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Salicylic acid improves cold and freezing tolerance in pea

Salisilik asit bezelyede soğuk ve donma toleransını artırır

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ABSTRACT

The most significant crop losses worldwide occur due to unfavorable temperatures such as heat, drought, cold, and freezing. Bioregulator substances like salicylic acid can play important roles in the growth, development, and stress responses of plants. In this study, changes in stem/root length and relative water content of peas under cold and freezing stress, as well as antioxidant system indicators such as proline, malondialdehyde, hydrogen peroxide, chlorophyll and ion leakage levels were investigated. The expressions of genes coding for the *TOP2* and *PDH47* enzymes, which play important roles in the replication, transcription, and repair of DNA molecules, were also examined in root and stem tissues in the presence of two different concentrations of salicylic acid under cold and freezing stress. The results have shown that the application of salicylic acid, when added to the growth medium, can have positive effects on the cold resistance of pea plants. Salicylic acid likely achieves some of its effects by increasing the activity of superoxide dismutase, one of the most important enzymes taking a role in combating reactive oxygen species. The data obtained indicate that salicylic acid also increased the expressions of *TOP2* and *PDH47* genes, which can both change the topology of DNA, possibly facilitating the transcription of genes taking a role in antioxidative defense. Salicylic acid also reduced the levels of reactive oxygen species hydrogen peroxide and maintained cell membrane integrity, which leads to a decrease in ion leakage and an increase in water-holding capacity. With this study, the mechanisms of action of salicylic acid in cold stress tolerance have been further elucidated, and its potential use in agricultural cultivation has been evaluated.

Key Words: *Pisum sativum* L., salicylic acid, cold stress, freezing stress

ÖZ

Dünya çapında en önemli ürün kayıpları; sıcak, kuraklık, soğuk ve donma gibi olumsuz hava sıcaklıklarından kaynaklanmaktadır. Salisilik asit gibi biyodüzenleyici maddeler bitkilerin büyümesinde, gelişmesinde ve stres tepkilerinde önemli roller oynayabilirler. Bu çalışmada soğuk ve donma stresi altında bezelyelerin gövde/kök uzunluğu ve bağıl su içeriğindeki değişimler ile prolin, malondialdehit, hidrojen peroksit, klorofil ve iyon sızıntısı seviyeleri gibi antioksidan sistem göstergeleri araştırılmıştır. DNA moleküllerinin replikasyonu, transkripsiyonu ve onarımında önemli rol oynayan *TOP2* ve *PDH47* enzimlerini kodlayan genlerin ifadeleri de soğuk ve donma stresi altında, iki farklı konsantrasyonda salisilik asit varlığında, kök ve gövde dokularında incelenmiştir. Sonuçlar, büyüme ortamına eklendiğinde salisilik asit uygulamasının bezelye bitkilerinin soğuğa karşı direnci üzerinde olumlu etkileri olabileceğini göstermiştir. Salisilik asit muhtemelen bazı etkilerini reaktif oksijen türleriyle mücadelede rol alan en önemli enzimlerden biri olan süperoksit dismutazın aktivitesini arttırarak sağlamaktadır. Elde edilen veriler salisilik asidin aynı zamanda DNA topolojisini

değiştirebilen *TOP2* ve *PDH47* genlerinin ifadesini de arttırdığını ve muhtemelen antioksidatif savunmada rol alan genlerin transkripsiyonunu kolaylaştırdığını göstermektedir. Salisilik asit aynı zamanda reaktif oksijen türü olan hidrojen peroksit düzeylerini azaltmış ve hücre zarı bütünlüğünü korumuştur, bu da iyon sızıntısında azalmaya ve su tutma kapasitesinde artışa yol açmıştır. Bu çalışma ile salisilik asidin soğuk stresine toleranstaki etki mekanizmaları bir adım daha aydınlatılarak tarımsal alanda kullanım potansiyeli değerlendirilmiştir.

Anahtar Kelimeler: *Pisum sativum* L., salisilik asit, soğuk stresi, donma stresi

Introduction

Among the causes of low crop yield, biotic factors such as diseases, insects, and weeds contribute to some extent, however usually the most significant losses occur due to unfavorable temperatures such as heat, drought, cold, and freezing (Mahajan & Tuteja, 2005). Cold and freezing stresses share fundamental mechanisms but involve different components. Cold stress results in various adverse effects, including a decrease in enzyme activities, membrane stiffening, destabilization of protein complexes, stabilization of RNA secondary structures, accumulation of reactive oxygen species, inhibition of photosynthesis, and cell membrane leakage, which can lead to cell death in advanced stages (Ruelland & Zachowski, 2010). Whereas the primary cause of freezing stress is the formation of ice within tissues. Freezing stress manifests its major harmful effects through damage caused by intense dehydration in cell and organelle membranes. During freezing stress, key genetic responses involve an increase in CBF gene expression, initiating transcriptional regulation, and mRNA degradation regulated by small RNAs through pre-mRNA splicing, which helps in the adaptation to low temperatures (Chinnusamy et al., 2007; Sunkar et al., 2007).

Agricultural plants most affected by cold stress are those planted in early spring or harvested in late autumn (Ceyhan, 2006; Duke, 2012; Tekin & Ceyhan, 2020). Pea (*Pisum sativum* L.) is one of the plants sown in early spring. Pea is one of the most cultivated legume crops globally after beans, chickpeas and black-eyed peas (FAOSTAT, 2022). The plant is highly productive under suitable weather conditions, and plays crucial roles in sustainable agriculture worldwide (Özdemir, 2002). Peas are a cool-season crop, exhibiting excellent growth in cool and moist conditions, but their development slows down in hot and dry weather. While it can withstand temperatures near freezing, it can be damaged by spring frosts (Alan, 1984; Ceyhan, 2006).

Biochemical and genetic responses of peas to cold and freezing stress have been explored in limited studies. One of the earliest studies suggested that the application of abscisic acid (ABA) and conditioning at 2°C increased freezing tolerance and induced the production of a 24 kDa protein in peas (Welbaum et al., 1997). Another study examined the effects of low and high temperatures on peas, revealing that while fluorescence was unaffected at 10 and 35 °C, it caused reversible damage to the photosystem at 4 and 40 °C (Georgieva & Lichtenthaler, 1999). Major carbon and phosphate compounds of peas subjected to cold stress at 5-7 °C and high light were also compared with two cold-resistant alpine plants, *Ranunculus glacialis* and *Soldanella alpina* (Streb et al., 2003). Peas showed significant increases in sucrose, glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, and phosphoglycerate levels, while the ATP/ADP ratio decreased. Alpine plants exhibited changes in ascorbate and glutathione levels, suggesting their role in stress metabolism. Research has also been conducted on pea seeds and epicotyl mitochondria regarding cold tolerance, revealing their ability to survive without damage at temperatures ranging from -3.5 °C to 40 °C. This resilience was attributed to a lower phosphatidylethanolamine/phosphatidylcholine ratio, fewer unsaturated fatty acids, and increased production of heat shock protein HSP22 and PsLEAm protein (Stupnikova et al., 2006). Another study investigated the relationship between cold stress and photosynthetic mechanisms in peas selected for cold resistance and tomatoes selected as cold-sensitive. The superiority of peas in stress conditions was explained by the different arrangements of thylakoid complexes in chloroplasts (Garstka et al., 2007).

Studies examining the relationship between the pea genome and cold stress have focused on the pea ABR17 gene, a member of the pathogenesis-related gene family PR10. The ABR17 gene was continuously expressed in *Arabidopsis* and conferred freezing stress tolerance, suggesting a potential role in plant-

pathogen interactions (Srivastava et al., 2006). The function of the gene has been suggested to be an RNase enzyme. Other genes found to function under cold stress in peas are the DNA-regulating *TOP2* and *PDH47*, whose expressions were also investigated in this study. *TOP2* is a type 2 DNA topoisomerase enzyme, which plays a roles in changing the topology of DNA and is involved in processes such as replication, transcription, recombination, and chromosome segregation. In plants, topoisomerases represent the least studied group of enzymes compared to bacteria, yeast, and animal systems (Singh, Sopory, & Reddy, 2004). Studies have shown that *TOP2* gene expression in peas can be increased by cold and salt stresses, as well as by salicylic acid and ABA treatments (Hettiarachchi et al., 2005).

Helicases, like topoisomerases, play important roles in DNA metabolism. Most helicases belong to the DEAD-box protein family and are involved in essential cellular processes such as DNA replication, repair, recombination, transcription, ribosome biogenesis, and translation initiation (Vashisht & Tuteja, 2006). The functions of helicases under stress conditions are not fully understood, but they are shown to regulate some metabolic pathways activated by stress, interact with topoisomerases, play regulatory roles in transcription and translation after being phosphorylated by protein kinases, and be involved in the repair of damaged DNA/RNA molecules (Vashisht & Tuteja, 2006). Compared to organisms such as humans, flies, worms, and yeast with determined genomic sequences, *Arabidopsis* contains the largest number (more than 50) of DEAD-box RNA helicase genes (Aubourg et al., 1999; Boudet et al., 2001). The first report suggesting the presence of cold-regulated DEAD-box helicase genes in plants and their involvement in stress signal transduction was related to *Arabidopsis* (Seki et al., 2001). In peas, a DEAD-box DNA helicase gene has been characterized and identified as *PDH47*, showing 93% similarity to the tobacco translation initiation factor eIF4A protein. *PDH47* demonstrated ATP-/Mg2+-dependent DNA unwinding and DNA-/Mg2+-dependent ATPase activity (Vashisht & Tuteja, 2005). The expression of *PDH47* in peas was significantly increased in shoots and roots under cold and salt stress but did not respond to heat stress. ABA treatment increased the expression only in roots but did not affect gene expression in shoots.

In recent years, various studies have intensively investigated the responses of plants to stress tolerance through the application of various external bioregulatory substances. Salicylic acid (SA), which is chemically close to aspirin, has been studied since the early 1990s for its role in regulating plant abiotic stress responses. In plant cells, cinnamic acid formed at the end of the shikimate metabolic pathway branches into three different pathways to produce rosmarinic acid, phenolics, and salicylic acid. Salicylic acid has been found to regulate cellular redox balance by modulating antioxidant enzyme activities and activating an alternative respiratory pathway. Additionally, it stimulates the expression of an RNA-dependent RNA polymerase gene involved in transcriptional post-silencing, suggesting a role in post-transcriptional gene silencing (Xie et al., 2001). Salicylic acid has been shown to enhance tolerance to various stresses, including heat, drought, salinity, UVB radiation, and heavy metals, in a wide range of plants through different metabolic pathways (Chen et al., 2023). Studies have also been conducted on the effects of external salicylic acid applications under cold and freezing stress in various plants. In these studies, it was determined that salicylic acid is involved in cold signal transduction using cGMP, phospholipids, Ca, ROS, NO, and MAPK signaling pathways. Salicylic acid generally plays a role in the enzymatic and non-enzymatic defense systems through the activation of transcription factors such as MYB, MYC, ABREB/A, and DREB1/2, promoting the accumulation of osmolytes, antifreeze proteins, heat shock proteins, dehydrins, and PR proteins (Saleem et al., 2021).

Studies investigating the effect of salicylic acid on pea development have also shed light on its working mechanism. For example, pea seeds coated with acetylsalicylic acid and hydrolyzed fish proteins achieved higher weight and height values after germination (Andarwulan & Shetty, 1999). The study also suggested that salicylic acid had stimulating effects on the antioxidant system, as indicated by increased total phenolic content, proline content, guaiacol peroxidase, and glucose-6-phosphate dehydrogenase enzyme activities. In another study, salicylic acid application to peas increased cell wall lignification, phenolic substances (mainly lignin) through pentose phosphate and shikimate metabolic pathways, and the production of NADPH2 and proline

(McCue et al., 2000). In peas, (Singh, Mishra, et al., 2004) demonstrated that salicylic acid increased the expression of the chloroplast translation elongation factor *tufA* gene, suggesting its role in environmental stress adaptation. Another study focused on the regulation of the PIP2-PLC (phospholipase C) enzyme, responsible to produce IP3 and DAG, two second messengers involved in signal transduction. The activity of the enzyme was found to be associated with acclimatization to heat, ABA applications, and the concentration of salicylic acid in the environment (Liu, Huang, et al., 2006; Liu, Liu, et al., 2006).

It is well known that salicylic acid provides stress resistance in plants under cold and freezing stress. The relationship between salicylic acid and the responses of pea tissues under various stresses has begun to be elucidated with different indicators, but the effect of salicylic acid application under cold and freezing stress in peas has been the subject of limited number of studies within our knowledge. In this study, changes in stem/root length and relative water content of pea under cold and freezing stress, as well as antioxidant system indicators such as proline, malondialdehyde, hydrogen peroxide, and chlorophyll levels, ion leakage, and the expressions of genes coding for the *TOP2* and *PDH47* enzymes, which play important roles in the replication, transcription, and repair of DNA molecules, were examined in root and stem tissues in the presence of two different concentrations of salicylic acid under cold and freezing stress. Pea (*Pisum sativum* L.) is one of the agricultural plants sown in early spring and most affected by cold stress (Duke, 2012). Extreme and unexpected weather conditions have become very common in recent years throughout the world due to climate change (Zomer et al., 2017). Therefore in this study, the mechanisms of action of salicylic acid in cold stress tolerance have been further elucidated, and its potential use for stress mitigation in agricultural production has been evaluated.

Materials and Methods

In this study, the pea variety Emerald, developed and registered by Istanbul Seed Company was used.

Plant growth

Pea seeds were sterilized with a 2% sodium hypochlorite solution, then germinated in 200 ml polypropylene containers containing sterile perlite, with three seeds each, and irrigated with ½ Hoagland solution (Hoagland & Arnon, 1950) every three days. The seed development continued for 15 days in a plant growth chamber with a 16-hour light/8-hour dark cycle, at 23°C, and 50% humidity until stress application.

Cold stress applications

Cold stress was applied by lowering the temperature to 4°C after 15 days of seedling development when the seedlings were at the stage 4 of leaf development (fourth stipule unfolded), and leaf samples for analysis were always collected from the forth stipule on the 1st and 4th days of cold stress (24 hours and 96 hours after the initiation of cold stress). Freezing stress was induced by lowering the temperature to -0.5°C, and samples were collected during the same periods as cold stress. Plants treated with salicylic acid (at concentrations of 50 and 100 µM) were germinated by irrigating with ½ Hoagland solution containing salicylic acid of the specified concentrations as soon as they were placed on perlite. Cold and freezing stresses, as well as sample collection periods, were applied as described above. Control plants kept under normal conditions spent an additional 1 and 4 days in the same environment after the 15-day germination period, ensuring that they experienced the same duration as the stressed plants in the culture environments.

Experiment was performed in randomized complete block design with treatments performed in triplicate, with each replicate consisting of four polypropylene containers containing three seeds each. This resulted in 12 pea seedlings prepared per treatment, and with replicates, there were a total of 36 seedlings for each treatment.

Determination of stem/root length and relative water content

After completing 15 days of development, plants subjected to stress with or without the presence of salicylic acid, along with control plants, were uprooted from perlite, washed under tap water, and root and stem lengths were determined. Root and stem tissues were separated, weighed, and after drying at 60°C for 48 hours, weighed again to determine dry

weights. Relative water content (RWC) was calculated according to the formula RWC(%) = (Fresh weight - Dry weight) / (Turgid weight - Dry weight) X 100, from Smart and Bingham (1974). The turgid weight was calculated by soaking tissues in distilled water at room temperature for 24 hours.

Determination of proline content

The determination of proline content started with the homogenization of 0.3 g leaf samples taken from the forth stipule in liquid nitrogen, followed by dissolution in 1 ml of 3% sulfosalicylic acid (Bates, et al., 1973). Then, 0.1 ml of the sample, after centrifugation, was mixed with 0.2 ml acid ninhydrin, 0.2 ml of 96% acetic acid, and 0.1 ml of 3% sulfosalicylic acid. The mixtures were held at 96°C for 1 hour, and after centrifugation and mixing with 1 ml of toluene, the absorbance of the upper phase was read at 520 nm.

Determination of hydrogen peroxide content

The amounts to be determined according to Bergmeyer (2012) started with the liquid nitrogen homogenization of 0.5 g leaf tissue and dissolution in 1.5 ml of 100 mM potassium phosphate buffer (pH 6.8). In the samples where 0.25 ml of supernatant was collected after centrifugation, the enzymatic reaction was initiated by mixing with 1.25 ml peroxidase solution (83 mM potassium phosphate buffer, pH 7.0, 0.005% (w/v) o-dianisidine, 40 µg peroxidase/ml) at 30°C. After 10 minutes, the reaction was stopped by adding 0.25 ml of 1 N perchloric acid, and the absorbance of the supernatant was measured at 436 nm after centrifugation.

Determination of malondialdehyde (MDA) content

The determination of MDA content for assessing cell membrane damage followed the Ohkawa et al. (1979) method. The homogenization of 0.2 g leaf tissue in liquid nitrogen was followed by adding 1 ml of 5% trichloroacetic acid (TCA). After centrifugation, the same volume of 20% TCA containing 0.5% thiobarbituric acid (TBA) was added, and the mixture was kept at 96°C for 25 minutes. The samples were cooled on ice, and the absorbance was read at 532 nm. Non-specific absorbance at 600 nm was determined and subtracted from the initial absorbance value.

Ion leakage

Ion leakage, determined according to the method of Nanjo et al. (1999), involved shaking six leaves in 15 ml tubes containing 5 ml of 0.4 M mannitol. Electrical conductivity was recorded as C1 using a Mettler Toledo MPC 227 conductivity meter. After boiling in water for 15 minutes and cooling the samples to room temperature, C2 readings were taken, and the leakage-related conductivity was calculated using the formula [(C1/C2) X 100].

Determination of chlorophyll content

Chlorophyll amounts were determined using the method of Lichtenthaler and Wellburn (1983). This involved the liquid nitrogen homogenization of 3 g leaf tissue, centrifugation, and determination of the supernatant at different absorbance values. Chlorophyll a (mg/L) and chlorophyll b (mg/L) were calculated using the following formulas:

- Chlorophyll a (mg/L) = 15.65 Abs666 – 7.340 Abs653
- Chlorophyll b (mg/L) = 27.05 Abs653 – 11.21 Abs666

Determination of superoxide dismutase (SOD) enzyme activities

Enzyme activities were determined according to the method of Beauchamp and Fridovich (1971). The homogenization of 0.2 g leaf samples in a glass-glass homogenizer mixed with homogenization buffer on ice was followed by centrifugation, and the supernatant was stored at -80°C until use. The protein content of the extracts was determined by the Bradford (1976) method. A native polyacrylamide gel consisting of a separation part and stacking part with a 30% (29:1) acrylamide-bis solution was prepared for use in a Bio-Rad midi gel apparatus. After loading the samples, electrophoresis was conducted at 8 V/cm, monitoring the tracking dye. Subsequently, the gel apparatus was disassembled, and for the determination of different isozymes of the SOD enzyme, first KCN and hydrogen peroxide were applied, and then negative activity staining with NBT was performed to visualize the enzyme isozyme bands.

Determination of gene expressions for TOP2 and PDH47 genes

The mRNA sequences of the *TOP2* and *PDH47* genes with GenBank accession numbers Y14559.1

and AY167670.1 were obtained from the National Center for Biotechnology Information (NCBI) database. The expression at the transcription level of these genes was investigated using the semi-quantitative reverse-transcription PCR (RT-PCR) technique. The pea actin gene with the GenBank accession number X68649.1 was used as an internal control.

RNA isolation and reverse transcription PCR (RT-PCR)

RNA isolation from pea leaves was performed using Qiagen RNeasy plant mini kits based on guanidine-isothiocyanate lysis and silica-membrane purification methods. The quantity of the obtained total RNAs was determined spectrophotometrically, and their quality was assessed by separating and visualizing them with 2% agarose gel electrophoresis. cDNA libraries were created using the Thermo First Strand cDNA Synthesis Kit (Thermo, USA) from the obtained RNA molecules. From this library, the *TOP2* and *PDH47* genes with NCBI accession numbers Y14559.1 and AY167670.1 were amplified by PCR using primers designed with the PrimerPremier 5.0 program from CA, USA, providing the most suitable conditions for amplification. The obtained bands were separated on a 0.8% agarose gel and visualized using the Biolab UV Tech gel imaging system. The bands were analyzed numerically using the ImageJ software developed by the National Institute of Health (NIH, USA) to determine differences in gene expression levels.

Statistical analyses

The data obtained in the study were evaluated using the SPSS 16.0 program. Differences between applications were determined by comparing means with the One Way Anova and Tukey Test.

Results and Discussion

Under cold and freezing stress conditions, the dry weights of pea roots and stems remained unchanged under any application of salicylic acid (data not shown). Different temperature treatments did not have a significant effect on tissue lengths on the 1st stress day, only causing the stems under cold and freezing stress to be shorter on the 4th day compared to those developed under normal conditions (Table 1). Root lengths increased under cold stress only in the presence of 100µM salicylic acid, similar to the findings of Golovatskaya et al. (2023), which demonstrated the improvement of morphological features in potato plants under cold stress with salicylic acid application and attributed it to the stimulation of the enzymatic/non-enzymatic defense system by salicylic acid.

Chilling and freezing stress for the duration of one day did not change the relative water contents of leaves, whereas freezing stress for 4 days decreased relative water contents, and the application of SA in both concentrations significantly increased RWC (Table 1). Total chlorophyll contents were not affected by SA application and cold stress, whereas freezing stress decreased chlorophyll content by up to 55% compared to normal growth conditions (Table 1). Chilling and freezing stress increased proline levels in both tissues in the range of 1 to 2.5-fold. Proline levels showed significant decreases upon SA application (Table 1). SA decreased proline levels even in control shoots grown under normal conditions. Nearly 3-fold and 2-fold decreases were observed in shoots upon SA treatment under 1 and 4 days of chilling stress, respectively. Nearly 2-fold and 1.5-fold decreases were observed in roots upon SA treatment under 1 and 4 days of chilling stress, respectively. Significant decreases in proline levels were also observed in shoot tissues on the 4th day of freezing stress upon SA treatment. In this study, proline content of pea plants decreased with cold stress, whereas in previous studies, it was reported that the proline content of pea plants increased with cold stress and other stresses (Gökmen & Ceyhan, 2015; Tekin & Ceyhan, 2020).

Table 1. Plant morphological responses, leaf relative water content (RWC), proline and total chlorophyll levels.

Treatments	Shoot Length (cm)	Root Length (cm)	RWC (%)	Total Chlorophyll (µg/g DW)	Proline (nmol/g DW)	
	1st Day of Stress					
					Shoot	Root
C*	5,74 ± 0,22$_{a,b}$	8,36 ± 0,44$_{a,b}$	99 ± 0,7$_{a,b}$	24,22 ± 2,89$_{a,b}$	188,66 ± 13,21$_{a,c}$	73,20 ± 6,71$_{a}$
SA50	6,22 ± 0,19$_{b}$	9,32 ± 0,59$_{b,c}$	100 ± 0,8$_{b}$	22,36 ± 1,80$_{a,c}$	141,24 ± 12,94$_{b}$	74,91 ± 7,19$_{a,b}$
SA100	6,19 ± 0,37$_{b}$	9,14 ± 0,58$_{a,b,c}$	100 ± 0,6$_{b}$	23,87 ± 1,32$_{a,b}$	136,86 ± 12,38$_{b}$	70,62 ± 7,08$_{a}$
C st4**	5,21 ± 0,24$_{a,c}$	8,69 ± 0,58$_{a,b,c}$	99 ± 1,1$_{a,b}$	30,18 ± 3,12$_{b}$	463,40 ±16,21$_{d}$	102,31 ± 9,89$_{b,c}$
SA50 st4	5,97 ± 0,42$_{a,b}$	9,36 ± 0,49$_{b,c}$	98 ± 1,5$_{a,b}$	27,74 ± 3,54$_{a,b}$	168,21 ± 14,37$_{a,b}$	47,60 ± 6,61$_{a}$
SA100 st4	6,44 ± 0,33$_{b}$	10,14 ± 0,51$_{c}$	100 ± 0,6$_{b}$	27,92 ± 2,85$_{a,b}$	149,48 ± 13,71$_{a,b}$	57,47 ± 5,94$_{a}$
C st-	4,40 ± 0,25$_{c}$	8,92 ± 0,48$_{a,b,c}$	99 ± 0,7$_{a,b}$	16,05 ± 1,19$_{c,d}$	208,59 ± 14,33$_{c}$	149,14 ± 13,15$_{d}$
SA50 st-	4,41 ± 0,34$_{c}$	7,77 ± 0,38$_{a}$	100 ± 0,8$_{b}$	15,75 ± 1,28$_{d}$	176,3 ± 13,72$_{a,b,c}$	117,01 ± 12,87$_{c}$
SA100 st-	4,67 ± 0,37$_{c}$	8,61 ± 0,49$_{a,b}$	97 ± 0,8$_{a}$	16,02 ± 0,15$_{c,d}$	165,72 ± 13,35$_{a,b}$	117,27 ± 12,93$_{c}$
	4th Day of Stress					
C	7,35 ± 0,46$_{a}$	9,72 ± 0,23$_{a}$	95 ± 1,2$_{a}$	31,15 ± 3,21$_{a}$	145,53 ± 12,99$_{a}$	73,54 ± 9,29$_{a,b,c}$
SA50	7,52 ± 0,31$_{a}$	9,52 ± 0,33$_{a}$	98 ± 1,5$_{a,b}$	28,92 ± 3,48$_{a}$	113,06 ± 12,85$_{a}$	56,01 ± 7,96$_{a}$
SA100	8,09 ± 0,42$_{a}$	11,51 ± 0,34$_{b}$	100 ± 0,8$_{b}$	31,29 ± 3,09$_{a}$	134,71 ± 13,28$_{a}$	64,95 ± 7,87$_{a}$
C st4	5,32 ± 0,27$_{b}$	7,61 ± 0,34$_{c}$	100 ± 0,5$_{b}$	30,63 ± 3,39$_{a}$	418,30 ± 17,32$_{b}$	106,96 ± 11,69$_{d}$
SA50 st4	5,52 ± 0,38$_{b}$	8,40 ± 0,45$_{c,d}$	100 ± 0,8$_{b}$	30,09 ± 3,56$_{a}$	190,98 ± 13,94$_{c}$	73,54 ± 8,23$_{a,b,c}$
SA100 st4	5,43 ± 0,26$_{b}$	8,12 ± 0,33$_{c,d}$	97 ± 1,3$_{a,b}$	32,36 ± 4,03$_{a}$	224,49 ± 13,87$_{c,d}$	66,50 ± 3,77$_{a,b}$
C st-	4,88 ± 0,44$_{b}$	9,28 ± 0,51$_{a}$	66 ± 1,8$_{c}$	17,03 ± 0,94$_{b}$	274,23 ± 14,47$_{e}$	99,48 ± 8,34$_{d}$
SA50 st-	4,94 ± 0,37$_{b}$	9,15 ± 0,48$_{a,d}$	76 ± 2,3$_{d}$	17,83 ± 1,37$_{b}$	212,37 ± 13,16$_{c,d}$	89,95 ± 7,78$_{b,c,d}$
SA100 st-	4,59 ± 0,34$_{b}$	8,63 ± 0,37$_{a,c,d}$	77 ± 2,1$_{d}$	18,01 ± 1,67$_{b}$	243,81 ± 13,66$_{d,e}$	93,30 ± 8,91$_{c,d}$

*C, SA50 and SA100 represent plants irrigated with Hoagland solution containing 0µM, 50µM and 100µM salicylic acid in nutrient media, respectively. ** The abbreviations next to the treatments; st4 represents cold stress applied at 4°C, and st- represents freezing stress applied at -0.5°C.

The letters at the bottom right of the results indicate statistically significant differences (p≤0.05) compared to control plants grown at room temperature. 1st and 4th days of stress should be examined separately.

Ion leakage levels increased with the intensity of stress on non-SA treated control plants except freezing-stressed roots on the 4th day, which had similar values to cold-stressed roots on the 4th day (Table 2). The levels in both tissues under chilling and freezing stress for 1- and 4-day durations were always lower with SA application, with cold-stressed shoots on the 1st day, shoots and roots under normal conditions, and shoots under freezing stress on the 4th day being significant.

Table 2. Cell membrane integrity parameters ion leakage and malondialdehyde (MDA) levels and the level of reactive oxygen species hydrogen peroxide (H_2O_2).

Treatments	Ion Leakage (%)		MDA (nmol/g DW)		H_2O_2 (nmol/g DW)	
	1st Day of Stress					
	Shoot	Root	Shoot	Root	Shoot	Root
C*	17,34 ± 1,94$_{a,b,c,d}$	16,88 ± 1,40$_{a}$	0,019 ± 0,001$_{a}$	0,012 ± 0,001$_{a,b,c}$	0,079 ± 0,006$_{a}$	0,125 ± 0,013$_{a}$
SA50	14,87 ± 1,39$_{a}$	14,52 ± 1,37$_{a}$	0,018 ± 0,001$_{a}$	0,013 ± 0,002$_{a,b,c}$	0,102 ± 0,017$_{a,b}$	0,184 ± 0,017$_{b}$
SA100	15,01 ± 0,76$_{a}$	15,04 ± 0,88$_{a}$	0,019 ± 0,001$_{a}$	0,012 ± 0,001$_{a,b,c}$	0,112 ± 0,013$_{a,b}$	0,202 ± 0,012$_{b,c}$
C st4**	19,58 ± 1,26$_{c,d}$	31,36 ± 3,30$_{b,c}$	0,020 ± 0,002$_{a}$	0,014 ± 0,001$_{b,c}$	0,129 ± 0,014$_{b,c}$	0,199 ± 0,014$_{b,c}$
SA50 st4	15,46 ± 1,28$_{a,b,c}$	26,52 ± 3,21$_{b}$	0,020 ± 0,002$_{a}$	0,010 ± 0,001$_{a}$	0,130 ± 0,011$_{b,c}$	0,176 ± 0,016$_{b}$
SA100 st4	15,17 ± 1,33$_{a,b}$	24,81 ± 2,97$_{b}$	0,020 ± 0,003$_{a}$	0,011 ± 0,001$_{a,b}$	0,097 ± 0,005$_{a}$	0,100 ± 0,013$_{a}$
C st-	21,16 ± 1,97$_{d}$	40,07 ± 2,96$_{d}$	0,018 ± 0,001$_{a}$	0,018 ± 0,002$_{d}$	0,151 ± 0,015$_{b,c}$	0,206 ± 0,011$_{b,c}$
SA50 st-	17,33 ± 1,86$_{a,b,c,d}$	36,36 ± 3,72$_{c,d}$	0,017 ± 0,002$_{a,b}$	0,015 ± 0,001$_{c,d}$	0,082 ± 0,007$_{a}$	0,234 ± 0,010$_{c}$
SA100 st-	19,44 ± 1,54$_{b,c,d}$	37,92 ± 3,08$_{c,d}$	0,012 ± 0,002$_{b}$	0,015 ± 0,001$_{c,d}$	0,125 ± 0,016$_{b,c}$	0,197 ± 0,017$_{b,c}$
	4th Day of Stress					
	Shoot	Root	Shoot	Root	Shoot	Root
C	20,12 ± 1,79$_{a}$	24,20 ± 1,46$_{a,b}$	0,018 ± 0,002$_{a,b}$	0,010 ± 0,00$_{a}$	0,091 ± 0,003$_{a,b}$	0,158 ± 0,020$_{a,b}$
SA50	12,74 ± 0,89$_{b}$	18,39 ± 1,32$_{a}$	0,017 ± 0,001$_{a}$	0,010 ± 0,00$_{a}$	0,090 ± 0,002$_{a,b}$	0,164 ± 0,032$_{a,b}$
SA100	12,96 ± 1,27$_{b}$	18,35 ± 1,27$_{a}$	0,016 ± 0,00$_{a,c}$	0,010 ± 0,001$_{a}$	0,052 ± 0,001$_{a}$	0,189 ± 0,031$_{b}$
C st4	23,45 ± 2,22$_{a}$	37,33 ± 2,73$_{c}$	0,021 ± 0,001$_{b}$	0,018 ± 0,001$_{b}$	0,182 ± 0,016$_{c}$	0,208 ± 0,024$_{b,c,d}$
SA50 st4	22,29 ± 2,16$_{a}$	35,14 ± 2,73$_{c,d}$	0,016 ± 0,00$_{a,c}$	0,014 ± 0,001$_{c}$	0,144 ± 0,022$_{c,d}$	0,197 ± 0,025$_{b,c}$
SA100 st4	22,49 ± 1,90$_{a}$	34,7 ± 2,00$_{c,d}$	0,020 ± 0,001$_{b}$	0,016 ± 0,00$_{d}$	0,139 ± 0,008$_{c,d}$	0,100 ± 0,019$_{a}$
C st-	25,66 ± 3,21$_{a}$	34,07 ± 3,79$_{c,d}$	0,014 ± 0,00$_{c,d}$	0,012 ± 0,001$_{e}$	0,104 ± 0,028$_{b,d}$	0,279 ± 0,031$_{d}$
SA50 st-	13,55 ± 1,45$_{b}$	28,28 ± 3,31$_{b,d}$	0,014 ± 0,001$_{c,d}$	0,011 ± 0,00$_{a,e}$	0,104 ± 0,009$_{b,d}$	0,272 ± 0,027$_{c,d}$
SA100 st-	23,59 ± 2,34$_{a}$	30,68 ± 1,89$_{b,c,d}$	0,013 ± 0,001$_{d}$	0,010 ± 0,00$_{a}$	0,103 ± 0,019$_{b,d}$	0,265 ± 0,029$_{c,d}$

*C, SA50 and SA100 represent plants irrigated with Hoagland solution containing 0 µM, 50µM and 100µM salicylic acid in nutrient media, respectively. ** The abbreviations next to the treatments; st4 represents cold stress applied at 4°C, and st- represents freezing stress applied at -0.5°C.

The letters at the bottom right of the results indicate statistically significant differences ($p \leq 0.05$) compared to control plants grown at room temperature. 1st and 4th days of stress should be examined separately.

Shoot MDA levels were not significantly affected by neither stress nor SA application, with reductions in 100 µM SA treated shoots under the 1st day of freezing stress and 50 µM treated shoots under the 4th day of chilling stress being exceptions (Table 2). Root tissues were more responsive to chilling and freezing stresses in terms of MDA levels. Levels increased for both stresses under both durations. SA applications in the 50 µM concentration reduced MDA levels significantly under chilling stress conditions. Both chilling and freezing stress increased H_2O_2 levels in both tissues in both durations (Table 2). Under normal conditions, SA applications increased tissue H_2O_2 levels, except for shoot tissue on the 4th day, in which the level was not changed with 50 µM SA application and decreased significantly upon 100 µM SA application. However, the H_2O_2

levels decreased upon SA application in both tissues under both chilling and freezing stresses. Magnitudes of significant decreases changed in the range of 1.1 to 2.1-fold, under 1 day long freezing stress and 4 days long chilling stress applied root tissue, respectively.

Comparative analysis of all antioxidative stress indicators revealed that salicylic acid played a crucial role in combating stress by preserving cell membrane integrity, reducing ion leakage, and increasing water-holding capacity. Similar observations in terms of ion leakage, MDA and relative water content levels were also made by Mutlu et al. (2016), Wang et al. (2020), and Golovatskaya et al. (2023) in barley, wheat, and potato plants, respectively. In pea tissues under stress, especially under cold stress, proline levels significantly increased. However, it was determined that salicylic acid did not contribute to the role of osmo protectant proline in the stress response system. Nevertheless, Wang et al. (2022) showed that salicylic acid application increased proline accumulation under freezing stress in winter wheat seeds, indicating that salicylic acid's effects may vary in different plant species.

SOD enzyme activity was detected in both pea tissues in the form of several or one of the Mn SOD1, CuZn SOD1, and CuZn SOD2 isozymes as determined by KCN/H_2O_2 treatment (data not shown). Shoots exhibited more intense activity when compared to roots under any treatment. Total SOD activity in shoot tissues was comprised of the activities of all three isozymes. In root tissues, activities of Mn SOD1 and CuZn SOD1 were undetectable under normal conditions and cold stress, whereas CuZn SOD2 was the only isozyme active under freezing stress (data not shown). In shoot tissue, cold stress increased SOD activity on the 1st day of stress for all treatments and only in non-SA treated seedlings on the 4th day of stress (Figure 1). SA application with one-day duration increased SOD activity in a concentration-dependent manner in shoot tissue under normal conditions and only upon 100 µM SA application on the 4th day. Significant decreases in shoot SOD activities were observed under the 4th day of cold stress upon SA treatments. In root tissue, increasing stress intensities increased SOD activity in an SA application-independent manner (Figure 1). Unlike root tissue, freezing stress always caused reductions in shoot SOD activity. In summary, SOD enzyme activity showed significant increases, especially on the 1st day of cold stress, compared to plants under normal conditions, but activities decreased with prolonged stress or intensified freezing stress. However, SOD enzyme activities showed a significant increase in the presence of salicylic acid at different isoenzyme levels. Similarly, Tekin & Ceyhan (2020) reported that an increase in SOD activity was observed in pea plants exposed to cold stress. Salicylic acid has also been shown to increase SOD enzyme activity in alfalfa, cucumber, barley, and potato plants under cold stress (Golovatskaya et al., 2023; Ignatenko et al., 2021; Mutlu et al., 2016; Wang et al., 2023).

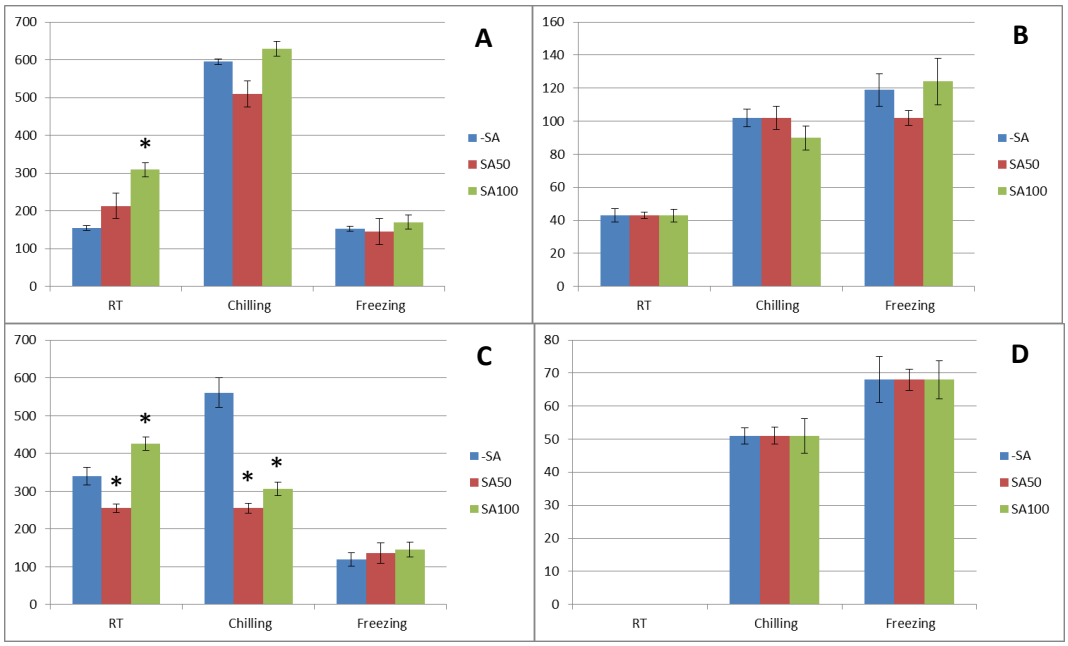

Figure 1. Superoxide dismutase (SOD) enzyme activities in shoots at day 1 (A), in roots at day 1 (B), in shoots at day 4 (C), in roots at day 4 (D) RT, Chilling and Freezing represent room temperature, cold stress applied at 4°C, and freezing stress applied at -0.5°C, respectively. Columns show the total activity of MnSOD1, CuZnSOD1 and CuZnSOD2 isoenzymes. *Indicates significant differences ($p \leq 0.05$) compared to the respective control.

Shoot *PDH47* expressions increased with the intensity of stress on day 1 of stress application without SA treatment (Figure 2). SA treatments significantly increased *PDH47* expressions under normal growth conditions up to 10-fold and under cold and freezing stress applications in a range of 1.2 to 4-fold.

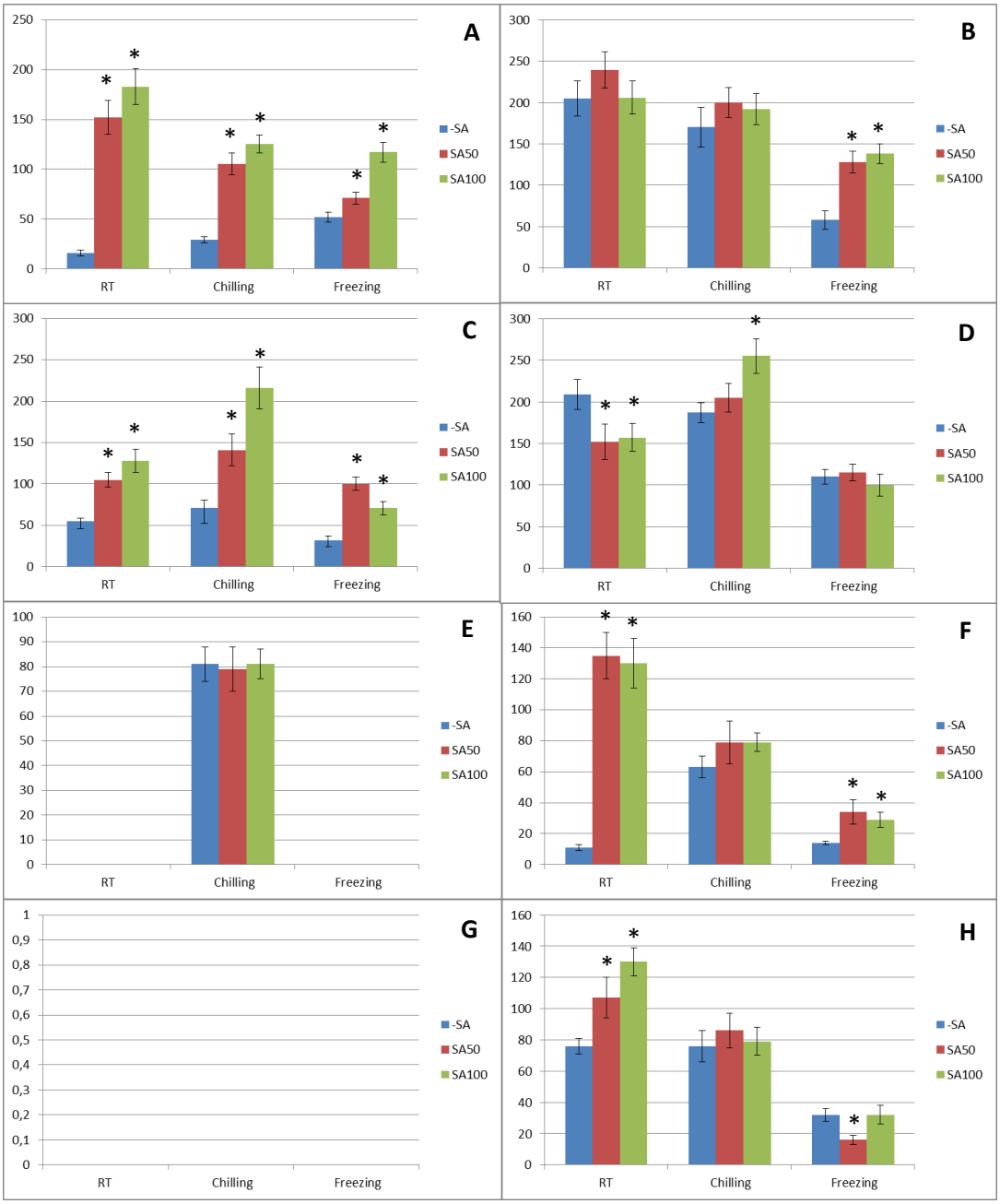

Figure 2. *PDH47* and *TOP2* gene expressions respective to internal control actin gene. *PDH47* expression on day 1 shoot (A), day 1 root (B), day 4 shoot (C), and day 4 root (D). *TOP2* expression on day 1 shoot (E), day 1 root (F), day 4 shoot (G) and day 4 root (H). RT, Chilling and Freezing represent room temperature, cold stress applied at 4°C, and freezing stress applied at -0.5°C, respectively. *Indicates significant differences (p≤0.05) compared to the respective control.

Unlike non-SA treated controls, *PDH47* activity decreased with the intensity of stress application under SA treatments. The lowest expression was observed under normal conditions on non-SA treated seedlings and the highest expression was on 100 µM SA-treated explants under normal conditions. SA-dependent expression patterns for shoots were similar on day 4 in normal and stress conditions, being higher under SA treatments compared to non-SA treated seedlings. However, unlike a steady decline in the intensity of stress as on day 1, all treatment groups showed higher expressions under chilling stress compared to room temperature and freezing stress on day 4.

Root tissue also exhibited strong *PDH47* expression under normal and stress conditions, while the expression was mostly unaffected from SA treatments (Figure 2). SA application significantly increased the expression only under 1 day freezing stressed roots and under 4 days chilling stressed roots compared to non-SA treated controls, while the application significantly decreased the expression levels under normal conditions compared to non-SA treated control on the 4th day. The levels of *PDH47* expression were lowest under freezing

stress for each treatment compared to expressions under normal conditions and cold stress.

Shoot *TOP2* expression was absent under every condition and application tested and appeared only under 1-day long cold stress treatment at the same intensity independent from SA application (Figure 2). Unlike shoot tissue, *TOP2* activity was present in root tissue under every treatment for both durations. SA application in both concentrations usually increased *TOP2* expression under normal and stress conditions, except for 4 days of freezing stress. Freezing stress did not affect *TOP2* expression level in non-SA treated seedlings under 1-day treatment; however, it decreased the expression under 4-days treatment compared to control plants under normal conditions. In SA treated plants, *TOP2* expression decreased gradually with increasing intensity of stress.

Investigations into the expression of *TOP2*, *PDH47*, and actin genes under cold and freezing stresses with salicylic acid applications revealed that all three genes were induced by stress and responded to the presence of salicylic acid in the environment. Hettiarachchi et al. (2005) demonstrated that *TOP2* gene expression increased under cold and salt stresses, as well as with applications of salicylic acid and ABA from phytohormones. The reason for the increase in *TOP2* gene expression under stress when DNA replication and cellular activities decrease has not been fully explained, but it has been suggested that it may be related to chromatin modeling and the need for DNA to adopt appropriate topology for the expression of stress-regulated genes. Significant increases were observed in the expression of the *PDH47* gene in stem and root tissues under salinity and cold stress in peas (Vashisht et al., 2005). When the *PDH47* gene was transferred to indica rice, the plant acquired resistance features under drought stress, demonstrating this by increasing relative water content with proline and reducing internal hydrogen peroxide levels (Singha et al., 2020; Singha et al., 2017). The data obtained in this study suggest that the expressions of these genes, which are believed to function in ribosome biogenesis, transcription, translation, and repair of damaged DNA/RNA molecules and potentially play an active role in stress tolerance, can be increased with salicylic acid. These increases might have positive effects observed on the antioxidative defense system.

Conclusions

This study has shown that the application of salicylic acid, when added to the growth medium, can have positive effects on the cold resistance of pea plants. Salicylic acid likely achieves some of its effects by increasing the activity of superoxide dismutase, one of the most important enzymes taking a role in combating reactive oxygen species. The data obtained indicates that salicylic acid increased the expressions of *TOP2* and *PDH47* genes, which can both change the structure of the DNA molecule. By altering the topology of DNA, they likely facilitate the transcription of genes encoding defense enzymes under both cold and freezing stress. Salicylic acid probably activates other enzymatic antioxidative system members such as CAT and POD, reducing the levels of reactive oxygen species such as hydrogen peroxide, which was also examined in this study. In this way, salicylic acid maintains cell membrane integrity, leading to a decrease in ion leakage and an increase in water-holding capacity. In summary, salicylic acid has been shown to induce various changes in pea metabolism, some of which were examined in this study, and these changes have been determined to have positive effects on the plant's cold (4°C) and freezing stress (-0.5°C) response systems at a level that can be utilized in agricultural cultivation.

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Disclosure Statement

The authors declare that they have no competing interests.

Contributions

Ufuk Celikkol Akcay, Hande Nur Kumbul and İbrahim Ertan Erkan equally contributed to experimental design, performance of analyses and the preparation of the manuscript.

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