Effect of protein hydrolysate-based biostimulants on chlorophyll content in wheat leaves

Wpływ biostymulatorów białkowych na zawartość chlorofilu w liściach pszenicy

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Abstrakt

W artykule zbadano wpływ dolistnego nawożenia biostymulatorów białkowych na poziom chlorofilu i karotenoidów w pszenicy ozimej. Otrzymane wyniki wskazują na znaczący wzrost barwników fotosyntetycznych dla próbek II i III, które stanowiły rośliny traktowane biostymulatorami zawierającymi hydrolizat kolagenu i salicylan sodu oraz hydrolizat kolagenu i keratyny w połączeniu z askorbinianem tytanu (odpowiednio Bio-2 i Bio-3). W pracy potwierdzono również, że efektywność ekstrakcji chlorofilu i karotenoidów zależy od rodzaju rozpuszczalnika. Uzyskane wyniki pozwalają sformułować wniosek, iż najkorzystniejszym rozpuszczalnikiem do ekstrakcji barwników fotosyntetycznych z liści pszenicy jest etanol.

Abstract

This paper investigates the effect of foliar fertilisation with protein biostimulators on chlorophyll and carotenoid levels in winter wheat. The results obtained show a significant increase in photosynthetic pigments for samples II and III, which were plants treated with biostimulants containing collagen hydrolysate and sodium salicylate and collagen and keratin hydrolysate in combination with titanium ascorbate (Bio-2 and Bio-3, respectively). The study also confirmed that the extraction efficiency of chlorophyll and carotenoids depends on the type of solvent. The results obtained allow the conclusion to be drawn that the most favourable solvent for the extraction of photosynthetic pigments from wheat leaves is ethanol.

Słowa kluczowe: biostymulatory, pszenica ozima, chlorofile, karotenoidy, analiza spektrofotometryczna

Keywords: biostimulants, winter wheat, chlorophylls, carotenoids, spectrophotometric analysis

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1. Introduction

In recent years, the growth and productivity of crop plants have been significantly affected by abiotic stresses. Regions with significant crop production, such as central Europe, south-central Asia, south-eastern South America and the south-eastern United States, are facing increasingly frequent occurrences of high temperatures and drought [1]. Biostimulants are essential for sustainable crop production in the face of climate change. Natural products such as seaweed extracts, humic substances, hydrolysate proteins and products containing amino acids or microorganisms contain bioactive substances that can improve nutrient efficiency, abiotic stress tolerance and/or crop quality traits, irrespective of their nutrient content [2,3]. Biostimulants also contribute to improved seed germination and induce plant biological activity [4]. One type of biostimulant is amino acids, which are produced by chemical synthesis or by hydrolysis of plant and animal proteins. Amino acids play a key role in construction, metabolism and transport. Tryptophan is known to be a precursor of the hormones responsible for stem and root elongation [5]. Glycine and glutamic acid are essential substrates for tissue formation and chlorophyll synthesis [5]. Proline, however, affects pollen fertility, thereby contributing to an increased yield [5]. Amino acids allow plants to make better use of their natural production potential, which is often suppressed by various stresses (e.g. drought, hail, frost). Supplying the plant with amino acids in the form of ready-to-use preparations during or before a biotic or abiotic stress factors ensures that the plant develops evenly and efficiently despite adverse conditions [6].

Chlorophylls are a widespread group of photosynthetic pigments found in higher plants, algae and cyanobacteria [7]. Chlorophyll molecules are esters of a dicarboxylic acid (chlorophyllins) and four five-membered pyrrole rings linked by methyl groups (-CH=) (protoporfirin ring) [8]. Chlorophyll is an essential pigment that plays a key role in the normal course of photosynthesis, in which the conversion of light energy into chemical bond energy is enabled by the absorption of light

quanta in redox reactions [7, 9]. Numerous genetic, morphological, physiological and abiotic factors affect its content in plant leaves [8]. Therefore, a change in chlorophyll content is one of the most obvious symptoms of plant stress [10]. The absorption properties of pigments allow their qualitative and quantitative analysis [8]. Both chlorophyll and carotenoids are lipid soluble compounds. Therefore, they can be extracted from living plant tissue containing water using organic solvents such as acetone, alcohols, ethers [11]. To accurately and precisely determine the content of chlorophylls and carotenoids, they must first be extracted from a fresh sample of plant tissue. The most common method for the determination of photosynthetic pigments in plant material is their extraction with solvents followed by spectrophotometric analysis. This method is reliable but time-consuming and requires high precision [8]. Many factors can affect the activity of a solvent, including the time needed for extraction, the amount of plant material or the percentage of moisture in the plant material form [12]. The efficiency of plant dye extraction depends on the type of solvent, which should selectively absorb the chemical compound of interest and not (or only slightly) absorb the other compounds present in the sample. The choice of solvent is therefore crucial [13]. The solvent extraction methods using polar aprotic solvents like acetone, dimethyl sulfoxide (DMSO), and N, N-dimethylformamide (DMF) and polar protic solvents like ethanol and methanol have been utilized widely [14].

The aim of the study was to investigate the effect of foliar application of protein biostimulants on the chlorophyll and carotenoids contents in winter wheat seedlings. In addition, the study tested several common organic solvents used for the determination of photosynthetic pigments in order to select the most effective one for wheat.

2. Materials and methods

2.1. Plant material

The study material consisted of 30-days winter wheat seedlings (*Triticum aestivum* L.) grown under controlled conditions in a Royal Room growbox (200x200x100cm). The trial was carried out in the Łukasiewicz Research Network – Lodz Institute of Technology in Lodz, Poland. The winter wheat seed was obtained from Plant Breeding Strzelce (Poland). The wheat was grown in 58x40.5x7cm plastic trays (approximately 180 seeds per tray) in horticultural soil (pH: 5.5-6.5, EC<1.5 mS/m, organic matter: 2%). Plant growth conditions during the experiment included 6 hours of artificial lighting (Lumatek Attis 200W LED FULL SPECTRUM ATS200W), day/night temperature 21/19ºC respectively and relative humidity around 50%. At the 3-4 leaf emergence stage (about 14 days after sowing), foliar application of biostimulants prepared by the Łukasiewicz-Lodz Institute of Technology (Łukasiewicz-LIT) from Poland was carried out. The physical and chemical properties of three biostimulant formulations (Bio-1, Bio-2, Bio-3) are presented in Table 1. During the experimentation, solutions of biostimulants including protein hydrolysates obtained through scientific collaboration with the Leather and Footwear Research Institute Division (INCDTP, Romania), titanium ascorbate synthesized in the Łukasiewicz-LIT, and commercially available sodium salicylate obtained from Pol-Aura Sp. z o.o. (Poland) were used. The antifungal preparation Afrodyta was applied together with the biostimulant (in the dose recommended by the manufacturer). The nitrogen content of the samples was determined using the Kjeldahl method. The plants were collected 30 days after sowing and the chlorophyll and carotenoids of the shoots were determined spectrophotometrically. Samples: SI, SII and SIII are treated with Bio-1, Bio-2 and Bio-3 biostimulants, respectively. The control sample of the experiment were untreated plants (C).

Tab. 1. Composition of applied biostimulants

No.	Protein Hydrolysate	Active Substance	Total N	Code
1	Collagen (0.50%)	Sodium salicylate (0.03%)	1.35%	Bio-1
2	Collagen (0.50%)	Titanium ascorbate (0.01%)	1.00%	Bio-2
3	Keratin (0.50%), Collagen (0.50%)	Sodium salicylate (0.03%)	1.20%	Bio-3

2.2. Photosynthetic pigment contents determination

Photosynthetic pigment contents determinations were conducted by selecting fully developed wheat leaves at random from each wheat variety cultivated during the experiment. For the measurement of chlorophyll and carotenoids, 0.5 g of fresh leaves were used. The leaves were crushed in a mortar with 3 ml of solvent, and a small amount of sand and $CaCO_3$. The solution was quantitatively transferred to centrifuge tubes by rinsing the mortar and pestle with 2 ml of solvent. The homogenized sample mixture was centrifuged at 10 000 rpm for 10 minutes at room temperature. Then 0.5 ml of the supernatant was removed and 4.5 ml of solvent was added. Chlorophyll content was determined using UV-9200 by RayLeigh spectrophotometer at wavelengths appropriate to the solvent used. All chemicals and solvent were of analytical grade. The contents of chlorophyll a, chlorophyll b and carotenoids were calculated according to formulae available in the literature [15-17]. These formulae are shown in Table 2.

Tab. 2. Formulas for determining the contents of photosynthetic pigment

Type of solvents	Equation/Formula
Acetone	$Ch_a=12.25A_{663} - 2.79A_{645}$ $Ch_b=21.5A645-5.1A665$ $C_{x+c}= (1000A_{470}-1.82C_a-85.02C_b)/198$
Ethanol	$Ch_a=13.36A_{663}-5.19A_{645}$ $Ch_b =27.43A_{645}-8.12A_{663}$ $C_{x+c}= (1000A_{470}-2.13C_a-97.63C_b)/209$
Diethyl-ether (DEE)	$Ch_a=10.05A_{663}-0.97A_{645}$ $Ch_b=16.36A_{645}-2.43A_{663}$ $C_{x+c}= (1000A_{470}-1.43C_a-35.87C_b)/205$

3. Results and discussion

The experiments investigated the effect of foliar application of biostimulants developed at Łukasiewicz-LIT (biostimulant composition in Table 1) on photosynthetic pigment contents in wheat leaves. The contents of chlorophyll a, chlorophyll b and carotenoids was determined in each of the samples tested (SI, SII, SIII and C) in the spectrophotometric analyses. In addition, in order to select the optimum solvent for the extraction of these pigments from wheat leaves, the study compared the effectiveness of three commonly used solvents, e.i. acetone, ethanol and diethyl ether (DEE). The results of the spectrophotometric analyses are summarized in Table 3. The concentration of photosynthetic pigments was determined from the absorbances measured, according to the formulae in Table 2.

Tab. 3. Spectrophotometric determination of absorbance for chlorophyll a, chlorophyll b, carotenoids with various extracting solvents.

Sample	Type of solvents								
	Acetone			Ethanol			Diethyl-ether		
	A663	A645	A470	A663	A645	A470	A663	A645	A470
SI	0.31±0.01	0.10±0.01	0.26±0.01	0.59±0.01	0.20±0.01	0.56±0.01	0.58±0.05	0.19±0.05	0.37±0.02
SII	0.57±0.02	0.19±0.01	0.47±0.01	0.82±0.02	0.28±0.01	0.75±0.03	1.46±0.20	0.51±0.02	0.99±0.05
SIII	0.59±0.02	0.20±0.01	0.50±0.02	0.68±0.02	0.23±0.01	0.66±0.02	1.51±0.25	0.53±0.05	0.99±0.04
C	0.27±0.01	0.10±0.01	0.24±0.01	0.42±0.01	0.15±0.01	0.41±0.01	0.32±0.01	0.12±0.01	0.23±0.02

Figure 1 compares the content of chlorophyll a in all the samples tested after application of the biostimulants, depending on the solvent used. The main conclusion from this experiment is that the highest contents of chlorophyll a values were obtained for SII and SIII. The obtained results most probably result from the biostimulating properties of the applied substances. SII were treated with a Bio-2 containing collagen and titanium ascorbate. Literature data indicate that collagen hydrolysate is primarily a source of nitrogen for plants. Furthermore, the composition comprises amino acids such as glycine, serine, and proline, which exhibit heightened accumulation in response to environmental stresses within plants [18]. Collagen is a fibrous protein with amphiphilic properties and the ability to

buffer pH fluctuations, chelate micronutrient ions, adhere to leaves, and act as a reservoir of organic nitrogen and amino acids. In addition, titanium ascorbate was used as an active ingredient in Bio-2. The literature acknowledges the positive impacts of titanium ascorbate on plants, leading to increased iron ion activity, enhanced pollen grain vigour and a higher nutrient uptake rate [19]. The effects of foliar-applied organic titanium salts on plants are documented extensively in review publication [20].

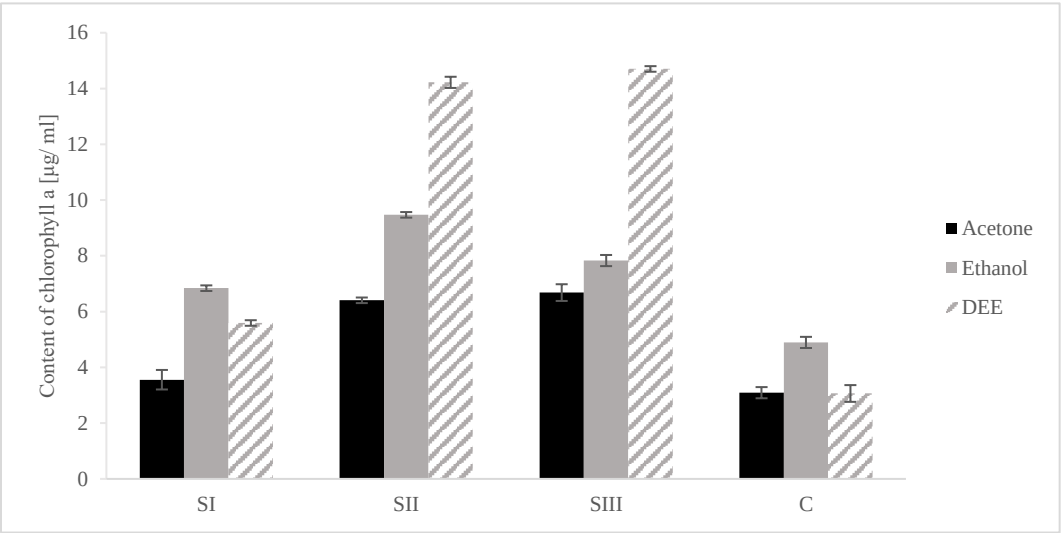

Fig. 1. Comparison of chlorophyll a content in the tested sample after application of biostimulants depending on the solvent used

On the other hand, SIII were treated with Bio-3, which contained collagen, keratin and sodium salicylate. The hydrolysate proteins used provide a rich source of nitrogen in the formula. Keratin is rich in cysteine (a sulphur-containing amino acid), which distinguishes it from other biopolymers. The cysteine and cystine content of the amino acid sequence is 7-12%. Keratin preparations exhibit a substantial concentration of amino acids, namely, glycine, alanine, serine, and valine. Meanwhile, their composition of methionine, lysine and tryptophan is relatively low [21]. An additional active ingredient utilized in the formulation Bio-3 is salicylic acid, an endogenous plant growth regulator with the capacity to

stimulate the systemic acquired resistance (SAR). This substance regulates processes including seed germination, growth of roots and leaves, synthesis of proteins and chlorophyll, flowering of plants, resistance to pathogens, as well as the transport of metabolites, particularly divalent metal cations [22]. Salicylic acid stabilizes cell membrane structure and permeability, facilitating increased nutrient uptake by increasing nitrate concentration in the plant. Research indicates that salicylic acid and its derivatives have a beneficial effect in reducing the impact of abiotic stressors on the growth, development and yield of plants [23]. Another important observation was that there are considerable differences in chlorophyll a concentration depending on the solvent used. Similar observations have been made in several reports [24,25,26]. In most cases, the highest extraction of selected pigments is observed with the DEE solvents, especially in SII and SIII. In other cases, the results obtained for DEE and ethanol are at a similar level and were 5.59 µg/ml (DEE) and 6.84 µg/ml (ethanol) for sample I and 3.06 µg/ml (DEE) and 4.89 µg/ml (ethanol) for the control sample. This observation is supported by the polar nature of the chlorophyll molecule, which allows greater solubility in DEE, which is characterized by a large dipole moment [25]. Similar observations were made for chlorophyll b for each sample.

In turn, Figure 2 showed the content of carotenoids in all the samples tested after application with the biostimulants, depending on the used solvent. The results collected in this graph show a similar relationship as in Figure 2. The highest values of photosynthetic pigment were obtained for SII and SIII, confirming the positive effect of the biostimulants. Much lower carotenoids contents were obtained in the C and SI samples.

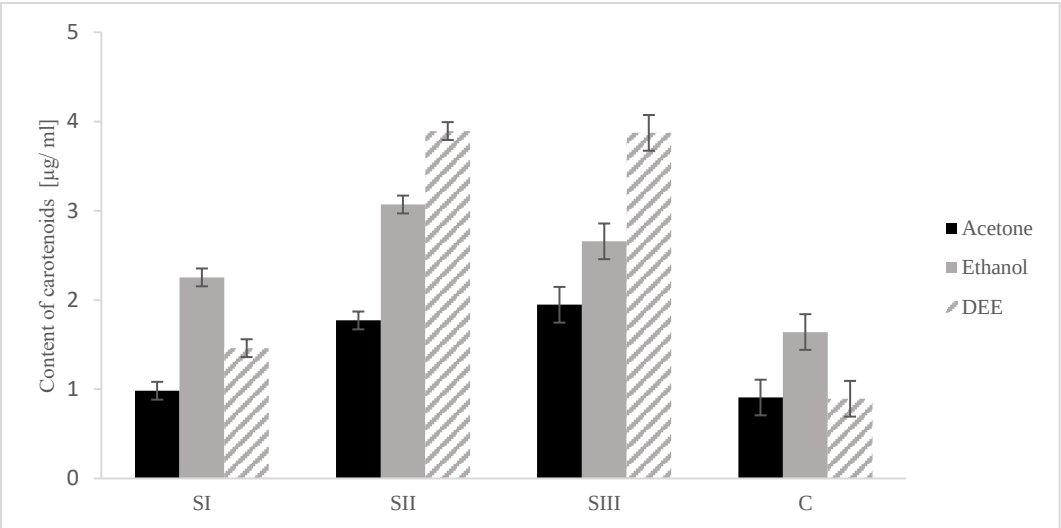

Fig. 2. Comparison of carotenoids content in the tested sample after application of biostimulants depending on the solvent used

The next part of the research was to compare commonly used organic solvents in order to select the most appropriate extractant for the determination of photosynthetic pigment contents in wheat leaves. Standardization of chlorophyll extraction is very difficult due to the variable chlorophyll content in leaves and between different plants. In addition, extraction efficiency is influenced by solvent polarity, solution pH, light, temperature. Studies Saito [27] showed the dependence of the absorption coefficients of pure chlorophyll a and chlorophyll b on the used solvent . In addition, trends in solvent usability vary according to the plant species studied. When choosing a solvent, a compromise must be made between selecting a solvent for efficient quantitative extraction of chlorophyll and using a solvent best suited for spectrophotometric assays. For example, acetone is an ideal solvent for chlorophyll determination as it gives very sharp chlorophyll absorption peaks [24, 28]. However, this compound is a poor extractant of chlorophyll for some plants. Furthermore, acetone is volatile and highly flammable. In addition, acetone reacts with polystyrene and polymethylacrylate making latex or plastic gloves and plastic cuvettes unsuitable [25]. Solvents such as methanol and ethanol are often more

effective extractants than acetone [10,24]. The toxicity of methanol unfortunately rules out this solvent in terms of safety for the test worker. A much safer solvent for chlorophyll determination is ethanol [25]. Although flammable, it is not highly toxic and is suitable for use in the laboratory [24]. A fourth common solvent used for green dye assays is diethyl ether (DEE) [25]. It is not the solvent of first choice for laboratory work because it is volatile, flammable, explosive and narcotic. Furthermore, it should be noted that plastic cuvettes and the majority of plastic laboratory equipment lack resistance to diethyl ether [25]. Other solvents used for the determination of chlorophylls in leaves can be found in the literature, such as chloroform, dimethyl sulphoxide (DMSO) and dimethyl formamide (DMF), but are not routinely used [29].

Acetone, ethanol and diethyl ether were used to extract photosynthetic dyes. In all samples tested, the contents of chlorophyll a were higher than those of other photosynthetic pigments (Fig. 3). For the control sample and for SI, the highest content of chlorophyll a during extraction with ethanol was observed, respectively 4.89 μg/ml and 6.84 μg/ml. However, for SII and SIII, the highest chlorophyll a content was determined using DEE. The most effective extractant chlorophyll b was found to be DEE. The contents of chlorophyll b varied from 1.13 μg/ml. for the control sample to 4.99 μg/ml for SIII. The effectiveness of the organic solvents used in this case presents itself in the following order: DEE>acetone>ethanol. In contrast, the highest carotenoid concentrations were determined during extraction of DEE (3.89 μg/ml SII and 3.87 μg/ml SIII). In contrast, for SI and the control sample, ethanol proved to be the most effective carotenoids extender. The solubility of photosynthetic dyes depends on the structure of the compound. Chlorophyll a and chlorophyll b molecules are non-polar. The long hydrocarbon (phytol) tail attached the porphyrin ring makes chlorophyll fat-soluble and insoluble in water. Chlorophyl b is more polar than Chlorophyll a due to the difference in their structure. The solubility also differs for both the pigments. Chlorophyll a is less soluble in polar solvents. According to the results (Fig. 3) obtained in the

experiment and taking into account the physico-chemical properties of the solvents, the most favourable pigment for the extraction of photosynthetic pigments is ethanol.

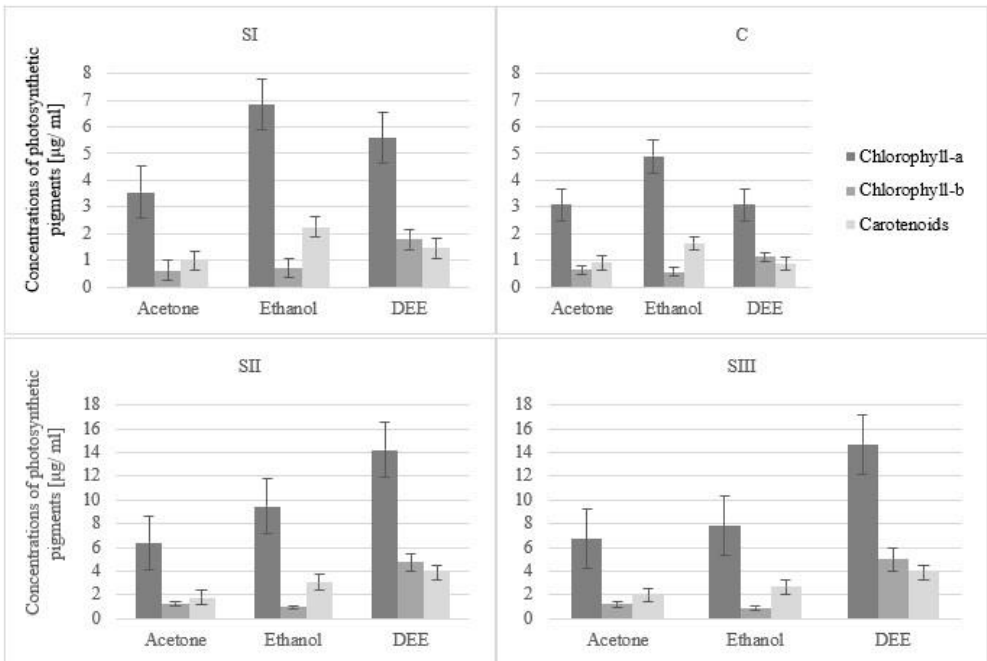

Fig. 3. The average concentrations (µg/ml) of chlorophyll a, b and carotenoids in winter wheat (Samples: C, SI, SII, SIII).

4. Conclusions

In sustainable crop production, biostimulants play an important role in improving plant growth and crop quality. Assimilation area and chlorophyll content are important parameters to assess plant growth. The chlorophyll content of leaves is a key factor in determining the rate of leaf photosynthesis. Since the nitrogen content of a leaf is made up of photosynthetic and non-photosynthetic nitrogen components, determining the chlorophyll content is a better characterisation of the photosynthetic capacity of the plant than testing the nitrogen content of the leaf. In this work, the effect of foliar application of protein biostimulants on the amount of photosynthetic pigments in wheat leaves was investigated. The highest levels of

chlorophyll and carotenoids were found in SII and SIII, plants treated with Bio-2 (containing collagen and titanium ascorbate) and Bio-3 (containing collagen, keratin and sodium salicylate). The results of the study thus confirmed the beneficial effect of protein biostimulants in increasing the efficiency of biochemical processes occurring in the leaves of the plant under study. The work also confirmed that the extraction efficiency of plant pigments depends on the type of solvent used. In most cases, the highest extraction of selected pigments is observed with DEE solvent, followed by ethanol and acetone. However, as the properties of ether (volatile, flammable, explosive) cause some inconveniences when working with this solvent, ethanol was chosen for the determination. Studies have shown that ethanol can be successfully used as a solvent for the determination of photosynthetic pigments.

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