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Chicken feather protein hydrolysate as a biostimulant improves the growth of wheat seedlings by affecting biochemical and physiological parameters

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Abstract: We derived a protein hydrolysate with rich content, which includes proteinogenic and nonproteinogenic amino acids, from chicken feathers by using an alkaline hydrolysis procedure with high yield success. After foliar application of aqueous solutions (0.05%, 0.075%, and 0.1%) of chicken feather protein hydrolysate (CFPH) to the seedlings of wheat (*Triticum aestivum* L. 'Altındane' and 'Bezostaya'), their effects were evaluated on important physiological and biochemical parameters. Treating plants with CFPH stimulated the expression of RuBisCo and increased root and shoot lengths, fresh and dry weights of the seedlings, and photosynthetic pigment content. In addition, applying CFPH at 0.075% and 0.1% concentrations decreased the content of reactive oxygen species, such as $O_2^{\cdot-}$ and H_2O_2, and the levels of lipid peroxidation (as malondialdehyde) and phenolic compounds, while augmenting free proline content. On the contrary, CFPH application ameliorated the antioxidant parameters, including enzymatic and nonenzymatic antioxidants. CFPH stimulated the activities of guaiacol, ascorbate, and glutathione peroxidase while it did not affect those of superoxide dismutase and catalase. Enzyme activity results agreed well with the isoenzyme expression profiles. In conclusion, the application of CFPH improved the parameters under consideration in wheat seedlings, suggesting that CFPH has the potential to be used as a suitable biostimulant in plant cultivation.

Key words: Chicken feather, protein hydrolysate, RuBisCo, biostimulant, antioxidant, wheat

1. Introduction

Human population growth and declines in cultivable productive soil around the world have triggered a variety of efforts to improve plant cultivation yield and quality, including development of more low-cost, environmentally sustainable, and reasonable approaches (Ertani et al., 2009, 2015; Popko et al., 2015). Increased crop production, however, mostly depends on the use of chemical fertilizers to reinforce the mineral nutrient requirements of plants (Colla et al., 2015). In recent years, researchers have increasingly recommended the use of biostimulants, including protein hydrolysates (PHs), as alternative inorganic fertilizers to improve crop cultivation (Colla and Rouphael, 2015; Popko et al., 2015); moreover, it has been suggested that biostimulants may serve as a favorable treatment in reducing the need for inorganic fertilizer and the pollution in agricultural lands (Colla et al., 2015). Widespread acknowledgment that increasingly intensive use of inorganic fertilizers impairs soil quality has led to a steady increase in the number of studies on the application of PHs, including small-sized peptides, free amino acids, and some nutrient elements, due to their beneficial effects on crop cultivation (Schiavon et al., 2008; Colla et al., 2015). Studies have shown that low-molecular-weight peptides and free amino acids in PH compounds are the most advantageous features of biostimulant treatments because almost all plant tissues can lightly absorb them (Morales-Pajan and Stall, 2003; Cerdán et al., 2009; Colla et al., 2015). In addition, PHs could improve plant productivity by affecting plant metabolism. Studies have suggested that, when applied to plant leaves, PHs could stimulate uptake effectiveness of nutrient elements and soil water (Cerdán et al., 2009; Halpern et al., 2015) and upgrade some of the biochemical mechanisms associated with protein synthesis, photosynthesis, lignification, and abiotic stress tolerance (Andarwulan and Shetty, 1999; Schiavon et al., 2008; Colla et al., 2015), resulting in improved growth, development, and productivity of crop plants (Ertani et al., 2009, 2014; Colla et al., 2015). For example, foliar application of fish protein hydrolysate enhanced seed performance, plant growth, enzyme activities such as guaiacol peroxidase and glucose-6-phosphate dehydrogenase, and the content of free proline and phenolics in pea seedlings (Andarwulan and Shetty, 1999). It has also been shown that PHs could stimulate tolerance to stresses such as drought (Feitosa de Vasconcelos et al., 2009) and play a role as chelating

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agents of cationic nutrient minerals for plants (Ashmead, 1986). Some studies suggested that the ameliorative effects of PH treatment depended on the concentration, plant species, environmental conditions, phenological stage, time of application, and leaf permeability (Kurbanoğlu et al., 2004; Kunicki et al., 2010; Ertani et al., 2014). PHs can be produced by enzymatic and/or chemical hydrolysis of unavailable proteins from animal- or plant-derived waste materials (Colla et al., 2015; Colla and Rouphael, 2015).

In the poultry industry, chicken feathers are a large-volume waste product and their excessive accumulation can lead to environmental pollution (Taşkın and Kurbanoğlu, 2011; Taşkın et al., 2012). In recent years, some researchers have revealed that chicken feathers could serve as a significant PH resource (Taşkın and Kurbanoğlu, 2011; Taşkın et al., 2012). The feathers contain nutritional minerals and keratin, along with high amounts of glutamate, cysteine, glycine, arginine, and phenylalanine (Taşkın et al., 2012; Veerabadran et al., 2012). Researchers have studied the potential use of chicken feather protein hydrolysates (CFPHs) as a general growth substrate for bacteria (Taşkın and Kurbanoğlu, 2011) and fungal development (Taşkın et al., 2012), as an alternative organic fertilizer, and as a biocontrol agent for the cultivation of crop plants (Gurav and Jadhav, 2013). CFPH, for example, exhibited a stimulative effect on seed germination and seedling growth of ryegrass when applied to soil (Gousterova et al., 2012). Its foliar application increased the number of fingers per hand, bunch weight, and hands per bunch in banana plants (Gurav and Jadhav, 2013), and significantly increased plant biomass in rapeseed (Popko et al., 2015). Although several studies have assessed the effect of CFPH on certain physiological parameters of plants, to this point almost no information has been produced regarding its role in relation to biochemical mechanisms, including reactive oxygen species (ROS) and nonenzymatic antioxidant compound (e.g., glutathione and ascorbate) levels, or antioxidant enzyme (e.g., superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase) and RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase) activities, especially for foliar application. A detailed evaluation of these parameters will provide more valuable data about CFPH's impact on crop plants as a potential organic fertilizer. In this study, we aimed to investigate the effects of foliar application of CFPH on the biochemical parameters known as indicator mechanisms in plant responses, as well as on physiological growth parameters in wheat (*Triticum aestivum* L.).

2. Materials and methods

2.1. Preparation of chicken feather protein hydrolysate

The hydrolysis processes for chicken feathers (CFs) were carried out using a modified method employed by Taşkın and Kurbanoğlu (2011) and Taşkın et al. (2012). Briefly, after washing them with distilled water, chicken feathers were dried at 70 °C until a constant weight. Then they were ground with a Waring blender until becoming a feather powder. One hundred grams of CF powder was added to 250 mL of KOH solution (2 N) in a glass tube and the tube containing the CF powder was subjected to hydrolysis processes for 60 h with gentle stirring (150 rpm) on a shaker. The obtained alkaline extract was neutralized with 10 N H_3PO_4 solution and then filtrated with Whatman filter paper in order to remove the particles that were not hydrolysable. Then it was dried at 80 °C until it became a powder and was referred to as chicken feather protein hydrolysate (CFPH). Aqueous solutions (0.05%, 0.075%, and 0.1%, w/v) of CFPH were prepared from this dried powder by dissolving in sterile pure water. The final pH value of the solutions was adjusted to 6–6.5. To determine the content of amino acids, 10 g of CFPH was used. This process was carried out by Düzen Norwest Laboratory (Environmental, Food, and Veterinary Health Services Training and Consulting Trade Co., Ankara, Turkey). For this, a Varian CP-3800 gas chromatograph (Varian Inc., Palo Alto, CA, USA) and EZ Rapid Amino Acid Analysis Kit (Phenomenex Inc., Torrance, CA, USA) were used.

2.2. Plant material and CFPH application

In this study, we used two varieties of wheat *(Triticum aestivum* L. 'Altındane' and 'Bezostaya'). Wheat seeds were obtained from the East Anatolian Agricultural Research Institute (Erzurum, Turkey). Sterilized seeds were sowed into a hydroponic system including half-strength Hoagland solution. CFPH was sprayed once on the leaves of 11-day-old wheat seedlings at concentrations determined in preliminary work (0.05%, 0.075%, and 0.1% w/v). For the control group, the same amount of pure water was sprayed on the plants instead of CFPH. After 3 days, the seedlings were harvested for use in physiological and biochemical experiments.

2.3. Determination of plant growth and soluble protein and pigment contents

To determine plant growth, the root and shoot lengths of wheat seedlings were measured and their dry weights were quantified after incubation for 48 h at 70 °C. Protein content was determined according to the method of Bradford (1976) and results were expressed as fresh tissue (mg/g). Chlorophyll a and b and carotenoid contents were detected spectrophotometrically in fresh leaves according to the method of Lichtenthaler (1987) and were expressed as mg/g fresh tissue.

2.4. Determination of RuBisCo expression via western blot analysis

After SDS-PAGE of proteins, polypeptides were transferred to a nitrocellulose membrane (0.45 mm) using a buffer

containing 13 mM Tris (pH 7.2), methanol (10%), and 190 mM glycine over 50 min at 15 V. 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 overnight at 4 °C. After the membrane was rinsed with Tween-20 (0.1%), it was incubated in powdered milk (2%) containing Tween-20 (0.1%) and the monoclonal antibody of RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase; EC 4.1.1.39) for 1 h at ambient conditions. Then the membrane was rinsed with Tween-20 (0.1%) and incubated in powdered milk (2%) containing Tween-20 (0.1%) and a secondary antibody for 1 h at ambient conditions. Finally, the membrane was rinsed with Tween-20 (0.1%) and distilled water. Interacting antibodies were determined using a chemiluminescent substrate (Towbin et al., 1979).

2.5. Determination of soluble sugar content

Soluble sugar content was measured according to the phenol-sulfuric method (Dubois et al., 1956). Dried leaves (1 g) were powdered and 5 mL of ethanol (70%) was added to a tube containing the powdered leaves. The mixture was incubated in a water bath for 45 min at 75 °C. The tubes were centrifuged for 10 min at 3500 × *g* and the obtained supernatant was diluted at a rate of 1/10 with ethanol (70%). Then a reaction mixture (3 mL) was prepared to include 100 µL of the supernatant, 300 µL of saturated phenol, and 2.6 mL of concentrated H_2SO_4. The absorbance of the mixture was monitored spectrophotometrically at 480 nm for pentose and at 488 nm for hexoses. Data are expressed as mg/g dry tissue in comparison with a standard chart prepared using pure fructose and glucose.

2.6. Determination of ROS and lipid peroxidation levels

Endogenous H_2O_2 level was measured by the method of Hu et al. (2005). In brief, 0.5 g of tissue was homogenized in 10 mL of cold acetone and centrifuged at 5000 × *g* for 15 min at 4 °C. Next, 0.5 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 3000 × *g* for 10 min at 4 °C. The obtained pellet was washed twice with cold acetone and dissolved in 3 mL of 1 M H_2SO_4. After filtration, absorbance measurement was carried out at 415 nm versus a blank. Data were expressed as ng/g fresh tissue. Superoxide anion ($O_2^{\cdot-}$) content was determined 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 and placed in 5 mL of 500 µM XTT (pH 7.0) with or without 3.5 U/mL superoxide dismutase. Two hours later, the homogenate was centrifuged at 10,000 × *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 a standard curve to calculate the content of superoxide. The lipid peroxidation level (LPO) was determined by measuring the content of malondialdehyde (MDA). The content of MDA was measured according to the method of Heath and Packer (1968). Briefly, 0.5 g of tissue was homogenized in 5 mL of 1% TCA and centrifuged at 12,000 × *g* for 20 min. One milliliter of supernatant obtained was mixed with 4 mL of 0.5% TBA in 20% TCA. The reaction mixture was incubated for 30 min in a boiling water bath, and the reaction was terminated in an ice bath. The samples were centrifuged once more at 5000 × *g* for 10 min. The absorbance of the supernatant was followed at 532 nm and it was corrected by subtracting nonspecific absorbance at 600 nm. MDA level was expressed as nmol/g fresh tissue.

2.7. Determination of glutathione, ascorbic acid, proline, and phenolic compounds

Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were determined enzymatically using the method of Griffith (1980) with slight modification. Fresh tissue (0.2 g) was homogenized in 2 mL of 5% metaphosphoric acid and centrifuged at 12,000 × *g* for 20 min. For total GSH, 150 µL of the obtained metaphosphoric extract was mixed in an Eppendorf tube with 1050 µL of KH_2PO_4 (50 mM, pH 7.5), 200 µL of EDTA (2.5 mM), 200 µL of DTNB (1 mM), 200 µL of GR (0.3 unit), and 200 µL of NADPH (1 mM M) in a final volume of 2 mL at 25 °C. The reaction was started with the addition of 200 µL of NADPH, and the increase in absorbance at 412 nm was monitored for 3 min at 25 °C. For GSSG, 150 µL of the metaphosphoric extract was neutralized with 96 µL of 1 M triethanolamine. Then 8 µL of 2-vinylpyridine was added to the extract. The mixture was allowed to incubate for 60 min at 25 °C. Then 228 µL 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 µL of EDTA (2.5 mM), 200 µL of DTNB (1 mM), 200 µL of GR (0.3 unit), and 200 µL of NADPH (1 mM M) in a final volume of 2 mL at 25 °C. The reaction was started with the addition of 200 µL of NADPH, and 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 µM) and GSSG (0.8–40 µM) prepared in 2% (w/v) metaphosphoric acid. Reduced GSH was total GSH – GSSG. The contents of reduced (AsA) and oxidized (DHA) ascorbate were determined as described by Okamura (1980). Briefly, 0.2 g of powdered sample in liquid nitrogen was extracted in 2 mL of 5% TCA. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4 °C. Ten microliters of 5 M NaOH was added

to 400 µL of supernatant and this was divided into two equal parts. The first mixture was combined with 200 µL of KH_2PO_4 (150 mM, pH 7.4) and 200 µL of distilled water. The second mixture was combined with 200 µL of KH_2PO_4 (150 mM, pH 7.4), 100 µL of DTT (10 mM), and 100 µL of N-ethylmaleimide (0.5%). The two mixtures were added separately to 400 µL of 10% TCA, 400 µL of 44% H_3PO_4, 400 µL of 2,2'-dipyridyl (4% in 70% ethanol), and 150 µL 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 compounds, the plant tissue (0.2 g) was homogenized in 2 mL of KH_2PO_4 (pH 7.4) and the obtained homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C. The total phenol content in the extracts was determined using Folin–Ciocalteu reagent (FCR) and gallic acid as the 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. The blank was formed from a similar sample that did not contain the extract. Total phenol content was determined with the use of an external standard curve and expressed as µg 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.

2.8. Activity assay of antioxidant enzymes

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 12,000 × *g* 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) and Agarwal and Pandey (2004). One unit of superoxide dismutase (SOD, EC 1.15.1.1) activity was defined as the amount of enzyme that inhibited 50% of the 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 the 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 substrates. One unit of GPX is defined as the amount of enzyme that increases the 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 disrupting 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, and 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 µM 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. The 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) as described by Foyer and Halliwell (1976) with minor modifications. Data were expressed as EU/min/mg protein.

2.9. Native PAGE for isoenzyme staining of antioxidant enzymes

Native proteins were run on PAGE under nondenaturing 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 the 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). The 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. CAT isoenzymes were monitored according to the method of Weydert and Cullen (2010). After 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. GR staining was carried out by incubation 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 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).

2.10. Statistical analysis

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

3. Results and discussion

Because excessive formations of toxic salts such as NaCl that arise during the hydrolysis process are not suitable

for plant life, the hydrolysis process is the most important step in the production of PHs to be used for plants. In the production of animal-derived PHs, chemical hydrolysis processes carried out in acidic, alkaline, or both conditions are more commonly used. Although acid hydrolysis is realized at high temperatures such as 120 °C and high pressure (about 220.6 kPa), alkaline hydrolysis is a simpler process that carries many advantages (Pasupuleti and Braun, 2010). In this study, we carried out hydrolysis processes on chicken feathers using a modified method employed by Taşkın and Kurbanoğlu (2011) and Taşkın et al. (2012), who applied KOH as a hydrolysis agent and then neutralized it with H_3PO_4. This process allows the formation of phosphate salt (KH_2PO_4) containing 2 macronutrient minerals (K and P) for plants. When the obtained CFPH was analyzed, we determined that the protein yield and the contents of amino acids resembled those of Taşkın and Kurbanoğlu (2011) (the relevant data are not reproduced here). The CFPH also contained almost all of the essential nutrients (macro and micro) that plants require (Taşkın et al., 2012). In this study, we determined that the total protein yield of the CFPH was 72.8% (w/w) and we identified 18 amino acids in the CFPH, including both basic and rare ones (Table 1). CFPH also contained some nonproteinogenic amino acids such as citrulline and ornithine at significant levels (Table 1). Therefore, in terms of these properties, we determined that CFPH hydrolyzed according to this method had the potential to be used as a suitable organic fertilizer for foliar applications in plant cultivation.

3.1. Effect of CFPH on plant growth parameters, soluble protein, and free sugar

Owing to the fact that soil microorganisms can interfere with the absorption of small peptides and amino acids, foliar application of PHs as biofertilizers has become increasingly common (Ashmead, 1986; Morales-Pajan and Stall, 2003). We therefore applied different aqueous solutions (0.05%, 0.075%, and 0.1% w/v) of CFPH to the seedling leaves of wheat cultivars Altındane and Bezostaya. After applying CFPH, the changes in plant dry and fresh weights and plant height parameters in the seedlings were determined. In scientific studies, evaluations of dry and fresh weights as well as plant height are among the parameters reflecting the growth and development of a plant and have been frequently used as important indicators after the application of organic fertilizers such as PHs. CFPH applications significantly increased root and shoot lengths in both cultivars compared to the controls (Table 2) and generally increased the parameters depending on the applied concentrations of CFPH. The same applications also increased the fresh and dry weights in both cultivars and, among the CFPH applications, the 0.075% concentration had the most pronounced effect (Tables 2 and 3). Our findings showed that foliar application of CFPH could promote the plant growth parameters studied in the wheat cultivars. Gousterova et al. (2012) studied the effect of CFPH on different parameters, including seed germination and growth of ryegrass, and reported that CFPH at a low concentration exhibited a remedial effect on the parameters. Our study also indicated that a particularly low concentration (0.075%) of CFPH had a more expressive effect on the growth parameters studied. Consistent with our results, other studies have shown that plants benefit most from PHs at low concentrations (Ertani et al., 2014; Colla et al., 2015). In addition, CFPH applications elevated the endogenous levels of soluble proteins and sugars in the same cultivars as compared to their controls (Table 2). The increases depended slightly on the concentrations of CFPH and a concentration of 0.075% had a more pronounced effect than other concentrations, especially on protein content. Schiavon et al. (2008) likewise reported that PH treatment could enhance the accumulation of free sugars and proteins in maize plants. PHs have also been determined to increase N assimilation and hence protein metabolism. The rich amino acid and mineral nutrient content of CFPH explains the ameliorative effect of its application on the parameters studied (Taşkın and Kurbanoğlu, 2011). In addition, we concluded that the chicken feather hydrolysis method we used in this study also contributed significantly to the effects of CFPH.

Table 1. Free amino acid composition of CFPH.

Amino acids	g/100 g	Amino acids	g/100 g
Ala	4.83	Leu	6.86
Arg	2.53	Lys	1.18
Asn	0.011	Met	0.42
Asp	4.51	Phe	3.78
Cys	0.15	Pro	13.1
Glu	8.63	Ser	5.40
Gln	0.00	Thr	1.07
Gly	6.65	Trp	0.028
His	0.32	Tyr	1.60
Ile	3.50	Val	5.40
Total protein (%)		Total N (%)	
72.8		11.7	

3.2. Effect of CFPH on photosynthetic pigments

To determine the effects of CFPH's foliar application on photosynthesis and thus to show that the changes in growth and development are related to photosynthetic activity, we measured both the chlorophyll and carotenoid contents and the expression level of RuBisCo in the

Table 2. Lengths of root and shoot (mm plant^{-1}), weights of fresh and dry (mg plant^{-1}), and contents (mg g^{-1} FW) of soluble protein and sugar.

	CFPH (%)	Root length	Shoot length	Dry weight	Protein	Sugar
Altındane	0.0	15.99 ± 0.28c	18.51 ± 0.28d	0.023 ± 0.0011b	19.72 ± 0.41c	5.97 ± 0.15c
	0.050	17.69 ± 0.27b	19.36 ± 0.21c	0.026 ± 0.0011ab	20.55 ± 0.63b	6.54 ± 0.16b
	0.075	18.41 ± 0.40ab	22.22 ± 0.50a	0.028 ± 0.0012a	21.44 ± 0.36a	7.22 ± 0.11a
	0.1	18.93 ± 0.35a	21.20 ± 0.46b	0.026 ± 0.0012ab	20.39 ± 0.44b	7.01 ± 0.15ab
Bezostaya	0.0	17.25 ± 0.23c	24.42 ± 0.25c	0.021 ± 0.0002c	16.47 ± 0.31c	7.66 ± 0.10c
	0.050	18.51 ± 0.33b	26.03 ± 0.28b	0.024 ± 0.0010ab	17.85 ± 0.33b	8.43 ± 0.09b
	0.075	19.28 ± 0.20a	27.70 ± 0.37a	0.026 ± 0.00016a	18.82 ± 0.35a	9.16 ± 0.11a
	0.1	19.36 ± 0.35a	27.79 ± 0.28a	0.023 ± 0.0004b	18.21 ± 0.21ab	9.35 ± 0.15a

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

Table 3. Contents of chlorophyll and carotenoid (mg g^{-1} FW).

	CFPH (%)	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll	Carotenoid
Altındane	0.0	3.35 ± 0.10c	2.04 ± 0.05c	6.54 ± 0.17c	0.86 ± 0.005c
	0.050	3.48 ± 0.06bc	2.16 ± 0.03b	6.89 ± 0.11b	0.87 ± 0.008bc
	0.075	3.66 ± 0.08a	2.30 ± 0.05a	7.29 ± 0.13a	0.90 ± 0.005a
	0.1	3.55 ± 0.07ab	2.22 ± 0.04ab	7.10 ± 0.07ab	0.89 ± 0.003b
Bezostaya	0.0	3.82 ± 0.08bc	2.22 ± 0.03c	7.20 ± 0.08c	0.94 ± 0.006c
	0.050	3.88 ± 0.05b	2.27 ± 0.02bc	7.33 ± 0.06bc	0.95 ± 0.004b
	0.075	4.06 ± 0.07a	2.42 ± 0.02a	7.74 ± 0.09a	0.99 ± 0.003a
	0.1	3.91 ± 0.04ab	2.30 ± 0.02b	7.45 ± 0.05b	0.97 ± 0.002a

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

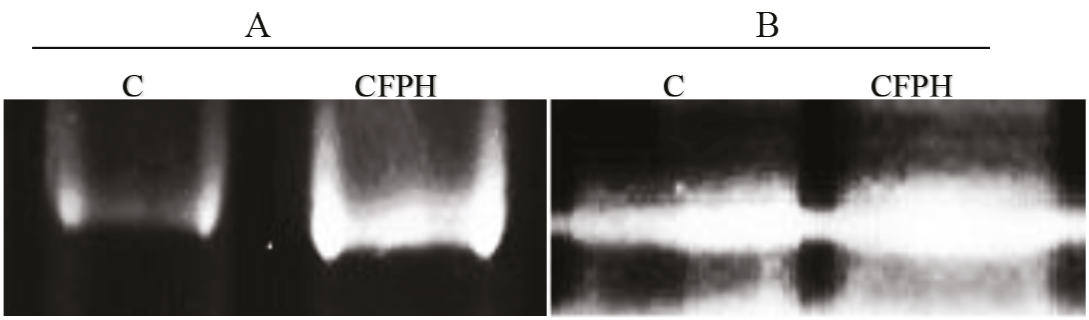

Figure 1. Effect of CFPH (0.1%) on the expression of RuBisCo in two cultivars of wheat. A- Altındane; B- Bezostaya; C- Control.

seedling leaves of wheat cultivars (Tables 2 and 3; Figure 1). RuBisCo expression level is directly related to plant yield and growth development. CFPH applications increased the chlorophyll (Chl-*a* and Chl-*b*) and carotenoid contents in both cultivars compared to their controls (Table 3). The increases in both parameters followed an almost regular uptrend that depended on the concentrations of CFPH and generally had the highest values at the 0.075% concentration. We found that CFPH applications had a nonsignificant influence on the Chl-*a*/*b* ratio (Table 3). The increased contents of total sugar, proteins (Table 2), and photosynthetic pigments (Table 3) explain the growth performance in the CFPH-applied wheat seedlings. Some researchers have reported findings that correspond with ours. For instance, studies have showed that animal-derived PH treatments promote chlorophyll content in crops including beans, corn, soybeans, and tomatoes (Kurbanoğlu et al., 2004; Horii et al., 2007; Cerdán et al., 2009). The mechanisms by which PHs enhance the content of photosynthetic pigments, such as chlorophyll and carotenoid, remain largely unclear (Horii et al., 2007). However, some researchers have asserted that cytokinin-like compounds in PHs trigger the biosynthesis of photosynthetic pigments (Vitoria and Mazzafera, 1997; Milazzo et al., 1999). Although we do not yet know whether CFPH contains cytokinin-like compounds, our research has shown that CFPH contains high levels of glutamate (8.63%) and proline (13.08%) (Table 1), which are also precursor amino acids involved in the biosynthesis of chlorophyll (Gough et al., 2003) and cytokinin (Milazzo et al., 1999). Proline as an amino acid has an important place in plant cells, and it is synthesized by a series of reduction reactions of glutamate and then stimulates certain anabolic pathways, including pentose phosphate, shikimate, and phenylpropanoid pathways (Hare and Cress, 1997; Andarwulan and Shetty, 1999). Milazzo et al. (1999) reported that elevated levels of endogenous proline in fish PH could bolster the production of cytokinin via the steps in the pentose phosphate pathway in melon (*Cucumis melo*). Based on the fact that the effect of CFPH on improving the enzymatic antioxidative parameters (Figures 2–6) was generally more pronounced at its 0.1% concentration, we preferred to measure the change in RuBisCo expression (the expression level of its large subunit by western blotting) at the concentration of 0.1% (Figure 1). It was shown that CFPH at 0.1% concentration significantly stimulated RuBisCo activity in both cultivars studied (Figure 1). This finding powerfully supports the contention that CFPH application improves carbon fixation and organic matter synthesis in photosynthesis. In addition, this finding can explain why CFPH applications generally increased the studied parameters, including plant length and dry weight, as well as protein, sugar, and carotenoid content of wheat cultivars; increased carbon assimilation via RuBisCo activity serves as an important indicator of plant growth and development for increased crop productivity (Parry et al., 2013). Evaluated together, the results suggest that CFPH application stimulates both the light and the carbon fixation reactions of the photosynthetic processes, resulting in increased protein and sugar content, and plant biomass (as plant length and dry weight).

3.3. Effect of CFPH on contents of ROS, MDA, proline, and phenolics

All of the CFPH applications significantly decreased the levels of ROS ($O_2^{\cdot-}$ and H_2O_2) and lipid peroxidation (as MDA) in both cultivars compared to their controls (Table 4). The concentration of 0.075% of CFPH was generally more effective on these parameters than other concentrations. Previous reports have indicated that fish PHs exhibit powerful antioxidant activity in in vitro conditions, including DPPH free radical-scavenging activity and reducing power (Fakhfakh et al., 2011; Gurav and Jadhav, 2013). However, although researchers have evaluated the response of antioxidant enzymes in PH-treated plants (Colla et al., 2015), they have not yet studied the levels of ROS ($O_2^{\cdot-}$, H_2O_2) and lipid peroxidation in the same plants. Reducing the ROS levels that are produced even in the course of normal metabolism in the cells is important because ROS products have the most damaging (and potentially lethal) effects on DNA structures, polyunsaturated lipids, and other biomolecules (Karuppanapandian et al., 2011). CFPH applications contributed to the consolidation of plant antioxidant responses by lowering both the ROS and lipid peroxidation levels in wheat cultivars. On the other hand, CFPH applications increased the content of free proline in wheat cultivars compared to their controls (Table 4). CFPH contains 13.1 g/100 g of proline and therefore the increase in proline levels in CFPH-treated wheat seedlings is not surprising. Previous studies have shown that exogenous application of PHs can elevate free proline content in plant tissues (Milazzo et al., 1999; Colla et al., 2015), and proline, proline analogs, or proline-rich PHs can stimulate plant growth and development (Milazzo et al., 1999; Kurbanoğlu et al., 2004). However, the reduction of phenolic content in the same plants treated with CFPH has been an interesting result (Table 4). There is a limited study asserting the reduction of phenolic content in animal-derived PH-treated plants (Horii et al., 2007), whereas there is intensive evidence that indicates increased phenolic content associated with PH treatment (Colla et al., 2015). In PH-treated soybean, for instance, phenolic content increased significantly, while it decreased in PH-treated tomato plants (Horii et al., 2007). Banana seedlings treated with feather PH that is rich in amino acids and minerals also exhibited an increase in the contents of phenolics and flavonoids (Gurav and Jadhav, 2013).

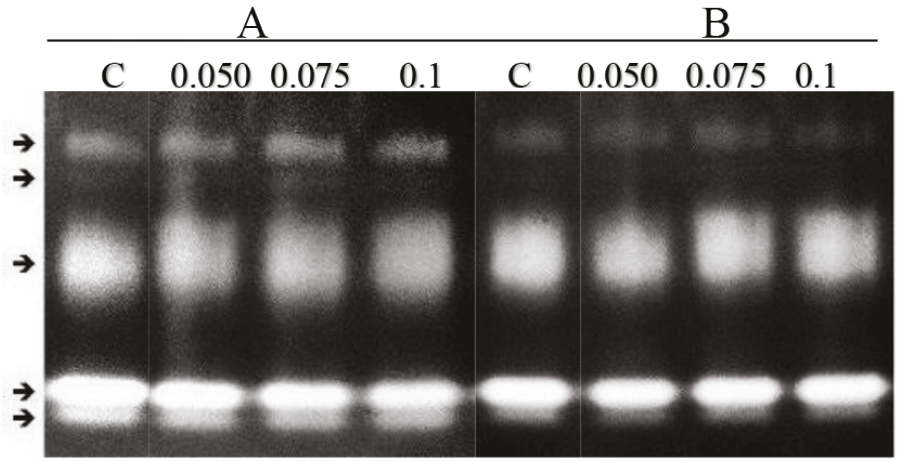

Figure 2. Effect of CFPH on the expression of SOD isoenzymes. A- Altındane; B- Bezostaya; C- Control.

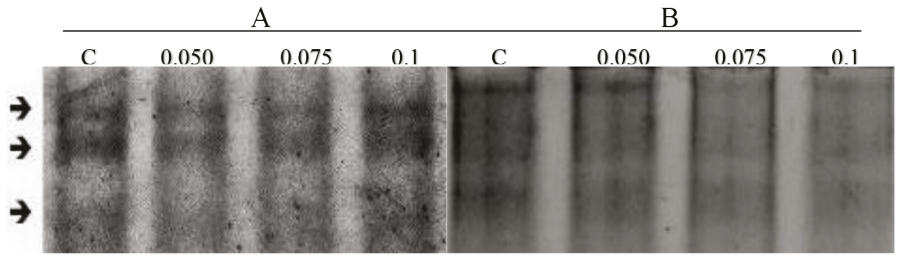

Figure 3. Effect of CFPH on the expression of CAT isoenzymes. A- Altındane; B- Bezostaya; C- Control.

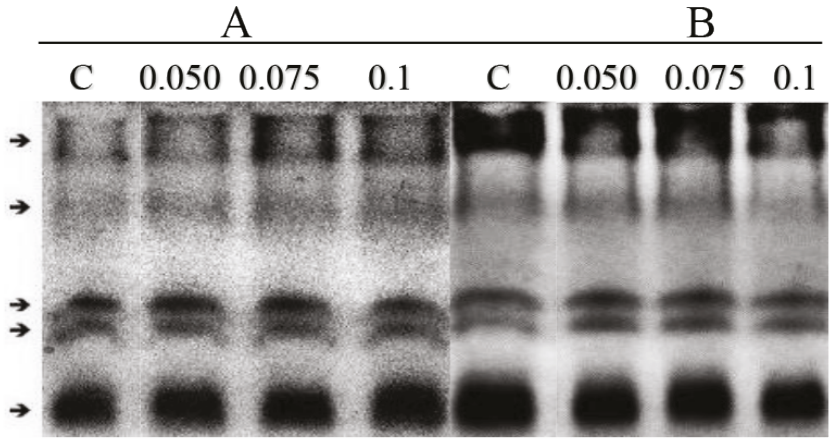

Figure 4. Effect of CFPH on the expression of GPX isoenzymes. A- Altındane; B- Bezostaya; C- Control.

Polyphenolic compounds are widely distributed in plants and are recognized as a type of secondary metabolites that have the most potent antioxidative properties (Colla et al., 2015). Especially under stress conditions, plant cells consume a significant proportion of the carbon (CO_2) assimilated during photosynthesis for the biosynthesis of secondary metabolites, including phenolics (Rice-Evans et al., 1997). We can thus draw the conclusion that the decreased ROS level (Table 4) and the increased enzymatic and nonenzymatic antioxidant capacity (Table 5) in CFPH-treated seedlings may explain the decrease in phenolic content (Table 4). The most important goal

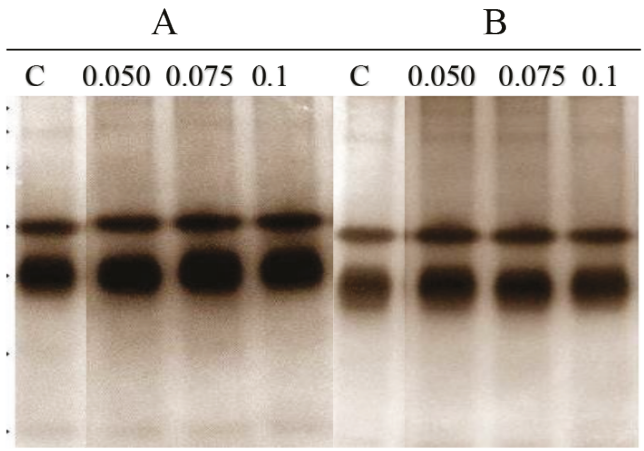

Figure 5. Effect of CFPH on the expression of APX isoenzymes. A- Altındane; B- Bezostaya; C- Control.

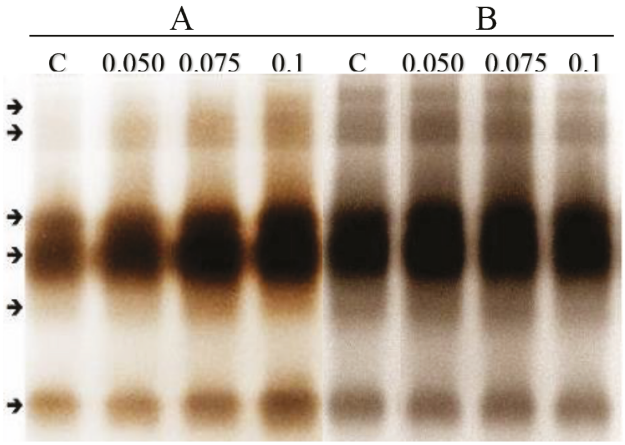

Figure 6. Effect of CFPH on the expression of GR isoenzymes. A- Altındane; B- Bezostaya; C- Control.

here could be that the carbon assimilated at a high cost in photosynthesis is used for the biosynthesis of primary metabolites (sugars, chlorophyll, and proteins), rather than secondary metabolites such as phenolics. Such a preference will further increase plant growth and development and consequently enhance plant productivity. Therefore, the decreased ROS level and the increased antioxidant capacity in the CFPH-treated wheat seedlings may be the reason for the decrease in phenolic content (Table 4). In our study, the increase in primary metabolite products, such as proteins (e.g., RuBisCo) and chlorophyll, via the application of CFPH supports this approach.

3.4. Effect of CFPH on enzymatic and nonenzymatic antioxidants

The evaluation of antioxidant system parameters serves as an important means of determining the possible effects of exogenous factors on a plant. For this reason, we determined the changes in both enzymatic (SOD, CAT, GPX, APX, and GR activities) and nonenzymatic (ASA, DHA, GSH, and GSSG) antioxidant parameters in the CFPH-applied wheat cultivars (Tables 5 and 6). CFPH applications did not significantly affect SOD and CAT activities in the Altındane and Bezostaya cultivars compared to the controls (Table 5). Our findings regarding SOD and CAT activities are illustrated in Figures 2 and 3, which show the accumulation of SOD and CAT isoenzymes. Figure 2, for instance, indicates that the SOD-3 isoenzyme slightly increased at 0.075% and 0.1% CFPH application in the Bezostaya cultivar. Figure 3, however, shows that CFPH at 0.075% and 0.1% slightly raised ($P < 0.05$) the CAT activity in the Altındane and Bezostaya cultivars. Feitosa de Vasconcelos et al. (2009) showed

Table 4. Contents of ROS (ng g^{-1}), MDA (nmol g^{-1}), phenolics, and proline (**µg** g^{-1}).

	CFPH (%)	$O_2^{\cdot-}$	H_2O_2	MDA	Proline	Phenolics
Altındane	0.0	35.1 ± 0.23a	51.4 ± 0.54a	2.97 ± 0.04ab	82.6 ± 3.4b	882 ± 24a
	0.050	32.1 ± 0.23b	51.6 ± 0.66a	2.85 ± 0.04b	88.5 ± 3.0abc	761 ± 13b
	0.075	30.8 ± 0.29c	49.3 ± 0.65b	2.60 ± 0.04c	96.7 ± 5.1a	707 ± 21b
	0.1	31.1 ± 0.24cb	51.1 ± 0.61a	2.73 ± 0.02c	94.9 ± 4.7ac	735 ± 26b
Bezostaya	0.0	27.1 ± 0.26a	45.7 ± 0.48a	2.67 ± 0.04a	108.5 ± 4.9b	958 ± 48a
	0.050	26.0 ± 0.11a	45.1 ± 0.65a	2.41 ± 0.03b	116.3 ± 4.3b	809 ± 23b
	0.075	24.2 ± 0.13b	42.6 ± 0.58b	2.12 ± 0.01c	139.6 ± 2.6a	829 ± 34b
	0.1	25.9 ± 0.15ab	43.1 ± 0.50b	2.30 ± 0.02bc	134.3 ± 4.6a	766 ± 34.b

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

Table 5. Activities (U mg $protein^{-1}$) of antioxidant enzymes.

	CFPH (%)	SOD	CAT	GPX	APX	GR
Altındane	0.0	35.28a	77.33ab	1643b	30.27b	9.16c
	0.050	34.94a	75.89b	1974a	40.68a	13.07b
	0.075	34.08a	79.08a	1932a	40.98a	14.85a
	0.1	33.44a	78.64a	2046a	41.25a	15.76a
Bezostaya	0.0	29.04a	64.86ab	1824a	39.08d	20.75b
	0.050	30.18a	63.22b	1721a	42.60c	25.70a
	0.075	30.46a	66.48a	1744a	44.78b	25.40a
	0.1	29.71a	65.14a	1718a	46.44a	25.75a

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

Table 6. Contents of nonenzymatic antioxidants (**µg** g^{-1} FW).

	CFPH (%)	Total ASA	ASA	DHA	ASA / DHA	Total GSH	GSH	GSSG	GSH / GSSG
Altındane	0.0	710b	445c	265a	1.68	2288b	1407d	820b	1.72
	0.050	924a	738b	186c	3.97	2864a	2048b	965ab	2.12
	0.075	997a	798a	198bc	4.03	3009a	2314a	1018a	2.28
	0.1	986a	758ab	231b	3.28	2822a	1974cb	955ab	2.07
Bezostaya	0.0	970c	660c	310a	2.13	1890b	1146bc	744a	1.54
	0.050	1054b	789b	266c	2.97	2392a	1777a	615b	2.89
	0.075	1181a	899a	282bc	3.19	2558a	1985a	573c	3.47
	0.1	1155a	859ab	296ab	2.91	2515a	1782a	734a	2.43

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

that the application of amino acid-based biostimulants increased SOD, CAT, and APX activities, helping plants overcome stressful conditions. In the Altındane cultivar, all CFPH applications stimulated GPX activity compared to the control, while GPX activity did not significantly change ($P < 0.05$) in the Bezostaya cultivar (Table 5). GPX activity reached its highest level with an increase of 25% at the concentration of 0.1% in the Altındane cultivar (Table 5). These findings are reflected in Figure 4, which shows the change in GPX isoenzymes. According to Figure 4, CFPH applications increased all GPX isoenzymes in the Altındane cultivar while slightly decreasing GPX-1 and GPX-5 isoenzymes in the Bezostaya cultivar (Figure 4). On the other hand, almost all CFPH applications gradually enhanced the APX and GR activities in both cultivars (Table 5). These findings are reflected in Figures 5 and 6, which show the changes in APX and GR isoenzymes, respectively. However, the isoenzyme findings indicate that CFPH-induced increases in APX and GR activity were more pronounced in the Altındane cultivar than in Bezostaya. The results show that CFPH applications generally increased antioxidant enzyme activities, and that the 0.1% concentration had a more pronounced effect than other concentrations on the increase in enzyme activities. Evaluated together, the findings obtained from both the ROS (Table 4) and the antioxidant enzyme activities (Table 5; Figures 2–6) indicate that CFPH application decreases ROS levels while increasing all enzyme activities except CAT in the wheat cultivars.

Nonenzymatic antioxidants, such as ASA and GSH, on the other hand, play a very important role both directly and indirectly in the detoxification of ROS. Among these substances, ASA and GSH crucially contribute to the continuation of cellular redox and the ascorbate-glutathione cycle, which detoxifies H_2O_2 (Karuppanapandian et al., 2011). We found that all of the CFPH applications significantly increased ($P < 0.05$) the levels of total ASA and ASA in both cultivars, whereas the same applications decreased the DHA level compared to the controls (Table 6). When analyzing the proportions of ASA/DHA, we determined that this finding was more obvious at 0.075% and 0.1% concentrations (Table 6). Interestingly, our analysis produced a similar result regarding the effect of CFPH applications on GSH and GSSG levels (Table 5). The AsA/DHA and GSH/GSSG ratios are among the best indicators of the ascorbate-glutathione circle. Increases in these ratios signal excess reductions in ASA and GSH, which scavenge ROS in cellular environments (Karuppanapandian et al., 2011). Our findings regarding the nonenzymatic antioxidants indicate that CFPH foliar applications stimulated AsA/DHA and GSH/GSSG ratios in both cultivars (Table 6). In a general sense, decreases in the ROS levels combined with increases in antioxidant enzyme activities suggest that CFPH plays a role in both protecting against damages from ROS and controlling antioxidative defense systems in plant cells.

In conclusion, based on our alkaline procedure with chicken feathers, we determined that CFPH includes 18 common and rare proteinogenic and 2 nonproteinogenic amino acids (citrulline and ornithine) at significant levels. Its foliar applications (0.05%, 0.075%, and 0.1%) to the leaves of wheat cultivars (Altındane and Bezostaya) promoted indicator parameters of plant growth and development in both cultivars. In addition, CFPH application increased chlorophyll and carotenoid pigment content. CFPH application at concentrations of 0.075% and 0.1% generally had the most pronounced effect on the studied parameters. The 0.1% concentration stimulated RuBisCo expression in the cultivars, and we deduced that CFPH application could stimulate carbon fixation and organic matter synthesis in photosynthesis. Furthermore, CFPH application, especially at the 0.075% concentration, decreased the levels of ROS ($O_2^{\cdot-}$ and H_2O_2) and LPO, and augmented the content of free proline while reducing that of phenolic compounds in the wheat cultivars. In contrast to the ROS parameters, CFPH application ameliorated the antioxidant system parameters, including enzymatic and nonenzymatic antioxidants. For example, CFPH application, especially at the 0.1% concentration, stimulated GPX, APX, and GR activities while not significantly affecting SOD and CAT activities in the two cultivars of wheat. The results obtained regarding these activities agreed well with the findings obtained from the electrophoretic isoenzyme profiles (native PAGE). In the wheat cultivars, CFPH application improved plant growth and photosynthetic parameters while consolidating the plant antioxidant system under examination.

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