RNA extraction

Materials:

- RNeasy® Plant Mini kit (Cat. no. 74904) from Qiagen
- Buffer RLT
- Buffer RW1
- Buffer RPE
- RNase-free water
- RNeasy spin column
- 2.0 ml collection tube
- Frozen leaf tissues of tomato (Solanum lycopersicum L.)
- Pestle and mortar
- Liquid nitrogen
- 1.5 ml tubes
- 70% ethanol
- Pipette
- Centrifuge (chilled)
- -80oC freezer
- Nanodrop spectrophotometer (Thermo Scientific™ NanoDrop 2000 and 2000c)

Methods:

1. Frozen plant tissues were ground to a fine powder in liquid nitrogen using a cooled pestle and mortar.
2. Total RNA was isolated from the leaf tissues using the RNeasy® Plant Mini kit from Qiagen according to the manufacturer's protocol.
3. 350 L of buffer RPE was added to each 1.5 ml tube containing 100 mg of the plant tissue sample (as lysis buffer).
4. The samples were homogenized and centrifuged for 3 minutes at 12,600 rpm in a chilled centrifuge.

5. The lysate was transferred to a new 1.5 ml tube, and 700 µL of 70% ethanol was added and mixed thoroughly by pipetting.
6. 700 µl of the lysate was transferred to the RNeasy spin column and centrifuged for 15 seconds at 12,600 rpm.
7. 350 µl of Buffer RW1 was added as a wash buffer and centrifuged again for 15 seconds at 12,600 rpm.
8. The column was then filled with 500 µl of Buffer RPE and centrifuged for 15 seconds at 12,600 rpm.
9. Another 500 µl of Buffer RPE was added to the column to dry the column membrane and centrifuged for 2 minutes at 12,600 rpm again.
10. 80 µl of RNase-free water was added to the column, and the column was centrifuged at 12,600 rpm for a minute to elute the RNA from the membrane.
11. The eluted RNA was stored in a labelled tube in a -80oC freezer before the next phase of the experiment.
12. The concentration of the RNA was measured using a Nanodrop spectrophotometer.
13. The entire process was carried out on the ice to prevent RNA degradation.

Reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate the expression levels of the SDH and GAPDH (as a control/housekeeping gene), procedures based on Nosarzewski et al. (2012) were used. The detection of SDH and GAPDH genes required two independent stages: reverse transcription of RNA to create first-strand cDNA and two semi-nested PCR steps for gene identification . Table 3.1 had shown the PCR primers used in this study.

Table 3.1: The consensus primer sequences (SDH and GAPDH).

Primer		Sequence	Expected size of DNA product (bp)
SDH	F	5'- GGATGAAGGCTGTCGGTATTT -3'	106
	R	5'- GCACATTCATGCCCAATCAC -3'	
GAPDH	F	5'-AATTGGCCGATTGGTTGCTC-3'	73
	R	5'-GAAGGGATCGTTCACTGCGA-3'	

First-strand synthesis of RNA

RNA, being a single-stranded molecule, cannot be amplified directly. To overcome this limitation, reverse transcription was performed to convert the RNA into double-stranded DNA, making it amenable to amplification. The reverse transcription reaction was carried out using a full RT kit (Promega GoScript® Reverse Transcription System), and a master mix was prepared. The master mix was prepared in a biosafety cabinet (BSC) to minimize cross-contamination. To maintain the integrity of the RT reagents, which are temperature-sensitive, they were kept on ice until ready for use. The amount of master mix made was determined by the number of samples analyzed. For example, if 10 samples were to be examined, a master mix for 11 samples was created. The master mix was then transferred to a 1.5 ml microcentrifuge tube, and 0.2 ml PCR tubes were labelled accordingly for the number of samples analyzed. Table 3.2 provides a summary of the number of reagents required for each sample.

Table 3.2: The master mix content.

Reagent	Volume per reaction (µL)	Final concentration
Nuclease-free water	4.4	Up to total volume
5X RT buffer	3	1X
dNTP mix (10mM each dNTP)	0.2	500µM (125µM each)
Oligo(dT)$_{20}$ primer (10µM)	1	0.25µM
Rnase inhibitor (RNasin)	1	20 unit
Final total	**9**	

After all the reagents were added, the mixture was vigorously and quickly pipetted and centrifuged for 2-5 seconds to eliminate any undesired air bubbles. Each 0.2 mL PCR tube received 5 µL of extracted RNA, 9 µL of master mix, and 1 µL of Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase enzyme. To avoid early breakdown, the enzyme was introduced last. The tubes were gently centrifuged to eliminate air bubbles and allow the ingredients to mix thoroughly. The tubes were then transferred to a thermal cycler (Multigene , Labent, Internationl. Inc, USA) and programmed as described in Table 3.3.

Table 3.3: Thermal cycler program settings for the RT reaction.

Reaction step	Time (min)	Temperature (^{0}C)
Heating	15	50.0
Reverse transcription	60	50.0
Hold	∞	4.0

After completion, the samples were taken out and cooled on ice. They were either tested again using semi-nested PCR to check for the presence of SDH and GAPDH genes or stored in a freezer at -20 degrees Celsius.

The detection of SDH and GAPDH(control, Housekeeping) genes:

The first round of PCR is used to amplify the SDH and GAPDH cDNA. The procedure for creating the PCR master mix is outlined in Table 3.4. The samples were put in 0.2 mL PCR tubes, and a 1.5 mL microcentrifuge tube was used for the master mix.

Table 3.4: The master mix content for each tube.

Reagent	Volume per reaction (µL)	Final concentration
Nuclease-free water	30.6	-
$MgCl_2$ (25mM)	5	2.5mM
dNTP mix (10mM each dNTP)	1	0.2mM each
SDH F primer	1	
Or		0.2mM
GAPDH F primer		
SDH R primer	1	
Or		0.2mM
GAPDH R primer		
5X Taq Green Buffer	10	1X
Final total	**48.6**	

The mixture was blended well and then carefully spun to remove any air bubbles. The 1 µl RT product was first added to the PCR tubes, followed by 48.6 L of the master mix. Then, 0.4 µL of Taq DNA polymerase (Promega GoTaq® Polymerase, from Germany) was added to the mixture. After that, the tubes were again gently centrifuged to eliminate any air bubbles that had formed. Finally, the tubes were put in a thermal cycler set to a specific program, as described in Table 3.5.

Table 3.5: The thermal cycler program settings for the first round of semi-nested PCR.

Reaction step	Time (min)	Temperature (^{0}C)	Cycles
Initial denaturation	5	95	1
Denaturation	1	94	
Annealing	1	55	35
Extension	1	72	
Final extension	10	72	1
Hold	∞ / to end	4	1

The sample tubes were removed from the heat cycler when the reaction was finished. They were frozen at -20ºC or immediately visualized using agarose gel electrophoresis.

Agarose gel electrophoresis

The cDNA amplification of the SDH and GAPDH genes was evaluated using agarose gel electrophoresis. A 1.6% gel was prepared by mixing 1.6 g of agarose powder with 100 mL of 0.5X TBE buffer and heating it in a microwave for 1 minute and 40 seconds. After cooling, 10 µL of SYBR® Safe DNA gel stain was added, and the mixture was poured into a casting tray and left to solidify for 30 minutes. The gel was then run in a horizontal electrophoresis tank with 0.5X TBE as the buffer, with a 100 bp DNA ladder in the first well. Electrophoresis was run for 1 hour at 100 volts, and the results were visualized and photographed using a Gel Documentation System.

Real-time PCR assays

Real-time PCR was performed using a Bio-Rad CFX96 machine on a 25 µL reaction containing 1 µL of first-strand cDNA, 10 µL of KAPA SYBR® FAST qPCR Kit (KR0389, KAPA Biosystems, USA), 2 µL of 10M SDH F primer, 2 µL of 10M SDH R primer, and nuclease-free ddH2O was used for the PCR. SDH gene-specific primers and GAPDH housekeeping gene primers were selected as a reference for measuring the amount of total RNA in the sample (Expósito-Rodríguez

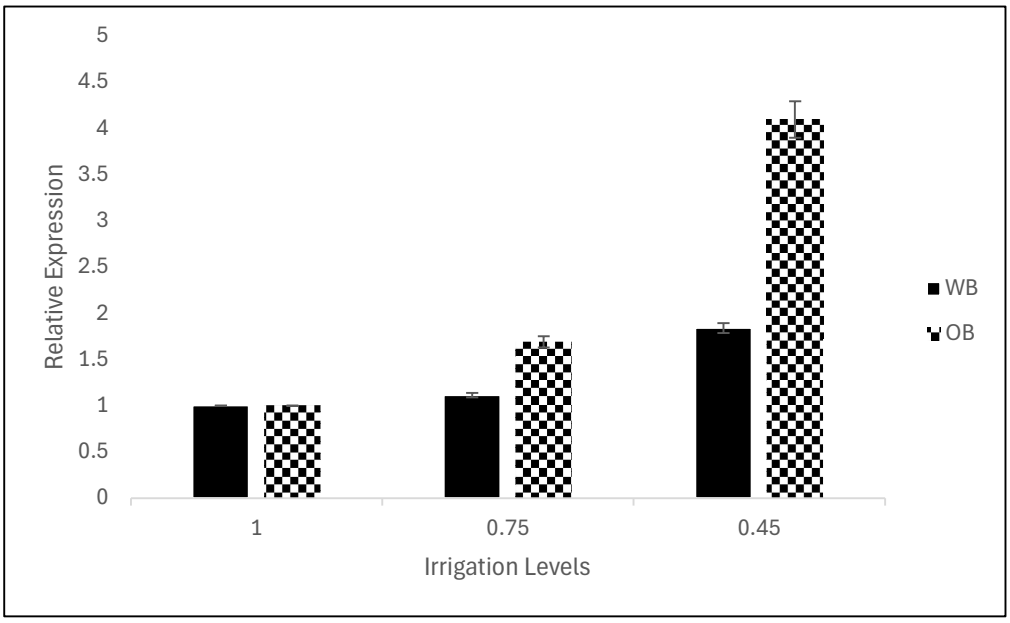
et al., 2008). Three replicate PCR runs were performed for each sample and each gene. The samples were denatured for 5 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C on the CFX96 Real-Time PCR System (Bio-Rad, CA). The relative gene expression was calculated using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001).

Relative expression of SDH in WB with biochar, and Relative expression of SDH in WO without biochar.

100% = normal irrigation, 75% = medium level of irrigation, and 45% = severe drought

9 replicates /treatment

	100%	75%	45%
WB	1	1.113222	1.838889
OB	1	1.688889	4.094444
SE	0	0.022947	0.052293
	0	0.060149	0.197744



REffects of Biochar Treatment on SDH Activity After 14 Days, in OB without biochar, and in WB with biochar.

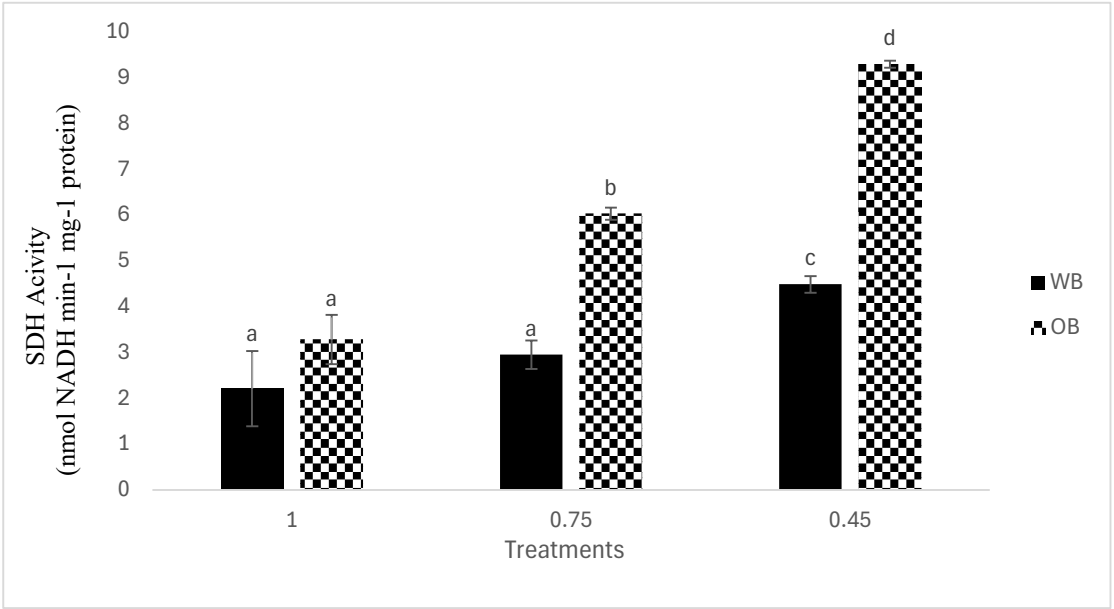
100% = normal irrigation, 75% = medium level of irrigation, and 45% = severe drought.

Means (n=9) were separated by Fisher's Least Significant Difference at P≤0.05.

		100%	75%	45%
SDH AC	WB	2.211945	2.95	4.488
	OB	3.286333	6.033	9.296
		1	0.75	0.45
S.E				
	WB	0.822093	0.312143	0.180216
	OB	0.539819	0.132204	0.076328

inregation	iochar treatmen	Replicates			Avearge	Standard Deviation	Stanadard Error
100%	OB	3.924597	3.721308	2.213094	3.286333	0.934993568	0.539818788
	WB	3.359946	2.657298	0.61859	2.211945	1.423907618	0.822093447
75%	OB	5.85	5.96	6.29	6.033333	0.22898326	0.132203547
	WB	3.37	2.34	3.14	2.95	0.54064776	0.31214313
45%	OB	9.286	7.512	11.09	9.296	1.789020961	1.032891734
	WB	4.434	4.89	4.14	4.488	0.37790475	0.218183409

a b c d

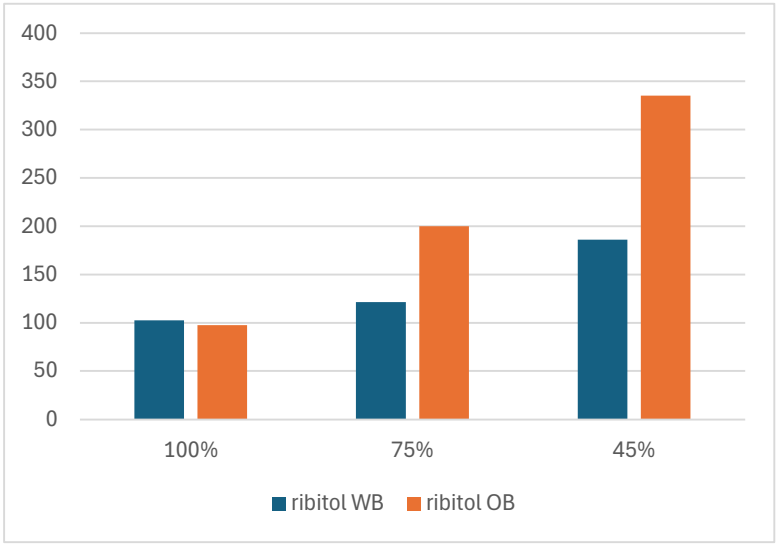


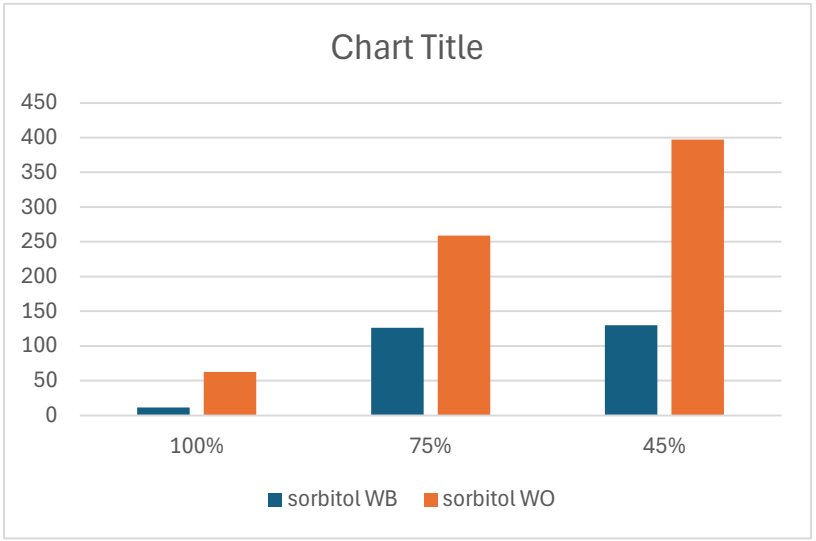
Effects of Biochar Treatment on Ribitol (A) and Sorbitol (B) Concentration After 14 Days

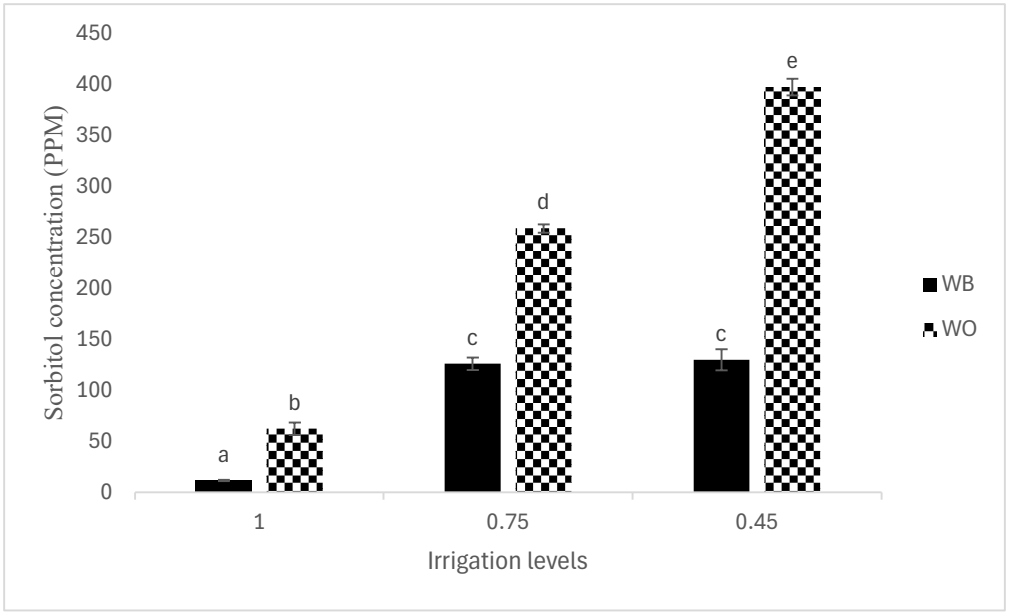
Considering Different Irrigation Levels (100%, 75%, and 45%) drought

	Ribitol						Sorbitol					
				AV	SD	SE				AV	SD	SE
WB-1	101.4465	103.306	102.3763	102.3763	0.759138	0.379569	10	13	11.5	11.5	1.224745	0.612372
WB-2	113.8655	128.959	121.4123	121.4123	6.161896	3.080948	141.123	111.123	126.123	126.123	12.24745	6.123724
WB-3	177	172	209.066	186.022	16.42193	8.210963	125	130	134.622	129.874	3.929175	1.964588
OB-1	97.533	97.533	97.533	97.533	0	0	77.438	47.438	62.438	62.438	12.24745	6.123724
OB-7	193.689	205.928	199.8085	199.8085	4.996551	2.498275	248.702	268.702	258.702	258.702	8.164966	4.082483
OB-4	330.3705	340.3705	335.3705	335.3705	4.082483	2.041241	400.01	390.009	401.09	397.0363	4.988598	2.494299

	ribitol			sorbitol		
	WB	OB		WB	WO
100%	102.3763	97.533	100%	11.5	62.438
75%	121.4123	199.8085	75%	126.123	258.702
45%	186	335.3705	45%	129.874	397.036






450
400
350
300
250
200
150
100
50
0
Sorbitol concentration (PPM)
a
b
c
d
c
e
1
0.75
0.45
Irrigation levels
WB
WO