Biochemical responses of 5 buckwheat (*Fagopirum esculentum* Moench.) cultivars to seed treatment by *Azospirillum brasilense*

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Abstract. Cultivation condition have a large effect on efficiency of buckwheat. Drought, high temperatures and their fluctuations, salinity, oxygen deficit in the soil, ultraviolet radiation, and abnormal nutrient content in the soil are the most common reasons of decreasing productivity of plants. Suboptimal parameters of the cultivation technology can also cause abiotic stress. Plant can decrease its efficiency from 30% to 50% depend on stress conditions. Using bacterial cultures is one of the preventive approaches to overcoming the negative impact of stressors. Microorganisms produce biologically active substances that stimulate plant growth, increase their resistance to abiotic factors. They are growth regulators and long-acting anti-stressants as well. Malondialdehyde (MDA) is formed as a result of the oxidative degradation of polyunsaturated fatty acids. Fructans are polysaccharides that are derived from D-fructose residues found in higher plants, green algae and bacteria. Fructans are involved in the adaptation of plants to the action of abiotic stressors and are valuable nutrients. The effect of *Azospirillum brasilense* pre-sowing treatment of buckwheat seeds on physiological and biochemical processes of MDA and fructan content was researched. Seed treatment with *Azospirillun brasilense* reduced the content of MDA in Ukrayinka and Syn 3/02. Sofia and Olga had a low level of MDA, but seed treatment increase it. Seed treatment increased the efficiency of photosynthesis (Fv / Fm) in Syn 3/02 from 0.58 to 0.72, in other varieties this effect was negligible. All cultivars have a strong relation between MDA and fructan content, that shows their participation in responses on cultivation conditions. Efficiency of photosynthesis in flowering–seed formation stage (BBCH 65–75) was close to maximum in field condition (0.70 ± 0.05) and seed treatment can increase it.

Key words: buckwheat, malondialdehyde (MDA), fructans, fluorescence.

INTRODUCTION

Limiting factor in buckwheat productivity is its demand for soil nutrients.

Seed microbial preparation of buckwheat can improve plant productivity as a result of improved mineral nutrition and synthesis of organic compounds (Bhattacharyya & Jha, 2012; Bano et al., 2013). The concentration of phenolic compounds and antiradical activity in the plant depends on the stage of development. Itsnumber increases as the seeds germinate (Beitane et al., 2018), but then decreases. Additional sources of

antistressants are needed for improvement stress resistance during active vegetation. *Azospirillum* spp. can be associated with buckwheat root system, increase its productivity and increase the amount of antistressants (Tummaramatti et al., 2014; Singh et al., 2015). The accumulation of fructans is the basic strategy of plants to counteract the unfavorable factors. Seed treatment by *Azospirillum brasilense* increases fructan content but the level of sucrose in plants decreases (Bagheri & Jafari, 2012). Genotypes with high fructan accumulation in stress condition can decrease malondialdehyde in plant (He et al., 2015). Seed treatment with microbial preparations can lead to a decrease in MDA concentration in some plant species may have species and cultivar reaction (Taran et al., 2016).

Malondialdehyde is a marker of the oxidative degradation of unsaturated fatty acids. However, the peroxide radical can also interact with neutral fatty acid molecules (Munne-Bosch & Pinto-Marijuan, 2016). Unsaturated fatty acids in membrane phospholipids can be oxidized in this way, but also free unsaturated fatty acids, residues of unsaturated fatty acids (Kumar & Ebel, 2016). The oxidation of unsaturated fatty acids is controlled by enzymes. The fact that the body has a normal physiological level (background) of malondialdehyde (MDA), diene conjugates (DC), other products of lipid peroxidation, indicates that there is a strict control of lipid oxidation by the entire hierarchical system of hierarchical regulation the DNA turn (You & Nam, 2014). The physiological role of oxidation reactions is to regulate the renewal and permeability of biological membrane lipids, the interaction of eicosanoids - mediators (local hormones) or signaling substances that play an important biological role in the organism (Zhang et al., 2016). Such important membrane processes as electron transfer in the respiratory chain, oxidative phosphorylation, methylation, hydroxylation of a number of endogenous and exogenous substrates by enzymatic systems of the endoplasmic reticulum, and, even, cell division, are accompanied by changes of the level of malondialdehyde (Huang et al., 2015). Malondialdehyde can modify proteins in cells and it leads to irreversible changes (Fenaille et al., 2002).

Fructans are polysaccharides that are derived from D-fructose residues found in higher plants, green algae and bacteria (Harding et al., 2017). Fructans are complex sugars that are important for the plant (Pollock et al., 2017). Fructans are distinguished by several main types: inulin, levan, neo-inulin and neo-levan (Le Roy et al., 2008). The synthesis of fructans from Sucrose involves at least two enzymes – *sucrose:sucrose 1-fructosyltransferase (1-SST)* and *fructan:fructan 1-fructosyltransferase (1-FFT)* (Kanabus et al., 1991; Ritsema & Smeekens, 2003). *1-SST* catalyzes the production of a Glc-Fru-Fru trisaccharide that can be extended with Fructose residues in various ways by *1-FFT* (Kanabus et al., 1991). The amount of D-fructose residues depends on species of the plants and condition of vegetation (Hellwege et al., 1998). Degree of polymerization (DP) of fructans increases under stress conditions in response to a stress factor (Quezada et al., 2017). It should be noted that fructans have an extremely important nutritional value for humans. Consuming a balanced content of fructans of different types (oligofructose and short-chain fructo-oligosaccharides) can increase the body's immune responses and improve the body's overall homeostasis (Roberfroid, 2004).

Chlorophyll fluorescence is one of the most popular techniques in plant physiology because of the ease with which the user can gain detailed information on the state of photosystem II (PSII) at a relatively low cost. It has had a major role in understanding

the fundamental mechanisms of photosynthesis, the responses of plants to environmental change, genetic variation, and ecological diversity (Murchie & Lawson, 2013). The method consists in detecting chlorophyll fluorescence which occurs if illuminated by bright light plants will be adapted to the darkness, known as the Kautsky effect (Kautsky & Hirsch, 1931). Light energy is absorbed by chlorophyll molecules in the leaves can be used for one of three processes: the photochemical reaction, the excess energy is dissipated as heat or re-emitted as fluorescence. These three processes are competitive because the effectiveness of one leads to changes in the other two. Thus the change in intensity of chlorophyll fluorescence gives information on efficiency of flow photochemical reactions (Maxwell & Johnson, 2000).

MDA and fructan content are markers of oxidative stress that occur under cultivation conditions. Determining of MDA level and fructan content in plants allows to establish cultivar responses to cultivation conditions in a specific region. Adverse conditions can affect the efficiency of biochemical processes of photosynthesis. Accumulation of antistressants in treated plants may increase resistance of photosynthesis systems to changing environmental conditions in the field. The reactions of different crops to seed treatment with *Azospirillum brasilense* are often described in the literature, but the evaluation of MDA, fructans and the induction of fluorescence of chlorophyll in buckwheat is poorly understood.

MATERIALS AND METHODS

Field studies were conducted in the Educational-Scientific Laboratory Demonstration Collection Field of Crops of the Department of Plant Science (Kyiv, Ukraine; 50º 22´ N, 30° 30´ E). The investigation was performed in field conditions. The soil of the experimental field was grey forest light loam soil with 2.32–3.01% humus content in the arable soil layer, pH_{KOH} − 5.8–6.1, hydrolyzed nitrogen − 62–83 mg, phosphorus − 75–120 mg, and potassium − 42–101 mg per 1 kg of soil. Cropping system is common to the northern forest-steppe of Ukraine.

Climate conditions
Field experiments were conducted in 2017–2018 (2 seasons). Daily average temperature in buckwheat vegetation in 2017 was 17.8 °C (average multi-annual +15.5 °C), but 2018 was hotter (19.4 °C) and dry. Air temperature in the first decade of May (sowing period) has varied over the years (13.6 °C in 2017, 16.9 °C in 2018). Temperature in first half of buckwheat vegetation (May-June) was abnormally in all region (Mazurenko et al., 2020), that could impact on its organogenesis and biochemistry processes. Summary precipitation in vegetation period was 146 mm in 2017 and 245 mm in 2018 (multi-annual 254 mm). Buckwheat fell into arid conditions with moisture deficiency in 2017.

Cultivation and sampling
Cultivar sensitivity to pre-sowing treatment with *Azospirillum brasilense* was established in 2-factore field experiment. First factor was 5 buckwheat (Fagopyrum esculentum Moench.) cultivars. There was Sofia, Olga, Ukrayinka, Antariya, Syn 3/02 varieties observed. Second factor of the cultivation of buckwheat included seed treatment with *Azospirillum brasilense, norm* 1 L t^{-1} *seed (Az. br.)* and control (without

Azospirillum brasilense, Wt). Pre-sowing treatment of seeds with strain of bacteria *Azospirillum brasilense* (1 mL contained 2×10^9 colony-forming units (cfu) bacteria of the genus *Azospirillum*) was performed.

The experiment was established in 4 replications. The size of elementary plots was 36 m^2 (24 m^2 to harvesting). Tillage system included disking after preceding crop harvesting (winter wheat) and ploughing on 18–20 cm in autumn. Disking for moisture saving was carried out in early spring and pre-sowing cultivation to a depth of 3–4 cm was conducted before sowing. $P_{45}K_{30}$ (superphosphate, 18% P; potassium chloride, 60% K) was applied before ploughing in autumn and N_{30} (ammonium nitrate) before sowing. Buckwheat was sown with 15–cm inter-row spacing with rate 400 grains per square meter. Sowing time (1st decade of May) depended on soil temperature (optimum 10–12 °C). Pesticides did not apply. Buckwheat was harvested when 65–75 % seeds was brown.

Leaf samples for MDA and fructan analyses are taken from the middle tier. Samples was weighed (100 mg), homogenized with the addition of phosphate buffer (pH 7.4, 1 mL) and centrifuged for 15 minutes at 15,000 rpm. The required aliquot was selected to determine the MDA and polyfructan content.

Analysis of the level of accumulation of malondialdehyde

Activity of enzymes of the antioxidant protection system in the plants was determined by the level of malondialdehyde (MDA). A lipid peroxidation test is based on the concentration of a colour complex formed as a result of the reaction of malondialdehyde (MDA) with two molecules of thiobarbituric acid (TBA) (Vladimirov & Archakov 1972). Supernatant was obtained after centrifugation. 300 μL of the supernatant mixed with 900 μL of 5% three chloroacetic acid and 300 μL of 0.8% thiobarbituric acid. This mixture was incubated a half an hour (90 °C). Optical density was determined at a wavelength of 532 nm on a SF-26 spectrophotometer.

Fructan content sampling

The content of polyfructans was determined by the ability of ketotsugars to stain in acid with resorcinol. 100 μL of the extract (supernatant) was mixed with 100 μL of 0.1% alcohol solution of resorcinol and 100 μL of concentrated HCI. Mixture boiled 10 minutes (80 °C) for observing purple colour. Optical density was measured on an Eppendorf biofotometr plus at 550 nm.

The concentration of polyfructans was determined according to the calibration graph (calibration reactions with resorcinol fructose solutions; concentration of 0.62, 1.25 2.5 and 5 mg mL^{-1}). Measurements were made in three replications.

Chlorophyll fluorescence sampling

Samples for induction of chlorophyll fluorescence were taken from the middle tier. Leaves were placed in moistened paper and kept without access to light for 15 minutes (dark adaptation) Then, the fixed part of the leaf is irradiated with light of wavelength 470 ± 15 nm. Under the influence of light the chlorophyll fluorescence is excited. Fluorescence signal is isolated with red filter and enters on the photo detector (wavelength 670 nm) which converts it into an electrical signal and amplified. An electrical signal sensor device is displayed and then stored and transferred to a computer for further analysis. The intensity of the induction of chlorophyll fluorescence (IFH) was

determined using a portable fluorometer 'Floratest' (Romanov et al., 2007). Fast and slow phase of chlorophyll fluorescence was determined for 3 min. Several physiological indices on the IFH curve were identified. There are minimal level fluorescence (Fo), maximum fluorescence (Fm), photochemical activity of FS II(Fv/Fo), potential quantum efficiency of photosynthesis (Fv/Fm) and index vitality (Fm/Fst).

Statistical analysis

Statistical analysis of the data was made using program Statistica 6.0 (StatSoft I.N.C.). Probability of the difference between the arithmetic mean of indicators was established using Student's test. The differences are considered to be significant at a value $P \leq 0.05$.

RESULTS AND DISCUSSION

MDA and fructan content in buckwheat

Buckwheat cultivars had different sensitivity to environmental conditions. Level of MDA was different in different cultivars (Fig. 1). MDA level above 0.3 µM g^{-1} of crude mass was formed by cultivars Ukrainka, Antaria and Syn 3/02. It should be noted that bacterial treatment did not have effect on the overall level of MDA in Antaria and Syn 3/02, but MDA level decreased in Ukrainka.

Sofia and Olga accumulated significantly less MDA. Sofia without treatment accumulated the least MDA, but *Azospirillum brasilense* treatment increased it. Olga also had this tendency, but the average MDA level was higher.
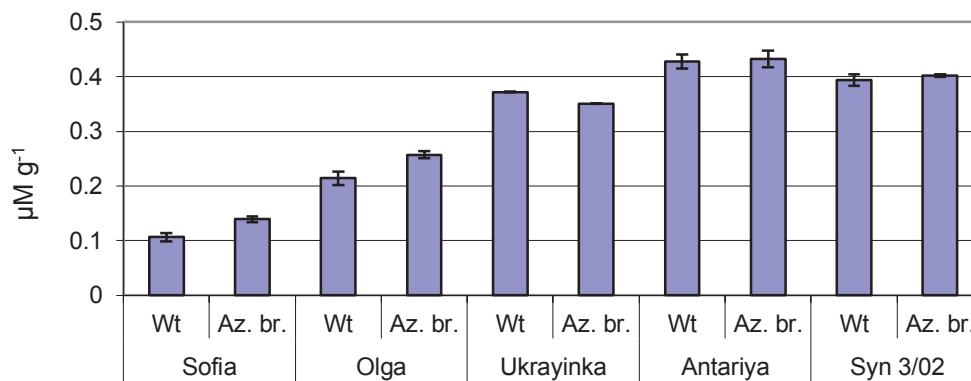

Figure 1. MDA levels a during grow up of buckwheat depending on the variant of the pre-sowing treatment of seeds: Wt – treatment with sterile water; Az. br. – treated with *Azospirillum brasilense*.

Fukami et al. (2018) showed that inoculation with *Azospirillum brasilense* decreases MDA content in roots but increase it in leaves. Tummaramatti et al. (2014) noted that *Azospirillum* spp. have a positive effect on growth processes, which is manifested in the increase of dry and wet weight of plants. However, their resistance to abiotic stress may be reduced with improved nutrition.

Tolerant genotypes under normal conditions have a much lower MDA content than sensitive ones. Certain genotypes do not change the level of MDA in stressful conditions (He et al., 2015).

The content of polyfructans in plants of one species is approximately constant. Changing their concentration may indicate stress or counteract it (Fig. 2). Content of fructans with or without treatment did not make a significant difference in Ukrainian and Olga. Fructan content was reduced by *Azospirillum brasilense* treatment in Syn 3/02 but exceeded the other cultivars. Significant increase in fructan content by treatment was observed in Sofia and Olga.
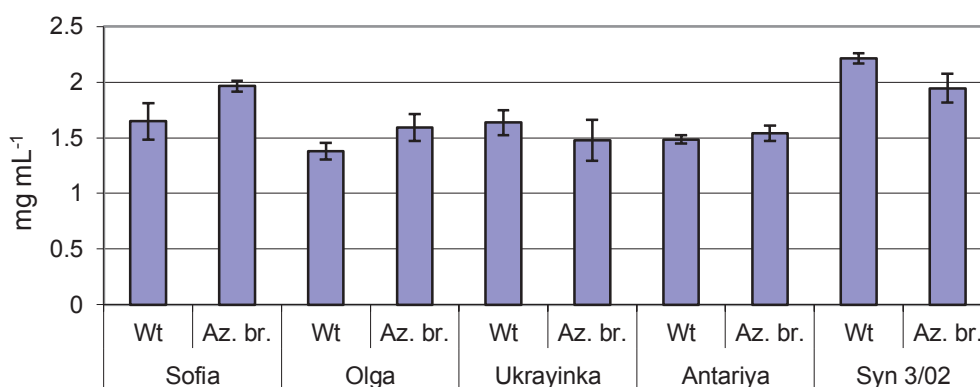

Figure 2. Fructan content of buckwheat depending on the variant of the pre-sowing treatment of seeds: Wt – treatment with sterile water; Az. br. – treated with *Azospirillum brasilense*.

On the other hand, improving nutrition can have a positive effect on the synthesis of metabolites that can counteract stress and bind ROS. The principle of counteracting oxidative stress is based on the properties of fructans react with peroxidases to form fructan radicals and water (Van den Ende & Valluru, 2008; Bolouri-Moghaddam et al., 2010). Fructans can also be a signaling mechanism of stress in plant cells (Van den Ende and El-Esawe, 2014). Content of fructans in sensitive varieties can be significantly reduced, and in resistant it increases. High fructan content does not always characterize genotype tolerance (Nemati et al., 2018).

MDA accumulation has a strong relationship (Table 1) with fructan content in plant. Increasing the fructan content with increasing levels of MDA may indicate adaptive properties of cultivar to environmental conditions. Sensitive to *Azospirillum* spp. cultivars increase the level of polysaccharides have a higher resistance to oxidative stress.

Table 1. Correlation between fructan content and MDA level

Cultivar	Untreated	Treated
Sofia	0.69	0.92
Olga	0.90	0.83
Ukrainka	0.89	0.87
Antariya	0.99	0.87
Syn 3/02	0.94	0.81

All correlation significant at $P \leq 0.01$.

Chlorophyll fluorescence of buckwheat leaves
Efficiency of photosynthesis was dependent on *Azospirillum brasilense* treatment and manifested differently in different cultivars (Table 2).

Table 2. Indicators of photosynthesis activity

Treatment	Cultivar	Fo, r.u.	Fm, r.u.	Fv/Fo	Fv/Fm	Fm/Fst
Without treatment	Sofia	488 ± 41	1,733 ± 134	2.55 ± 0.02	0.70 ± 0.01	3.16 ± 0.09
	Olga	475 ± 12	1,557 ± 89	2.28 ± 0.15	0.68 ± 0.03	3.17 ± 0.12
	Ukrayinka	408 ± 10	1,403 ± 53	2.44 ± 0.06	0.70 ± 0.01	3.13 ± 0.04
	Antariya	427 ± 20	1,733 ± 98	3.06 ± 0.06	0.75 ± 0.01	2.98 ± 0.17
	Syn 3/02	454 ± 46	1,334 ± 26	1.94 ± 0.14	0.58 ± 0.04	3.73 ± 0.11
Azospirillum brasilense	Sofia	439 ± 18	1,387 ± 76	2.16 ± 0.16	0.71 ± 0.02	3.21 ± 0.1
	Olga	446 ± 23	1,573 ± 66	2.53 ± 0.08	0.72 ± 0.01	3.31 ± 0.12
	Ukrayinka	346 ± 32	1,077 ± 128	2.11 ± 0.07	0.69 ± 0.02	2.89 ± 0.17
	Antariya	421 ± 2	1,312 ± 112	2.11 ± 0.16	0.66 ± 0.04	3.08 ± 0.12
	Syn 3/02	336 ± 4	1,205 ± 10	2.59 ± 0.04	0.72 ± 0.01	2.90 ± 0.05

F_o – minimal level fluorescence; Fm – maximum fluorescence; Fv/Fo – photochemical activity of *FS* II; Fv/Fm – potential quantum efficiency of photosynthesis; Fm/Fst – index vitality; *r.u.* – relative units.

Minimal level fluorescence (Fo) ranged from 336 to 488 relative units in observed samples. This indicator characterizes the amount of excitation energy that is lost during migration on the pigment matrix. Concentration of clotophyllles that are not bonded to the reaction centers (RCs) are also relevant to this indicator. Treated plants have a lesser energy loss in compare with without treatment. Higher difference between treated and untreated variants was observed in Syn 3/02 (26%) and Ukrayinka (15%). Sofia, Antariya and Olga decreased his Fo parameter, but they did not have significant difference.

Maximum fluorescence level (Fm) shows the fluorescence intensity of chlorophyll at the 'closed' reaction centers of FS II, when all electron acceptor QA (a bound quinone) are restored and unable to receive electrons from the RC. Seed treatment *Azospirillum brasilense* reduced Fm by 20–24% in Sofia, Ukrayinka and Antaria. Other cultivars reduced Fm without significant difference.

Largest difference (indicator Fv/Fo) between treated and untreated variants was observed in Syn 3/02. Increase in photosynthetic activity of photosystem II was observed in cultivar Olga, but it has a lesser improvement. Other cultivars have the highest Fv/Fo for untreated variants and decreased this parameter for *Azospirillum brasilense* treatment.

Lazar (1999) indicated that Fv/Fm reaches 0.82 under optimal conditions. There was Fv/Fm from 0.66 to 0.75 relative units in researched cultivars. It may indicate the effect of stress factor on plants. Improvement of photosynthetic activity of buckwheat plants was observed in Olga (+3%) and Syn 3/02 (+9%) for treatment. Antaria had the highest Fv/Fm without treatment, but reduced it in treated variant. Bagheri & Jafari (2012) have indicated that seed treatment by *Azospirillum brasilense* reduces the negative effects in the activity of the photosynthetic apparatus and decreases reduction of Fv/Fm.

Fm/Fst indicator varied quite strongly in Olga and Syn 3/02 in treated and untreated variants. Fm/Fst characterizes the efficiency of the dark phase of photosynthesis, it can be noted that in plants treated with bacteria of the genus *Azospirillum brasilense* although there was an improvement of this process on 2–5%.

CONCLUSIONS

MDA levels and fructan content are important indicators of the relationship of plants to the environment. *Azospirillum* spp. treatment stimulates a lot of processes in plant, but effect on accumulation of antistressants was different in buckwheat cultivars.

According to MDA level, it can be noted that Olga and Sofia have a better oxidative resistance than other cultivars. High fructan content in Sofia indicates high potential for tolerance in different field conditions.

Pre-sowing seed treatment of *Azospirillum brasilense* causes an increase in the efficiency of functioning of the photosynthetic apparatus of buckwheat plants. Features of its action depend on varietal characteristics of plants. Positive influence on all stages of photosynthesis was noted in Olga variety. In other varieties, this effect was not manifested by all the parameters studied. This issue needs further study.

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