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Seed Priming Improves Biochemical and Physiological Performance of Wheat Seedlings under Low-Temperature Conditions

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Abstract: Wheat is a widely cultivated cereal throughout the world and stress caused by low temperatures significantly affects all stages of wheat development. Seed priming is an effective method to produce stress-resistant plants. This work was carried out to determine whether different priming methods (hormo-, halo-, osmo-, and hydropriming) can increase the resistance of wheat to low-temperature conditions (10 °C). The effect of priming on growth, as well as the biochemical and physiological performance of wheat seedlings were monitored. In general, priming had a significant stimulatory effect on the monitored characteristics. Hormo- and halopriming had a positive effect on the growth, vigor index, and total soluble protein content of wheat seedlings. Additionally, hormopriming reduced the malondialdehyde (MDA) content in wheat seedlings compared to unprimed seeds. A dominant effect on antioxidant enzymes (superoxide-dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase, and pyrogallol peroxidase) was recorded after seed priming with KNO_3. The effectiveness of priming was also confirmed through the increased content of phenolic compounds (including flavonoids), and total antioxidant activity. The HPLC analysis showed increased content of chlorogenic acid, catechin, 4-hydroxy benzoic acid, sinapic acid, rutin, naringin, and quercetin in primed wheat seedlings compared to unprimed grown seedlings under low-temperature conditions with the best effects achieved by hormo- and hydropriming. It is concluded that seed priming can be regarded as a promising approach for increasing the resistance of wheat seedlings to low-temperature stress.

Keywords: antioxidant enzymes; cereals; HPLC; priming agents; temperature stress

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1. Introduction

Since it is produced on more land than any other crop and accounts for approximately 26% of the worldwide production of cereals, *Triticum aestivum* L. (wheat) is one of the most important crops for humanity [1,2]. Wheat grains are a rich source of phytochemicals, including vitamin E, resistant starch, β-glucan, carotenoids, lignans, inulin, sterols, phytates, and phenolic acids [3]. Therefore, wheat is the leading grain in consumption primarily due to its nutritional value but also due to relatively easy harvesting and processing [4]. Despite the increasing demand for wheat production, cultivation of wheat is challenged with difficulties since wheat is sensitive to various abiotic stresses [5]. The most important limiting factors for worldwide production include late frost, high temperature, drought, and heavy rainfall, and these abiotic stresses can significantly reduce crop yield as well as their quality [6–8]. Recently, low-temperature stress has been pointed out as a serious challenge for cereal production [9], causing damage to wheat production in the United States [10,11], Australia [12–14], Europe [7], and China [15].

Temperature stresses are among the most pivotal abiotic stresses, which inhibit the development of many crops by limiting metabolic processes [16]. Heat stress inhibits germination by preventing imbibition, thereby preventing plant sprouting due to loss of seed vitality [17]. A serious negative effect on wheat growth, productivity, and grain yield quality has been recorded after heat stress [18]. Low-temperature stresses can stop wheat growth by forming ice crystals causing mechanical injuries and metabolic dysfunction, resulting in different metabolic changes (including disruption of metabolic regulations, accumulation of osmolytes, modification of carbohydrate metabolism and photosynthetic properties), and inactivation of many metabolic enzymes [19,20]. By increasing the content of reactive oxygen species (ROS) and malondialdehyde (MDA), as well as by inhibiting photosynthesis, low temperature inhibits the growth of wheat [21]. Xiao et al. [22] suggest that low-temperature stress (even short term) severely impairs wheat growth by limiting photosynthetic capacity through reduced leaf area. The negative impact of temperature stress on the growth and yield of wheat was recorded by Shah et al. [23]. In cereals, low-temperature stress in the vegetative phase limits main reactions in plant cells, resulting in wilting, leaf chlorosis, and necrosis [24,25]. Low temperatures were found to significantly reduce the number of ears per plant, as well as the number of grains per ears in wheat, leading to a decrease in crop quality [26,27]. By developing defense mechanisms to survive under low-temperature conditions, plants have increased their tolerance to stress [20,28]. This process is achieved by molecular, biochemical, and physiological changes, starting with membrane transformation into a rigid structure [29,30]. It has been observed that the concentration of proteins and the activity of peroxidases increased under the influence of low temperature [31]. In addition, improved expression of genes and antioxidant capacity increases crop tolerance to low temperature [32] together with the activation of the biosynthesis of phenolic compounds and the prevention of their oxidation [33]. In addition, the accumulation of soluble sugars significantly contributes to the protection and stabilization of cell membranes during cold acclimatization [32]. To maintain yield stability and crop quality, and to reduce the inhibitory impact of low-temperature stresses, it is necessary to develop methods that improve plant resistance in critical growth stages [27].

The priming technique can be an important method for the production of stress-resistant plants [34,35]. The priming method represents the process of seed hydration with solutions of different osmotic potentials that trigger certain metabolic processes (protein synthesis, repair, or synthesis of new mitochondria) that initiate germination but do not allow the emergence of a radicle [36]. In addition, seed priming improves seedling rooting and growth, vigor index, and ultimately crop yield [37]. Several different approaches to priming methods are commercially applied, including halo-, osmo-, hydro-, chemo- hormo-, or biotic priming [36]. The effects depend on the potential of the agents for priming, crop types, and stress intensity [34]. It was previously confirmed that the priming method increases the wheat tolerance to high temperature [18] and waterlogging [38]. Priming improves the resistance of plants by enabling seed germination, by modulating the expression of genes involved in the gibberellic and abscisic acid synthesis or stress tolerance [17,39]. Greater resistance of primed seeds is also associated with improved activation of DNA and RNA repair processes, antioxidant capacities, and ethylene biosynthesis [39,40].

There is little information on wheat resistance to low temperatures during germination, seedling emergence, or potential modeling of wheat tolerance to given abiotic stress by priming agents. The effects of different priming agents on germination and early seedling growth of wheat under low temperature (10 °C) were evaluated in this study. With the assumption that the biochemical and physiological changes in wheat seeds, caused by priming agents, can be important for seedling tolerance to low temperatures, we aimed to identify the most prominent priming agent for wheat seeds under low-temperature conditions. Therefore, the specific objectives were to investigate the ability of various priming agents to improve the germination potential and early seedling growth, as well as to quantify the low-temperature stress influence on the physiological and biochemical changes of seedlings grown from unprimed compared to primed seeds.

2. Materials and Methods

2.1. Design of Experiment

The winter wheat (*Triticum aestivum* L. cv. Belija) seeds obtained commercially were stored under optimal conditions (temperature 10–15 °C, air humidity 11–13%). The preparation and priming of wheat seeds were carried out according to Kanjevac et al. [41]. The applied priming agents were for hormopriming 1 mM gibberellic acid (GA_3) or 1 mM indol-3-acetic acid (IAA), for halopriming 1% KNO_3 or 1% $MgSO_4$, for osmopriming 1% H_2O_2 or 0.01% ascorbic acid (AA), and for hydropriming distilled water (H_2O). Seeds were primed for 12 h in the appropriate solution. After priming, seeds were dried (at room temperature for the next 48 h). Unprimed seeds (untreated) were set as a control sample. Dried primed seeds, together with the untreated ones, were placed in Petri boxes with filter paper and distilled water, and placed in a climate chamber (temperature 10 °C, photoperiod 16/8 h, air humidity 60%). With a moderate-continental climate in Serbia, chosen temperature corresponds to the temperature stress in late autumn which is the seeding time for this wheat cultivar. The low-temperature stress (10 °C) is applied until the two-leaf stage of tested wheat cultivar.

2.2. Seed Moisture Content

Different priming agents can lead to changes in seed moisture content and consequently to different biochemical–physiological performances of seedlings. To exclude this possibility, the seed moisture content was evaluated. The moisture content in wheat seeds was determined according to the oven method using a low constant temperature (101–105 °C) for 17 h [42]. Seed moisture content (SMC) was estimated for unprimed seeds before germination; for primed seeds after 12 h of different priming agents' application, as well as after application of priming followed by desiccation before germination.

2.3. Germination, Growth, and Vigor Index

After the appearance of a 2 mm long radicle, wheat seeds were considered germinated. The number of germinated seeds was constant after the third day of incubation, and the total percentage of germination (GP) was calculated [43]. The seedling weight vigor index (SWVI) and seedling length vigor index (SLVI) was evaluated according to Bojović et al. [44].

2.4. Total Protein Concentration

To evaluate total soluble protein content in wheat leaves, the bovine serum albumin (BSA) was used as a standard solution [45]. The obtained values were expressed according to the fresh mass of plant material (mg g^{-1} FW).

2.5. Lipid Peroxidation

The extraction and lipid peroxidation determination in wheat leaves using MDA as a marker was carried out according to Jakovljević et al. [46]. The absorbance of the supernatant was determined at 532 and 600 nm, and content of MDA was expressed as nM g^{-1} FW.

2.6. Extraction and Analysis of Enzymes

The antioxidant enzymes were extracted from wheat leaves according to Jakovljević et al. [47]. Superoxide-dismutase (SOD) activity was determined by the spectrophotometric method according to Beauchamp and Fridovich [48]. One unit of SOD is the amount of enzyme that inhibits the NBT reduction by 50%. Catalase (CAT) activity was determined by the spectrophotometric method described in Goth [49]. One unit of CAT represents the amount of enzyme that decomposes 1 μM H_2O_2 per minute under assay conditions. Ascorbate peroxidase (A-POX) and guaiacol peroxidase (G-POX) activity was determined according to Jia et al. [50] while activity of pyrogallol peroxidase (P-POX) was determined according to Kukavica et al. [51]. Activity of all investigated enzymes is expressed as U mg^{-1} of protein.

2.7. Preparation of Plant Extracts

The aboveground part of the wheat seedlings was dried for 7 days (room temperature, air humidity 55–60%). The dried aboveground parts of the wheat seedlings were crushed to obtain plant powder. For the preparation of plant extracts, dry plant powder (1 g) was extracted with methanol (20 mL) (98%); after 48 h, extracted samples were filtered and then evaporated. The dry extract dissolved in methanol at a concentration of 1 mg/mL was used for further analysis.

2.8. Total Phenolic Compounds

Total phenolic content was estimated by the spectrophotometric method according to Singleton et al. [52]. A total of 0.5 mL of plant extract, 2.5 mL Folin–Ciocalteu reagent, and 2 mL $NaHCO_3$ were incubated for 15 min at 45 °C, after which the absorbance was determined at 765 nm. The results were expressed as gallic acid equivalents (mg of GA g^{-1} of extract).

2.9. Total Flavonoids

The mixture of 1 mL of plant extract and 1 mL of $AlCl_3$ dissolved in methanol was incubated (1 h at room temperature) and then the absorbance was determined at 415 nm [53]. The results were expressed as rutin equivalents (mg of RU g^{-1} of extract).

2.10. Total Antioxidant Activity

Serial dilutions of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) with methanol were made from 1 mg/mL plant extracts to obtain concentrations from 500 to 0.97 µg mL^{-1}. The diluted solutions were mixed with the DPPH [54]. After incubation (30 min, room temperature, dark), the absorbance was determined (at 517 nm). The results were expressed as a percentage of DPPH radical inhibition, based on differences in the absorbance of plant extract samples and control samples (samples without plant extract solutions).

2.11. Quantification of Phenolic Acids and Flavonoids

2.11.1. Chemicals

Gallic acid, 4-hydroxy benzoic acid (4-HBA), 3.4-dihydroxy benzoic acid (3.4-DHBA), 3.5-dihydroxy benzoic acid (3.5-DHBA), catechin, chlorogenic acid, syringic acid, caffeic acid, epicatechin, p-coumaric acid, sinapic acid, ferulic acid, naringin, rutin, myricetin, quercetin, naringenin, apigenin and crysin were from Sigma-Aldrich (Steineheim, Germany). Formic acid and acetonitrile (HPLC grade) were purchased from J.T. Baker (Gliwice, Poland). Water was obtained from a Millipore Milli-Q system (Watford, UK).

2.11.2. Instruments

To determine the individual phenolic acids and flavonoids, the HPLC system (Shimadzu, Kyoto, Japan) consisted of degasser DGU-20A3, analytical pumps LC-20AT, manual injector 7125 and SPD M20A diode array detector, and CBM-20A system controller. A reversed-phase Hypersil GOLD aQ (150 × 4.6 mm, particle size 5 µm) column (Bellefonte, PA, USA) was used for separation. The chromatographic data were processed using LC Solution computer software (Shimadzu).

2.12. Statistical Analysis

The results were obtained with the SPSS for Windows (version 21) and expressed as the mean value of at least five repetitions ± standard error (SE) and presented in tables or figures. Data were analyzed using the ANOVA test ($p \leq 0.05$). To determine the existence of differences between individual treatments, Tukey's tests were used ($p \leq 0.05$).

3. Results

3.1. Seed Moisture Content

Seed moisture content significantly affects physiological processes in seed. Based on this, seed moisture content was measured to confirm that the selected priming agents did not cause different seed moisture content after priming. The wheat seed moisture content measured immediately after priming with different priming agents ranged from 32.34 to 33.33%, without significant differences among the applied agents (Figure 1). Additionally, it was shown that the primed seed moisture content measured after desiccation was similar, while the control unprimed seed moisture content was significantly lower and amounted to 9.5%.

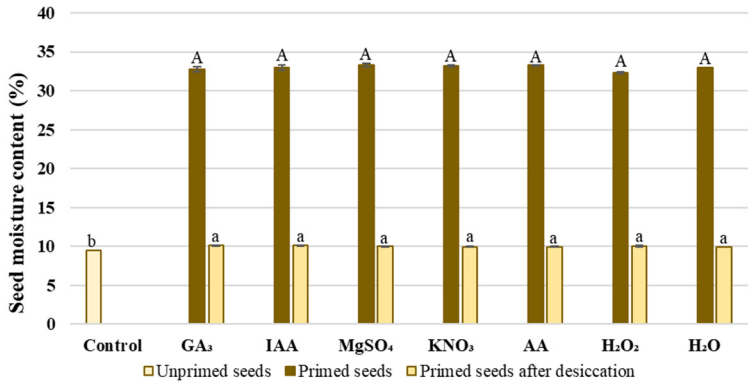

Figure 1. The moisture content in unprimed wheat seeds, in primed seeds immediately after priming and in primed seeds after desiccation before germination; the values represent the means of five replicates ± standard error. Different small letters above bars indicates significant differences between unprimed seeds and primed seeds after desiccation. Same capital letters indicate significant differences after application of different priming agents using Tukey test ($p \leq 0.05$).

3.2. Germination and Growth Characteristic

The low temperature did not affect the germination of wheat seeds since high germination percentage were recorded (>98%) in the shortest period (within 3 days) without significant differences between primed and unprimed seeds (Figure 2).

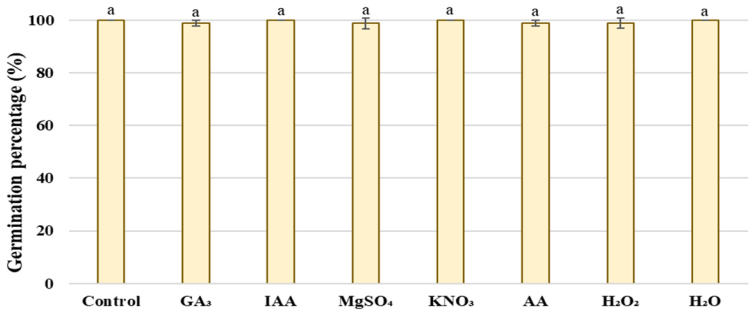

Figure 2. Effect of different priming treatments on germination percentage (GP) (%) of wheat. Same letters above bars indicates that differences were not significant ($p > 0.05$) using Tukey test.

The application of priming had a significant impact on the monitored growth characteristics (Table 1). In general, applied agents had a stimulating effect on shoot and root growth compared to the unprimed seeds. The priming treatment with GA_3 stands out as the treatment with the dominant effect (15.90 cm). The highest value for root length was recorded in the treatment with AA (10.71 cm) while priming treatment with KNO_3 had the most prominent effect on fresh (146.00 mg) and dry mass (25.50 mg). Observed through the values obtained for SLVI (Table 1), the priming treatment with GA_3 stands out as the treatment with the most prominent impact (SLVI = 2572.13). In the case of the seedling

weight vigor index (Table 1), the most favorable impact was recorded after treatment with KNO_3 (SLWI = 14.60).

Table 1. Effect of different priming treatments on shoot and root length (cm), fresh and dry weight (mg), seedling weight vigor index (SWVI), and seedling length vigor index (SWVI) of wheat.

Treatments	Root Length	Shoot Length	Fresh Weitgh	Dry Weitgh	SLVI	SWVI
Control	8.39 ± 0.23^{c}	9.43 ± 0.12^{f}	99.00 ± 0.00^{e}	19.30 ± 0.00^{c}	1728.00 ± 0.00^{e}	9.90 ± 0.00^{h}
GA_3	10.11 ± 0.20^{ab}	15.90 ± 0.23^{a}	126.00 ± 0.00^{bc}	22.10 ± 0.00^{b}	2572.13 ± 28.87^{a}	12.46 ± 0.14^{cd}
IAA	9.56 ± 0.27^{abc}	13.70 ± 0.27^{b}	135.00 ± 0.00^{ab}	20.60 ± 0.00^{bc}	2326.00 ± 0.00^{b}	13.50 ± 0.00^{b}
$MgSO_4$	10.41 ± 0.37^{a}	11.80 ± 0.21^{c}	124.00 ± 0.00^{bcd}	21.40 ± 0.00^{bc}	2194.57 ± 26.43^{c}	12.25 ± 0.15^{de}
KNO_3	9.89 ± 0.31^{ab}	11.79 ± 0.15^{c}	146.00 ± 0.00^{a}	25.50 ± 0.00^{a}	2168.00 ± 0.00^{c}	14.60 ± 0.00^{a}
AA	10.71 ± 0.26^{a}	11.17 ± 0.21^{cd}	129.00 ± 0.00^{bc}	21.50 ± 0.00^{bc}	2162.84 ± 25.16^{c}	12.75 ± 0.15^{c}
H_2O_2	8.61 ± 0.31^{c}	10.15 ± 0.25^{ef}	112.00 ± 0.00^{de}	19.30 ± 0.00^{c}	1854.43 ± 24.57^{e}	11.07 ± 0.13^{f}
H_2O	9.04 ± 0.26^{bc}	10.55 ± 0.15^{de}	119.00 ± 0.00^{cd}	21.70 ± 0.00^{b}	1959.00 ± 0.00^{d}	11.90 ± 0.00^{e}

Note: Different letters indicates that differences were not significant ($p > 0.05$) using Tukey.

3.3. Total Soluble Proteins

In the conditions of low-temperature stress, in seedlings that were previously primed, a significant effect on the synthesis of total soluble proteins was determined compared to unprimed seedlings (Table 2). The measured protein concentrations in the leaves of wheat seedlings ranged from 98.51 to 172.96 mg g^{-1} FW, and the best effects were achieved after treatment with $MgSO_4$ and AA.

Table 2. Effect of different priming treatments on total soluble protein concentration (mg g^{-1} FW) and malondialdehyde (MDA) content (nM g^{-1} FW) in leaves of wheat seedlings.

Treatments	Total Soluble Proteins	MDA
Control	98.51 ± 2.19^{d}	7.84 ± 0.06^{c}
GA_3	137.40 ± 5.93^{bc}	4.55 ± 0.17^{ab}
IAA	156.07 ± 1.39^{ab}	4.35 ± 0.39^{a}
$MgSO_4$	172.96 ± 7.15^{a}	4.94 ± 0.06^{ab}
KNO_3	119.84 ± 1.18^{cd}	5.03 ± 0.34^{ab}
AA	165.40 ± 4.73^{a}	5.42 ± 0.11^{b}
H_2O_2	128.96 ± 6.94^{c}	5.03 ± 0.11^{ab}
H_2O	136.07 ± 8.67^{bc}	4.65 ± 0.00^{ab}

Note: Different letters indicates that differences were not significant ($p > 0.05$) using Tukey.

3.4. Malondialdehyde Content (MDA)

The accumulation of MDA indicates damage of cells and formation of ROS under stressful conditions. In this regard, lower values of MDA content under stressful conditions may represent the tolerance capacity of plants. Compared to the control, the application of all priming agents significantly reduced MDA content and increased cell membrane stability (Table 2) with the most prominent effect achieved by hormopriming treatments (GA_3 and IAA).

3.5. Antioxidant Enzymes Activity

The pre-sowing effect on the antioxidant enzyme activity depended on the applied priming agents (Figures 3 and 4). Hormopriming caused a decrease in SOD and A-POX activity, and a significant increase in P-POX, G-POX, and CAT. SOD activities remained unaffected after seed halopriming with KNO_3 compared to the control, while a stimulatory effect of this treatment on the activity of G-POX, P-POX, and CAT was noticed. Halopriming treatments differed in their effectiveness, since $MgSO_4$ had a positive effect on CAT, while all other tested enzymes showed lower activity compared to the control. Osmopriming with H_2O_2 caused a significant decrease in SOD, A-POX, and CAT activity, while AA caused a significant decrease in SOD, A-POX, and G-POX. Hydropriming caused an increase in CAT activity.

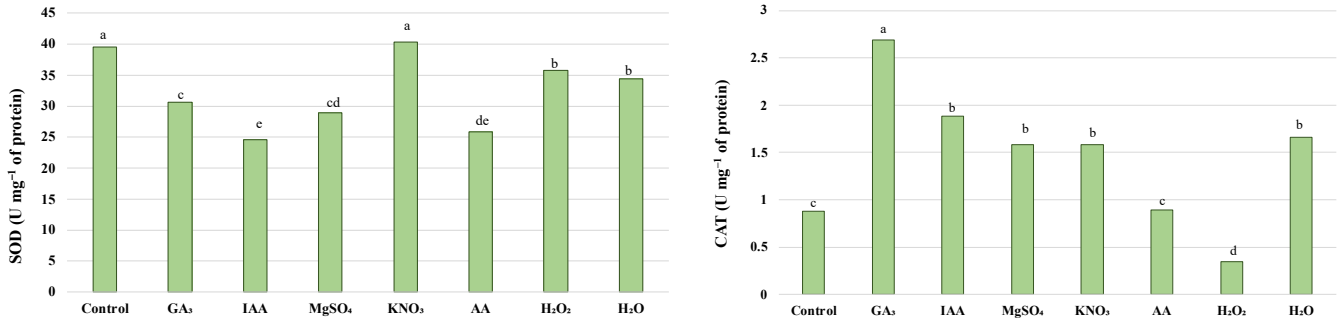

Figure 3. Activity of SOD and CAT in leaves of wheat seedlings after different priming treatments. Different letters above bars indicates that differences were not significant ($p > 0.05$) using Tukey.

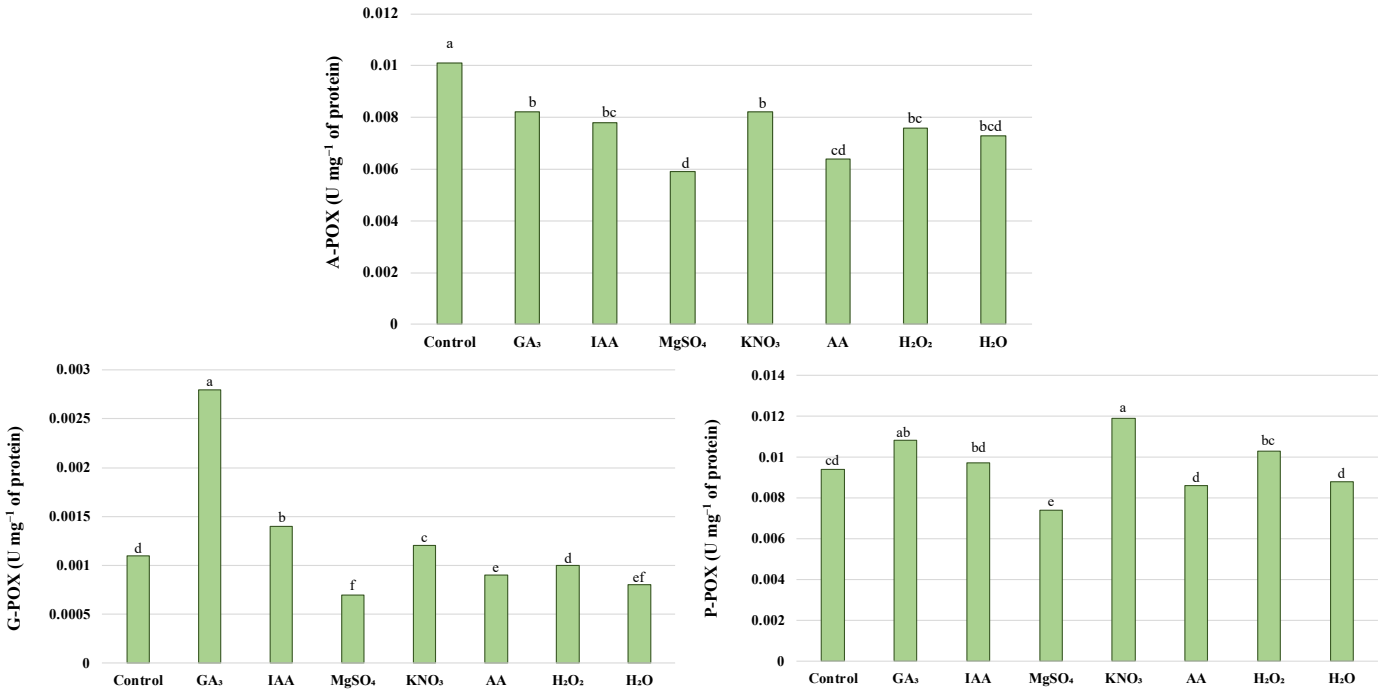

Figure 4. Activity of A-POX, G-POX, and P-POX in leaves of wheat seedlings after different priming treatments. Different letters above bars indicates that differences were not significant ($p > 0.05$) using Tukey.

3.6. Total Phenolic Content and Concentration of Flavonoids

The measured values for phenolic compounds (Table 3) ranged from 19.28 to 22.50 mg of GA g^{-1} of extract. The highest values were recorded after treatment with KNO_3 (22.50 mg of GA g^{-1} of extract) and AA (21.17 mg of GA g^{-1} of extract), without significant differences between treatments.

Table 3. Total phenolic content (mg of GA g^{-1} of extract) and concentration of flavonoids (mg of RU g^{-1} of extract) after different priming treatments in the aboveground part of wheat seedlings.

Treatments	Total Phenolic Content	Total Flavonoids
Control	19.28 ± 0.17^{b}	25.39 ± 0.02^{e}
GA_3	20.51 ± 0.30^{b}	26.45 ± 0.02^{bcd}
IAA	19.80 ± 0.43^{b}	26.41 ± 0.12^{cd}
$MgSO_4$	20.13 ± 0.48^{b}	26.35 ± 0.03^{d}
KNO_3	22.50 ± 0.65^{a}	26.61 ± 0.02^{acd}
AA	21.17 ± 0.34^{ab}	26.74 ± 0.05^{ab}
H_2O_2	19.23 ± 0.14^{b}	26.88 ± 0.03^{a}
H_2O	20.84 ± 0.42^{ab}	26.66 ± 0.10^{abc}

Note: Different letters indicates that differences were not significant ($p > 0.05$) using Tukey.

The measured concentrations of the flavonoid concentration ranged from 25.39 to 26.88 mg of RU g^{-1} of extract, whereby all priming treatments significantly increased the concentration of flavonoids. The most favorable effect was after treatment with H_2O_2 and AA.

3.7. Antioxidant Activity

The neutralization of free DPPH radicals by plant extracts, depending on the applied treatments, is shown in Table 4. In general, wheat seedlings, obtained from the primed seed, had a higher antioxidant capacity compared to the unprimed seeds, whose activity directly depended on the concentration of the extract. The most significant influence on the inhibition of free DPPH radicals was recorded in plant extracts obtained from seeds primed with KNO_3, followed by plants obtained from seeds primed with H_2O and AA.

Table 4. DPPH inhibition (%) in the aboveground part of wheat seedlings after different priming treatments.

Extract Concentration (μg mL^{-1})	Treatments							
	Control	GA_3	IAA	$MgSO_4$	KNO_3	AA	H_2O_2	H_2O
500	51.23 ± 0.24^{d}	45.90 ± 0.83^{e}	51.43 ± 0.12^{cd}	52.25 ± 0.95^{cd}	61.89 ± 0.71^{a}	56.66 ± 1.01^{b}	54.71 ± 0.47^{bc}	57.17 ± 0.47^{b}
250	29.82 ± 0.65^{e}	29.51 ± 0.47^{e}	31.35 ± 0.24^{de}	31.97 ± 0.35^{d}	39.04 ± 0.53^{a}	34.12 ± 0.30^{bc}	33.09 ± 0.4^{cd}	35.45 ± 0.36^{b}
125	23.67 ± 0.18^{d}	23.26 ± 0.10^{d}	23.87 ± 0.18^{d}	23.97 ± 0.12^{cd}	26.74 ± 0.53^{a}	24.08 ± 0.10^{cd}	25.10 ± 0.10^{bc}	25.72 ± 0.30^{ab}
62.5	20.59 ± 0.41^{b}	20.90 ± 0.12^{b}	21.11 ± 0.12^{b}	21.62 ± 0.30^{ab}	22.54 ± 0.12^{a}	21.72 ± 0.24^{ab}	21.41 ± 0.30^{ab}	21.00 ± 0.41^{b}
31.25	18.85 ± 0.71^{a}	19.26 ± 0.12^{a}	19.98 ± 0.10^{a}	19.98 ± 0.18^{a}	20.29 ± 0.12^{a}	19.88 ± 0.35^{a}	19.57 ± 0.30^{a}	19.47 ± 0.35^{a}
15.62	17.32 ± 0.89^{a}	18.75 ± 0.18^{a}	19.26 ± 0.12^{a}	19.16 ± 0.30^{a}	19.16 ± 0.30^{a}	18.65 ± 0.24^{a}	18.34 ± 0.18^{a}	18.44 ± 0.47^{a}
7.81	17.11 ± 0.89^{a}	18.55 ± 0.18^{a}	19.06 ± 0.12^{a}	18.65 ± 0.24^{a}	18.55 ± 0.30^{a}	18.44 ± 0.24^{a}	18.03 ± 0.24^{a}	17.93 ± 0.53^{a}
3.9	16.60 ± 0.71^{b}	18.24 ± 0.24^{ab}	18.85 ± 0.12^{a}	18.44 ± 0.24^{a}	18.34 ± 0.30^{ab}	18.24 ± 0.24^{ab}	17.83 ± 0.24^{ab}	17.73 ± 0.53^{ab}
1.9	16.29 ± 0.77^{b}	18.03 ± 0.24^{ab}	18.55 ± 0.10^{a}	18.24 ± 0.24^{a}	18.14 ± 0.30^{ab}	18.03 ± 0.24^{ab}	17.62 ± 0.24^{ab}	17.32 ± 0.53^{ab}
0.97	15.98 ± 0.83^{b}	17.52 ± 0.18^{ab}	17.21 ± 0.12^{ab}	17.62 ± 0.24^{ab}	17.93 ± 0.30^{a}	17.73 ± 0.18^{ab}	17.42 ± 0.24^{ab}	17.11 ± 0.53^{ab}

Note: Different letters indicates that differences were not significant ($p > 0.05$) using Tukey.

3.8. Concentration of Phenolic Acids and Flavonoids

HPLC analysis confirmed that the application of the priming method caused significant changes in the concentration of both individual phenolic acids and flavonoids in wheat leaves (Table 5). In seedlings grown from unprimed seeds, the most abundant among phenolic acids were ferulic acid (7.06 μg of g^{-1} of dry extract), catechin (6.80 μg of g^{-1} of dry extract), and caffeic acid (1.11 μg of g^{-1} of dry extract). The concentration of most abundant phenolic acid in control (particularly ferulic acid and caffeic acid) decreased when plants were grown from primed seeds; however, synthesis of other phenolic acids increased, and the particular response depended on the applied priming agent. In general, except for ferulic and caffeic acid, seed priming caused an increase in the concentration of all detected phenolic acids under low-temperature conditions. Most significant effects were achieved for gallic acid, 4-HBA, and chlorogenic acid after hormopriming with GA_3, and for gallic acid, sinapic acid, and catechin after hydropriming. In the case of flavonoids, in control plants, naringin (1.72 μg of g^{-1} of dry extract) and rutin (1.20 μg of g^{-1} of dry extract)

were the most abundant. The stimulatory effect of the seed priming on flavonoid synthesis was recorded since an increase in the concentration of all detected flavonoids under low-temperature conditions can be seen compared to the control. After seed priming with GA_3 concentration of naringin and quercetin was four-times higher, while hydropriming caused a 2.5-times increased synthesis of rutin.

Table 5. The qualitative and quantitative composition of phenolic acids and flavonoids (μg of g^{-1} of dry extract) of wheat seedlings after different priming treatments.

Compound	Treatments							
	Control	GA_3	IAA	$MgSO_4$	KNO_3	AA	H_2O_2	H_2O
Gallic acid	0.32 ± 0.05^{d}	0.46 ± 0.05^{ab}	0.23 ± 0.02^{c}	0.29 ± 0.01^{cd}	0.46 ± 0.03^{abd}	0.34 ± 0.04^{bc}	0.27 ± 0.00^{c}	0.57 ± 0.03^{a}
3.4-DHBA	[#] nd	nd	nd	nd	nd	nd	nd	nd
3.5-DHBA	nd	nd	nd	nd	nd	nd	nd	nd
4-HBA	0.06 ± 0.01^{d}	0.77 ± 0.01^{a}	0.42 ± 0.07^{b}	0.27 ± 0.05^{bc}	0.32 ± 0.03^{bc}	0.20 ± 0.01^{cd}	0.25 ± 0.05^{bc}	0.38 ± 0.01^{b}
Catechin	6.80 ± 0.33^{bc}	6.59 ± 0.62^{c}	8.46 ± 0.80^{bc}	6.52 ± 0.16^{c}	8.26 ± 0.45^{bc}	8.74 ± 0.18^{b}	8.53 ± 0.13^{bc}	10.9 ± 0.24^{a}
Chlorogenic acid	0.34 ± 0.12^{b}	3.77 ± 0.56^{a}	2.95 ± 0.32^{a}	3.98 ± 0.19^{a}	3.56 ± 0.22^{a}	0.39 ± 0.14^{b}	0.23 ± 0.01^{b}	0.31 ± 0.03^{b}
Caffeic acid	1.11 ± 0.10^{ab}	0.42 ± 0.10^{c}	1.31 ± 0.18^{a}	0.43 ± 0.08^{c}	0.24 ± 0.10^{c}	0.93 ± 0.01^{ab}	0.70 ± 0.03^{bc}	1.19 ± 0.09^{a}
Syringic acid	nd	nd	nd	nd	nd	nd	nd	nd
Epicatechin	0.37 ± 0.10^{acd}	0.54 ± 0.03^{ab}	0.51 ± 0.14^{ac}	0.27 ± 0.07^{bcd}	0.09 ± 0.03^{d}	0.18 ± 0.06^{cd}	0.38 ± 0.02^{acd}	0.64 ± 0.01^{a}
p-Coumaric acid	0.69 ± 0.07^{ab}	0.69 ± 0.01^{ab}	0.26 ± 0.10^{c}	0.55 ± 0.05^{b}	0.63 ± 0.06^{ab}	0.84 ± 0.02^{a}	0.69 ± 0.02^{ab}	0.85 ± 0.04^{a}
Ferulic acid	7.06 ± 0.33^{a}	1.41 ± 0.11^{e}	0.31 ± 0.19^{f}	4.30 ± 0.13^{d}	6.57 ± 0.1^{ab}	6.12 ± 0.02^{bc}	5.53 ± 0.11^{c}	6.55 ± 0.19^{ab}
Sinapic acid	0.83 ± 0.02^{cd}	0.33 ± 0.01^{d}	0.37 ± 0.23^{d}	1.07 ± 0.22^{bc}	1.37 ± 0.18^{abc}	1.69 ± 0.06^{ab}	1.33 ± 0.06^{abc}	1.77 ± 0.03^{a}
Rutin	1.20 ± 0.03^{bcd}	1.03 ± 0.10^{cd}	0.37 ± 0.06^{d}	2.35 ± 0.42^{ab}	2.73 ± 0.50^{a}	2.25 ± 0.19^{abc}	1.99 ± 0.15^{abc}	3.10 ± 0.08^{a}
Naringin	1.72 ± 0.38^{d}	5.41 ± 0.34^{a}	3.67 ± 0.08^{b}	2.23 ± 0.07^{cd}	2.68 ± 0.15^{c}	2.82 ± 0.05^{bc}	2.56 ± 0.05^{cd}	2.90 ± 0.05^{bc}
Myricetin	nd	nd	nd	nd	nd	nd	nd	nd
Quercetin	0.17 ± 0.02^{d}	0.65 ± 0.01^{a}	0.18 ± 0.02^{d}	0.26 ± 0.02^{cd}	0.27 ± 0.02^{cd}	0.38 ± 0.05^{bc}	0.42 ± 0.03^{b}	0.47 ± 0.01^{b}
Naringenin	nd	nd	nd	nd	nd	nd	nd	nd
Apigenin	nd	nd	nd	nd	nd	nd	nd	nd
Crysin	nd	nd	nd	nd	nd	nd	nd	nd

[#] nd—not detected. Different letters indicates that differences were not significant ($p > 0.05$) using Tukey.

4. Discussion

Stress caused by low temperatures seriously affects the distribution of species in nature, plant life cycle, as well as agricultural productivity [55,56]. The resistance of plants to temperature stress (low or high temperature), depends on plant species strength, and duration of stress [57]. Fluctuations of temperature can alter cellular homeostasis negatively affecting biochemical and physiological processes [58]. Suboptimal temperatures are the main limiting factor leading to crop reduction or complete failure [59] and the serious negative impact of low temperatures on wheat production was recorded [60]. In recent years, the priming method has been an effective technique for increasing crop tolerance to temperature stresses [61–63], which is achieved by modulating metabolic activities before germination, such as genetic and structural repair/protection, increased protein accumulation, activation of osmotic enzymes and activation of antioxidant capacity [39,64].

At the sowing stage, low-temperature stress leads directly to wheat crop failure by inhibiting/reducing germination and plant growth. Indirect low-temperature stress reduces plant density in the field [65]. Furthermore, temperature stress, caused by low temperature, reduces root length and biomass, thereby inhibiting normal nutrient and water uptake, resulting in a reduction in aboveground mass [61]. Similarly, an inhibitory effect on the reduction of size and area of leaves, as well as on shoot biomass was recorded [66]. In the reproductive phase, low temperature leads to significant functional and structural deformities, exemplified by a reduction in the number of ears, grains per ear, and ultimately yield [67]. The growth, organogenesis, and metabolic reactions in plants are also inhibited by low temperature, therefore increasing crop tolerance to cold is considered a key solution to increase optimization of their production and nutritional quality [68]. To the best of our knowledge, our study is the first report on the germination of a tested wheat cultivar (*T. aestivum* L. cv. Belija) under low-temperature conditions. Based on the obtained data, the germination of this cultivar can be considered independent of low temperature. Although there are no differences in germination, priming significantly contributed to growth as shown by SLVI and SWVI, so the application of GA_3 and KNO_3 as priming can contribute

to the morphology of the tested wheat cultivar under conditions of temperature stress. GA_3, as a growth regulator, induces various physiological responses in plants, thereby stimulating the germination and growth [69]. Application of GA_3 induces plant development by increasing the content of amino acids in the embryo as well as by stimulating hydrolytic enzymes for breaking down starch necessary for embryo development [70]. In addition, gibberellic acid has a dynamic potential in increasing crop tolerance to abiotic stresses [71,72]. The beneficial effects of KNO_3 as a priming agent may be associated with increased imbibition, stimulation of cell division and elongation, synthesis and/or repair of genetic material, the activation of enzymes for mobilization, including amylases, dehydrogenases, and phosphatases [73].

Under temperature stress, plants produce ROS which consequently leads to the creation of oxidative stress [74]. Oxidative stress leads to the oxidation of macromolecules such as proteins, lipids, and nucleic acids, thus causing not only cell damage but even complete cell destruction (death) [75]. The resulting changes are highly toxic but can be rapidly detoxified by various plant cellular mechanisms [76]. Seed priming can stimulate plants to eliminate oxidative stress, whose regulatory role is related to the neutralization of ROS, improvement of antioxidant defense mechanisms, accumulation of metabolites such as soluble proteins, or increased expression of genes responsible for the secondary metabolism [77,78]. In our study, a significant accumulation of proteins under low-temperature conditions was observed after the priming of wheat seeds, compared to unprimed. As a defense strategy, a high concentration of these proteins may enhance the ability of cells to retain water and can provide repair and structural protection of cell membranes against damage caused by abiotic stress [79]. A significant stimulating effect of priming treatment on the content of total soluble proteins under stressful conditions was recorded by Ashraf et al. [80]. In addition to higher protein content in primed wheat seedlings, seedlings obtained from primed seeds were also reported to produce lower MDA content. This is probably due to reduced free radical damage and greater membrane protection, thus preventing unsaturated fatty acid damage and electrolyte leakage, which is in agreement with Moori et al. [81]. According to Nawaz et al. [82], the lower MDA concentration can be attributed to the reorganization of cell membranes that improves membrane repair and antioxidant systems after priming, which synergistically eliminates oxidative damage. Superoxide dismutase rapidly catalyzes the dismutation of superoxide radicals into hydrogen peroxide, later converted into H_2O by the activity of catalase and peroxidases [83]. In general, higher activities of antioxidant enzymes and reduced levels of MDA in membranes of primed wheat seedlings indicate the vital role of priming in improving antioxidant capacity under low-temperature conditions. Taking all priming agents into account, the dominant protective effect is achieved with KNO_3 which unaffected SOD and stimulated the activity of G-POX, P-POX, and CAT. This can indicate the significant role of KNO_3 as a pre-sowing treatment in improving the defense potential of wheat seedlings under low temperatures. Zhang et al. [84] suggest that the implementation of priming promotes expression of genes and achieves a stimulatory plant response to a given stress. Similarly, Liu et al. [85] showed that halopriming can improve wheat cold tolerance through the regulation of ROS metabolism, effectively maintaining membrane integrity under cold stress. This is in agreement with previous studies where it was found that the application of priming reduced the harmful effects of oxidative stress in wheat by increasing the activity of antioxidant enzymes [23,86]. Higher activities of antioxidant enzymes in primed seedlings were recorded earlier under different abiotic stress conditions [72,87,88].

Temperature stress leads to the increased production of secondary metabolites [89] and the higher content of phenolic compounds can be regarded as an adaptive mechanism in plant protection from oxidative damage, which consequently increases stress tolerance [90]. Phenolic compounds, including flavonoids, have a significant function in the elimination of ROS and plant defense responses to environmental limitations [91,92]. In this study, the application of pre-sowing treatments in the form of priming, low-temperature stress, and their interaction were significant for increasing the content of phenolics and flavonoids,

and for antioxidant activity. Priming with KNO_3, H_2O, and AA increased the total phenolic content and concentration of flavonoids, which consequently improved the antioxidant capacity to remove free DPPH radicals in seedlings treated with these agents. Ashraf et al. [93] confirmed that priming can increase the concentration of phenolic compounds that are significant initiators of antioxidant potential. Similar notices were recorded by Hatami et al. [94].

The profile of wheat phenolic compounds was previously presented by Wang et al. [95] and Gupta et al. [3]. Frequently identified phenolic acids were syringic, p-hydroxybenzoic, ρ-coumaric, vanillic, gallic, ferulic, caffeic, and sinapic acids. Frequently identified flavonoids were quercetin, apigenin, luteolin, tricin, and chrysoeriol. A similar compound profile was identified in our study. Kowalska et al. [96] confirmed that phenolic compounds present in wheat show great variability in concentration depending on environmental factors. Exposure to low temperatures affects a wide range of phenolics in wheat seedlings. According to Ma et al. [97], stimulation of the synthesis of phenolic acids can be one of the key factors regarding the resistance of wheat. An increase in the phenolic acid content of wheat grain during temperature stress was observed by Shamloo et al. [98]. HPLC results of our study suggest that the priming method had a prominent effect on the production of all detected phenolic compounds (except for ferulic acid) under low-temperature stress compared to seedlings obtained from unprimed seeds, with the dominant effect observed after priming with GA_3 and H_2O. Considering the importance of phenolic acids in the abiotic stress mitigation [99], it could be presumed that seed priming can reduce the negative effects of cold stress on the biochemistry and physiology of wheat seedlings through the induction of phenolic acid and flavonoid synthesis (including gallic acid, chlorogenic acid, 4-HBA, catechin, epicatechin, caffeic acid, p-coumaric acid, sinapic acid, naringin, rutin, and quercetin). Similar observations were recorded by Mnafgui et al. [100] and Mahmood et al. [101]. Bearing in mind that phenolic acids and flavonoids are of great importance in the prevention or treatment of various diseases due to their antioxidant, antiangiogenic, antiproliferative, anticarcinogenic, and proapoptotic properties [102], the application of priming method on wheat seeds for the production of bioactive secondary metabolites can have potential practical importance.

5. Conclusions

It is shown that the implementation of priming agents can be an effective pre-sowing treatment for wheat plants to improve their ability to cope with adverse effects caused by low temperatures. In short, the application of priming significantly stimulated the growth of wheat seedlings. The accumulation of soluble proteins increased, and the MDA content decreased compared to seedlings obtained from unprimed seeds. The results confirmed that seed priming strengthens the antioxidant capacity of wheat under conditions of low temperature through the activity of antioxidant enzymes, the secondary metabolites' content (phenolic acids and flavonoids), and total antioxidant capacity. Overall, this study highlights the potential implication of priming with KNO_3 and GA_3 for enhancing the defense mechanism of wheat seedlings under low-temperature conditions. Future research should consist of field experiments in multiple years and locations to further verify the effect of priming under limited environmental conditions.

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