Plant Physiology and Biochemistry 145 (2019) 205–215

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Fructan and antioxidant metabolisms in plants of *Lolium perenne* under drought are modulated by exogenous nitric oxide

Athos Poli Rigui[a,b], Victória Carvalho[a,c], André Luiz Wendt dos Santos[d], Annette Morvan-Bertrand[e], Marie-Pascale Prud'homme[e], Maria Angela Machado de Carvalho[b], Marília Gaspar[b,*]

[a] Programa de Pós-Graduação em Biodiversidade Vegetal e Meio Ambiente, Instituto de Botânica, São Paulo, Brazil
[b] Núcleo de Pesquisa em Fisiologia e Bioquímica, Instituto de Botânica, CEP, 04301-902, São Paulo, SP, Brazil
[c] Núcleo de Pesquisa em Plantas Ornamentais, Instituto de Botânica, CEP, 04301-902, São Paulo, SP, Brazil
[d] Departamento de Botânica, Universidade de São Paulo, CEP, 05508-090, São Paulo, SP, Brazil
[e] Ecophysiologie Végétale Agronomie et Nutritions N.C.S. Normandie Univ, UNICAEN, INRA, EVA, 14000, Caen, France

ARTICLE INFO

Keywords:
Antioxidant enzyme activity
Fructan enzyme activity
Perennial ryegrass
S-nitrosoglutathione
Stress signaling
Water deficit

ABSTRACT

Drought is a major environmental factor that can trigger oxidative stress and affect plant growth and productivity. Previous studies have shown that exogenous nitric oxide (NO) can minimize oxidative stress-related damage through the modulation of antioxidant enzyme activity. Fructan accumulation also has an important role in drought tolerance, since these carbohydrates participate in osmoregulation, membrane protection and oxidant scavenging. Currently, there are few studies investigating NO-regulated fructan metabolism in response to abiotic stresses. In the present study, we sought to determine if treating plants of *Lolium perenne* with *S*-nitrosoglutathione (GSNO), a NO donor, improved drought tolerance. Two-month-old plants received water (control), GSNO and reduced glutathione (GSH) as foliar spray treatments and were then maintained under drought or well-watered conditions for 23 days. At the end of drought period, we evaluated growth, pigment content and antioxidant and fructan metabolisms. None of these conditions influenced dry mass accumulation, but the leaves of plants treated with GSNO exhibited a slight increase in pigment content under drought. GSNO treatment also induced 1-SST activity, which was associated with a 3-fold increase in fructan content. GSNO-treated plants presented higher GR activity and, consequently, increased GSH levels. *L. perenne* cv. AberAvon was relatively tolerant to the water stress condition employed herein, maintaining ROS homeostasis and mitigating oxidative stress, possibly due to fructan, ascorbate and glutathione pools.

1. Introduction

Drought represents a major environmental factor that is known to impact growth and productivity of plants (Ahmad et al., 2015; Fàbregas and Fernie, 2019). It is predicted that climate changes will result in drought periods, that are expected to be more intense and frequent in the subtropical band, resulting in expansion of dry areas to the Poles (Seneviratne et al., 2012; Trenberth et al., 2014). In fact, it has been proposed that these changes could modify the carbon balance in European grasslands (Soussana and Luescher, 2007), consequently reducing primary production on temperate pastures (De Boeck et al., 2008).

Plant responses to abiotic stresses include the modulation of transcriptional, proteomic and/or metabolic pathways, which together contribute to cell protection (Fàbregas and Fernie, 2019). Previous studies have shown that under stress there is an increase in reactive oxygen species (ROS) production, including the generation of hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), which can potentially react with and damage cell structures, proteins, lipids, carbohydrates and nucleic acids, and in some cases result in cell death (Gill and Tuteja, 2010; Khan et al., 2015). On the other hand, ROS also function as crucial components of signaling pathways involved in the regulation of several physiological processes in plants (Mittler, 2017). The antioxidant defense system responsible for ROS homeostasis is composed of enzymatic (e.g. ascorbate peroxidase, catalase and glutathione reductase) and non-enzymatic (e.g. ascorbate, glutathione and sugars) components (Gill and Tuteja, 2010; Fàbregas and Fernie, 2019).

Under drought conditions, *Festuca arundinacea* (Jiang and Huang, 2001), *Oryza sativa* (Wang et al., 2005), *Poa pratensis* (Bian and Jiang,

* Corresponding author.
E-mail address: gasparmarilia@ibot.sp.gov.br (M. Gaspar).

https://doi.org/10.1016/j.plaphy.2019.10.029
Received 15 July 2019; Received in revised form 17 October 2019; Accepted 19 October 2019
Available online 21 October 2019

2009; Xu et al., 2011), *Dactylis glomerata* (Ji et al., 2014), *Guzmania monostachia* (Abreu et al., 2018) and *Agropyron cristatum* (Sheikh-Mohamadi et al., 2017, 2018) have all been reported to present upregulated antioxidant enzyme expression and activity, thus supporting the role of ROS homeostasis when challenged with this stress.

Nitric oxide (NO) is another important molecule involved in drought signaling pathways (Salgado et al., 2017), interacting with several stress signaling molecules. It is well established that under drought NO regulates the synthesis and sensing of the phytohormone abscisic acid (ABA), modulating seed germination, stomatal closure, photosynthesis, among others (Prakash et al., 2018; Begara-Morales et al., 2019). Additionally, NO derivatives, including peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3) and *S*-nitrosoglutathione (GSNO), the main form of NO storage in cells, modulate gene expression and enzyme activity through post-translational modifications such as nitration and *S*-nitrosylation, consequently altering metabolic activities in response to abiotic stresses (Zaffagnini et al., 2016; Salgado et al., 2017). Indeed, NO can regulate the activity of some enzymes from antioxidant metabolism, promoting an increase in the levels of non-enzymatic antioxidant compounds (e.g. reduced glutathione; GSH), thereby protecting plants against oxidative damage (Yang et al., 2015; Begara-Morales et al., 2016, 2019; Hasanuzzaman et al., 2018a; Nguyen et al., 2018; Nabi et al., 2019). Previously, it was shown that ascobate peroxidase (APX) and gluthatione reductase (GR) activities increased, due to protein *S*-nitrosylation, in *Antiaris toxicaria* seeds treated with NO, resulting in a reduction of oxidative damage during desiccation (Bai et al., 2011). In addition, exogenous treatment with sodium nitroprusside (SNP), a NO donor, was shown to prevent oxidative damage to the photosynthetic apparatus and control ROS accumulation, as observed in *Crambe abyssinica* under water deficit (Batista et al., 2018).

Several studies have shown that sugars, such as raffinose oligosaccharides, can scavenge ROS (Nishizawa et al., 2008; Schneider and Keller, 2009; Hernandez-Marin and Martínez, 2012; Soares et al., 2018). Fructans are another class of oligo and polysaccharides capable of scavenging ·OH and other ROS (Peshev et al., 2013; Soares et al., 2018). These sugars originate from sucrose and are stored in the plant vacuoles (Wagner et al., 1983; Darwen and John, 1989), and have been described as antioxidants. Based on these findings, it has been proposed that vacuoles should be included in the cellular antioxidant network (Peshev and Van den Ende, 2013; Soares et al., 2018).

Furthermore, fructan degree of polymerization (DP) has also been associated with antioxidant capacity. For example, in wheat seedlings exposed to drought, it was shown that the accumulation of high DP fructan ensured a rapid ·OH elimination and protection against oxidative damage (Nemati et al., 2018). In addition, fructans also influence tissue water retention and membrane stabilization during drought (Pilon-Smits et al., 1995, 1999; Hincha et al., 2007). Despite evidences associating fructan metabolism regulation and NO to abiotic stresses, studies on this subject are still limited. In fact, until now, only one study using wheat plants has shown that exogenous NO treatment upregulated the expression and activity of the enzymes involved in fructan synthesis, and that this response increased protection against damages caused by low temperatures (Li et al., 2013).

Perennial ryegrass (*Lolium perenne* L.) is one of the most predominant species in temperate grasslands, due to its rapid establishment, high herbage production, defoliation tolerance and high digestibility by grazing cattle (Meuriot et al., 2018). However, its cultivation may be affected by the predicted increase in drought episodes, due to climate change (Westermeier et al., 2016; Buttler et al., 2019). *L. perenne* presents a complex and diverse fructan metabolism, with four synthesis enzymes already characterized: sucrose:sucrose 1-fructosyltransferase (1-SST) (Chalmers et al., 2003), fructan:fructan 1-fructosyltransferase (1-FFT) (Lasseur et al., 2006), fructan:fructan 6G-fructosyltransferase (6G-FFT) (Lasseur et al., 2006), and sucrose:fructan 6-fructosyltransferase (6-SFT) (Lasseur et al., 2010). Additionally, two hydrolysis enzymes, 1-fructan exohydrolase (1-FEH) and 6-fructan exohydrolase (6-FEH), were isolated and characterized (Marx et al., 1997; Lothier et al., 2007, 2014). Hence, due to the extensive knowledge about fructan metabolism in *L. perenne*, this species constitutes an interesting model to investigate fructan metabolism regulation by NO and its role in drought tolerance.

In this study, we treated *L. perenne* plants with the NO donor, GSNO, and respective controls, prior to exposing the plants to drought condition. We evaluated the potential NO-mediated antioxidant and fructan metabolism regulation and determined how these responses could contribute to drought tolerance in this species.

2. Material and methods

2.1. Plant material and treatments

Seeds of *Lolium perenne* L. cv. AberAvon were obtained from the Laboratory Écophysiologie Végétale, Agronomie & Nutritions (UMR INRA-UCN 950), Université de Caen, France, placed in 280 mL pots (12 seeds per pot) containing sand and vermiculite (3:1) and kept in a greenhouse at Instituto de Botânica, São Paulo, Brazil (23° 38′ 26.4″ S, 46° 37′ 22.0″ W). The plants were irrigated with a modified half strength EVA nutrient solution containing: 1 mM NH_4NO_3, 0.4 mM KH_2PO_4, 0.15 mM K_2HPO_4, 3 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM K_2SO_4, 0.2 mM NaFe EDTA, 14 μM H_3BO_3, 5 μM $MnSO_4$, 3 μM $ZnSO_4$, 0.7 μM $CuSO_4$, 0.7 μM $(NH_4)_6Mo_7O_{24}$ and 0.1 μM $CoCl_2$ (Prud'homme et al., 1992), every 7 days. After two months, 90 pots were placed inside three separate transparent plastic boxes (65 L), covered with porous plastic to allow gas exchanges with the environment. Plants placed in each box were sprayed with 60 mL of either water, 100 mM reduced glutathione (GSH) or 100 mM *S*-nitrosoglutathione (GSNO). Fifteen pots in each box were maintained under well-irrigated condition (control) while the remaining 15 were submitted to drought by watering suspension. At the start of the experiment, all pots were watered to field capacity and five pots from each treatment were weighed every 48 h, in order to monitor soil water evaporation during watering suspension. Well-irrigated plants received 15 mL of water after each weight assessment to maintain the soil water status, and water, GSH or GSNO were sprayed on the leaves every 48 h. After spraying, the boxes were covered and reopened the next day to allow excess water to evaporate from the solutions. After 23 days of watering suspension, plant leaves from each treatment were sampled and separated into three biological replicates (5 pots per replicate), immediately frozen in liquid nitrogen, pulverized and stored at −80 °C. Subsequent biochemical analyses were performed in triplicate (Fig. S1).

2.2. Analyses of plant and soil water status

Four plants of each treatment were collected and separated in leaf sheaths and leaf blades to determine fresh and dry weight, and water content, using the equation

$$WC = [(FW/DW) \text{ x } 100]$$

where *WC* represents the water content, *FW* the fresh weight and *DW* the dry weight of the samples.

For sap osmotic potential analysis, frozen pulverized leaves were weighed in 1.5 mL tubes with a perforated bottom, and the sap was collected in another intact tube by centrifugation at 16000 *g*. Osmotic potential was measured using a vapor pressure osmometer (model 5520, VAPRO, Wescor, Logan, UT). Osmolarity was converted from mmol kg^{-1} into MPa using the Van't Hoff equation: $MPa = \text{mmol } kg^{-1} \times 2.58 \times 10^{-3}$ (Santa-Cruz et al., 2002). A dew point psychrometer (Model WP4, Decagon Devices, Inc., Pullman, WA) was used to determine soil water potential. Soil from rhizosphere was collected from three pots of each treatment and transferred to circular sampling

capsules with a 12.5 m^3 capacity.

2.3. Chlorophyll and carotenoids quantification

Chlorophyll extracts were obtained from 100 mg of powdered leaves homogenized in pure acetone and ultrasonicated for 15 min. The extracts were centrifuged at 8000 *g* for 10 min at 4 °C and the supernatants collected. This procedure was repeated once and the supernatants were pooled and measured spectrophotometrically at 662, 645 and 470 nm for chlorophyll *a*, chlorophyll *b* and carotenoids content, respectively (adapted from Munné-Bosch and Lalueza, 2007). Pigment concentrations were determined according to the equations presented by Lichtenthaler and Buschmann (2001).

2.4. S-nitrosothiols quantification

S-nitrosothiols (SNO) content was analyzed using the method of Saville (1958), and followed the modifications described by Frungillo et al. (2013). Using 100 mg of fresh leaves homogenized in 100 mM phosphate buffer, pH 7.2, the SNO content was estimated by hydrolysis in the presence of mercuric salts, yielding equivalent amounts of nitrous acid. The nitrous acid was then quantified spectrophotometrically at 550 nm, by monitoring changes in brilliant azo dye absorbance, obtained by reaction with sulphanilamide and N-(1-naphthyl) ethylenediamine.

2.5. H_2O_2 and ·OH levels

ROS levels were measured in 100 mg of powdered leaves homogenized in 1 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 12000 *g* for 20 min at 4 °C (Velikova et al., 2000). For H_2O_2 content, 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI were added to the leaf extract (1:2:1, v/v/v), and the reaction mixture was incubated on an ice bath, in the dark, for 10 min. Each sample was measured spectrophotometrically at 350 nm and H_2O_2 content was determined based on a standard H_2O_2 curve, adapted from Junglee et al. (2014).

For ·OH content, 200 mg of frozen leaves were homogenized in 1.5 mL of a 6 mM potassium phosphate buffer (pH 7.4) containing 15 mM 2-deoxy-D-ribose, and the homogenates were centrifuged at 16000 *g*, 4 °C for 25 min. The supernatants were collected and incubated at 37 °C for 2 h (adapted from Beligni and Lamattina, 2002). The reaction between ·OH, present in the leaf extract, and 2-deoxy-D-ribose yields malondialdehyde (MDA), which is quantified by mixing equal parts of the extract with 1% (w/v) thiobarbituric acid (TBA) in 50 mM NaOH and 2.8% (w/v) TCA. After combining all the reaction components, the mixture was incubated at 100 °C for 20 min (Halliwell et al., 1988), the absorbance was measured at 532 and 600 nm, and MDA content was calculated according to Heath and Packer (1968).

2.6. Lipid peroxidation

Lipid peroxidation extraction was achieved by adding 1 mL of 0.1% (w/v) TCA to 100 mg of powdered leaves and centrifuging the samples at 15600 *g* for 10 min at 4 °C. Assays were conducted according to Boaretto et al. (2014). Absorbance readings and MDA content determinations were described previously in section 2.5.

2.7. Ascorbate and glutathione content

Total, reduced and oxidized ascorbate levels were quantified according to Gillespie and Ainsworth (2007). Ascorbate was extracted from 100 mg of powdered leaves by adding 1 mL of 6% TCA. Reduced ascorbate content was estimated by the reduction of the ferric to ferrous ion by ascorbate, which forms a complex with α-ά-bipyridyl and absorbs at 525 nm. The total ascorbate pool was quantified by adding dithiothreitol (DTT) and *N*-ethylmaleimide prior to the α-ά-bipyridyl reaction, thus promoting the reduction of oxidized ascorbate (DHA) to the anion form. The oxidized pool was calculated by subtracting the reduced ascorbate pool from the total ascorbate pool.

Reduced, oxidized and total glutathione were quantified as described by Israr et al. (2006), with some modifications. Briefly, 100 mg of powdered leaves was added to 2 mL 0.1% (w/v) sulfosalicylic acid, and then centrifuged at 11000 *g* for 20 min at 4 °C. To quantify the reduced pool, sample extracts were mixed with 0.5 mM EDTA and 0.3 mM 5,5′dithio-bis-(2-nitrobenzoic acid) (DTNB), both being previously diluted in potassium phosphate buffer (pH 7.0). After 5 min, an aliquot was transferred to a microplate and the absorbance was measured at 412 nm. Total glutathione was quantified by adding 50 µL of 0.5 mM NADPH, previously diluted in 100 mM potassium phosphate buffer (pH 7.0), and 1 µL of glutathione reductase to the initial mixtures. After 20 min, aliquots of the solutions were transferred to a microplate and the absorbance was measured. The oxidized pool was calculated by subtracting the reduced glutathione pool from the total glutathione pool.

2.8. Antioxidant enzyme activities

Extracts for measuring antioxidant enzyme activities were obtained by homogenizing 200 mg of powdered leaves in 2 mL of 1M potassium phosphate buffer (pH 7.5), 1 mM EDTA and 50 mM NaCl. The homogenates were centrifuged at 11000 *g* for 15 min at 4 °C (modified from Souza et al., 2013). All the enzyme activities were determined in the same extract, using different substrates. Data were collected for 2 min in a spectrophotometer with a reading interval of 15 s. Glutathione reductase (GR, EC 1.8.1.7) activity was determined by the rate of NADPH consumption at 340 nm, according to Schaedle and Bassham (1977). Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by the rate of H_2O_2-dependent ascorbate oxidation at 290 nm, according to Weng et al. (2007).

2.9. Soluble carbohydrate contents

To determine soluble carbohydrate content, 200 mg of powdered plant material was homogenized in 80% ethanol, and maintained at 80 °C for 15 min. The sample was subsequently centrifuged at 10000 *g* for 15 min and the supernatant was collected and transferred to a fresh tube. This process was repeated once more with the pellet, and a third extraction was performed using pure water at 60 °C for 15 min (adapted from Lothier et al., 2007). All of the resulting supernatants were pooled and concentrated to dryness in a SpeedVac, resuspended in 150 µL deionized water and analyzed for reducing sugar (Somogyi, 1945) and total fructose content (i.e. sum of free fructose and fructosyl residues included mainly in sucrose and fructans), for determination of fructan content (Jermyn, 1956).

2.10. Fructan enzyme activities

The protein extraction for determining fructan enzyme activities was performed as described by Lasseur et al. (2006), and the enzymatic assays were conducted according to Table 1. Products formed following the incubations were analyzed using high performance anion exchange chromatography and pulse amperometric detector (HPAEC/PAD) equipped with a 4 × 250 mm CarboPac PA-1 column on a Dionex system (model ICS 3000, Dionex, Sunnyvale, CA, USA), as described by Asega et al. (2008). Enzyme activities were calculated by direct measurement of the products, using the external standard method.

2.11. Statistical analyses

Shapiro-Wilk normality tests indicated that all the data followed a normal distribution. Two-way ANOVA analyses were used to compare

Table 1
Substrates, incubation time and the enzyme product analyzed for determination of fructan enzyme activities.

Enzyme	EC	Substrate	Final concentration	Incubation time	Product analyzed
1-SST	2.4.1.99	Sucrose	100 mM	2 h	1-kestrotriose
INV	3.2.1.26	Sucrose	100 mM	2 h	Fructose
1-FFT	2.4.1.100	1-kestotriose	100 mM	2 h	Nystose
6G-FFT	2.4.1.243	Sucrose + 1-kestotriose	100 + 100 mM	2 h	6G-kestotriose
FEHs (1-FEH + 6-FEH)	3.2.1.153 3.2.1.154	Fructans from *Lolium perenne*	5%	2 h	Fructose

the means between the different leaf spray treatments (water, GSH and GSNO) and soil water conditions (well-irrigated control and drought). The level of significance was set at $p \leq 0.05$. One-way ANOVA analyses were used to compare the means of the different spray treatments under the same irrigation condition. When significant differences were detected, a post-hoc Tukey's honestly significant difference (HSD) test was also performed, with the level of significance set at $p \leq 0.05$. Principal component analysis (PCA) was carried out using the following parameters: leaf water potential, all enzyme activities and SNO, H_2O_2, ·OH, GSSG, GSH, AsA, DHA, MDA, reducing sugar and fructan levels. The PCA was performed from the correlation matrix with data standardized by Z-transform. The randomization test (999 permutations) was used to choose the PCA interpretation dimension, with the level of significance set at $p \leq 0.05$. All analyses were conducted with the Past3 program (Hammer et al., 2001).

3. Results

3.1. Plant and soil water status

No significant changes in leaf biomass were observed at the end of the experimental drought treatment (Fig. 1A–B). However, under this condition, plants exhibited significant reductions in leaf water content, 1.4–1.7-fold lower (Fig. 1C–D), as well as significantly reduced leaf water potential, presenting values that were 1.5–1.9-fold lower than control plants (Fig. 2A). Under well-irrigated condition, the leaf water potential was 7.5% higher (less negative) in the GSNO spray treatment when compared to water and GSH treatments. As expected for hydrated soils, the soil water potential was zero for the well-irrigated control condition, while these values fell to approximately −4 to −5 MPa under drought condition (Fig. 2B). Except for the GSNO under well-irrigated condition, none of the other spray treatments caused any significant changes in the water status of the soil or plants.

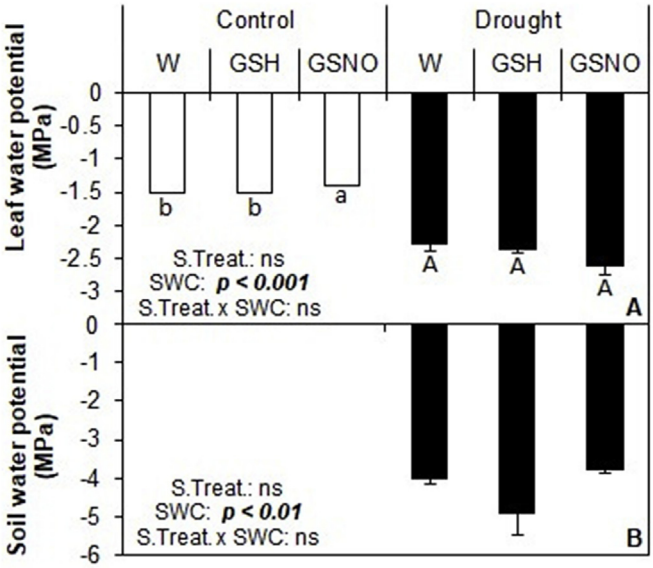

Fig. 2. Leaf water potential (A) and soil water potential (B) of *Lolium perenne* submitted to foliar spraying with water (W), 100 mM reduced gluthatione (GSH) or 100 mM *S*-nitrosogluthatione (GSNO) and then grown under well-irrigated (Control) and watering suspension (Drought) conditions for 23 days. Bars represent the average ± standard error. The *p*-values from ANOVA results are presented and significant effects (i.e. $p < 0.05$) are shown in bold. S. Treat (isolated effect of spraying treatments), SWC (isolated effect of soil water conditions) and S. Treat × SWC (interaction between spraying treatments and soil water conditions). Lower case letters compare treatments in the well-irrigated condition and upper case letters compare treatments in the drought condition ($p < 0.05$). Letters were added only when statistical differences were observed.

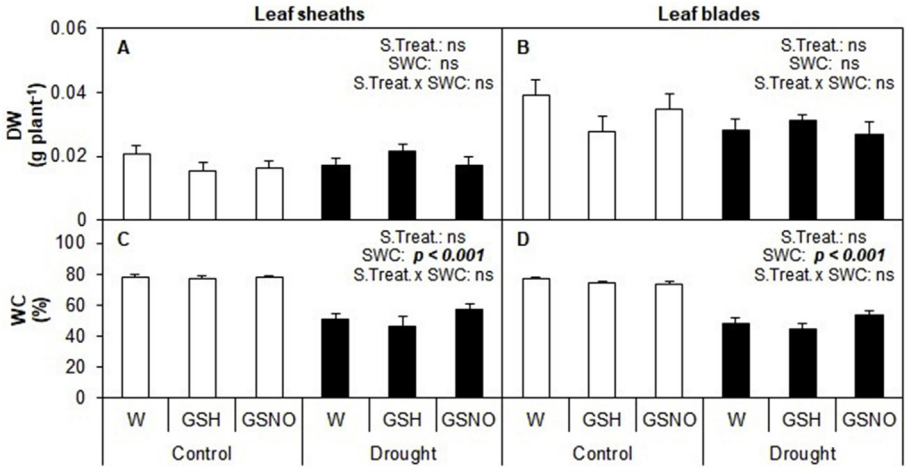

Fig. 1. Dry weight (DW) of leaf sheaths (A) and leaf blades (B) and water content (WC) of leaf sheaths (C) and leaf blades (D) of *Lolium perenne* submitted to foliar spraying with water (W), 100 mM reduced gluthatione (GSH) or 100 mM *S*-nitrosogluthatione (GSNO) and then grown under well-irrigated (Control) and watering suspension (Drought) conditions for 23 days. Bars represent the average ± standard error. The *p*-values from ANOVA results are presented and significant effects (i.e. $p < 0.05$) are shown in bold. S. Treat (isolated effect of spraying treatments), SWC (isolated effect of soil water conditions) and S. Treat × SWC (interaction between spraying treatments and soil water conditions). Lower case letters compare treatments in the well-irrigated condition and upper case letters compare treatments in the drought condition ($p < 0.05$). Letters were added only when statistical differences were observed.

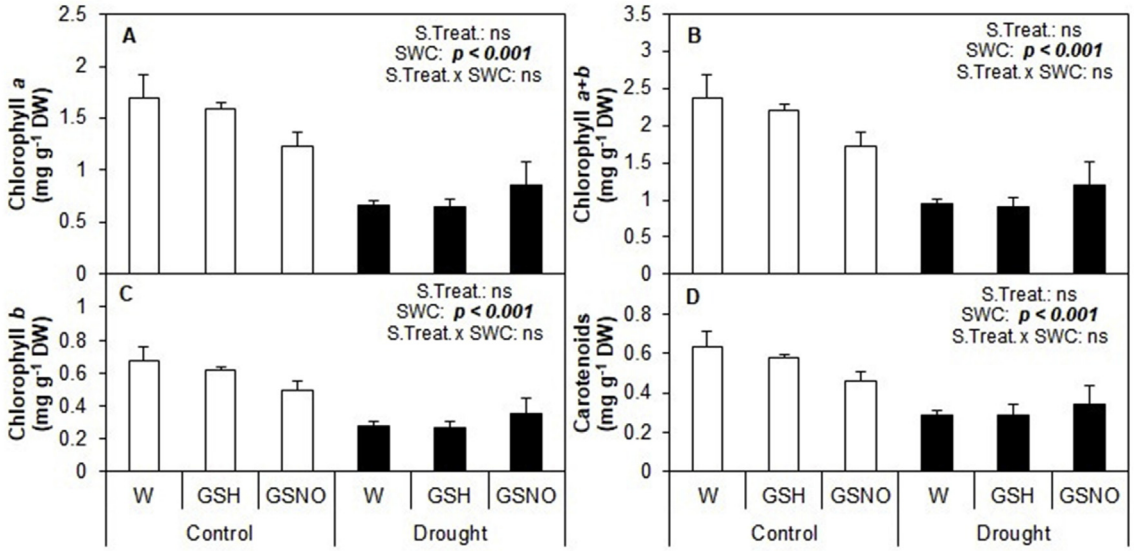

Fig. 3. Chlorophyll *a* (A), chlorophyll *b* (B), chlorophyll *a* + *b* (C) and carotenoids (D) contents of *Lolium perenne* submitted to foliar spraying with water (W), 100 mM reduced gluthatione (GSH) or 100 mM *S*-nitrosogluthatione (GSNO) and then grown under well-irrigated (Control) and watering suspension (Drought) conditions for 23 days. Bars represent the average ± standard error. The *p*-values from ANOVA results are presented and significant effects (i.e. $p < 0.05$) are shown in bold. S. Treat (isolated effect of spraying treatments), SWC (isolated effect of soil water conditions) and S. Treat × SWC (interaction between spraying treatments and soil water conditions). Lower case letters compare treatments in the well-irrigated condition and upper case letters compare treatments in the drought condition ($p < 0.05$). Letters were added only when statistical differences were observed.

3.2. Chlorophyll and carotenoids contents

As shown in Fig. 3A–D, the drought condition resulted in significant decreases in chlorophyll and carotenoids contents when compared to well-irrigated *L. perenne* plants. However, plants under drought and treated with GSNO, showed a slight increase in total chlorophyll ($a + b$) (Fig. 3B) and carotenoids (Fig. 3D), when compared to control plants sprayed with water, under the same conditions.

3.3. S-nitrosothiols content, oxidative markers and antioxidant metabolism

Under drought, GSH and GSNO foliar spray treatments promoted a non-significant 2-fold increase in SNO content (Fig. 4A). On average, plants exposed to drought after water foliar spray presented lower H_2O_2 (Fig. 4B) and ·OH (Fig. 4C) levels, reduced by approximately 41% and 33%, respectively, with no significant differences related to GSH or GSNO foliar application. Moreover, under well-irrigated condition and GSNO treatment, H_2O_2 and ·OH leaf contents were reduced by 23% and 19%, respectively, when compared to water sprayed leaves. Lipid peroxidation levels were not affected by water availability or foliar spray treatments (Fig. 4D).

Under drought, the levels of ascorbate (AsA) (Fig. 5A) decreased regardless of foliar spray treatments. Well-irrigated and drought stressed plants treated with GSH exhibited both reduced levels of DHA, 39% and 82% lower, respectively, (Fig. 5C), positively affecting the AsA:DHA ratio (Fig. 5E). Under well-irrigated condition, an increase of 77% in DHA was observed for GSNO compared to water-treated plants. GSSG content (Fig. 5D) increased in all foliar spraying-treated plants under well-irrigated condition with the highest accumulation in GSNO-treated plants. However, drought led to significant reductions in GSSG levels, regardless of the spraying treatment. Meanwhile, GSH content was not affected by any of the treatments applied (Fig. 5B). Consequently, due to the substantial reductions in GSSG content, the GSH:GSSG ratio was 2.3–10.3-fold higher in plants exposed to drought, when compared to well-irrigated control plants (Fig. 5F). APX activity (Fig. 5G) was not significantly affected by the different treatments. Finally, drought significantly increased GR activity, but no significant differences were detected among spraying treatments (Fig. 5H).

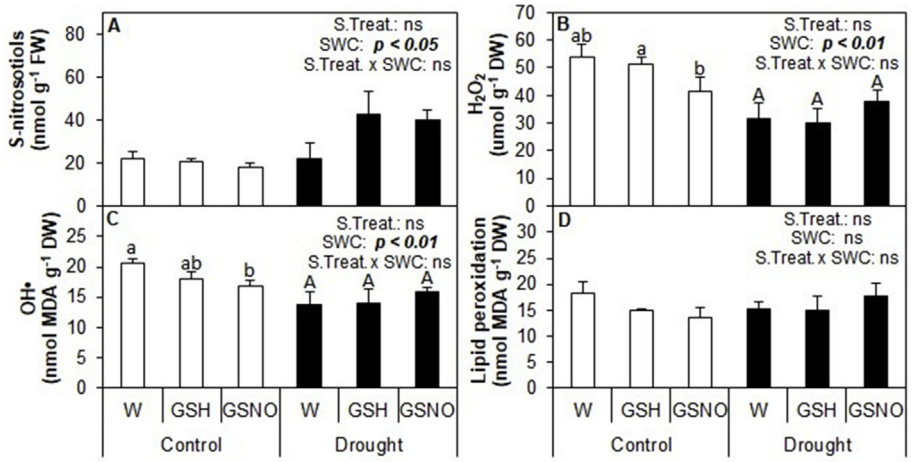

Fig. 4. *S*-nitrosotiols (A), H_2O_2 (B), ·OH (C) and lipid peroxidation (D) content of *Lolium perenne* submitted to foliar spraying with water (W), 100 mM reduced gluthatione (GSH) or 100 mM *S*-nitrosogluthatione (GSNO) and then grown under well-irrigated (Control) and watering suspension (Drought) conditions for 23 days. Bars represent the average ± standard error. The *p*-values from ANOVA results are presented and significant effects (i.e. $p < 0.05$) are shown in bold. S. Treat (isolated effect of spraying treatments), SWC (isolated effect of soil water conditions) and S. Treat × SWC (interaction between spraying treatments and soil water conditions). Lower case letters compare treatments in the well-irrigated condition and upper case letters compare treatments in the drought condition ($p < 0.05$). Letters were added only when statistical differences were observed.

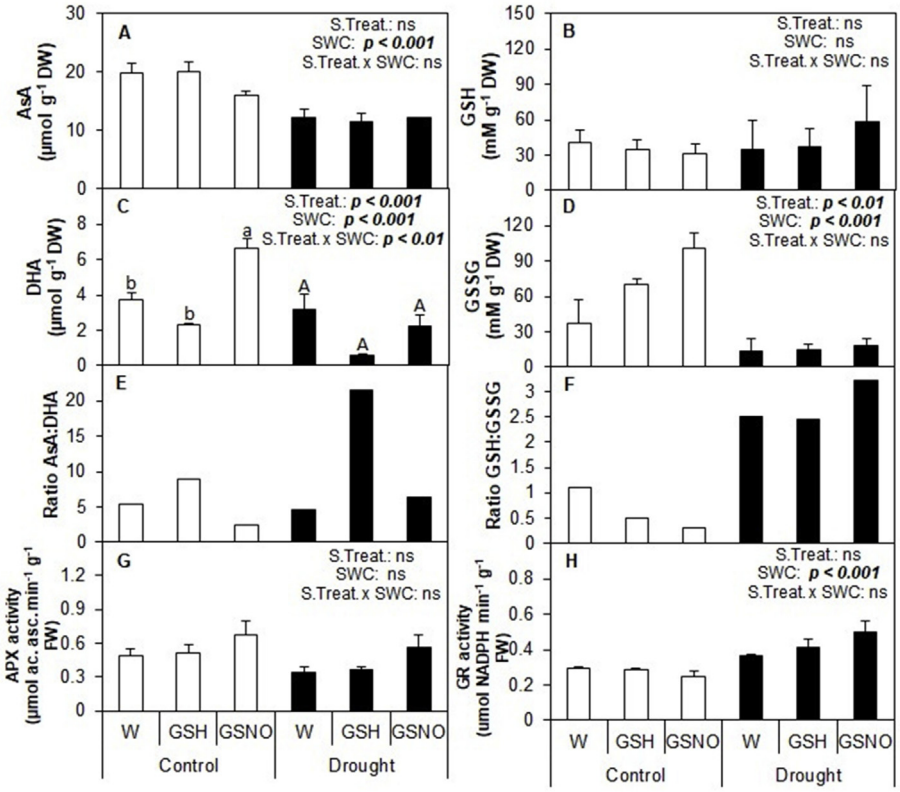

Fig. 5. Ascorbate (AsA) (A), GSH (B), DHA (C), GSSG (D) contents, ratios of AsA:DHA (E) and GSH:GSSG (F), and activities of APX (G) and GR (H) of *Lolium perenne* submitted to foliar spraying with water (W), 100 mM reduced gluthatione (GSH) or 100 mM *S*-nitrosogluthatione (GSNO) and then grown under well-irrigated (Control) and watering suspension (Drought) conditions for 23 days. Bars represent the average ± standard error. The *p*-values from ANOVA results are presented and significant effects (i.e. $p < 0.05$) are shown in bold. S. Treat (isolated effect of spraying treatments), SWC (isolated effect of soil water conditions) and S. Treat × SWC (interaction between spraying treatments and soil water conditions). Lower case letters compare treatments in the well-irrigated condition and upper case letters compare treatments in the drought condition ($p < 0.05$). Letters were added only when statistical differences were observed.

3.4. Soluble sugar contents and fructan metabolism

Under drought condition fructan content increased significantly (Fig. 6A), with plants treated with GSNO displaying values that were 2.6-fold higher than well-irrigated control plants. Reducing sugar contents was significantly affected by drought (Fig. 6B).

Concomitant to the increase in fructan content, a significant increase in 1-SST activity was observed in drought stressed plants sprayed with GSH (1.5-fold) and GSNO (1.7-fold) (Fig. 7A), although a slight decrease of 6G-FFT activity was observed under the same conditions (Fig. 7B). On the other hand, these treatments induced an increase in 6G-FFT activity under well-irrigated condition (Fig. 7B). As shown in Fig. 7D, FEH activity was not affected by drought, but under well-irrigated condition, GSNO spray treatment inhibited the activity of this enzyme by 14%. Although there was a tendency for GSNO treatment to reduce 1-FFT activity in plants from both soil water conditions (Fig. 7C), and to slightly increase INV activity in well-irrigated plants (Fig. 7E), no significant changes were detected.

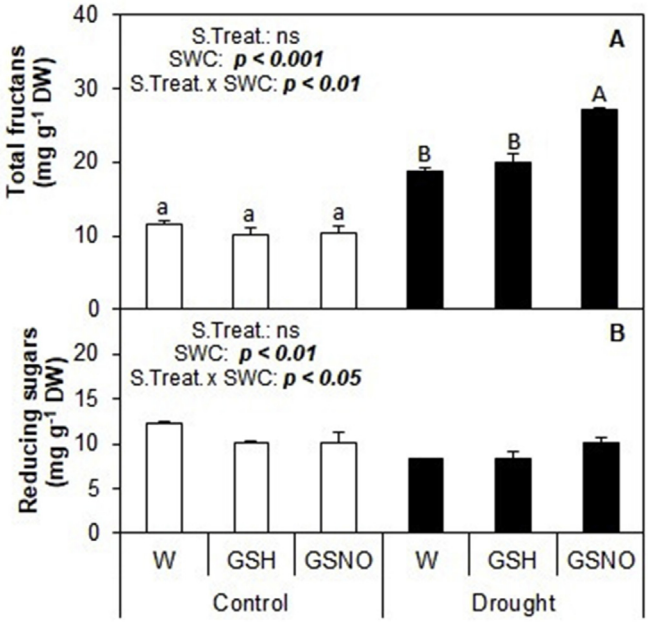

Fig. 6. Fructan (A) and reducing sugar (B) contents of *Lolium perenne* submitted to foliar spraying with water (W), 100 mM reduced gluthatione (GSH) or 100 mM *S*-nitrosogluthatione (GSNO) and then grown under well-irrigated (Control) and watering suspension (Drought) conditions for 23 days. Bars represent the average ± standard error. The *p*-values from ANOVA results are presented and significant effects (i.e. $p < 0.05$) are shown in bold. S. Treat (isolated effect of spraying treatments), SWC (isolated effect of soil water conditions) and S. Treat × SWC (interaction between spraying treatments and soil water conditions). Lower case letters compare treatments in the well-irrigated condition and upper case letters compare treatments in the drought condition ($p < 0.05$). Letters were added only when statistical differences were observed.

3.5. Principal component analysis

Principal component analysis summarized 59% of the total data variability on the two first axes (Fig. 8), a value considered significant, based on the randomization test ($p < 0.005$). All samples from well-irrigated plants were positioned on the positive side of Axis 1, which is indicative of a correlation with leaf water potential (LWP, $r > 0.8$), AsA (ASC, $r > 0.7$), GSSG ($r > 0.7$) and H_2O_2 ($r > 0.7$). Conversely, all samples under drought were located on the negative side of Axis 1, being correlated with total fructan content (FRUC, $r < -0.9$) and GR activity ($r < -0.8$). Concerning Axis 2, all samples of the well-irrigated controls, two of the GSH-treated, one of the GSNO-treated, all of the drought GSNO-treated and two of the GSH-treated were correlated with ·OH ($r > 0.7$) and reducing sugars (RS, $r > 0.65$). Additionally, two of the well-irrigated GSNO-treated, all of the water-treated, and one drought GSH-treated samples were correlated with 6G-FFT activity

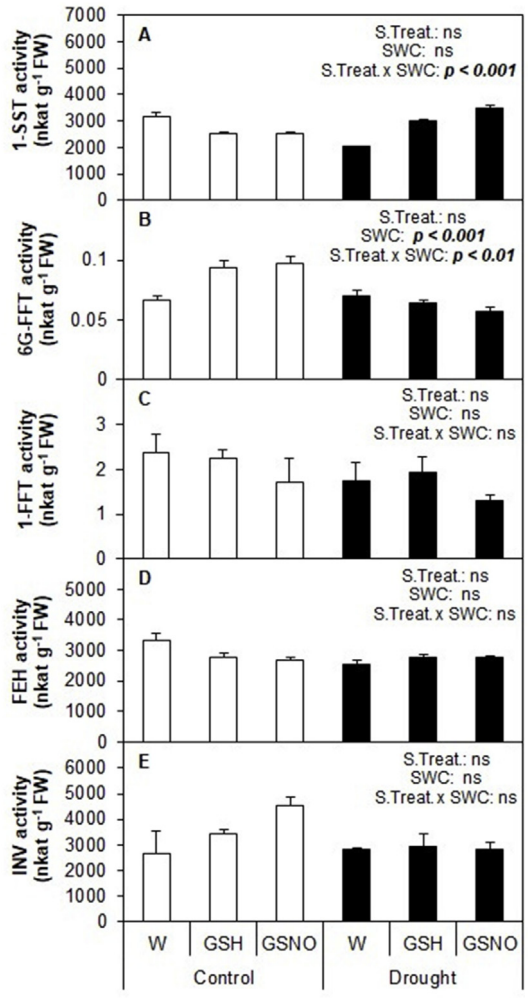

Fig. 7. Activities of 1-SST (A), 6G-FFT (B), 1-FFT (C), FEHs (D) and INV (E) of *Lolium perenne* submitted to foliar spraying with water (W), 100 mM reduced gluthatione (GSH) or 100 mM *S*-nitrosogluthatione (GSNO) and then grown under well-irrigated (Control) and watering suspension (Drought) conditions for 23 days. Bars represent the average ± standard error. The *p*-values from ANOVA results are presented and significant effects (i.e. $p < 0.05$) are shown in bold. S. Treat (isolated effect of spraying treatments), SWC (isolated effect of soil water conditions) and S. Treat × SWC (interaction between spraying treatments and soil water conditions). Lower case letters compare treatments in the well-irrigated condition and upper case letters compare treatments in the drought condition ($p < 0.05$). Letters were added only when statistical differences were observed.

(6GFFT, $r < -0.5$). In summary, the PCA analysis revealed that the spraying treatments had distinct effects on fructan and antioxidant metabolisms, associated with soil water condition.

4. Discussion

Leaf water content and leaf water potential data (Figs. 1 and 2) showed that suspending irrigation for 23 days was sufficient to induce water deficit in two-month-old plants of *L. perenne*. In fact, soil water potential (Fig. 2) was lower than leaf water potential, preventing water absorption by the roots. Pretreating the plants with GSH or GSNO did not affect water content, when comparing with water-sprayed plants under drought. Hence, the NO donor, at least in the concentrations used herein, did not prevent water loss in *L. perenne* plants, different from what was previously described for wheat and sugarcane under water deficit (García-Mata and Lamattina, 2001; Silveira et al., 2016, 2017; Hasanuzzaman et al., 2018b). In the present study, foliar pigment content of the NO donor-treated plants was affected to the same extent by drought (Fig. 3), suggesting that NO does not alleviate the negative effects on photosynthesis, observed during drought stress, and opposite to what was observed with crambe (Batista et al., 2018), wheat and maize plants (Prabhu et al., 2018).

Fructans are osmorregulators and the metabolism of these sugars is modulated under drought conditions in several Poaceae and Asteraceae species (De Roover et al., 2000; Feng et al., 2009; Garcia et al., 2011, 2015), including *L. perenne* (Amiard et al., 2003). Changes in fructan metabolism in response to drought usually involve increased hydrolysis activity, decreased fructan DP and increased osmoregulation capacity (Portes et al., 2008; Garcia et al., 2011, 2015). However, the observed induction of 1-SST activity in plants of *L. perenne* (Fig. 7A) could also lead to the accumulation of fructan-oligosaccharides, a result that was reported earlier in chicory roots (De Roover et al., 2000). Furthermore, the role of fructan accumulation in drought tolerance has been previously discussed for *L. perenne* (cv. Bravo) (Amiard et al., 2003), and osmotic adjustment, cell membrane stability and soluble carbohydrate accumulation have all been correlated with drought tolerance in *L. perenne* (Chai et al., 2010).

For *L. perenne* cv. AberAvon, the low ROS content and the maintenance of lipid peroxidation status (Fig. 4B–D) suggest that plants were able to prevent oxidative damage, both in GSH and GSNO spraying treatments. The increased fructan content may be an underlying mechanism associated with this response, since fructans stabilize the cell membrane during desiccation (Livingston et al., 2009) and can mitigate oxidative damage by scavenging ·OH (Peshev et al., 2013). A similar study with wheat seedlings submitted to water deficit also demonstrated an increase in fructan synthesis and resulted in higher stress tolerance, presumably through increased ROS scavenging and maintenance of lipid peroxidation status (Nemati et al., 2018). In a drought-sensitive wheat cultivar, increased fructan synthesis activity did not increase fructan content, but rather increased fructan DP. On the other hand, a drought-tolerant cultivar with higher 1-FFT and 1-SST activities, showed both, increases in fructan content and DP (Nemati et al., 2018). Although an increase in fructan DP was not observed for *L. perenne* (data not shown), fructan content did increase under drought, most notably in the GSNO treatment, suggesting that NO regulated fructan metabolism, contributing to drought tolerance in the AberAvon cultivar.

Both GSH and GSNO treatments induced the accumulation of SNOs in leaves (Fig. 4A), similar to what was reported for sugarcane under water deficit (Silveira et al., 2016). As previously suggested by Silveira et al. (2016), exogenous GSH could promote GSNO formation due to the increased NO release under stress conditions, and the drought-related increased SNO content could, in turn, modulate protein *S*-nitrosylation (Lindermayr et al., 2005). However, under drought, only 1-SST and 6G-FFT were modulated by NO *in vivo*, while in well irrigated plants, only FEH was under NO regulation (Fig. 7A and B). Even though fructan enzymes have putative *S*-nitrosylation and nitration sites in their sequences (Table S1), the post-translational regulation of these enzymes by SNOs is still not clear. Protein *S*-nitrosylation largely depends on the microenvironment of the reactive cysteine residue, and on the proximity of basic or acidic residues (Zaffagnini et al., 2016). Although NO modulated the activity of some fructan enzymes on *in vitro* experiments (data not shown), the cellular chemical environment and other endogenous factors may have not favored *S*-nitrosylation of fructan and antioxidant enzymes *in vivo* in our experimental conditions. In addition, the use of DTT as a reducing agent, during the fructan enzyme extraction procedure, not only disrupts disulfide bridges, but can also remove NO from binding sites (Raju et al., 2012; Sehrawat et al., 2013), further complicating the assessment of the effects of *S*-nitrosylation *in vivo*. Provided NO has been shown to directly regulate the expression of genes from different metabolic pathways (Begara-Morales et al., 2014; Moro et al., 2017), fructan enzyme activites could be also regulated at the transcriptional level.

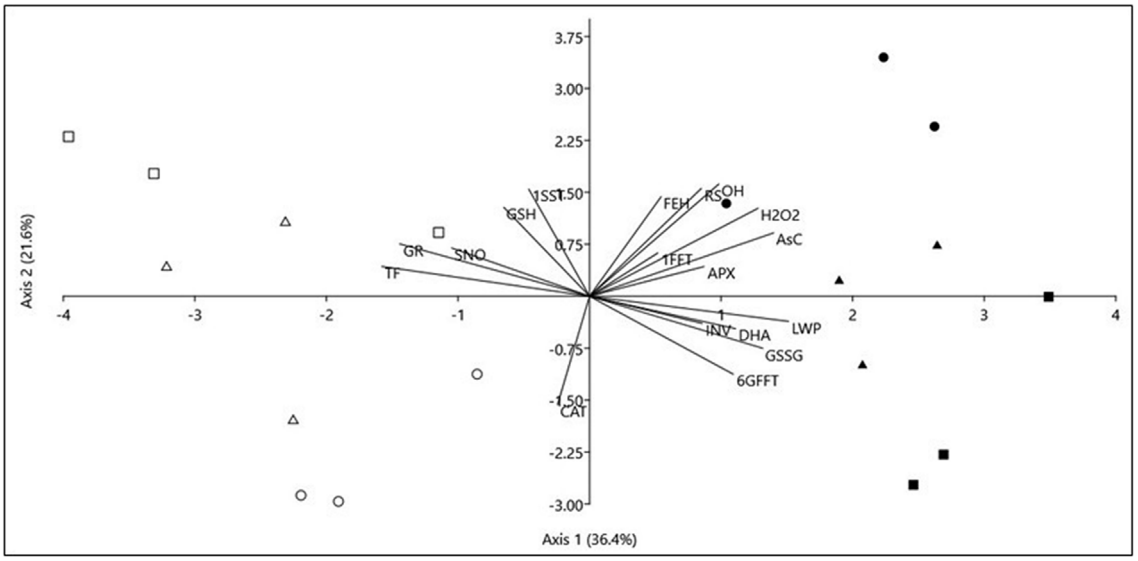

Fig. 8. PCA bi-plot of *Lolium perenne* submitted to foliar spraying with water, 100 mM GSH or 100 mM GSNO and then grown under well-irrigated and drought conditions, and biochemical and physiological parameters. Leaf water potential (LWP), H_2O_2 (H2O2), ·OH (OH), reduced glutathione (GSH), oxidized glutathione (GSSG), ascorbate (ASA), dehydroascorbate (DHA), *S*-nitrosotiols (SNO), total fructan (TF) and reducing sugars (RS) contents and 1-SST (1SST), 6G-FFT (6GFFT), 1-FFT (1FFT), FEHs (FEH), INV, APX and GR activities. Empty symbols indicate samples under well-irrigated soil condition and filled symbols indicate samples under drought condition. Different symbols indicate the different spraying treatments: circle for water, triangle for GSH and square for GSNO.

Likewise, GSH pretreatment modulated 1-SST activity in *L. perenne*. It was previously shown that glutathione can modify protein structure and function by changing the thiol-disulfide balance (Noctor et al., 2011). Disulfide bonds between cysteine residues can inhibit the activity of some enzymes, as observed with bovine vacuolar ATPase (Feng and Forgac, 1992). Previous studies have shown that fructan enzymes, 1-FEH IIa from *C. intybus* (Verhaest et al., 2005) and 6-SFT from *Pachysandra terminalis* (Lammens et al., 2012), both contain disulfide bonds, which could serve as an additional redox regulation mechanism for fructan metabolism under stress conditions. Besides, we cannot exclude the possibility that these enzymes can be regulated by glutathionylation, a reversible post-translational modification which can occur spontaneously, especially in presence of ROS/RNS (Zaffagnini et al., 2012, 2016).

Under watering suspension, the redox status of glutathione shifted from an oxidized to a reduced state, as evidenced by the GSH:GSSG ratio (Fig. 5B, D, F). This response is probably due to an increase in GR activity (Fig. 5G), which would be expected to improve the recovery of GSH and decrease GSSG levels. Similar to glutathione, ascorbate was also shifted toward a more reduced state, especially in the plants pretreated with GSH, which is justified by the fact that DHA can be chemically reduced to ascorbate by GSH (Fig. 5A, C, E). According to Foyer and Noctor (2011), plants with elevated GR activity exhibit increased foliar AsA content and improved oxidative stress tolerance. Indeed, higher AsA and GSH contents, along with the induction of the AsA-GSH cycle enzymes, were found to be associated with lower oxidative damage during osmotic stress in a drought-resistant wheat variety (Lascano et al., 2001). Additionally, a positive correlation between AsA content and drought tolerance was observed for different wheat genotypes (Roy et al., 2017). Therefore, under drought conditions employed in our study, *L. perenne* maintains an antioxidant pool under reduced state, that together with fructan accumulation, ensures greater drought resistance through the prevention of oxidative damage.

It has been reported that SNP treatment, prior to or concomitant with drought stress, can modulate the activity of antioxidant enzymes in *L. perenne*, *Poa pratensis, Cynodon dactylon* and *Oryza sativa*, thus minimizing the deleterious effects of stress (Farooq et al., 2009; Boogar et al., 2014). *S*-nitrosylation could be involved in the NO-induced increase in the activity of AsA-GSH pathway enzymes, as previously shown for seeds of *Antiaris toxicaria* under desiccation (Bai et al., 2011). Although no significant differences were detected in this work, slightly higher APX and GR activities (Fig. 5G and H) were observed in GSNO-treated plants under drought, suggesting that exogenous NO could upregulate the activity of both enzymes, and perhaps contribute to the maintenance of ascorbate-glutathione status in *L. perenne*.

As shown in Fig. 8, the PCA clearly indicated the existence of two distinct groups, corresponding to well-irrigated and drought-stressed plants. Plants of *L. perenne* responded to drought stress by increasing the activities of 1-SST and GR, which consequently increased fructan and GSH contents. These molecules act to scavenge ROS and stabilize cell structures, thereby preventing oxidative stress and providing protection against desiccation. The higher SNO content detected under drought is associated with GSH and GSNO treatments, but did not seem to modulate the activity of the fructan enzymes evaluated in this study. Further assessments of the involvement of *S*-nitrosylation on fructan metabolism responses to drought should be performed to confirm this hypothesis.

5. Conclusions

L. perenne cv. AberAvon, reported as having high soluble sugar content, was relatively tolerant to the water stress imposed. A slight association between drought tolerance and GSNO spraying treatment was detected and related to increased fructan synthesis and content. Despite drought imposition, *L. perenne* mitigated oxidative stress through the modulation of fructan, ascorbate and glutathione pools (Fig. 9).

The regulation of fructan metabolism by GSNO and GSH seems to be complex and may involve hormone or other signaling pathways. Future studies focused on effectively detecting *S*-nitrosylation and *S*-glutathionylation of fructan enzymes need to be conducted to elucidate the molecular mechanisms underlying fructan metabolism regulation by GSH and GSNO.

Authors contribution

The study was conceived by MG and MAMC. APR, VC and ALWS performed the experiments. APR computed and analyzed data. MAMC,

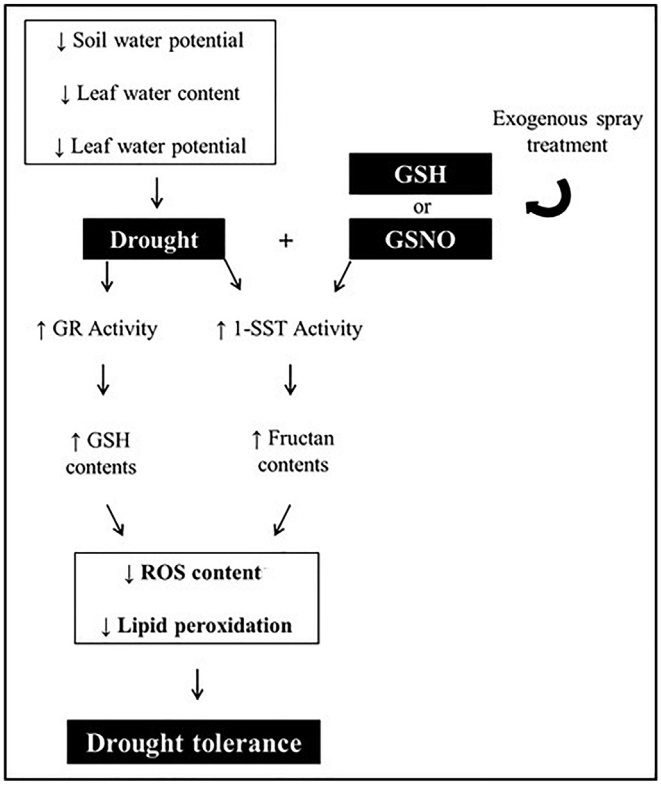

Fig. 9. Summary of the main results and conclusions obtained with plants of *Lolium perenne* submitted to drought and treated with GSH and GSNO.

AMB and MPP supervised the fructan analyses. APR, MAMC and MG wrote the manuscript. All the authors revised the manuscript.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

We thank Ione Salgado and Lucas Siqueira Cardinelli for helping with quantification of the SNO contents and statistical analyses, respectively. We also thank Eduardo Purgatto for technical support. Athos Poli Rigui was financially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Programa de Doutorado Sanduíche no Exterior (PDSE/CAPES).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.029.

References

Abreu, M.E., Carvalho, V., Mercier, H., 2018. Antioxidant capacity along the leaf blade of the C3-CAM facultative bromeliad *Guzmania monostachia* under water deficit conditions. Funct. Plant Biol. 45, 620–629. https://doi.org/10.1071/FP17162.

Ahmad, Z., Waraich, E.A., Ahmad, R., Iqbal, M.A., Awan, M.I., 2015. Studies on screening of maize (*Zea mays* L.) hybrids under drought stress conditions. J. Adv. Bot. Zool. 2, 1–5.

Amiard, V., Morvan-Bertrand, A., Billard, J.P., Huault, C., Keller, F., Prud'homme, M.P., 2003. Fructans, but not the sucrosyl-galactosides, raffinose and loliose, are affected by drought stress in perennial ryegrass. Plant Physiol. 132, 2218–2229. https://doi.org/10.1104/pp.103.022335.

Asega, A.F., Nascimento, J.R.O., Schroeven, L., Van den Ende, W., Carvalho, M.A.M., 2008. Cloning, characterization and functional analysis of 1-FEH cDNA from *Vernonia herbacea* (Vell.) Ruby. Plant Cell Physiol. 49, 1185–1195. https://doi.org/10.1093/pcp/pcn094.

Bai, X., Yang, L., Tian, M., Chen, J., Shi, J., Yang, Y., Hu, X., 2011. Nitric oxide enhances desiccation tolerance of recalcitrant Antiaris toxicaria seeds via protein *S*-nitrosylation and carbonylation. PLoS One 6https://doi.org/10.1371/journal.pone.0020714. 20714.

Batista, P.F., Costa, A.C., Müller, C., Silva-Filho, R.O., Barbosa, F.S., Merchant, A., Mendes, G.C., Nascimento, K.J.T., 2018. Nitric oxide mitigates the effect of water deficit in *Crambe abyssinica*. Plant Physiol. Biochem. 129, 310–322. https://doi.org/10.1016/j.plaphy.2018.06.012.

Begara-Morales, J.C., Sánchez-Calvo, B., Luque, F., Leyva- Pérez, M.O., Leterrier, M., Corpas, F.J., Barroso, J.B., 2014. Differential transcriptomic analysis by RNA-Seq of GSNO-responsive genes between Arabidopsis roots and leaves. Plant Cell Physiol. 55, 1080–1095. https://doi.org/10.1093/pcp/pcu044.

Begara-Morales, J.C., Sánchez-Calvo, B., Chaki, M., Valderrama, R., Mata-Pérez, C., Corpas, F.J., Barroso, J.B., 2016. Protein *S*-nitrosylation and *S*-glutathionylation as regulators of redox homeostasis during abiotic stress response. In: Gupta, D.K., Palma, J.M., Corpas, F.J. (Eds.), Redox State as a Central Regulator of Plant-Cell Stress Responses, pp. 365–386.

Begara-Morales, J.C., Chaki, M., Valderrama, R., Mata-Pérez, C., Padilla, M.N., Barroso, J.B., 2019. The function of *S*-nitrosothiols during abiotic stress in plants. J. Exp. Bot. 70, 4429–4439. https://doi.org/10.1093/jxb/erz197.

Beligni, M.V., Lamattina, L., 2002. Nitric oxide interferes with plant photo-oxidative stress by detoxifying reactive oxygen species. Plant Cell Environ. 25, 737–748. https://doi.org/10.1046/j.1365-3040.2002.00857.x.

Bian, S., Jiang, Y., 2009. Reactive oxygen species, antioxidant enzyme activities and gene expression patterns in leaves and roots of Kentucky bluegrass in response to drought stress and recovery. Sci. Hortic. 120, 264–270. https://doi.org/10.1016/j.scienta.2008.10.014.

Boaretto, L.F., Carvalho, G., Borgo, L., Creste, S., Landell, M.G., Mazzafera, P., Azevedo, R.A., 2014. Water stress reveals differential antioxidant responses of tolerant and non-tolerant sugarcane genotypes. Plant Physiol. Biochem. 74, 165–175. https://doi.org/10.1016/j.plaphy.2013.11.016.

Boogar, A.R., Salehi, H., Jowkar, A., 2014. Exogenous nitric oxide alleviates oxidative damage in turgrasses under drought stress. South Afr. J. Bot. 92, 78–82. https://doi.org/10.1016/j.sajb.2014.02.005.

Buttler, A., Mariotte, P., Meisser, M., Guillaume, T., Signarbieux, C., Vitra, A., Preux, S., Mercier, G., Quezada, J., Bragazza, L., Gavazov, K., 2019. Drought-induced decline of productivity in the dominant grassland species *Lolium perenne* L. depends on soil type and prevailing climatic conditions. Soil Biol. Biochem. 132, 47–57. https://doi.org/10.1016/j.soilbio.2019.01.026.

Chai, Q., Fang, J., Merewitz, E., Huang, B., 2010. Growth and Physiological Traits Associated with Drought Survival and Post-drought Recovery in Perennial Turfgrass Species, vol. 135. pp. 125–133.

Chalmers, J., Johnson, X., Lidgett, A., Spangenberg, G., 2003. Isolation and characterisation of a sucrose:sucrose 1-fructosyltransferase gene from perennial ryegrass (*Lolium perenne*). J. Plant Physiol. 160, 1385–1391. https://doi.org/10.1078/0176-1617-01107.

Darwen, C.W., John, P., 1989. Localization of the enzymes of fructan metabolism in vacuoles isolated by a mechanical method from tubers of Jerusalem artichoke (*Helianthus tuberosus* L.). Plant Physiol. 89https://doi.org/10.1104/pp.89.2.658. 658-563.

De Boeck, H.D., Lemmens, C., Zavalloni, C., Gielen, B., Malchair, S., Carnol, M., Merckx, R., Van den Berge, J., Ceulemans, R., Nijs, I., 2008. Biomass production in experimental grasslands of different species richness during three years of climate warming. Biogeosciences 5, 585–594. https://doi.org/10.5194/bg-5-585-2008.

De Roover, J., Vandenbranden, K., Van Laere, A., Van den Ende, W., 2000. Drought induces fructan synthesis and 1-SST (sucrose: sucrose fructosyltransferase) in roots and leaves of chicory seedlings (*Cichorium intybus* L.). Planta 210, 808–814. https://doi.org/10.1007/s004250050683.

Fàbregas, N., Fernie, A.R., 2019. The metabolic response to drought. J. Exp. Bot. 70, 1077–1085. https://doi.org/10.1093/jxb/ery437.

Farooq, M., Basra, S.M.A., Wahid, A., Rehman, H., 2009. Exogenously applied nitric oxide enhances the drought tolerance in fine grain aromatic rice (*Oryza sativa* L.). J. Agron. Crop Sci. 195, 254–261. https://doi.org/10.1111/j.1439-037X.2009.00367.x.

Feng, B., Yu, H., Hu, Y., Gao, X., Gao, J., Gao, D., Zhang, S., 2009. The physiological characteristics of the low canopy temperature wheat (*Triticum aestivum* L.) genotypes under simulated drought condition. Acta Physiol. Plant. 31, 1229–1235. https://doi.org/10.1007/s11738-009-0358-4.

Feng, Y., Forgac, M., 1992. A novel mechanism for regulation of vacuolar acidification. J. Biol. Chem. 267, 19769–19772.

Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 155, 2–18. https://doi.org/10.1104/pp.110.167569.

Frungillo, L., de Oliveira, J.F.P., Saviani, E.E., Oliveira, H.C., Martínez, M.C., Salgado, I., 2013. Modulation of mitochondrial activity by *S*-nitrosoglutathione reductase in *Arabidopsis thaliana* transgenic cell lines. Biochim. Biophys. Acta 1827, 239–247. https://doi.org/10.1016/j.bbabio.2012.11.011.

Garcia, P.M.A., Asega, A.F., Silva, E.A., Carvalho, M.A.M., 2011. Effect of drought and rewatering on fructan metabolism in *Vernonia herbacea* (Vell.) Rusby. Plant Physiol. Biochem. 49, 664–670. https://doi.org/10.1016/j.plaphy.2011.03.014.

Garcia, P.M.A., Hayashi, A.H., Silva, E.A., Figueiredo-Ribeiro, R.C.L., Carvalho, M.A.M., 2015. Structural and metabolic changes in rhizophores of the Cerrado species *Chrysolaena obovata* (Less.) Dematt. as influenced by drought and re-watering. Front. Plant Sci. 6, 721. https://doi.org/10.3389/fpls.2015.00721.

García-Mata, C., Lamattina, L., 2001. Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol. 126, 1196–1204. https://doi.org/10.1104/pp.126.3.1196.

Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic

stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/j.plaphy.2010.08.016.
Gillespie, K.M., Ainsworth, E.A., 2007. Measurement of reduced, oxidized and total ascorbate content in plants. Nat. Protoc. 2, 871–874. https://doi.org/10.1038/nprot.2007.101.
Halliwell, B., Grootveld, M., Gutteridge, J.M., 1988. Methods for the measurement of hydroxyl radicals in biochemical systems: deoxyribose degradation and aromatic hydroxylation. Methods Biochem. Anal. 33, 59–90. https://doi.org/10.1002/9780470110546.ch2.
Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Paleontol. Electron. 4, 1–9.
Hasanuzzaman, M., Nahar, K., Alam, M.M., Bhuyan, M.H.M.B., Oku, H., Fujita, M., 2018a. Exogenous nitric oxide pretreatment protects *Brassica napus* L. seedlings from paraquat toxicity through the modulation of antioxidant defense and glyoxalase systems. Plant Physiol. Biochem. 126, 173–186. https://doi.org/10.1016/j.plaphy.2018.02.021.
Hasanuzzaman, M., Nahar, K., Rahman, A., Inafuku, M., Oku, H., Fujita, M., 2018b. Exogenous nitric oxide donor and arginine provide protection against short-term drought stress in wheat seedlings. Physiol. Mol. Biol. Plants 24, 993–1004. https://doi.org/10.1007/s12298-018-0531-6.
Heath, R.L., Packer, L., 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125, 189–198. https://doi.org/10.1016/0003-9861(68)90654-1.
Hernandez-Marin, E., Martínez, A., 2012. Carbohydrates and their free radical scavenging capability: a theoretical study. J. Phys. Chem. B 116, 9668–9675. https://doi.org/10.1021/jp304814r.
Hincha, D.K., Livingston, D.P., Premakumar, R., Zuther, E., Obel, N., Cacela, C., Heyer, A.G., 2007. Fructans from oat and rye: composition and effects on membrane stability during drying. Biochim. Biophys. Acta 1768, 1611–1619. https://doi.org/10.1016/j.bbamem.2007.03.011.
Israr, M., Sahi, S., Datta, R., Sarkar, D., 2006. Bioaccumulation and physiological effects of mercury in *Sesbania drummondii*. Chemosphere 65, 591–598. https://doi.org/10.1016/j.chemosphere.2006.02.016.
Jermyn, M.A., 1956. A new method for the determination of ketohexoses in presence of aldohexoses. Nature 177, 38–39. https://doi.org/10.1038/177038a0.
Ji, Y., Zhang, X., Peng, Y., Huang, L., Liang, X., Wang, K., Yin, G., Zhao, X., 2014. Osmolyte accumulation, antioxidant enzyme activities and gene expression patterns in leaves of orchardgrass during drought stress and recovery. Grassl. Sci. 60, 131–141. https://doi.org/10.1111/grs.12052.
Jiang, Y., Huang, B., 2001. Drought and heat stress injury to two cool-season turfgrasses in relation to antioxidant metabolism and lipid peroxidation. Crop Sci. 41https://doi.org/10.2135/cropsci2001.412436x. 36-442.
Junglee, S., Urban, L., Sallanon, H., Lopez-Lauri, F., 2014. Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. Am. J. Anal. Chem. 5, 730–736. https://doi.org/10.4236/ajac.2014.511081.
Khan, M.I.R., Asgher, M., Fatma, M., Per Tasir, S., Khan Nafees, A., 2015. Drought stress vis a vis plant functions in the era of climate change. Clim. Change Environ. Sustain. 3, 13–25. https://doi.org/10.5958/2320-642X.2015.00002.2.
Lammens, W., Le Roy, K., Yuan, S., Vergauwen, R., Rabijns, A., Van Laere, A., Strelkov, S.V., Van den Ende, W., 2012. Crystal structure of 6-SST/6-SFT from *Pachysandra terminalis*, a plant fructan biosynthesizing enzyme in complex with its acceptor substrate 6-kestose. Plant J. 70, 205–219. https://doi.org/10.1111/j.1365-313X.2011.04858.x.
Lascano, H.R., Antonicelli, G.E., Luna, C.M., Melchiorre, M.N., Gómez, L.D., Racca, R.W., Trippi, V.S., Casano, L.M., 2001. Antioxidant system response of different wheat cultivars under drought: field and in vitro studies. Aust. J. Plant Physiol. 28, 1095–1102. https://doi.org/10.1071/PP01061.
Lasseur, B., Lothier, J., Djoumad, A., Coninck, B., Smeekens, S., Laere, A.V., Morvan-Bertrand, A., Van den Ende, W., Prud'homme, M.P., 2006. Molecular and functional characterization of a cDNA encoding fructan:fructan 6G-fructosyltransferase (6G-FFT)/fructan:fructan 1-fructosyltransferase (1-FFT) from perennial ryegrass (*Lolium perenne* L.). J. Exp. Bot. 57, 2719–2734. https://doi.org/10.1093/jxb/erl034.
Lasseur, B., Lothier, J., Wiemken, A., Van Laere, A., Morvan-Bertrand, A., Van den Ende, W., Prud'homme, M.P., 2010. Towards a better understanding of the generation of fructan structure diversity in plants: molecular and functional characterization of a sucrose:fructan 6-fructosyltransferase (6-SFT) cDNA from perennial ryegrass (*Lolium perenne*). J. Exp. Bot. 62, 1871–1885. https://doi.org/10.1093/jxb/erq388.
Li, C., Li, T., Zhang, D., Jiang, L., Shao, Y., 2013. Exogenous nitric oxide effect on fructan accumulation and FBEs expression in chilling-sensitive and chilling-resistant wheat. Environ. Exp. Bot. 86, 2–8. https://doi.org/10.1016/j.envexpbot.2011.12.032.
Lichtenthaler, H.K., Buschmann, C., 2001. Chlorophylls and carotenoids: measurement and characterization by UV-VIS spectroscopy. Curr. Protoc. Anal. Chem. 1https://doi.org/10.1002/0471142913.faf0403s01. F43.1.-F43.8.
Lindermayr, C., Saalbach, G., Durner, J., 2005. Proteomic identification of *S*-nitrosylated proteins in arabidopsis1[w]. Plant Physiol. 137, 921–930. https://doi.org/10.1104/pp.104.058719.
Livingston III, D.P., Hincha, D.K., Heyer, A.G., 2009. Fructan and its relationship to abiotic stress tolerance in plants. Cell. Mol. Life Sci. 66, 2007–2023. https://doi.org/10.1007/s00018-009-0002-x.
Lothier, J., Lasseur, B., Le Roy, K., Van Laere, A., Prud'homme, M.P., Barre, P., Van den Ende, W., Morvan-Bertrand, A., 2007. Cloning, gene mapping, and functional analysis of a fructan 1-exohydrolase (1-FEH) from *Lolium perenne* implicated in fructan synthesis rather than in fructan mobilization. J. Exp. Bot. 58, 1969–1983. https://doi.org/10.1093/jxb/erm053.
Lothier, J., Van Laere, A., Prud'homme, M.P., Van den Ende, W., Morvan-Bertrand, A., 2014. Cloning and characterization of a novel fructan 6-exohydrolase strongly inhibited by sucrose in *Lolium perenne*. Planta 240, 629–643. https://doi.org/10.1007/s00425-014-2110-6.
Marx, S.P., Josef, N., Frehner, M., 1997. Hydrolysis of fructan in grasses: a β-(2-6)-linkage specific fructan-β-fructosidase from stubble of *Lolium perenne*. New Phytol. 135, 279–290.
Meuriot, F., Morvan-Bertrand, A., Noiraud-Romy, N., Decau, M.L., Escobar-Gutiérrez, J., Gastal, F., Prud'homme, M.P., 2018. Short-term effects of defoliation intensity on sugar remobilization and N fluxes in ryegrass. J. Exp. Bot. 69, 3975–3986. https://doi.org/10.1016/10.1093/jxb/ery211.
Mittler, R., 2017. ROS are good. Trends Plant Sci. 22, 11–19. https://doi.org/10.1016/j.tplants.2016.08.002.
Moro, C.F., Gaspar, M., Silva, F.R., Pattathill, S., Hahn, M.G., Salgado, I., Braga, M.R., 2017. *S*-nitrosoglutathione promotes cell wall remodelling, alters the transcriptional profile and induces root hair formation in the hairless root hair defective *6 (rhd6)* mutant of *Arabidopsis thaliana*. New Phytol. 213, 1771–1786. https://doi.org/10.1111/nph.14309.
Munné-Bosch, S., Lalueza, P., 2007. Age-related changes in oxidative stress markers and abscisic acid levels in a drought-tolerant shrub, *Cistus clusii* grown under Mediterranean field conditions. Planta 255, 1039–1049. https://doi.org/10.1007/s00425-006-0412-z.
Nabi, R.B.S., Tayade, R., Hussain, A., Kulkami, K.P., Muhammad, I., Mun, B.G., Yun, B.W., 2019. Nitric oxide regulates plant responses to drought, salinity, and heavy metal stress. Environ. Exp. Bot. 161, 120–133. https://doi.org/10.1016/j.envexpbot.2019.02.003.
Nemati, F., Ghanati, F., Gavlighi, H.A., Sharifi, M., 2018. Fructan dynamics and antioxidant capacity of 4-day-old seedlings of wheat (*Triticum aestivum*) cultivars during drought stress and recovery. Funct. Plant Biol. 45, 1000–1008. https://doi.org/10.1071/fp18008.
Nguyen, K.H., Mostofa, M.G., Watanabe, Y., Tran, C.D., Rahamn, M.M., Tran, L.P., 2018. Overexpression of GmNAC085 enhances drought tolerance in Arabidopsis by regulating glutathione biosynthesis, redox balance and glutathione-dependent detoxification of reactive oxygen species and methylglyoxal. Environ. Exp. Bot. 161, 242–254. https://doi.org/10.1016/j.envexpbot.2018.12.021.
Nishizawa, A., Yabuta, Y., Shigeoka, S., 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol. 147, 1251–1263. https://doi.org/10.1104/pp.108.122465.
Noctor, G., Queval, G., Mhamdi, A., Chaouch, S., Foyer, C.H., 2011. Glutathione. Arabidopsis Book, vol. 9, e0142. https://doi.org/10.1199/tab.0142.
Peshev, D., Van den Ende, W., 2013. Sugars as antioxidants in plants. In: Tuteja, N., Gill, S.S. (Eds.), Crop Improvement under Adverse Conditions. Springer Science + Business Media, New York, pp. 285–308 Tuteja, N., Gill, N., Singh, S. Crop Improvement Under Adverse Conditions.
Peshev, D., Vergauwen, R., Mioglia, A., Hideg, E., Van den Ende, W., 2013. Towards understanding vacuolar antioxidant mechanisms: a role for fructans? J. Exp. Bot. 64, 1025–1038. https://doi.org/10.1093/jxb/ers377.
Pilon-Smits, E.A.H., Ebskamp, M.J.M., Paul, M.J., Jeuken, M.J.W., Weisbeek, P.J., Smeekens, S.C.M., 1995. Improved performance of transgenic fructan accumulating tobacco under drought stress. Plant Physiol. 107, 125–130. https://doi.org/10.1104/pp.107.1.125.
Pilon-Smits, E.A.H., Terry, N., Sears, T., Van Dun, K., 1999. Enhanced drought resistance in fructan-producing sugar beet. Plant Physiol. Biochem. 37, 313–317.
Portes, M.T., Figueiredo-Ribeiro, R.C.L., Carvalho, M.A.M., 2008. Low temperature and defoliation affect fructan metabolizing enzymes in different regions of the rhizophores of *Vernonia herbacea*. J. Plant Physiol. 165, 1572–1581. https://doi.org/10.1016/j.jplph.2008.01.004.
Prabhu, B.M., Ramteke, P.W., Shukla, P.K., Mishra, P., Attri, A., Singh, B., Pagire, G.S., 2018. Evaluation of response of exogenous nitric oxide on photosynthetic enzymes and pigments of C3 and C4 plants grown under drought stress. J. Pharmacogn. Phytochem. 7, 2606–2612.
Prakash, V., Singh, V.P., Tripathi, D.K., Sharma, S., Corpas, F.J., 2018. Crosstalk between nitric oxide (NO) and abscisic acid (ABA) signaling molecules in higher plants. Environ. Exp. Bot. 161, 41–49. https://doi.org/10.1016/j.envexpbot.2018.10.033.
Prud'homme, M.P., Gonzalez, B., Billard, J.P., Boucaud, J., 1992. Carbohydrate content, fructan and sucrose enzyme activities in roots, stubble and leaves of ryegrass (*Lolium perenne* L.) as affected by source/sink modification after cutting. J. Plant Physiol. 140, 282–291. https://doi.org/10.1016/S0176-1617(11)81080-1.
Raju, K., Doulias, P.T., Tenopoulou, M., Greene, J.L., Ischiropoulos, H., 2012. Strategies and tools to explore protein *S*-nitrosylation. Biochim. Biophys. Acta 1820, 684–688. https://doi.org/10.1016/j.bbagen.2011.05.009.
Roy, S., Arora, A., Chinnusamy, V., Singh, V.P., 2017. Endogenous reduced ascorbate: an indicator of plant water deficit stress in wheat. Indian J. Plant Physiol. 22, 365–368. https://doi.org/10.1007/s40502-017-0308-x.
Salgado, I., Oliveira, H.C., Gaspar, M., 2017. Plant nitric oxide signaling under environmental stresses. In: Pandey, G.K. (Ed.), Mechanism of Plant Hormone Signaling under Stress. Wiley, New Jersey, pp. 345–361.
Santa-Cruz, A., Martinez-Rodriguez, M.M., Perez-Alfocea, F., Romero-Aranda, R., Bolarin, M.C., 2002. The rootstock effect on the tomato salinity response depends on the shoot genotype. Plant Sci. 162, 825–831. https://doi.org/10.1016/S0168-9452(02)00030-4.
Saville, B., 1958. A scheme for the colorimetric determination of microgram amounts of thiols. Analyst 83, 670–672. https://doi.org/10.1039/AN9588300670.
Schaedle, M., Bassham, J.A., 1977. Chloroplast glutathione reductase. Plant Physiol. 59, 1011–1012. https://doi.org/10.1104/pp.59.5.1011.
Schneider, T., Keller, F., 2009. Raffinose in chloroplasts is synthesized in the cytosol and transported across the chloroplast envelope. Plant Cell Physiol. 50, 2174–2182. https://doi.org/10.1093/pcp/pcp151.

Sehrawat, A., Abat, J.K., Deswall, R., 2013. RuBisCO depletion improved proteome coverage of cold responsive *S*-nitrosylated targets in *Brassica juncea*. Front. Plant Sci. 4, 342. https://doi.org/10.3389/fpls.2013.00342.

Seneviratne, S.I., Nicholls, N., Easterling, D., Goodess, C.M., Kanae, S., Kossin, J., Luo, Y., Marengo, J., McInnes, K., Rahimi, M., Reichstein, M., Sorteberg, A., Vera, C., Zhang, X., 2012. Changes in climate extremes and their impacts on the natural physical environment. In: Field, C.B., Barros, V., Stocker, T.F., Qin, D., Dokken, D.J., Ebi, K.L., Mastrandrea, M.D., Mach, K.J., Plattner, G.K., Allen, A.P., Tignor, M., Midgley, P.M. (Eds.), Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation. A Special Report of Working Groups I and II of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge and New York, pp. 109–230.

Sheikh-Mohamadi, M.H., Etemadi, N., Arab, M.M., Aalifar, M., Arab, M., 2018. Physiological and Ascorbate-Glutathione pathway-related genes responses under drought and heat stress in crested wheatgrass. Sci. Hortic. 242, 195–206. https://doi.org/10.1016/j.scienta.2018.07.037.

Sheikh-Mohamadi, M.H., Etemadi, N., Nikbakht, A., Arab, M., Majidi, M.M., Pessarakli, M., 2017. Antioxidant defence system and physiological responses of Iranian crested wheatgrass (*Agropyron cristatum* L.) to drought and salinity stress. Acta Physiol. Plant. 39, 245. https://doi.org/10.1007/s11738-017-2543-1.

Silveira, N.M., Frugillo, L., Marcos, F.C.C., Pelegrino, M.T., Miranda, M.T., Seabra, A.B., Salgado, I., Machado, E.C., Ribeiro, R.V., 2016. Exogenous nitric oxide improves sugarcane growth and photosynthesis under water deficit. Planta 244, 181–190. https://doi.org/10.1007/s00425-016-2501-y.

Silveira, N.M., Marcos, F.C., Frungillo, L., Moura, B.B., Seabra, A.B., Salgado, I., Machado, E.C., Hancock, J.T., Ribeiro, R.V., 2017. S-nitrosoglutathione spraying improves stomatal conductance, Rubisco activity and antioxidant defense in both leaves and roots of sugarcane plants under water deficit. Physiol. Plant. 160, 383–395. https://doi.org/10.1111/ppl.12575.

Soares, C., Carvalho, M.E.A., Azevedo, R.A., Fidalgo, F., 2018. Plants facing oxidative challenges - a little help from the antioxidant networks. Environ. Exp. Bot. 161, 4–25. https://doi.org/10.1016/j.envexpbot.2018.12.009.

Somogyi, M., 1945. A new reagent for the determination of sugars. J. Biol. Chem. 160, 61–63.

Soussana, J.F., Luescher, A., 2007. Temperate grasslands and global atmospheric change: a review. Grass Forage Sci. 62, 127–134. https://doi.org/10.1111/j.1365-2494.2007.00577.x.

Souza, S.R., Blande, J.D., Holopainen, J.K., 2013. Pre-exposure to nitric oxide modulates the effect of ozone on oxidative defenses and volatile emissions in lima bean. Environ. Pollut. 179, 111–119. https://doi.org/10.1016/j.envpol.2013.03.065.

Trenberth, K.E., Dai, A., Van der Schrier, G., Jones, P.D., Barichivich, J., Briffa, K.R., Sheffield, J., 2014. Global warming and changes in drought. Nat. Clim. Chang. 4, 17–22. https://doi.org/10.1038/nclimate2067.

Velikova, V., Yordanov, I., Edreva, A., 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. Plant Sci. 151, 59–66. https://doi.org/10.1016/S0168-9452(99)00197-1.

Verhaest, M., Van den Ende, W., Le Roy, K., De Ranter, C.J., Van Laere, A., Rabijns, A., 2005. X-ray diffraction structure of a plant glycosyl hydrolase Family 32 protein: fructan 1-exohydrolase IIa of *Cichorium intybus*. Plant J. 41, 400–411. https://doi.org/10.1111/j.1365-313X.2004.02304.x.

Wagner, W., Keller, F., Wiemken, A., 1983. Fructan metabolism in cereals: induction in leaves and compartmentation in protoplasts and vacuoles. Z. Pflanzenphysiol. 112, 359–372. https://doi.org/10.1016/S0044-328X(83)80053-1.

Wang, F.Z., Wang, Q.B., Kwon, S.Y., Kwak, S.S., Su, W.A., 2005. Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase. J. Plant Physiol. 162, 465–472. https://doi.org/10.1016/j.jplph.2004.09.009.

Weng, X., Zheng, C.J., Xu, H.X., Sun, J., 2007. Characteristics of photosynthesis and functions of the water–water cycle in rice (*Oryza sativa*) leaves in response to potassium deficiency. Physiol. Plant. 131, 614–621. https://doi.org/10.1111/j.1399-3054.2007.00978.x.

Westermeier, P., Wosnitza, A., Willner, E., Feuerstein, U., Luesink, W., Schulze, S., Schum, A., Hartmann, S., 2016. Variation in drought tolerance of perennial ryegrass (*Lolium perenne* L.). In: Róldan-Ruiz, I., Baert, J., Reheul, D. (Eds.), Breeding in a World of Scarcity, pp. 63–68.

Xu, L., Han, L., Huang, B., 2011. Antioxidant enzyme activities and gene expression patterns in leaves of Kentucky bluegrass in response to drought and post-drought recovery. J. Am. Soc. Hortic. Sci. 136, 247–255. https://doi.org/10.21273/JASHS.136.4.247.

Yang, H., Mu, J., Chen, L., Feng, J., Hu, J., Li, L., Zhou, J.M., Zuo, J., 2015. S-nitrosylation positively regulates ascorbate peroxidase activity during plant stress responses. Plant Physiol. 167, 1604–1615. https://doi.org/10.1104/pp.114.255216.

Zaffagnini, M., Bedhomme, M., Marchand, C.H., Morisse, S., Trost, P., Lemaire, S.D., 2012. Redox regulation in photosynthetic organisms: focus on glutathionylation. Antioxidants Redox Signal. 16, 567–586. https://doi.org/10.1089/ars.2011.4255.

Zaffagnini, M., De Mia, M., Morisse, S., Di Giacinto, N., Marchand, C.H., Maes, A., Lemaire, S.D., Trost, P., 2016. Protein S-nitrosylation in photosynthetic organisms: a comprehensive overview with future perspectives. Biochim. Biophys. Acta 1864, 952–966. https://doi.org/10.1016/j.bbapap.2016.02.006.