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In vitro assessment of salt tolerance in 'Balady' apple (*Malus domestica*) seedling clones

Samar A. Abdelaziz[1], Emam A. Abdel-Rahim[2], Mohamed M. Rashed[2], Rania A. Mahmoud[3]

[1]*Deciduous Fruits Research Department, Biochemistry Central Laboratory, Horticulture Research Institute (HRI), ARC , Giza, Egypt*
[2]*Biochemistry Department, Faculty of Agriculture, Cairo University, Giza, Egypt*
[3]*Deciduous Fruits Research Department, Biotechnology Central Laboratory , Horticulture Research Institute (HRI), ARC , Giza, Egypt*

Soil salinization has become a significant threat to some regions' apple production. This study aimed to improve the detection and selection process for salt stress tolerance in 'Balady' apple (*Malus domestica*) seedling clones using advanced tissue culture techniques. Open-pollinated mature seeds of the local Egyptian 'Balady' apple were collected from a single tree, and then each surface-sterilized seed was cultivated independently and assigned a distinct serial number. After three consecutive subcultures, salt stress was induced by supplementing the proliferation medium with graded NaCl concentrations (0, 1000, 2000, and 3000 ppm). The micro-propagated individuals were not identical according to similar indices based on Inter Simple Sequence Repeats; the lowest percentage of 84.9% was scored between the mother plant and the MS5 clone. The MS5 clone exhibited superior growth parameters under elevated salinity levels compared to other tested clones, highlighting its resilience and adaptability. The MS5 clone, the most salt-tolerant clone, showed the lowest levels of reduced sugars and total phenols and minimal overall antioxidant activity. However, it demonstrated stimulated enzymatic antioxidant activity at the highest salt concentration. Data from three common differentially expressed genes revealed that protein phosphatases PP2C-77a and ABA-insensitive transcription factor ABI5-5b were downregulated. Also, the serine/threonine-protein kinase SAPK3 was overexpressed in the MS5 genotype under severe salt stress conditions. A unique 290 bp positive marker was identified in the salt-tolerant MS5 clone using the HB12 ISSR primer, providing molecular basis for its tolerance. This study confirmed the viability of *in vitro* screening for salt-tolerant genotypes within apple seedling populations.

Keywords: Apple; Salinity; *In vitro;* Seedling clones; Antioxidant activity; ISSR

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CORRESPONDENCE TO
Samar A. Abdelaziz,
Deciduous Fruits Research Department, Biochemistry Central Laboratory, Horticulture Research Institute (HRI), ARC , Giza, Egypt
Email: abdelaziz_samar@yahoo.com
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INTRODUCTION

Renowned for its exceptional nutritional value, particularly its antioxidant and mineral contents, the apple (*Malus domestica*) is widely cultivated and holds substantial economic significance globally. It ranks third in fruit intake worldwide (Li *et al*., 2023). In temperate climates around the world, apples are commonly farmed. Abiotic stressors linked to climate change have caused a recent drop in apple production culture (Dutta *et al*., 2022). Abiotic stresses in agriculture arise from interactions between plants and their environmental conditions, including non-living factors that adversely affect growth and yield. These stresses include potentially adverse effects such as salinity, drought, flooding, chilling, metal toxicity, nutrient deficiency, UV exposure, and air pollution (Rehman *et al*., 2005). Three main abiotic factors that affect apple tree growth and fruit output are drought, cold, and high salinity (Li *et al*., 2023).

In extensive orchards worldwide, water is frequently the greatest limiting issue. Soil salinization has become a major threat to apple production in some regions. Salinity is a critical agricultural issue in Egypt, impacting traditionally cultivated lands and newly reclaimed soils due to high evaporation rates, saline water tables, and limited rainfall (Hellal *et al*., 2012). Utilization of tissue culture techniques for quantifying stress tolerance of various crops has been increasing rapidly. Tissue culture systems help evaluate tolerance to environmental stresses because stress conditions can be easily controlled *in vitro* (Errabii *et al*., 2006). Because apples are highly heterozygous genetically, their seedling populations segregate heavily. Dai *et al*. (2013) reported that developing a genotype with high regeneration capacity and sensitivity to Agrobacterium from the *in vitro* seedling population of apples is feasible. Therefore, this research aimed to facilitate the detection and selection of salt stress tolerance in local Egyptian 'Balady' apple (*Malus domestica*) seedling clones through tissue culture technique.

MATERIALS AND METHODS

This study was conducted at the Horticulture Research Institute's Biotechnology Laboratory at the Agricultural Research Center in Egypt in cooperation with the Biochemistry Department of Cairo University's Faculty of Agriculture.

In vitro culture

Establishment stage: Open-pollinated mature seeds of the local Egyptian 'Balady' apple (*Malus domestica* Volus) were collected in spring 2022 from ripped fruits of a single tree at the experimental orchard of

Horticulture Research Institute and then used as plant materials. The seeds were surface-sterilized using a sequential process: a 1-minute rinse in 70% ethanol followed by a 15-minute treatment with 15% commercial bleach (Clorox, 5.25% sodium hypochlorite) containing 0.01% Tween-20, as described by Pooler and Scorza (1995). On the surfaces of the establishment medium that the embryos' sides were in contact with, seeds were inoculated vertically. Every seed was cultivated independently and assigned to a distinct serial number. Two culture media were evaluated for seedling establishment: Woody Plant Medium (WPM) as proposed by Lloyd and McCown (1980), and Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Ascorbic acid (2 mg/L), pantothenic acid (1 mg/L), casein hydrolysate (200 mg/L), 3% sucrose, and 0.7% purified agar were all present in each free growth regulator medium (Rizzo *et al*., 1998). The cultures were placed at 25±1°C with a 16-hour photoperiod of roughly 1500–2000 Lux light intensity after being stratified in the dark for 60 days at 5°C. Data on the percentage of germination of cultured seeds, shoot height (cm), number of leaves, root length (cm), and fresh weight (g) of seedlings were recorded after four weeks.

Multiplication stage: Regenerated shoots that had emerged throughout the establishment stage were excised from the cotyledons and cultured preliminary on solid MS medium supplemented with 1.0 mg/L BA, 0.1 mg/L IBA, and 30 g/L sucrose (Welander, 1985) for three consecutive subcultures. After four weeks of the third subculture, data on shoots' number, average height (cm), and fresh weight (g) were recorded.

Molecular identification

Plant tissues were ground using liquid nitrogen to a fine powder, and then bulked DNA was extracted using the 'QIAGEN' DNeasy plant Mini Kit according to the manufacturer's protocol. PCR amplification was performed using ten ISSR primers, HB 10 (GA)6CC, HB 12 (CAC)3GC, HB 14 (CTC)3GC, UBC-811 (GA)8C, UBC-812 (GA)8A, 17898-B (CA)6GT, 17899-A (CA)6AG, 17899-B (CA)6GG, 844 A (CT)8AC and 844 B (CT)8GC. The amplification reaction was carried out in a volume of 25 µl containing 2.5 µl of primer (10 pmol), 2.5 µl of template DNA (25 ng µl-1), 12.5 µl of 'Willowfort' Cosmo PCR red master mix and 7.5 µl of nuclease-free water. The thermocycler (T100 Thermal Cycler, BioRad, USA) was programmed for an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C (denaturation), 1 min at 45°C (annealing) and 1 min at 72°C (extension) then a final extension step at 72°C for 5 min (Kumar *et al*., 2010). The PCR products were separated on 1.5% agarose gels, and fragment sizes were estimated with 100 bp ladder markers. DNA bands on gels were scored as one "1" if present or zero "0" if absent for all samples, then a binary statistical matrix was constructed. Similarity matrix correlations between clones were calculated using XLSTAT software.

***In vitro* evaluation of salinity tolerance**

Growth responses: In the fourth subculture, salinity stress was achieved by adding different concentrations of NaCl (0, 1000, 2000 and 3000 ppm) to the proliferation medium (Mohamed *et al*., 2022). Explants of each clone were cultured at the various tested salt concentrations. Each treatment consisted of five replicates (jars) with 3 explants per jar. Shoot multiplication was evaluated by counting the number of shoots, measuring the average height (cm) of shoots and determining cluster fresh weight per explant after four weeks of subculture.

Biochemical responses

Total polyphenols content: The Folin–Ciocalteu colorimetric method was applied to measure the total polyphenols content as g gallic acid /100g F.W (%) in accordance with Singleton and Rossi (1965). Antioxidant activity: The antioxidant activity was determined using the 'Ferric Reducing Antioxidant Power' assay by Oyaizu (1986). Ascorbic acid was used in the reference, and the sample reducing power was expressed as g ascorbic acid/100g F.W (%) comparable to the standard. Total free amino acids content: According to Jayaraman (1985), the ninhydrin reaction was performed to quantify the total free amino acid content. Reducing sugar content: The 3,5-dinitrosalicylic acid (DNSA) method was used to calculate the amount of reducing sugars as g glucose/100g (%) F.W (Miller, 1959).

Regulation of salt tolerance adaptive mechanisms

Gene expression analysis: Total RNA was extracted using the 'GeneDireX' Total RNA Isolation Kit (Plant) according to the manufacturer's protocol. RNA concentration, integrity, and purity were assessed using NanoDrop Spectrophotometry (MicroDigital, Korea). Differential expression of three targeted genes (Li *et al*., 2019) and β-ACTIN as a reference gene (Table 1) was confirmed by qPCR using Applied Biosystems StepOne™ Real-Time PCR (Thermo Fisher Scientific, USA). Reverse transcription was performed with the 'Thermo Scientific' Revert Aid First Strand cDNA Synthesis Kit. The reverse transcription reaction product is stored at -20°C for less than one week.

Table 1. Primer sequences of the targeted genes used for expression analysis

Name	Forward primer (5'– 3')	Reverse primer (5'– 3')
PP2C-77a	AGTGGATGCTGAGATTGGAGGAG	TGCTACGATAATATGCGTTGGAC
ABI5-5b	CACTTTGGCTCCGAGTTCAGG	AGGCACGGGTGACACGGATG
SAPK3	GATTTAGTGAAGACGAGGCAAGG	AGCAATGTATGCAGGTGTTCCA
β-ACTIN	CTGAACCCAAAGGCTAATCG	ACTGGCGTAGAGGGAAAGAA

Willowfort *HERA*^{PLUS} SYBR® Green qPCR Kit was used for all assays. The thermal profile was set as follows: 5 min at 95°C, 40 cycles with 20 s at 94°C, 20 s at 60°C, and 20 s at 72°C (Li *et al*., 2019). ROX was set as the reference dye. Ct value data were analyzed for gene expression.

Enzymatic antioxidants activity

Extraction for antioxidant enzyme assays: Using a pre-chilled mortar and pestle, 0.1 g of plant material was ground and then suspended in an ice-cold extraction buffer made up of 1 ml of 100 mM sodium phosphate buffer (pH 7.0) with 0.5% (w/v) polyvinylpyrrolidone (PVP). The supernatant was used to measure enzyme activity after the homogenates were centrifuged in a chilled centrifuge at 10,000 Xg for 20 minutes at 4°C. At 25°C, enzyme activity was measured using spectrophotometry. Superoxide dismutase (SOD) enzyme activity: Nishikimi *et al*. (1972) method was used to measure the activity of superoxide dismutase (SOD). Superoxide radicals are generated in PMS-NADH systems by the oxidation of NADH and assayed by the reduction of NBT. The absorbance of the reaction mixture at 560 nm indicated increased superoxide anion scavenging activity. Peroxidase (Px) enzyme activity: Peroxidase (Px) activity was assayed by measuring spectrophotometry of the increasing absorbance due to the oxidation of guaiacol, which measured at 436 nm (Malik and Singh, 1980). Catalase (CAT) enzyme activity: The protocol for the catalase activity assay was carried out in accordance with Hadwan *et al*. (2024). The procedure entails incubating enzyme-containing samples with a carefully chosen concentration of H_2O_2 for a specified incubation period. Subsequently, a solution containing ferrous ammonium sulfate (FAS) and sulfosalicylic acid (SSA) is added to terminate the enzyme activity. A distinctive maroon-colored ferrisulfosalicylate complex is formed. The formation of this complex is a direct result of the reaction between FAS and any residual peroxide present. This leads to the generation of ferric ions when coordinated with SSA. The complex has a maximum absorbance of 490 nm.

Statistical analysis

Data were analyzed using ANOVA within a completely randomized factorial design framework. Significant differences between treatments were assessed using LSD values at a 0.05 confidence level (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

***In vitro* culture**

Establishment stage: Regarding the effect of establishment media, the data presented in Table 2 revealed that the MS medium showed a 72.2% germination percentage. In comparison, the WP medium reported 69.4% for tested 'Baldy' apple seeds, with insignificant differences between both culture media. Concerning the effect of culture media on growth parameters of *in vitro* established 'Baldy' apple seedlings, Table 3 represents the growth parameters of 51 seedlings, 26 out of 36 on MS and 25 out of 36 in total on WP medium. Seedlings gained between 0.15 and 0.39 g as fresh weight on the MS medium and between 0.11 and 0.37 g on the WP medium. The root length was 2.5 to 10 cm for seedlings growing on MS medium and 3 to 13 cm for those ever-increasing on WP medium. The height of the shoot recorded minimum values of 0.7 and 1 cm for seedlings grown on MS and WP medium, respectively, and up to 6 cm for both tested culture media. For the number of leaves, Table 3 shows the seedlings had a minimum of 3 leaves on both tested culture media and up to 7 and 8 leaves for those grown on MS and WP medium, respectively. Generally, the type of culture media did not significantly affect the recorded growth parameters. These results agree with Bakr *et al*. (2015), who reported that culture media type did not significantly affect the growth parameters of immature peach embryos.

Table 2. Effect of culture media on germination of *in vitro* established 'Baldy' apple seeds

Media	Germination %
MS	72.22
WP	69.44
LSD 5%	10.90

Multiplication stage: After three subsequent subcultures, only 12 clones remained, six of which were *in vitro* established by seeds on each tested media and could survive and grow sufficiently. In contrast, others had insufficient regeneration rates or were lost due to vitrification. The data (Table 4) declared that MS7 had the highest number of shoots, 8.80, followed by MS24 and WP1 clones, which gave 6.47 and 6.06, respectively, and shoots No, with no significant differences between both. MS8 clone recorded the shortest shoots with an average of 1.36 cm. On the other hand, the maximum fresh weight was 2.50 g gained by WP1, with an insignificant difference with WP16, which weighed 2.14 g, while MS2 was the least (0.64 g). These results are partially in harmony with the data of Welander (1985), who stated that the total number of shoots on MS medium reached 5.2 shoots, and the length of shoots reached 1.6 cm, while Caboni *et al*. (2000) indicated that the average shoot number of apple culture was between 1.1 and 7.1.

Molecular identification

ISSRs amplification analysis: Bands produced using the 17899-B ISSR primer were monomorphic across all individuals (Table 5). The pattern obtained by primer HB14 showed the maximum number of twelve DNA fragments (292-2958 bp molecular size) with only five polymorphic bands detected (41.6%). The results of primer HB12 indicated that ten DNA fragments were amplified with molecular sizes ranging from 173 bp to 1707 bp, of which nine bands were polymorphic, representing the maximum polymorphism percentage of 90%. No specific markers were detected by 17898-B or 17899-B primers and for MS1, MS2, MS7, WP1, WP5, and WP15 clones. Five positive markers, 701 bp UBC-812, 290 bp HB 12, 1076 bp 17899-A, 1008 bp 17899-A, and 1270 bp 844 A, were detected in mother-plant MS5, MS18, MS24 and WP20 clones, respectively. A maximum number of three specific fragments with molecular sizes of 668, 292, and 429 bp were specified as negative markers for the WP20 clone using HB 10, HB 14 and UBC-812 primers, respectively.

Genetic similarity: According to the similarity index (Table 6), none of the twelve micro-propagated individuals exhibited complete genetic similarity either among themselves or to the mother plant. The most dissimilar individual to the mother plant was MS5, representing 84.9% similarity. Inter-simple sequence repeat PCR (ISSR-PCR) is a fast-genotyping technique based on length variation in the regions between microsatellites. The method requires no species-specific prior knowledge of microsatellite location or composition. Tiny amounts of DNA are needed, making this method ideal for organisms of conservation concern or where the quantity of DNA is minimal due to organism size. Inter-simple sequence repeat (ISSR) markers are highly polymorphic, relatively easy to develop, and inexpensive compared to other methods. It is a reliable and cost-effective technique for assessing genetic diversity between closely related individuals. It is worth noting that an Inter Simple Sequence Repeat (ISSR) technique was successfully used as a fingerprinting tool to evaluate the genetic stability of *in vitro* preserved 'Balady' apple shoot tip explants under different sugar concentrations and low temperatures (El-Homosany and Shimaa, 2016).

***In vitro* evaluation of salinity tolerance**

Growth responses: All growth parameters, number of shoots, average shoot height, and fresh weight, were adversely affected by salt, with all means gradually decreasing with increasing NaCl concentrations in the proliferation medium recording the lowest values (3.04 shoots, 1.46 cm, and 0.53 g, respectively) under adverse salt stress compared to control with 4.43 shoot, 2.08 cm and 1.12 g for shoot count, average shoot height and fresh weight, respectively (Table 7). Increasing NaCl concentration up to 3000 ppm had an insignificant effect on all recorded growth parameters of MS2, MS5, MS24, and WP8 clones. Comparing these four clones under severe salinity, MS2 and WP8 had the lowest number of shoots (2.50), while MS5 and MS24 were the best, with 4.33 and 4.67 shoots, respectively. On the other hand, both MS24 and WP8 had the lowest average shoot height (1.24 and 1.24 cm, respectively), while MS2 and MS5 were the best, with 1.74 and 1.92 cm, respectively. In addition, MS2 was the least considered fresh weight (0.35 g) compared to MS5, MS24 and WP8 clones, with 1.09, 0.67 and 0.58 g in descending order. MS5 maintained a relatively higher number of shoots, average shoot height, and fresh weight, together with 3000 ppm NaCl, indicating the superiority of the MS5 clone under severe salt stress conditions. On the contrary, the MS18 clone recorded a maximum fresh weight of 2.30 g under control conditions. It was reduced by 87.39% to obtain 0.29 g as affected by the highest salt concentration, accompanied by a relatively low proliferation rate with 1.83 shoots of 1.10 cm tall compared to other tested clones, indicating the susceptibility of the MS18 clone to salinity. Also, the MS1 clone had a lower proliferation rate under severe

Table 3. Effect of culture media on growth parameters of *in vitro* established 'Baldy' apple seedlings

Clones	Fresh weight (g)		Root length (cm)		Shoot height (cm)		No. of leaves	
	MS	WP	MS	WP	MS	WP	MS	WP
1	0.35	0.37	9.0	10.0	4.0	6.0	4	6
2	0.34	0.23	9.0	7.0	4.0	2.3	6	4
3	0.37	0.29	7.0	6.0	3.0	4.0	3	4
4	0.22	0.21	3.0	6.0	2.5	3.0	4	3
5	0.26	0.32	8.0	4.5	3.0	5.0	5	4
6	0.31	0.16	9.0	3.5	3.5	2.0	5	8
7	0.36	0.33	4.0	9.0	5.0	3.0	4	7
8	0.22	0.25	8.5	6.0	4.0	2.0	5	3
9	0.29	0.34	8.0	5.0	3.5	4.5	5	4
10	0.23	0.19	10.0	8.5	4.0	4.0	4	3
11	0.25	0.21	5.0	5.0	6.0	1.5	6	4
12	0.34	0.11	9.5	3.0	3.5	2.5	5	4
13	0.25	0.30	9.0	4.5	4.0	1.5	6	4
14	0.22	0.20	8.5	5.0	3.5	1.0	4	5
15	0.28	0.13	7.0	7.0	4.0	1.0	4	5
16	0.23	0.23	6.5	7.5	2.0	3.0	5	5
17	0.15	0.24	4.5	13.0	2.0	4.0	4	5
18	0.27	0.21	4.9	7.0	3.5	3.0	5	6
19	0.16	0.22	3.5	3.5	2.0	3.0	6	4
20	0.20	0.22	6.5	8.0	3.0	3.0	4	5
21	0.39	0.27	5.5	7.0	3.0	3.8	6	4
22	0.23	0.27	4.5	5.5	3.5	3.0	5	6
23	0.25	0.21	6.5	5.0	3.5	3.0	6	4
24	0.22	0.22	5.0	6.0	0.7	1.0	3	5
25	0.34	0.22	6.0	8.0	2.0	3.0	7	5
26	0.27		2.5		1.0		4	
Mean	0.27	0.24	6.55	6.42	3.22	2.92	4.81	4.68
LSD 5%	0.04		1.26		0.68		0.64	

Table 4. Growth parameters of *in vitro* 'Baldy' apple regenerated clones by the third multiplication

Clones	Shoot number	Shoot height (cm)	Fresh weight (g)
MS1	3.40	2.23	1.49
MS2	4.00	2.27	0.64
MS5	2.67	2.31	1.24
MS7	8.80	2.68	1.43
MS18	2.47	2.76	1.67
MS24	6.47	2.27	1.19
WP1	6.06	2.06	2.50
WP5	3.70	1.98	1.38
WP8	2.67	1.36	1.25
WP15	2.20	2.11	1.92
WP16	3.57	2.00	2.14
WP20	3.00	2.28	1.64
LSD 5%	1.87	0.50	0.53

Table 5. Molecular size (bp), total amplified fragments, number of polymorphic bands, specific markers of 'Baldy' apple genotypes based on ISSRs

Primer	Molecular size (bp)	Amplified fragments	Polymorphic bands	Specific markers	
				Presence	Absence
HB 10	288-1488	9	1	--	WP20
HB 12	173-1707	10	9	MS5	MS5, WP8
HB 14	292-2958	12	5	--	MS18, MP, WP20
UBC-811	414-1912	10	4	--	WP16
UBC-812	429-1350	9	6	MP	MP, MS5, WP20
17898-B	286-1407	8	2	--	--
17899-A	278-1619	10	4	MS18, MS24	--
17899-B	685-1188	3	0	--	--
844 A	379-1701	8	3	WP20	--
844 B	395-1694	10	5	--	MS18

Table 6. Similarity indices among 'Baldy' apple clones based on ISSRs data analyses

Genotypes	MP	MS1	MS2	MS5	MS7	MS18	MS24	WP1	WP5	WP8	WP15	WP16	WP20
MP	1.000												
MS1	0.923	1.000											
MS2	0.918	0.966	1.000										
MS5	0.849	0.912	0.920	1.000									
MS7	0.892	0.940	0.934	0.947	1.000								
MS18	0.918	0.925	0.933	0.880	0.921	1.000							
MS24	0.903	0.924	0.932	0.892	0.920	0.932	1.000						
WP1	0.910	0.959	0.966	0.899	0.927	0.940	0.966	1.000					
WP5	0.897	0.918	0.926	0.886	0.927	0.913	0.925	0.946	1.000				
WP8	0.910	0.932	0.940	0.872	0.927	0.913	0.912	0.946	0.973	1.000			
WP15	0.897	0.945	0.953	0.872	0.927	0.913	0.925	0.959	0.973	0.973	1.000		
WP16	0.932	0.939	0.947	0.880	0.934	0.933	0.905	0.940	0.940	0.953	0.953	1.000	
WP20	0.931	0.924	0.932	0.865	0.907	0.919	0.904	0.925	0.898	0.912	0.912	0.946	1.000

Table 7. Effect of NaCl concentrations on growth parameters of *in vitro* multiplied 'Baldy' apple clones

Treatments (B)		No. of shoots					Shoot height (cm)					Fresh weight (g)				
Clones (A)		Co	S1	S2	S3	Mean	Co	S1	S2	S3	Mean	Co	S1	S2	S3	Mean
MS1		3.83	2.00	2.00	1.17	2.25	2.45	2.17	2.08	1.75	2.11	1.10	0.27	0.18	0.16	0.43
MS2		3.50	2.17	2.67	2.50	2.71	1.94	2.53	2.02	1.74	2.05	0.74	0.35	0.52	0.35	0.49
MS5		3.50	4.33	3.83	4.33	4.00	2.57	1.78	1.26	1.92	1.88	1.04	1.48	0.80	1.09	1.10
MS7		8.30	3.83	3.83	2.67	4.71	2.68	1.60	1.67	1.55	1.87	0.83	0.59	0.60	0.35	0.60
MS18		2.83	2.17	2.67	1.83	2.38	1.83	1.08	1.26	1.10	1.32	2.30	0.46	0.70	0.29	0.94
MS24		6.00	4.33	4.17	4.67	4.79	1.54	1.84	1.33	1.24	1.49	0.69	0.42	0.53	0.67	0.58
WP1		6.00	4.33	3.67	4.67	4.67	2.50	1.13	1.39	1.26	1.57	1.42	0.45	0.67	0.64	0.80
WP5		5.17	3.00	2.33	2.83	3.33	1.94	1.61	1.89	1.72	1.79	1.28	0.77	0.45	0.50	0.75
WP8		2.83	2.50	3.17	2.50	2.75	1.17	1.25	1.05	1.28	1.19	0.88	0.65	0.58	0.58	0.67
WP15		2.83	5.83	3.33	4.00	4.00	2.08	1.57	1.07	1.46	1.55	1.46	0.88	0.60	0.85	0.95
WP16		4.50	3.17	3.00	2.50	3.29	2.05	1.01	1.17	1.17	1.35	1.07	0.71	0.66	0.50	0.74
WP20		3.83	3.50	3.33	2.67	3.33	2.21	1.25	1.45	1.31	1.56	0.60	0.67	0.85	0.36	0.62
Mean		4.43	3.43	3.17	3.04		2.08	1.57	1.47	1.46		1.12	0.67	0.59	0.53	
LSD 5%	A	0.83					0.35					0.21				
	B	0.48					0.20					0.12				
	A x B	1.67					0.70					0.44				

Co, S1, S2, and S3 are control, 1000, 2000 and 3000 ppm, respectively

salt stress conditions, with 1.17 shoots weighing 0.16 g. The clones MS2, MS7 and WP16 had a vitrified appearance, so they were excluded from further analysis to ensure the accuracy of the results. Similar results were observed for apple rootstock MM.106 (Bahmani *et al*., 2012) and some apple varieties (Shibli *et al*., 2000), where shoot length, number of shoots and fresh weight were decreased with increased salt levels of NaCl. According to Liu *et al*. (2008) and Sotiropoulos (2007), apple shoots were damaged by salt stress, as seen by the large number of necrotic patches in the salt-treated leaves after treatment, compared to very few in the control.

All growth parameters, number of shoots, average shoot height, and fresh weight, were adversely affected by salt, with all means gradually decreasing with increasing NaCl concentrations in the proliferation medium recording the lowest values (3.04 shoot, 1.46 cm, and 0.53 g, respectively) under adverse salt stress comparing to control with 4.43 shoot, 2.08 cm and 1.12 g for shoots count, average shoot height and fresh weight, respectively (Table 7). Increasing NaCl concentration up to 3000 ppm had an insignificant effect on all recorded growth parameters of MS2, MS5, MS24, and WP8 clones. Comparing these four clones under severe salinity, both MS2 and WP8 had the lowest number of shoots (2.50 shoot) while MS5 and MS24 were the best with 4.33 and 4.67 shoot, respectively. On the other hand, both MS24 and WP8 had the lowest average shoot height (1.24 and 1.24 cm, respectively), while MS2 and MS5 were the best with 1.74 and 1.92 cm, respectively. In addition, MS2 was the least considerable fresh weight (0.35 g) compared to MS5, MS24 and WP8 clones with 1.09, 0.67 and 0.58 g in descending order. The MS5 maintained relatively higher number of shoots, average shoot height and fresh weight together up to

3000 ppm NaCl indicating the superiority of MS5 clone under severe salt stress conditions. On contrary, MS18 clone recorded the maximum fresh weight of 2.30 g under control conditions and was reduced by 87.39% to obtain 0.29 g as affected by the highest salt concentration which was accompanied with a relatively low proliferation rate with 1.83 shoot of 1.10 cm tall comparing to other tested clones, indicating susceptibility of MS18 clone to salinity. Also, MS1 clone had a lower proliferation rate under severe salt stress conditions with 1.17 shots weighing 0.16 g. The clones MS2, MS7 and WP16 had vitrified appearance so they were excluded from further analysis to ensure accuracy of the results. Similar results had been observed for apple rootstock MM.106 (Bahmani *et al*., 2012) and some apple varieties (Shibli *et al*., 2000), where shoot length, number of shoots and fresh weight were decreased with increased salt levels of NaCl. According to Liu *et al*. (2008) and Sotiropoulos (2007), apple shoots were damaged by salt stress, as seen by the large number of necrotic patches that were seen in the salt-treated leaves at the conclusion of treatment, compared to very few in the control.

Biochemical responses

Antioxidant capacity: The antioxidant capacity of *in vitro* multiplied 'Baldy' apple clones affected by salt treatments was represented in total phenol content and antioxidant activity (Table 8). Increasing NaCl concentration up to 3000 ppm caused a remarkable reduction in total phenol content and total antioxidant activity (0.33 and 3.44%, respectively) compared to control (0.46 and 4.93%, respectively). Generally, the WP15 clone recorded the highest average of total phenol content (0.55%) accompanied by the maximum overall antioxidant activity of 6.74%, while MS5 was the least (0.31 and 2.74% for total phenol content and total antioxidant activity, respectively). Under control conditions, the WP1 clone had a minimum of 0.16% total phenol content and 1.93% total antioxidant activity. In comparison, MS18 had the maximum of both (0.67% and 8.69%, respectively), which were gradually reduced with increasing NaCl concentrations up to 3000 ppm by 73.13% for total phenol content and 78.25% for total antioxidant activity compared to the control. Data on antioxidant capacity revealed that the MS24 clone was the least affected by salt treatments in this concern. Because of their ability to protect biological tissues from oxidative damage, polyphenols are the most essential phytochemicals that promote health (Pandey and Rizvi, 2009).

The carbon skeleton of phenols is highly diversified, resulting from many classes of chemicals and metabolites that serve a variety of vital roles in plant growth and development. Since one of the effects of exposure to salinity is the generation of ROS, including superoxide radicals ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$), tolerance to salt stress has frequently been linked to oxidative stress that damages different biological components, including proteins, nucleic acids, and membrane lipids (Valko *et al*., 2006; Ashraf, 2009). Their effective ROS scavenging systems shield plants from harmful oxidative reactions (Sharma *et al*., 2010). Plants produce a variety of secondary metabolites, including phenolic compounds and other antioxidant chemicals, to cope with detrimental environmental conditions (Waśkiewicz *et al*., 2012).

Compatible solutes (osmolytes): Total free amino acids content recorded the highest mean value of 54.70 mg/100g using 2000 ppm NaCl to induce salt stress (Table 9). MS18 clone showed the maximum average of total free amino acids (71.71 mg/100g). It had the highest content (71.96 mg/100g) under control conditions and maintained higher levels with elevated stress severity. On the contrary, the MS1 clone gave the minimum average of total free amino acids (18.34 mg/100g), where it had the least content of control (16.27 mg/100g) and kept the lowest values under salt stress conditions. Otherwise, the rest of the clones gained an average range of total free amino acids content between 48.77 and 58.13 mg/100g. Salinity had a limited effect on means of reducing sugar content, while different clones observed a broad sense of variation in response to salt treatments (Table 9). MS5 clone recorded the lowest average of reducing sugars (0.88%). It had the lowest content of control (0.58%) and maintained lower levels with elevated stress severity. On the other hand, the MS18 clone gained the maximum content (5.54%) of reducing sugar under control conditions followed by WP20 (4.28%) which maintained the highest levels with elevated stress severity to obtain the maximum mean of 4.28%, while reducing sugars content of MS18 was adversely affected by adding NaCl to the culture medium. Plant water uptake is disrupted, and osmotic stress is caused by salinity (Ismail *et al*., 2014). Reducing cell osmotic potential and stabilizing cellular structure requires the buildup of osmotic adjustment substances, such as soluble sugars (Apse and Blumwald, 2002).

Table 8. Effect of NaCl concentrations on total phenols content (%) and total antioxidant activity (%) of *in vitro* multiplied 'Baldy' apple clones

Treatments (B) / Clones (A)		Phenols (%)					Antioxidant activity (%)				
		Co	S1	S2	S3	Mean	Co	S1	S2	S3	Mean
MS1		0.37	0.34	0.51	0.37	0.40	2.60	2.90	6.64	3.30	3.86
MS5		0.30	0.29	0.28	0.35	0.31	2.46	2.60	2.55	3.36	2.74
MS18		0.67	0.48	0.24	0.18	0.39	8.69	4.87	2.30	1.89	4.44
MS24		0.55	0.52	0.50	0.55	0.53	4.97	4.65	4.84	4.91	4.48
WP1		0.16	0.50	0.45	0.48	0.40	1.93	5.59	4.16	4.29	3.99
WP5		0.58	0.59	0.63	0.27	0.52	4.03	3.95	3.50	3.85	3.83
WP8		0.43	0.42	0.62	0.10	0.39	5.30	5.10	6.11	1.14	4.41
WP15		0.47	0.50	0.69	0.52	0.55	6.25	6.21	8.18	6.30	6.74
WP20		0.65	0.37	0.39	0.17	0.40	8.14	5.12	5.13	1.96	5.09
Mean		0.46	0.45	0.48	0.33		4.93	4.55	4.82	3.44	
LSD 5%	A	0.01					0.38				
	B	0.01					0.26				
	A x B	0.03					0.77				

Co, S1, S2, and S3 are control, 1000, 2000 and 3000 ppm, respectively.

Table 9. Effect of NaCl concentrations on total free amino acids (mg/100g F.W) and reducing sugars (%) contents of *in vitro* multiplied 'Baldy' apple clones

Treatments (B) / Clones (A)		Free amino acids (mg/100g)					Reducing sugars (%)				
		Co	S1	S2	S3	Mean	Co	S1	S2	S3	Mean
MS1		16.27	18.99	19.07	19.01	18.34	2.11	1.96	3.59	2.59	2.56
MS5		50.06	49.78	51.58	55.72	51.79	0.58	0.99	0.96	0.99	0.88
MS18		71.96	73.61	78.85	62.42	71.71	5.54	1.98	1.66	1.82	2.75
MS24		47.48	48.19	48.23	51.17	48.77	1.70	1.57	1.64	3.25	2.04
WP1		46.18	56.81	64.38	46.31	53.42	0.78	2.45	1.83	1.33	1.60
WP5		49.86	50.23	50.27	51.87	50.56	1.16	1.79	1.68	2.30	1.73
WP8		53.11	57.97	58.83	62.60	58.13	1.49	1.11	1.05	0.98	1.16
WP15		55.27	54.32	56.62	60.25	56.62	3.16	2.44	2.57	1.31	2.37
WP20		50.35	52.88	64.45	51.06	54.69	4.28	4.30	4.83	3.71	4.28
Mean		48.95	51.42	54.70	51.16		2.31	2.07	2.20	2.03	
LSD 5%	A	3.51					0.15				
	B	2.34					0.10				
	A x B	7.02					0.29				

Co, S1, S2, and S3 are control, 1000, 2000 and 3000 ppm, respectively.

Water inside cells and around cellular components, such as proteins and lipid membranes, is thought to be partially replaced by this carbohydrate by creating hydrogen bonds with lipids and proteins (Elbein *et al*., 2003). Moreover, additional soluble N-containing substances like polyamines, soluble proteins, and other amino acids may shield plant tissues from osmotic stress (Rai, 2002). Plants synthesize osmolytes to promote osmotic balance at the cellular level.

Regulation of salt tolerance adaptive mechanisms

Based on morphological and biochemical results, four 'Baldy' apple clones, MS5, MS18, MS24, and WP1, representing different levels of salt tolerance, sensitive, moderate, and tolerant, were selected for further analysis.

Gene expression analysis: Three common differentially expressed genes (DEGs), protein phosphatases PP2C-77a, ABA-insensitive transcription factor ABI5-5b and serine/threonine-protein kinase SAPK3, were analysed (Figure 1). Data revealed that PP2C-77a and ABI5-5b were downregulated. At the same time, SAPK3 was overexpressed in the MS5 genotype under severe salt stress conditions, while MS24 showed downregulation of both PP2C-77a and SAPK3 but slight overexpression of ABI5-5b as affected by 3000 ppm NaCl. On the other hand, the three tested DEGs were upregulated in the WP1 genotype in response to the highest salt concentration, while MS18 showed overexpressed PP2C-77a and ABI5-5b but downregulated SAPK3 under severe salinity. Li *et al*. (2019) observed that the elevated expression of the tested common DEGs modulates the expression of

multiple genes involved in salt stress tolerance. PP2C-77a was involved in the high-salinity-stress response through SOS1, SOS2 and SOS3 regulation. Comparatively, overexpression of ABI5-5b and SAPK3 caused upregulation of only SOS3 and SOS1, respectively. Also, overexpression of the three upregulated common differentially expressed genes (DEGs), PP2C-77a, ABI5-5b and SAPK3, individually in apple calli promoted the consistent upregulation of DREB6, CBF1 and ZAT10. It increased mass weight and antioxidant ability, implying these five common DEGs are involved in multiple pathways and improved comprehensive resistance to stress. The common DEGs were predominantly associated with antioxidant-, drought-, or high-salinity- stress regulation pathways. According to Li *et al*. (2019), the overexpression of five common DEGs, PP2C-37b, PP2C-77a, ABI5-5b, SAPK3 and HPt3a, induced the transcription of the CSD1, APX2a, DHAR3, and GPX6 antioxidant enzyme genes which promoted superoxide enzyme activities and decreased the H2O2 concentration of apple calli under abiotic stress conditions, which also suggests involvement of these five common DEGs in multiple pathways and improvement of comprehensive resistance to stress. On the other hand, it was also observed examples of the suppression of abiotic stress-inducible genes, such as CAT1, DREB2B, CBF3, ICE1, and NHX1, by these five common DEGs, PP2C-37b, PP2C-77a, ABI5-5b, SAPK3, and HPt3a. Positive functions on abiotic stress resistance genes, such as NHX1 for high-salinity stress, were not well reflected under the overexpression of the five common DEGs, showing that specificity and coordination coexist in multiple regulatory (Li *et al*., 2019).

Enzymatic antioxidants activity: As shown in Figure 2, activities of the three tested antioxidative enzymes, superoxide dismutase (SOD), peroxidase (Px), and catalase (CAT), were stimulated over control as affected by elevated salt concentrations up to 3000 ppm NaCl in both MS5 and MS24 genotypes. On the other hand, the MS18 genotype showed higher peroxidase activity but lower superoxide dismutase and catalase activities under severe salinity relative to control. At the same time, WP1 had higher superoxide dismutase but lower both peroxidase and catalase in response to the highest concentration of 3000 ppm NaCl relative to control. Osmotic stress and ionic toxicity are the two types of stress factors that plants experience in a salty environment.

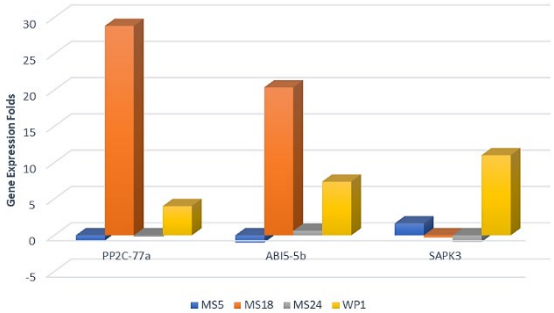

Figure 1. Changes in regulation of the targeted genes, PP2C-77a, ABI5-5b and SAPK3, for selected 'Balady' apple genotypes, MS5, MS18, MS24 and WP1, grown under severe salinity stress (3000 ppm NaCl) relative to control (0 ppm NaCl).

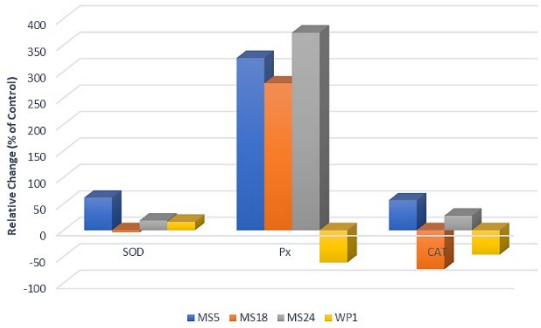

Figure 2. Changes (%) in the activity of the targeted antioxidative enzymes, SOD, Px and CAT, for selected 'Balady' apple genotypes, MS5, MS18, MS24 and WP1, grown under severe salinity stress (3000 ppm NaCl) relative to NaCl-untreated control.

While the latter harms the physiological process of plant metabolism, the former prevents plants from absorbing water. Furthermore, both may generate reactive oxygen species (ROS), which harm cell membrane structure (Slama *et al*., 2015). Under salt stress, different plants have various primary ROS scavenging substances. Even within the same plant, responses to salt stress vary across tissues (Wang *et al*., 2022). The ability of plants to scavenge ROS under stress may be reflected in changes in Px, SOD, and CAT activities. To get rid of the harmful H_2O_2 and phenol amine, SOD can dismutate O_2^- to O_2 or H_2O_2, CAT can catalyze H_2O_2 to H_2O and O_2, and Px can direct the oxidation of phenol or amine compounds with H2O2 as an electron acceptor (Sudhakar *et al*., 2002). According to Ahmad *et al*. (2016), under salt stress, SOD, Px, and CAT activities increased to scavenge ROS quickly.

CONCLUSION

This investigation confirmed the feasibility of *in vitro* screening for a high salt-tolerant genotype among the apple seedling population. Perennial plant seedling populations with high genetic heterozygosity

segregate densely and produce wildly disparate individuals, suggesting that this strategy could be applied to other perennial woody plants. The findings underscore the potential of integrating molecular and biochemical markers with tissue culture techniques to accelerate the development of salt-tolerant apple genotypes, particularly in regions with salinity-prone soils.

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