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Phytostimulator Application after Cold Stress for Better Maize (*Zea mays* L.) Plant Recovery

Karolina Ratajczak *, Hanna Sulewska, Katarzyna Panasiewicz, Agnieszka Faligowska and Grażyna Szymańska

Department of Agronomy, Poznań University of Life Sciences, 11 Dojazd St., 60-632 Poznań, Poland
* Correspondence: karolina.ratajczak@up.poznan.pl

Abstract: Phytostimulators are attracting considerable attention for replacing mineral fertilizers, which are of environmental concern, being especially forbidden in organic farming. The benefit of applying such products based on microorganisms (e.g., algae extract) or minerals of nano-meter-sized particle (e.g., nanofertilizers) is that plants can uptake them faster than soil fertilizers, targeting plant growth by regulating their phytohormones, as well as improving plant tolerance to unfavorable environmental conditions (e.g., cold stress). The aim of this study was to test and evaluate the effects of three commercial phytostimulators, called biostimulants (a seaweed-based extract—Kelpak®, mineral nanoparticles—Nano Active®, zinc nanoparticles—Dynamic Cresco®) on yield, chlorophyll content, level of CO_2 assimilation and the effectiveness of PSII under cold stress. The values of all chlorophyll fluorescence and photosynthetic parameters significantly decreased under cold, which indicated a strong inhibition of light-phase photosynthesis in maize leaves. Predicted by the regression analysis minimum, 20 days was enough for maize plants to recover from the inhibition caused by stress damages in their photosynthetic apparatus. At the final measurement in maize growth stage BBCH 65, all the tested phytostimulators showed significant effects in increased values of effective quantum yield of photosystem II, maximum photosynthetic efficiency of PSII and electron transport rate. At this stage, Dynamic Cresco® and Nano Active® treatment significantly increased the value of maximum net photosynthetic rate (15.37% and 18.85%, respectively) and leaf chlorophyll content (7.8% and 8.7%, respectively). The application of Dynamic Cresco® significantly promoted total dry weight by 43.4% in comparison to control under stress growth conditions with cold. These phytostimulators can be used to enhance yield and physiological status of plants after abiotic stress (such as cold) to improve crop productivity, especially in organic farming.

Keywords: nanoparticles; seaweed extract; abiotic stress; chlorophyll fluorescence; gas exchange

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1. Introduction

In critical periods, yields may be significantly reduced as a result of the impact on plants not only of high temperatures associated with heat waves, but also of low temperatures associated with early spring or late frosts. These phenomena, called extreme ones, are among the greatest threats posed by climate change [1,2]. The losses caused by them, according to analyses for Europe, are to be the main reason of greater yield variability in Europe [3], causing reduction in the global cereal production of maize and wheat by 3.8% and 5.5%, respectively [4]. It is indicated that adaptation to a greater frequency of extreme events may be much more difficult for farmers than to changes resulting from changes in climate standards [5].

Early spring planting to maximize the duration of the growing season that potentially increases yield and participates in water deficit avoidance in summer, preventing fungal growth and mycotoxin production in grain and reducing drying costs [6], has increased the likelihood that maize plants will spend some portion of early development under suboptimal temperature conditions [7]. The optimal temperature for root, shoot

and leaf rapid elongation in maize is between 20 and 30 °C. At suboptimal temperatures (approximately 15 °C), plants can acclimate rapidly, but growth is retarded. At temperatures below 5 °C, neither growth nor photosynthesis occur, and plants depend on defense mechanisms to avoid damage and to survive. Chilling commonly refers to a temperature range between 5 and 15 °C, when plants are still capable of adapting developmental processes in order to survive more unfavorable temperature conditions, such as during cold spells [8]. During the initial maize plant growth in spring months, not only in central Europe (e.g., Poland), but also in other countries of northern Europe, cold days occur where low temperatures cause chilling stress which decreases water potential in different organs of maize, which is a plant susceptible to such occurrences [9]. These cold days are extremely dangerous when accompanied by high insolation (increased PAR intensity), which may cause low-temperature photoinhibition of photosynthesis in maize [10]. Marocco et al. [7] indicated three main categories of chilling stress, in which one of them is classified as 'strong chilling stress' (2–10 °C) in the light, when cold-induced water stress occurs because the rate of transpiration exceeds the rate of water uptake by roots due to inhibition of root hydraulic conductivity.

Low temperatures which induce cold stress are difficult to predict and, commonly, a reaction to them is possible only thanks to the visible symptoms of stressed plants. It is important to search for solutions which enable plants to recover after stress. One such method is the application of formulations that stimulate plant growth and that are widely used by not only in agriculture practice but also floriculture, horticulture, on vegetable plantations and in orchards. The broad term agricultural biostimulants is defined by the European Biostimulants Industry Council (EBIC, 2019) as "substance(s) and/or micro-organisms whose function when applied to crops or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality"[11]. The diversity of plant responses to biostimulants is so wide that it cannot be fully explained by the current understanding of plant processes [12]; particularly, it is difficult because of the complexity of the applied extracts and the wide range of molecules contained in the solution [13]. One of the terms, variants and synonyms of the term "biostimulant" is "phytostimulators", which is "a category that includes microorganisms that promote plant growth usually by hormonal action" [14] or "microorganism with the ability to produce or change the concentration of growth regulators such as indole acetic acid, gibberellic acid, cytokinins and ethylene" [15]. The moment of biostimulant application is very important; it should be used at plant development stages crucial for prospective yield quality and quantity, e.g., during young seedlings sprouting, flowering and fruit setting (preventive method). In hardly predictable situations, e.g., low temperatures, drought, chemical contamination with herbicides or pesticides, they can be applied as an intervention method, even after stress, for better plant recovery [16].

At present, plant production and protection should be based on stimulation growth and development, especially when improving agricultural technology becomes insufficient to fully use the biological potential of the cultivated variety. Searching for new biostimulants or products that are considered and marketed as "natural" (the substitution of chemical inputs), such as phytostimulators, is conducted by scientists, breeders and crop protection companies as the most prospective and promising method to meet this aim, simultaneously with environment protection and supporting the safety of the agri-food chain [17,18], also to facilitate the transition towards agroecological production systems and organic farming principles [19].

In this context, the phytostimulators qualified by Chemirol company to "biostimulants and fertilizers" category, used in the experiment constitute a promising alternative solution to the use of synthetic products in improving productivity and crop quality, as well as assisting plants to cope with abiotic stress [20] and promoting plant yield and health [21]. One of the phytostimulators used in this study was produced from seaweed concentrate, belonging to the species *Ecklonia maxima*, whose bioactive ingredients are pol-

ysaccharides, carbohydrates, proteins and mineral elements naturally enriched in phytohormones, but also able to promote the endogenous biosynthesis of aux-ins, cytokinin and gibberellins [22]. The other two preparations used in our experiment were foliar fertilizers [23,24] prepared using nanotechnology, which targeted plant growth by regulating phytohormones such as indoleacetic acid (IAA), gibberellic acid (GA3) and abscisic acid (ABA), as well as increasing antioxidants such as vitamin C with increased guaiacol peroxidase (POD) and superoxide dismutase (SOD) activity to accelerate plant growth [25].

Preparations of different composition are produced by manufacturers, but as they are used as biostimulants in agriculture, minimum active doses, time of application and the method of application depend on the plant species and the purpose for which they are used [26,27]; therefore, the recommendations are actually valuable for agricultural practice. Thus, the aim of the study was to determine the possibility of regeneration of maize plant after cold stress and phytostimulator application. We hypothesized that maize plants may respond differently to the selected preparations in situations of such abiotic stress; hence, it would be worthwhile to examine the physiological state associated with maize response for academic and practical reasons.

2. Materials and Methods

2.1. Plant Materials and Growing Conditions

Seeds of maize (*Zea mays* L., hybrid 'Ronaldinio', KWS Saat SE) were germinated for 9 days at 26°C, then grown for 3 weeks in pots filled with soil (with the composition 28.2 g P_2O_5, 6 g K_2O, 48.6 g Mg per pot, pH 6.4 in 1M KCl) at 25–30 °C with a 14/10h light/dark periodicity in greenhouse of the Department of Agronomy at the University of Life Sciences (Poznań, Poland). Plants were watered regularly (350 mL tap H_2O per pot per 72h) and fertilized using universal Biohumus Max® (fertilizer resulting from the digestion of organic matter by California earthworms). Throughout the experiment period, seedlings were watered up to the pot capacity to maintain the equal optimal level of soil moisture of 20–22%. The cold treatment was carried out for 4 days in cold room with an air temperature 5–6 °C (day) with short-term decreases in night temperature to −1.2 °C. Directly after severe staining of the surface of the leaf blade into a purple color and characteristic crumpled symptoms on maize leaves, the cold stress was stopped. After the two-day period at 11/12 maize leaves stage, phytostimulators were applied as single spraying using a set of TeeJet flat spray nozzles (type DGTJ60 11003) at 0.35MPa pressure at 5–6 °C, 60% to 80% relative humidity. Foliar treatment was performed directly on the leaves without protecting the soil surface.

2.2. Phytostimulators

The manufacturer of Kelpak® (*Ecklonia maxima* extract) declares that it is accredited by all recognized ecological and quality certification departments around the world, including for the perfection of production standards and use in organic farming. In 2011, Kelpak® was accredited by BCS Öko-Garantie GmbH in Nürnberg, Germany, confirming the compliance of organic production. Currently, Chemirol company, the direct supplier of the preparation in Poland, declares that Kelpak® has been approved for production and organic farming under the number (EC) Nos. 834/2007, 889/2008, AMS 7 CFR Part 205 and NOP/USDA.

Nano Active® (MgO-4.0%, CaO-2.0%, Ca-36.0%, Fe-0.02%, Mn-0.01%, Zn-0.002%) has been qualified by IUNG Puławy (Institute of Cultivation, Fertilization and Soil Science—National Research Institute in Puławy, Poland—dealing with the assessment of product compliance with the requirements set out in the regulations of organic farming) for use in organic farming under the number NE/384/2017. Nano Active® involves appropriate selection of deposits of several minerals, the size, surface area and the activity of its nanoparticles. Production of this fertilizer is subject to complex grinding processes using nano mills. It has been described that in this way, with size measured in nanometers, activated

nutrient substances are able to provide to the plant a considerably larger amount of key nutrients (Nano Active®, 2023) [23]. Another, Dynamic Cresco® (Zn-8.0%) used in this study contains zinc acetate, an ingredient that stimulates plants for the production of auxin, phytohormone corresponding, e.g., for the growth of lateral roots and root hairs (Dynamic Cresco®, 2023) [24].

2.3. Phytostimulator Treatments

Phytostimulator application was performed according to the following treatments: 1. Kelpak (K) 6,7ml/ L H_2O (2L/300L H_2O/ha). 2. Nano Active (N) 6,7g/L H_2O (2kg/300L H_2O/ha), 3. Dynamic Cresco (D) 4ml/L H_2O (0,8L/200L H_2O/ha). Control treatment (C1) was sprayed and watered with distilled H_2O, growing under normal growth greenhouse conditions without cold stress. Second control (C2) under stress was only sprayed and watered with distilled H_2O. All measurements of plants' physiological condition were performed in the middle of all the same fully expanded leaf at the following times: initial measurement before stress in BBCH 19, 10 leaf stage (1D), treatments application in BBCH 19, 10 leaf stage (TA), 2 days after treatments application in BBCH 19, 11 leaf stage (2DATA), 9 days after treatments application in BBCH 19, 11 leaf stage (9DATA), 27 days after treatments application in BBCH 65 (27DATA). Plants regeneration measurements were taken from 9 to 27 days after treatments application.

2.4. The Photochemical Efficiency of Photosystem II (PSII)

An indirect method of chlorophyll fluorescence measurements was used to determine the efficiency of photosynthesis, using OS5p fluorometer (Opti-Sciences, Inc., Hudson, NH, USA). Apparatus settings were adjusted according to the manufacturer's instructions (OS5p User's Guide, The standard in Plant Stress Measurement, Opti-Sciences, 040113) and previous experiments on maize plants [28]. Prior to fluorescence measurements, the upper surface of the leaf was pre-darkened with leaf clips to ensure complete relaxation of all reaction centers, for 30 min at an intensity of light pulse 15000 μmol photons m^{-2} s^{-1} and a wavelength of 660 nm. The assessed parameter was maximum photosynthetic efficiency of PSII (Fv/Fm), which was calculated using formula: Fv/Fm = (Fm − F0)/Fm [29], on the basis of the measured parameters: minimal fluorescence (F0), maximal fluorescence (Fm), variable fluorescence (Fv). ETR (electron transport rate) parameter was estimated from chlorophyll fluorescence and was defined as: ETR = ΦPSII ×0.84×0,5×PPFD [30], where ΦPSII is the effective quantum yield of photosystem II in the light; PPFD (μmol photons m^{-2} s^{-1}) is the photosynthetic photon flux density incident on the leaf, 0.5 is a multiplication factor because transport of a single electron requires the absorption of 2 light quanta, 0.84 is the specific fraction of incident quanta absorbed by the leaf (ETR-factor) [31,32]. The samples were light adapted using photosynthetic active radiation (PAR) clips for 30 min before the ΦPSII and ETR were measured.

2.5. Photosynthetic Activity

Leaf gas exchange was determined at 8 levels of photosynthetic photon flux density, PPFD (0, 50, 100, 200, 400, 700, 1000, 1500 $\mu molm^{-2}s^{-1}$) at 360 ppm CO_2 concentration, leaf temperature of 25 ± 1 °C and gas flow rate of 200 μmol s^{-1}. Irradiance was increased gradually to increase the incident PAR to 1500 $\mu molm^{-2}s^{-1}$, adjusted automatically by a red–blue light-emitting diode as light source (LCP Narrow Lamp, ADC BioScientific Ltd., Hoddesdon, UK). Relation of maximum net photosynthetic rate (PN, μmol CO_2 $m^{-2}s^{-1}$) to a saturation irradiance (I) was measured by a portable photosynthetic gas analysis system LCpro-SD (ADC BioScientific Ltd., Hoddesdon, UK) using a narrow leaf chamber (area: 5,8 cm^2). For each treatment, plot of PN against PAR suggested an asymptotic regression model, similar to the rectangular hyperbola model proposed by Thornley [33] for modelling leaf photosynthesis as a function of PAR. Therefore, for each treatment PN (Y) as a function of PAR (X) was modeled using the rectangular hyperbola in general form as:

$$Y = \frac{\propto X\, P_{Nmax}}{\propto X + P_{Nmax}} - R_D + \varepsilon, \quad (1)$$

where Y is PN, α is the initial slope of PAR response curve at low PAR, often called photosynthetic efficiency or quantum yield, X is PAR incident on leaf surface, P N max is asymptote of photosynthesis at high PAR, RD is dark respiration rate and ε is the error term assumed to be normally distributed with zero mean, constant variance and independent of one another. The compensation irradiance at PAR level where Y is zero was calculated from the fitted model [34]. The setting protocols and methods of measurement were selected in accordance with the manufacturer's instructions and previous experiments [35,36].

2.6. The Determination of Leaf Chlorophyll Content

Chlorophyll Content Meter CCM-200plus (OPTI-SCIENCE, USA) was used to estimate the chlorophyll content index (CCI) on the same leaves which were used for chlorophyll fluorescence measurements. CCM-200plus measures the chlorophyll absorbance and calculates chlorophyll content index, which is proportional to the concentration of chlorophyll in the sample.

2.7. Yield

After harvesting, the plants (leaves, shoots, panicles, cobs) were weighed and their fresh and dry weights were evaluated.

2.8. Statistical Analysis of Data

Statistical analysis was performed by using software STATISTICA, version 10. Analysis of variance (ANOVA) was used to determine the significance of differences between the means, Tukey's test was used as post hoc test to evaluate significance of differences at p =0.05. The Pearson procedure with level of significance α = 0.05 was applied to calculate the correlation coefficient. The experimental design was a split-plot in time with treatment as the main factor and measuring times as sub-factor [37]. Throughout the experiment, the data were collected based on three biological replicates (one plants in each pot) and at least 3 technical replicates (measurements). The experiment was repeated twice independently. Both independent experiments expressed the same trends; therefore, the data were pooled and analyzed. In addition, ANOVA was used to discriminate the main sources of variation of the various characteristics tested in this study. The heat map and cluster analyses were based on the mean values noted during the experiment. Similarities between the photochemical efficiency of photosystem II, photosynthetic activity, leaf chlorophyll content and the experimental treatments and yield were analyzed. A cluster analysis was conducted to group similar parameters according to the considered treatments. Euclidean distance measurements and Ward's hierarchical clustering were used to determine the dendrogram.

3. Results

Analysis of variance (Table 1) showed a strong influence of the treatment on all chlorophyll fluorescence and photosynthetic parameters, with the exception of dark respiration rate. Time of measurement was considered as a factor and was highly significant for all parameters. The interaction treatment × time exerted a strong influence on the observed variance.

Table 1. Analysis of variance for the effect of phytostimulators and time of measuring. Df=degree of freedom, ΦPSII: effective quantum yield of photosystem II in the light, Fv/Fm: maximum photosynthetic efficiency of PSII, ETR: electron transport rate, CCI: chlorophyll content index, P N max: asymptote of photosynthesis at high PAR, RD: dark respiration rate.

Source of Variation	df	F-value					
		ΦPSII	**Fv/Fm**	**ETR**	**CCI**	**PNmax**	**RD**
Phytostimulator (P)	4	3.1*	8.7**	3.1*	3.9**	18.5**	0.69 n.s
Time (T)	4	23.4**	48.0**	23.4**	150.1**	82.5**	7.3**
P × T	16	2.4**	4.2**	2.4**	1.0 n.s	4.2**	0.29 n.s
Error	125						
Total	149						

* = $p \leq 0.05$, ** = $p \leq 0.001$, n. s = not significant.

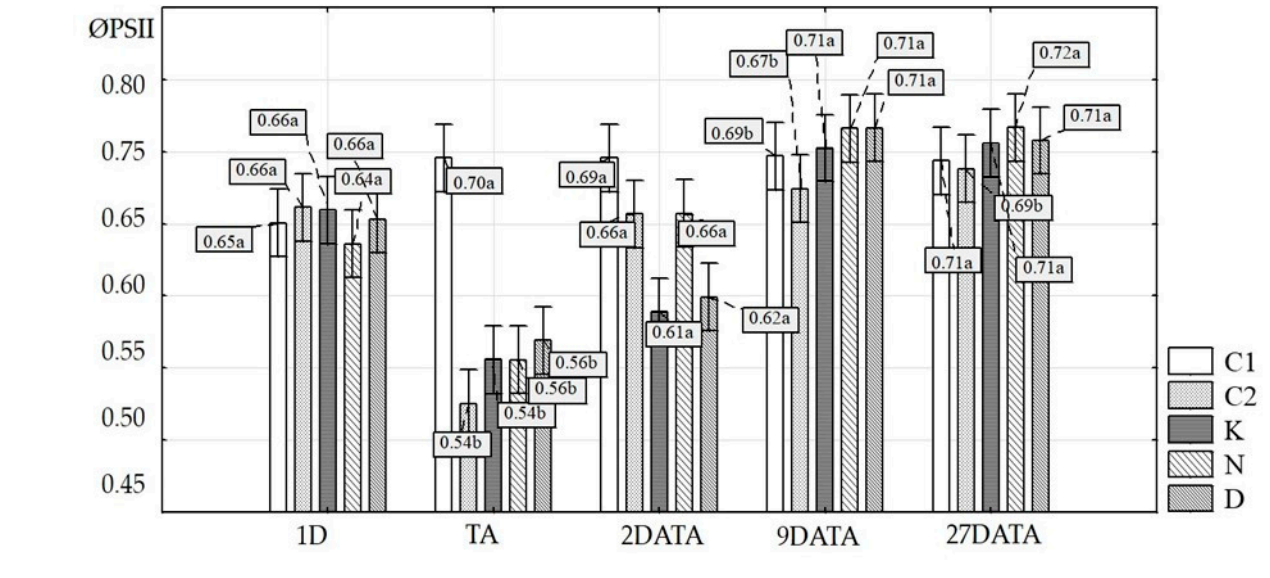
3.1. Chlorophyll Fluorescence Parameters

Growth and cold-stress-dependent variations in the maximum photosynthetic efficiency of PS II, effective quantum yield of photosystem II under steady light condition and electron transport rate in response to phytostimulator and time of measurements for leaves of maize are presented in Figure 1.

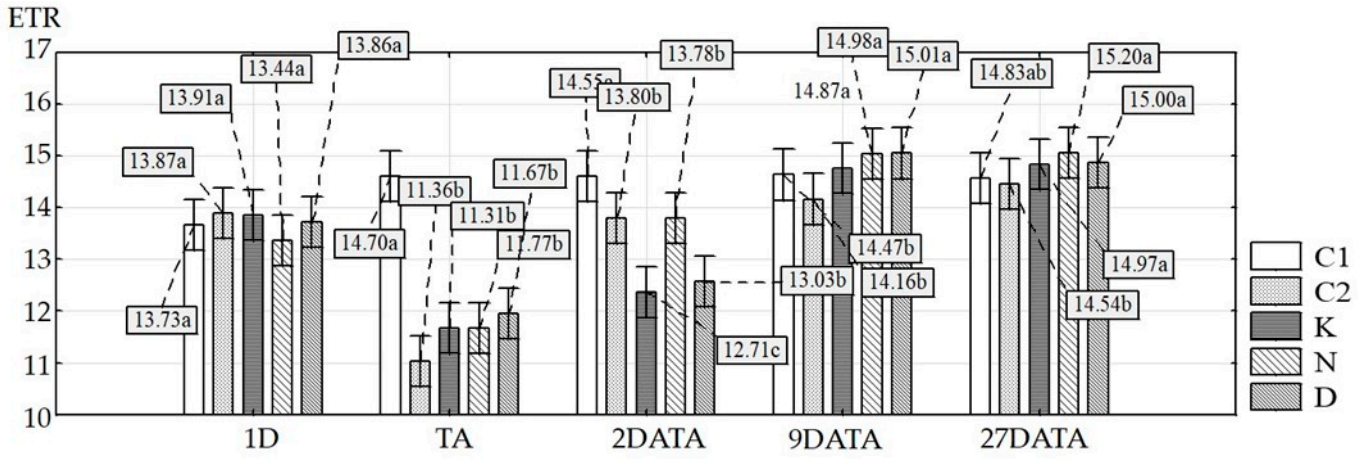
The levels of the fluorescence parameters Fv/Fm, ΦPSII and ETR were fairly similar before exposure to cold conditions. After exposure, significant effects of stress were found for all the mentioned parameters. Exposure to cold conditions had a tremendous effect on the maximum photosynthetic efficiency of PSII. The efficiency for control treatment without stress was 0.78. A 4-day cold exposure resulted in a reduction in Fv/Fm to 0.59, 24.4% of control without stress (Figure 1).

There were significant differences in Fv/Fm in maize leaves in different treatments at 2 days after phytostimulator application. Phytostimulators increased Fv/Fm value. K and N objects had higher Fv/Fm value than control, which increased by 2.7 and 2.7%, respectively. The D treatment increased to the greatest extent the Fv/Fm value, which was at a similar level as control without cold exposure. At 9 days after treatment application, a maximum photosynthetic efficiency of PS II increased slightly in all treatments, but the differences were small. The D treatment matched the control without cold exposure at the level of 0.78 Fv/Fm value. At the final measurement (BBCH 65-flowering growth stage), all phytostimulators tested showed higher Fv/Fm values than control, even higher than compared to control without cold exposure (Figure 1).

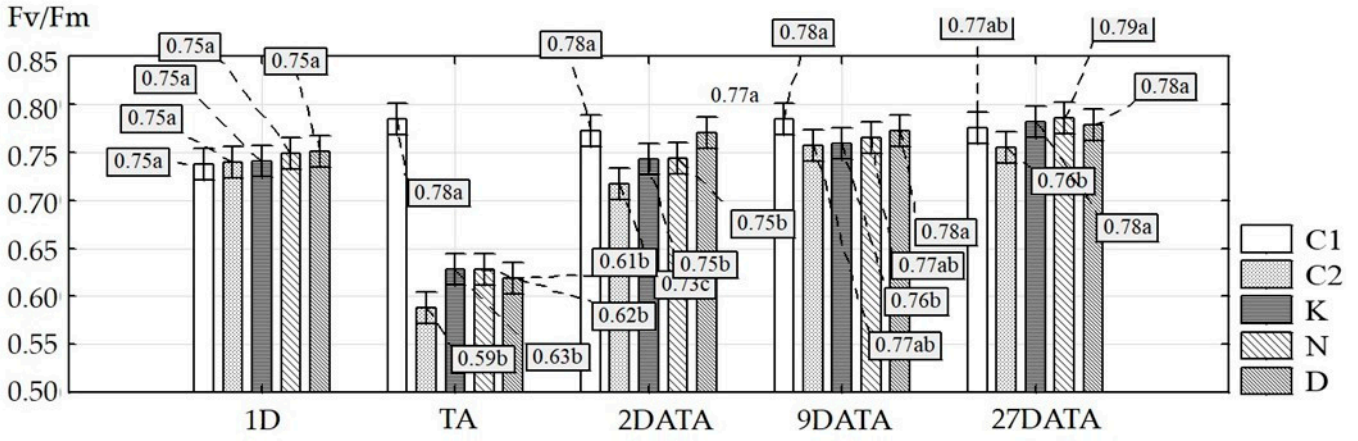
Similar to the above parameter, potential light-use efficiency (ΦPSII) and electron transport rate (ETR) in maize leaves were reduced after 4 days of cold exposure. At 2 days after application treatments, effective quantum yield of photosystem II in the light was at the level of 0.61 (K)–0.66 (N) and the differences were insignificant compared to controls, but after K treatment, electron transport rate was reduced by a 1.1 value compared to control (C1). The significant differences appeared at further growth stages. Both at BBCH 19 and BBCH 65, phytostimulator treatments showed higher effective quantum yield of photosystem II and electron transport rate compared to controls (Figure 1). Analysis of variance (Table 1) showed a strong influence of the treatment on all chlorophyll fluorescence and photosynthetic parameters, with the exception of dark respiration rate. Time of measurement was considered as a factor and was highly significant for all parameters. The interaction treatment × time exerted a strong influence on the observed variance.



(**a**)



(**b**)



(**c**)

Figure 1. Effects of phytostimulators and time of measurements on chlorophyll fluorescence parameters of maize leaves. (**a**) Effective quantum yield of photosystem II in the light, (**b**) electron transport rate, (**c**) maximum photosynthetic efficiency of PSII, different treatments and the values followed by a different letter show statistically significant differences within treatments at $p < 0.05$. Vertical bars mean ± SE, control treatment under normal growth conditions without cold stress (C1), second control treatment under stress growth conditions with cold (C2), Kelpak® (K), Nano Active®

(N), Dynamic Cresco® (D), initial measurement before stress (1D), treatments application (TA), 2 days after treatments application (2DATA), 9 days after treatments application (9DATA), 27 days after treatments application (27DATA).

3.2. Gas Exchange Parameters and Leaf Chlorophyll Content

There were significant differences in maximum net photosynthetic rate of maize leaves (P N max) in different treatments (Figure 2). Compared to C2, maximum net photosynthetic rate increased faster in phytostimulator treatments D and N. At 2 days after treatments application, N and D treatments promoted P N max by 28.8 and 30.4 pp more than C2. This revealed that under N and D treatment, maize plants could recover better after cold exposure, and it furthermore affected P N max. In addition, P N max values of N and D treatment were at a similar level as C1 (without cold exposure) and the differences amounted to 3.95 and 3.29, respectively, and were not significant. At 9 days after treatments application, compared to C2, P N max increased faster in K treatment; it promoted P N max by 16.2 pp. The differences between C2 and K treatment amounted to 5.56 and were significant. This may show a slower regeneration of maize plants after seaweed concentrate application, compared to the phytostimulatory effects of nanotechnology fertilizers, in which the increase in P N max value was shown already 2 days after application. At 27 days after treatment application, all of them increased in P N max significantly and the values of N and D treatments were even larger (N-39.0, D-37.4) than C1 (36.82), but the differences were not significant. K treatment improved P N max value by 3.55 compared to C2.

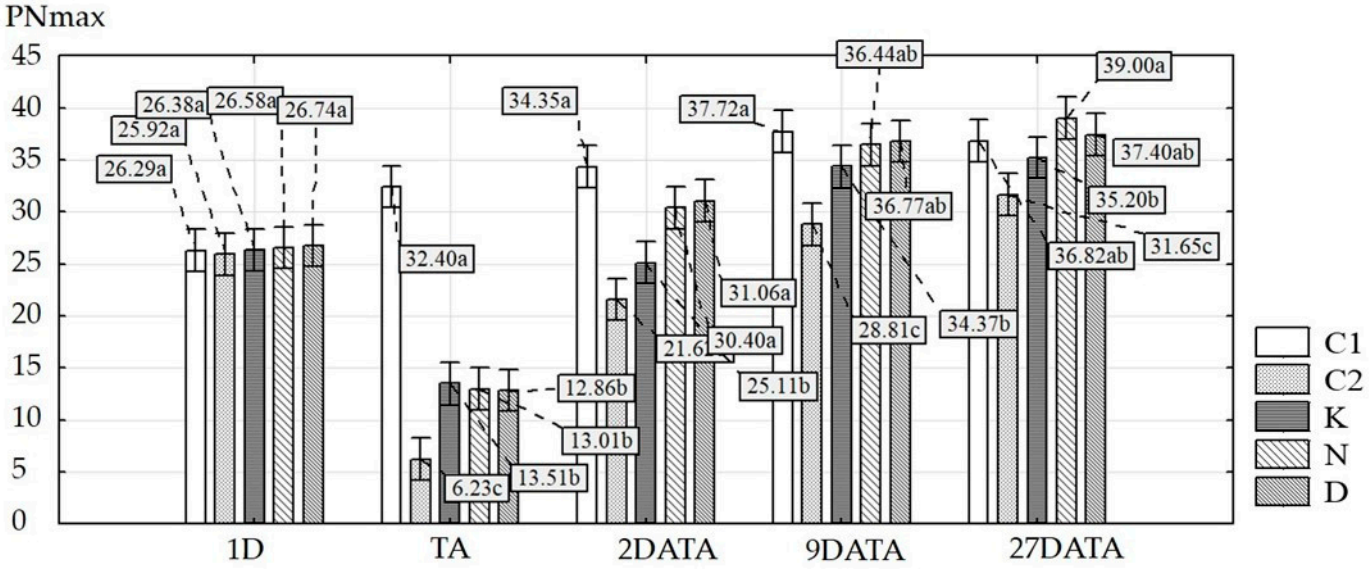


Figure 2. Effects of phytostimulators and time of measurements on maximum net photosynthetic rate of maize leaves. Different treatments and the values followed by the different letter show statistically significant differences within treatments at $p < 0.05$. Vertical bars mean ± SE, control treatment under normal growth conditions without cold stress (C1), second control treatment under stress growth conditions with cold (C2), Kelpak® (K), Nano Active® (N), Dynamic Cresco® (D), initial measurement before stress (1D), treatments application (TA), 2 days after treatments application (2DATA), 9 days after treatments application (9DATA), 27 days after treatments application (27DATA).

Phytostimulators affected chlorophyll content index (CCI) (Table 2). The most similar level of chlorophyll content index to C1 had N and D treatments, but the differences were not significant in BBCH 65 growth stage of maize plants. After treatment application, the increasing values of CCI independent of treatment were shown, but significant differences were obtained for the D treatment; the CCI value increased by 3.15 in comparison to C2

and it reached the same CCI value level as C1. Sampling time also affected CCI. Compared to the initial measurement (BBCH 19, 10 leaf stage), chlorophyll content index decreased after cold exposure by a 1.11 value. At further growth stages, CCI increased to 26.90 (BBCH 65), less than 60% of the BBCH 19 (Table 3).

Though treatment application increased the rate of dark respiration (RD), it showed no significant differences in different treatments. After cold exposure rate of dark respiration decreased, it then increased in further growth stages, and the highest value was obtained at BBCH 65; it increased by a 0.53 value, compared to the initial growth of maize, and the differences were significant (Table 3).

Table 2. Effects of phytostimulators on chlorophyll content index and dark respiration rate in maize leaves. CCI: chlorophyll content index, RD: dark respiration rate; in each column, values followed by different letters indicate statistically significant ($p \leq 0.05$) differences, control treatment under normal growth conditions without cold stress (C1), second control treatment under stress growth conditions with cold (C2), Kelpak® (K), Nano Active® (N), Dynamic Cresco® (D).

Treatment	CCI			RD
	(BBCH 19, 10)	(BBCH 11)	(BBCH 65)	
C1	11.35^{a}	12.77^{a}	16.71^{a}	1,00^{a}
C2	8.90^{b}	9.18^{b}	13.62^{b}	0,88^{a}
K	9.58^{b}	10.01^{b}	14.14^{b}	0,89^{a}
N	9.70^{ab}	10.00^{b}	14.92^{ab}	1,06^{a}
D	9.47^{b}	12.33^{a}	14.78^{ab}	0,87^{a}

Table 3. Effects of time of measurements (days) on chlorophyll content index and dark respiration rate in maize leaves. CCI: chlorophyll content index, RD: dark respiration rate; in each column, values followed by different letters indicate statistically significant ($p \leq 0.05$) differences, initial measurement before stress (1D), treatments application (TA), 2 days after treatments application (2DATA), 9 days after treatments application (9DATA), 27 days after treatments application (27DATA).

Days of Measurement	CCI	RD
1D	10.91^{c}	0.84^{b}
TA	9.80^{c}	0.62^{b}
2DATA	10.86^{c}	0.86^{b}
9DATA	15.75^{b}	1.01^{ab}
27DATA	26.90^{a}	1.37^{a}

3.3. Dry Weight of Maize Plant

Phytostimulators N and D significantly increased dry weight of leaves compared to C2. Moreover, the D treatment had higher dry weight of leaves, shoots and cobs, their values were at the same level as C1. The N treatment had the same dependence only for dry weight of leaves. The K treatment increased all studied characters compared to C2, but the differences were not significant (Table 4). The yields according to dry weight of whole plants were in a trend of C1>D>N>K>C2, and the application of phytostimulator type increased yield by 15.5–71.2% compared to control with cold exposure (C2). In particular, the D treatment showed a more obvious effect; dry weight was only 3.2% less than C1 (Table 5).

Table 4. Effects of phytostimulator treatments on dry weight of parts of maize plants. In each column, values followed by different letters indicate statistically significant ($p \leq 0.05$) differences, control treatment under normal growth conditions without cold stress (C1), second control treatment under stress growth conditions with cold (C2), Kelpak® (K), Nano Active® (N), Dynamic Cresco® (D).

Treatment	Dry Weight (g/plant)			
	Leaf	Shoot	Panicle	Cob
C1	8.84 ± 2.56^{a}	10.53 ± 2.38^{a}	0.82 ± 0.23^{a}	3.92 ± 1.07^{a}
C2	4.66 ± 0.78^{b}	6.33 ± 0.71^{b}	0.41 ± 0.10^{b}	2.23 ± 0.92^{b}
K	5.60 ± 1.19^{b}	6.58 ± 2.06^{b}	0.52 ± 0.16^{b}	3.06 ± 0.88^{ab}
N	7.50 ± 1.94^{a}	8.63 ± 1.80^{ab}	0.62 ± 0.15^{ab}	2.76 ± 0.93^{ab}
D	7.57 ± 2.10^{a}	11.33 ± 3.53^{a}	0.51 ± 0.13^{b}	3.94 ± 0.58^{a}

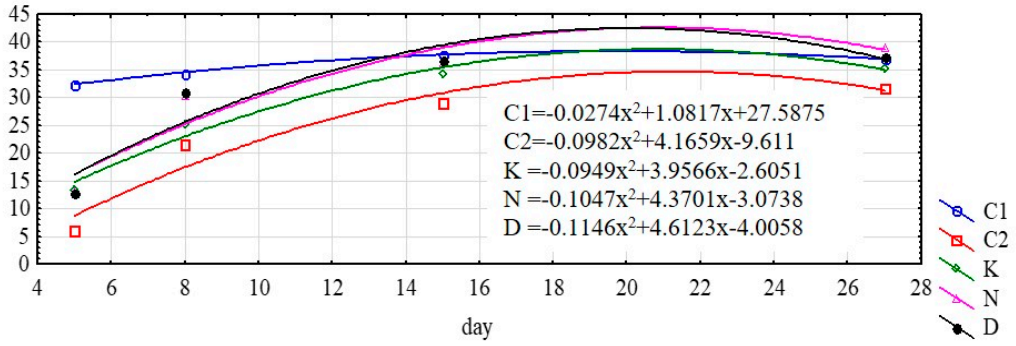
Table 5. Effects of phytostimulator treatments on dry weight of whole maize plant. In each column, values followed by different letters indicate statistically significant ($p \leq 0.05$) differences, control treatment under normal growth conditions without cold stress (C1), second control treatment under stress growth conditions with cold (C2), Kelpak® (K), Nano Active® (N), Dynamic Cresco® (D), dry weight of whole plant (g/plant) (DW (g/plant), dry weight of whole plant (g/plant) compared to control treatment under normal growth conditions without cold stress (DW (%) C1), dry weight of whole plant (g/plant) compared to second control treatment under stress growth conditions with cold (DW (%) C2).

Treatment	DW (g/plant)	DW (%) C1	DW (%) C2
C1	24.11 ± 4.22^{a}	-	-
C2	13.64 ± 1.11^{d}	43.4	-
K	15.76 ± 3.16^{cd}	34.7	15.5
N	19.51 ± 2.97^{bc}	19.2	43.0
D	23.35 ± 5.28^{ab}	3.2	71.2

3.4. Prediction of Maize Plant Recovery Day after Stress

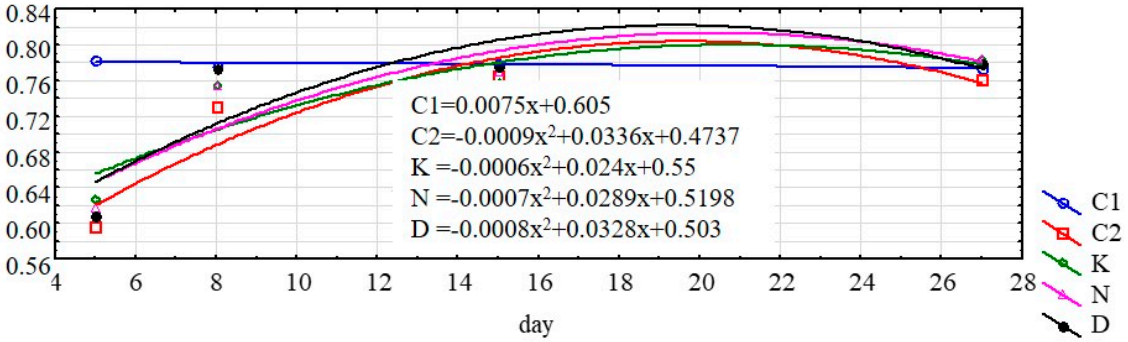
Results showed that regression of calculated variables in the dependence of chlorophyll fluorescence and photosynthetic parameters on the growth stage of maize had a parabolic progressive relation (Figure 3). After 20 days (directly 7 days before BBCH 65), irrespective of treatment, studied parameters (P N max, Fv/Fm, ETR) obtained the highest values; in further growth stages, parameters declined. Based on these results, it can be assumed that a regeneration process after cold stress occurred in all maize plants irrespective of treatment, but the application of the phytostimulators K, N and D definitely improved the functioning of PSII, electron transport and photosynthesis at high PAR.

(a)



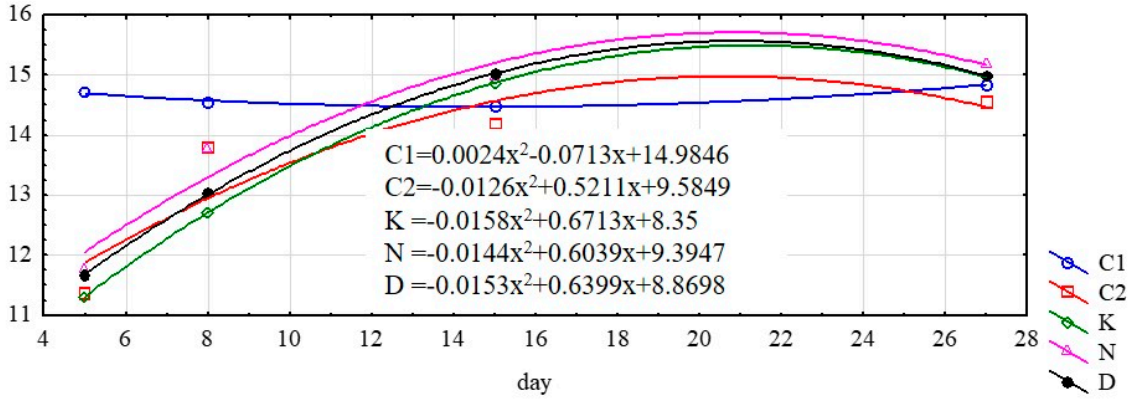
	X_{opt}	Y_{max}	R^2
C1	19.7	38.3	76
C2	21.2	34.6	82
K	20.8	38.6	83
N	20.8	42.5	79
D	20.0	42.4	74

(b)



	X_{opt}	Y_{max}	R^2
C1	-	-	81
C2	18.7	0.78	79
K	20.0	0.79	80
N	20.6	0.81	80
D	20.5	0.83	71

(c)



	X_{opt}	Y_{max}	R^2
C1	14.9	14.5	34
C2	20.7	15.0	82
K	21.2	15.5	93
N	21.0	15.7	90
D	20.9	15.6	92

Figure 3. Effects of phytostimulators and time of measurements on maximum net photosynthetic rate (P N max) (**a**), maximum photosynthetic efficiency of PS II (Fv/Fm) (**b**) and electron transport rate (ETR) (**c**). Control treatment under normal growth conditions without cold stress (C1), second control treatment under stress growth conditions with cold (C2), Kelpak® (K), Nano Active® (N), Dynamic Cresco® (D), X optimum (X_{opt}), Y maximum (Y_{max}), determination coefficient (R^2).

The influence of the phytostimulator used in maize plants on the physiological state of the plants in correlation with the yield was visualized using a heat map (Figure 4).

The comparison of the characteristics of all treatments showed that the control treatment under normal growth conditions without cold stress (C1) and Dynamic Cresco® (D) treatment were the most similar to each other. There were also strong similarities between the above treatments and the Nano Active® (N) treatment. In comparison with the other experimental treatments, the N treatment, where plants were sprayed with Nano Active, was characterized by much higher values of activity of the parameters under analysis, except CCI and DW. Results from the heat map showed that second control treatment under stress growth conditions with cold presented low values of the parameters characterizing yield (DW, CCI). This effect may have been caused by the poor physiological state of the plants, as indicated by low values of the physiological parameters of maize. Such parameters included maximum net photosynthetic rate (P N max), maximum photosynthetic efficiency of PS II (Fv/Fm), electron transport rate (ETR) and dark respiration rate (RD). The highest value of yield (DW), comparable to control, was showed by the D treatment. Higher values of maximum net photosynthetic rate were found in this experimental treatment.

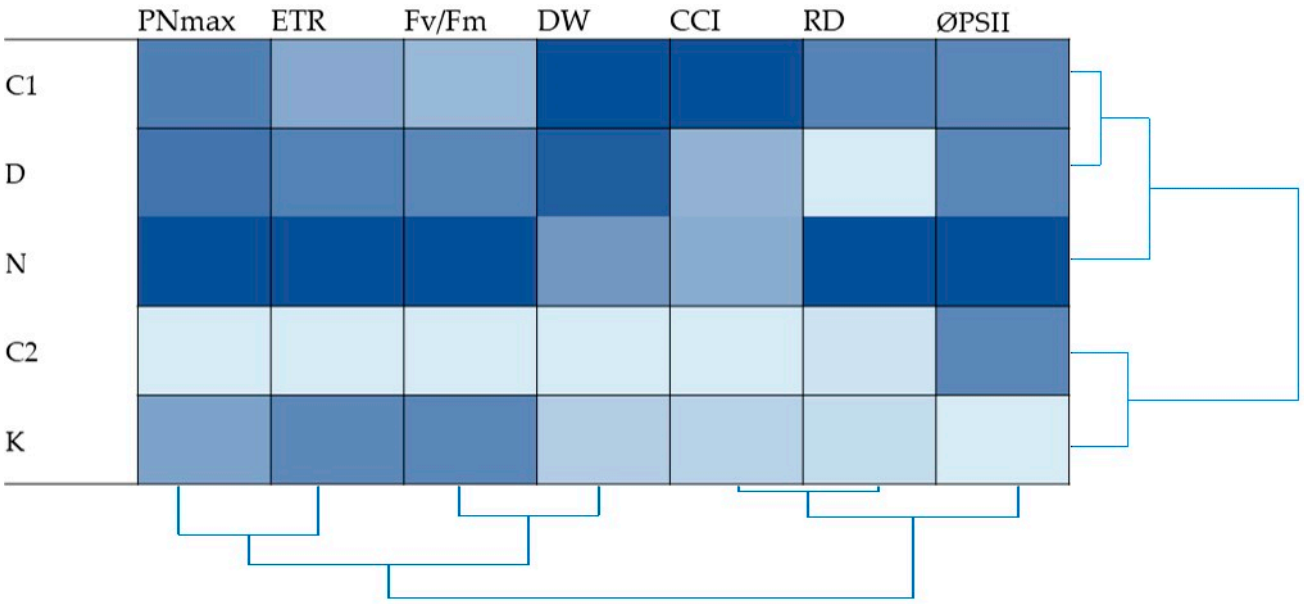


Figure 4. Effects of phytostimulators on maximum net photosynthetic rate (P N max), maximum photosynthetic efficiency of PS II (Fv/Fm), electron transport rate (ETR), chlorophyll content index (CCI), dark respiration rate (RD), effective quantum yield of photosystem II in the light (ΦPSII), dry weight of whole plant (g/plant) (DW) at BBCH 65, control treatment under normal growth conditions without cold stress (C1), second control treatment under stress growth conditions with cold (C2), Kelpak® (K), Nano Active® (N), Dynamic Cresco® (D).

4. Discussion

Chlorophyll fluorescence parameters determined under control conditions were higher than in maize plants grown after cold exposure. After 4 days, impacts of 5–6 °C daytime ambient temperature, with short-term decreases in night temperature to −1,2 °C, values of determined parameters decreased. This may indicate that low temperatures induced photoinhibition, while maize was shown in many studies to be highly susceptible to this occurrence [38]. Photoinhibition can be caused by a sudden increase in radiation (PAR) many times larger than its current level, or even moderate-intensity PAR, at the same time as the impact of stress factors limiting the rate of CO_2 assimilation in the dark phase of photosynthesis [39,40]. In our climatic conditions, often such a factor is low positive temperature (cold). Exposure to low temperature (cold stress) impairs chloroplast function, thereby reducing photosynthetic capacity, and may result in cell death [41]. Upon severe cold in chloroplasts, photosynthetic electron transport in PS II is slowed down, phosphorylation is restricted and active oxygen is generated [42,43]. Especially dangerous is the blockade of electron transport, favoring the conversion of O_2 released in a highly active form of singlet oxygen ($1O_2$), having the ability to oxidize the chlorophyll in the reaction center of PS II [40]. The activities of enzymes involved in CO_2 fixation are strongly inhibited, increasing the proportion of captured energy, which cannot be used for photosynthesis. As a result, the amount of excess energy dissipated as heat in the antennae of photosystem II (PSII) increases and the trapping efficiency of PSII decreases [7]. It can be assumed that low-temperature photoinhibition is becoming a serious threat to the growth and yield of maize plants. In cold conditions with a strong irradiation PAR, mechanisms to dissipate excess excitation energy are no longer sufficient (e.g. through the accumulation of xanthophyll zeaxanthin [44], anti-oxidative defenses [45], cyclic electron transport [46]) and systems of antioxidant are not able to protect plant cells against the harmful effects of toxic O_2 radicals and PSII reaction centers are subject to permanent damage, a process called photoinhibition [6]. Especially in maize, in contrast to C3 plants, the recycling of antioxidants associated with photosynthesis is restricted because of the cellular portioning of antioxidants [45]. The physiological response mechanism to chilling

is a decrease in CO_2 assimilation and photosynthetic activity as a result of stomata closure and loss of enzyme activities of CO_2 concentration mechanisms [47]. Reduction in the CO_2 assimilation capacity by 60% under chilling stress has been reported in rice plants, contributing to a lower growth rate and grain yield [48].

The mechanisms and effects of chilling stress, and especially the impact of low-temperature photoinhibition on the function of photosynthetic apparatus, can be studied using the methods of luminescence; thus, chlorophyll fluorescence provides a tool to classify the impact of low temperature on basic physiological processes [46]. Chlorophyll fluorescence parameters are highly sensitive, convenient, non-invasive and rapid indicators to monitor the photosynthetic productivity of individual leaves and plants [49]. Optimal values of Fv/Fm amounted to 0.83–0.85 and values lower than this could suggest that a sample has been exposed to some type of biotic or abiotic stress factor, reducing the capacity for photochemical quenching of energy within PSII [32]. Additionally, a lower level of ΦPSII indicates the weak electron transport ability of the photosynthetic apparatus and a lower proportion of absorbed light energy to be used for photochemical reaction [29].

Upon exposure to cold stress, mean values of maximum photosynthetic efficiency of PSII, electron transport rate and effective quantum yield of PSII decreased in our experiment, while these parameters characterized performance of photochemical reactions in PSII. The reason for a decline in Fv/Fm values is the decreasing light absorption in the thylakoid electron transport of PSII and increasing excitation energy quenching in the light-harvesting antennae [50]. More recent studies also found that low temperature (5°C and 10°C) significantly reduced the ratio of Fv/Fm in both maize genotypes tested [51].

It can be assumed that stress factors seriously reduced the efficiency of the reaction of the dark phase of photosynthesis. The obtained data are confirmed in the papers by Huner et al. [52] and Borowski [53].

For the light conditions, the maximum conversion efficiency of photon energy PAR to chemical energy is described by ΦPSII and electron transport rate, which is directly related to ΦPSII. In studied maize leaves after cold exposure, values of these parameters also decreased.

Moreover, we found that maximum net photosynthetic rate (P N max parameter) was also affected by the applied cold stress. We could even deal with chronic photoinhibition, because when the stress is more severe or prolonged, a decrease in P N max parameter is to be expected, similar to findings made by Lootens et al. [54], in which they proved that prolonged cold stress of 2 °C combined with high irradiance at 500 μmol (photon)$m^{-2}s^{-1}$ (comparable to a morning stress in springtime) resulted in significant decline in the maximum photosynthetic rate.

In the present study, chlorophyll content index was markedly reduced after cold exposure by 28% in BBCH 11, compared to control. Wu et al. [51] showed that the Chl a, Chl b and total chlorophyll contents were markedly reduced by 47.2%, 60.5%, 53.4%, respectively, when the maize seedlings were exposed to 5°C compared to 25°C, assuming that it might be associated with lower enzyme activities. The negative impacts of chilling stress on chlorophyll contents have been reported in many studies, indicating that reduction in chlorophyll content levels can increase the level of energy dissipation, which decreases PSII efficiency [55,56].

In our experiment, we did not investigate the mechanisms' ability to mitigate the abiotic stresses but tried to determine whether the applied phytostimulators significantly improved maize tolerance to chilling stress. Plants' exposure to cold stress was stopped when the changes in leaves were strongly visible as discoloration. After 2 days, plants were subjected to a regeneration process after the application of seaweed extract and two nanofertilizers as a solution for better plant recovery. Then, the same tested leaves were subjected to assessment of the ability of the photosynthetic apparatus to overcome the negative effects of low temperature. After 2 days of treatment application (time of initial

regeneration), values for the majority of parameters significantly increased. Moreover, after approximately 20 days of regeneration, chlorophyll fluorescence parameters in maize leaves had recovered to control levels, indicating that the damages caused by low-temperature on the integrity and functioning of photosystems were reversible, which was in agreement with findings of Riva-Roveda et al. [6]. It should be emphasized that seaweed concentrate and fertilization increased chlorophyll parameters compared to control and even exceeded the control without exposure to cold stress. The results are similar with those of Dong et al. [57], which showed that new coated fertilizers with inorganic material increased chlorophyll fluorescence. Maximum net photosynthetic rate also significantly increased, especially in treatment with fertilizers, and at the final growth stage exceeded control. A similar observation was made by Dong et al. [57] and Haghighi et al. [58] on maize for effects of biological and new coated release fertilizers. Some reports showed that nanofertilizers stimulated plant photosynthesis rate, e.g., in waterweed by 35% [59] or by 15.8% in sugar beet [60]. However, published literatures regarding the potential of nanofertilizers in enhancing chlorophyll fluorescence or photosynthetic parameters are rare.

The CCI values increased significantly after one of the nanotechnology fertilizer (D) treatment applications in comparison with the control. The changes in the leaf anthocyanin content, induced by low growth temperature, are an effective strategy for modulating the light available to the chloroplasts [61] and are thought to mask chlorophyll or act as a filter, preventing excess light absorption by the leaf [62]. After the application of treatments, this characteristic's changes were not visible. Zaefyzadeh et al. [63] showed that CCI values in stress conditions was lower than under normal conditions, which the authors explained by the relative degradation of chlorophyll induced by drought stress. Similarly, it was proved in our research under low temperature conditions. It can be assumed that maize leaves recovered by increased chlorophyll content after D treatment. This increase in chlorophyll production could be the result of decreasing radical content that damages chloroplasts and causes chlorophyll degradation. It was proved by researchers that the ratio of pigment contents (chlorophyll a, b and total carotenoids and xanthophylls) increased for cold-intolerant cultivars in comparison with the cold-tolerant ones [54] and higher chlorophyll contents were also found in maize grown at 14°C in comparison to 24°C [64].

The results of this study showed that dry weight of all parts of maize were in a trend of C1>D>N>K>C2. New phytostimulators contain natural substances or fertilizers that have different effects and mechanisms on crop growth [27,65,66]. Different studies were conducted with the use of preparations based on phytohormones, e.g., seaweed extract as an organic biostimulator, and the results showed both stimulation and inhibition [67] effects on the growth of plants dependent on the method of application, the species, a variety of crop or even concentration [68]. Seaweed extracts and commercial seaweed plant fertilizers are used as agricultural biostimulants to produce better yields and to enhance crop productivity without any adverse effects [69]. Some older studies indicated that this bioregulator does not destroy the crop but stimulates the growth of somatic tissue [70]. The recent study made by Rengasamy et al. [71] revealed the positive effect of phlorotannin eckol, isolated from *E. maxima* on the seedling of maize. Among the different crops studied, maize showed the greatest extent of response to the use of this biostimulator by increasing its yield by over 21% compared to control [72]. Our research also showed a good effect on maize growth after the exposure to stress induced by low temperature; it insignificantly increased yield by 15.5% compared to control, so the application after stress showed positive effect. Another report even points out that the date of application has greater importance than the size of dose [73].

Different substances of natural origin (which could be synthesized), their mixture and bio-extracts are classified as biostimulators [18] and some improved by new technology fertilizer products are named just biostimulators. The new nanotechnology fertilizers have started to attract attention in agriculture and some results indicate that

such fertilizers can have important effects on plant growth and yield, e.g., cucumber [74], pepper [75], maize [57] or soybean [76]. Their mechanism of action is well known—when augmented with plant nutrients, they can increase plant-uptake efficiency of nutrients and reduce the adverse impacts of conventional fertilizer application [76]. In both fertilizers tested, Dynamic Cresco and Nano Active, the ingredient is zinc, which is responsible for various physiological processes, including hormonal regulations, gene regulation and expressions and cell membrane integrity [77]. Our results of dry weight of whole plants were in a trend of C1>D>N>K>C2, and the application of nanotechnology fertilizer type significantly increased yield compared to control with cold exposure (C2), and even the D treatment did not show differences compared to control without stress (C1). This is in accordance with the chlorophyll content, which was on a higher level in fertilizer treatments. In turn, a sufficient amount of chlorophyll means greater production of photosynthates responsible for the growth and development of the plant. Those results are also compatible with chlorophyll fluorescence and photosynthetic parameters. This indicated that nanotechnology fertilizers may have affected processes through its transportation capabilities in terms of penetration and movements within the plant systems. These processes occurred during maturation of leaves, which is accompanied by a large number of functional and anatomic changes, resulting in a reversal of transport direction from importing to exporting [78]. Gupta et al.'s [79] findings confirmed the phytostimulatory effect of nanoparticles (AgNPs) on rice seedling growth and their involvement in regulating the generation of ROS and its scavenging towards growth stimulation. In the present study, different nanoactive ingredients might have influenced the mechanisms of nanofertilizers' action, which could result in an acceptable reactivity, which could increase the effective uptake of nutrient elements for the growth and development of maize plants and their metabolisms [80].

5. Conclusions

Cold stress resulted in a significant decrease in the values of all chlorophyll fluorescence and photosynthetic parameters, which indicates a strong inhibition of light-phase photosynthesis in maize leaves. The values of the parameters considerably increased in maize plant leaves independent of treatment after the recovery. The application of seaweed extract and nanofertilizers promoted growth, development and photosynthetic activity in maize and the phytostimulators have potential to improve crop production. In the light of the present study results, foliar application of seaweed extract and nanofertilizers may be an efficient method of mitigating the negative effects of cold stress in maize plants, especially in organic farming. The outcome of this research would be beneficial for other studies involving the application of such phytostimulators under field conditions.

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