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ORIGINAL RESEARCH PAPER in PHYSIOLOGY

Effects of the Protein Hydrolysate Pretreatment on Cucumber Plants Exposed to Chilling Stress

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Abstract

This study aimed to evaluate the effects of the protein hydrolysate Naturamin WSP on the antioxidant defense system and oxidation-related damage of young cucumber plants exposed to chilling stress. Low positive temperatures have a negative effect on plant growth and performance, and besides visible alterations, such as inhibited growth, significant changes occur at the cellular level. Plants grown at low temperature typically suffer from oxidative damage, which leads to increased lipid peroxidation. Moreover, chilling-stressed plants accumulate more proline to protect their cell membranes. The application of biostimulants such as the protein hydrolysate Naturamin WSP can alleviate some of the adverse effects caused by low temperature. Our results indicated an increased activity of guaiacol peroxidase (GPOD) in all plants treated with the biostimulant regardless of the temperature of cultivation. The mitigation of damages caused by chilling stress might be explained by an enhanced anti-oxidative defense, as demonstrated by the activity of guaiacol peroxidases and increased proline concentrations in Naturamin WSP-treated plants.

Keywords

antioxidative defense system; antioxidative enzyme; Cucumis sativus L.; low positive temperature stress; osmolytes; soluble protein

1. Introduction

Cucumber (*Cucumis sativus* L.) is an intensively cultivated salad crop worldwide. It is a cold-sensitive plant that suffers from low nonfreezing temperatures of approximately and below 10 °C (Shibaeva et al., 2018). Under the climatic conditions of Bulgaria, cucumber plants are exposed to chilling stress during winter months. Chilling stress could severely inhibit the growth and development of cucumber. Visible symptoms of chilling injuries include wilting, leaf and internal tissue discoloration, surface pits, and leaf necrosis (Lukatkin et al., 2012). The extent of the damage caused by chilling stress depends on temperature, duration of exposure, plant developmental stage, and other factors (Borowski, 2009; Phansak et al., 2021). The most frequently reported chilling-provoked changes at the cellular level include alterations in gene expression, protein turnover, lipid and carbohydrate composition, membrane permeability, and solute leakage (Kuk & Shin, 2007). Some of these changes and damages can be attributed to chilling-induced oxidative stress in the plant cells (Gill & Tuteja, 2010; Lukatkin, 2002).

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and their quenching in cells. Plant cells possess an effective anti-oxidative defense system that involves both nonenzymatic and enzymatic antioxidant components (Dumanović et al., 2021; Noctor & Foyer, 1998). Insufficient inactivation of ROS leads to oxidative damage, which affects macromolecules and

biological structures. Furthermore, such damage often results in enhanced lipid peroxidation and electrolyte leakage from the cells.

Currently, two main approaches are used to increase the tolerance of cucumber plants to chilling stress: (***i***) grafting on rootstocks from more chilling tolerant plant species such as pumpkin (Fu et al., 2021; Xu et al., 2017) and (***ii***) application of different products such as plant growth promoters, plant growth regulators, and biostimulants, with anti-stress properties (Borowski, 2009; Ghanbari & Kordi, 2019; Zhao et al., 2017).

The European Biostimulant Industry Council (EBIC) defines biostimulants as fertilizing products used for stimulating plant nutrition, apart from the nutrient content of the product, to improve one or more of the following characteristics of plants or plant rhizosphere: (***i***) nutrient use efficiency, (***ii***) tolerance to abiotic stress, (***iii***) quality, or (***iv***) availability of confined nutrients in the soil or rhizosphere (European Biostimulants Industry Council, 2019; Ricci et al., 2019).

Protein hydrolysates (PH) are one of the most common groups of biostimulants containing "mixtures of free amino acids, low molecular weight peptides, and other nitrogen-containing organic substances" (Colla et al., 2017). In recent years, the number of publications on the protective effects of protein hydrolysates on plants exposed to different stress factors has increased significantly (Colla et al., 2017; Nardi et al., 2016). However, information regarding the effects of protein hydrolysates on plants exposed to chilling stress is very limited. Recently, Cholakova-Bimbalova et al. (2019) reported that the application of a protein hydrolysate increased the photosynthetic performance of maize plants exposed to chilling stress.

One possible explanation for the protective effects of protein hydrolysates is related to the role of free amino acids and low-molecular-weight peptides as signal molecules and precursors for essential metabolites. Teixeira et al. (2017) showed that after foliar application of free amino acids (phenylalanine, cysteine, and glycine) to young soybean plants, the activities of some key antioxidative enzymes, such as superoxide dismutase, catalase, and peroxidase, increased significantly. However, the present information regarding the mode of action of protein hydrolysates in stress-exposed plants is still insufficient. The aim of the current study was to evaluate the effects of a protein hydrolysate on cucumber plants exposed to chilling stress, with special attention paid to their antioxidant defense system and oxidation-related damages.

2. Material and Methods

2.1. Growth Conditions and Experimental Design

Cucumber plants (*Cucumis sativus* L. 'Kaliopa') were grown in climatized growth chambers at the Department of Plant Physiology, Biochemistry, and Genetics, Agricultural University, Plovdiv. The seeds were imbibed in distilled water in a Petri dish for 24 hr and then sown in pots filled with perlite enriched with a modified Hoagland nutrient solution (1/2 strength). The volume of each pot was 2 L. The plants were cultivated in a controlled environment: photoperiod, 14/10 hr day/night; PPFD, 200 μmol m^{-2} s^{-1}; temperature, 25 ± 1 °C / 20 ± 1 °C (day/night), and relative air humidity, 60% ± 5%. Half of the plants were sprayed with 0.1% water solution of the biostimulant Naturamin WSP (a protein hydrolysate; Daymsa, Spain) when the first true leaf reached full development (18 days after germination). The concentration was chosen according to the manufacturer's recommendations. Control plants were sprayed with distilled water.

After treatment, the plants were transferred to two growth chambers with different temperature regimes and identical light and air humidity conditions. The experimental design of the study included four variants:

1. Untreated plants grown at 25 ± 1 °C / 20 ± 1 °C (day/night) (control).
2. Untreated plants grown at 10 °C.
3. Pretreated plants with Naturamin WSP grown at 25 ± 1 °C / 20 ± 1 °C (day/night).

4. Pretreated plants with Naturamin WSP grown at 10 °C.

The plants were grown under the aforementioned temperature regimes for 5 days and then cultivated at an optimal temperature (25 °C) for 7 days. Each variant had three replicates (pots), with three plants per pot. The duration of the experiment was 12 days. Biochemical analyses were performed on the cucumber plants at the end of the chilling exposure period and after 7 days of recovery. The experiment was repeated twice.

2.2. Plant Growth Analysis

The plants were harvested at the end of the experimental period and their fresh biomass and leaf area were determined. The leaf area of the plants was measured using a leaf area meter (NEO-2; Technical University, Bulgaria).

2.3. Soluble Protein Determination

Soluble protein content was determined according to Bradford (1976) using a standard curve prepared with bovine serum albumin and Coomassie blue, which reacts with basic amino acid residues, especially arginine, in response to protein concentration. Fresh plant samples (0.5 g) were ground with quartz sand, 5 mL ice-cold extraction buffer (pH 7.8), and 200 mg PVP (polyvinylpyrrolidone). Samples were centrifuged at 4 °C at 13,500 rpm for 10 min. The clear supernatant was used for analysis immediately. Absorbance was measured spectrophotometrically at 595 nm.

2.4. Guaiacol Peroxidase (GPOD) Activity

Guaiacol peroxidase (GPOD) activity was determined spectrophotometrically according to Bergmeyer et al. (1974). The reaction mixture, which contained 2.3 mL of KH_2PO_4 buffer (pH 7.0), 300 µL of H_2O_2, 300 µL of 8 mM guaiacol, and 100 µL of extract, was placed in a cuvette. Absorbance was measured at 436 nm against a blank with the same components but without the tissue extract (E = 26.6 mM cm^{-2}). The results are expressed as U mg g^{-1} fresh weight (FW).

2.5. Lipid Peroxidation

Lipid peroxidation was estimated according to the method of Heath and Packer (1968) by determining thiobarbituric acid (TBA) reactive compounds. The plant leaves were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 10,000 rpm for 20 min. One milliliter of the supernatant was mixed with 4 mL of TCA (20%) containing 0.5% w/v of TBA. The mixture was heated at 95 °C for 30 min, cooled on ice, and centrifuged at 10,000 rpm for 15 min. The absorbance was measured at 532 nm and corrected at 600 nm. The concentration of TBA reactive compounds was calculated using the 155 mM cm^{-1} extinction coefficient.

2.6. Proline Content

Proline content was determined colorimetrically using Toluol according to Bates et al. (1973). One gram of the primary leaves was homogenized with 3% sulfosalicylic acid and centrifuged at 10,000 rpm for 10 min. The absorbance of each extract was measured spectrophotometrically at 520 nm.

2.7. Statistical Analysis

Statistical analysis of the data was performed using one-way ANOVA (for $p < 0.05$) and main comparison at a 95% confidence level was performed using Tukey's test.

3. Results

As expected, the cucumber plants exposed to chilling stress showed retarded growth and partial wilting. The results of the biometric parameters are presented in Table 1. The untreated plants grown under the chilling temperature regimes had the lowest fresh biomass and leaf area (Variant 2). The plants exposed to chilling stress and pretreated with Naturamin WSP (Variant 4) showed relatively higher values of fresh weight and leaf area than the plants of Variant 2. A similar trend was observed after the recovery period. The values of the fresh biomass and leaf area of plants grown at 25 °C and treated with Naturamin WSP were significantly higher (Variant 3) than those of other variants.

The soluble protein contents in the leaves of cucumber plants are presented in Table 2. Exposure of the plants to chilling stress reduced the protein content in their leaves (Variant 2) compared to the leaves of control plants (Variant 1). The reductions were 6% and 9% during the chilling stress and recovery periods, respectively.

Pretreatment with the biostimulant Naturamin WSP preserved, to some extent, the protein content in the leaves of plants exposed to chilling stress (Variant 4). Their protein content was significantly higher (by 10%) compared to that of untreated plants exposed to chilling stress (Variant 2) at the end of the stress period. However, pretreatment with the biostimulant increased the protein content in the leaves of plants grown at 25 °C (Variant 3) by 7%–19%.

Chilling stress significantly increased the content of proline (Table 3) in the leaves of the cucumber plants by 26% (Variant 2) compared to the control plants (Variant 1).

Pretreatment with Naturamin WSP increased the proline content in plants exposed to chilling stress (Variant 4). It was 17% higher than that of plants exposed to chilling stress without Naturamin WSP treatment (Variant 2). The proline content in the leaves of nonstressed plants pretreated with the biostimulant (Variant 3) was higher than that in the control plants but lower than that in plants exposed to chilling stress. The proline accumulation observed in the leaves of cucumber plants during the chilling period was similar to that observed at the end of the recovery period.

Table 1 Fresh weight (FW, in g) and leaf area (LA, in cm^2) of cucumber plants (cv. Kaliopa) grown at different temperature regimes with or without Naturamin WSP treatment.

Variants	Exposure period		Recovery period	
	FW	LA	FW	LA
(1) 25 °C (control)	5.54a	105.0a	8.40a	165.0b
(2) 10 °C	3.87c	85.7c	4.52c	98.2d
(3) 25 °C + Naturamin WSP	5.85a	112.6a	8.95a	187.1a
(4) 10 °C + Naturamin WSP	4.12b	91.1b	4.98b	105.0c

The different superscript letters after the mean values (a, b, c, d) indicate statistically significant differences between variants at $p < 0.05$.

Table 2 The soluble protein content (mg g^{-1} fresh weight) in the leaves of cucumber plants (cv. Kaliopa) grown at different temperature regimes with or without Naturamin WSP treatment.

Variants	Exposure period	Recovery period
(1) 25 °C (control)	2.73c	3.19b
(2) 10 °C	2.57d	2.89c
(3) 25 °C + Naturamin WSP	3.26a	3.42a
(4) 10 °C + Naturamin WSP	2.82b	2.98c

The different superscript letters after the mean values (a, b, c, d) indicate statistically significant differences between variants at $p < 0.05$.

Table 3 The proline content (μg g^{-1} fresh weight) in the leaves of cucumber plants (cv. Kaliopa) grown at different temperature regimes with or without Naturamin WSP treatment.

Variants	Exposure period	Recovery period
(1) 25 °C (control)	80.78d	83.91d
(2) 10 °C	102.03b	117.81a
(3) 25 °C + Naturamin WSP	90.16c	93.59c
(4) 10 °C + Naturamin WSP	119.69a	124.69a

The different superscript letters after the mean values (a, b, c, d) indicate statistically significant differences between variants at $p < 0.05$.

Table 4 Lipid peroxidation rate (nmol MDA g^{-1} FW) in the leaves of cucumber plants (cv. Kaliopa) grown at different temperature regimes with or without Naturamin WSP treatment.

Variants	Exposure period	Recovery period
(1) 25 °C (control)	7.82d	5.66d
(2) 10 °C	13.72a	10.08a
(3) 25 °C + Naturamin WSP	9.84c	6.27c
(4) 10 °C + Naturamin WSP	10.62b	9.45b

The different superscript letters after the mean values (a, b, c, d) indicate statistically significant differences between variants at $p < 0.05$.

The effects of chilling and biostimulant pretreatment on lipid peroxidation in leaves are presented in Table 4. The lowest content of TBA-reactive compounds was found in the leaves of the control plants (Variant 1). After the 5-day chilling period, the content of TBA-reactive compounds in the leaves was 75% higher (Variant 2) than that in the control (Variant 1). In the plants exposed to chilling stress, a higher level of TBA-reactive compounds was observed during the recovery period.

The content of TBA reactive compounds was higher in the leaves of plants exposed to chilling stress and pretreated with Naturamin WSP (Variant 4) than that in the leaves of control plants but was significantly lower (23%) than that in the leaves of Variant 2. Interestingly, the application of the biostimulant enhanced the content of TBA reactive compounds in the leaves of plants grown at 25 °C by 26% (Variant 3), but the effect reduced during the recovery period (11%).

The GPOD activity in the leaves of cucumber plants is presented in Table 5. Chilling stress more than doubled the GPOD activity of Variant 2 compared to that of the control (Variant 1). This effect was maintained during the recovery period. Interestingly, the foliar application of Naturamin WSP considerably enhanced GPOD activity compared to the variants without Naturamin WSP treatments (Variants 1 and 2) regardless of the temperature regime (Variants 3 and 4).

Table 5 The activity of guaiacol peroxidase (U g^{-1} fresh weight) in the leaves of cucumber plants (cv. Kaliopa) grown at different temperature regimes with or without Naturamin WSP treatments.

Variants	Exposure period	Recovery period
(1) 25 °C (control)	2.02d	1.95d
(2) 10 °C	4.31c	3.97c
(3) 25 °C + Naturamin WSP	8.82a	5.26b
(4) 10 °C + Naturamin WSP	8.21b	5.57a

The different letters after the mean values (a, b, c, d) indicate statistically significant differences between variants at $p < 0.05$.

4. Discussion

This study aimed to evaluate the effects of the protein hydrolysate Naturamin WSP on the growth and performance of cucumber plants exposed to chilling stress. The results showed that a 5-day-long chilling stress caused significant growth retardation (Table 1) and oxidation-related damage to the leaves of the plants. Lower biometric parameters (Table 1), reduced leaf protein content (Table 2), and enhanced lipid peroxidation rate (Table 4) were indicators of this damage in cucumber plants exposed to chilling stress. These results agree with earlier data on the effects of chilling stress on cucumber plants (Borowski, 2009; Ghanbari & Kordi, 2019; Shibaeva et al., 2018). In addition, our study demonstrated that the pretreatment of the cucumber plants with the biostimulant Naturamin WSP alleviated, to some extent, the negative effects of chilling stress on plant growth (Table 1) and affected several biochemical parameters (Table 2–Table 5). These observations corroborate the results of Botta (2013) and Cholakova-Bimbalova et al. (2019), who reported a positive influence of foliar amino acid application on lettuce and maize plants exposed to chilling stress, respectively.

Chilling stress can cause several morphological and functional disorders in plants. The most frequently reported negative effects of chilling stress are loss of membrane integrity and solute leakage, which are common consequences of oxidative stress in plant cells (Gill & Tuteja, 2010; Lukatkin, 2002). Therefore, it seems reasonable to explain the observed positive effects of the biostimulant Naturamin WSP, mainly through the support of the cellular antioxidative defense system of cucumber plants exposed to chilling stress.

Peroxidases are well-known enzymes whose activities are enhanced in plants under oxidative stress. They detoxify H_2O_2 using a vast array of aromatic substrates. The GPOD activity in the leaves of cucumber plants exposed to chilling stress was strongly enhanced (Table 5). This stimulation may be explained by the possible upregulation of enzyme gene expression, which has been recently demonstrated in cucumber plants exposed to chilling stress (Pan et al., 2020).

Different plant biostimulants, including protein hydrolysates, have antioxidant properties (Tkaczewska et al., 2020), and they can modulate plant anti-oxidative defense network. Teixeira et al. (2017) revealed that the foliar application of single and combined amino acids can act as signaling molecules in soybean plants, and the application of small doses was sufficient to increase the activity of antioxidative enzymes. Ertani et al. (2013) and Rouphael et al. (2017) reported that protein hydrolysates can modulate peroxidase activity, mitigating the growth inhibition of salt- and alkaline-stressed plants. Therefore, the biostimulant-induced increase in GPOD activity in the cucumber plants could be explained by the aforementioned properties of protein hydrolysates.

Plants accumulate water-soluble compounds with low molecular weights, called osmolytes, to overcome the negative effects of different environmental stress factors. This group of substances includes betaines, sugars, polyols, polyamines, and amino acids (mostly proline) (Giri, 2011). Proline has multifunctional roles: not only is it a compatible osmolyte, it is also an effective scavenger of reactive oxygen species. It is also used as a metabolite that stabilizes subcellular structures, modulates cell redox homeostasis, supplies energy, and can act as a signaling molecule in a range of metabolic pathways under stress conditions (Ejaz et al., 2020). Therefore, we assumed that chilling stress could lead to a significant increase in proline accumulation in the leaves of plants exposed to chilling stress (Table 3).

Plant biostimulants, including protein hydrolysates, have been shown to modulate proline accumulation in plants exposed to different stress factors (Colla et al., 2017; Ertani et al., 2013). Other studies have revealed that the application of protein hydrolysates could stimulate carbon and/or nitrogen metabolism, even in oxidative stressed plants (Rouphael et al., 2017). Therefore, we support the report of Omoarelojie et al. (2021) that biostimulants may contribute to proline accumulation in plants exposed to stress by providing the necessary metabolites (glutamate or ornithine) for its biosynthesis.

5. Conclusion

Protein hydrolysates are a promising group of substances that can mitigate the adverse effects of low-temperature stress in susceptible plant species such as cucumber. The plant-derived product named Naturamin WSP can alleviate, to some extent, the negative impact of oxidative stress caused by low temperatures. Our results indicate that the activation of the antioxidant enzyme system in the biostimulant-treated cucumber plants, as demonstrated by the enhanced activity of guaiacol peroxidases and the higher content of the amino acid proline, have a role in the mitigation of chilling stress damage in young cucumber plants.

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