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The role of exogenous glutamine on germination, plant development and transcriptional expression of some stress-related genes in onion under salt stress

Kamile Ulukapi[1], *Ayse Gul Nasircilar*[2,*]

[1] Department of Plant and Animal Production, Vocational High School of Technical Sciences, Akdeniz University, 07059 Antalya, Turkey

[2] Department of Mathematics and Science Education, Faculty of Education, Akdeniz University, 07059 Antalya, Turkey

ABSTRACT

The purpose of this research was to explore the role of glutamine (Gln) on plant germination and growth under salinity [0, 50, 100, 150, 200 mM sodium chloride (NaCl)], and on the activation of some stress-related genes [*CuZn-Superoxide Dismutase (SOD), Mn-SOD, L-ascorbate oxidase (AO), DNA Polymerase Delta 1 (POLD)-1, Chaperon (CHAPE) and Heat Shock Protein (HSP)-21*], genetic template stability and photosynthetic pigment activation. Under salt stress, 2 mM Gln application reduced the mean germination time (MGT) (4.51 days), increased the coefficient of velocity of germination (CVG) (75.85), germination index (GI) (1.46) and germination percentage (GP) (82%), indicating that it was the best application for germination under stress. Gln promoted the development of aboveground plant organs. 3 mM Gln increased the number of leaves from 5.3 to 13.7 and 4.3 to 6.6 under unstress and stress conditions, respectively. Under salt stress, Gln increased photosynthetic pigments and genomic template stability (GTS) (80%). At 2 mM Gln, total chlorophyll and carotenoid content increased from 1.59 mg · g^{-1} TA to 2.23 mg · g^{-1} TA and from 0.28 mg · g^{-1} TA to 0.37 mg · g^{-1} TA, respectively. The effect on gene expression levels varied with the concentration. The application of 2 mM Gln, which enhanced germination and vegetative parameters under stress, caused a raise in CuZn-SOD gene expression and a 43% decrease in the transcriptional expression of the HSP-21 gene as an indicator of the functionality of the repair mechanism. The improved growth of Gln-treated plants under stress suggests that Gln is involved in bridging the energy deficit of the plant by acting as an alternative fuel for metabolic activities under stress.

Keywords: *Allium cepa* L., gene expression, genetic template stability, growth attributes, salinity

Abbreviations: AO, L-ascorbate oxidase; Car, carotenoids; CHAPE, chaperon; Chl, chlorophyll; CuZn-SOD, CuZn-superoxide dismutase; CVG, coefficient of velocity of germination; GTS, genomic template stability; GI, germination index; Gln, glutamine; GP, germination percentage; HSP-21, heat shock protein-21; MGT, mean germination time; Mn-SOD, Mn-superoxide dismutase; PCR, polymerase chain reaction; POLD1, DNA Polymerase Delta 1; RAPD, Randomly Amplified Polimorphic DNAs.

INTRODUCTION

Glutamine (Gln) is one of the primary products of ammonia assimilation and one of the most important nitrogen donors in living organisms. As one of the most abundant free amino acids in plants, Gln provides about 28% of cellular nitrogen, along with histidine, tryptophan, asparagine, purines, pyrimidines, amino sugars and *p*-aminobenzoate (Kan et al., 2015; Yoneyama and Suzuki, 2020). Gln, known to play an

*Corresponding author.
e-mail: nasircilar@akdeniz.edu.tr (Ayse Gul Nasircilar)

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effective role in coping with abiotic stress conditions, is a precursor metabolite involved in the synthesis of other amino acids and is very important for plant growth and development (Hildebrandt et al., 2015; Muthuramalingam et al., 2020). It also plays a role in facilitating the scavenging of reactive oxygen derivatives, the biosynthesis of osmolytes and the induction of some transcription factors of the genes responsible for stress reactions under abiotic stress conditions (Kan et al., 2015; Nam et al., 2015; Miranda et al., 2017). Gln application was found effective in reducing chromosome aberrations in onion under salinity (Çavuşoğlu et al., 2020). The best germination results in onion seeds primed with ten different amino acids were obtained as a result of treatment with Gln. Gln increased the germination rate and vigour index, shortened the germination time and also enhanced the chlorophyll and carotenoid amounts (Abdelkader et al., 2023). Additionally, external melatonin application in maize (Jiang et al., 2016) and silicon treatment in lettuce (Alves et al., 2020) are examples of other chemicals used to alleviate the negative impacts induced by salt stress. These examples, which can be multiplied further, are important in demonstrating the effects of externally applied chemicals under stress conditions.

Onion (*Allium cepa* L.) is cultivated in more than 175 countries and is the third-most extensive produced after tomato and potato. In addition to its use as a spice and vegetable, it is also used in pharmaceutical preparations due to its phytochemical content. It is widely used throughout the world, and it is estimated that the demand for onions will rise due to economic development, urbanisation and population growth (Cope, 2005; Baloch et al., 2014; Kumar et al., 2022; FAO, 2023). *Allium cepa* (2n = 16), which has a relatively small genome, is a glycophytic vegetable with high economic importance and is sensitive to salt (Alam et al., 2023). Stress sources are among the most important factors causing yield loss in onion, which is a highly susceptible species to biotic and abiotic stresses factors (Ghodke et al., 2020). Salt stress, which is one of the abiotic stresses that adversely influences plant growth, affects 7.6% of the total area and causes production losses ranging from 18% to 43% (Singh, 2015; Correa-Ferreira et al., 2019). Abiotic stress conditions negatively affect plant growth and yield by altering reactive oxygen metabolism in plants (Chaki et al., 2020). Ascorbate oxidase (AO), Mn-superoxide dismutase (Mn-SOD) and CuZn-superoxide dismutase (CuZn-SOD) are among the antioxidant defence components that scavenge reactive oxygen compounds in plant mitochondria (Millar et al., 2001). Superoxide dismutase (SOD) enzymes catalyse the reduction of the superoxide radical anion to less reactive compounds. Besides its important role in the metabolic regulation of stress, CuZn-SOD also has a crucial function in maintaining intracellular haemostasis (Michonneau et al., 2023). CuZn-SOD, one of the SODs that protect plants against oxidative damage, is localised in the chloroplast and cytosol, whereas Mn-SOD is mainly found in the mitochondria and peroxisomes (Kumar et al., 2013; Del Río and López-Huertas, 2016). Ascorbate oxidase (AO) is one of two enzymes involved in the oxidation of ascorbate, which plays a role in the elimination of harmful reactive oxygen species (Asada and Takahashi, 1987). It plays an active role in cell metabolism associated with cell expansion (Smirnoff, 1996). In addition, it has also been found to increase tolerance to drought (Fotopoulos et al., 2008) and salt stress (Yamamoto et al., 2005). Heat shock proteins-21 (*HSP21*) are responsible for chloroplast development (Zhong et al., 2013) and chaperonins (CHAPE) for protein folding, metabolic regulation and reply to abiotic stress (Pareek et al., 2021). *HSP21* is thought to play a protective role in the plant against oxidative stress and heat stress by stabilising photosystem II during photosynthesis reactions (Sun et al., 2020). Gln is known to activate some genes responsible for signalling and stress-response formation under stress conditions (Kan et al., 2015).

Nowadays, overcoming stresses has become increasingly important. Understanding the genome and stress-related gene expression will help elucidate the mechanisms of responses generated by plants under different stress conditions. Besides, determining the role of exogenous applications in stress-related gene activities will help determine applications to provide stress tolerance. This study intends to clarify the role of Gln, which is the main source of nitrogen for plants under normal conditions, in stress-related gene activation, genomic stability and plant growth under stress conditions.

MATERIALS AND METHODS

Plant material and germination condition

The *Allium cepa* bulbs (Göver seed onion) used in the study were provided by a commercial company, and the same size (approximately 2 cm in diameter) was used in the experiments. The germination study consisted of two parts. First, onions were germinated at 50, 100, 150, and 200 mM salt (NaCl/Sigma, Missouri, USA) concentrations to determine the salt threshold value at which germination was inhibited. After determining the 150 mM NaCl concentration as the threshold value, the second stage of the study was carried out by adding different concentrations of Gln (Sigma, Missouri, USA) to the media containing 150 mM NaCl. In all experiments, plastic containers were used in which styrofoam plates with holes large enough to fit onions were placed. The solutions, prepared as indicated in the experimental design (Table 1), were poured into the plastic containers, and the styrofoam was placed in the plastic containers at such a distance that the bottoms of the bulbs were in contact with the water. The experiment was conducted in 4 replicates of 25 bulbs per replicate, giving 100 bulbs per treatment. Germination experiments were

Table 1. Schematic representation of the experiment.

	Treatments								
First stage	Control	50 mM NaCl	100 mM NaCl	150 mM NaCl	200 mM NaCl	1 mM Gln	2 mM Gln	3 mM Gln	4 mM Gln
Second stage	150 mM NaCl + 1 mM Gln			150 mM NaCl + 2 mM Gln		150 mM NaCl + 3 mM Gln		150 mM NaCl + 4 mM Gln	

Gln, glutamine.

accomplished in a dark and controlled climate room at 20 ± 2°C. After emergence, a 16:8 (light:dark) photoperiod was applied and the experiments were continued for 14 days according to the International Seed Testing Association (ISTA) (1985).

The number of germinating bulbs was recorded daily throughout the experiment; the germination percentage (GP), the mean germination time (MGT), the coefficient of velocity of germination (CVG), and the germination index (GI) were calculated at the end of the experiment. Shoot length (SL, mm), root length (RL, mm), shoot fresh and dry weight (SFW, SDW, g), root fresh and dry weight (RFW, RDW, g), and total number of leaves (TNLs) were measured in ten randomly selected plants from each group to determine the impact of Gln on plant growth under salinity and normal circumstances.

Germination data were calculated using the following formulas:

GP (%) = number of germinated seeds/total number of seeds × 100 (Gosh et al., 2014).

MGT: ΣD n/Σn D = days counted from the beginning of the experiment (Ellis and Roberts, 1981; Sivritepe, 2012)

D, day; *n*, number of seeds germinated on related day.

CVG: N1 + N2 + …. + Nx/100 × N1T1 + …… + NxTx (Kotowski, 1926)

N, number of seeds germinated daily; *T*, number of days related to *N*.

GI: (14 × n1) + (13 × n2) + ….. + (1 × n14) (modified from Benech et al., 1991)

n1, n2,…, n14 – number of seeds germinated per day for 14 days from the first day of germination; 14, 13…., and 1 are the weights of the germinated seeds daily.

Photosynthetic pigment analysis

The determination of the pigment in onion leaf tissue exposed to salt stress and Gln was performed according to Doğanlar et al. (2010). In this context, 200 mg of fresh onion leaves were homogenised in 8 mL of 80% acetone using a Daihan HG-15-A homogeniser. The solutions were centrifuged at 3,000 rpm for 15 min. The absorbance of the supernatants was measured at 645, 652, 663 and 470 nm UV-vis spectrometer (Hitachi U-1800 spectrophotometer). Photosynthetic pigment concentrations were calculated according to the following formulas:

Total chlorophyll: $A_{652} \times 27.8 \times 20$/mg leaf weight

Chlorophyll a (Chl a): $(11.75 \times A_{663} – 2.35 \times A_{645}) \times 20$/mg leaf weight

Chlorophyll b (Chl b): $(18.61 \times A_{645} – 3.96 \times A_{663}) \times 20$/mg leaf weight

Carotenoids (Car): [(1000 × A_{470}–2.27 × Chl a–81.4 × Chl b)/227] × 20/mg leaf weight

A – absorbance.

Genomic template stability

DNA was isolated from 100 mg of the plant samples using the GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific™, Waltham, MA, USA) according to the kit protocol. The concentration and purity of DNA were measured using a microvolume spectrophotometer (OPTIZEN, Nano Q Lite, Daejeon, Republic of Korea). As a result of DNA isolation, total DNAs obtained from the control and treatment groups were equalized with nuclease-free water before the randomly amplified polymorphic DNAs-polymerase chain reaction (RAPD-PCR) reaction and used as template DNA.

For the RAPD-PCR analysis, a total reaction volume of 40 µL was prepared in PCR tubes using Dreamtaq 2X PCR Master Mix (Thermo Scientific™), 12 RAPD primers (Table 2) and equalised DNA samples. The reaction mixture (40 mL) for the amplification of genomic DNA is as follows: Dreamtaq 2X PCR Master Mix (20 mL), 3.85 µL of primer intermediate stocks (0.5 µM) were added for each primer and DNAs from template DNA stocks prepared at 100 ng DNA per 1 µL were brought to the final volume with nuclease-free water. PCR was performed on the Applied Biosystems® ProFlex™ PCR instrument (Waltham, MA USA). PCR was performed under PCR conditions of 1 cycle 98°C 1 min, 42 cycles 94°C 20 s, 37°C 20 s and 72°C 1 min and 1 cycle 72°C 10 min PCR conditions.

After DNA amplification, 12 µL of the PCR products and 6 µL of the DNA Ladder were loaded into wells containing 2% agarose gel + ethidium bromide and run in 2 × TAE (Tris 1.6 M, acetic acid 0.8 M, EDTA 40 mM) buffer at 70 volts for approximately four and a half hours. DNA Ladder of 100–10000 base pairs was used as a molecular weight standard according to the manufacturer's instructions. The bands obtained were

Table 2. RAPD PCR primers and sequences.

Primer		Primer sequence	
RAPD2	5′	ACGGTACCAG	3′
RAPD7	5′	TTCCGAACCC	3′
OPPO5	5′	CCCCGGTAAC	3′
OPI18	5′	TGCCCAGCCT	3′
12OPC06	5′	GAACGGACTC	3′
14OPC09	5′	CTCACCGTCC	3′
19OPC14	5′	TGCGTGCTTG	3′
21OPC16	5′	CACACTCCAG	3′
OPW10	5′	TCGCATCCCT	3′
30OPAF05	5′	CCCGATCAGA	3′
25OPN01	5′	CTCACGTTGG	3′
S237	5′	ACCGGCTTGT	3′

PCR, polymerase chain reaction; RAPD, randomly amplified polymorphic DNAs.

photographed using a UV transilluminator (Infinity Capture, Vilber Lourmat, Quantum, France), and the size of the bands was determined using the Bio-Profil Bio1D++ programme (Vilber Lourmat, Quantum, France) and were analysed by molecular weight (base pairs).

Genomic template stability [GTS (%)]; GTS% = [1–(a/n)] × 100 was calculated using this formula.

where *n*, number of bands determined in the DNA profile of the control group; a, total changes in the DNA profiles.

Gene expression

Homogenisation of tissues

Onion leaf tissues were homogenised using the Tissue Lyser LT system (Qiagen, Germantown, Maryland, USA). The lysis process was repeated thrice until the samples were completely homogenised. Afterwards, the samples were kept at room temperature, and the RNA isolation stage was initiated.

RNA isolation and cDNA synthesis

RNA was isolated using a combination of TRIzol™ Reagent and PureLink RNA Mini Kit (Thermo Fisher Scientific, USA). The samples were brought to room temperature and placed in Bioer Thermo Shakers; 500 µL of TRIzol reagent was added and incubated at 400 rpm for 5–10 min at room temperature. Then, 100 µL of chloroform (Merck, Darmstadt, Germany) was added to these homogenised samples and incubated for 2–3 min and centrifuged at 12000 × *g* for 15 min at 4°C using MIKRO 200 R (Hettich Instruments, Beverly, USA). After centrifugation, the RNA phase, which was transparent and colourless at the top of the three different phases, was collected into new sterile 1.5 mL eppendorph tubes. To the material in these tubes, 70% ultrapure ethanol (Merck, Darmstadt, Germany) was added up to the volume collected in the tubes and the samples were mixed by vortexing. This mixture was loaded as 700 µL onto the PureLink RNA Mini Kit columns. The columns were centrifuged sequentially at 12000 *g* for 30 s, and the RNAs were loaded onto the columns. These columns were then washed with washing solutions 1 and 2. After washing, the columns were centrifuged once at 12000 *g* for 3 min to dry the columns, and the columns were placed in sterile 1.5 mL eppendorph tubes; 60 µL of the solution given in the kit was pipetted into the centre of the membrane in the column. Then, pure RNAs were collected in the eppendorph tube by centrifugation at 12000 *g* for 1 min. The purity of the collected RNAs was determined by OPTIZEN NanoQ micro-volume spectrophotometer (Mecasys, Daejeon, Republic of Korea). The obtained RNAs were equilibrated to 750 ng · 10 µL with DNAse and RNAse free ultrapure water (Sigma, Missouri, USA) for the next step. Complementary DNA (cDNA) synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) according to the kit protocol using the Applied Biosystems® ProFlex™ PCR System thermal cycler (step 1: 25°C, 10 min; step 2: 37°C, 120 min; step 3: 85°C, 5 min). Obtained cDNAs were stored at –20°C for further analysis.

qRT PCR analysis

In this study, the relative expression levels of *CuZn-SOD, Mn-SOD, AO, DNA Polymerase Delta 1 (POLD)-1, CHAPE* and *HSP21* genes in the control and salt-stressed onion leaf tissues were analysed by qRT-PCR. The primer pairs used for these genes are listed in Table 3.

For the determination of gene expressions, cDNAs were amplified using Applied Biosystems QuantStudio 5 Real-Time PCR according to the Power Syber Green qPCR Master Mix (Thermo Scientific) protocol. Gene expressions were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Endogenous control β-actin and 18S mRNA expressions were used as calibration and correction factors with the multiple control method. Protocol: Step 1 for the qRT-PCR reaction: enzyme activation: 95°C, 10 min; Step 2: denaturation: 95°C, 15 s; primer annealing chain extension: 60°C, 1 min; Step 3: melting curve: 95°C, 15 s, 60°C, 1 min, 95°C, 15 s.

Statistical analysis

Analysis of variance (ANOVA) was used in the Minitab 21 package programme (Pennsylvania, USA) to compare the mean differences between the plant growth and the germination attributes of Gln applications; the applications were compared using the Tukey test ($p < 0.01$ and $p < 0.05$). In addition, the distribution of Gln treatments according to germination and growth attributes was visualised separately and together by using linear projection in the Orange statistical program.

Table 3. Genes, gene names, sequences and literature.

Gene ID	Gene name	Primer sequences (5′→3′)	Reference
SOD	Superoxide dismutase	F TTCCTCCAGCATTCCCAGTG R ATGGCTTGACACATGGTGCT	Ghodke et al. (2020)
SOD-2	Superoxide dismutase 2, mitochondrial	F GGCGAAGCAAACAGCCTCAT R AGTATCGCCGAACGAGTGGA	Ji et al. (2021)
AO	L-ascorbate oxidase-like	F TGATGTTTGTGCTGTCTTTCGG R ACCGTGAAAGTGTTTGTGCT	Ghodke et al. (2020)
POLD1	DNA Polymerase Delta 1, catalytic subunit	F AGACGACTCGCTGTGTATTGCT R CCAGTAACTCGTGCCATCTCCA	Lyu et al. (2020)
Chape	Chaperone	F TCCTGGCAAGTCTGCTTTGA R GGCTCTAATTCCTCGCGTTT	Ghodke et al. (2020)
HSP21	21 kDa protein	F TAGATGGATGGCGAACTCGG R TCTTCTTCTTCGCACTCCT	Ghodke et al. (2020)
β-*Actin*	β-actin	F TCCTAACCGAGCGAGGCTACAT R GGAAAAGCACTTCTGGGCACC	Sun et al. (2013)
18S	18S ribosomal RNA	F GAATGACTCCTGGCAATG R GATTGGAATGACGCTATACA	Liu et al. (2015)

Protocol: Step 1 for the qRT-PCR reaction: enzyme activation: 95°C, 10 min; Step 2: denaturation: 95°C, 15 s; primer bonding-chain elongation: 60°C-1 min, Step 3: melting curve: 95°C, 15 s, 60°C, 1 min, 95°C, 15 s.

The analysis of GTS values was compared using SPSS 18 software at a significance level of $p < 0.05$, one-way ANOVA and independent group *t*-test.

In the study, the difference between the means of expression values obtained by the $2^{-\Delta\Delta Ct}$ method in the qRT-PCR array studies was determined by one-way ANOVA and the groups in which the means were determined by the Tukey HSD test. Analyses were performed using SPSS 20, University Licence (IBM, New York, USA), and $p \leq 0.05$ was used.

RESULTS

Germination and growth attributes

This part of the research was accomplished in two stages. In the first stage, a threshold value was determined according to the rate at which increasing salt levels suppressed vegetative growth. The most dramatic reduction in growth parameters was observed in plants treated with 150 mM NaCl. Shoot and RL decreased from 300.78 mm to 55.37 mm and from 335.30 mm to 54.16 mm, respectively, compared to the control. Therefore, 150 mM NaCl was defined as the threshold concentration, and the vegetative changes caused by Gln treatments in onion under salt-stress conditions were examined at this salt level.

Firstly, when the germination results were examined (Figure 1), the GP in the control experiment was 100%. In the bulbs exposed to 150 mM NaCl, it decreased to 64% and the best result was obtained from the 2 mM Gln group under salt-stress conditions (82%). Under normal conditions, 4 mM Gln application showed its positive effect by increasing the MGT (3.48 days) and GI (1.79). Under salt stress, the MGT decreased from 6.48 days to 4.51 days as a result of 2 mM Gln application, the CGV was 75.85, and the GI was 1.46, indicating that the 2 mM Gln application was the best application under salinity.

It was determined that different Gln concentrations affected vegetative growth under unstressed and salt-stress conditions. Under unstressed conditions, 2 mM Gln increased all parameters except root characteristics above the control level. In addition, although 3 mM Gln was not as successful as 2 mM Gln in promoting plant growth, it increased the TNLs, which is an important criterion for producers, by 150.7% compared to the control group (13.7). It was also found that 3 mM Gln applied under stress conditions was the most effective concentration in reducing the negative effects of salinity. In addition to plant growth, it increased the TNLss to 6.6 and provided a 53.5% increase compared to 150 mM NaCl. The findings of the ANOVA showed that Gln had statistically significant effects on all variables (Table 4).

The distribution of the treatments according to the changes in the germination and growth characteristics separately and together is presented as a linear projection in Figure 2. In addition, a heat map was used to show the distribution of the treatments with a dendrogram as well as the increase and decrease of the examined parameters according to the treatments (Figure 2). According to the germination data only, the control, 50 mM NaCl, 100 mM NaCl, Gln treatments and 150 mM NaCl + 2 mM Gln formed a separate group. According to vegetative data only, it can be seen that the control group was completely separated; on the other hand, under severe stress conditions, the Gln-treated groups and severe stress treatments were clustered. When all parameters were analysed together, it was determined that the control group was completely separated, the 150 mM and 200 mM NaCl groups were clustered and the groups treated with 1, 3 and 4 mM Gln under stress

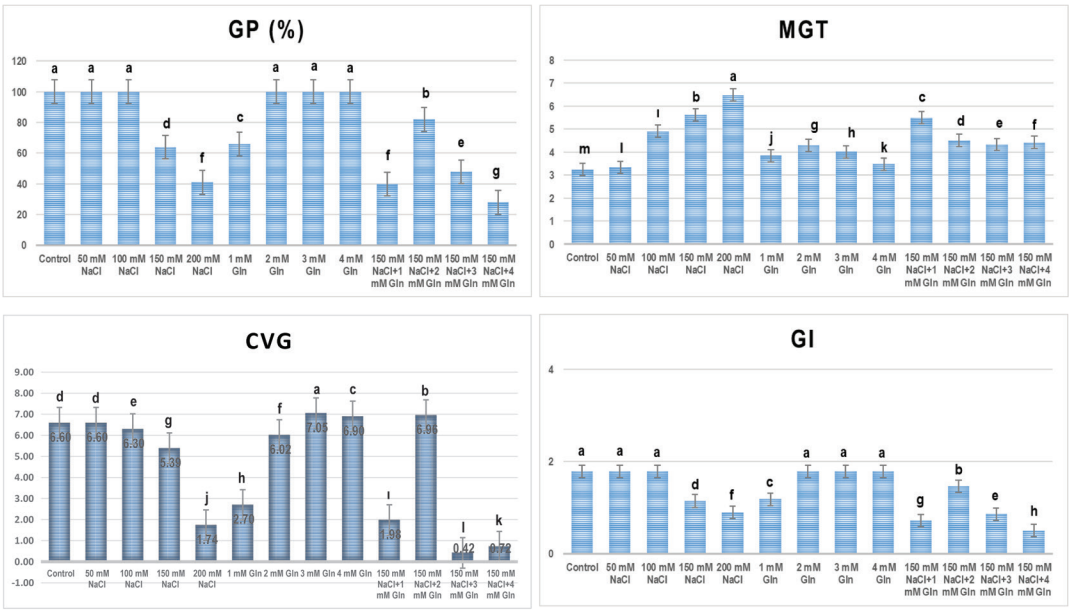


Figure 1. Changes in GP, MGT, CVG, and GI caused by salt stress and Gln treatments. Statistically significant differences between treatments are shown in the bar graphs with different letters. According to one-way ANOVA (Tukey test), statistically significant differences were determined between the experimental groups at the $p \leq 0.05$ level. ANOVA, analysis of variance; CVG, coefficient of velocity of germination; GI, germination index; GP, germination percentage; MGT, mean germination time.

Table 4. Changes in the vegetative growth of onions at different Gln concentrations under salt-stress and non-stress conditions.

Treatments	SL (mm)	RL (mm)	SFW (g)	RFW (g)	SDW (g)	RDW (g)	TLN (no.)
Control	300.78 ab	335.30 a	4.7717 bc	5.3486 a	0.2974 b	0.2578 b	5.4 c
50 mM NaCl	200.50 abc	174.67 b	3.3849 c	1.8111 bcd	0.2423 b	0.1269 cd	6.0 c
100 mM NaCl	176.14 bcd	99.98 bcd	3.0034 c	0.9761 cd	0.3421 b	0.0514 de	4.7 c
150 mM NaCl	55.37 de	54.16 cd	1.6138 c	0.7861 cd	0.1272 b	0.0980 de	4.3 c
200 mM NaCl	35.96 e	30.95 d	0.6784 c	0.2976 d	0.1009 b	0.0250 e	3.6 c
1 mM Gln	265.67 ab	78.67 bcd	5.6750 abc	2.0207 bcd	0.3935 b	0.1229 cd	6.7 bc
2 mM Gln	316.00 a	131.33 bc	11.1293 a	3.1972 b	5.0274 a	0.2022 bc	11.6 ab
3 mM Gln	269.00 ab	70.00 cd	10.4772 ab	2.8864 bc	3.4157 a	0.9267 a	13.7 a
4 mM Gln	243.67 ab	68.67 cd	6.4551 abc	2.2977 bcd	4.5882 a	0.2567 b	6.6 bc
150 mM NaCl+ 1 mM Gln	64.67 de	79.67 bcd	1.3471 c	1.8282 bcd	0.1246 b	0.1017 de	3.7 c
150 mM NaCl+ 2 mM Gln	65.33 de	78.00 cd	1.6087 c	1.3310 bcd	0.1228 b	0.1293 cd	6.0 c
150 mM NaCl+ 3 mM Gln	81.67 cde	84.67 bcd	1.7282 c	1.8951 bcd	0.1594 b	0.1341 cd	6.6 bc
150 mM NaCl+ 4 mM Gln	77.67 cde	64.00 cd	0.8713 c	1.1304 bcd	0.1030 b	0.1166 cde	5.7 c
	**	**	**	**	**	**	**

Statistically, the differences between the applications are shown with different letters (Tukey). ** $p \leq 0.001$, * $p \leq 0.005$, ns: not significant. Gln: glutamine, SL: shoot length, RL: root length, SFW: shoot fresh weight, RFW: root fresh weight, SDW: shoot dry weight; RDW: root dry weight; TLN: total leaf number.

conditions were in the same group. These results are also consistent with the dendogram formed in the heat map. Furthermore, when the heat map was analysed, it was found that the control, 50 mM NaCl, 100 mM NaCl, 1 mM, 2 mM, 3mM and 4 mM Gln groups were separated from the others in terms of both germination and vegetative development, and good results were obtained. Besides, the closest application to this large group was the 150 mM NaCl + 2 mM Gln.

Photosynthetic pigment analysis

In this context, the contents of the total Chl, Chl a, Chl b and Car, which are informative parameters under stress conditions, were examined (Figure 3). Under normal

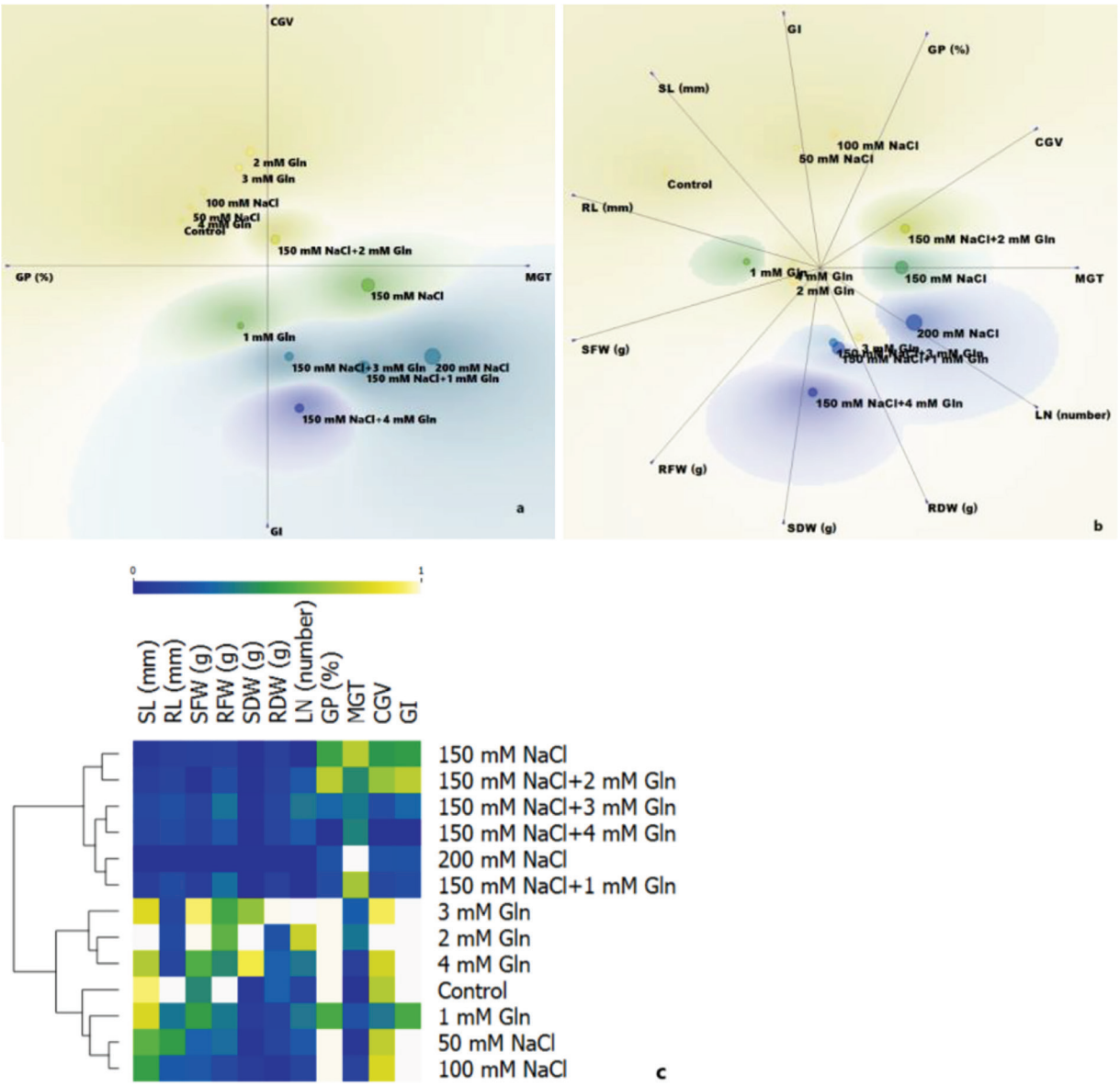


Figure 2. Linear projection of the distribution of applications according to plant growth and germination values and visualisation of the classification of applications according to the change in plant characteristics with heat map. (A) Linear projection according to germination attributes, (B) linear projection according to germination and vegetative attributes (C) heat map. By using the principal component analysis data in linear projection (A and B), a two-dimensional projection is presented in which different applications are best separated according to variables. As it can be followed from the scale on the heat map (C), the change of colours gives information about the effect of the applications on the variables. The lowest values are shown in dark blue. The change of colour towards white in the heat map showed that the values increased.

conditions, the application of Gln did not increase the levels of total Chl, Chl a, Chl b and Car above the control plants. Although the Chl a/b ratio increased in all the Gln applications compared to the control group, it reached its highest value in the 3 mM and 4 mM Gln application groups. Under salt-stress conditions, it was found that the photosynthetic pigment content was enhanced in all the Gln applications compared to the 150 mM NaCl group. It was found that the application of 4 mM Gln (total Chl: 2.81 mg · g^{-1} TA, Car: 0.49 mg · g^{-1} TA) gave the best results, especially in terms of total Chl and Car.

GTS

RAPD analysis was performed to assess the impact of Gln, applied under salinity conditions, on the stability of the genomic template of the onion. In the current study, increased stress intensity caused DNA damage and decreased GTS. The sequences of 8 different primers (Figure 4) that gave significant results from 12 primers

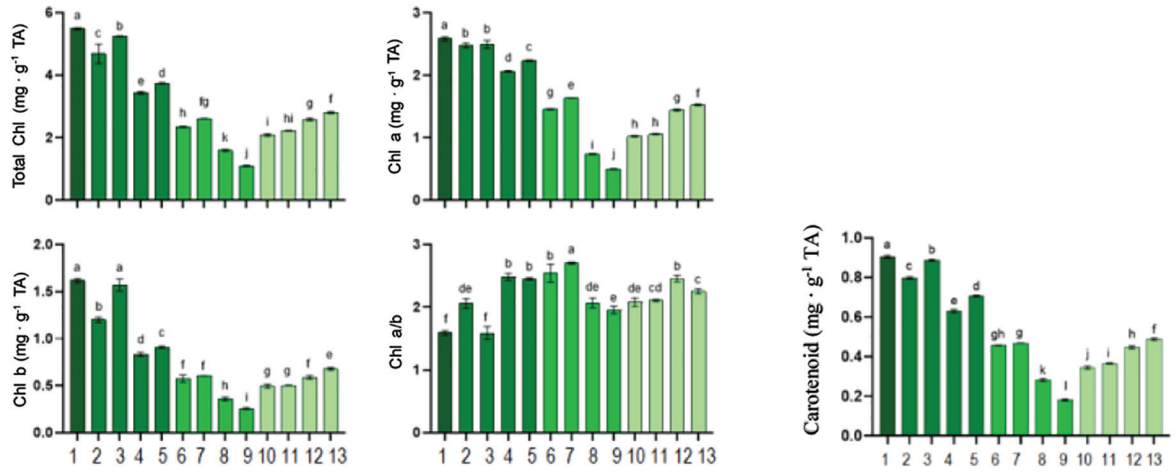


Figure 3. Total Chl, Chl a, Chl b, Chl a/b and carotenoid amounts determined in leaf tissues of control and experimental groups of onion. Data are given as mean ± standard deviation, $n = 5$. The averages shown with different letters on the graph are statistically different from each other. One-way ANOVA, TUKEY HSD test, $p \leq 0.05$ (1: Control, 2: 1 mM Gln, 3: 2 mM Gln, 4: 3 mM Gln, 5: 4 mM Gln, 6: 50 mM NaCl, 7: 100 mM NaCl, 8: 150 mM NaCl, 9: 200 mM NaCl, 10: 150 mM NaCl + 1 mM Gln,11: 150 mM NaCl + 2 mM Gln, 12: 150 mM NaCl + 3 mM Gln, 13: 150 mM NaCl + 4 mM Gln). ANOVA, analysis of variance; Chl a, chlorophyll a; Chl a/b, chlorophyll a/b ratio; Chl b, chlorophyll b; total Chl, total chlorophyll.

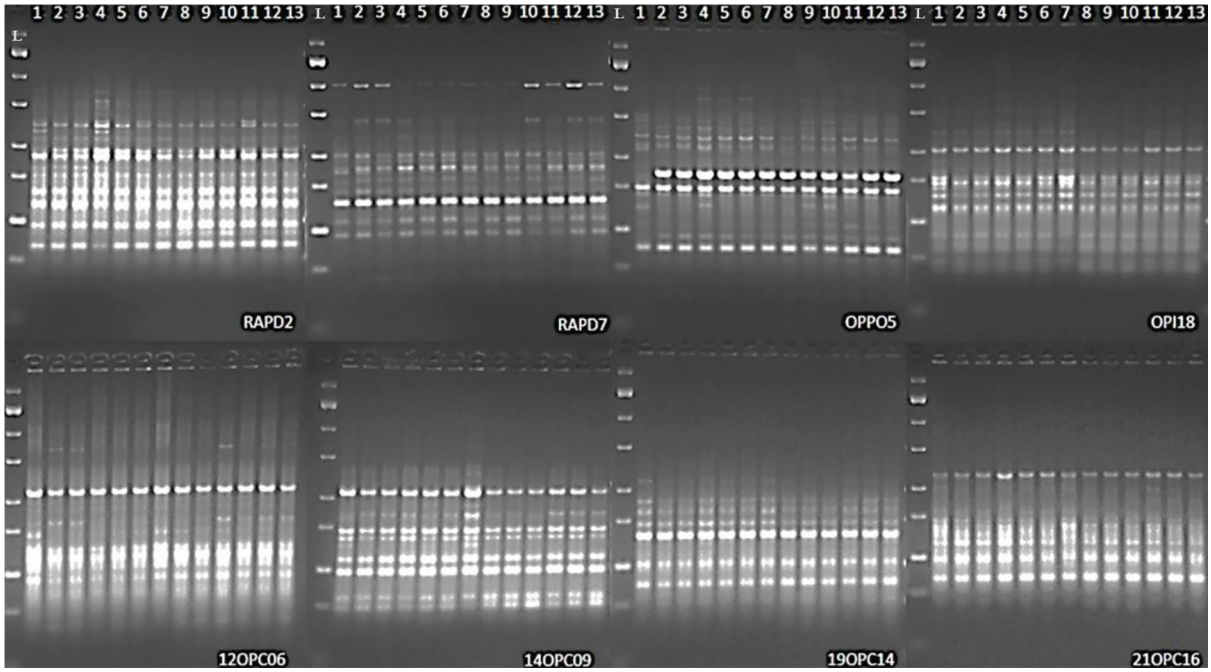


Figure 4. Gel image of the primers included in the analysis. (1: Control, 2: 1 mM Gln, 3: 2 mM Gln, 4: 3 mM Gln, 5: 4 mM Gln, 6: 50 mM NaCl, 7: 100 mM NaCl, 8: 150 mM NaCl, 9: 200 mM NaCl, 10: 150 mM NaCl + 1 mM Gln, 11: 150 mM NaCl + 2 mM Gln, 12: 150 mM NaCl + 3 mM Gln, 13: 150 mM NaCl + 4 mM Gln). L (Ladder): Fermantas zip ruler DNA ladder 100 bp.

used in the determination of DNA polymorphisms in plant samples are presented in Table 2. The gel images of these primers are given in Figure 4, and also the gel images of the 4 monomorfic primers that were not included in the analysis. The graph showing the genomic pattern stability (GTS) in the control and treatment groups is shown in Figure 5. The GTS was determined in the experimental groups in comparison to the control plants according to the number of bands they gave. The highest band changes and the lowest GTS among the groups were found in the 150 mM NaCl group with 59 ± 7.56% ($F = 2.547$; sd = 12.91; $p < 0.01$). DNA stability increased with Gln treatment. DNA stability was over 80% in 2, 3, 4 mM Gln applications and the best stability results were obtained in onions treated with 3, 4 mM Gln (Figure 5).

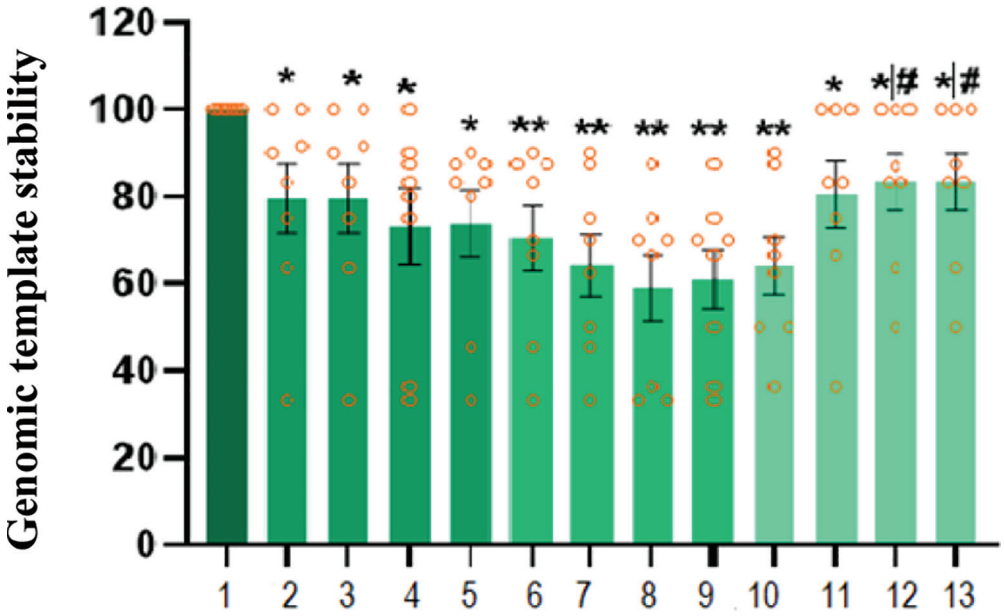


Figure 5. GTS in the control and treatment groups. n = 8, data mean ± standard error. Comparison of treatment groups compared to control, data are statistically different, one-way ANOVA test, *$p \leq 0.05$, **$p \leq 0.01$; #comparative applications vs. 150 mM NaCl, data are statistically different, independent groups t-test, $p \leq 0.05$ (1: Control, 2: 1 mM Gln, 3: 2 mM Gln, 4: 3 mM Gln, 5: 4 mM Gln, 6: 50 mM NaCl, 7: 100 mM NaCl, 8: 150 mM NaCl, 9: 200 mM NaCl, 10: 150 mM NaCl + 1 mM Gln,11: 150 mM NaCl + 2 mM Gln,12: 150 mM NaCl + 3mM Gln, 13: 150 mM NaCl + 4mM Gln). ANOVA, analysis of variance; GTS, genomic template stability.

Gene expression

In this study, the expression levels of *CuZn-SOD, Mn-SOD, AO, POLD1, CHAPE* and *HSP21* genes in onion leaf tissues were analysed by qRT-PCR method. Descriptive statistics of *CuZn-SOD, Mn-SOD, AO, POLD1, CHAPE* and *HSP21* gene expressions was calculated by the $2^{-\Delta\Delta Ct}$ method using the multiple control method of β-*actin* and *18S* endogenous control genes in the leaf tissues. The calculated relative gene expressions of the *CuZn-SOD, Mn-SOD, AO, POLD1, CHAPE* and *HSP21* genes compared to the control are given in Figure 6. It was determined that the expression of the antioxidant defence (*CuZn-SOD, Mn-SOD, AO*), DNA damage repair gene (*POLD1*) and heat-shock molecular chaperone (*CHAPE, HSP21*) genes in non-stressed conditions was quite low compared to stressed conditions. The highest expression of all genes (except *HSP21*) was found at 150 mM NaCl. *CuZn-SOD* expression increased approximately 6.5 fold at 150 mM NaCl and 150 mM NaCl+ 2 mM Gln compared to the control. While *Mn-SOD* increased 13.3 fold at 150 mM NaCl, it was raised 8.5-fold at 150 mM NaCl + 2 mM Gln. The highest level of expression was observed for the *CHAPE* gene, with an increase of approximately a 31.3 fold at 150 mM NaCl and a 19.8 fold increase at 150 mM NaCl + 1 mM Gln. *AO* expression increased approximately 10-fold in 150 mM NaCl compared to control. In 150 mM NaCl + 2 mM Gln applications, the expression level reached under salt stress decreased approximately 6-fold. Increases in *POLD-1* expression was upregulated 12.5 fold in 150 mM NaCl and 7 fold in 150 mM NaCl + 2 mM Gln. *HSP-21* expression was upregulated 3 fold in 150 mM NaCl and 0.5 fold in 150 mM NaCl + 2 mM Gln.

DISCUSSION

Gln is one of the most plentiful free amino acids in plants, acting as a nitrogen and ammonium transporter in the synthesis of nitrogenous organic substances such as proteins and nucleotides. The effects of exogenous applications of Gln, which functions as an important metabolic fuel in plants in natural conditions, on plant growth and metabolism have not been fully elucidated yet, and there are restricted studies on this subject. Moreover, the limited number of researches have reported that Gln also plays a role in metabolic pathways responsible for the stress response under stress circumstances (Kan et al., 2015; Han et al., 2022). Onion, which has a relatively small genome (2n = 16), is a suitable material for studies to understand the stress mechanism (Alam et al., 2023). Therefore, in the current study, the onion was used to understand the morphological, genomic and transcriptomic effects of Gln on plant metabolism under both salt-stress and normal conditions. In a study investigating the effects of different amino acids on germination and growth parameters of onion, the positive effect of Gln was noted. Gln enhanced the GP of onion under stress-free conditions and promoted root and shoot elongation (Abdelkader et al., 2023). In the present research, the vegetative development of onions after exogenous Gln

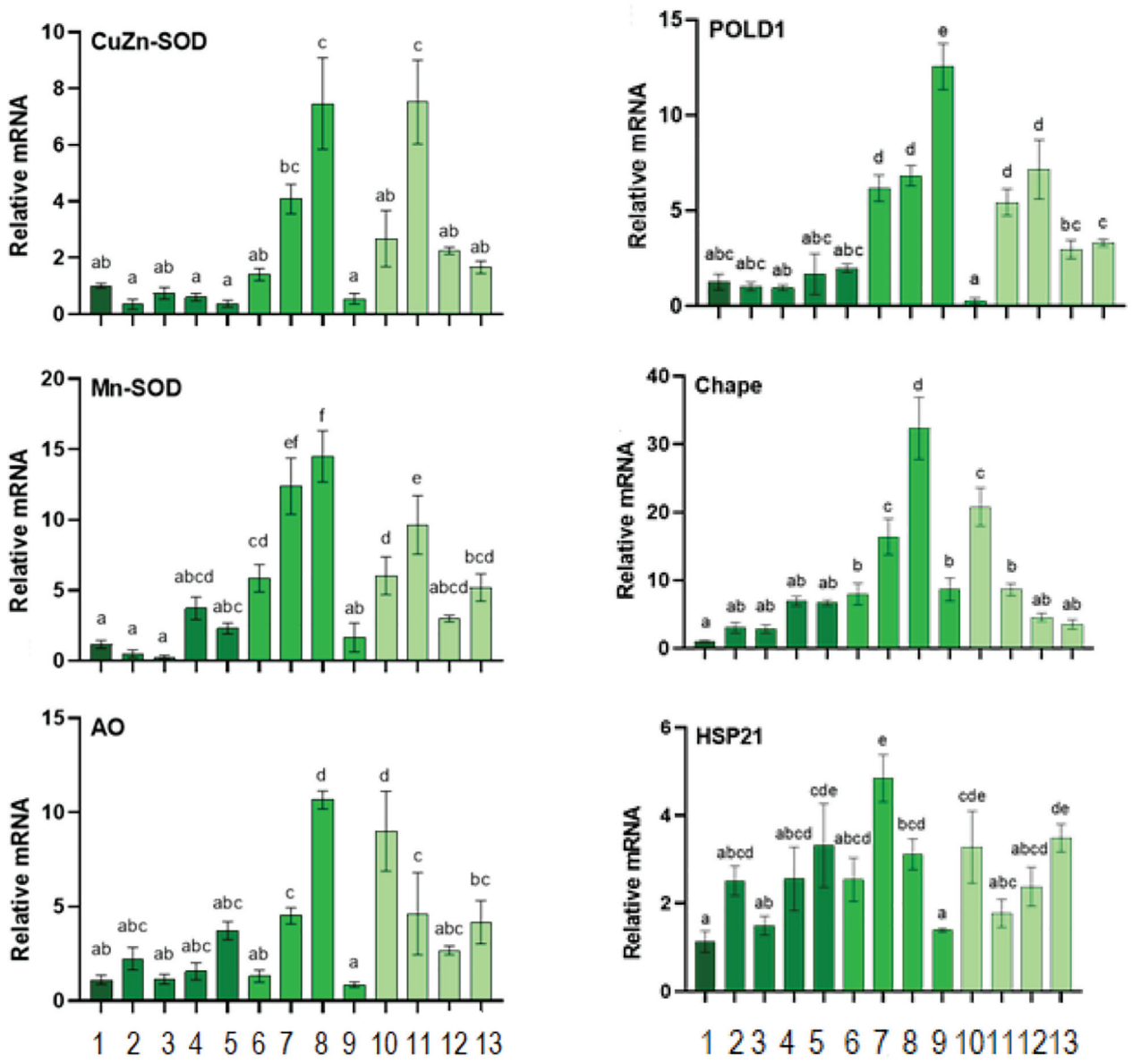


Figure 6. Relative fold increased values of antioxidant defence genes *CuZn-SOD, Mn-SOD, AO*, DNA damage repair gene *POLD1*, heat-shock molecular chaperone *CHAPE* and *HSP21* gene expressions in leaf tissues in experimental groups (data are β-*actin* and normalised to *18S* mRNA level by the multiple control method). Data are given as mean ± standard error, $n = 5$. The averages shown with different letters on the graph are statistically different from each other. One-way ANOVA, Tukey HSD test, $p \leq 0.05$ (1: Control, 2: 1 mM Gln, 3: 2 mM Gln, 4: 3 mM Gln, 5: 4 mM Gln, 6: 50 mM NaCl, 7: 100 mM NaCl, 8: 150 mM NaCl, 9: 200 mM NaCl, 10: 150 mM NaCl + 1 mM Gln,11: 150 mM NaCl + 2 mM Gln,12: 150 mM NaCl + 3mM Gln, 13: 150 mM NaCl + 4 mM Gln). ANOVA, analysis of variance.

applications is consistent with the results obtained from genomic stability and transcriptomic analysis and is compatible with the above-mentioned functions of Gln. In the non-stress condition, 2 mM Gln had a stimulating effect on the above-the-ground parts of the plants. This resulted in a considerable improvement, particularly in fresh shoot weight and leaf number. It was also found that the application of 2 mM Gln to all vegetative parameters reversed the adverse impact of stress in plants exposed to salinity (150 mM NaCl) (Table 2). The GP was not altered by the Gln application under unstressed conditions. However, it was increased 2-fold, and the MGT was shortened under salinity (Figure 1). Moreover, the TNL, which is an important yield criterion in onions, increased in both stressed and non-stressed conditions compared to the control group. The effects of Gln application on germination vary depending on whether the plant is in a salt-stressed or non-stressed condition, as well as on the application concentration and genotype. In a study conducted with different carrot cultivars, although the pre-application of Gln had no effect on the germination properties of orange carrot under unstress circumstances, a concentration of 1 mM Gln had a positive influence on the germination

characteristics under salinity (Üstüner et al., 2023). Moreover, the increase in the amount of chlorophyll and carotenoids (Figure 3) and the improvement in genomic stability (Figure 4) in stressful situations have proven that Gln plays a critical role in the nutritional and metabolic activities in current research. Similarly, Abdelkader et al. (2023) showed that Gln priming under stress-free conditions increased the amount of carotene in onion by 50%. This result obtained in onion was also shown in rice and poplar (Kan et al., 2015; Han et al., 2022) and supports the view of Gln as a potential nitrogen source, as the researchers stated.

The stimulating effects of exogenously applied Gln *in vitro* and *in vivo* have been demonstrated in different plants. Gln treatment promoted shoot regeneration in banana (Husin et al., 2014) and cucumber (Vasudevan et al., 2004) under *in vitro* conditions. Similarly, it was found to increase shoot biomass in rice (Kan et al., 2015) and maize (Hassan et al., 2020) seedlings. Although it promotes shoot growth, it has a positive or negative bidirectional effect on root development. This effect varied according to the plant species and the application technique. For example, 2 mM Gln increased root activity in poplar (Han et al., 2022). Consistent with the present study, Gln inhibited the root growth in rice (Kan et al., 2015), *Arabidopsis* (Kim et al., 2010), ginseng (Jung et al., 2006) and cherry (Sarropoulou et al., 2016). In the present study, the inhibitory effects of root growth in the non-stressed condition and the stimulatory effects in the stressed condition were determined. As stated by Jung et al., (2006), excessive accumulation of Gln, especially in the root tips, may be the cause of the inhibitory effect. The increase in the expression of some genes under stress conditions is related to the defence mechanism. According to growth parameters, plants developed tolerance to stress conditions and genomic stability increased when an appropriate concentration of Gln was applied. In a study conducted on *Salvadora persica* with nanoparticles (Fouda et al., 2021), which have a stimulating effect on plant growth, the nanoparticles reduced the percentage of GTS compared to the control plants, and the highest GTS value was determined as 85.1% in the 0.5 mg · L^{-1} Fe_3O_4 application. Similarly, in the current study, Gln applications under stress-free conditions decreased the genomic pattern stability, and GTS was determined as 80% in 1 mM and 2 mM Gln. It increased GTS by having the opposite effect under salt-stress conditions and increased it above 80% (Figure 4). A number of researches have shown that salt stress has a negative influence on the genetic pattern stability (Alotaibi, 2021; Hussien, 2022; Coşkun, 2023a,b). However, it has also been shown that some applications result in an increase in GTS. Consistent with the positive effect of Gln detected in the current research, Coşkun, (2023a) determined that salt stress reduced GTS to 47.2% in cucumber, and this rate could be increased up to 79.5% with grafting. Similarly, Coşkun, (2023b) found that grafting had a positive effect on GTS in drought stress. Spermidine, melatonin (Yadu et al., 2018), and glycinebetaine (Yadu et al., 2017), which were used to reduce fluoride toxicity in *Cajanus cajan* L., improved the GTS and alleviated the adverse effects of $\underline{F}^-$.

Different concentrations of Gln treatment under salt-stress conditions caused transcriptional upregulation of different stress-related genes. The 2 mM Gln concentration caused a rise in the expression of the *CuZn-SOD, Mn-SOD* and *POLD1* genes, while the 1 mM Gln increased the expression of the *AO* and *CHAPE* genes. The 4 mM Gln concentration caused transcriptional induction in *HSP21* (Figure 6). Compared with other organisms, *HSP21* is one of the most abundant HSPs in plants, localised in plastids and responsible for chloroplast development (Zhong et al., 2013). In the current research, total chlorophyll and carotenoid content gradually increased with Gln application under stress conditions and reaching the highest level at 4 mM Gln (Figure 3). The fact that *HSP21* gene expression, which is involved in the polymerase-dependent transcription of plastid-encoded RNA, was detected at the highest level in the same application group and is related to its function in chloroplasts and supports the conclusion of Zhong et al. (2013) that *HSP21* is required for chloroplast development under heat-stress conditions in *Arabidopsis*. The *HSP21* gene has been shown to protect photosystem II from oxidative stress in tomato (Neta-Sharir et al., 2005) and tobacco (Guo et al., 2007) and to stimulate colour change during fruit ripening in tomato (Neta-Sharir et al., 2005). On the other hand, the effects of small HSP family members on ripening were also determined in Kyoho grapes (Wang et al., 2019; Guo et al., 2020). HSPs, which help to maintain the three-dimensional structure of proteins under various stress conditions, support plant survival. The *HSP21* gene, which is expressed at high levels in poplar under salt stress, was found to confer tolerance to salinity (Yer et al., 2018). Given the upregulation of HSP21 expression under salt-stress conditions by application of Gln, it is thought to have the potential to be put into practice as an exogenous application for fruit ripening under stress conditions. CHAPE, a sub-member of the HSP family, are genes responsible for protein folding, regulation of metabolism and response to abiotic stress (Pareek et al., 2021). It has been shown that the upregulation of some genes, including *CHAPE* genes, under heat-stress conditions in coffee is associated with heat tolerance, and that CHAPE in particular have this effect by attenuating the inhibition of photosystem II (Martin et al., 2016). Similarly, in *Rhododendron*, an ornamental plant, upregulation of CHAPE was found to protect the photosynthetic apparatus and reduce photosynthetic damage by increasing heat tolerance. In sorghum, proposed as a model plant for abiotic stress tolerance, the identified CHAPE and HSPs were found to confer abiotic stress tolerance by aiding protein folding in the

plastids (Nagaraju et al., 2021). In the current study, the most effective concentration for activating the *CHAPE* gene was found to be 1 mM Gln. The fact that it was less induced than the 150 mM salt stress was interpreted as Gln treatments helping to maintain the structure of protein folding under stress conditions (Figure 6).

SOD is a primary defence mechanism responsible for maintaining intracellular homeostasis by preventing the accumulation of radicals in cells under stress conditions (Kumar et al., 2020). SODs, which protect plant cells from oxidative damage, are localised in different regions of the cell. CuZn-SOD is found in the chloroplast, cytosol and extracellular space, whereas Mn-SOD is mainly found in mitochondria and peroxisomes (Kumar et al., 2013; Del Río and López-Huertas, 2016). It was found that CuZn-SOD and APX gene transfer into salt-sensitive sweet potato and overexpression of these genes increased tolerance to salt stress (Yan et al., 2016). A linear relationship was found between increasing salinity in rice and the transcription of the *Mn-SOD* gene. It was found that SOD enzyme activity also increased due to this increase (Çelik et al., 2019). In the current research, gene expressions were differentially regulated according to Gln concentrations. Similarly, Mn-SOD that belongs to the same gene family was upregulated, and Fe-SOD was downregulated during pepper ripening (González-Gordo et al., 2020). It is not surprising that different gene families produce conflicting patterns of regulation in the formation of stress responses as well as in fruit ripening. Kumar et al. (2020) found that Mn-SOD can be used as a biochemical marker for heat stress in wheat. The increase in the transcriptional expression of this gene observed in the current study and the compatibility of the data on the vegetative development of plants suggest that it can also be used as a biomarker in salt-stress studies. Extensive research on different plants will be conducted for this purpose. Overexpression of *AO* in transgenic tobacco caused a decrease in stomatal conductance, low water loss and high water content in the plant. Researchers have demonstrated that the signal transduction related to stomatal closure is related to the degree of expression of *AO* (Fotopoulos et al., 2008). It is thought that stress-related events first occur in the apoplasmic cell membrane. The fact that ascorbate oxidase is an apoplastic enzyme strengthens the idea that it is associated with stress (Yamamoto et al., 2005). Ascorbic acid is the most abundant antioxidant in the apoplast and is oxidized by *AO*. Therefore, it should be determined whether changes in *AO* expression affect tolerance to stress. Consistent with the results of this study, it was stated that the suppressed expression of apoplastic *AO* under salt stress conditions in tobacco and Arabidopsis resulted in relatively low levels of hydrogen peroxide accumulation and, as a result, high seed yield (Yamamoto et al., 2005). In the present study, the suppression of *AO* expression by Gln application was interpreted as being related to plant tolerance. When evaluated with vegetative growth parameters, it was concluded that under salt-stress conditions, Gln acts as a metabolic fuel, supplementing the energy deficit caused by salt stress and promoting the vegetative growth of the plant.

CONCLUSIONS

Gln, a precursor molecule in the synthesis of nitrogenous organic substances, has been shown to activate some genes responsible for intracellular signalling under stress conditions. In this study, the changes caused by Gln application in the expression of some genes involved in stress response in onion under normal and salt stress conditions were investigated in relation to germination and vegetative development. It was found that pre-treatment with Gln under salt stress conditions had a positive effect on all germination parameters. Although pre-application of Gln did not have an important effect on germination under normal conditions, it supported the vegetative development and increased the number of leaves, which is an important yield criterion especially in onions. The number of leaves, which was 5.4 in the control, increased by more than two-fold to 13.6 with Gln application. Under salt stress, Gln supported the development of the above-the-ground plant organs and increased the photosynthetic pigment content and genomic pattern stability at all application concentrations (80%). Transcriptional induction of stress- relevant genes in the presence of Gln under salt stress remained at a lower expression level except for *CuZn-SOD* when compared to 150 mM salt stress. On the other hand, considering the growth parameters, the fact that the plant showed better growth than the plants under salt stress suggests that Gln helps to fill the energy deficits of the plant by acting as an alternative fuel for metabolic activities under stress conditions.

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AUTHOR CONTRIBUTIONS

U.K. and N.A.G. – conceptualisation. U.K. and N.A.G. – methodology. U.K. and N.A.G. – investigation. U.K. – statistical analysis. U.K. and N.A.G. – supervision. U.K. and N.A.G. – writing - original draft. U.K. and N.A.G. – writing - review and editing.

CONFLICT OF INTEREST

The authors confirm that this article's content has no conflict of interest.

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