was measured.

Table 1. Chemical characteristics of the two types of water used in the research.

Characters		Types of irrigation water	
		river water	well water
Ec, pH	EC (ملیموز) بدرجة 25م	0.57	3.2
	PH	7.22	7.68
dissolved ions (ppm)	Na^{+}	73	431
	Ca^{+2}	53	234
	Mg^{+2}	6	22
	SO_4^{-2}	32	154
	NO^{3-}	5	73
	Cl^{-}	Nil	194

Second, Gene expression measurement

A study of gene expression was carried out in the leaves of wheat and barley plants at the flowering stage, in response to the salinity of the irrigation water. The study was conducted in the Scientific Progress Laboratory / Baghdad, and the method included the following stages:

1- RNA Purification

RNA was isolated from sample according to the protocol of TRIzol™ Reagent as the following steps:

A-Sample Tissues lysis:

For each tube, 1mL from TRIzol™ Reagent was added per 50-100 mg of sample and gently mixed by vortex.

B-For three phase's separations

- For each tube, 0.2 mL of chloroform was added to the lysate, then the tube cap was secured.
- All mixes were Incubated for 2–3 minutes then centrifuged for 10 minutes at 12,000 rpm, the mixture was separated into a lower organic phase, interphase, and a colorless upper aqueous phase.
- The aqueous phase containing the RNA was transferred to a new tube.

C-For RNA precipitation

- 0.5 mL of isopropanol was added to the aqueous phase and incubated for 10 minutes then centrifuged for 10 minutes at 12,000 rpm.
- Total RNA was precipitated and formed a white gel-like pellet at the bottom of the tube
- Supernatant was then discarded.

D-For RNA washing

- For each tube, 0.5mL of 70% ethanol was added and vortex briefly then centrifuged for 5 minutes at 10000 rpm.
- Ethanol then aspirated and air-dried the pellet.

E-For RNA solubility

- **Pellet** was rehydrated in 100µl of Nuclease Free Water then incubated in a water bath or heat block set at 55–60°C for 10–15 minutes.

2- Determine RNA yield

- **Fluorescence Method**

Quantus Fluorometer was used to detect the concentration of extracted RNA in order to detect the quality of samples for downstream applications. For 1 µl of RNA and 200µl of diluted QuantiFlour Dye was mixed. After 5min incubation at room temperature in dark place, RNA concentration values were detected. Table 2.

Table 2. RNA concentration in wheat and barley plants

plant species / Irrigation water type	wheat *Triticum aestivum*		barley *Hordeum vulgare*	
	river water	well water	river water	well water
RNA concentration (ng/µl)	3	3	2	2.3

3-Primer preparation

The primers, Table 3. Were supplied by Macrogen Company in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

Table 3. Primers used in the reaction

Primer Name	Sequence 5`- 3`	Annealing Temp. (ºC)
α-actin-F	GGCACACTGGTGTCATGGT	58
α-actin-R	GCGCCTCATCACCAACATA	
HvWRKY33-F	CTGCAACTTTCCCAGGTACT	
HvWRKY33-R	GGGTCGCTGTGATCTTTCT	

4-Reaction Setup and Thermal Cycling Protocol

- **One Step RT-PCR**

The components shown in Table 4 were used.

Table 4. Components of the RT- qPCR Master mix One Step

Master mix components	Stock	Unit	Final	Unit	Volume 1 Sample
qPCR Master Mix	2	X	1	X	5
RT mix	50	x	1	x	0.25
MgCl2					0.25
Forward primer	10	µM	1	µM	0.5
Reverse primer	10	µM	1	µM	0.5
Nuclease Free Water					2.5
RNA		ng/µl		ng/µl	1
Total volume					10
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template				

5-The program used to amplify the prefixes

The program shown in Table 5 was used.

Table 5. The software used and the reaction steps followed to amplify the primers

Steps	Temp.°C	Time m: s	Cycle
RT. Enzyme Activation	37	15:00	1
Initial Denaturation	95	05:00	
Denaturation	95	00:20	40
Annealing	58	00:20	
Extension	72	00:2	

Third: Analysis Gene Expression: to measure gene expression, data were analyzed using the using method of (Livak and Schmittgen, 2001).

Relative quantification

Folding =2-ΔΔCT

ΔCT =CT gene – CT House Keeping gene

ΔΔCT =ΔCT Treated or Control – ΔCT Control

Fourth- Statistical analysis:

The results of the research were analyzed statistically by applying the Minitab program according to the ANOVA test, and the arithmetic means were compared according to the Dunkin's multiple range test at the level of probability $p \leq 0.05$ (Al-Rawi and Khalaf Allah, 2000).

Results and discussion

1-RNA Extraction

RNA extraction plays an important role in various experiments in plant molecular biology, such as gene expression and transcriptomics. However, obtaining RNA of sufficient quality and quantity can be very challenging, especially for plant species because their biochemical

composition can hinder and even prevent extraction. For example, the presence of proteins associated with the DNA has an effect on the concentration of the sample and thus reduces the efficiency of extraction, and that polysaccharides cause contamination of the sample and affect the purity of RNA because of their precipitation with it, as they have almost similar chemical and physical properties, While polyphenols bind and oxidize with nucleic acids (Mattheus et al., 2003), the quality of the reagents used in the RNA extraction process contributes to increasing the extraction efficiency (Portillo et al., 2006).

2- Effect of irrigation water type on WRKY33 gene expression

Gene expression was studied in the leaves of wheat and barley plants at the flowering stage using real-time polymerase chain reaction (RT-qPCR) to estimate and detect the gene expression level of the WRKY33 gene. The α-actin gene was used as a reference gene, depending on the method of relative quantitative expression, as the data are presented in relation to the reference gene, and to compare the levels of RNA transcripts, a comparison was made between the values of the threshold limit (cut-off point) ct, which is the number of cycles required for fluorescence emission (fluorescent) from the dye to reach the threshold level to detect the interaction. reference gene, where the Δct value is calculated from the difference between the ct of the target gene and the reference gene (Xiayu et al., 2013) and according to the following equation:

Δ Ct (sample) = Ct (sample)mean – Ct (reference)mean

This technique is based on a one-step reaction, which means that the entire reaction of cDNA construction and amplification takes place in a single tube (Maris and Juan, 2005). By measuring the gene expression in the leaves of the wheat plant, it was found that irrigation with well water caused a decrease in the ct value of the reference gene α-actin, which amounted to 23.37 compared to river water, which gave a higher value of 23.84. When measuring gene expression in barley, the ct value of the reference gene α-actin decreased when irrigated with well water, amounting to 22.675 compared to river water, which gave 24.1, Table 6. As Figure 1 shows, curve The value of the threshold limit ct for the reference gene α-actin in the polymerase reaction, as A shows the Threshold value, which reached 0.035 at a temperature of 74.19 ° C, as the fluorescence of the target samples exceeded the threshold limit, which indicates a reaction of the samples in the polymerase chain device, while B shows the number of cycling cycles in the device.

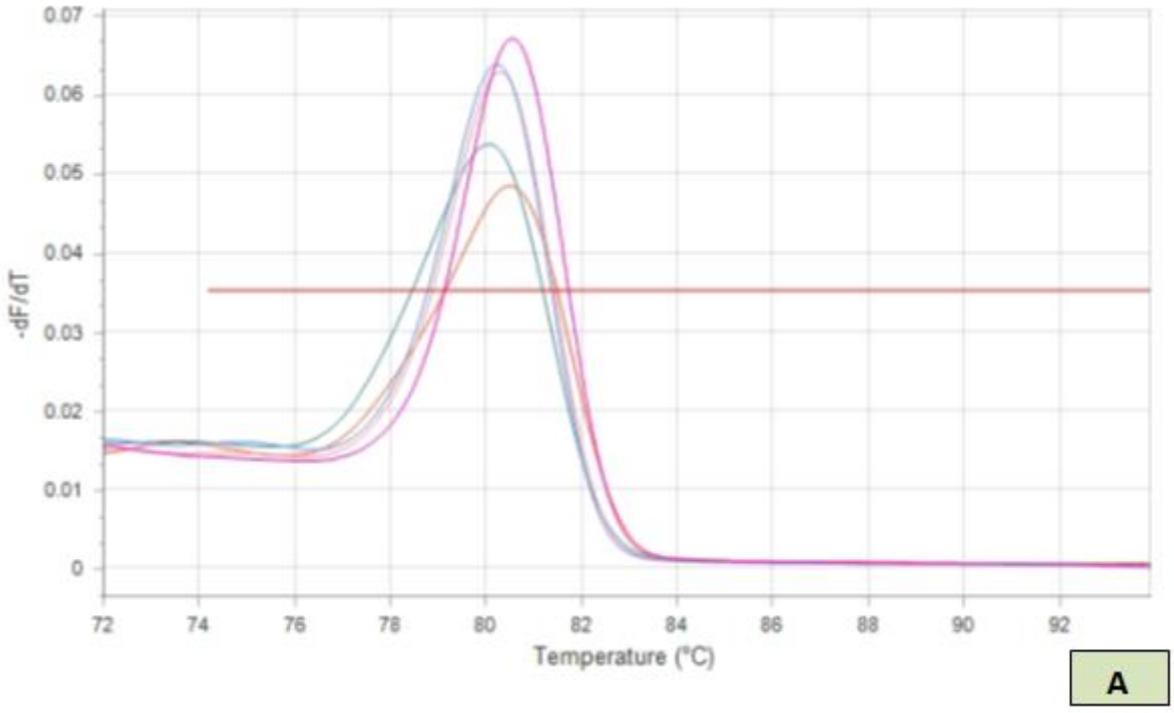
It was also evident that there was a decrease in the ct value of the studied gene WRKY33 when irrigated with well water for wheat plants, as it reached 27.03 compared to irrigated with river water, which gave 28.3, while the gene expression increased significantly when irrigated with well water, reaching 1.75 compared to irrigated with river water, which gave 1.15. In barley, there was a decrease in the ct value of the studied gene WRKY33 when irrigated with well water, reaching 28.2 compared to irrigated with river water, which gave 29.11. The gene expression also decreased significantly when irrigated with well water, reaching 0.75 compared to river water, which gave 1.00, Table 6. Figure 2 shows the curves of the ct threshold value of the studied gene WRKY33 in the polymerase reaction, as A shows the Threshold value of 0.024 and it started at a temperature of 79.8 ° C, while B indicates the number of cycling cycles in the device, as the samples gave a reaction inside the polymerization device through the fluorescent flashes that appeared in the device. The results are consistent with (Aras et al., 2019) in his study on the cherry plant *Prunus spp*., the expression of WRKY33 gene was significantly increased in salinity.

It is clear from the results of this study that there was an increase in the expression of the WRKY33 gene when irrigated with well water compared to irrigation with river water. In the barley plant, the expression of the WRKY33 gene decreased when irrigated with well water compared to irrigation with river water, because the barley plant is one of the most salt-tolerant

field crops, as mentioned by Munns et al. (2006), It has more salinity-tolerant properties compared to other crop plants (Kumar et al., 2013). Plants are tolerant to salinity through certain mechanisms, and most studies agree that the mechanism of exclusion of sodium ion is the likely mechanism for the tolerance of salinity in wheat (Munns and James, 2003). Barley uses two different mechanisms to deal with long term ion toxic stress; One is to exclude Na^+ and Cl^- from uptake by radicals and the other is tissue tolerance (sequestration of Na^+ in the vacuole and retention of K^+) which helps maintain ionic balance (Tavakkoli et al., 2011; Shabala et al., 2010). Sequestration of Na^+ and Cl^- in the vacuole is a tissue tolerance mechanism to reduce ion toxicity while counteracting the osmotic effects of plants (Munns and Tester, 2008).

Table 6. Effect of irrigation water type on gene expression of wheat and barley plants

WRKY33 and α-actin genes	Ct for the target gene		Ct for the reference gene		ΔCt		ΔΔCt		gene expression	
Irrigation Water type / plant species	river water	Well water	river water	Well water	river water	Well water	river water	Well water	river water	Well water
Triticum aestivum	28.3	27.03	23.84	23.37	4.465	3.66	0.78	0.805	1.15 b	1.75 a
Hordeum vulgare	29.11	28.2	24.1	22.675	5.01	5.52	0.01	0.535	1.00 b	0.75 c



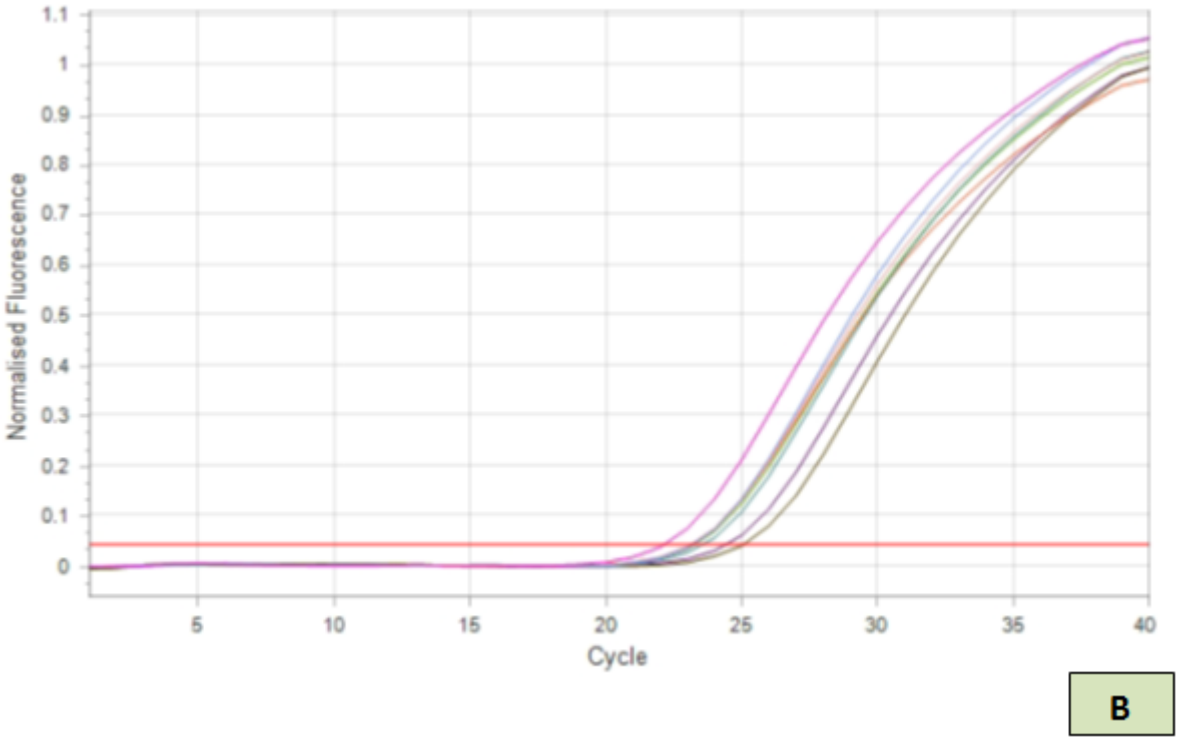
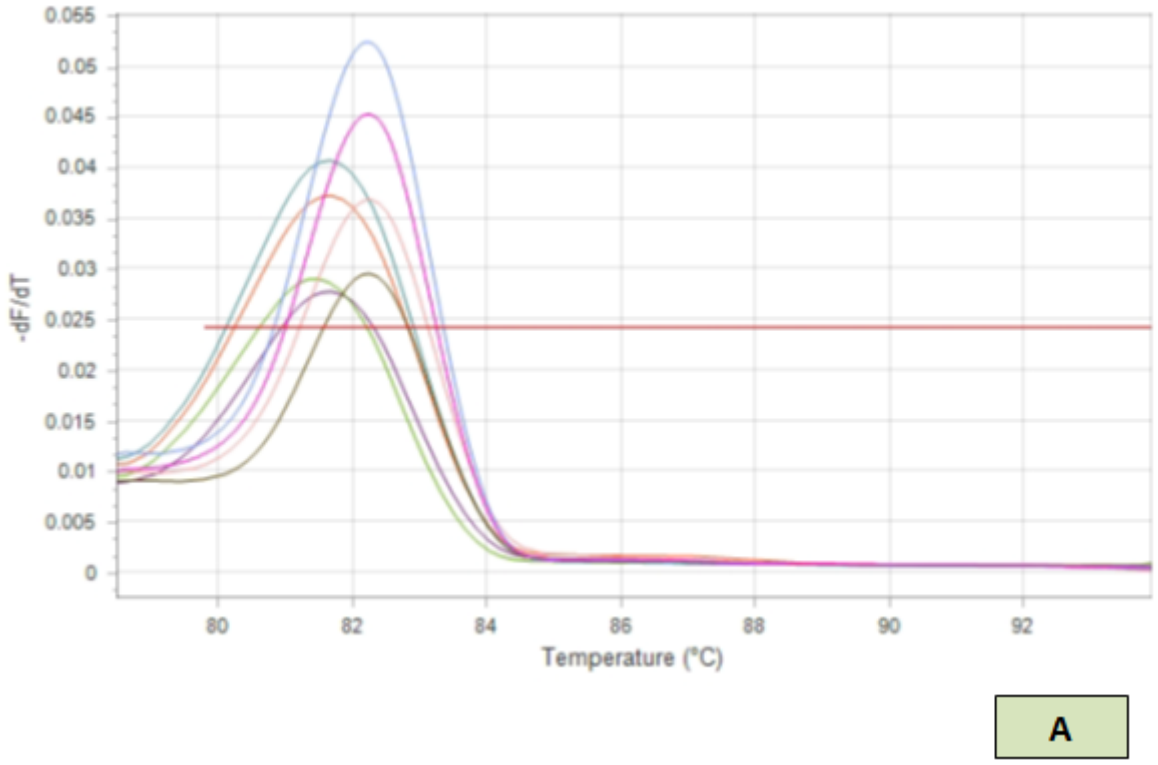


Figure 1. Curves of the threshold value (ct) in RT-PCR for the reference gene α-actin

A. Threshold

B. Cycling



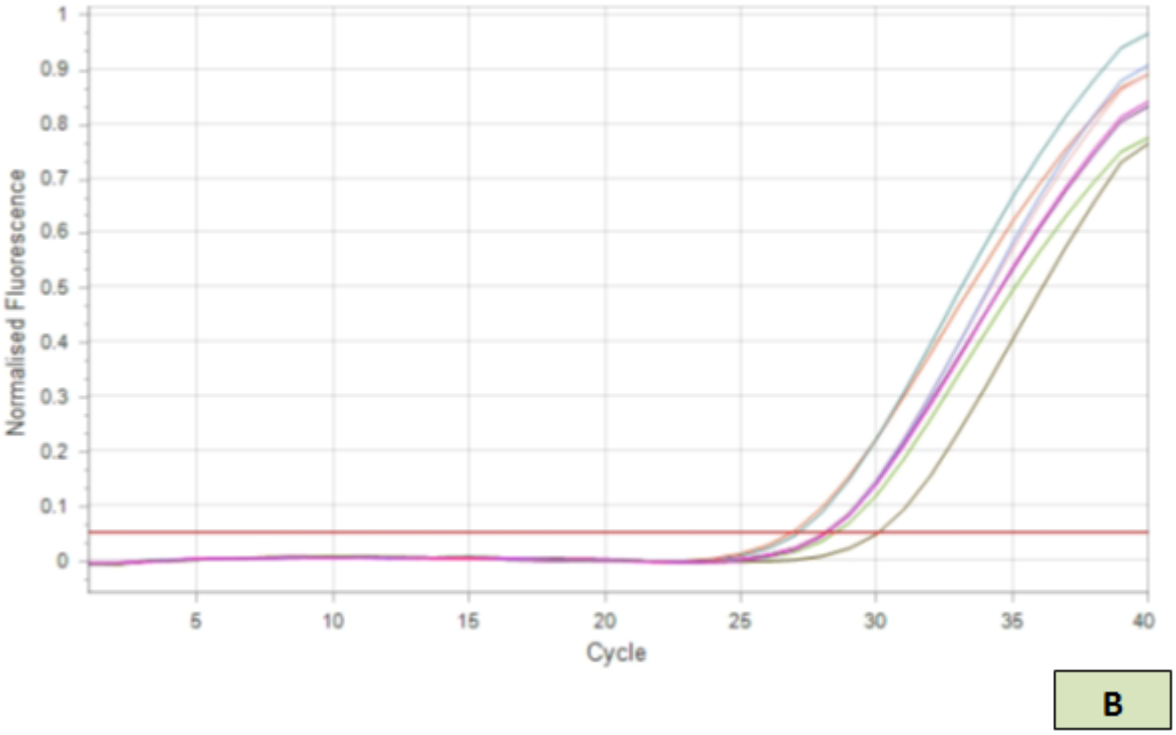


Figure 2. Curves of the threshold value (ct) in RT-PCR for the WRKY33 gene

A. Threshold

B. Cycling

Conclusions

Through the results of the current study, it is clear that the well water caused an increase in the expression of the WRKY33 gene in the leaves of the wheat plant, while it caused a decrease in the expression of the WRKY33 gene in the leaves of the barley plant, compared to irrigation with river water. Therefore, the studied barley variety is more tolerant to salinity than the studied wheat variety.

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