Chicken Feather Protein Hydrolysate Ameliorates Cold Resistance by Upregulating Physiologic and Biochemical Responses of Wheat (*Triticum Aestivum* L.)

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Research Article

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Abstract

Chicken feathers are one of the waste products of the poultry industry and millions of tons of waste feathers produced by poultry processing plants are increasingly causing a serious waste problem in almost every country. We produced a chicken feather protein hydrolysate (CFPH) and investigated its effect on the low-temperature stress response of two wheat cultivars (*Triticum aestivum* L., cvs. Altındane and Bezostaya). CFPH obtained by an alkaline process contained 19 proteinogenic and 3 non-proteinogenic amino acids, as well as helpful salts for plants. The aqueous solution of CFPH (0.1%) was applied to the seedling leaves before cold stress and then the seedlings (treated and untreated) were transferred to cold conditions (5/2 ^{o}C, day/night) for 3 days. The CFPH treatment increased Rubisco expression, and the contents of photosynthetic pigment, soluble sugar, and free proline while decreasing phenolic content in the seedling leaves of both cultivars under cold stress. Moreover, CFPH treatment suppressed the levels of reactive oxygen species (like $O_2.^-$ and H_2O_2), and lipid peroxidation (as malondialdehyde) whose contents were increased by the cold alone. The cold alone stimulated SOD, CAT, GPX, APX, and GR activities but CFPH treatment further stimulated all the antioxidant enzyme activities in both cultivars, except APX and GR activities in Altındane. This finding of the activities in both cultivars was generally supported by the accumulation in the isoenzyme profiles of the same enzymes on native PAGE. In addition, CFPH treatment caused an improvement in ASA/DHA and GSH/GSSG ratios, and plant growth parameters that are impaired by the cold. We propose that CFPH derived from waste chicken feathers not only supports the growth and development of wheat under normal conditions as a biostimulant but also ameliorates its tolerance to low-temperature stress.

Introduction

Today, the productivity in agricultural output mostly relies on synthetic fertilizers to support the mineral nutrient requirement of plants (Colla et al. 2015). Despite increasing demand for plant productivity, its decline due to intense environmental stresses such as salinity, drought, temperature, and organic and inorganic contaminants has led to the quest for new approaches that are more cost-effective, sustainable, and compatible with the natural environment. In recent years, it has been suggested as an alternative approach to use the biostimulants containing protein hydrolyzate (PH) to increase plant performance in crop cultivation, instead of synthetic fertilizers that disrupt the soil ecosystem and human health. (Ertani et al. 2009; Colla et al. 2015; Popko et al. 2015; Genç and Atıcı 2019). Low molecular size peptides and free amino acids in PHs are considered to be one of the most advantageous approaches for biostimulant treatments because of their easy uptake by almost all plant tissues (Morales-Payan et al. 2003; Cerdán et al. 2009; Colla et al. 2015). The foliar treatment of fish protein hydrolysate (FPH), for instance, improved plant productivity by stimulating seed performance, plant growth, antioxidant enzyme activity, and free proline and phenolic compound contents in pea seedlings (Andarwulan and Shetty 1999). PHs could also mitigate plant tissue injuries under oxidative, osmotic, and drought stresses (Feitosa de Vasconcelos et al. 2009), and serve as a chelating agent of cationic nutrient minerals in plants (Lucini et al. 2015). For example, Feitosa de Vasconcelos et al. (2009) propounded that a

biostimulant including free amino acids ameliorated the activity of antioxidant enzymes in certain crops under drought stress. Similarly, PH derived from an animal source induced growth and development in certain plants grown under cold stress (Botta 2013).

Chicken feathers are a large number of waste products in the poultry industry, and their excessive accumulation leads to environmental pollution (Taşkın and Kurbanoğlu 2011; Taşkın et al. 2012; Mézes et al. 2015). Since the keratin content of chicken feathers takes a long time to decompose in nature, physical, chemical and/or biological pretreatment is needed in practical uses, which must be adjusted according to the intended use (Mézes et al. 2015). Some researchers pointed out that chicken feathers could be evaluated as a significant PH resource. Chicken feathers comprise important mineral elements such as Ca, P and Mg, and protein keratin containing high amounts of glutamate, cysteine, glycine, arginine, and phenylalanine (Taşkın and Kurbanoğlu 2011). In several studies, therefore, chicken feather protein hydrolysate (CFPH) was evaluated as a general substrate for bacterial and fungal growth (Taşkın and Kurbanoğlu 2011; Taşkın et al. 2012), and also both an alternative organic fertilizer and a biocontrol agent for crop cultivation (Gurav and Jadhav 2013; Mézes et al. 2015; Genç and Atıcı 2019). CFPH, in addition, exhibited a stimulating effect on seed germination and seedling growth of ryegrass, rapeseed, and maize (Gousterova et al. 2012; Popko et al. 2015). Its foliar application raised the number of fingers per hand, bunch weight, and hands per bunch in banana plants (Gurav and Jadhav 2013).

Crop plants encounter numerous undesirable environmental conditions, including low-temperature stress, during their cultivation. In cold-sensitive crops, low temperature causes a variety of disorders in physiological and biochemical processes including the antioxidative metabolism and photosynthesis machinery at the cellular level, resulting in a serious decline in the growth and development, and productivity of plants (Atıcı and Nalbantoglu 2003; Esim and Atıcı 2015). For instance, the uncontrollable overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl anion (.OH), and superoxide anion ($O_2.^-$) radical disrupts cellular biochemical processes in plant cells exposed to cold stress. ROS lead to directly or indirectly the denaturation of cellular proteins and the irreparable degradations in DNA, and specifically attacks polyunsaturated fatty acids in cell membranes, leading to the formation of malondialdehyde (MDA) and other lipid peroxidation products. Lipid peroxidation (LPO) disrupts the structure of polyunsaturated fatty acids including phospholipids, resulting in irregular permeability in all cell membranes and eventual cell death if its intracellular level is not kept down. Once LPO begins in a membrane, it proceeds as a chain reaction and leads to significant degradations in all cell membranes. In addition, ROS interfering with proteins lead to the formation of peptide fragments containing aldehyde and ketone groups (Stadtman and Levine 2003). Such modified protein derivatives with multiple carbonyl groups, described as advanced glycation end products (AGE), carry as highly oxidizing properties as ROS. On the other hand, the ROS-induced oxidative stress can be mitigated by enzymatic antioxidants such as superoxide dismutases (SOD), catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), guaiacol peroxidases (POX), or non-enzymatic antioxidants such as glutathione (GSH) and ascorbic acid (ASA). The antioxidant enzymes with a high catalytic activity can decrease efficaciously ROS levels in cell compartments. ASA and GSH also are directly or indirectly

efficient on ROS scavenging as co-substrates for APX and glutathione peroxidase (GPX) (Foyer and Noctor 2005). In most studies, a strong correlation between cold acclimatization and the up-regulation of the antioxidant system has been suggested to be in especially cold-resistant plants (Esim and Atıcı 2015; Tiryaki et al. 2019). However, more efforts are needed to unravel the mechanisms of plant response to low temperatures, an uncontrollable climate factor. Our previous study also showed that the foliar treatment of different CFPH doses ameliorated important physiological and biochemical parameters such as seedling biomass, the antioxidant system including non-enzymatic antioxidants, and Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase) activity while decreased ROS and LPO levels (Genç and Atıcı 2019). In this study, we hypothesized that CFPH having a high usage potential in plant cultivation as a biostimulant could be evaluated to improve the cold resistance of crops, and therefore investigated the effects of its foliar treatment in response to cold stress of wheat (*Triticum aestivum* L.). Our study is important in terms of making an important contribution to environmental health by using CFPH derived from chicken feather that is waste for the environment, as well as showing that this product can be used to increase the stress response of cultivated plants.

Materials And Methods

Preparing CFPH

The hydrolysis process on chicken feathers (CF) was realized by using a modified method employed by Taşkın and Kurbanoğlu (2011) and Taşkın et al. (2012). Briefly, chicken feathers were dried at 70 °C until a constant weight after washing with distilled water. Then, it was ground with a Waring blender until becoming a feather powder. A 100 g of CF powder was added into a 250 mL of KOH solution (2 N) in a glass tube and the tube including CF powder was subjected to hydrolysis processes for 60 h with gently stirring (150 rpm) on a shaker. The obtained alkaline extract was neutralized with H_3PO_4 (10 N) and then infiltrated by Whatman filter paper to remove the particles that are non-hydrolyzable. Then, it was dried at 80 °C until it became powder and called chicken feather protein hydrolysate (CFPH). Aqueous solutions (0.1%, w/v) of CFPH were prepared from the obtained dried powder by dissolving in sterile pure water. The final pH value of the solutions was adjusted to 6-6.5. Amino acid content determination was carried out by Düzen Norwest Laboratory (Environmental, Food, and Veterinary Health Services Training and Consulting Trade Co., Ankara, Turkey) using Varian CP-3800 gas chromatograph (Varian Inc., Palo Alto, Calif., USA) and rapid amino acid analysis kits (Phenomenex Inc., Torrance, Calif., USA).

Plant material and CFPH application

Altındane and Bezostaya varieties of wheat (*Triticum aestivum* L., cvs.) were used as plant material and seeds were obtained from Karadeniz Agricultural Research Institute and Geçit Kuşağı Agricultural Research Institute. After seed sterilization, prepared soils with sand+peat mixture (3:1) were filled into pots, and swelled seeds with distilled water were planted in equal amounts in each pot. Seedlings were grown in a climate chamber under controlled conditions (20,000 lux, 70% humidity, at 23/20 ^{o}C and 14/10 h, light-dark) and irrigated daily with an equal amount of distilled water and also once with

Hoagland nutrient solution. The aqueous solution of CFPH (0.1%, w/v) was sprayed once to the leaves of 11-day seedlings. Also, the same amount of distilled water was used for the untreated group. Twelve hours after the application, the CFPH-treated and the untreated seedlings (control) were transferred in a climate chamber at 5/2 ^{o}C (15,000 lux, 50% humidity, and 14/10 h, light-dark). After 3 days, all the seedlings were harvested to use for physiologic and biochemical experiments.

Determining plant growth, soluble sugar, and pigment content

Plant growth was determined by measuring root and shoot lengths of seedlings and then their dry weights were determined after incubation for 48 h at 70 °C. Chlorophyll and carotenoid content were detected as spectrophotometrically at fresh leaves according to the method of Lichtenthaler (1987) and were submitted as mg/g fresh tissue. Soluble sugar content was measured according to the phenol-sulphuric method (Dubois et al. 1956). Dried leaves (1 g) were powdered and 5 mL of ethanol (70%) was added to the powdered leaves. The mixture in a tube was incubated in a water bath for 45 min at 75 °C. The tubes were centrifuged for 10 min at 3500xg and obtained supernatant was diluted at a rate of 1/10 with ethanol (70%). Then, a reaction mixture (3 mL) was prepared to include 100 mL of the supernatant, 300 mL of saturated phenol, and 2.6 mL of concentrated H_2SO_4. The absorbance of the mixture was monitored as spectrophotometrically at 480 nm for pentose and 488 nm for hexoses. Data is submitted as mg/g dry tissue in comparison with a standard chart prepared by using pure fructose and glucose.

Determining Rubisco expression via Western Blot analysis

After SDS-PAGE of total soluble proteins, polypeptides were transferred in a nitrocellulose membrane (0.45 mm) using a buffer containing 13 mM Tris (pH: 7.2), methanol (10%), and 190 mM glycine for 50 min at 15 volts. The membrane was enclosed in a buffer (Tris-HCl; 20 mM, pH 7.6) including Tween-20 (0.1%), NaCl (140 mM), bovine serum albumin (3%), and powdered milk (2%), and incubated throughout a night at 4 °C. After the membrane was bathed with Tween-20 (0.1%), it was incubated in powdered milk (2%) including Tween-20 (0.1%) and the monoclonal antibody of Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39) for one hour at room conditions. Subsequently, the membrane bathed with Tween-20 (0.1%) was incubated in powdered milk (2%) including Tween-20 (0.1%) and secondary antibody for 1 hour at room conditions. Then, the membrane was bathed with Tween-20 (0.1%) and distilled water, respectively. Interacting antibodies were determined by using a chemiluminescent substrate (Genç and Atıcı 2019).

Determining ROS and lipid peroxidation level

Endogenous H_2O_2 level was measured by the method of Hu et al. (2005). Tissue (0.5 g) was homogenized in 10 mL of cold acetone and centrifuged at 5000xg for 15 min at 4 °C. One-half mL of supernatant was mixed with 0.15 mL of 5% $Ti(SO_4)_2$ and 0.3 mL of 19% NH_4OH. The mixture was centrifuged at 3000xg for 10 min at 4 °C. The obtained pellet was washed two times with cold acetone and dissolved in 3 mL of 1 M H_2SO_4. After filtering, absorbance measurement was carried out at 415 nm

versus blank. Data were expressed as ng/g fresh tissue. Superoxide anion ($O_2^{\cdot -}$) content was determined by using XTT {(2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} (Frahry and Schopfer 2001). Fresh tissue (0.5 g) was ground in a 5 mL of 500 mM XTT (pH 7.0) with or without 3.5 U/mL superoxide dismutase. Two hours later, the homogenate was centrifuged at 10000x*g* for 10 min at 4 °C. Then, 1 mL of the supernatant was mixed with 0.9 mL of 65 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride. After the mixture was incubated at 25 °C for 15 min, 1 mL of the mixture, 1 mL of 17 mM anhydrous amino benzene sulfonic acid, and 1 mL of 17 mM 1-naphthylamine were mixed and incubated at 25 °C for 20 min. A 3 mL aliquot of butyl alcohol was added to the mixture and the absorbance was measured at 530 nm. Sodium nitrite ($NaNO_2$) was used for the standard curve to calculate the content of superoxide. Lipid peroxidation level (LPO) is determined by measuring malondialdehyde (MDA) level (Heath and Packer 1968). Tissue (0.5 g) was homogenized in 5 mL of 1% TCA and centrifuged at 12000xg for 20 min. One mL of the supernatant obtained was mixed with 4 mL of 0.5% TBA (thiobarbituric acid) in 20% TCA (trichloroacetic acid). The reaction mixture was incubated for 30 min at a boiling water bath, and the reaction was terminated in an ice bath. The samples were once more centrifuged at 5000xg for 10 min. The absorbance of the supernatant was followed at 532 nm and it was corrected by subtracting non-specific absorbance at 600 nm. MDA level was expressed as nmol/g fresh tissue.

Determining non-enzymatic antioxidants

Reduced glutathione (GSH) and oxidized glutathione (GSSG) content were determined enzymatically by using the method of Griffith (1980). Fresh tissue (0.2 g) was homogenized in 2 mL of 5% meta-phosphoric acid and centrifuged at 12000×*g* for 20 min. For total GSH, 150 mL of the obtained meta-phosphoric extract was mixed in an Eppendorf tube with 1050 mL of KH_2PO_4, (50 mM, pH 7.5), 200 mL of EDTA (2.5 mM), 200 mL of DTNB (1 mM), 200 mL of GR (0.3 unit), 200 mL of NADPH (1 mM M) in a final volume of 2 mL at 25 °C. The reaction was started with the addition of 200 mL of NADPH, and the increase in absorbance at 412 nm was monitored for 3 min at 25 °C. For GSSG, 150 mL of the meta-phosphoric extract was neutralized with 96 mL of 1 M triethanolamine. Then, 8 mL of 2-vinylpyridine was added to the extract. The mixture was allowed to incubate for 60 min at 25 °C. Then, 228 mL of the derivatized extract was added to the reaction medium consisting of 0.972 mL of KH_2PO_4, (50 mM, pH 7.5), 200 mL of EDTA (2.5 mM), 200 mL of DTNB (1 mM), 200 mL of GR (0.3 unit), 200 mL of NADPH (1 mM M) in a final volume of 2 mL at 25 ^{o}C. ReaThe reactions started with the addition of 200 mL of NADPH, and the increase in absorbance at 412 nm was recorded for 3 min at 25 °C. Calibration curves were drawn using standards of GSH (1.6–80 mM) and GSSG (0.8–40 mM) prepared in 2% (w/v) meta-phosphoric acid. Reduced GSH was total GSH-GSSG. The contents of reduced (AsA) and oxidized (DHA) ascorbate were determined as described by Genç and Atıcı (2019). Briefly, a 0.2 g powdered sample in liquid nitrogen was extracted in 2 mL of 5% TCA. The homogenate was centrifuged at 12000x*g* for 20 min at 4 ^{o}C. Ten mL of 5 M NaOH was added to 400 mL of supernatant and it was equally divided into two parts. The first mixture was combined with 200 mL of KH_2PO_4 (150 mM, pH 7.4) and 200 mL of distilled water. The second mixture was combined with 200 mL of KH_2PO_4 (150 mM, pH 7.4), 100 mL of

DTT (10 mM), and 100 mL of N-ethylmaleimide (0.5%). In both mixtures were added separately to 400 mL of 10% TCA, 400 mL of 44% H_3PO_4, 400 mL of 2,2¢-dipyridyl (4% in ethanol 70%) and 150 mL of 3% $FeCl_3$. Both samples were incubated at 37 °C for 60 min and the absorbance of the samples was recorded at 525 nm. Standard curves of AsA and DHA were prepared in 5% (w/v) TCA. The first mixture gives reduced ASA and the second mixture gives total ASA. DHA is extracted from II to I (DHA= II-I). For soluble phenolic compound, fresh plant tissue (0.2 g) was homogenized in 2 mL of KH_2PO_4 (pH: 7.4), and obtained homogenate was centrifuged at 12000x*g* for 15 min at 4 °C. Total phenol content was determined using Folin-Ciocalteu reagent (FCR) and gallic acid as standard (McDonald et al. 2001). The samples of the extract (0.5 mL) were added to 2.5 mL of 10% (v/v) FCR after 6 min, 2 mL of sodium carbonate (Na_2CO_3, 7.5%) was added to the mixture. After 1 h of reaction, the absorbance of the mixture was measured at 765 nm in a spectrophotometer. Blank is formed from a like sample that does not contain extract. Total phenol content was determined with the use of an external standard curve and expressed as mg gallic acid/mg fresh weight of tissues. Soluble proline content was determined according to the method of Bates et al. (1973). Total proline content was determined with the use of an external standard curve and expressed as micromoles per gram of fresh weight.

Activity assay of enzymatic antioxidants

Fresh tissue (0.2 g) was ground in 2 mL of extraction buffer (0.1 M KH_2PO_4 buffer, pH: 7.0) containing 0.2% polyvinylpyrrolidone and 1 mM EDTA, and the homogenate was centrifuged at 12000xg for 15 min at 4 °C. Protein content was determined according to the method of Bradford (1976). A protein standard curve was generated using bovine serum albumin. Antioxidant enzyme activities were assayed according to the method of Nakano and Asada (1981). One unit of superoxide dismutase (SOD, EC 1.15.1.1) activity was defined as the amount of enzyme that inhibited 50% photoreduction of nitroblue tetrazolium chloride. The activity was expressed as EU/min/mg protein. Guaiacol peroxidase activity (GPX, EC 1.11.1.7) was assayed by determining absorbance increase at 470 nm caused by tetraguaiacol which is a product of the reaction in which guaiacol and H_2O_2 are used as substrate. One unit of GPX is defined as the amount of enzyme that increased absorbance at a rate of 0.01 within 1 min at 25 °C, and data are expressed as EU/min/mg protein. Catalase (CAT, EC 1.11. 1.6) activity is based on the measurement of the decrease in absorbance at 240 nm when CAT provides the conversion of H_2O_2 to O_2 and H_2O. One unit of CAT is determined as the amount of enzyme disrupted 1 mM H_2O_2 within 1 min at 25 °C, and data are expressed as EU/min/mg protein. For ascorbate peroxidase (APX, EC 1.11.1.11) activity, the reaction mixture (3 mL) contained 0.5 mM ascorbic acid (AsA), 2 mM H_2O_2, 0.1 mM EDTA in 50 mM KH_2PO_4 buffer (pH 7.0). One unit of APX activity is defined as the amount required to decompose 1 mM oxidized ASA/min/mg protein. Glutathione reductase (GR, EC 1.6.4.2) activity was determined by monitoring glutathione-dependent oxidation of NADPH at 340 nm. Reaction mixture included 0.2 mM NADPH, 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM oxidized glutathione (GSSG) and 100 mM Tris-HCl (pH 7.8). Data were expressed as EU/min/mg protein.

Native PAGE for isoenzyme staining of antioxidant enzymes

Native proteins were run on PAGE under non-denaturing conditions as suggested by Laemmli (1970). For SOD activity staining, the gel was incubated in 0.2 M sodium acetate buffer (pH 5) containing 30 mM H_2O_2 and 10 mM guaiacol in dark for 30 min at 37 °C, and then proteins were monitored after incubation for 30 min in 0.05 M phosphate buffer (pH 7.8) containing 1 mM EDTA (Weydert and Cullen 2010). GPX isoenzymes were monitored according to Weydert and Cullen (2010). Activity staining was realized after incubation for 30 min in 0.2 M sodium acetate buffer (pH 5.0) containing 30 mM H_2O_2 and 10 mM guaiacol. For CAT isoenzymes, the gel was incubated in 30 mM H_2O_2 for 10 min, it was stained with 2% $FeCl_3$ and 2% K_3FeCN_6 solutions (Weydert and Cullen 2010). GR staining was carried out by incubating in a reaction solution including 250 mM Tris-HCl buffer (pH 8.4), 2 mM EDTA, 1 mM NADPH, 2 mM DTNB and 4 mM GSSG (Rao et al. 1996). For APX activity, the gel was at first incubated for 30 min in 0.05 M phosphate buffer (pH 7.0) containing 2 mM ascorbic acid and then incubated for 20 min in the same buffer containing 4 mM ASA and 2 mM H_2O_2. After this, staining was performed with 50 mM phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.4 mM NBT (Rao et al. 1996).

Statistical Analysis

After each experiment was repeated at least three times, the statistical analysis of data obtained was conducted using SPSS 13.0, and means were compared by Duncan's Multiple Range Test at a 0.05 level of confidence.

Results

Effect of CFPH application on seedling growth

Cold stress inhibited root (15% and 15%) and shoot (9% and 24%) length, and fresh weight (17% and 24%) in Bezostaya and Altındane, respectively compared to control. The inhibition rates were higher in the cold-susceptible cultivar (Altındane) than that of the resistant one (Bezostaya) (Table 1). In contrast, CFPH + cold treatment improved significantly ($P < 0.05$) the cold-induced inhibitions on the same parameters compared to cold alone (Table 1). The improvements caused by CFPH generally ranged between 10% and 25% in the same parameters. CFPH + cold treatment increased seedling fresh weight by 25% in Altındane and 13% in Bezostaya (Table 1). However, the same treatment affected negatively seedling dry weight in especially Altındane while cold application alone increased it compared to control.

Table 1
Root and shoot lengths (mm.plant^{-1}), and dry and fresh weights (mg.plant^{-1})

		Root length	Shoot length	Fresh Weight	Dry Weight
Altindane	**Control**	16.01 ± 0.169b	19.51 ± 0.211d	233 ± 23ab	21.05 ± 0.15d
	Cold	13.61 ± 0.318e	14.86 ± 0.384g	178 ± 7.1d	25.61 ± 0.90a
	CFPH + Cold	15.04 ± 0.275c	16.58 ± 0.177ef	196 ± 7.4c	24.82 ± 0.10b
Bezostaya	**Control**	17.92 ± 0.165a	23.09 ± 0.214c	245 ± 6.7a	22.91 ± 0.25c
	Cold	15.20 ± 0.184c	21.13 ± 0.409e	203 ± 8.8c	25.43 ± 0.10a
	CFPH + Cold	17.56 ± 0.184a	22.18 ± 0.439de	223 ± 14.7bc	26.63 ± 0.92a

Differences between groups with the same letters in a column are insignificant compared to $P < 0.05$ significance level. ±: SE

Effect Of Cfph Treatment On Freezing Injury

According to the results from electrical conductivity measurement, cold alone increased dramatically freezing injury (FI) in the leaves of both varieties compared to control plants (Fig. 1). But CFPH + cold application decreased significantly FI% in the leaves of the same varieties compared to the cold application alone. In addition, CFPH + cold reduced LT50 value from − 6.7°C to -7.8°C in the sensitive cultivar, and from − 6.7°C to -8.6°C in the resistant cultivar.

Rubisco Activity And Photosynthetic Pigment Content

It was shown that cold application alone significantly inhibited Rubisco activity (large subunit accumulation) compared to control, whereas CFPH + cold treatment stimulated it compared to cold alone in both the cultivars (Fig. 2). In addition, the cold application also decreased photosynthetic pigment content compared to the control (Table 2). For example, total chlorophyll and carotenoid contents diminished by 23% and 22% in Altındane and by 14% and 18% in Bezostaya, respectively. However, CFPH + cold treatment improved the stress-induced pigment inhibition by about 10% in both varieties.

Table 2

Effect of CFPH on contents of chlorophyll (mg.g^{-1} FW), free proline (µg/ FW), sugar (mg.g^{-1} FW), carotenoids (µg.g^{-1} FW) and phenolic compounds (µg.g^{-1} FW)

		Chlorophyll	Free proline	Total sugar	Carotenoids	Phenolics
Altindane	**Control**	6.78 ± 0.11b	82.55 ± 3.4d	5.97 ± 0.106e	860 ± 8b	882 ± 23.5b
	Cold	5.20 ± 0.07e	114.42 ± 7.7cb	8.54 ± 0.144c	668 ± 6d	1105 ± 52.7a
	CFPH + Cold	5.70 ± 0.10d	125.33 ± 5.04b	8.91 ± 0.264c	733 ± 4c	753 ± 26.5c
Bezostaya	**Control**	6.89 ± 0.08a	108.44 ± 4.12c	7.66 ± 0.103d	935 ± 6a	958 ± 47.8b
	Cold	5.87 ± 0.07d	137.5 ± 4.90b	10.43 ± 0.147b	765 ± 5c	1088 ± 90.1a
	CFPH + Cold	6.22 ± 0.03c	158.39 ± 6.57a	13.31 ± 0.206a	836 ± 3b	877 ± 28.5b

Differences between groups with the same letters in a column are insignificant compared to P < 0.05 significance level. ±: SE; FW: Fresh weight

Table 3 ROS ($O_2^{\cdot-}$ and H_2O_2) and MDA contents

		$O_2^{\cdot-}$ (ng/g FW)	H_2O_2 (ng/g FW)	MDA (nmol/g FW)
Altindane	**Control**	35.11±0.7c	51.38±3.4dc	2.97±0.38b
	Cold	44.62±1.2a	64.03±1.7a	3.93±0.40a
	CFPH+Cold	39.23±0.9b	58.34±2.9b	3.27±0.35b
Bezostaya	**Control**	27.10±0.6e	45.71±1.8e	2.67±0.42c
	Cold	33.96±1.1c	55.69±1.7bc	3.52±0.29ab
	CFPH+Cold	31.06±1.0d	49.36±1.2d	3.12±0.23b

Differences between groups with the same letters in a column are insignificant compared to P <0.05 significance level. ±: SE; FW: Fresh weight

Proline, Sugar, And Phenolic Contents

Cold alone in Altındane and Bezostaya increased free proline (39% and 26%, respectively), total sugar (43% and 36%, respectively), and phenolic compound (25% and 14%, respectively) contents compared to their controls. In addition, CFPH + cold treatment also stimulated protein and sugar contents while decreasing phenolic content compared to cold alone (Table 2).

Ros And Mda Contents

Cold stress alone led to significant increases in ROS content in both varieties, whereas the same parameters were decreased in the seedlings treated by CFPH in the seedlings under cold stress compared to cold alone (Table 3). For instance, cold alone increased $O_2.^-$ and H₂O₂ contents by 27% and 26% in Altındane, and 25% and 22% in the Bezostaya, respectively. However, CFPH + cold treatment decreased the same parameter levels in the varieties (Table 3). For example, $O_2.^-$ anion and H₂O₂ were decreased by 12% and 8% in Altındane and 9% and 11% in the Bezostaya compared to the cold alone, respectively. In addition, cold application alone in Altındane and Bezostaya increased MDA content by 32% and 31%, respectively whereas CFPH + cold treatment significantly decreased its level (17% and 12% in Altındane and Bezostaya, respectively) in the seedlings compared to cold alone.

Non-enzymatic Antioxidants

Cold application in Altındane and Bezostaya decreased ASA content by 18% and 21% while increased DHA content by 63% and 64%, respectively (Table 5). However, CFPH + cold treatment in Altındane and Bezostaya increased ASA content (80% and 40%, respectively) while decreased DHA content (47% and 35%, respectively). The cold application did not affect GSH content in Altındane while reduced in Bezostaya compared to control. GSSG content, however, significantly increased by cold stress in Altındane (82%) and Bezostaya (68%) compared to cold alone. But CFPH + cold treatment increased GSH content in both varieties while did not have a significant effect on GSSG content. In addition, it can be seen in Table 5 that ASA/DHA and GSH/GSSG rates decrease by cold application alone but increase by CFPH application.

Enzymatic Antioxidants

The cold application alone stimulated SOD, CAT, GPX, APX, and GR activities in both varieties compared to control plants (Table 4). In the study, the activities of the same enzymes were also evaluated with native PAGE. It was seen that the cold alone also caused the accumulation in the isoenzyme profile of the antioxidant enzymes compared to control (Figs. 1–7) and the findings of these two studies supported each other. CFPH + cold treatment, however, stimulated SOD, CAT, and GPX activities while inhibited APX and GR activities in Altındane cultivar compared to cold application. But the same application generally stimulated the antioxidant enzyme activities studied in Bezostaya cultivar compared to the cold application (Table 4). Comparing the findings with the profiles of the same isoenzyme, it can be seen that CFPH + cold treatment exhibited an inhibiting effect on SOD and CAT accumulation while a stimulating effect GPX, APX, and GR accumulation (Figs. 1–7). In addition, it was evaluated that both cold and CFPH treatment decreased or increased the activities of antioxidant enzymes studied, especially by affecting the accumulation of low molecular-weight isoenzymes.

Table 4

Effect of CFPH on antioxidant enzyme activities (U.mg protein^{-1})

		SOD	CAT	GPX	APX	GR
Altindane	**Control**	36.01 ± 1.4c	69.56 ± 2.4d	1522 ± 83e	30.27 ± 0.18e	9.16 ± 0.23f
	Cold	46.79 ± 1.4a	88.64 ± 0.8a	2623 ± 101bc	43.87 ± 0.20c	16.84 ± 0.42e
	CFPH + Cold	44.38 ± 1.3b	86.45 ± 0.9a	2558 ± 128c	55.49 ± 0.40a	22.08 ± 0.25c
Bezostaya	**Control**	30.86 ± 1.1d	68.63 ± 2.0d	1944 ± 75d	39.08 ± 0.32d	20.75 ± 0.23d
	Cold	36.30 ± 1.4c	78.16 ± 1.4c	2761 ± 94b	49.27 ± 0.40b	25.36 ± 0.22b
	CFPH + Cold	37.31 ± 1.6c	82.94 ± 1.6b	2928 ± 44a	54.25 ± 0.30a	29.53 ± 0.40a

Different letters in the column of a cultivar express important differences at $P < 0.05$ level. ± means standard error. FW: Fresh weight

Table 5

Effect of CFPH on non-enzymatic antioxidants contents (µg.g^{-1} FW)

		ASA	DHA	ASA/DHA	GSH	GSSG	GSH/GSSG
Altindane	**Control**	445 ± 5	265 ± 2e	1.68	140.7 ± 2c	82.0 ± 3e	1.72
	Cold	366 ± 4	433 ± 5ab	0.85	141.2 ± 1cb	149.5 ± 3.4c	0.94
	CFPH + Cold	657 ± 3b	281 ± 3c	2.13	171.7 ± 1a	153.6 ± 3bc	1.12
Bezostaya	**Control**	660 ± 3ab	310 ± 1d	2.13	114.6 ± 1.2c	94.4 ± 1.9d	1.21
	Cold	521 ± 6b	507 ± 4a	1.02	95.6 ± 01.9d	159.1 ± 2ab	0.60
	CFPH + Cold	731 ± 4a	329 ± 3bc	2.22	169.1 ± 1.6b	164.3 ± 1.2a	1.03

Different letters in the column of a cultivar express important differences at $P < 0.05$ level. ± means standard error. FW: Fresh weight

Discussion

Waste recycling of poultry feathers which often causes significant environmental problems ensures the recovery of essential nutrients for plants and is also one of the indispensable strategies for dealing with waste. Poultry feathers are a rich source of nitrogen and sulfur and other nutritional minerals for plants (Popko et al. 2015). On the other hand, the hydrolysis process is one of the most important steps in obtaining animal-derived protein hydrolysates (PH) to be used for plant growth. Alkaline hydrolysis providing some advantages is a pretty simple process and is more commonly used (Nustorova et al. 2006; Genç and Atıcı 2019). For this reason, as in our previous study, we carried out hydrolysis processes of chicken feathers using KOH as a power hydrolysis agent and then the getting mixture neutralized with H_3PO_4 (Taşkın et al. 2012; Genç and Atıcı 2019). This process leads to the formation of KH_2PO_4 salt including two valuable minerals like K and P for plants. The protein yield and amino acid content of the protein hydrolysate acquired at the end of the process was determined to be almost the same as the data presented by Genç and Atıcı (2019). Also, it was proposed that chicken feather protein hydrolysate (CFPH) involved nearly all of the essential nutrients that plants require (Taşkın et al. 2012; Genç and Atıcı 2019). It was shown that the total protein yield of CFPH was about 73% (w/w) and identified 19 proteinogenic amino acids in the CFPH. Apart from this, CFPH contained several non-proteinogenic amino acids like ornithine, cystine, and citrulline. Therefore, it was suggested that CFPH hydrolyzed with the method expressed has the potential to be used as a suitable organic fertilizer for foliar applications in plant cultivation (Genç and Atıcı 2019).

Soil microorganisms can interfere with the absorption of small peptides and amino acids by plant roots, so foliar treatment of protein hydrolysates as biofertilizers (PHs) or biostimulants is more recommended than soil application (Morales-Pajan and Stall 2003; Genç and Atıcı 2019). In our previous study, when different aqueous solutions of CFPH (0.05%, 0.075%, and 0.1% w/v) were applied to seedling leaves of the same wheat cultivars grown under normal conditions (20/22 ^{o}C, day/night), it was determined that 0.1% treatment of CFPH was the most effective dose on growth, development and antioxidant parameters in the wheat seedlings (Genç and Atıcı 2019). After CFPH treatment (0.1%), it was first determined the change of plant dry and fresh weight and plant height parameters in the seedlings. In similar studies, the evaluation of plant dry and fresh weights, and plant height among the parameters reflecting growth and development for a plant has been frequently used as an important evaluation indicator (Genç and Atıcı 2019). In addition, Gousterova et al. (2012) proposed that CFPH treatments displayed an improving effect on similar parameters in mostly low concentrations. Our findings showed that the foliar treatment of CFPH (0.1%) could ameliorate the plant growth parameters studied in both wheat cultivars exposed to cold stress. For instance, the cold stress decreased seedling growth in the context of root and shoot length, and fresh weight in Bezostaya and Altındane varieties of wheat. Also, the inhibitory effect of cold was more pronounced in Altındane. However, the inhibitions caused by cold on seedling growth, except for dry weight was mitigated by CFPH + cold treatment. The stimulating effect of CFPH on wheat varieties under cold stress can be attributed to the important amino acids that it contains as a biostimulant. Because CFPH is rich in especially common and rare amino acids such as proline (13.08%), glutamate (8.63%), leucine (6.86%), serine (5.4%), valine (5.4%), alanine (4.83%), aspartate (4.51%), phenylalanine (3.8%), isoleucine (3.5%), arginine (2.5%), methionine (0.42%), histidine

(0.32%), cysteine (0.15%), tryptophan (0.028%) as well as non-proteinogenic amino acids like ornithine, cystine and citrulline (Genç and Atıcı 2019).

Low temperature leads to the elevation of electrolyte leakage in plant cells, and such increases of more than a certain threshold level are directly related to freezing injury in plant tissues. The primary cause of freezing-induced injury in plant cells is ice formation in the apoplastic area because of dehydration of the intracellular environment and physical damage by ice crystals (Atıcı nad Nalbantoglu 1999, Tiryaki et al. 2019). The cold application alone increased freezing injury (FI%) while CFPH + cold treatment decreased both FI% and LT50 value in the leaves of the two varieties (Fig. 1). It can be seen that CFPH treatment-induced tolerance to the freezing injury in detached wheat leaves under cold stress. In both cold-sensitive and tolerant plants including wheat, mitigation of frost damage in leaves has been associated with increased tolerance to low-temperature stress (Atıcı and Nalbantoglu 2003; Esim and Atıcı 2015; Tiryaki et al. 2019).

We measured both the chlorophyll and carotenoid content, and the expression level of Rubisco in the seedling leaves of wheat cultivars to determine the effects of CFPH's foliar application on photosynthesis. These parameters are directly related to plant yield and growth development (Genç and Atıcı 2019). The cold application alone decreased Rubisco activity and photosynthetic pigment contents (chlorophyll and carotenoids), whereas CFPH + cold treatment improved the inhibitions on these parameters in both cultivars (Fig. 2 and Table 2). In parallel with these findings, several studies have proposed that animal-derived PH treatments enhanced chlorophyll content in some crops including beans, corn, soybeans, tomatoes, and wheat under normal conditions (Kurbanoğlu et al. 2004; Horii et al. 2007; Cerdán et al. 2009; Genç and Atıcı 2019). In this study, it is seen that CFPH (0.1%) treatment also contributed significantly to the improvement of the photosynthetic machinery, which deteriorates at low temperatures. How PHs stimulate photosynthetic pigment contents and Rubisco activity is still not fully understood (Horii et al. 2007). However, cytokinin-like compounds in PHs can trigger photosynthetic pigment biosynthesis. It is not yet known if CFPH contains cytokinin-like compounds, CFPH contains high levels of the precursor amino acids glutamate (8.63%) and proline (13.08%), which play a role in the biosynthesis of chlorophyll and hormone cytokinin (Milazzo et al. 1999). In addition, the high levels of proline in fish protein hydrolysate were asserted to stimulate cytokinin synthesis via certain steps in the pentose phosphate pathway in melon (Milazzo et al. 1999). On the other hand, CFPH treatment stimulated Rubisco accumulation (the large subunit) in both cultivars exposed to cold (Fig. 2). This finding can support the hypothesis that CFPH treatment under cold stress promotes carbon fixation and organic matter synthesis in the photosynthesis and can explain why CFPH treatment enhanced the studied parameters including plant length and dry weight, and protein, sugar, and carotenoid contents of both wheat cultivars (Tables 1 and 2).

Both wheat cultivars elevated the content of osmotic compounds such as free proline and total sugar, and phenolic compounds with antioxidant properties in response to the cold application. But CFPH + cold treatment further stimulated protein and total sugar content while interestingly decreasing phenolic content (Table 2). It was asserted that PH treatment enhanced total free sugar accumulation in maize

plants (Schiavon et al. 2008) and free proline content in plant tissues (Milazzo et al. 1999; Colla et al. 2015). Also, proline, proline analogs, or proline-rich PHs could stimulate plant growth and development (Milazzo et al. 1999; Kurbanoğlu et al. 2004). We think that the chicken feather hydrolysis method we use, which causes CFPH to have rich amino acid and mineral nutrient content, also contributes to these determined effects of CFPH (0.1%) (Taşkın and Kurbanoğlu 2011). On the other hand, decreasing phenolic content in the same seedlings treated with CFPH during cold exposure is an interesting result (Table 2). Horii et al. (2007) determined a decreased phenolic content in animal-derived PH-treated tomato plants while an increase in soybean plants. In addition, banana seedlings treated with PH from feathers appeared also an increase in the contents of phenolics and flavonoids (Gurav and Jadhav 2013). It is known that plant cells, especially under stress conditions, consume a significant proportion of the assimilated carbon (CO_2) via photosynthesis for the biosynthesis of secondary metabolites including phenolics (Rice-Evans et al. 1997). According to our findings, it can interpret that the decrease in ROS level (Table 2) and increase in enzymatic and non-enzymatic antioxidant capacity (Tables 4 and 5) in seedlings treated with CFPH under cold stress increase the low-temperature tolerance in wheat seedlings, resulting in a decrease in phenolic content. Following such an arrangement, the biosynthesis of primary metabolites such as Rubisco and chlorophyll may have increased, resulting in a significant contribution to the improvement of growth and development of both wheat varieties.

Reducing ROS levels in cells even during the normal course of metabolism is important because ROS and other radicals whose production is caused by ROS are very much harmful and have potentially deadly effects on many biomolecules like nucleic acids and polyunsaturated lipids (Karuppanapandian et al. 2011). Fish PHs were shown to have a powerful antioxidant activity like free radical-scavenging activity and ferric reducing/antioxidant power in vitro conditions (Fakhfakh et al. 2011; Gurav and Jadhav 2013). In addition, Genç and Atıcı (2019) showed to yield lower ROS content the chicken feathers PH hydrolysate treatments in wheat under normal conditions. Our findings also demonstrate that ROS contents such as $O_2.^-$ and H_2O_2, and MDA levels were upregulated by cold alone in both varieties, but the same parameters were downregulated in the seedlings treated by CFPH under cold stress (Table 3). This result indicated that CFPH contributed to the ameliorating plant antioxidant responses by lowering ROS, and LPO (as MDA) during cold exposure in both wheat cultivars.

Since antioxidant system pathways in plants play a fundamental role in response to abiotic stresses such as low temperature, we evaluated changes in both enzymatic and non-enzymatic antioxidant parameters in CFPH-treated wheat varieties (Tables 4 and 5). SOD, CAT, GPX, APX, and GR activities were enhanced by cold alone in both varieties and these increases were corroborated by the accumulation in the isoenzyme profile of the same enzymes on native PAGE. On the other side, CFPH + cold treatment had different effects on the enzyme activities, in short, it increased all antioxidant enzyme activities in Bezostaya cultivar, but inhibited APX and GR activities while stimulating SOD, CAT, and GPX activities in Altındane (Table 4). According to native PAGE data, CFPH + cold treatment resulted in a reduction in the accumulation of SOD and CAT, and an increase in that of GPX, APX, and GR, especially in low molecular weight isoenzymes on the native PAGE gel (Figs. 1–7). Feitosa de Vasconcelos et al. (2009) determined

that amino acid-based biostimulants treatment stimulated SOD, CAT, and APX activities, resulting in improving stressful conditions in maize and soybean under drought stress. In addition, our findings may indicate that low molecular-weight isoforms rather than the higher molecular weight of antioxidant enzymes play a crucial role in response to the cold stress response of wheat. As known, non-enzymatic antioxidants also make a very important contribution both directly and indirectly to ROS detoxification. Among these, ASA and GSH have a crucial role in the regulation of cellular redox situation and the ascorbate-glutathione cycle detoxifying H_2O_2 (Karuppanapandian et al. 2011). We determined that cold application decreased the DHA and GSSH content of Altındane and Bezostaya cultivars. However, CFPH + cold treatment increased their overall contents and caused an improvement in ASA/DHA and GSH/GSSG ratios (Table 5). High rates of AsA/DHA and GSH/GSSG are among the best indicators of the ascorbate-glutathione circle (Karuppanapandian et al. 2011). Our findings on non-enzymatic antioxidants indicate that CFPH foliar treatments ameliorated AsA/DHA and GSH/GSSG ratios in both cultivars under cold stress.

Conclusions

Foliar application of CFPH (0.1%) containing 19 proteinogenic and 2 non-proteinogenic amino acids derived from chicken feathers by an alkaline process improved the deteriorated parameters of plant growth in both wheat cultivars (Altındane and Bezostaya) under cold stress. While the same treatment decreased phenolic compounds, it increased the free proline and total sugar content in parallel with the increase in photosynthetic pigments and Rubisco activity. In addition, CFPH treatment diminished ROS and LPO levels by stimulating the antioxidant system players including enzymatic (SOD, CAT, GPX, APX, and GR) and non-enzymatic (Asa and GSH) antioxidants. We propound that the foliar treatment of CFPH as a biostimulant could increase cold stress tolerance in wheat seedlings.

Declarations

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Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

Conflict of interest The authors declare no competing interests

Data availability All data generated or analyzed during the current study are presented in this article.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ökkeş Atıcı and Ebru Gezgincioğlu. The first draft of the manuscript was written by Ökkeş Atıcı, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

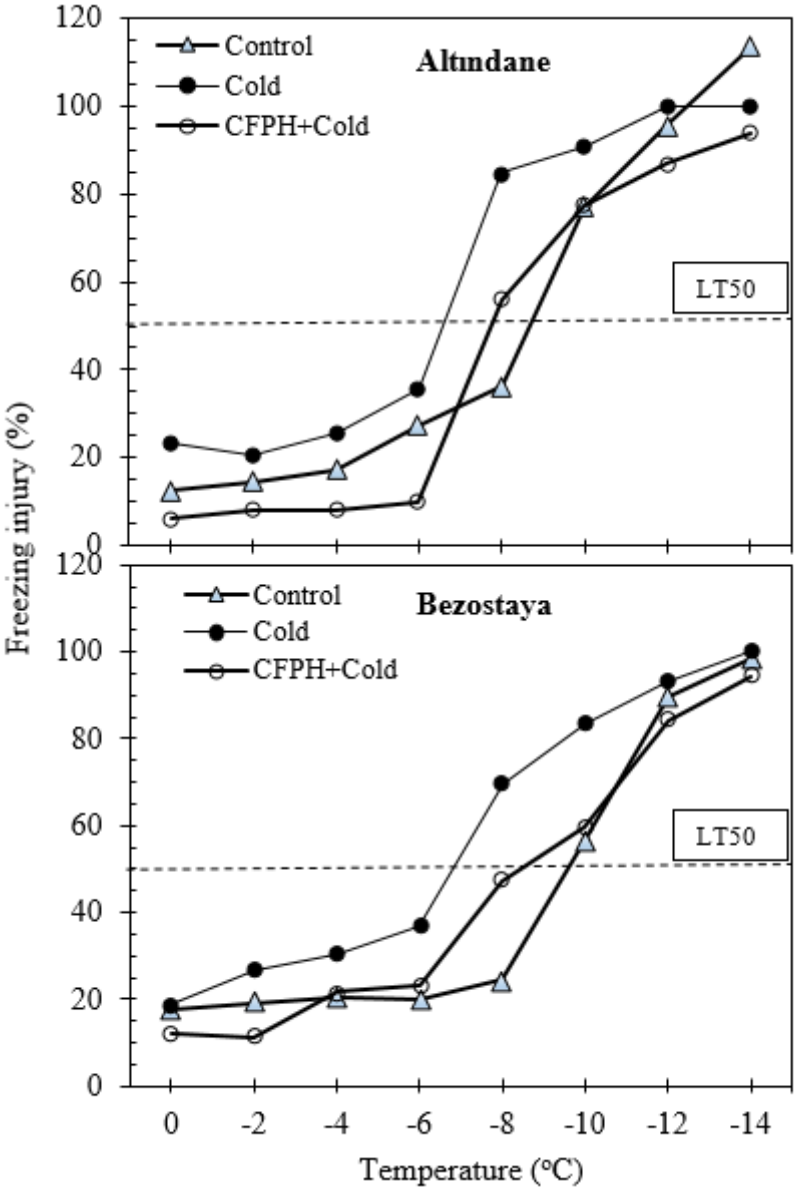

Figure 1

Effect of CFPH treatment on freezing injury (%)

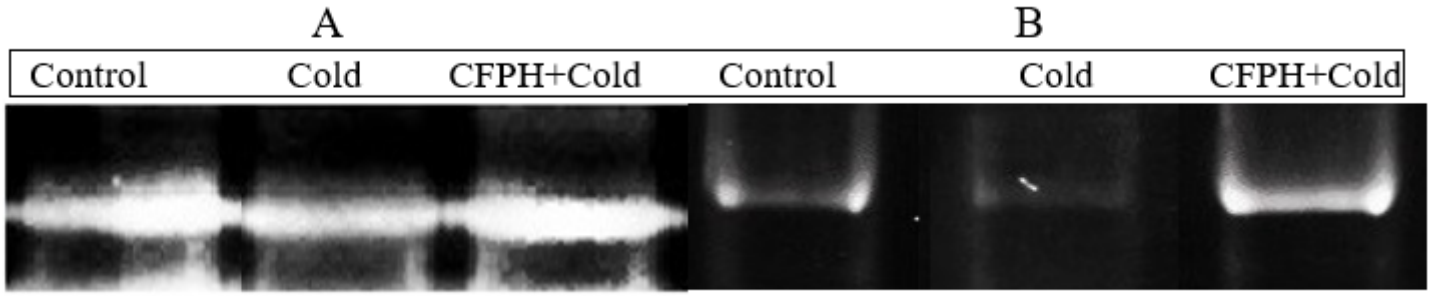

Figure 2

The accumulation of Rubisco in two cultivars of wheat. A: Altındane, B: Bezostaya

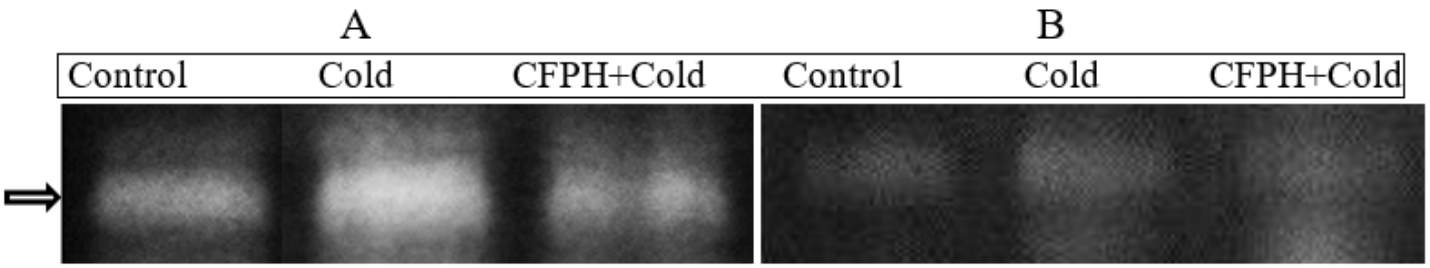

Figure 3

SOD isoenzymes in wheat cultivars. A: Altındane, B: Bezostaya

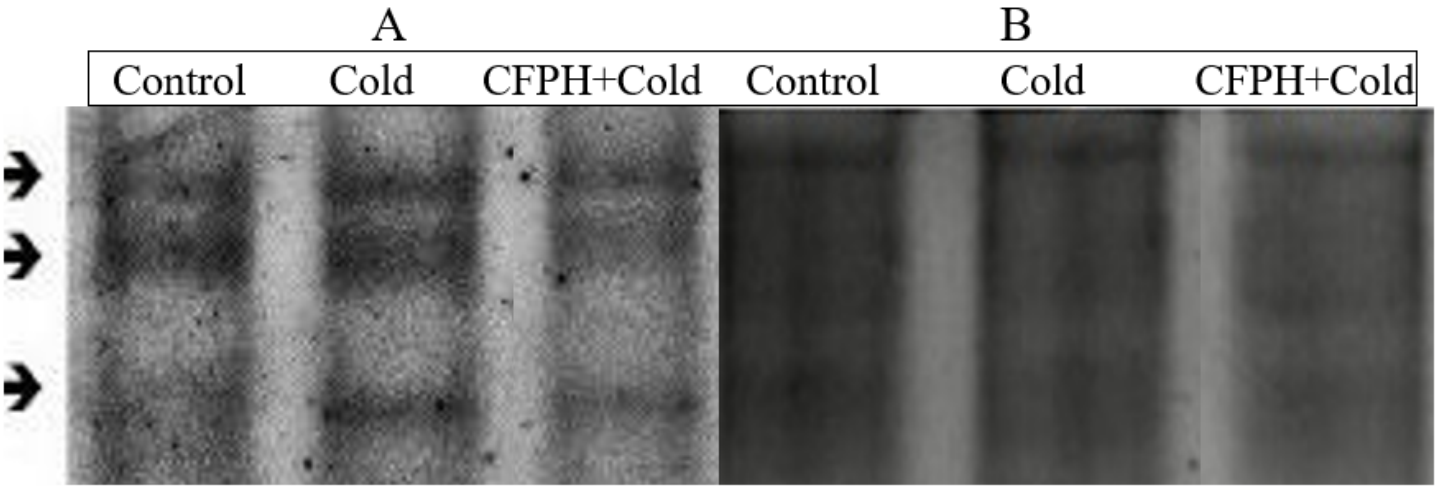

Figure 4

CAT isoenzymes in wheat cultivars. A: Altındane, B: Bezostaya

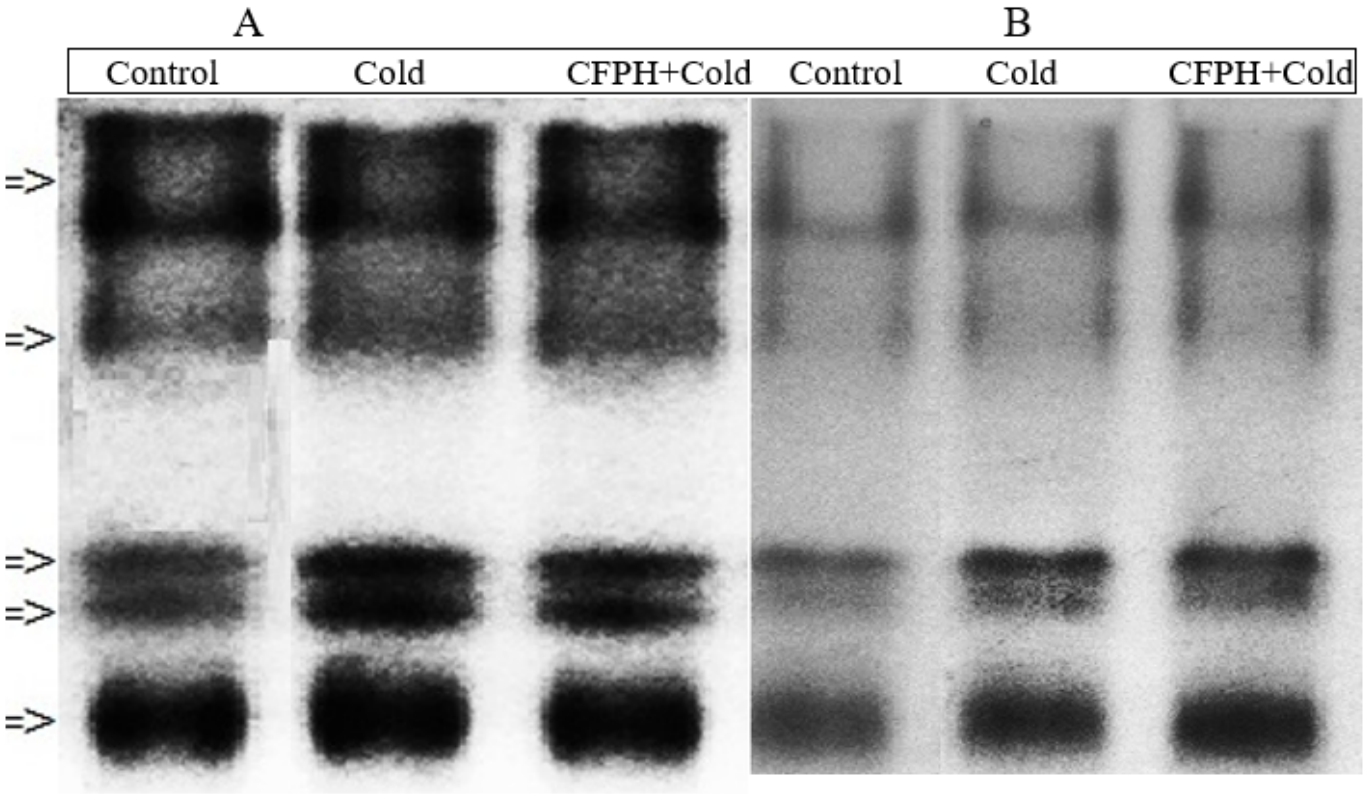

Figure 5

GPX isoenzymes in wheat cultivars. A: Altındane, B: Bezostaya

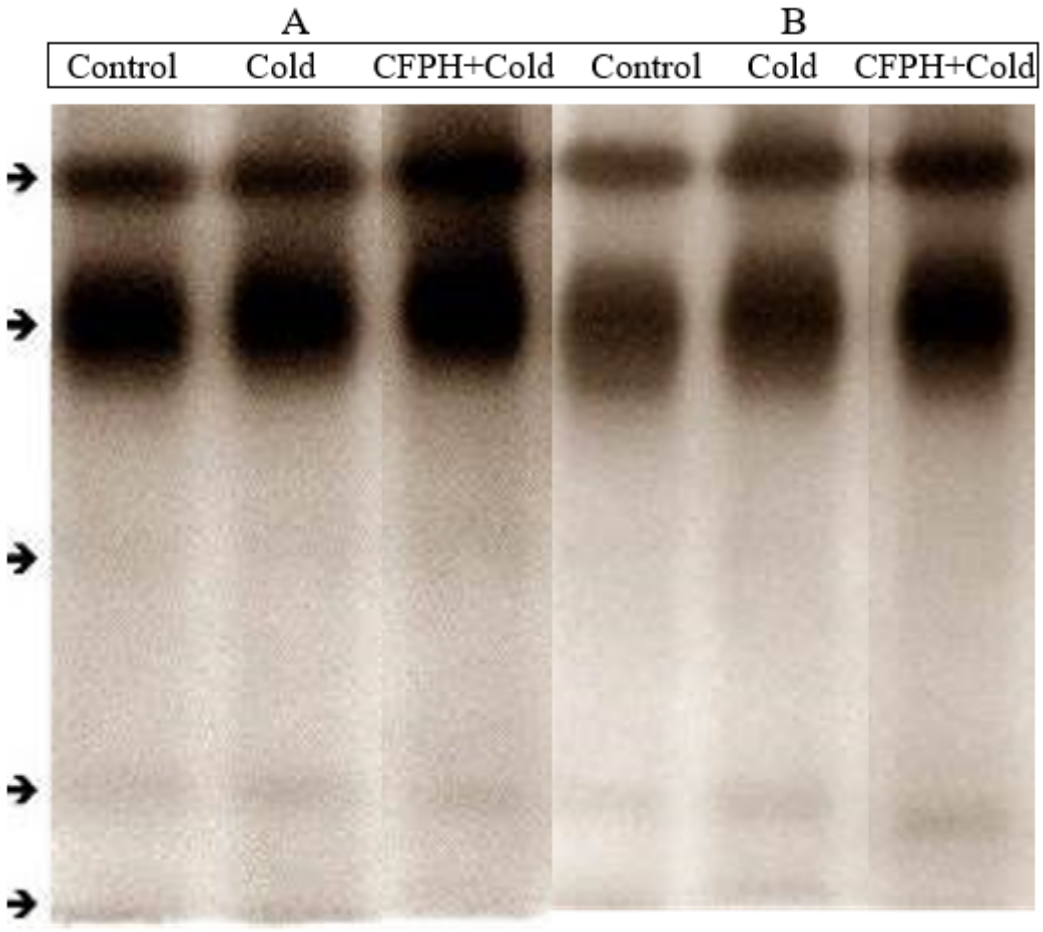

Figure 6

APX isoenzymes in wheat cultivars. A: Altındane, B: Bezostaya

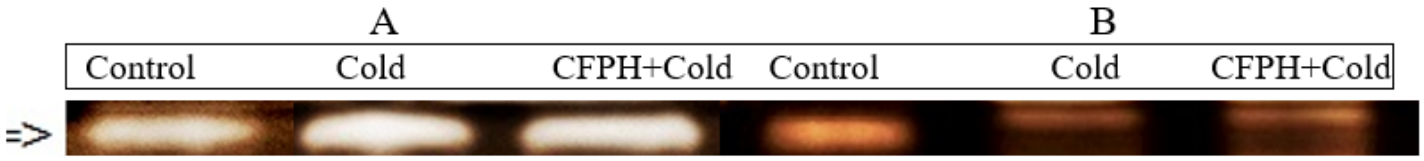

Figure 7

GR isoenzymes in wheat cultivars. A: Altındane, B: Bezostaya