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Combined and single osmopriming effects on wheat (*Triticum aestivum* L.) performance

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Abstract: Osmopriming has been shown to improve the germination and growth of bread wheat (*Triticum aestivum* L.). This study explores the impact of various priming agent NaCl (3g l^{-1}), proline (1 mM), ZnSO4 (1 mM), and their combination on wheat performance during the summer season (Jul-Aug 2022) at the greenhouse of Payame Noor University, Tabriz. Wheat seeds treated with a combination of priming agent demonstrated significantly enhanced performance compared to untreated seeds. Chlorophyll fluorescence measurements taken 35 days post-cultivation revealed a higher Photosystem Performance Index (PIabs) in osmoprimed seeds, particularly those treated with combined priming agent. Furthermore, primed plants demonstrated elevated concentrations of chlorophyll a, b, and carotenoids. Osmopriming also modulated the oxidative status of enzymes such as glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD). Genetic analysis showed that osmopriming could influence the expression of *NHX2*, a gene linked to improving plant growth, water uptake, and yield in stress conditions.

Key words: priming, antioxidant capacity, phenolic compounds, gene expression, fluorescence, wheat (*Triticum aestivum* L.)

Rastni učinki kombiniranega in enovrstnega tretiranja semen krušne pšenice (*Triticum aestivum* L.) z ozmotiki

Izvleček: Tretiranje semen krušne pšenice (*Triticum aestivum* L.) z ozmotiki izboljša kalitev in rast. V raziskavi so bili preučevani učinki različnih obravnavanj z ozmotiki kot so NaCl (3 g l^{-1}), prolin (1 mM), ZnSO4 (1 mM) in njihovih kombinacij na rast pšenice v poletni rastni sezoni (julij-avgust 2022) v rastlinjaku na Payame Noor University, Tabriz. Zrna pšenice, tretirana s kombinacijami osmotikov so pokazala značilno boljšo rast kot netretirana. Meritve fluorescence klorofila, opravljene 35 dni po gojenju v loncih so pokazale večje vrednosti indeksa učinkovitosti fotosinteze (PIabs) v primeru z ozmotiki tretiranih semen, še posebej tistih tretiranih s kombinacijo osmotikov. Z ozmotiki pred kalitvijo tretirane rastline so imele povečane vsebnosti klorofila a, b in karotenoidov. Predobravnava z ozmotiki je vzpodbudila aktivnost antioksidacijskih encimov kot so glutation peroksidaza (GPX), katalaza (CAT) in superoksid dizmutaza (SOD). Geneteske analize nakazujejo, da ima predobravnavanje semen z ozmotiki pred kalitvijo pozitivni učinek na parametre uspešne rasti krušne pšenice.

Ključne besede: predobravnavanje, antioksidacijska sposobnost, fenolne spojine, ekspresija genov, fluorescenca, krušna pšenica (*Triticum aestivum* L.)

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1 INTRODUCTION

Bread wheat (*Triticum aestivum* L.), a vital cereal crop, is extensively cultivated and accounts for over 20 % of the global population's daily protein intake (Rai-Kalal & Jajoo, 2021; Singhal, Pandey, & Bose, 2021). However, challenges such as poor seed properties, suboptimal soil conditions, and biotic and abiotic stresses can severely impact wheat productivity (Adnan et al., 2020; Amoah et al., 2019; da Costa et al., 2011; Dalil, 2014).

Expanding the range of wheat production is crucial, and developing stress-tolerant wheat cultivars through selective breeding is highly recommended (Amoah et al., 2019; Lobato et al., 2009). Seed priming techniques such as hydropriming, osmopriming, nanopriming, and mix priming offer cost-effective and efficient methods to enhance crop speed and stand in the field (Adnan et al., 2020). Osmopriming wheat seeds with osmotic components can improve stand establishment and reduce the time between seed sowing and seedling emergence (Farooq et al., 2019). Various priming agent, such as PEG, KNO_3, $K3PO_4$, $CaCl_2$, and NaCl, have been utilized for wheat seed priming (Amin, Khan, & Khalil, 2012). The properties and effectiveness of priming solutions differ based on the crop species. (Rai-Kalal & Jajoo, 2021). However, the study on the impact of combined priming agent on plant performance during seed germination and growth is limited.

The seed industry is actively seeks potent priming agents that can enhance plant resilience in challenging field conditions (Srivastava et al., 2010). Nonetheless, seed osmopriming, a chemical treatment for seeds, raises environmental and health concerns due to its detrimental effects on the environment and human health (Hasan et al., 2016). The adaptability of seed priming relies on the selection of suitable priming agents and understanding their mechanisms (Islam, Mukherjee, & Hossin, 2012). Factors such as economic costs, the nature of pretreatment agents, priming exposure duration, and crop species influence the effectiveness of pretreatment (Bisen et al., 2015). Despite its constraints, seed priming has demonstrated promise in improving seed germination, growth, and resilience to abiotic stresses such as salinity, drought, and heat within agriculture (Siyar et al., 2020). In previous research, we refined the osmopriming technique for wheat seeds. This study encompassed experiments conducted in Petri dishes, where different concentrations of NaCl, $ZnSO_4$, proline, and trehalose were used. The findings indicated a significant increase in wheat seed germination when exposed to 3 and 10 g l^{-1} NaCl concentrations, 1 and 20 mM ZnSO4, 1 and 10 mM proline, and 0 and 1 mM trehalose. These concentrations were validated using the surface-response method and experiments. Utilizing three priming reagents, individually or in combination, resulted in a marked increase in seed germination. Notably, the most efficacious treatments included NaCl (3 g l^{-1}), Proline (1 mM), and $ZnSO_4$ (1 mM) for 12 hours (Yavari et al., 2022). Therefore, the present investigation aimed to examine optimized concentrations and evaluate their potential in improving the germination, emergence, and early stand establishment, as well as specific physiological attributes of wheat in soil.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND EXPERIMENTAL CONDITIONS

We utilized NaCl (3 g l^{-1}), proline (1 mM), $ZnSO_4$ (1 mM), and combinations thereof (NaCl (3 g l^{-1}) + proline (1 mM) + $ZnSO_4$ (1 mM)) as osmopriming agents, which were optimized in our previous Petri dish experiments (Yavari et al., 2022). Seeds were soaked in osmopriming agents for 12 hours and subsequently sown at a depth of 0.5 cm in soil-filled vases. Both non-primed and primed seeds were sown in three replicates of 50 seeds each in the greenhouse of Payame Noor University, Tabriz, during the summer season (July–August 2022). Each vase received daily irrigation of 10 ml of water. The performance indexes of the seeds were measured on the 35^{th} day.

2.2 FLUORESCENCE ANALYSIS AND PHOTOSYNTHETIC PIGMENTS

An analysis of leaf fluorescence was performed at room temperature using a plant efficiency analyzer (PEA, Packet-PEA, Hansatech Instruments Ltd., England). The efficiency of the oxygen-evolving complex on the donor side of PSII (*Fv/Fo*) and the maximum quantum yield of photosystem II (*Fv/Fm*) were determined. In this context, Fm represents the maximal intensity of chlorophyll fluorescence, *Fv* stands for variable chlorophyll fluorescence, and *F*o indicates minimal fluorescence. Spectrophotometry was employed to determine the content of photosynthetic pigments, including chlorophyll a/b and carotenoids. Samples were homogenized with methanol, centrifuged at 1000 rpm, and the resulting supernatants were used for analysis. Calculation was performed based on the method described by Lichtenthaler and Wellburn (1983) (Lichtenthaler & Wellburn, 1983).

2.3 COMPATIBLE SOLUTE CONTENT MEASUREMENTS

The process of extracting leaf samples was carried out using a sodium phosphate buffer solution. (PBS, 50 mM, pH = 6.8). Following centrifugation (15000 g, 20 min), protein content was quantified using an auto-analyzer device (Abbott Alcyon 300). To determine sugar content, 200 µl of the supernatant was mixed with anthrone-sulfuric reagent (1 ml), boiled in a hot water bath (10 min, 100 °C). After cooling, the solution was measured for absorbance at 650 nm. Total soluble sugars were calculated using a glucose standard curve (Sigma). Starch analysis followed the method described by Magné et al. (2006) (Magné, Saladin, & Clément, 2006). Starch was dissolved in a 4 : 1 (v/v) mixture of 8 N HCl/dimethylsulfoxide and the solution was then mixed with an iodine HCl solution and absorbance was measured at 600 nm. In order to measure the amount of starch present, a standard curve of starch obtained from Merck was utilized. Proline content was assessed using the method outlined by Bates et al. (1973) (Bates, Waldren, & Teare, 1973). Leaf samples were homogenized in sulfosalicylic acid (3 % w/v, 4 °C) and centrifuged (3000 g, 20 min). The supernatant was mixed with acid ninhydrin and glacial acetic acid for 1 hour in a hot water bath, and proline content was calculated at 520 nm using a proline (Sigma) standard curve.

2.4 PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY AND RELATED METABOLITES

The activity of PAL was measured using the modified method of Zucker (1965). Briefly, leaf samples were homogenized in PBS (50 mM, pH 7.0) supplemented with polyvinyl polypyrrolidon (PVPP) (2 % w/v), EDTA (2 mM), β-mercaptoethanol (18 mM) and Triton X-100 (1 % v/v). The cinnamic acid formation was monitored by spectrophotometry at 290 nm, representing PAL activity (one unit (U) activity equals one nmol cinnamic acid per hour produced by the enzyme). The Velioglu et al. (1998) method was used to measure total phenolic content. (Velioglu et al., 1998). A standard curve was created using gallic acid, and the results were expressed as milligrams per gram of fresh mass. Total flavonoid content was determined using the method outlined by Meda et al. (2005) (Medaet al., 2005). In brief, 5 ml of aluminum chloride (2 %) in methanol was mixed with 5 ml of leaf extracts (0.02 mg ml^{-1}). The total flavonoid content of the extract was determined using a standard curve of quercetin and expressed as mg quercetin equivalent (QE) 100 g^{-1} extract after 10 minutes.

2.5 ASSAY OF ANTIOXIDANT ENZYMES AND RELATED METABOLITES

SOD and CAT activity were determined using the method previously reported by Habibi and Hajiboland (2012) (Habibi and Hajiboland, 2012). Glutathione peroxidase (GSH-Px) activity was assessed using the modified method by Flohé and Günzler (1984). (Flohé & Günzler, 1984). To determine the extent of lipid peroxidation in membranes, the concentration of malondialdehyde (MDA) was measured. Leaf samples were homogenized in thiobarbituric acid (1 ml, 0.1 %) and centrifuged at 12,000 × g for 10 min. A 1, 1 , 3, 3-tetra ethoxy propane-based standard curve was used to quantify MDA, and the absorbance was measured at 525 nm.. The hydrogen peroxide (H_2O_2) content was assayed according to the procedures described by Velikova et al. (2000) (Velikova, Yordanov, & Edreva, 2000). H_2O_2 content was determined using a standard curve.

2.6 RNA EXTRACTION, CDNA SYNTHESIS AND RT-PCR ANALYSIS

The Trizol reagent was used to isolate total RNA from both primed and non-primed plant leaves.cDNA synthesis was carried out using the cDNA Reverse Transcription Kit (Applied BiosystemsTM) according to the protocol. The quality of the synthesized cDNA was verified using a 1 % agarose gel. Forward (F-ATTTTGCTCGGGTTGGTTCTGGTT) and reverse (R-GTGCAGGGACTTCGGTGACGC) primers targeting the NHX2 gene were employed. The actin gene of wheat served as an internal standard.

2.7 STATISTICAL ANALYSIS

The results were derived from three independent series of experiments. Chlorophyll fluorescence parameters were analyzed using the PEA Plus V1.10 software. GraphPad Prism (version 9.4.1) was used to perform statistical analysis, and differences among treatments were assessed by one-way ANOVA at a significance level of $p < 0.05$. (Refer to Table 1).

Table 1: Results of variance analysis of parameters

Variables	Nonprime	Combination	NaCl (3 g l^{-1})	Proline (1 nM)	ZnSO4 (1 mM)
Germination (%)	76 ± 7.21	98 ± 2	89.33 ± 1.15	93.33 ± 7.02	82 ± 12.16
Chl*a* content (mg g FM^{-1})	22.43 ± 0.11	23.51 ± 1.51	30.96 ± 1.66	27.22 ± 1.51	25.75 ± 2.07
Chl*b* content (mg g FM^{-1})	11.25 ± 0.96	22.93 ± 2.66	28.01 ± 0.39	27.73 ± 1.76	12.05 ± 1.31
Carotenoid content (mg g FM^{-1})	4.15± 0.87	5.33± 0.32	3.006± 0.83	5.89± 0.22	5.1 ± 0.32
Seedling length (mm)	421.2 ± 24.69	456.86 ± 3.28	431.93 ± 20.51	451.8 ± 8.74	384.4 ± 5.92
RWC (%)	59.11 ± 1.23	85.87 ± 7.21	92.24 ± 5.51	84.91 ± 10.32	101.02 ± 4.86
PI abs	1.39 ±0.04	2.207 ± 0.01	1.129 ± 0.07	0.43 ± 0.08	2.25 ± 0.05
Fv/Fm	0.67 ± 0.11	.08 ± 0.02	0.77 ± 0.06	0.68 ± 0.09	0.79 ± 0.009
Fv/Fo	2.28 ± 1.13	4.16 ± 0.75	3.58 ± 1.25	2.35 ± 0.97	3.89 ± 0.2
Starch content (mg g FM^{-1})	124.25 ± 3.5	138.79 ± 0.66	144.18 ± 1.95	140.77 ± 6.40	131.04 ± 0.40
Soluble sugars content (mg g FM^{-1})	7.74 ± 0.86	20.2 ± 0.22	13.51 ± 1.08	7.77 ± 0.95	16.60 ± 3.46
Protein content (mg dl^{-1})	34 ± 1	47.5 ± 1.5	71 ± 3	49.5 ± 1.5	36.5 ± 5.5
Proline content (µM g FM^{-1})	3.66 ± 0.33	9.25 ± 0.21	14.77 ± 0.67	9.33 ± 0.10	4.69 ± 0.40
SOD activity (U ml^{-1} protein)	0.038 ± 0.0005	0.017 ± 0.001	0.026 ± 0.001	0.023 ± 0.002	0.021 ± 0.0002
GPX activity (U g^{-1} protein)	1.81 ± 0.08	1.88 ± 0.08	1.26 ± 0.05	2.64 ± 0.18	2.52 ± 0.47
Flavonoid content (mg QE g FM^{-1})	1.56 ± 0.16	21.63 ± 0.08	16.75 ± 0.17	16.53 ± 0.12	17.18 ± 0.16
Phenol content (mg GEA g FM^{-1})	22.33 ± 0.83	25.06 ± 2.85	25.16 ± 0.95	25.2 ±.5	25.73 ± 0.35
PAL activity (µmol cinamic cid g^{-1} protein min^{-1})	0.49 ± 0.04	0.57 ± 0.02	0.71 ± 0.02	0.71 ± 0.018	0.51 ± 0.108
CATactivity (µmol H_2O_2 mg^{-1} protein min^{-1})	17.55 ± 1.95	33.15 ± 1.95	17.55 ± 1.95	6.85 ± 0.95	25.35 ± 1.95
H_2O_2 content (µM g FM^{-1})	61.77 ± 0.76	42.03 ± 2.28	44.31 ± 3.04	25.31 ± 0.76	42.79 ± 1.52
MDA content (nM g FM^{-1})	32.5 ± 2.5	16.5 ± 1.5	30.5 ± 4.5	24.5 ± 1.5	27.5 ± 1.5

3 RESULTS AND DISCUSSION

3.1 COMBINED AND INDIVIDUAL OSMOPRIMING SIGNIFICANTLY ENHANCE SEED GERMINATION INDICES AND PHOTOSYNTHETIC FUNCTION

The efficacy of priming agent as seed osmopriming agents on germination depends on concentration and priming duration. Wheat seeds primed with NaCl (3 g l^{-1}), proline (1 mM), $ZnSO_4$ (1 mM) and their combination exhibited improved germination rates. A combined priming treatment resulted in a significant increase in seed germination compared to untreated seeds. (Figure 1A). Previous studies have highlighted the beneficial effects of NaCl (Mirza, 2021), proline (Ambreen et al., 2021) and $ZnSO_4$ (Rehman et al., 2022) in wheat. For instance, seed priming with $ZnSO_4$ (0.1 and 0.5 M) enhanced plant water relations, grain yield, seedling growth, and stand establishment in wheat compared to non-primed seeds (Rehman et al., 2022). Similarly, proline priming improved germination rate (GR) and relative germination energy (RGE) under salinity stress in rice seeds (Hua-long et al., 2014) with the most effective concentration being 25 mM (Feghhenabi et al., 2020). Our findings align with these results, showing maximum germination in wheat seeds primed with different concentrations of NaCl, proline and $ZnSO_4$. Additionally, combined osmopriming exhibited significant improvement in germination compared to individual priming. This observation is consistent with the synergistic effect of combined $Mg(NO_3)_2$ and $ZnSO_4$ under drought stress compared to individual and non-priming treatments (Singhal et al., 2021). Relative water content (RWC), a crucial physiological parameter, reflects plant water status and stress tolerance (Singhal et al., 2021). Non-priming treatments exhibited the lowest RWC values, while combined priming resulted in the highest RWC values (Figure 1B). The effectiveness of combined osmopriming likely arises from the synergistic interaction of NaCl, proline and $ZnSO_4$, which collectively contribute to seed germination and early growth. NaCl facilitates water up-

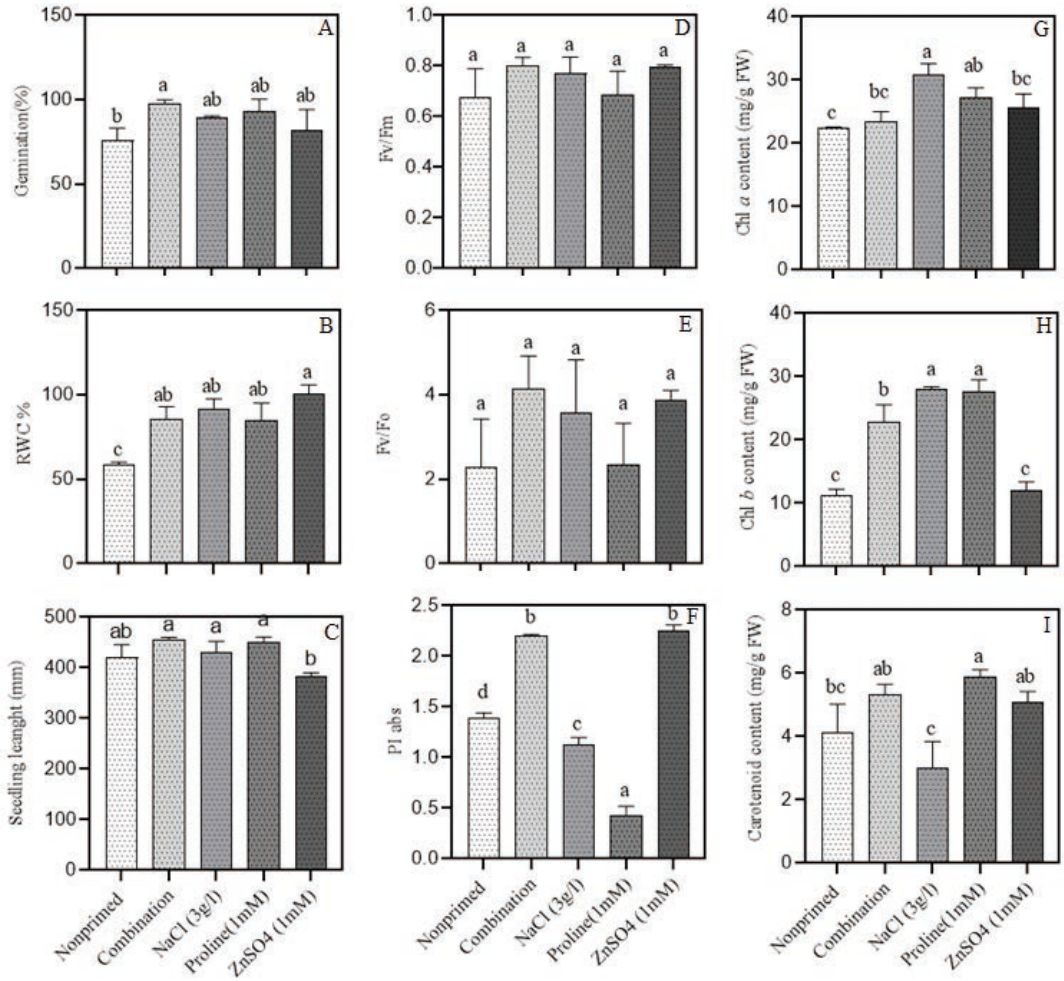

Figure 1: Screening the effect of different priming treatments on A) germination percentage, B) Relative water content (RWC) percentage, C) seedling length, D) The efficiency of oxygen-evolving complex on the donor side of PSII (Fv/Fo) (E) the maximum quantum yield of photosystem II (Fv/Fm), F) The Performance Index (PIabs), G) chlorophyll a, H) chlorophyll b, I) content of carotenoids . The error bars represent the standard deviation, and groups with the same letter are not significantly different from each other

take (Biswas et al., 2023), proline protects against osmotic stress and reactive oxygen species (ROS) (Kavi Kishor et al., 2022) and $ZnSO_4$ aids in enzyme activation and DNA synthesis, thereby enhancing seed performance. Bibi et al. (2017) reported a significant increase in plant growth and RWC of wheat under sodium nitroprusside priming (Bibi et al., 2017). However, our results suggest that applying $ZnSO_4$ hurt seedling length (Figure 1C) compared to other priming agents. Similar observations have been reported who noted retardation in the growth of seedlings treated with $ZnSO_4$ (Pavani et al., 2014; Rai-Kalal & Jajoo, 2021). This may be due to the high solubility of ZnSO4, which has minimal retention within the plant and results in inefficient Zn bioavailability over a prolonged period. (García-López et al., 2019; Prasad et al., 2012). Seed germination and seedling growth in wheat were negatively affected by seed priming with CuSO4 and ZnSO4, according to the results (Mim et al., 2021).

Figure 1D-F depicts the typical polyphasic rise (O–J–I–P) of fluorescence transients for NaCl, proline, $ZnSO_4$ and their combination after 30 days of cultivation. A non-significant increase in the ratio of *Fv/Fo* was observed in primed plants (Figure 1D). The *Fv/Fm*, representing the maximum quantum yield of photosystem II, remained unchanged under seed priming conditions (Figure 1E). However, a significant increase in the photosystem performance index (PIabs) was observed in treated plants (Figure 1F), suggesting that osmopriming can mitigate damage to the electron transport chain of PSII. Interestingly, the efficiency of the water-splitting complex increased in primed plants with combinational treatment and $ZnSO_4$ compared to unprimed plants. This

finding aligns with previous research on seeds primed with zinc oxide nanoparticles (Rai-Kalal & Jajoo, 2021).

It was found out that the osmopriming treatments resulted in the mildest increase in chlorophyll content. Notably, NaCl and proline treatments significantly influenced the content of photosynthetic pigment chlorophyll a compared to the control (Figure 1G). Furthermore, the largest increase in chlorophyll b was observed in treatments with NaCl, proline, and combination treatment (Figure 1H). Carotenoids content, which play a crucial role in plant photoprotection mechanisms, also significantly increased in the proline treatment compared to non-primed plants (Figure 1I). Additionally, osmopriming increased chlorophyll fluorescence in the I–P phase from the OJIP transient, possibly due to reduced availability of ferredoxin and NADP (Kalaji et al., 2016).

Osmopriming is a method that enhances plant tolerance to stress by improving photosynthesis. Studies have demonstrated that applying 0.9 MPa polyethylene glycol (PEG) as a priming agent at 18 °C for 30 hours can confer drought resistance in wheat reproductive stages. This effect is achieved by increasing the net photosynthetic rate and enhancing photo-protective and antioxidative mechanisms (Sherin, Aswathi, & Puthur, 2022). Primed plants under stress conditions exhibit higher levels of carotenoids, which are crucial for optimal growth and yield (Abid et al., 2018). Osmopriming preserves the structure and function of photosynthetic pigments and the photosynthetic apparatus (Sherin et al., 2022). In comparison, untreated plants may have a higher relative growth rate and yield of grains in barley during drought conditions (Kaczmarek egt al., 2017). Investigations into the effects of osmopriming, specifically with 30 % PEG 6000, on sunflower plants under water stress conditions have shown that it increases the net assimilation of CO_2 and improves photosynthesis. Priming also influences the accumulation of soluble sugars in sunflower plants, leading to higher yields even under water stress. This improved performance is correlated with a 40 % increase in chlorophyll levels in primed leaves (Bourioug et al., 2020; Sherin et al., 2022). Osmopriming with PEG 6000 has also improved drought tolerance in *Medicago sativa* L. by increasing PSII efficiency, enhancing plant height, leaf area and growth (Mouradi et al., 2016). Additionally, it improves growth and biomass in *Lens cullinaris* Medik by reducing oxidative damage through improved sugar and calcium accumulation (Farooq et al., 2020). Overall, osmopriming boosts seedling growth and germination by improving photosynthesis, carbohydrate production, energy, light absorption, CO_2 uptake, biomass, stress tolerance and antioxidant protection. (Sherin et al., 2022).

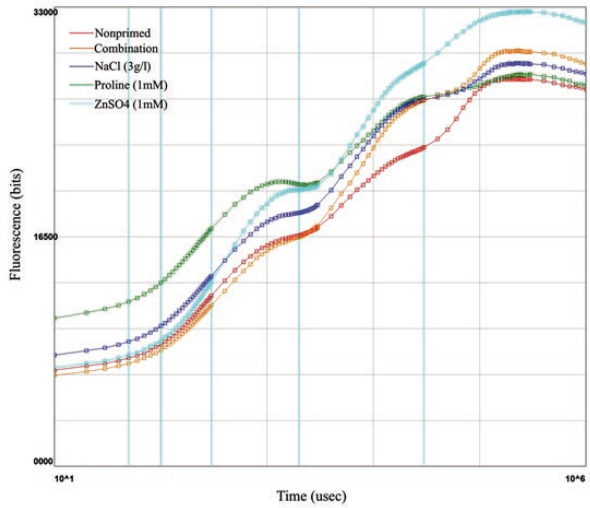

Figure 2: Effects of different priming treatments on the chlorophyll a fluorescence induction curve of wheat

3.2 COMBINED AND SINGLE OSMOPRIMING HAVE SHOWN SIGNIFICANT EFFECTS ON COMPATIBLE SOLUTES

When seeds are osmoprimed under low external water potential, they release organic solutes such as proline, glycine- free amino acids, and betaine (Ibrahim, 2016; Lemmens et al., 2019). Studies have demonstrated a significant correlation between starch content and germination index, seedling vigor index, shoot length, root length, and total seedling length (Salleh, Nordin & Puteh, 2020). Osmopriming can enhance the activities of acid invertase, alkaline invertase, and sucrose synthase (cleavage), as well as the contents of reducing sugars and starch in the grains of stressed plants (Kawatra, Kaur & Kaur, 2019). According to statistical data, the process of osmopriming using proline and NaCl resulted in the highest starch content (Figure 3A). Seed priming leads to enhanced accumulation of soluble sugars compared to non-primed seeds. The breakdown of starch into soluble sugars fuels seedling growth and germination. (Savvides et al., 2016). The highest soluble sugar content was found in NaCl, $ZnSO_4$, and combined-treated seeds, while the lowest was recorded in proline-treated seeds (Figure 3B). Khaing et al. (2020) indicated that 1 % K_2SO_4 significantly increased proline content in two wheat cultivars, Keumkang and Backjung (Khainget al., 2020). Consistent with these results, our data also showed that proline content increased in primed seeds compared with unprimed seeds (Figure 3C). There was a significant increase in soluble proteins (TSP) in treatments, except $ZnSO_4$,

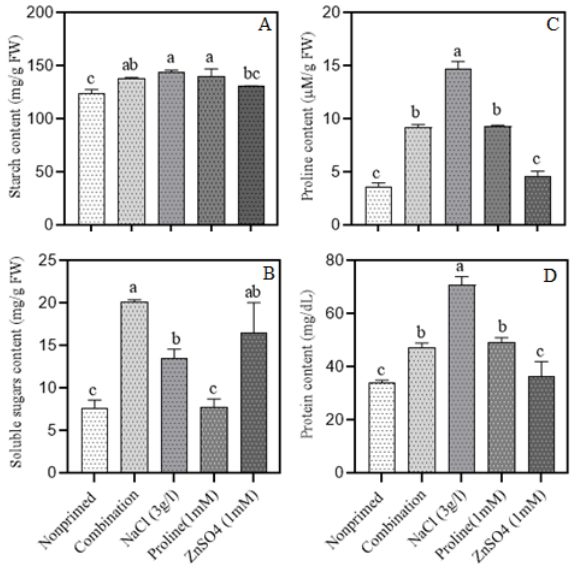

Figure 3: Effects of different priming treatments on A) starch content, B) soluble sugars, C) proline, D) protein. The error bars represent the standard deviation, and groups with the same letter are not significantly different from each other

compared to non-primed seeds (Figure 3D). Seed priming with combinational concentrations of Fe and Zn (4 and 8 mg l^{-1}) significantly increased soluble proteins in bread wheat compared to the control (Carvalho et al., 2019). Studies have shown that priming with $Mg(NO_3)_2$, $ZnSO_4$, and their combination can significantly improve the protein content of seeds (Choudhary et al., 2021). Priming also increased the total protein in amaranth seeds (Moosavi et al., 2009).

It is well established that secondary metabolites such as flavonoids and phenolic acids play a central role in defense mechanisms, signaling, and scavenging of free radicals, ultimately leading to increased nutritional values of crops (Kanjevac et al., 2022; Mousavi et al., 2021; Tohidi, Rahimmalek, & Arzani, 2017). In this study, the applied osmopriming treatments had a significant effect on PAL activity, phenolic and flavonoid content (Figure 4A-C). Previous studies have demonstrated increased flavonoid concentration in radish seedlings after priming with $MgSO_4$, IAA, and H_2O_2 (Kanjevac et al., 2022).

3.3 COMBINED AND INDIVIDUAL OSMOPRIMING EXERTED SIGNIFICANT EFFECTS ON THE ANTIOXIDANT DEFENSE SYSTEMS

Enzyme activity of GPX, CAT and SOD was assessed, revealing a prominent decrease in the activity of these enzymes in primed seeds, likely attributable to low levels of reactive oxygen species (ROS). CAT enzyme activity notably increased with combined and $ZnSO_4$ treatments but decreased with proline compared to non-primed seeds (Figure 4D). These findings align with Weisany et al. (2012), who suggested that the elevated CAT activity may result from the indirect requirement of zinc for H_2O_2 detoxification (Rai-Kalal & Jajoo, 2021; Weisany et al., 2012). Additionally, a decrease in SOD enzyme level was observed in treated seeds (Figure 4E), consistent with nanoparticle $ZnSO_4$-based seed priming effects (Rai-Kalal & Jajoo, 2021). However, in contrast to our results, Rai-Kalal (2021) showed enhanced SOD activity in $ZnSO_4$-primed plants compared to non-primed ones (Rai-Kalal & Jajoo, 2021). The activity of GPX increased in seed osmopriming with proline and $ZnSO_4$ (Figure 4F). Plants primed with $ZnSO_4$ exhibit higher GPX activity, which may be due to increased ROS generation from the greater solubility of toxic Zn^{2+} ions. (Rai-Kalal & Jajoo, 2021). Osmopriming-based high GPX/CAT activity likely contributes to restoring the antioxidant defense system for early seedling establishment. For instance, CAT activity upregulation during early germination and higher overall antioxidant activity in germinated seeds/seedlings than non-germinated ones have been reported (Chen & Arora, 2011).

Plant photosynthesis is linked to antioxidant defense mechanisms and osmolyte accumulation, particularly in response to various environmental stresses. Drought (water or moisture stress) increases photosynthetic pigment and proline content, indicating a responsive mechanism (Binodh et al., 2023). Similarly, under drought stress conditions, antioxidant enzymes such as superoxide dismutase, peroxidase and catalase are upregulated, suggesting an enhanced antioxidant defense system (Wang et al., 2019). Priming agent like proline, glycine betaine, and trehalose accumulate under salinity stress, playing a vital role in osmotic adjustment (Forough et al., 2018). This osmotic adjustment is crucial for plants to adapt to saline environments, as evidenced by changes in photosynthesis observed in halophyte plants (Nikalje et al., 2018). Furthermore, in maize plants subjected to water stress, the application of a combination of 24-epibrassinolide, spermine, and silicon enhances photosynthetic metabolites and antioxidant enzyme activity, leading to improved drought resistance and reduced accumulation of reactive oxygen species (Ghasemi et al., 2022). Similarly, alterations in photosynthesis in pigeon pea seedlings exposed to copper stress contribute to increased antioxidant defense mechanisms and osmolyte accumulation (Sharma et al., 2017). These changes are manifested through increased catalase and peroxidase enzyme activity, along with the production of priming agent like proline, glycine betaine, and trehalose (For-

ough et al., 2018). High antioxidant capacity is advantageous for plants as it helps desensitize photosynthesis to over-reduction in the photosynthetic electron transport (PET) chain and can alleviate over-reduction in water-water cycle activity. However, the precise influence of antioxidant capacity on retrograde signaling pathways is not fully understood. Exploring redox signaling pathways could provide valuable molecular insights for upregulating plant protective genes (Foyer & Shigeoka, 2011).

Malondialdehyde (MDA) is a reliable marker for assessing plant injury caused by stress, as it correlates with the degree of plant damage (Fayez & Bazaid, 2014). Under stress conditions, plants produce reactive oxygen species (ROS) that inhibit biomolecule production, leading to increased levels of MDA and cellular leakage. Monitoring MDA levels provides valuable insights into plant growth dynamics, enabling real-time assessment of stress conditions and facilitating preemptive measures against drought (Zhang et al., 2021). In our experimental setup, MDA levels exhibited a significant decrease in plants subjected to combine and proline priming compared to non-primed plants (Figure 4G). This reduction in MDA content in primed plants aligns with findings reported by Prabha Rai-Kalal et al. (Rai-Kalal & Jajoo, 2021). Regardless of the priming agent and stress imposition, hydrogen peroxide (H_2O_2) levels decreased under primed conditions. (Ellouzi, Sghayar, & Abdelly, 2017). Primed seeds demonstrated lower tissue H_2O_2 contents than the control (Figure 4H). Additionally, seeds subjected to osmopriming with melatonin OMel50 and OMel500 exhibited the lowest H_2O_2 accumulation during the experiment (Marta, Szafrańska, & Posmyk, 2016).

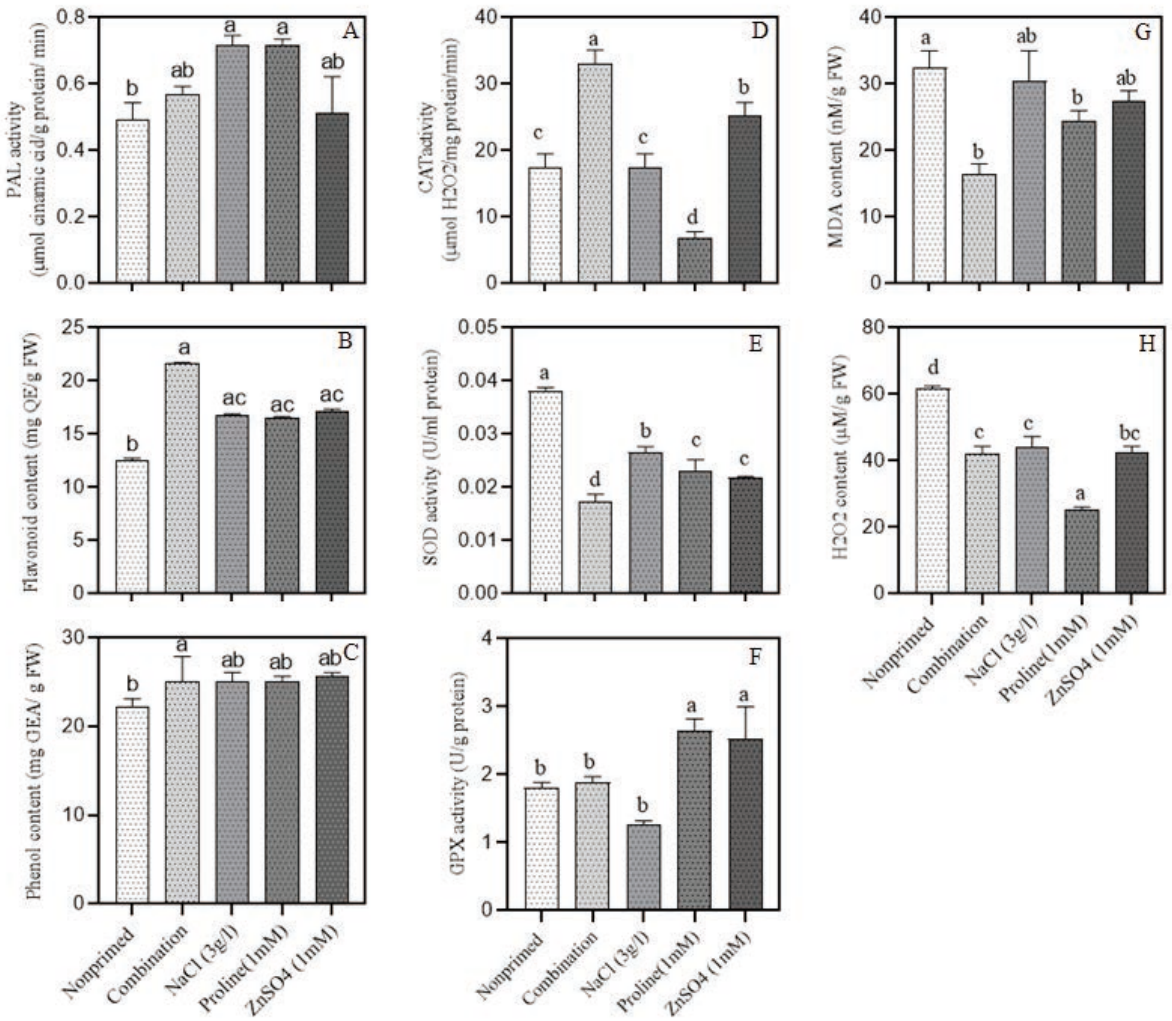

Figure 4: Effects of different priming treatments on A) PAL activity, B) flavonoid, C) phenol content, D) CAT E) SOD F) GPX activity, G) malondialdehyde (MDA), H) H2O2 content The error bars represent the standard deviation, and groups with the same letter are not significantly different from each other

3.4 OSMOPRIMING HAD AN IMPACT ON THE EXPRESSION OF THE *NHX2* ANTIPORTER GENE

Intracellular Na^+/H^+ (*NHX*) antiporters are crucial for maintaining cellular pH and the homeostasis of Na^+ and K^+ ions (Bassil et al., 2011; Xu et al., 2013). *NHX1* and *NHX2* are pivotal in regulating K^+ levels and intravacuolar pH, essential for cell expansion and flower growth (Xu et al., 2013). They enhance salt stress resistance by facilitating intracellular potassium partitioning, thereby regulating cellular pH and K+ homeostasis (Bassil et al., 2011). *NHX* genes in the wheat genome, particularly *NHX2*, are crucial for salinity tolerance across various plant species (Yarra, 2019). Transgenic plants expressing *NHX2* exhibit elevated levels of chlorophyll, relative water content, superoxide dismutase, ascorbate peroxidase, reduced hydrogen peroxide levels, and malondialdehyde content compared to wild-type plants (Bulle et al., 2016; Yarra, 2019). In this study, the potential role of the *NHX2* gene in wheat germination under optimized priming concentrations was investigated. The study revealed that priming seeds with NaCl and proline, as well as combinations of treatments, led to an increase in the expression of the *NHX2* gene in primed seeds. This increase may be attributed to the rise in sodium content under non-stressed conditions (Figure 5A-D). Recent research has shown that *NHX1* and *NHX2* are transporters located in the vacuole that play a key role in regulating the pH and potassium levels within the vacuole. These proteins are essential for facilitating the uptake of potassium at the tonoplast, maintaining osmotic balance and turgor pressure, and have a notable impact on stomatal function (Barragan et al., 2012). In response to 500 mM NaCl, the *NHX2* gene exhibited a similar pattern of expression, showing a significant increase in leaves of both non-primed and

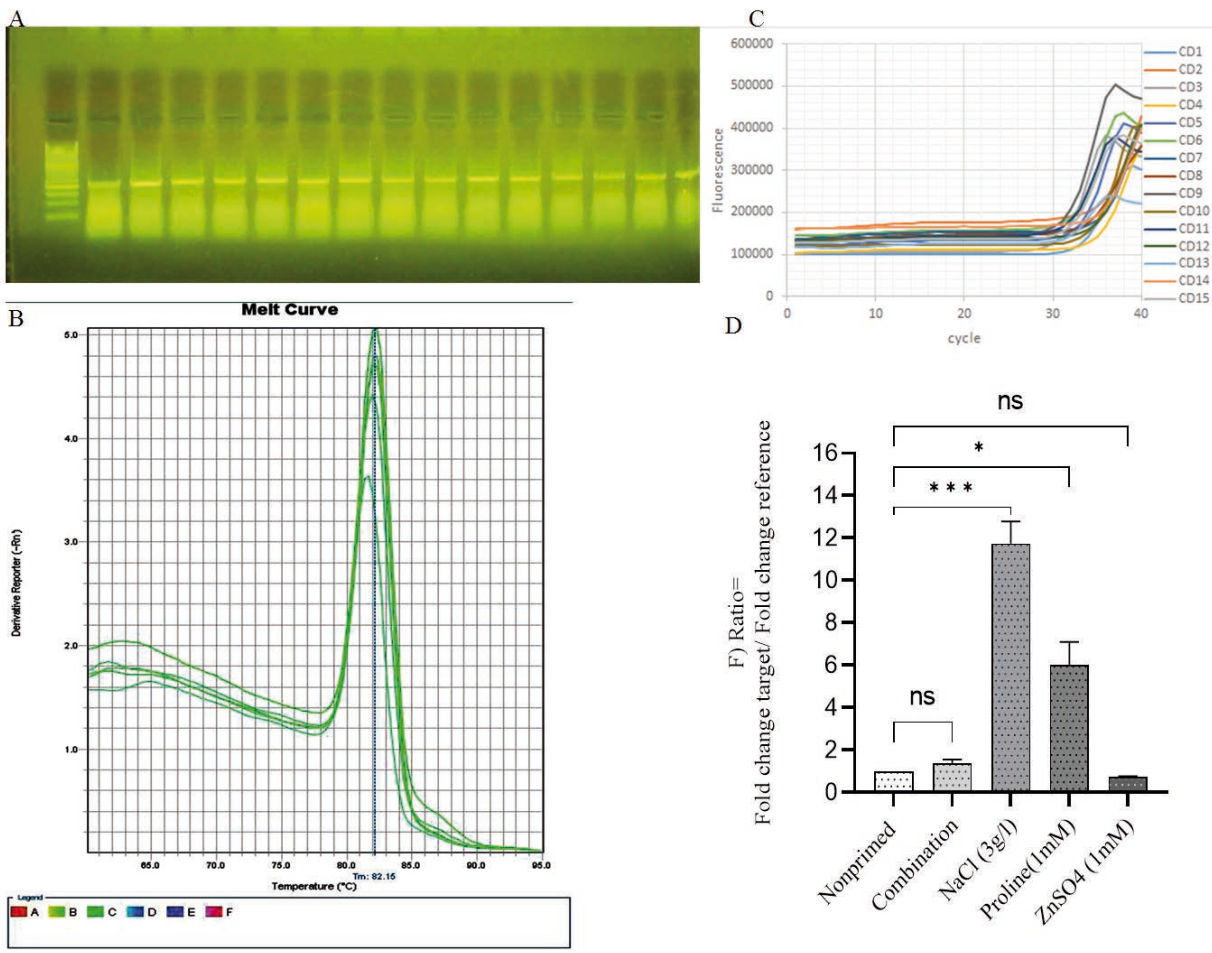

Figure 5: The effect of wheat seed osmopriming on NHX2 gene expression. A) Gel electrophoresis B) Melting curve. C) Real-time curve nonprime (CD1-CD3), combination (CD4-CD6), NaCl (3 g l-1) (CD7-CD9), proline (1 mM) (CD10-CD12), ZnSO4 (1 mM) (CD13-CD15). D) Gene expression graph compared to the group's average without prime (values are the average of 3 repetitions and the same letters indicate no significant difference between the averages at the $p < 0.05$ level)

primed plants (Janda et al., 2016). It was demonstrated that priming with jasmonic acid had a positive impact on the expression of *NHX2* gene in wheat plants under both saline and non-saline conditions (Sheteiwy et al., 2022).

4 CONCLUSION

Several indicators were selected to evaluate the impact of priming agents on wheat because stress responses are multifaceted and require a thorough assessment of plant physiological, biochemical, and molecular alterations. These indicators encompass photosynthetic pigments, protein levels, sugar and starch content, PAL activity, antioxidant enzymes, associated metabolites, RNA, and specific genes such as *NHX2* (E Sobhy et al., 2023; Faisal et al., 2023; Hosen et al., 2023). Photosynthetic pigments indicate the health and stress tolerance of plants, while protein levels show growth and stress reactions. Sugar and starch levels reveal energy availability, PAL activity is linked to stress defense mechanisms, and antioxidant enzymes indicate responses to oxidative stress. RNA and *NHX2* gene expression offer insights into molecular responses to stress. By examining these various indicators, researchers can obtain a thorough understanding of how priming agents affect wheat growth, stress tolerance, and overall productivity. This study evaluates the impact of combined and individually optimized osmopriming on wheat plant growth and development. Priming resulted in a significant increase in antioxidant enzymes, soluble sugars, and proteins, while reducing endogenous levels of H_2O_2 and MDA. It has been demonstrated that osmopriming treatments, such as hydro- and osmopriming with PEG solutions, improve germination attributes and seedling performance in various plant species (Debta et al., 2023; Mehboob et al., 2022). The results indicate that combinational osmopriming has the highest positive effect on wheat seed performance, enhancing the efficiency of PSII functioning, primary photochemistry, and biochemistry. Combined osmopriming treatments are an effective method for enhancing seed germination and seedling growth in crop production, especially in mitigating stress effects (Singhal et al., 2021). Combined osmopriming with Ca^{2+} and K^+ enhances salt tolerance in quinoa seeds and seedlings, improves growth, nodulation, chlorophyll fluorescence, and nutrient uptake in alfalfa under drought conditions, and significantly enhances seedling length and dry weights (Mamedi et al., 2022; Mirmazloum et al., 2020; Mouradi et al., 2016). Combined osmopriming with melatonin is more effective than treating with fungicides due to its enhanced germination capacity, reduced fungal incidence, and improved seed quality (Rosińska, Andrzejak, & Kakkerla, 2023). The effectiveness of this method is attributed to the synergistic interaction of NaCl, Proline, and $ZnSO_4$, which contribute to different aspects of seed germination and early seedling growth. NaCl improves water uptake, Proline protects against osmotic stress and reactive oxygen species, and $ZnSO_4$ aids enzyme activation and DNA synthesis.

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