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Effectiveness of cyanobacteria and green algae in enhancing the photosynthetic performance and growth of willow (*Salix viminalis* L.) plants under limited synthetic fertilizers application

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Abstract

The physiological response of plants to triple foliar biofertilization with cyanobacteria and green algae under the conditions of limited use of chemical fertilizers was investigated. Triple foliar biofertilization with intact cells of *Microcystis aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120, and *Chlorella* sp. significantly enhanced physiological performance and growth of plants fertilized with a synthetic fertilizer *YaraMila Complex* (1.0, 0.5, and 0.0 g per plant). This biofertilization increased the stability of cytomembranes, chlorophyll content, intensity of net photosynthesis, transpiration, stomatal conductance, and decreased intercellular CO_2 concentration. Applied monocultures augmented the quantity of N, P, K in plants, the activity of enzymes, such as dehydrogenases, RNase, acid or alkaline phosphatase and nitrate reductase. They also improved the growth of willow plants. This study revealed that the applied nontoxic cyanobacteria and green algae monocultures have a very useful potential to increase production of willow, and needed doses of chemical fertilizers can be reduced.

Additional key words: energy plant; gas exchange; mineral fertilization.

Introduction

Recently, the strong interest in crop production is focused on the use of microorganisms, including cyanobacteria and green algae, as biofertilizers, which are eco-friendly, can be an alternative to chemical fertilization, and offer economic and ecological benefits to farmers. Biofertilization allows them to reduce the use of chemical fertilizers and pesticides which are dangerous to environment and increase risk for human health (Sahu *et al.* 2012).

Researchers indicate that the biofertilization with cyanobacteria and green algae is able to increase rooting of grapes cuttings and germination of sunflower seeds, and improve plant growth, as observed in rice, barley, oats, tomato, radish, cotton, sugarcane, maize, chili, lettuce, wheat, gillyflower, grapevine, and corn (Spiller and Gunasekaran 1990, Romanowska-Duda *et al.* 2004, Thajuddin and Subramanian 2005, Song *et al.* 2005, Nilsson 2005, Karthikeyanb *et al.* 2007, Abd El-Moniem and Abd-Allah 2008, Shanan and Higazy 2009, Romanowska-Duda *et al.* 2010, Sahu *et al.* 2012, Shariatmadari *et al.* 2013, Grzesik and Romanowska-Duda 2014, Grzesik and Romanowska-Duda 2015). It is suggested that the increased growth of plants can be caused by ability of microalgae to restore soil's natural nutrient cycles, to build soil organic matter, and also by enriching plants with various nutrients, hormones, and secondary metabolites, which have the crucial impact on growth (Parkash *et al.* 2014, Uysal *et al.* 2015, Vasileva *et al.* 2016). Efficient atmospheric nitrogen-fixing strains of cyanobacteria (*Nostoc linkia, Anabaena variabilis, Aulosira fertilisima, Calothrix* sp*., Tolypothrix* sp*.,* and

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Abbreviations: A.PCC – *Anabaena* sp. PCC 7120; B-A – *Bio-Algeen S90*; Ch.sp – *Chlorella* sp.; ES – the environmental sample; GA_3 – gibberellic acid; IBA – indole-3-butyric acid; M.a – not sonicated monocultures of *Microcystis aeruginosa* MKR 0105
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Scytonema sp.) were identified from various agro-ecological regions and utilized for rice production (Prasad and Prasad 2001). The growth promotion in response to application of *Nostoc muscorum* (N-fixing cyanobacteria) could be attributed to the nitrogenous as well as nitrate reductase activities of the algae applied to the surface of plants, or the amino acids and peptides produced in cyanobacterial filtrate and/or other compounds that stimulated growth of plants (Kulk 1995, Adam 1999). Some strains of these blue-green algae are capable of abating various kinds of pollutants and are potentially biodegrading organisms (Subramanian and Uma 1996). According to Malliga *et al.* (1996) *Anabaena azollae* exhibited lignolysis and released phenolic compounds which induced profuse sporulation of an organism.

In spite of the mentioned data concerning biology of microalgae, information about the influence of particular cyanobacteria and green algae strains on physiological performance and development of individual plant species are still very limited. It concerns also the effects of foliar application of blue-green algae and green algae strains on growth, physiological performance, chemical composition, and energy properties of willow plants grown in soil poorly fertilized with synthetic nutrients.

Our research was performed to assess the ability of *Microcystis aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120 (cyanobacteria), and *Chlorella* sp. (green algae) monocultures, used as foliar biofertilizers, in order to improve the physiological performance, chemical composition, energy properties, growth, and yield of willow (*Salix viminalis* L.) plants, under conditions of limited use of synthetic fertilizers, in order to decrease the use of chemical fertilization and environment pollution.

Materials and methods

Plants: The plants of *Salix viminalis* L. were obtained from woody cuttings according to the procedure used for commercial production. The cuttings were rooted and the obtained plants were grown in 3-L pots, filled with poor quality soil, and placed outside. The soil contained minimal amounts of N, P, K, Mg, Fe, Mn, Cu, Zn, Mo, which were sufficient only for 1–2 weeks of growth. The plants were watered with tap water when needed, while temperature depended on weather.

Monocultures of *Microcystis aeruginosa* MKR 0105 (M.a), *Anabaena* sp. PCC 7120 (A.PCC), and *Chlorella* sp. (Ch.sp) were cultivated on *BG11* medium (*ATCC Medium 616*) at 27°C under *FAREL* lamp (18 W), according to the procedure elaborated by Romanowska-Duda *et al.* (2010).

Measuring the number of cells was performed microscopically with a Fuchs-Rosenthal hemocytometer at a magnification of 40 × (*Motic Microscope BA310E*, Poland). The chamber depth of 0.1 mm and a volume of 0.0001 ml had a grid of nine squares with 1 mm^2 area. Before each measurement, the sample was taken for 1 min to break up a group and an even distribution of cells on the grid of Fuchs-Rosenthal chamber. In order to obtain a clearer picture, 15 ml of liquid iodine was added to inoculate. Counts were made from the surface area of five squares. In each case three counts were performed for each sample and the result was averaged. The results were converted to a number of cells in 1 ml according to the method of Tukaj (2007). Prior to application, each monoculture was centrifuged at 4,000 × *g* for 2 min and suspended in water. The cell density used in the experiments was calculated to be 2.5×10^5 cells ml^{-1}(water).

An environmental sample contained a mixture of all algae strains and other water plants present in water, taken from a natural reservoir in the center of Poland.

Bio-Algeen S90 (*Schulzeand Hermsen GmbH*, Germany, B-A) of 1% concentration was the commercial ecological formulation made from a natural extract of brown algae (seaweed). B-A contains: N – 0.02%, P_2O_5 – 0.006%, K_2O – 0.096%, CaO – 0.31%, MgO – 0.021%, boron (16 mg kg^{-1}), iron (6.3 mg kg^{-1}), copper (0.2 mg kg^{-1}), manganese (0.6 mg kg^{-1}), zinc (1.0 mg kg^{-1}). The preparation contains also molybdenum, selenium, and cobalt. Gibberellic acid (GA_3) and indole-3-butyric acid (IBA) were a commercial product of *Sigma*.

A commercial synthetic fertilizer *YaraMila Complex* (*Yara*, Poland), consisted of 12% nitrogen (5% nitrate, 7% ammonium), 11% phosphorus (P_2O_5), 18% potassium (K_2O), magnesium (2.65% MgO), sulphur (19.9% SO_3), and trace elements: zinc (0.02%) and boron (0.015%).

Treatments: At the beginning of the growing season, two weeks after cutting, the plants grown in 3-L pots were divided into three plots. The soil in every plot was fertilized once with the synthetic fertilizer *YaraMila Complex* at dosages of 0.0, 0.5, or 1.0 g per plant [0.0, 0.17, and 0.33 g L^{-1}(soil), respectively]. Plants were grown in 3-L pots filled with universal soil. B-A was sprayed on leaves.

The selected batches of willow plants, within each plot, were sprayed three times during vegetation season, at three-week intervals, with monocultures of *M. aeruginosa* MKR 0105 (M.a), *Anabaena* sp. PCC 7120 (A.PCC), or *Chlorella* sp. (Ch.sp), which were not sonicated. The first application was made when shoot length was 5 cm. The results were compared to the physiological performance of plants treated similarly with GA_3 (10^{-6} M), IBA [50^{-6} g L^{-1} (H_2O)], B-A, the environmental sample (ES), and with water, which served as a control.

Assessment of plants: To assess dynamics of growth, the height of plants, total length of all shoots, and their number were measured every 3–4 weeks during the whole vegetation season. Fresh mass (FM) of shoots and their dry mass (DM, dried for 3 d at 130°C in hot-air oven) were measured at the end of experiments.

Relative chlorophyll (Chl) contents in the leaves were evaluated using *Minolta SPAD-502* chlorophyll meter (*Konica Minolta*, Japan) and expressed in SPAD units (Grzesik and Romanowska-Duda, 2014). Net photosynthetic rate (P_N), transpiration (E), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured using the portable photosynthesis measurements system *TPS-2* (*PP Systems*, USA) (Kalaji *et al*. 2014). Measurements were provided under field conditions at the end of July on fully developed leaves at morning hours (08:00–11:00 h). Temperature was about 20–25°C, air humidity about 70–80%, and light intensity *ca*. 1,100–1,300 μmol(photon) m^{-2} s^{-1}. Activities of acid (pH 6) (EC 3.1.3.2) and alkaline (pH 7.5) (EC 3.1.3.1) phosphatase [mU g^{-1}(FM) min^{-1}] and RNase (EC 3.1.27.5) [mU g^{-1}(FM) min^{-1}] in the leaves were examined according to the methods described by Knypl and Kabzinska (1977). Total dehydrogenase activity (EC 1.1.1.-) [mg(formazan) g^{-1} (leaf FM)] was evaluated by the procedure described by Gornik and Grzesik (2002), using spectrophotometer *UVmini-1240* (*Shimadzu*, Japan) for formazan determination at a wavelength of 480 nm. Enzyme activities were calculated on the base of FM. Nitrate reductase (EC 1.7.99.4) [μmol(NO_2) g^{-1}(DM) h^{-1}] was assessed using the procedure elaborated by Bergman *et al*. (1992).

Electrolyte leakage was measured at 20°C after placing leaf segments in test tubes and adding 3 ml of distilled water. Microcomputer conductivity meter *CC-551* (*Elmetron*, Poland) was used to measure electrolyte leakage from the leaves after 2 and 4 h (Gornik and Grzesik 2002).

Assessment of macroelement (N, P, and K) contents in plants, caloric value of shoots in the operating state, heat of combustion in the analytical state, and ash content in the working state were made by local specialized laboratories (*Carbochem*, Poland), having national certifications to perform such tests, and using Polish standards for these analyzes: PN-G-04511:1980, PN-ISO 1171:2002, PN-ISO 1928:2002, and PN-G-04584:2001.

Statistical analysis: All experiments were performed in three series and in three replicates for each treatment within each serie. During first two subsequent years, experiments were carried out outdoor in pots. Every replicate under such conditions contained 10 plants grown individually in separate 3-L pots. In the third year, the effect of selected treatments was tested under field conditions, on plots with the size of 3 x 3 m. Within each series, each replicate was set up in a completely randomized block design. Effects of the synthetic fertilizer and the treatments with microalgae on growth and physiological activity of plants, cultivated by both methods, were similar. Therefore, in order to reduce the number of repeating data, tables and figures give the results of experiments performed in containers. The obtained data, given as means from series and replicates, were processed applying analysis of variance (*ANOVA*), by *Statistica 12*. The means of chosen parameters were grouped employing the *Duncan*'s test at $\alpha = 0.05$ significance level.

Results

Vegetative growth of willow: The impact of fertilization with synthetic fertilizers and foliar application of microalgae on growth and physiological activity of plants was similar, independent of a fact whether they were cultivated in pots or under field conditions. This indicated that the treatments could be performed under all conditions of plant cultivation, including the field. The research showed that the application of triple foliar biofertilization of willow with intact cells of M.a, A.PCC, and Ch.sp resulted in increased plant height, total shoot length, FM and DM, and its physiological performance in comparison to B-A, ES, GA_3, IBA, and especially to the control, where the willow plants were sprayed only with tap water. These positive changes were found in all variants fertilized with the synthetic fertilizer *YaraMila Complex*, although their extent depended on the doses of this nutrient added to soil (1.0, 0.5, or 0.0 g per plant) and treatment. The presented study proved that biofertilization with M.a, A.PCC, and Ch.sp can be a powerful means of enriching plants with growth-promoting substances and improving willow crop yields.

The reduction of synthetic fertilizer (*YaraMila Complex*) dose by half (from 1.0 to 0.5 g per plant) resulted in a very slight decrease in growth of willow plants which were treated with cyanobacteria and green algae, while in the control (plants sprayed with water), it caused very significant reduction in the plant height (Fig. 1*A*–*C*), number of shoots, total shoot length (Table 1), and FM and DM (Fig. 1*D*,*E*). Biofertilization with these strains caused the increased total length of shoots by 16.7–29.7% at the fertilizer dose of 1.0 g per plant as compared with the control and by 69.2–85.8%, when this dose was reduced to 0.5 g per plant. Similar correlations of a lesser degree were observed after foliar application of B-A, GA_3, and IBA. The plants, which were not fertilized with *YaraMila Complex*, grew very poorly, and their growth increased by the treatment with cyanobacteria and green algae, although to a much lesser extent than that in the case when the fertilizer was used at a dose of 1.0 and 0.5 g per plant (Table 1, Fig. 1).

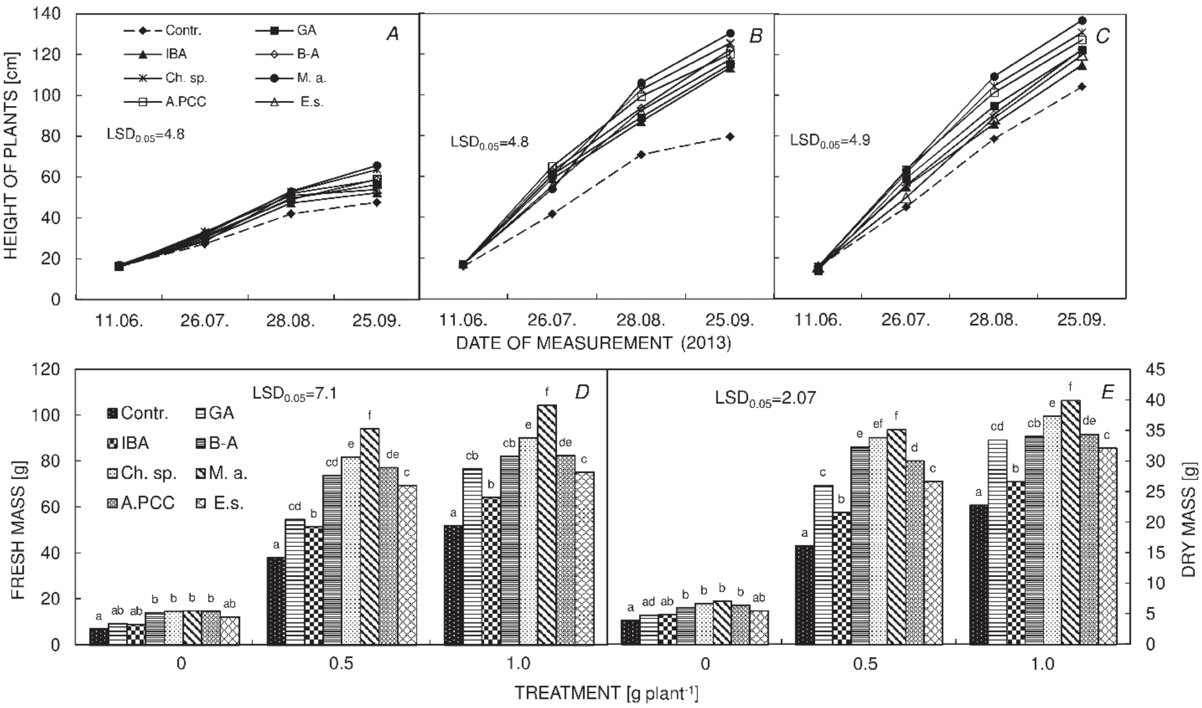

Fig. 1. (*A–C*) Dynamics of willow plant growth in 3-l L pots outdoor, as affected by triple foliar spray of leaves with GA_3 10^{-6}M (GA), IBA 50^{-6}g L^{-1}(H_2O) (IBA), *Bio-Algeen S90* (B-A), and intact monocultures of *Microcystis aeruginosa* MKR 0105 (M.a), *Chlorella* sp. (Ch.sp.), *Anabaena* sp. PCC 7120 (A.PCC.), and the environmental sample (E.s.). Doses of the complex fertilizer *YaraMila* added to soil: 0.0 g (*A*), 0.5 (*B*), and 1.0 g per plant (*C*). The LSD were calculated at the significance level of p=0.05. Effect of microalgae on fresh and dry mass of plants (*D*, *E*). The data marked with *the same letter* (separately for fresh and dry mass) are not significantly different according to *Duncan´*s multiple range test at the significance level of p=0.05.

Table 1. Number of shoots in willow plants grown in 3-L pots outdoor and increase in the total length of shoots as affected by triple foliar spray with biostimulators, intact monocultures of cyanobacteria*,* green algae or the environmental sample and soil fertilization with different doses of the synthetic fertilizers (0.0, 0.5, and 1.0 g per plant). *The data marked with *the same letter*, within number of shoots measurements and particular doses of fertilizer, are not significantly different according to *Duncan´*s multiple range test at the significance level of p=0.05. **The LSD were calculated at the significance level of p=0.05.

Applied stimulators, Cyanobacteria, or green algae	Number of shoots per plant Doses of fertilizer *YaraMila* [g plant^{-1}]			Increase in the length of shoots [%] Complex [g plant^{-1}]		
	0.0	0.5	1.0	0.0	0.5	1.0
Control	1.07^{a}	1.33^{a}	1.67^{b*}	100.0^{a}	100.0^{a}	100.0^{a}
GA_3 10^{-6} M	1.33^{a}	1.33^{a}	1.63^{b}	110.6^{c}	128.0^{c}	106.0^{b}
IBA50^{-6} g l^{-1}(H_2O)	1.67^{b}	1.68^{bc}	2.00^{c}	106.1^{b}	117.0^{b}	105.9^{b}
Bio-Algeen S90	2.00^{c}	2.10^{cd}	2.33^{d}	115.1^{d}	145.4^{e}	111.6^{c}
Chlorella sp.	2.10^{cd}	2.67^{e}	2.73^{e}	132.3^{g}	173.3^{g}	121.2^{e}
Microcystis aeruginosa	2.10^{cd}	3.00^{e}	3.00^{e}	135.3^{g}	185.8^{h}	129.7^{f}
Anabaena PCC 7120	2.10^{cd}	2.38^{de}	2.70^{e}	125.6^{f}	169.2^{f}	116.7^{d}
Environmental sample	1.70^{bc}	2.00^{c}	2.10^{cd}	120.2^{e}	136.3^{d}	108.6^{bc}
LSD $_{0.05}$**	0.33			4.0		

Our study showed that independently of the chemical fertilization, M.a enhanced plant growth a little more effectively than Ch.sp and A.PCC and that these three monocultures influenced more effectively plant growth than B-A and ES taken from the natural water reservoir. The beneficial impact of GA_3 (10^{-6} M) and IBA (50^{-6} g L^{-1}) on plant growth was intermediate, between the effectiveness of microalgae treatments, and the control. Efficiency of foliar biofertilization grew with an increasing number of willow treatments. It resulted in the increased height, number of shoots, and their total length compared with the control, probably due to the transfer of higher quantity of active compounds from microalgae strains and their increased accumulation in willow plants with each next application (Table 1, Fig. 1). Foliar biofertilization with these strains also improved plant health status (data not shown).

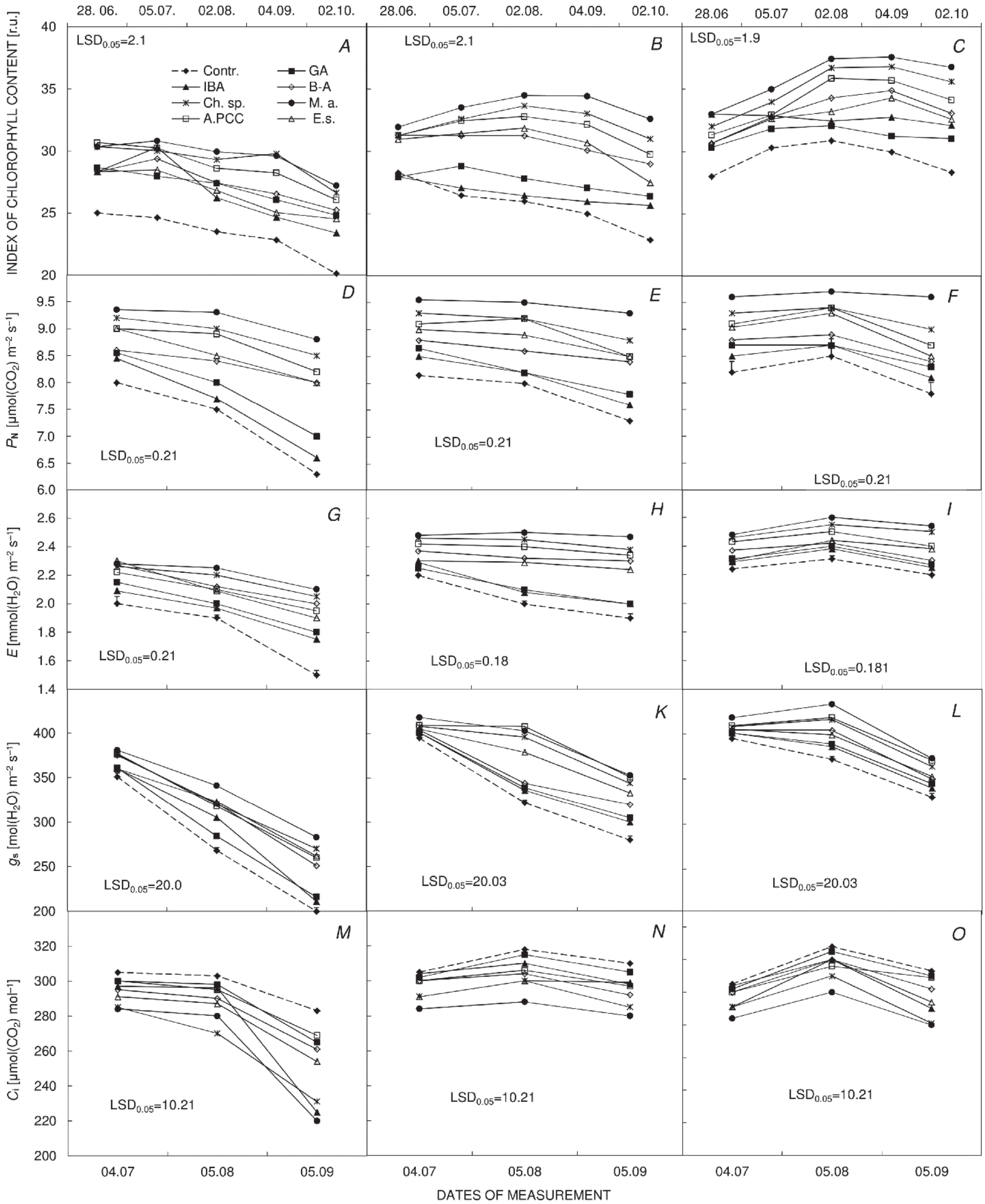

Fig. 2 (*A–C*) Index of chlorophyll content (in relative SPAD units) in leaves of willow plants, grown in 3-L pots outdoor, as affected by triple foliar spray with biostimulators, intact monocultures of cyanobacteria, green algae or the environmental sample and soil fertilization with different doses of the synthetic fertilizers [0.0 (*A*), 0.5 (*B*), and 1.0 g per plant (*C*)]. Effect of microalgae on plant gas-exchange parameters (*D–O*): net photosynthesis (P_N, *D–F*), transpiration (*E*, *G–I*), stomatal conductance (g_s, *J–L*), and intercellular CO_2 concentration (C_i, *M–O*). Doses of the synthetic fertilizers was similar to those of chlorophyll content (same order from left to right in each row, *i.e.* 0.0, 0.5, and 1.0 g per plant). The LSD were calculated at the significance level of p=0.0. Legend explanation is in Fig. 1.

Table 2. Activity of acid (pH 6.0) and alkaline (pH 7.5) phosphatase in willow leaves, as affected by triple foliar spray with biostimulators, intact monocultures of cyanobacteria, green algae or the environmental sample and soil fertilization with different doses of the synthetic fertilizers (0.0, 0.5, and 1.0 g per plant). *The data marked with *the same letters*, within particular enzyme are not significantly different, according to *Duncan´*s multiple range test at an alpha level of 0.05. **The LSD were calculated at the significance level of p=0.05. The LSD were calculated at the significance level of p=0.05.

Applied stimulators, cyanobacteria or green algae	Phosphatase (pH 6.0) [mU g^{-1}(FM)] Doses of fertilizer *YaraMila* [g per plant]			Phosphatase (pH 7.5) [mU g^{-1}(FM)] Complex [g per plant]		
	0.0	0.5	1.0	0	0.5	1.0
Control	0.29^{a}	0.41^{cd}	0.55^{fg*}	0.9^{a}	0.13^{b}	0.18^{de}
GA_3 10^{-6} M	0.36^{bc}	0.65^{ij}	0.64^{hij}	0.13^{b}	0.19^{ef}	0.22^{fg}
IBA 50^{-6} g l^{-1}(H_2O)	0.35^{b}	0.59^{gh}	0.61^{hi}	0.12^{ab}	0.17^{c-e}	0.19^{ef}
Bio-Algeen S90	0.40^{bc}	0.76^{k}	0.77^{k}	0.14^{bc}	0.26^{h}	0.25^{gh}
Chlorella sp.	0.46^{de}	0.83^{lm}	0.88^{m}	0.15^{b-d}	0.30^{i}	0.33^{ij}
Microcystis aeruginosa	0.50^{ef}	0.89^{mn}	0.94^{n}	0.18^{de}	0.35^{jk}	0.38^{k}
Anabaena PCC 7120	0.41^{cd}	0.81^{kl}	0.86^{lm}	0.14^{bc}	0.30^{i}	0.32^{ij}
Environmental sample	0.40^{bc}	0.63^{hij}	0.67^{j}	0.13^{b}	0.22^{fg}	0.25^{gh}
LSD $_{0.05}$**	0.05			0.03		

Table 3. Activity of RNase and total dehydrogenases in willow leaves, as affected by triple foliar spray with biostimulators, intact monocultures of cyanobacteria, green algae or environmental sample and soil fertilization with different doses of synthetic fertilizers (0.0, 0.5, and 1.0 g per plant). *The data marked with *the same letters*, within particular enzyme, are not significantly different, according to *Duncan´*s multiple range test at an alpha level of 0.05. **The LSD were calculated at the significance level of p=0.05.

Applied stimulators, cyanobacteria or green algae	RNase [mU g^{-1}(FM)]			Total dehydrogenases [mg(formazan) g^{-1}(leaf FM)]		
	Doses of fertilizer *YaraMila* [g per plant]					
	0.0	0.5	1.0	0.0	0.5	1.0
Control	1.50^{a}	2.0^{b}	2.60^{cd*}	0.43^{a}	0.62^{b}	0.77^{cd}
GA_3 10^{-6} M	2.00^{b}	3.13^{e}	3.40^{ef}	0.60^{b}	0.85^{d}	1.13^{e}
IBA 50^{-6} g l^{-1}(H_2O)	1.95^{b}	3.00^{de}	3.08^{e}	0.58^{b}	0.79^{d}	1.10^{e}
Bio-Algeen S90	2.25^{bc}	3.57^{f}	3.77^{fg}	0.62^{b}	1.18^{ef}	1.22^{e-g}
Chlorella sp.	2.60^{cd}	4.10^{gh}	4.43^{h}	0.69^{bc}	1.35^{hi}	1.44^{ij}
Microcystis aeruginosa	2.60^{cd}	4.43^{hi}	4.82^{i}	0.79^{d}	1.50^{jk}	1.62^{k}
Anabaena PCC 7120	2.25^{bc}	4.10^{gh}	4.41^{h}	0.66^{bc}	1.30^{f-h}	1.43^{h-j}
Environmental sample	2.15^{b}	3.57^{f}	3.77^{fg}	0.60^{b}	1.13^{e}	1.23^{eg}
LSD $_{0.05}$**	0.40		0.13	0.13		

Permeability of cytomembranes and physiological performance: Independently of soil feeding with synthetic fertilizer, the microalgae strains applied to leaves improved several physiological features which had the essential impact on willow plant development, although it depended on the fertilization level. Biofertilization of plants with the applied microalgae strains and substances increased the relative Chl content in leaves and intensified gas exchange (P_N, E, g_s, accompanied with decreased C_i) (Fig. 2). It limited electrolyte leakage from willow leaves, indicating lower permeability of cytomembranes, independently of the fertilization with *YaraMila Complex* (data not shown) and increased activity of acid and alkaline phosphatase (Table 2), dehydrogenases, RNase (Table 3), and nitrate reductase (Fig. 3). Similarly, as in the case of vegetative growth, the highest physiological performance was caused by M.a, A.PCC, and Ch.sp, while lower performance was found with B-A, ES, GA_3, IBA. The control plants treated with water exhibited the weakest growth and physiological performance (Tables 2, 3; Fig. 2).

The reduction of synthetic fertilizer dose (added to soil) from 1.0 g to 0.5 g(*YaraMila*) per plant did not change physiological performance in the willow plants treated with cyanobacteria and green algae, while in the plants sprayed with water only, it caused serious decreases in enzyme activities and gas-exchange parameters. In the plants nonfertilized with the synthetic fertilizer, physiological performance was also enhanced by treatment with cyanobacteria and green algae, though to a much lesser degree, as compared to the plants fertilized with doses of 1.0–0.5 g per plant. This was particularly evident during the second half of the growing season, when the nutrient content in soil significantly diminished (Tables 2, 3; Fig. 2).

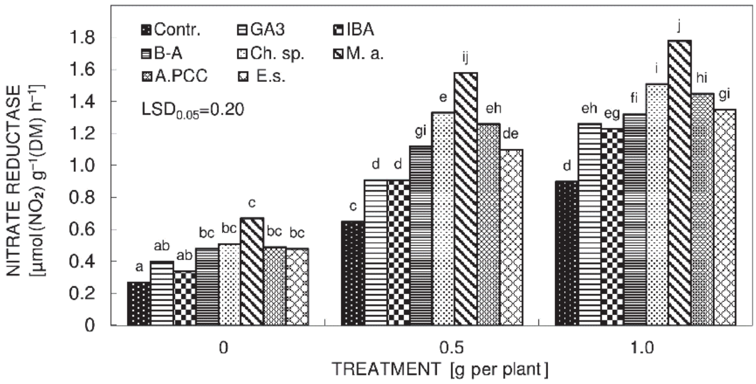

Fig. 3. Activity of nitrate reductase in willow leaves, as affected by triple foliar spray with biostimulators, intact monocultures of cyanobacteria*,* green algae or the environmental sample and soil fertilization with different doses of the synthetic fertilizers (0.0, 0.5, and 1.0 g per plant). Data marked with *the same letters* within column are not significantly different, according to *Duncan´*s multiple range test at an alpha level of 0.05**. The LSD were calculated at the significance level of p=0.05. Legend explanation is in Fig. 1.

Table 4. Content of N, P, and K in willow plants, as affected by triple foliar spray with biostimulators, intact monocultures of cyanobacteria*,* green algae or the environmental sample and soil fertilization with different doses of the synthetic fertilizers (0.0, 0.5, and 1.0 g per plant). *The data marked with *the same letters* within particular macronutrients, are not significantly different, according to *Duncan´*s multiple range test at an alpha level of 0.05. **The LSD were calculated at the significance level of p=0.05.

Applied stimulators, Cyanobacteria, or green algae	N [%]			P [g kg^{-1}(DM)]			K [g kg^{-1}(DM)]		
	Doses of fertilizer [g per plant]								
	0.0	0.5	0.1	0.0	0.5	1.0	0.0	0.5	1.0
Control	1.36^{a}	2.41^{d}	3.05^{g*}	1.220^{a}	1.830^{c}	2.266^{d-f}	14.450^{a}	20.024^{c}	25.910^{e}
GA_3 10^{-6} M	1.42^{bc}	2,48^{de}	3.12^{gh}	1.226^{ab}	1.841^{c}	2.271^{d-h}	14.550^{ab}	20.230^{cd}	26.017^{ef}
IBA 50^{-6} g $l^{-1}(H_2O)$	1.40^{ab}	2.47^{d}	3.11^{gh}	1.227^{ab}	1.835^{c}	2.268^{d-g}	14.530^{ab}	20.109^{cd}	26.004^{ef}
Bio-Algeen S90	1.47^{bc}	*2.58^{ef}*	3.18^{h}	1.246^{b}	2.256^{de}	2.285^{f-h}	14.754^{b}	20.327^{d}	26.218^{ef}
Chlorella sp.	1.49^{bc}	2.58^{ef}	3.18^{h}	1.247^{b}	2.257^{de}	2.292^{gh}	14.758^{b}	20.330^{d}	26.212^{f}
Microcystis aeruginosa	1.51^{c}	2.60^{f}	3.20^{h}	1.247^{b}	2.257^{de}	2.294^{h}	14.760^{b}	20.330^{d}	26.215^{f}
Anabaena PCC 7120	1.5^{bc}	2.58^{ef}	3.18^{h}	1.246^{b}	2.256^{de}	2.292^{gh}	14.751^{b}	20.324^{cd}	26.210^{ef}
Environmental sample	1.45^{bc}	2.51^{d-f}	3.15^{gh}	1.225^{ab}	2.247^{d}	2.280^{e-h}	14.680^{ab}	20.167^{cd}	26.090^{ef}
LSD $_{0.05}$**	0.10			0.249			0.300		

Nutrients composition and energetic values of willow plants: The applied monocultures of cyanobacteria*,* green algae, and B-A increased the quantity of micronutrients (N, P, K) in willow plants, as compared with the control. The effect depended on the quantity of the synthetic fertilizer and was the most intensive with its highest dose applied to soil (Table 4). The microalgae strains, B-A, and ES, applied to leaves, did not change the willow plant calorific value in the operating state, heat of combustion in the analytical state, and ash content in the working state, as compared with the control (Fig. 4).

Discussion

The use of organic fertilizers, biofertilizers, and other microbial products is beneficial because it allows limiting chemical fertilizer application, which is harmful for the environment. Cyanobacteria and green algae can play a crucial role in plant and soil fertility as nitrogen-fixing microorganisms and producers of several natural substances positively affecting growth. Thus, recently around the world, a considerable progress took a place in the development of cyanobacteria and green algae-based biofertilizer technology (Saadatnia and Riahi 2009).

Proper foliar biofertilization with cyanobacteria and green algae may allow limit markedly doses of chemical fertilizers by as much as 50%, without significant reduction of growth and biomass yield of willow. This suggestion was confirmed by research of Hegazi *et al.* (2010) performed on common bean; they suggested that ¼ to ½ of the recommended dose of nitrogen mineral fertilizer could be saved by using some species of N-fixing cyanobacteria.

Our results are in line with the studies of other researchers (Falch *et al.* 1995, Kreitow *et al.* 1999, Burja *et al.* 2001, Nain *et al.* 2010, and Rana *et al*. 2012) who demonstrated the stimulatory influence of green-blue algae on wheat development and their inhibitory effect on

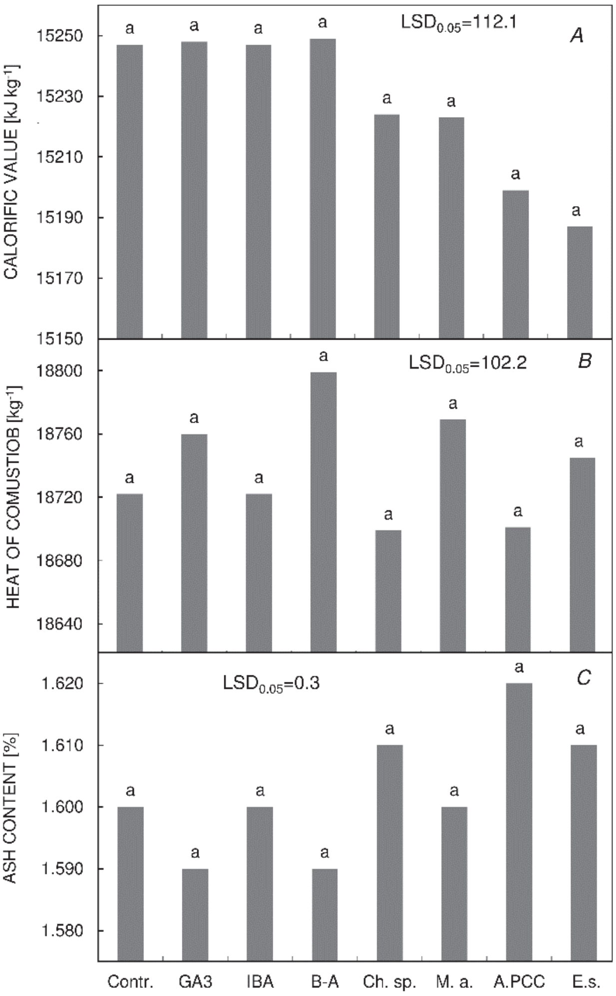

Fig. 4. Energy properties of plants (*A*, *B*) and ash content (*C*), as affected by triple foliar spray with biostimulators, intact monocultures of cyanobacteria, green algae or the environmental sample and fertilization with complex the fertilizer at dose of 1.0 g per plant. Data marked with *the same letters* within particular macroelements, are not significantly different, according to *Duncan´*s multiple range test at an alpha level of 0.05. The LSD were calculated at the significance level of p=0.05. Legend explanation is in Fig. 1.

pathogenic microflora growth, by synthesizing some active compounds, which inhibit the growth of bacteria and fungi. The presented results also agree with reports of Rastogi and Singha (2009) who indicated that some cyanobacterial secondary metabolites were toxic to living organisms. Diverse cyanotoxins may play ecological roles as allelochemicals, and could be employed for the commercial development of algaecides, herbicides, and insecticides. Bioelicitors obtained from *Ulva lactuca* (sea lettuce) reduced wilt development in tomato seedlings, caused by *Fusarium oxysporum f.* sp. *lycopersici.* (El Modafar *et al.* 2012). Their natural defense was accompanied by a systemic acquired resistance which seemed to be salicylic acid-dependent.

The increased length of shoots, branching and biomass of willow, caused by foliar feeding with the studied microalgae strains, could be caused by greater intensity of several physiological events and by plant enrichment with auxin (IAA), GA, cytokinins, amino acids, macronutrients (N, P, K, Ca, Mg), microelements (S, Zn, Fe, Mn, Cu, Mo, Co), polyamines, and several other secondary metabolites, which can be produced by cyanobacteria and green algae (Haroun and Hussein 2003, Masojídek and Prášil 2010, Chojnacka *et al*. 2010, Nunnery *et al.* 2010, Perez-Garcia *et al*. 2011, Pszczolkowski *et al*. 2012, Sahu *et al.* 2012, Markou and Nerantzis 2013). According to Hussain and Hasnain (2012), effectiveness of phytohormones of microbial origin is comparable to that of standard cytokinins and IAA. The natural supplements, produced by cyanobacteria, induced adventitious shoot formation in *Brassica oleracea*, similarly as it was observed in willow plants. Release of high amounts of IAA from some plant-interacting bacteria was also reported by Glick *et al.* (1999). Ability of symbiotic isolates of cyanobacteria to accumulate and release IAA was also reported by Sergeeva *et al*. (2002). According to them, IAA accumulation is stimulated by exogenous tryptophan and may proceed *via* the indole-3-pyruvic acid pathway. This blue-green algae may initiate phytohormone signals both when free-living and when *in planta*. *Nostoc* sp. stimulated mitotic activity in host cells close to the site of penetration (Bergman *et al.* 1992).

The hastened willow plant development resulting in higher biomass yield could be caused not only by metabolites and other natural biostimulators synthesized by cyanobacteria (Markou and Nerantzis 2013) but also by their ability to assimilate atmospheric nitrogen, even up to 20–25 kg ha^{-1} (Sahu *et al.* 2012). The N_2 assimilated from atmosphere could be delivered to willow tissues and cause increasing plant growth, as it was found in rice, wheat, gillyflower, grapevine, and corn (Spiller and Gunasekaran 1990, Obreht *et a*l. 1993, Haroun and Hussein 2003, Shanan and Higazy 2009, Romanowska-Duda *et al.* 2010, Pszczolkowski *et al*. 2012, Grzesik and Romanowska-Duda 2014). Enhanced growth of willow plants could be also caused by the ability of the blue-green algae to convert complex nutrients into simple ones available for plants (Sahu *et al*. 2012) and to increase water-holding capacity through their jelly structure (Roger and Reynaud 1982). It might be also improved by the use of dead cyanobacteria as a fertilizer reducing soil salinity (Saadatnia and Riahi 2009). Treatment with cyanobacteria could also increase the phosphate content in soil by excretion of organic acids (Wilson 2006).

Differentiated impact of the applied strains of *M. aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120, *Chlorella* sp., and *Bio-Algeen S90* (an extract from brown algae and seaweeds) on willow plant growth could be also caused by growth-promoting compounds, contained in the studied monocultures which so far has not been fully elucidated. The more positive influence of the used cyanobacteria and green algae monocultures than that of GA_3 and IBA could be caused by additional activity of a large number of growth-promoting substances present in these strains in addition to the mentioned hormones (Roger and Reynaud 1982, El Fouly *et al.* 1992, Mahmoud 2001, Haroun and Hussein 2003, Rodriguez *et al.* 2006, Masojídek and Prášil 2010, Chojnacka *et al*. 2010, Nunnery *et al*. 2010, Perez-Garcia *et al*. 2011, Pszczokowski *et al*. 2012, Markou and Nerantzis 2013).

Since in algae the amount of natural substances is relatively smaller as compared to synthetic mineral fertilizers, their foliar application seems to be the most appropriate way to increase the efficiency of biofertilization. During foliar fertilization, more than 90% of the compounds are utilized by a plant, while when they are supplied to soil, only 10% of them are absorbed by crops. Thus, foliar application can increase yields by 12–25% when compared to conventional fertilization (Ecochem 2017).

The presented study indicated close relations between the studied physiological activities (Chl content, membrane permeability, enzyme activity, gas exchange) and growth intensity of willow plants, in dependence on on microalgal biofertilization and soil fertilization, similarly as it was also observed in corn (Grzesik and Romanowska-Duda 2014, Grzesik and Romanowska-Duda 2015).

Higher Chl content in leaves (by 19%, compared with the control) was also observed by Khan *at al*. (2012) following foliar application of 0.5 mL L^{-1} of mixture of seaweed extract and amino acids to grapevines. Exogenous application of seaweed extract caused also 12% increase in Chl contents, photosynthesis, and respiration rates in leaves of 'Fuji' apple (Spinelli *et al*. 2009). Increased content of Chl in leaves could be caused by higher amount of nitrogen assimilated by cyanobacteria from atmosphere and delivered to plant tissues (Spiller and Gunasekaran 1990, Nilsson 2005, Karthikeyanb *et al*. 2007).

The, lower permeability of cytomembranes, caused by foliar biofertilization with the tested cyanobacteria and green algae strains, could be caused by deposition of phosphates (periplasmic enzymes) on the membrane or between the cytoplasmic membranes and a cell wall and the consequent interactions between them (Cheng *et al*. 1971).

The study indicated that foliar application of cyanobacteria and green algae strains caused increases in activity of several enzymes having important impact on plant development, including acid and alkaline phosphatase, independently of plant fertilization level. Both these enzymes are responsible for the distribution of phosphorus in plants and they catalyze hydrolysis of organic phosphorus. They are also considered to be a good indicator of the activity of secondary metabolites released from cyanobacteria and green algae, moreover, they show the mineralization potential of organic phosphorus or biological activity of soil (Dick and Tabatabai 1993). Cyanobacteria contain mainly acid and alkaline phosphatases which are able to detach phosphate residues from polyphosphates and release energy.

Cyanobacteria and green algae applications to willow plants stimulated ribonuclease (RNase) activity, similarly at both fertilization doses [0.5–1.0 g(*YaraMila Complex*) per plant] and to a lesser extent, when plants were not fertilized with the synthetic fertilizer. RNase constitutes a heterogeneous group of enzymes involved in the process of enzymatic degradation of various fractions of ribonucleic acid. Their activity increases during apoptosis, aging of plants, and seed germination. Booker (2004) indicated that ribonuclease modified the activity of particular genes by the specific degradation of mRNA transcripts, leading to changes in the concentration of molecules of these compounds. According to Lehmann *et al.* (2001), Šindelářová *et al*. (2005), and Srivastava *et al*. (2006), RNase activity increased after the attack of phytopathogens and under phosphorus-deficiency conditions. Stimulation of specific RNase activity may play an important role in increasing defense mechanisms in plant tissues, as it was also observed in willow and corn plants, in which the improved health status was associated with the enhanced RNase activity (Grzesik and Romanowska-Duda 2014, Grzesik and Romanowska-Duda, 2015).

The increased activity of dehydrogenases, caused by cyanobacteria and green algae treatments, are in line with reports of De-Mule *et al.* (1999) and De-Caire *et al.* (2000) who indicated a significant positive effects of algal species application to compost materials which led to a remarkably increased activity of dehydrogenase, as compared with the control variants. Cyanobacteria also increased activity of dehydrogenases and growth of Virginia fanpetal (*Sida hermaphrodita*) plants which were treated with these monocultures and biostimulator *Asahi SL* (*Arysta Lifescience*), to alleviate the negative influence of adverse impact of insufficient temperature and soil moisture content (Grzesik and Romanowska-Duda 2009).

Our results indicated that the applied cyanobacteria and green algae to the leaves can affect the content of microelements in plants. The greater amount of nitrogen in the plants treated with cyanobacteria could be caused by additional assimilation of atmospheric nitrogen by their intact cells and its subsequent transport to the willow tissues or by macroelements present in the cells of the applied strains (Spiller and Gunasekaran 1990, Nilsson 2005, Karthikeyanb et al. 2007). The higher content of N, P, K was also observed by Abd El Monien and Abd-Allach (2008) in grapevines foliar biofertilized with green algae *Chlorella vulgari.,* while multiple spray applications of a mixture of amino acids and a seaweed extract increased N, P, K, B, Fe, and Zn contents (Khan *et al*. 2012). Moreover, application of algae had a significant effect on nutrient uptake by wheat (Mohammadi *et al.* 2010). The correlation between nitrogen fixation and increased P uptake by plant was found by Swarnalakshmi *et al.* (2013) who evaluated novel biofilmed preparations, using *Anabaena torulosa* (cyanobacteria) as a matrix for agriculturally useful bacteria (*Azotobacter*, *Mesorhizobium*, *Serratia*, and *Pseudomonas*) in wheat. In spite the positive impact on growth and physiological activity, the applied strains neither reduced the energy properties of willow plants nor changed the ash content. Moreover, due to greater amount of N, P, K, this ash could be a useful fertilizer for ecological crops. The literature data concerning these issues are hard to find. The improved overall health, enhanced Chl content in leaves, intensified photosynthetic rate, and other plant gas-exchange parameters in leaves, as well as higher activity of the studied enzymes, integrity of cytomembranes and microelements quantity, caused by application of *Microcystis aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120, and *Chlorella* sp., could have the crucial influence on hastening growth of willow plant cultivated for energy purposes.

The observation indicated that the foliar application of selected strains of *M. aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120, and *Chlorella* sp. caused the increased growth and physiological performance of willow plants, independently of soil fertilization with synthetic fertilizers. Proper foliar biofertilization with these monocultures may allow a marked decrease in recommended doses of chemical fertilizers for willow plant cultivation (Al-Khiat 2006). Cyanobacteria are a bio-geo-chemically important part of the ecosystems, playing an important role in nitrogen and carbon cycling. Some algae strains also have the important ability to form affectionate symbiotic associations with a broad range of eukaryotic hosts belonging to different plant groups (Gorelova 2006). Nontoxic cyanobacterial and green algae cultures can be used in ecological and integrated willow cultivation and can facilitate environmental protection, by reducing the need to use toxic artificial fertilizers and pesticides (Hegazi *et al.* 2010). However, due to limited available data and an increased interest in this area, further research is needed to elucidate this phenomenon and to identify active compounds discharged by cyanobacteria and green algae and to determine their influence on particular plant species.

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