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Original article

PHOTOSYNTHETIC ACTIVITY AND ANTIOXIDANT CAPACITY OF PERENNIAL FORAGE GRASSES

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Analysis of the photosynthetic activity and the antioxidant capacity was carried out on the perennial forage grasses: English ryegrass (*Lolium perenne* L.), Tetramis cultivar, tall fescue (*Festuca arundinacea* Schreb.) Albena cultivar, meadow fescue (*Festuca pratensis* Huds.) selected population, red fescue (*Festuca rubra* L.) ecotype Ravnogor and smooth brome (*Bromus inermis* Leyss.) Nika cultivar. The analysis was performed during the summer and autumn periods of growth and development. As a basic physiological process, photosynthesis is tightly related to the plant tolerance to different stressors. In addition, tolerance to the secondary exerted oxidative stress directly depends on the antioxidant content. The analysis of the antioxidant capacity was performed by the methods of ferric and molybdate reduction. Photosynthetic activity and the amount of photoprotection were determined by chlorophyll fluorescence methods. The results show about a twofold higher increase in photosynthetic activity of the light phase in *F. arundinacea*, *F. pratensis* and *B. inermis* in summer, which increased with about 20% in *L. perenne, F. arundinacea*, 25% in *F. rubra* and decreased with about 15% in *B. inermis* in autumn. However, the levels of photoinhibition were comparatively low, indicating an absence of stress in practice. The antioxidant capacity was five times higher in the smooth brome from the Nika cultivar, as compared with the other grasses. The tall fescue from the Albena cultivar had the highest photosynthetic activity, uninhibited during the measurements under the high, as well as the low temperatures.

Key words: perennial forage grasses, antioxidant defence, photosynthetic activity, chlorophyll fluorescence parameters, ferric reducing antioxidant power, phosphomolybdate reduction

Abbreviations:
AAE – ascorbic acid equivalents
AO – antioxidant
CEF – cyclic electron flow
D1 – core protein from the reaction centre of PSII
FRAP – ferric reducing antioxidant power
φ_{Po} – quantum yield of the primary photochemical reaction
φ_{Eo} – quantum yield of the electron transport
φ_{Ro} – quantum yield of the reduction of end acceptors of PSI
φ_{Do} – quantum yield of the energy dissipation
PI_{ABS} – performance index based on absorptivity
LEF – linear electron flow
LHCII – light-harvesting complexes of PSII
NPQ – non-photochemical quenching
P680 – the reaction centre of PSII
P700 – the reaction centre of PSI
PAM – pulse-amplitude modulation
PF – prompt fluorescence
PIABS – performance index based on absorptivity
PM – phosphomolybdate
PSII, I – photosystem II, I
qE – energy and ΔpH-dependent quenching
qI – photoinhibitory quenching
qT – state transition quenching
qZ – zeaxanthin-dependent quenching
ROS – reactive oxygen species
TPTZ – 2,4,6-Tris(2-pyridyl)-s-triazine
WRB – world reference base

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A large part of the forage crops contains plants from the grass family (*Poaceae)*, with applications in the grain production, as well as pasture cultures (Kosolapov *et al.* 2021). The five investigated grain forage crops (*Lolium perenne, Festuca arundinacea, Festuca pratensis*, *Festuca rubra, Bromus inermis*) are perennial grass species. During their lifetime plants are exposed to multiple stressors, varying in their strength and duration (Keep *et al.* 2021). These stressors are grouped into two subdivisions: abiotic (physico-chemical factors) and biotic stress (impact of other organisms, such as pathogens, competitors, grazing animals, parasitic plants, and fungi). Consequently, plants have developed various mechanisms for the response against stress: physiological, anatomical, biochemical, and molecular (Al-Tawaha 2017).

One of the biochemical mechanisms for the plant‘s response against stressors is the antioxidant (AO) system. Two types of antioxidants exist, based on their biochemical character, either enzymatic (such as catalase; superoxide dismutase; glutathione peroxidase; ascorbate peroxidase) and non-enzymatic nature (mostly from the two big groups of the terpenoids and the phenols) (Szarka *et al.* 2012). A characteristic of the AO system is the protection against the secondary effect of the multiple stressors, namely the oxidative stress (Xie *et al.* 2019). The oxidative stress is a result of the elevated production of reactive oxygen species (ROS) (Hasanuzzaman *et al.* 2011). ROS are harmful highly reactive particles. They can easily damage the plant biomolecules like proteins, DNA and cause peroxidation to the phospholipids, thus disrupting the structure of the cell membranes (Apel & Hirt 2004). Most of them are radicals, but non-radicals, such as hydrogen peroxide and singlet oxygen also exist. Singlet oxygen is the first ROS particle, produced by spin inversion of the electron from the outer layer of oxygen (Krieger-Liszkay 2004). Afterward, the oxygen in its singlet form could be much more easily reduced, and subsequently, more harmful ROS particles further continue to form (Apel & Hirt 2004).

ROS are produced in small amounts during the plant physiological processes, the photosynthesis being in the first place (Choudhury *et al.* 2016). Under stress photosynthesis further increases the production of ROS, due to the unregulated photooxidative elements of the photosynthetic apparatus. Excited electrons mostly from the photosystems can reduce oxygen to ROS, and subsequently, water (Apel & Hirt 2004; Krieger-Liszkay 2004). To mitigate oxidative stress, plants synthesise large amounts of antioxidants, such as enzymes neutralising ROS and non-enzyme antioxidants that can also prevent their production (Hasanuzzaman *et al.* 2011).

In addition, the antioxidants are valuable substances in animal nutrition, since they protect against ROS in the animal organism, as well. Some of them are important vitamins that can be exclusively supplied from the food and they carry out key functions in animals (Miller *et al.* 1993). Moreover, plant polyphenols perform AO functions both in plants and animals, however, they are particularly important for the digestion of proteins by ruminant animals (Theodorou *et al.* 2006; Celi & Gabai 2015). In addition, the quality of meat, for example, the degree of lipid peroxidation also depends on the amounts of antioxidants in the forages (Corino & Rossi 2021).

To determine the potential response to different climatic conditions (July and October), five species of perennial grasses were studied, in relation to their level of AO capacity, as well as to their photosynthetic activity, as measured by the method of prompt fluorescence of chlorophyll *a*. It is well-known that photosynthesis is the main process, on which the plant productivity is based (Nowicka *et al.* 2018; Busch 2020). However, this process is also very susceptible to the conditions of the environment, due to its highest share in the generation of ROS (Choudhury *et al.* 2016; Walker *et al.* 2020).

Photooxidative damage occurs when plants have decreased ability to fixate CO_2, as compared with the light absorption at the photosystems (Durrant *et al.* 1990). This mainly occurs after exposure to stress, due to the decrease of photosynthetic activity and over-reduction of the photosynthetic components. In this case, photoprotective processes, like the fast-relaxing phase of the non-photochemical quenching (NPQ), activate to prevent the formation of the singlet oxygen (Adams *et al.* 2004). NPQ is a process, competing with photochemistry, which deflects the excessive unused light energy that can potentially damage the plants by generating ROS (Ruban 2017). In addition, the AO pigments, such as

the zeaxanthin from the xanthophyll cycle prevent further reduction of the singlet oxygen to other more harmful types of ROS (Krieger-Liszkay 2004). Finally, AO enzymes neutralise the already produced ROS (Maxwell & Johnson 2000). Consequently, photosynthesis, being basic for productivity, is also strongly inhibited under stress, allowing plants to survive the unfavourable factors on the account of yields (Demmig-Adams *et al.* 2012; Walker *et al.* 2020). Thus, the combined study of the AO capacity and the photosynthetic activity under both environmental conditions represented an interest.

The importance of the measured photosynthetic activity and AO capacity can be demonstrated in the strength of the relation between the climatic conditions, type of the grass species and the plant physiology. Photosynthetic activity could be an indirect indicator of productivity, to a certain extent. The correlation between AO capacity and the NPQ could be used to make assumptions about the species-related photoprotection mechanisms, and the responses of the grass forage crops to different climatic conditions. They could also be used to monitor the seasonal growth in the perennial grasses. The measurements could be important to study the adaptability to limiting environmental conditions and the response to such conditions. They can show the effect of such conditions upon the vitality of grasses in the frames of a year, as well as the longevity in the frames of three and more years.

MATERIAL AND METHODS

Measurements of the following five species (and cultivars) of perennial grain forage grasses were performed: English ryegrass (*Lolium perenne* L.) Tetramis cultivar (1), tall fescue (*Festuca arundinacea* Schreb.) Albena cultivar (2), meadow fescue (*Festuca pratensis* Huds.) selected population (3), red fescue (*Festuca rubra* L.) ecotype Ravnogor (4), smooth brome (*Bromus inermis* Leys.) cultivar Nika (5). They were planted in previous years, from previously produced seedlings and as a nursery collection in the experimental field of the Institute of Forage Crops, Pleven, under non-irrigated conditions on Chernozem (slightly to moderately leached) soil type by WRB (2006) (Shishkov 2019). The soil has a heavy sandy clay profile, without significant changes at a depth of up to 40 cm. Pleven (Central Northern Bulgaria) is located at the transition between the Northern and Middle climatic regions of the Danube Plain, and falls into the moderately continental climatic sub-region. The varieties were created in the IFC Pleven and are distinguished with high productivity and ecological stability under Bulgarian conditions (Katova *et al.* 2016; Katova 2023).

Perennial ryegrass is economically the most important grass species in the world. Tetramis is a new tetraploid variety, which is very early, persistent, winter hardy, drought tolerant and resistant to crown rust, and highly productive for forage and seed. The first Bulgarian smooth brome variety Nika is an octoploid (8n). Nika is suitable for hay, grazing, silage, erosion control and is stress tolerant to drought, cold and leaf diseases. The first Bulgarian tall fescue variety Albena is a hexaploid (6n). Albena is suitable for hay, grazing, silage, erosion control and is stress tolerant to drought, cold, leaf diseases, acidic, and saline soils. Nika is considered tolerant to drought, cold, leaf diseases, whereas Albena is tolerant to drought, cold, leaf diseases, soil acidity, and salinity (Katova 2023).

Fresh leaf samples were collected at two dates, as follows: during the summer period (26.07.2023; 10:00 h and air temperature 30°C), as well as in the autumn (18.10.2023; 10:00 h and air temperature 10°C). Meteorological data (Table 1) for Pleven, Bulgaria (July – October 2023) was obtained from the website bulletin of Bulgarian National Institute of Meteorology and Hydrology (Avaiable at: https://bulletins.cfd.meteo.bg/).

The photosynthetic activity was determined by the method of the prompt fluorescence (PF) of chlorophyll *a* (Strasser *et al.* 2004). The response to the stress of the photosynthetic apparatus (photoprotection) was obtained from the degree of NPQ of the chlorophyll fluorescence in dark-adapted leaves. NPQ was measured by means of the pulse amplitude modulated (PAM) fluorescence analysis (Murchie & Lawson 2013). The fluorometer FluorPen FP 110 (PSI, Brno, Czech Republic) was used for measurements performed by both fluorescence methods. The device is equipped with a light-emitting diode (LED), emitting a measuring pulse with a wavelength of 455 nm. The measurements were carried out

Table 1

Meteorological data (Bulgaria, Pleven, July–October 2023). Source: The website bulletin of Bulgarian National Institute of Meteorology and Hydrology (Avaiable at: https://bulletins.cfd.meteo.bg/)

Month	avg t°C	max t°C (date)	min t°C (date)	sum t°C	precipitation [mm]	% of norm
Jul	26.4	40.8 (25)	12.8 (28)	792.6	26.4	40.0
Aug	25.4	39.2 (4)	12.5 (9)	775.2	43.0	101.0
Sep	21.5	35.6 (23)	10.5 (29)	635.1	17.0	32.0
Oct	17.5	33.7 (21)	1.5 (17)	549.5	1.5	3.1

with the intensity of the actinic light 300 μmol/m^2/s (30%) and saturating pulse 3,000 μmol/m^2/s (100%). Dark-adapted for 30 min freshly collected leaves were used in both analyses of the chlorophyll fluorescence. Two existing pre-programmed protocols: NPQ1 (60 s light-adapted and 88 s dark-adapted measurement) and NPQ2, (200 s light-adapted and 390 s dark-adapted measurement) were used to measure the NPQ. Saturating pulses with intervals of 20 s / 1 min between them and intensity of 3,000 μmol/m^2/s (100%) were applied in these protocols.

The analyses of the AO capacity were carried out by means of the ferric reducing antioxidant power (FRAP) method, Benzie and Strain (1996), as well as by the method of the phosphomolybdate (PM) reduction, Prieto *et al.* (1999). Extraction of plant material was performed in the same way for both methods: 400 mg of fresh leaf material was used in both measurements. The material was ground in 10 mL of 80% ethanol. The samples were centrifuged (Sigma 1-14K, Germany) at 12,500 rpm for 15 min and 100 μL of supernatant was taken, and mixed up with 1.9 mL of fresh reagent. After collecting the entire supernatant, 2 mL of distilled water was added, and then the sample was intensely stirred and centrifuged again. The same quantities of the sample and the reagent were used in the analysis of the water extracts. The FRAP reagent was prepared immediately before the measurement by mixing up 0.3 M acetate buffer, 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), 20 mM $FeCl_3$, in the proportions 10:1:1. The acetate buffer was prepared with 0.31 g sodium acetate (trihydrate), 1.6 mL glacial acetic acid and distilled water up to 100 mL. TPTZ was dissolved in 40 mM HCl, and $FeCl_3.6H_2O$ in dH_2O to obtain the stock solutions of 10 mM and 20 mM, respectively. Immediately after the mixing up of the samples and the reagent the test tubes were placed on ice for 40 min. The absorbance was measured on a spectrophotometer at the wavelength of 593 nm. For the preparation of the phosphomolybdate reagent 4 mM of $(NH_4)_6Mo_7O_{24}$ (tetrahydrate), 28 mM of KH_2PO_4, as well as 0.6 M concentrated H_2SO_4 were dissolved in 100 mL dH_2O. The samples were incubated at 95°C for 90 min, after which the reaction was stopped on ice. The measurements were performed on a spectrophotometer at 695 nm. Results were presented as mM of ascorbic acid equivalents (AAE) per mg of fresh weight.

The statistical analysis was performed by means of the Tukey test and the statistically significant differences between the groups were represented at $p \leq 0.05$. For the analysis of the photosynthetic activity, five separate individual measurements (replicates) of every grass species were performed, and for the analysis of the AO capacity two repetitions of each sample were measured. The science libraries *numpy* and *scikit* from Python 3.8 were used for the statistical analysis, and the library *matplotlib* was used for the drawing of the graphs. The coding was performed in the Python development environment of Google, Colab. Colab is especially well suited to machine learning, data science, and education (Avaiable at: https://colab.research.google.com/).

RESULTS

Based on the meteorological data from Pleven (Table 1), the average monthly temperatures, me-

asured in July and October were, correspondingly: 26.4°C, 17.5°C. The average temperature at 26.07.23 was 30°C and at 18.10.23 was 13°C. The maximal and minimal temperatures were 40.8°C (25th July) and 12.8°C (28th July), as well as 33.7°C (21st October) and 1.5°C (17th October). The weather was stable for about two weeks before both measurements, and immediately after the measurements the average daily temperature decreased rapidly in July and increased in October. The precipitation was about 26.4 mm (40% of norm) in July and 1.5 mm (3.1% of norm) in October. The sum of the average monthly temperatures was, as follows: 792.6 in July and 549.5 in October.

Starting from the parameter which shows the efficiency of photosystem II (PSII), φ_{Po}, (Figure 1 A, B first frame) it had high values amongst all species, remaining high in the summer, as well as the autumn periods of measurement (Figure 1 A, B first frame). This parameter is the most stable and changes very little under stress. The grasses *F. rubra* and *L. perenne* can be distinguished with slightly lower values of this parameter in July. The measurements from both seasonal experiments indicate statistically significant inhibition of this parameter only in *F. rubra*. Its value was, however, only 10% lower, as compared with the highest value of the parameter in *B. inermis*. In July, four of the forages had almost similar and very high values of φ_{Eo} (the efficiency of the intersystem electron transport, Figure 1 A) (except for *F. rubra*, which had lower values of this parameter), whereas in October the highest value of the parameter was in *F. arundinacea*, and the lowest was again in *F. rubra* (Figure 1 B). In addition, a high similarity between the results was found in October. The quantum yield of the reduction of end acceptors of PSI (φ_{Ro}) showed the highest values in both *L. perenne* and *F. rubra* in summer (Figure 1 A, B third frame). Whereas this parameter was the highest in *L. perenne* in the summer, in the autumn its values remained insignificantly higher only in *F. rubra*, whereas in *L. perenne* φ_{Ro} decreased similarly to the values in the other crops. The most significant differences were observed amongst the

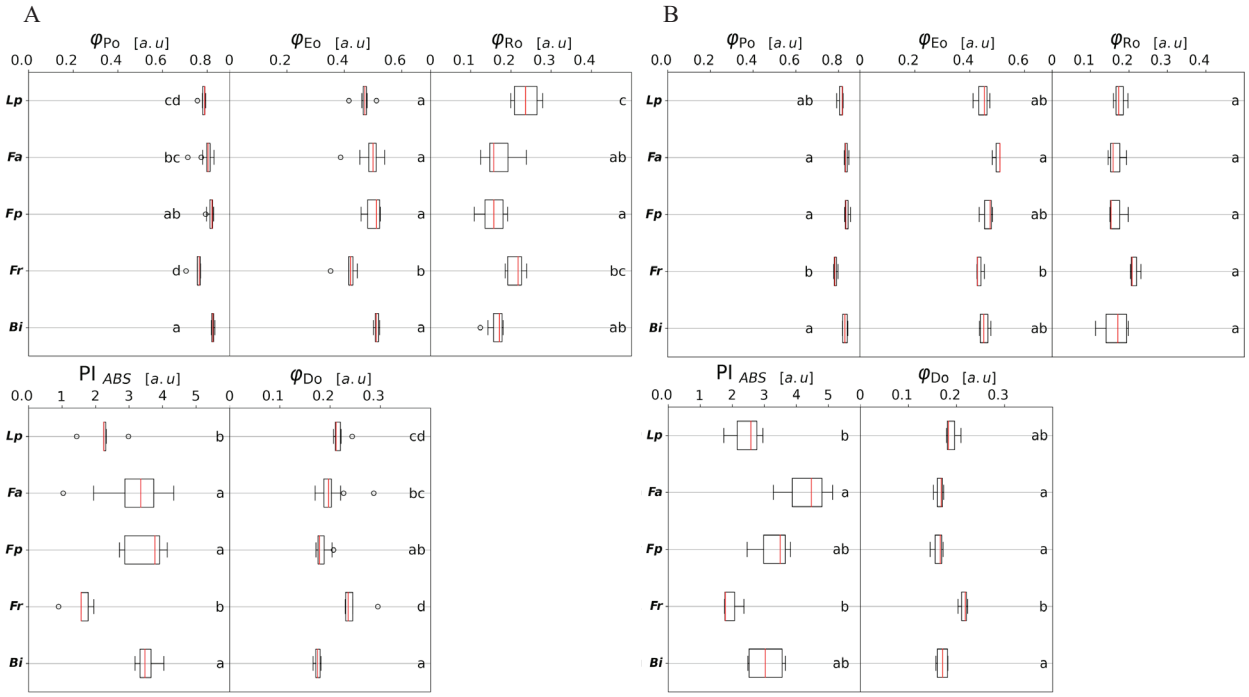

Figure 1. Measured and calculated parameters of the prompt fluorescence of chlorophyll *a*. The following parameters are represented on the graphs: quantum yields of the efficiency of the primary photochemical reaction of PSII (φ_{Po}), of the electron transport between the photosystems (φ_{Eo}), of reduction of the end acceptors (φ_{Ro}), of the energy dissipation (φ_{Do}) and the performance index on absorption basis (PI_{ABS}). Measurements were carried out in summer, 26.07 (A) and autumn, 18.10 (B) (see materials and methods). The abbreviations indicate the studied forage grasses as follows: Lp – English ryegrass (*Lolium perenne* L.) Tetramis cultivar, Fa – tall fescue (*Festuca arundinacea* Schreb.) Albena cultivar, Fp – meadow fescue (*Festuca pratensis* Huds.) selected population, Fr – red fescue (*Festuca rubra* L.) ecotype Ravnogor and Bi – smooth brome (*Bromus inermis* Leyss.) Nika cultivar. Lowercase letters indicate statistically significant differences at $p \leq 0.05$.

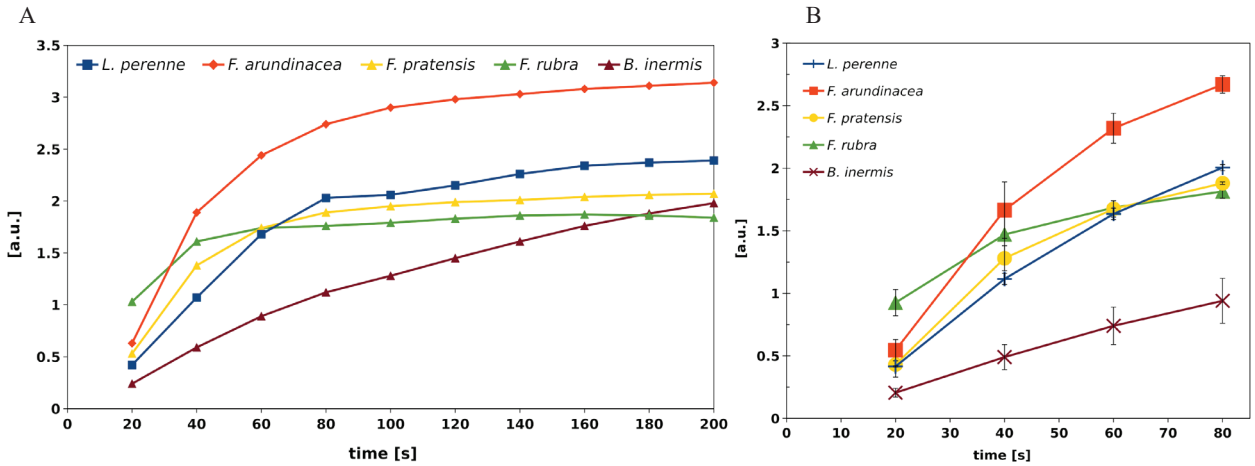

Figure 2. Measurements of the non-photochemical quenching (NPQ) of chlorophyll fluorescence by the method of the pulse-amplitude modulated (PAM) fluorescence. The presented results are from the summer period. The measuring protocol NPQ2 (A) has a duration of 200 sec light-adapted and 390 sec dark-adapted measurement. The measuring protocol NPQ1 (B) has a duration of 60 sec light-adapted and 88 sec dark-adapted measurement. The graphs represent the increase (activation) of NPQ during light-adaptation. The pre-loaded protocol from the device applies 10 saturating pulses of 3,000 μmol/m^2/s (100%) and the interval between them is 20 sec (see Material and Methods). This data was measured in July.

results from the measurements of the performance index (on the absorption basis, PI_{ABS}) (Figure 1 A, B fourth frame). The highest mean values were shown in *B. inermis* in summer and in *F. arundinacea* in autumn. Whereas in *B. inermis* and *F. pratensis* in July, the values of the parameter were the highest, PI_{ABS} in these two grasses was about 25% lower, as compared with PI_{ABS} in *F. arundinacea* in October. In autumn, the results from the performance index showed an increase of about 20% in *L. perenne*, *F. arundinacea*, 25% in *F. rubra* and decrease of about 15% in *B. inermis*, as compared with the summer results. The lowest values were again in *F. rubra* in both seasons, followed by *L. perenne*. The values in *F. rubra* were about more than 50% lower than the highest value in both seasons, which was a significantly larger difference, as compared with the results from the measurements of φ_{Po}. The results from both seasonal experiments demonstrated the highest values of energy dissipation (φ_{Do}) in *F. rubra*, followed by *L. perenne* (Figure 1 A, B fifth frame). The lowest values of the parameter were observed in *B. inermis* in summer, and the autumn results were the lowest in *F. pratensis*.

The NPQ, measured by the PAM analysis (Figure 2) showed higher values in *F. arundinacea*, whereas other crops had slightly lower values of the NPQ at the final (200 s of light-adaptation, Figure 2 A). The activation of the NPQ after the first 80 s was measured to be between 85 and 95% of the final value in four of the grasses (Figure 2 B). The fastest activation of NPQ was measured in *F. rubra* (over 95% after 80 s), however the final values were the lowest in this plant. *F. rubra* was the plant possessing the lowest photosynthetic activity (as measured by the performance index, PI_{ABS}) in both seasonal experiments. Results from *B. inermis* demonstrated the slowest activation of NPQ, where the activation was only 50% after 80 s. Results between the photosynthetic activity and the final values of NPQ were uncorrelated, and only in *F. arundinacea* the activity of the light phase and the light-adapted NPQ were notably higher. It should also be noted that the relaxation (the dark-adapted) phase from the NPQ measurement was very similar in the five studied forage grasses. The end values of the relaxed NPQ were also very low and very close to the mean value. The fastest relaxation was again most clearly seen only in *F. arundinacea* (data not shown).

Results from the measurements of the AO capacity by both methods (PM and FRAP) showed visible correlation (Figure 3). Significant differences in the results obtained from both methods were shown in samples of *B. inermis,* as well as in *F. pratensis* collected in July. The results from the FRAP method were higher as compared with the PM method in *B. inermis*, whereas in *F. pratensis* the results from the FRAP method were lower (Figure 3, light and dark red colours). In contrast, measurements from October (Figure 3, light and dark blue colours) clear-

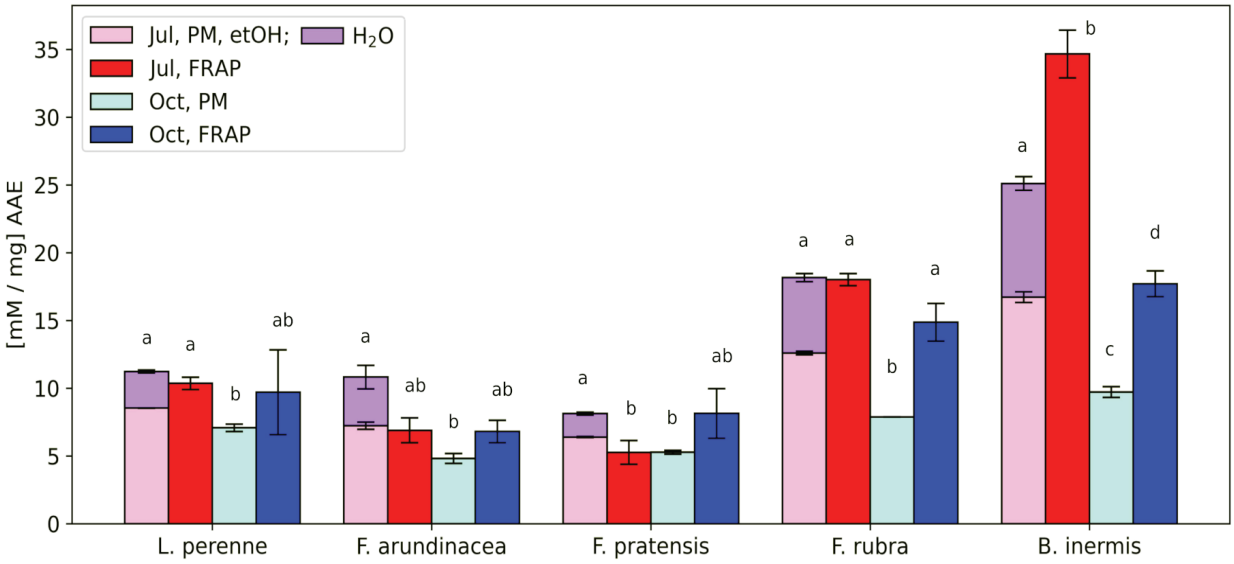

Figure 3. Measurements of the AOcapacity, by the methods of the phosphomolybdate reduction, PM (light colours), as well as by the ferric reducing antioxidant power method, FRAP (intensive colours), carried out in July (red) and October (blue). The results from the PM analysis, performed in the summer period are represented as two separate extracts, correspondingly: 80% ethanol (light red; etOH) and distilled water (violet, H_2O). They were shown in this way, due to the significantly higher values of the water extracts. The rest of the experiments demonstrated very low results of the measurements in dH_2O extracts. Thus, both extracts (80% etOH and dH_2O) were mixed up and the total sum of them was represented (see Material and Methods). Results were presented as mM equivalents of ascorbic acid (AAE) per mg fresh weight. Lowercase letters indicate statistically significant differences in total extracts at $p \leq 0.05$.

ly demonstrated that the results from the FRAP analysis were higher, as compared with the results from the measurements by the PM method in all of the five studied forages, even if the differences were insignificant as in *L. perenne, F. arundinacea* and *F. pratensis*. The highest values from both methods and both seasonal experiments were found in *B. inermis,* whereas the lowest values were measured in *F. pratensis,* followed closely by *F. arundinacea* (Figure 3, all colours). In addition, nearly the same results were obtained from the measurements of the total sum of ethanol and water (Figure 3, pink and violet) extracts of *F. rubra*, as well as of *L. perenne* by both methods for the determination of the AO capacity in July (they belong statistically to the same set). These two grasses also demonstrated the lowest levels of photosynthesis, as measured by the performance index (Figure 1, PI_{ABS}). It should be also noted that measurements on *F. arundinacea* and *F. pratensis* in July also showed similar values of the ethanol extracts as measured by the PM method, to the total extracts, as measured by FRAP (Figure 3, pink and red).

The results from the determination of AO capacity, as measured by the PM method demonstrated the decrease of the AO capacity in October (Figure 3, light blue), as compared with the results from July (Figure 3, light red). However, the results, as measured by FRAP were almost unchanged in *L. perenne, F. arundinacea, F. rubra*, decreased in *B. inermis* and even slightly (insignificantly) increased in *F. pratensis* measured in summer as compared with the autumn results (Figure 3, dark red and dark blue).

DISCUSSION

The first parameter of the prompt fluorescence method, the quantum efficiency of the primary photochemical reaction of PSII (φ_{Po}) is based on the relation between the variable fluorescence at the beginning of measurements and the maximal fluorescence (observed usually near the end, at the 300th ms) (thus it is also denoted as F_V/F_M) (Strasser *et al.* 2004). Its values usually remain constant (around 0.83–0.84, which is also accepted as a standard for the efficiency of capturing light energy from the PSII) (Maxwell & Johnson 2000), and significant changes occur only after more intense impact on physiology (Murchie & Lawson 2013; Guidi *et al.*

2019). The efficiency of PSII can be affected more significantly only by destructive processes, such as the photoinhibition of D1 core protein from the reaction centre of PSII, P680 (Tyystjärvi & Aro 1996). The over-reduced P680 in triplet form can easily generate singlet oxygen, the initial step of ROS production. The excessive amounts of this excited molecule cause photoinhibition of PSII, deactivating the core protein D1 (Prášil *et al.* 1992; Aro *et al.* 1993). Thus, photoinhibition as a protective mechanism in plants prevents further oxidative stress, on account of the degradation of D1 and the decreased efficiency of PSII.

The photoinhibition is reflected in the prompt fluorescence parameter, showing the PSII efficiency (φ_{Po}). Since a significant decrease of φ_{Po} in any measurement was not observed, it could be assumed that the production of singlet oxygen inside P680 was retained within the physiological boundaries, and thus the studied grasses remained unaffected by the photooxidative stress. Rintämaki *et al.* (1995) confirmed that φ_{Po} is mostly insensitive to influence from stressors. On the other hand, amongst other components of the photosynthetic apparatus, PSII is very sensitive to high temperatures (Berry & Bjorkman 1980; Inamullah & Isoda 2005). Due to that, the lack of significant inhibition in the studied crops demonstrates tolerance to heat stress (Figure 1 A). The substantially high values of φ_{Po}, even in *F. rubra*, can indicate lack of essential stress in both seasonal experiments and comparatively high levels of physiological activity under both high and chilling temperatures.

The electron transport between photooxidation components (as measured by φ_{Eo}) *w*as inhibited in *F. rubra* in the summer (Figure 1 A, second frame), as well as the PI_{ABS}, both indicating a lower activity of the linear electron flow (LEF) (Farias *et al.* 2016), as compared with the other four grasses. It is well-known that φ_{Eo} decreases strongly under temperature (either high or low) stress (Kalaji *et al.* 2016). Based on the measurements of φ_{Eo} and PI_{ABS} can be concluded that the strongest inhibition of the light phase under heat was in *F. rubra*, whereas the activity of LEF was maintained to the largest degree after chilling in *F. arundinacea* (Figure 1 A, B fourth frame), whereas the overall photosynthetic activity even increased (PI_{ABS}).

φ_{Ro} can be considered an indicator for the reduction of the PSI (Schansker *et al.* 2006). Whereas the higher values of this parameter show actively functioning P700, the lower values could be due to the more intensive processes after PSI (for example the fixation of CO_2 and overall Rubisco activity, including photorespiration), as well as the decreased function of PSI, the intensive production of ROS at PSI (from the Mehler reaction, the photorespiration) and even damages (Schansker *et al.* 2006). In addition, the mutual inhibition of the LEF and the increase of the activity of PSI can be an indication for the higher activity of the photoprotective mechanism of the cyclic electron flow (CEF) (Yang *et al.* 2017).

The results from the measurement of the parameter PI_{ABS} clearly distinguish the measured grasses by their total photosynthetic activity of the light phase. The parameter is complex and includes the more basic parameters of the photooxidative components in the calculation of the biophysical membrane potentials, shown by the Nernst equation (Strasser *et al.* 2004). Thus, the components of the light phase can be described. Its values often correlate with the measurements of the photosynthetic activity by the method of the CO_2 uptake, uncovering the connection between the light and the dark phases (Kalaji *et al.* 2014; 2016). Whereas this could be possible mostly when the plant is physiologically active and without stress exposure (Genty *et al.* 1989), some authors have shown that such dependence exists even under stress (van Heerden *et al.* 2007). In this way the decrease of PI_{ABS} could be an indicator for the early inhibition of the CO_2 fixation, either due to the stomatal closure or the inactivation of Rubisco. *B. inermis* and *F. pratensis* had the most active photosynthesis, as measured by this parameter in summer, whereas in autumn the photosynthesis in these two grasses was visibly lower. These observations demonstrate the better response to cold (chilling) temperatures of *L. perenne, F. arundinacea* and *F. rubra*, similar results in *F. pratensis* and a lower physiological activity in *B. inermis*. PI_{ABS} is used for monitoring the earliest stages of environmental (abiotic) stress (van Heerden *et al.* 2007; Oukarroum *et al.* 2016; Faseela *et al.* 2020), since the stomatal closure is one of the earliest responses against stressors (de Souza *et al.* 2013). A correlation could be potentially found between the results from the

photosynthetic activity (more specifically the performance index) and the productivity in terms of the yield of seeds and the dry biomass. The performance index is a sensitive parameter to changes in the physiology, as a response to the environment. Thus, it was proven useful as a selection tool for the production of new breeds and crop cultivars (Dąbrowski *et al.* 2019; Rastogi *et al.* 2020) and could be applied for the selection of forage grasses, as well.

The measurements of the productivity in these grasses were presented in a different article (Katova 2023). The grasses studied in both the cited and the current articles are the same; they were planted years ago and are still cultivated and investigated. Results showed the highest seed yields around 0.6 – 0.7 t/ha in *F. arundinacea* and *B. inermis,* and 0.5 – 0.7 t/ha in *L. perenne* (v. Tetramis). The dry matter yield was around 9 t/ha in *F. arundinacea* and *B. inermis* and around 8 – 11 t/ha in *L. perenne* (v. Tetramis). In contrast, *F. rubra* grows very slowly and is mostly used as a decorative plant (*L. perenne* and other *Festuca* sp. can be used sometimes as such).

The parameter, describing the energy dissipation (φ_{Do}) measures the unused energy by the photosynthetic apparatus (Strasser *et al.* 2004). Usually, its values are low in healthy plants and the increase shows the inability of the plant to utilise the light energy. This occurs under stress and is a result of damaging processes (Zhu *et al.* 2021). In contrast to NPQ as measured by the method of PAM, the unregulated loss of energy from PSII, denoted as q_{NO} in the method of PAM (Rohacek 2002) and φ_{Do} in the method of the prompt fluorescence, Strasser *et al.* (2004) shows damages mainly to PSII as well as production of ROS (Zhou *et al.* 2019; Stefanov *et al.* 2022). Having in mind the very high efficiency of the primary photochemical reaction, the dissipation of energy (measured by the means of φ_{Do}) is a stress indicator (Stefanov *et al.* 2022). The higher values of this parameter indicate processes, leading to leakage of the unused energy that can potentially damage the photosynthetic apparatus and produce ROS (Zhu *et al.* 2021). The highest values of this parameter in *F. rubra,* followed by *L. perenne* correlate to their lowest photosynthetic activity and indicate initial stress effects (Figure 1 A, B fifth frame).

Since the activation of NPQ is a stress response of plants, it could be assumed that NPQ and photosynthetic activity are inversely correlated. In the literature, it was often shown that along with the onset of stress, NPQ initially increases, whereas φ_{Po} decreases. However, as plants suffer damage from the stress, NPQ starts decreasing, as well (Itam *et al.* 2023). It can be seen that the highest value of NPQ was found in *F. arundinacea,* one of the three grasses with the highest photosynthetic activity. According to Ruban (2017) plants with elevated values of NPQ possess higher protection against photodamage and thus, they can sustain a more active photosynthesis. The grass with the slowest activation rate of NPQ was *B. inermis* (Figure 2). This result showed that grasses have different mechanisms for photoprotection, to respond to the photooxidative stress. In addition, grasses with the highest photosynthetic activity (*F. arundinacea, F. pratensis, B. inermis*) demonstrated very different levels of AO capacity.

The rate of activation of the NPQ is an indicator of the photoprotection mechanisms, due to which the plants respond to stress (Ruban 2017). In four of the grasses NPQ activated almost entirely (to maximal values) within the first minute (Figure 2). Knowing the fastest NPQ process is qE, with a rate of activation of about a minute, and also that qZ and qT activate more slowly (Nilkens *et al.* 2010), it can be assumed that qE was the predominant mechanism for photoprotection. qE is a fast, ΔpH-dependent process, relying on the activation of the quenching protein from PSII, the PSBS (Müller *et al.* 2001; Ahn *et al.* 2009; Ralph *et al.* 2010). The slowest NPQ phase, qI can be neglected, since the results showed only insignificant decrease in φ_{Po}. This could affirm that the mechanism behind qI, the photoinhibition, remained practically inactive.

On the other hand, the observed gradual activation of NPQ in *B. inermis* (Figure 2), indicates to a larger proportion of the slower processes involved in the NPQ, such as the de-epoxidation of violaxanthin to the photoprotective pigment zeaxanthin, denoted qZ (the so-called xanthophyll cycle, Nilkens *et al.* 2010*;* Jahns & Holzwarth 2012; Yang *et al.* 2015), but can also indicate to the state transition of the light-harvesting complexes of PSII (LHCII) from PSII to PSI, denoted qT.

qT represents the transition of phosphorylated LHCII from PSII to PSI, as well as their dissociation

as trimeric aggregates (Iwai *et al.* 2008). In addition, qT induces the activation of the CEF, decreasing the energy pressure on the PSII and transferring it to PSI (Minagawa 2011). Thus, higher activity of qT would correspond to higher activity of PSI, as measured by the quantum efficiency of end acceptors (φ_{Ro}). Many authors describe the relation between the phase I-P from the OJIP transient and the amounts of PSI in the leaf (Kalaji *et al.* 2014). Schansker *et al.* (2006) demonstrated the relation between OJIP phases and the different components of NPQ (qE, qT, qI) in pea leaves. Whereas the higher activity of PSI positively relates to this parameter, it could be also affected by many other alternative processes, such as the formation of ROS in PSI, due to stimulated Mehler reaction and photorespiration (Pollastrini *et al.* 2020). It is also important to note that growth conditions can affect the amount and ratio of the photosynthetic components, such as PSII, PSI, LHCII (Wientjes *et al.* 2013). Finally, the migration of LHCII in higher plants represents only up to 25%, whereas in algae it can be as high as 80% (Minagawa 2011). Thus, qT is considered to exert a much lower impact in higher plants (Tietz *et al.* 2017).

In conclusion, later activation of NPQ in combination with higher values of φ_{Ro} could suggest the higher impact of qT, whereas higher values of the pigment from the xanthophyll cycle zeaxanthin, could be an indication for the qZ, as the predominant NPQ component. Both qT and qZ are not excluding each other. In fact, low pH of the lumen is an activation signal for both the de-epo. The observations showed the increase of NPQ in *B. inermis* at the end of measurements to be almost the same as in the other species, except for *F. arundinacea*. The results from φ_{Ro} did not prove such an assumption, showing the highest activity of PSI in *L. perenne* in July (Figure 1 A, third frame). Moreover, results from measurements of φ_{Do} clearly showed higher energy dissipation in both grasses with the lowest photosynthesis *F. rubra,* followed by *L. perenne* and thus imply increased ratio of the processes competitive to photosynthesis (Figure 1, fifth frame). In contrast, the results from the AO capacity (Figure 3) showed the highest capacity in *B. inermis* (up to 5 times higher than the other grasses). However, the amount of the individual carotenoid pigments was not measured. Based on the above arguments, the mechanism of state transition as a reason for late activation of the NPQ can be practically excluded. Thus, the remaining assumption at this point would be the higher influence of qZ. Such zeaxanthin-dependent activation of NPQ (presented as a slower rate of NPQ activation) was documented by other authors (Johnson & Ruban 2011; Snellenburg *et al.* 2017; Murchie & Ruban 2019) in studies on Arabidopsis mutants. Concerning the highest level of AO capacity in *B. inermis*, it is possible some could be attributed to the higher content of the xanthophyll pigments (including lutein, zeaxanthin or violaxanthin), but it could be stated with certainty only after a measurement of the pigment content had been performed. It is well-known that zeaxanthin is one of the carotenoids with the highest antioxidant strength (Havaux *et al.* 2007) and therefore the increase of its content would have a higher reflection on the AO capacity. Carotenoid pigments have many functions in the plant organism: they act in photosynthesis, redirecting the light to the reaction centres, but they also protect plants from excess light and in addition, they quench the triplet chlorophylls and the singlet oxygen, preventing further formation of ROS and photooxidative damage (Niyogi *et al.* 1998).

The two methods used for the measurements of the AO capacity, namely PM and FRAP showed similar results in all of the studied grasses. However, differences in the values, which could be attributed to the differences in detection between both methods also exist. Since the redox-potential of the couple Fe^{3+}/Fe^{2+} (0.77 V) is higher, as compared with the Mo^{VI}/Mo^{V} (0.43 V) (Choirunnisa *et al.* 2016), the FRAP method could measure also some other substances, which are unable to be determined by the method of PM. Thus the values as measured by the FRAP method would in theory be higher as compared with PM (Dontha 2016). The results confirmed this assumption only for the measurements in October. The measurements of *F. arundinacea and F. pratensis* in July, however demonstrated the reverse tendency (Figure 3), whereas *L. perenne* and *F. rubra* showed similar results, and only in *B. inermis* this tendency was observed.

Other differences in detection between both methods exist, which could explain this discrepancy in the obtained results. In contrast to the antioxidants,

such as ascorbic acid, in which for oxidation and reduction an electron transfer occurs, the glutathione redox reactions require the transfer of hydrogen ions from the thiol group (Choirunnisa *et al.* 2016). Due to the latter the methods for determination of AO capacity differ in their ability to measure different antioxidants. There is also a difference in the solubility of the antioxidants, and those with terpenoid nature (tocopherol, carotenoids) tend to be more hydrophobic, whereas the ascorbic acid and glutathione are more hydrophilic, and the solubility of phenols can be found in between these two categories. Whereas both methods can detect hydrophilic and hydrophobic antioxidants in a similar way, they differ in their ability to measure glutathione (Prieto *et al.* 1999; Prior & Cao 1999). The PM method can determine the amount of the reduced glutathione (Prieto *et al.* 1999), whereas the method of FRAP does not allow the detection of thiols (including glutathione) (Prior & Cao 1999; Pinchuk *et al.* 2012; Dontha 2016). It is interesting to note, that during the separate measurement of extracts dissolved in 80% ethanol and distilled water it was observed that the higher values of the AO capacity, measured by the PM method in both *F. arundinacea and F. pratensis* were precisely due to the extracts, dissolved in water (Figure 3), whereas in *L. perenne* and *F. rubra,* the sum of ethanol and water extracts (by PM) was almost equal to the FRAP results. However, only the chemical analysis of these extracts can unambiguously prove the presumed involvement of the glutathione (or any other substance) in the results.

Concerning the AO function of the glutathione, it has non-enzymatic nature, but participates in some enzyme reactions, as well (for example as the substrate of the enzymatic antioxidant, the glutathione peroxidase) and is a key element of the AO defence in many organisms, including plants (Pisoschi *et al.* 2016). Along with the ascorbic acid (vitamin C) and the tocopherol (vitamin E), the glutathione is a part of the tocopherol-ascorbate-glutathione triad (Szarka *et al.* 2012). This mechanism unites the enzymatic and non-enzymatic AO defences and is of basic importance for the neutralisation of the lipid radicals, obtained in the destructive process of the lipid peroxidation (Faltin *et al.* 2010; Szarka *et al.* 2012). At the same time, for the regeneration of the glutathione, a constant supply of the reducing NADPH is required, a large part of which is generated in the light phase of the photosynthesis (Noctor *et al.* 2012; Dorion *et al.* 2021). Therefore, for maintaining low levels of the oxidative stress a delicate balance between the neutralisation of the ROS and the radicals and their production is necessary (Bela *et al.* 2015), both processes being regulated by the light phase of the photosynthesis. (Noctor *et al.* 2012).

CONCLUSIONS

The studied five grasses are cultivated perennially in the field of the "Institute of forage crops, Pleven". Summer is an especially critical period for the perennial grasses, due to the very high temperatures, occurring sometimes (above 40°C) and also the severe drought. Some grasses fall into the so-called summer "dormancy", in which the physiological activity remains minimal and concentrated around the roots. The studied grasses in this article can survive the heat and drought during summer without drying out of their aboveground parts. Both high and low temperatures during July and October did not significantly affect the efficiency of PSII of these grasses, which suggests absence of significant environmental stress impact. Results showed practically absence of photoinhibition, which was most noticeable in *F. rubra.* The results from July and October were very similar, implying insignificant effects from either high and low temperatures.

Out of the five grasses, *F. rubra* is mostly used as a decorative plant, since it grows very slowly and has lower yields. This was reflected in the results from the photosynthetic activity in both seasons was the lowest in this decorative plant, with almost twice as low as the grasses with the highest photosynthesis. The results of the most parameters of the prompt fluorescence were notably lower only in *F. rubra,* whereas among the results from the other parameters only PIABS showed significant difference between all of the grass species. On the basis of this parameter, grasses could be divided to such with higher (*F. arundinacea, F. pratensis* and *B. inermis*) and with lower photosynthetic activity (*L. perenne* and *F. rubra*). The performance index was higher in October, as compared with July mea-

surements, whereas the AO capacity was higher in the summer. A reverse relation between the results from the photosynthetic activity (PIABS) and the AO capacity could be found. The only exception is *B. inermis,* where the AO capacity was notably (fivefold) higher as measured by both methods (PM and FRAP). Higher AO capacity is a mechanism for response against the oxidative stress, formed after stress inhibition of photosynthesis. The rate of NPQ activation was very fast (1 min) in the four grasses, except *B. inermis*. *F. arundinacea* had the highest value of NPQ.

As representative crops *B. inermis* (Nika cultivar) was found to have very high AO capacity (as measured by both PM and FRAP), and *F. arundinacea* (Albena cultivar) was found to have especially high photosynthetic activity, as measured by the performance index. In addition, in *F. arundinacea* the NPQ values were the highest in summer, demonstrating the best response to high temperatures, whereas in *B. inermis* the NPQ activation was found to be about two times slower, as compared with the other four species. The tall fescue of the cultivar Albena had the highest photosynthetic activity of the light phase, more than two times as compared with the plants with the lowest activity in both summer and autumn experiments (*F. rubra*), and had especially high photosynthesis in autumn, suggesting its highest productivity and tolerance to low temperatures.

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