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Drought stress during the reproductive stage of two soybean lines

Abstract – The objective of this work was to evaluate the effects of drought stress in the reproductive stage (R3) on the physiological parameters and grain yield of two soybean (*Glycine max*) lines. The Vx-08-10819 and Vx-08-11614 soybean lines were grown in a greenhouse, where they were irrigated until they reached the R3 development stage. During three days, the weight of the pots was monitored daily in order to maintain 100, 60, and 40% field capacity (control and moderate and severe stress, respectively). The parameters gas exchange and chlorophyll *a* fluorescence, as well as chloroplast pigments, osmoregulatory solutes and antioxidant enzymes, were determined. After stress, the plants were rehydrated until the end of the reproductive stage (R8), to evaluate grain yield. Vx-08-10819 showed traits that contributed to drought tolerance, such as better water-use efficiency, modulation of leaf area, and enzymatic activity, as well as a more efficient photosynthetic apparatus and a lower lipid peroxidation rate than Vx-08-11614. In addition, Vx-08-10819 maintained its productivity even after the severe water deficit. By contrast, water limitations affected negatively the productivity of Vx-08-11614. The Vx-08-10819 soybean line can efficiently withstand drought periods during the reproductive stage, without any interferences on final grain yield.

Index terms: *Glycine max*, oxidative stress, photosynthesis, productivity, proline, water deficit.

Vanessa do Rosário Rosa[1], Adinan Alves da Silva[1], Danielle Santos Brito[2], José Domingos Pereira Júnior[2], Cíntia Oliveira Silva[1], Maximiller Dal-Bianco[3], Juraci Alves de Oliveira[2] and Cleberson Ribeiro[2]✉

[1] Universidade Federal de Viçosa, Departamento de Biologia Vegetal, Avenida Peter Henry Rolfs, s/nº, Campus Universitário, CEP 36570-900 Viçosa, MG, Brazil. E-mail: vanessadorosariorosa@gmail.com, adinan.alves64@gmail.com, oliveirascintia11@gmail.com

[2] Universidade Federal de Viçosa, Departamento de Biologia Geral, Avenida Peter Henry Rolfs, s/nº, Campus Universitário, CEP 36570-900 Viçosa, MG, Brazil. E-mail: sbritodanielle@gmail.com, josedoming@gmail.com, jalves@ufv.br, cleberson.ribeiro@ufv.br

[3] Universidade Federal de Viçosa, Departamento de Bioquímica e Biologia Molecular, Avenida Peter Henry Rolfs, s/nº, Campus Universitário, CEP 36570-900 Viçosa, MG, Brazil. E-mail: maximiller@gmail.com

✉ Corresponding author

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Estresse hídrico durante a fase reprodutiva de duas linhagens de soja

Resumo – O objetivo deste trabalho foi avaliar os efeitos do estresse hídrico no estádio reprodutivo (R3) sobre os parâmetros fisiológicos e o rendimento de grãos de duas linhagens de soja (*Glycine max*). As linhagens de soja Vx-08-10819 e Vx-08-11614 foram cultivadas em casa de vegetação, onde foram irrigadas até atingirem o estágio de desenvolvimento R3. Durante três dias, o peso dos vasos foi monitorado diariamente para manter 100, 60 e 40% da capacidade de campo (controle e estresses moderado e severo, respectivamente). Foram determinados os parâmetros trocas gasosas e fluorescência da clorofila *a*, além dos pigmentos cloroplastídicos, solutos osmorregulatórios e enzimas antioxidantes. Após o estresse, as plantas foram reidratadas até o final do estádio reprodutivo (R8) para avaliar o rendimento de grãos. Vx-08-10819 apresentou características que contribuíram para a tolerância à seca, como melhor eficiência no uso da água, modulação da área foliar e atividade enzimática, bem como aparato fotossintético mais eficiente e menor peroxidação lipídica que Vx-08-11614. Além disso, Vx-08-10819 manteve sua produtividade mesmo após deficiência hídrica severa. Em contraste, as limitações hídricas afetaram negativamente a produtividade de Vx-08-11614. A linhagem de soja Vx-08-10819 pode suportar eficientemente períodos de seca durante o estádio reprodutivo, sem que haja interferência no rendimento final de grãos.

Termos para indexação: *Glycine max*, estresse oxidativo, fotossíntese, produtividade, prolina, deficit hídrico.

Introduction

Current efforts in soybean [*Glycine max* (L.) Merr.] breeding largely focus on identifying genotypes with a high seed yield and drought tolerance (Nakagawa et al., 2018; Fried et al., 2019). Water availability is important throughout soybean development, and the need for water increases as the plant grows, reaching maximum demand at the reproductive stage (R1–R2, bloom; R3–R4, pod; and R5–R6, seed), which is the most drought sensitive (Nakagawa et al., 2018). Therefore, water scarcity during the soybean reproductive stage may induce changes in the plant metabolism, resulting in a smaller number and size of pods and seeds, lower seed weight, leaf senescence, and shortened plant life cycle, which may reduce productivity (Manavalan et al., 2009; Farooq et al., 2017).

In addition, the reduction in CO_2 availability and the low biochemical activity induced by water deficit may lead to the formation of reactive oxygen species (ROS), whose homeostasis is controlled by non-enzymatic and enzymatic antioxidant mechanisms (Pandey et al., 2016; Choudhury et al., 2017). The maintenance of a high antioxidant capacity for ROS elimination has been associated with increased plant tolerance to a variety of environmental stresses (Das & Roychoudhury, 2014). Therefore, drought-tolerant plants maintain cell homeostasis under stress condition, differently from sensitive plants that show several dysregulated pathways (Lima et al., 2019). Furthermore, under water deficit, plants may accumulate osmoregulatory molecules, such as proline and amino acids, which decrease cell osmotic potential, preserving water absorption and cell turgor pressure (Fang & Xiong, 2015). This mechanism allows drought-tolerant plants to maintain a higher photosynthetic rate, stomatal opening, and cell expansion under this type of stress.

Therefore, plants respond to water stress by integrating several mechanisms at the physiological and biochemical levels (Fang & Xiong, 2015). Understanding how these mechanisms act to maintain productivity may help to identify and select genotypes that are more productive under water deficit conditions, which is an important tool to meet the growing demand for food worldwide.

The objective of this work was to evaluate the effects of drought stress in the reproductive stage (R3) on the physiological parameters and grain yield of two soybean lines.

Materials and Methods

Thirteen soybean cultivars and lines – Embrapa 48, Vx-08-11644, Vx-08-10819, 11646, M-9350, Vx-08-770, 13241, MG/BR46 (Conquista), Vx-08-11614, 11540, M-9144, 9397, and 11377 – were grown in a greenhouse and then classified regarding productivity after water deficit. For the present study, the Vx-08-10819 and Vx-08-11614 lines were selected for presenting a productivity that was maintained and reduced, respectively, after water deficit.

The experiments were conducted in a greenhouse covered with transparent polyethylene and protected on the sides with 50% shading. During the experiment, the average temperature and humidity of the greenhouse was 27°C and 67%, respectively. Seeds of the Vx-08-10819 and Vx-08-11614 lines were germinated in Bioplant substrate (Bioplant Agrícola Ltda., Nova Ponte, MG, Brazil). Four seedlings were grown per 9-kg pot in a mixture of soil and sand (2:1), which was fertilized with 10 g of the 4% NO_3, 14% P_2O_5, and 8% K_2O. All the pots were weighed to maintain field capacity at 100%. Irrigation was interrupted when the plants reached the R3 growth stage of pod development, except in the control treatment. Then, during three days, the weight of the pots was monitored daily to maintain 60 and 40% field capacity (moderate and severe stress, respectively); the control was kept at 100% field capacity. Afterwards, three plants per pot were analyzed and leaf samples were harvested. The fourth plant was watered and kept with 100% field capacity until reaching the R8 growth stage of complete maturation, in order to evaluate yield.

Leaf water potential was evaluated on fully-expanded leaves in the morning, using the Model 1000 pressure pump (PMS Instruments, Albany, OR, USA). Six leaf discs with a 6-mm diameter were collected from fully-expanded leaves, weighed, and placed in water for saturation, in order to analyze the relative water content (RWC), which was obtained by the formula: Leaf RWC (%) = ((FW-DW) / (TW-DW)) × 100, where FW is fresh weight, TW is turgid weight, and DW is dry weight. The hydrated discs were then weighed again and dried to determine dry weight (Turner, 1981). After exposure to water deficit, total leaf area and specific total leaf area were evaluated using the LI-3100C area meter (LI-COR Biosciences, Lincoln, NE, USA).

Gas exchange parameters were determined on fully-expanded leaves at the same time that chlorophyll *a* fluorescence was measured using an open-flow infrared gas exchange analyzer system equipped with the LI-6400-40 leaf chamber fluorometer (LI-COR Biosciences, Lincoln, NE, USA). The net CO_2 assimilation rate (*A*), stomatal conductance (g_s), internal CO_2 concentration (Ci), and transpiration rate (*E*) were measured from 8:00 to 10:00 a.m. (solar time), when *A* was at its maximum, under artificial photosynthetically active radiation (i.e., 1,000 µmol photons m^{-2} s^{-1} at the leaf level and 400 µmol CO_2 mol^{-1} air), at 25°C, with vapor pressure deficit maintained at ≈1.0 kPa and amount of blue light set to 10% of the photosynthetic photon flux density to optimize stomatal aperture. The instantaneous water-use efficiency and carboxylation efficiency were calculated as *A*/*E* and *A*/Ci ratios, respectively. The slow phase of chlorophyll *a* fluorescence induction, fluorescence of a light-adapted sample measured briefly before the application of a saturation pulse (F), and maximum fluorescence of a light-adapted sample ($F_{m'}$) were obtained sequentially by applying a saturation pulse of actinic light (>3,000 µmol photons m^{-2} s^{-1}). The minimal fluorescence parameters of the illuminated plant tissue ($F_{0'}$) and the fraction of open photosystem II centers (qP) were calculated from F and $F_{m'}$. The effective quantum yield of photosystem II photochemistry (ΦPSII) was used to estimate the apparent electron transport rate (ETR). The photochemical quenching coefficient was calculated as $qP = (F_{m'} - F_s)/(F_{m'} - F_{0'})$, and the non-photochemical (NPQ) one as $NPQ = (F_m - F_{m'})/F_{m'}$.

Six-millimeter diameter leaf discs from fully-expanded leaves were immersed in dimethyl sulfoxide saturated with calcium carbonate. Then, the absorbances of the samples were evaluated at 665.1, 649.1, and 480 nm to calculate the concentrations of chlorophyll *a* and *b* and of carotenoids (Wellburn, 1994).

Aliquots of 100 mg frozen leaf material were homogenized in 3% sulfosalicylic acid and centrifuged at 6,300 *g* for 10 min. Proline was quantified following the protocol described by Bates et al. (1973). For amino acids, 10 mg frozen leaf material were homogenized in 0.2 mol L^{-1} sodium citrate buffer (pH 5.0), 5% ninhydrin, 0.002 mol L^{-1} potassium cyanide, and 60% (v/v) ethanol. Amino acids were analyzed at the wavelength of 570 nm, following the protocol described by Yemm & Cocking (1955).

Lipid peroxidation was estimated via the analysis of malondialdehyde accumulation (Cakmak & Horst, 1991). Briefly, samples of 100 mg frozen leaf material were homogenized in 1% trichloroacetic acid and centrifuged at 12,000 *g* for 15 min, at 4°C. Afterwards, they were added to a supernatant with 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid and then incubated in a water bath at 95°C. The reaction was stopped and centrifuged at 10,000 *g*, and absorbance was determined at 532 and 600 nm. The concentration of the malondialdehyde-TBA complex was estimated using a molar absorptivity coefficient of 155 mmol L^{-1} cm^{-1}.

Samples of 100 mg fresh leaves were ground in liquid nitrogen and homogenized in 0.1 mol L^{-1} potassium phosphate buffer (pH 6.8), 0.1 mmol L^{-1} ethylenediaminetetraacetic acid, 1.0 mmol L^{-1} phenylmethylsulfonyl fluoride, and 1.0% (w/v) polyvinylpolypyrrolidone. The analysis of the enzymes superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), peroxidase (EC 1.11.1), and ascorbate peroxidase (EC 1.11.1.11) followed the protocol described by Ribeiro et al. (2012). The enzyme activities were estimated by changes in absorbance during the first minute of the reaction at 30ºC and expressed by the molar extinction coefficients of catalase (240 nm, ε: 36 mol L^{-1} cm^{-1}), peroxidase (420 nm, ε: 2.47 mmol L^{-1} cm^{-1}), and ascorbate peroxidase (290 nm, ε: 2.8 mmol L^{-1} cm^{-1}). The protein content of the enzyme extracts was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Agronomic performance – including number of pods with one, two, or three grains; number of grains; number of pods per plant; and final yield – was evaluated at the end of the R8 stage for each cultivar after the control and moderate and severe water stress treatments; the experimental unit consisted of one plant per pot in five replicates.

The experiments were conducted in a completely randomized design in a 2x3 factorial arrangement, with five replicates of each cultivar. Data were statistically examined by the analysis of variance, and means were compared by Tukey's test, at 5% probability, using the AgroEstat 1.0 software (Barbosa & Maldonado Júnior, 2015).

Results and Discussion

The Vx-08-10819 and Vx-08-11614 soybean lines showed distinct physiological responses. Vx-08-10819 was the only one that maintained productivity after moderate and severe water deficiency, i.e., at 60 and 40% field capacity, respectively, in the reproductive stage. In comparison, the agronomic yield of JS 97-52, EC 538828, 'BR 16', and 'Embrapa 48' was reduced after water deficit during the vegetative stage and, particularly, the reproductive stage (Jumrani & Bhatia, 2018; Lima et al., 2019). Therefore, the water stress induced at the reproductive stage is more detrimental to agronomic performance than that at the vegetative stage. Jumrani & Bhatia (2018) found that, due to their plasticity and consequent recovery, the plants subjected to water stress at the vegetative stage showed a less affected seed yield; however, at the reproductive stage, there was no plasticity for plants to recover, resulting in a severe reduction in yield.

The stomatal closure observed for Vx-08-10819 contributed to maintaining the plant's water potential (Figure 1 A and B). Although the results of g_s and E were the same for this line and for Vx-08-11614 (Figure 2 B and C), the former might better adapt to stress conditions by reducing its total leaf area and specific total leaf area (Figure 1 C and D). This reduction decreases the leaf surface responsible for water loss, preserving leaf hydration (DaMatta & Ramalho, 2006).

The CO_2 assimilation rate was affected negatively by water deficit, especially in the Vx-08-11614 line (Figure 2 A). According to Manavalan et al. (2009), the reduction in g_s may limit the entry of CO_2 into chloroplasts, reducing the photosynthetic rate and, consequently, contributing to a lower biomass accumulation. The reduction in g_s has been considered a limiting factor for the photosynthetic process,

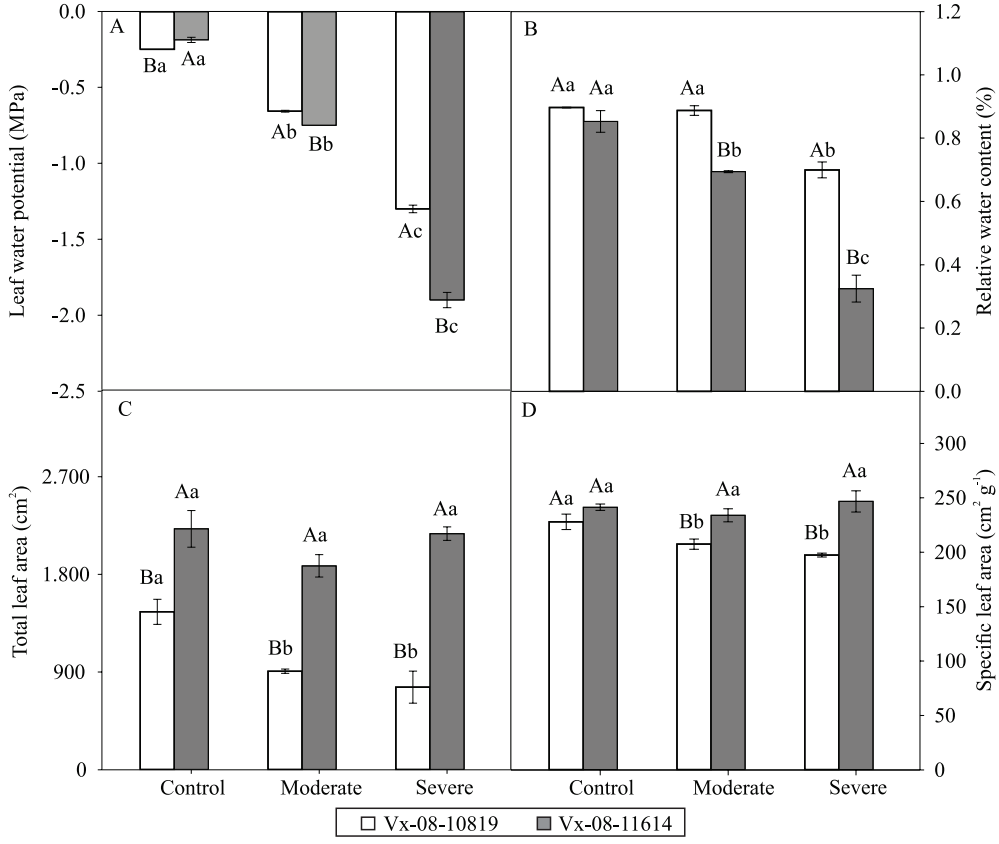

Figure 1. Leaf water potential (A), relative water content (B), leaf area (C), and specific leaf area (D) of the Vx-08-10819 and Vx-08-11614 soybean (*Glycine max*) lines at the reproductive stage after moderate (60% field capacity) and severe (40% field capacity) water deficit. The control treatment was maintained at 100% field capacity. Means followed by equal letters, uppercase between the lines for the same treatment and lowercase among treatments for the same line, do not differ by Tukey's test, at 5% probability. Bars represent standard deviation (n = 5).

mainly at the beginning of water deficit (Dias et al., 2018). However, under more severe water limitations, reductions in photosynthetic rates may occur due to the inhibition of metabolic processes, causing a lower efficiency in the use of energy by the plant's photochemical apparatus (Manavalan et al., 2009). Therefore, the reduction in ΦPSII (Figure 3 A) and the increase in regulated non-photochemical energy loss [Y(NPQ)] and in non-regulated non-photochemical energy loss [Y(NO)] (Figure 3 C and D) in the Vx-08-11614 line under moderate and severe water deficit suggest a decrease in the electron transport rate (Figure 3 B), leading to a lower energy conversion efficiency (Dias et al., 2018).

Although the rate of CO_2 assimilation also decreased under severe water deficit in the Vx-08-10819 line, it was still higher than that of Vx-08-11614 (Figure 2 A). In addition, ΦPSII, ETR, and qP (Figure 3 A, B, and E) were also higher for Vx-08-10819. These results confirm that the Vx-08-10819 line presents adaptation mechanisms to preserve the functionality of its photosynthetic apparatus in dry periods. This may be proved by the increase in carboxylation efficiency, instantaneous water use efficiency (Figure 2 E and F), and reduction in total leaf area (Figure 1 C and D), which improve plant water status and turgor maintenance (Kufa & Burkhardt, 2011). Moreover, a higher dissipation of the Y(NPQ) (Figure 3 C) of the

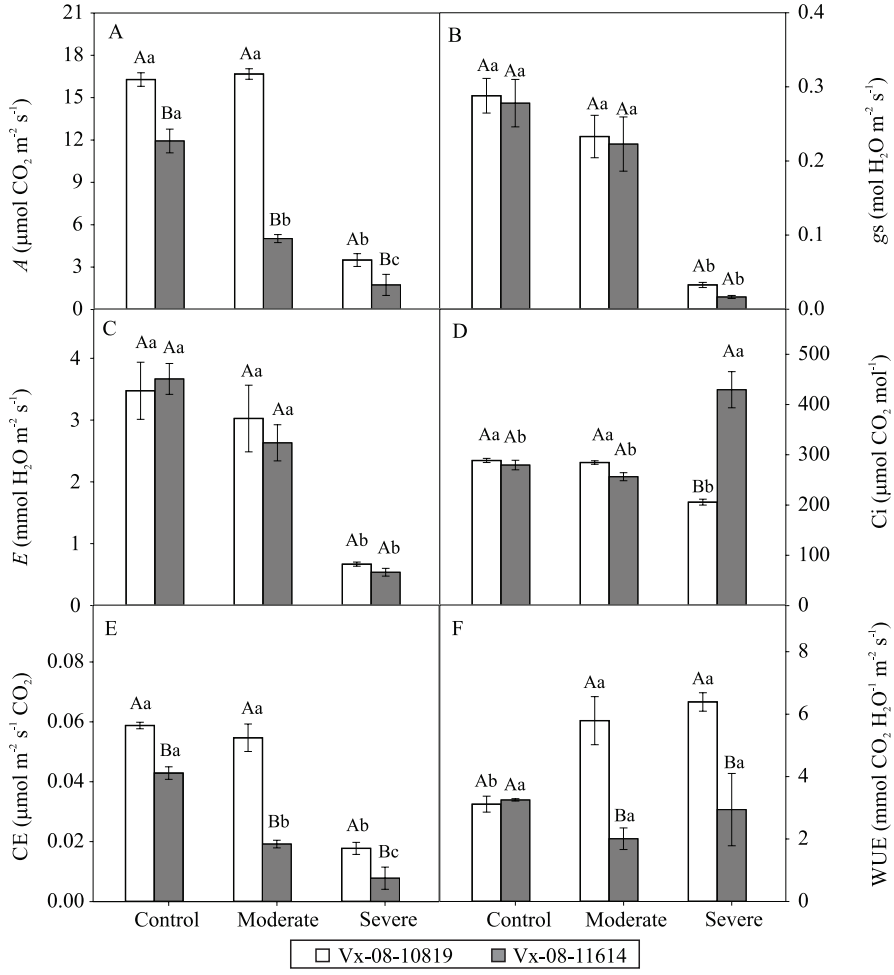

Figure 2. CO_2 net assimilation rate – *A* (A), stomatal conductance – *gs* (B), transpiration rate – *E* (C), internal CO_2 concentration – *C*i (D), carboxylation efficiency – CE (E), and instantaneous water use efficiency – WUE (F) of the Vx-08-10819 and Vx-08-11614 soybean (*Glycine max*) lines at the reproductive stage after moderate (60% field capacity) and severe (40% field capacity) water deficit. The control treatment was maintained at 100% field capacity. Means followed by equal letters, uppercase between the lines for the same treatment and lowercase among treatments for the same line, do not differ by Tukey's test, at 5% probability. Bars represent standard deviation (n = 5).

Vx-08-10819 line after severe water deficit indicates a greater efficiency in its protective mechanism. In general, the results of fluorescence variables show that the Vx-08-10819 line has a more efficient photosynthetic apparatus than Vx-08-11614 to tolerate photoinhibition conditions resulting from a low soil water availability (Rivas et al., 2016).

The reduction in the photosynthetic rate of the Vx-08-11614 line is also attributed to its lower contents of chlorophyll *a* and *b* and of carotenoids (Figure 4 A–C). The lower chlorophyll content of this line shows that it has a reduced efficiency in light absorption and transfer of radiant energy to reaction centers. In addition, the lower content of carotenoids reveals a low photoprotective capacity of the photochemical processes, since these antioxidants scavenge ROS to inhibit oxidative damage and to protect the photosynthetic apparatus (Luo et al., 2016).

High concentrations of total amino acids and proline were found in the leaf tissues of the Vx-08-11614 line after the water deficit treatments (Figure 4 D and E). In general, plants may tolerate water deficit through the accumulation of these solutes in cells that contribute to the maintenance of cell turgor, stomatal conductance, photosynthesis, and growth (Singh et al., 2015; Basu et al., 2016). However, the accumulation of total amino

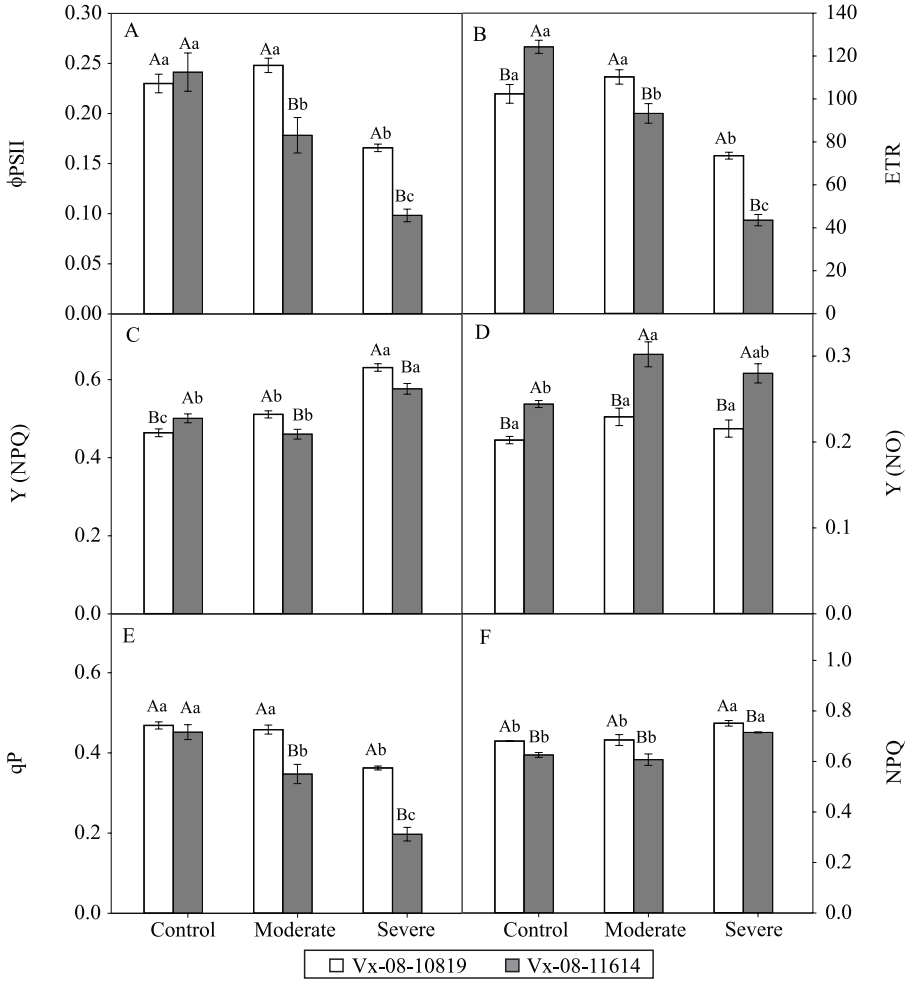

Figure 3. Effective quantum yield of photosystem II photochemistry – ΦPSII (A), electron transport rate – ETR (B), quantum yield of regulated non-photochemical energy loss – Y(NPQ) (C), quantum yield of non-regulated non-photochemical energy loss – Y(NO) (D), photochemical quenching coefficient – qP (E), and non-photochemical quenching coefficient – NPQ (F) of the Vx-08-10819 and Vx-08-11614 soybean (*Glycine max*) lines at the reproductive stage after moderate (60% field capacity) and severe (40% field capacity) water deficit. The control treatment was maintained at 100% field capacity. Means followed by equal letters, uppercase between the lines for the same treatment and lowercase among treatments for the same line, do not differ by Tukey's test, at 5% probability. Bars represent standard deviation (n = 5).

acids and proline was not able to maintain the water status of this line (Figure 1 A and B). A possible explanation is that, since the anabolism of these osmoregulatory molecules has a high energy cost (Kaur & Asthir, 2015), the Vx-08-11614 line may have directed the energy flow along this route; however, these effects were not noticeable. Additionally, the high accumulation of amino acids in this line under drought may be due to failures in protein synthesis and degradation (Khoyerdi et al., 2016), reflecting the damage induced by drought in the plant's metabolism.

The inefficient photosynthetic mechanism of the Vx-08-11614 line (Figure 2 A) and the increase in internal carbon concentration (Figure 2 D) and non-photochemical energy loss (Figure 3 D) after water deficit are favorable conditions for ROS formation and accumulation (Hazrati et al., 2016). However, stresses such as drought can activate the enzymatic antioxidant system, inducing protection against oxidative stress (Pandey et al., 2016; Choudhury et al., 2017). This system consists mainly of the enzymes superoxide dismutase, catalase, ascorbate peroxidase, and peroxidase (Tian et al., 2016). The activities of superoxide dismutase and catalase were lower in the Vx-08-11614 line after water stress (Figure 5 A and B). According to Das & Roychoudhury (2014), these enzymes are the main

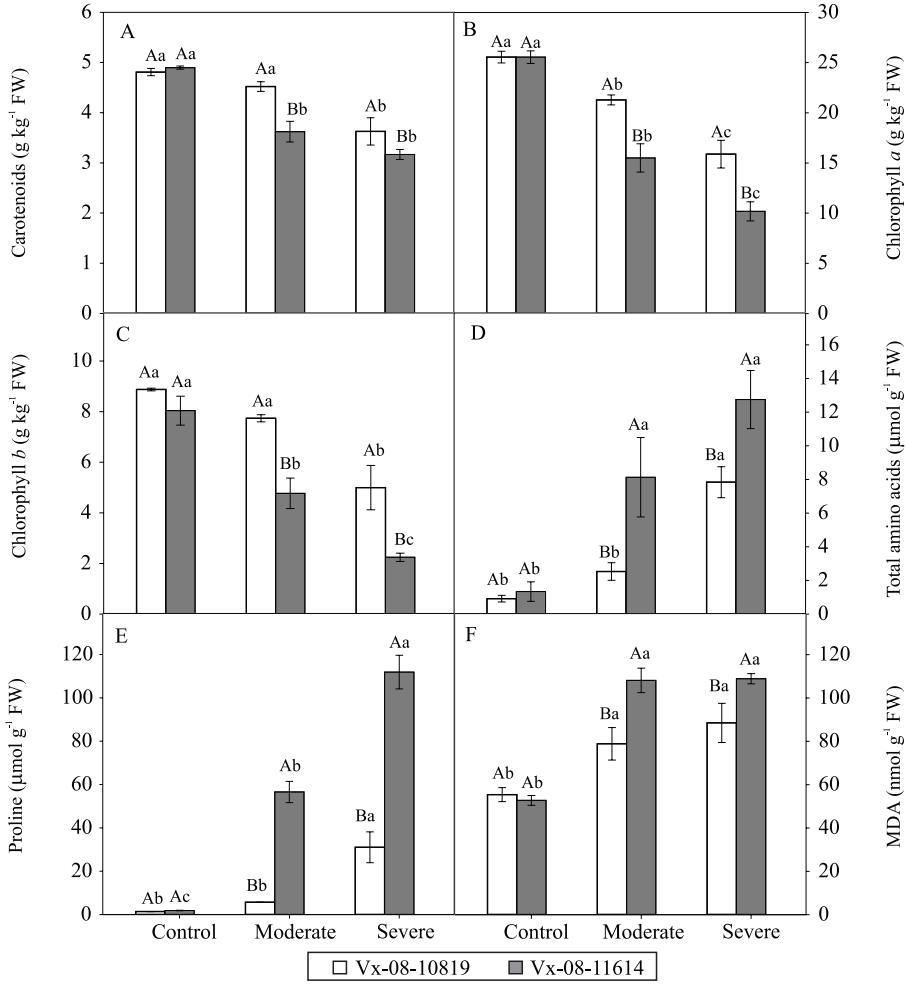

Figure 4. Contents of carotenoids (A), chlorophyll *a* (B), chlorophyll *b* (C), total amino acids (D), proline (E), and lipid peroxidation by MDA analysis (F) of the Vx-08-10819 and Vx-08-11614 soybean (*Glycine max*) lines at the reproductive stage after moderate (60% field capacity) and severe (40% field capacity) water deficit. The control treatment was maintained at 100% field capacity. Means followed by equal letters, uppercase between the lines for the same treatment and lowercase among treatments for the same line, do not differ by Tukey's test, at 5% probability. Bars represent standard deviation (n = 5). MDA, malondialdehyde.

scavengers of the superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), respectively. Therefore, the low activity of these enzymes may allow oxidative damage to membrane components and induce lipid peroxidation, as observed in the Vx-08-11614 line after the water deficit treatments (Figure 4 F).

The Vx-08-11614 line had a higher peroxidase activity in the control and moderate treatments (Figure 5 C). However, Vx-08-10819 showed a higher activity of the catalase enzyme in the moderate and severe treatments (Figure 5 B). The peroxidase and catalase enzymes have different affinities for H_2O_2. Catalase removes high concentrations of H_2O_2, while peroxidase enzymes are responsible for the fine modulation of the levels of this ROS (Mittler, 2002). In the present study, catalase activity reached 40 µmol H_2O_2 min^{-1} mg^{-1} protein in the Vx-08-10819 line (Figure 5 B), whereas peroxidase activity reached only 12 µmol H_2O_2 min^{-1} mg^{-1} protein in Vx-08-11614 (Figure 5 C). Therefore, catalase acts as a key enzyme, effectively eliminating high levels of H_2O_2 and reducing its oxidative damage (Ghanati et al., 2005), which, consequently, contributed to reduce the rate of lipid peroxidation in the Vx-08-10819 line (Figure 4 F).

Taken together, the obtained results suggest that the Vx-08-10819 line has a better metabolic and physiological fitness than Vx-08-11614 after exposure to moderate and severe water deficit, which led to a better agronomic performance at the end of the reproductive stage (R8). This better performance can be confirmed by the line's higher number of grains per plant, total number of grains, total number of pods, and number of pods with three grains (Figure 6 A–C and F). Therefore, the Vx-08-10819 line can

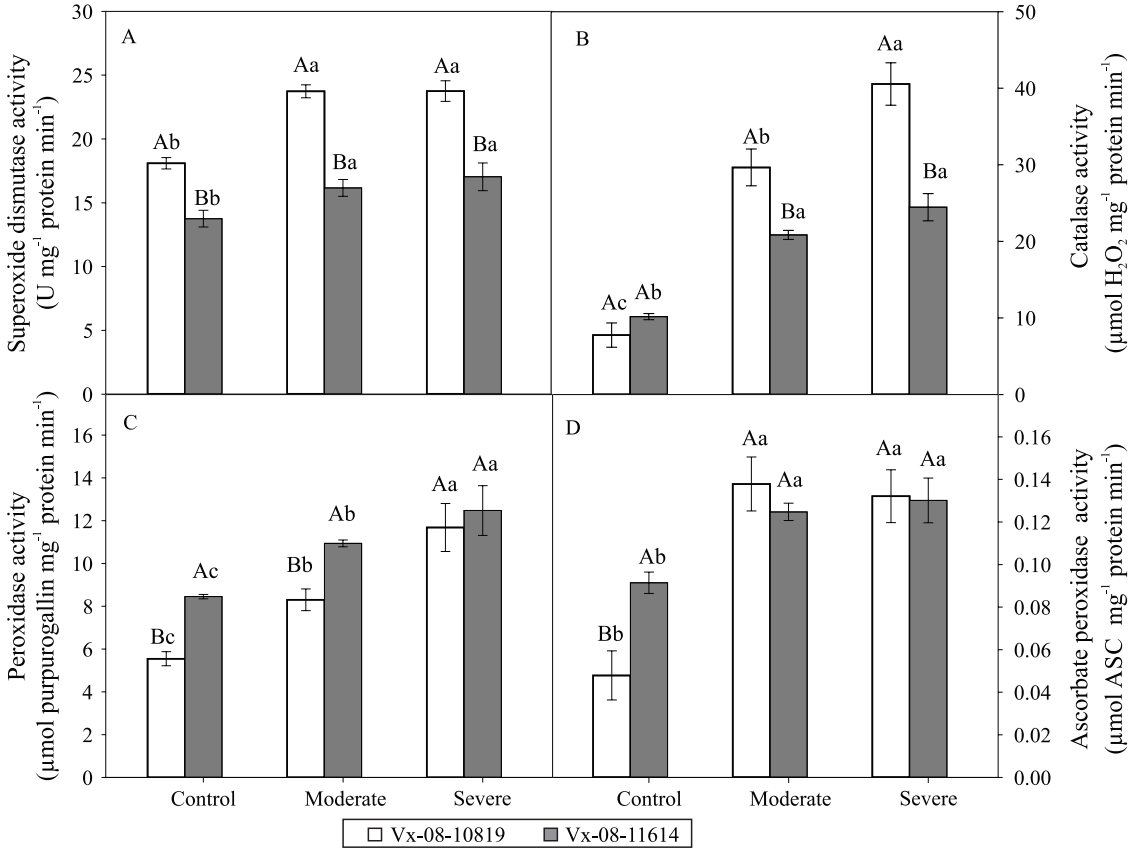

Figure 5. Activities of the antioxidant enzymes superoxide dismutase (A), catalase (B), peroxidase (C), and ascorbate peroxidase (D) in the Vx-08-10819 and Vx-08-11614 soybean (*Glycine max*) lines at the reproductive stage after moderate (60% field capacity) and severe (40% field capacity) water deficit. The control treatment was maintained at 100% field capacity. Means followed by equal letters, uppercase between the lines for the same treatment and lowercase among treatments for the same line, do not differ by Tukey's test, at 5% probability. Bars represent standard deviation (n = 5). ASC, ascorbic acid.

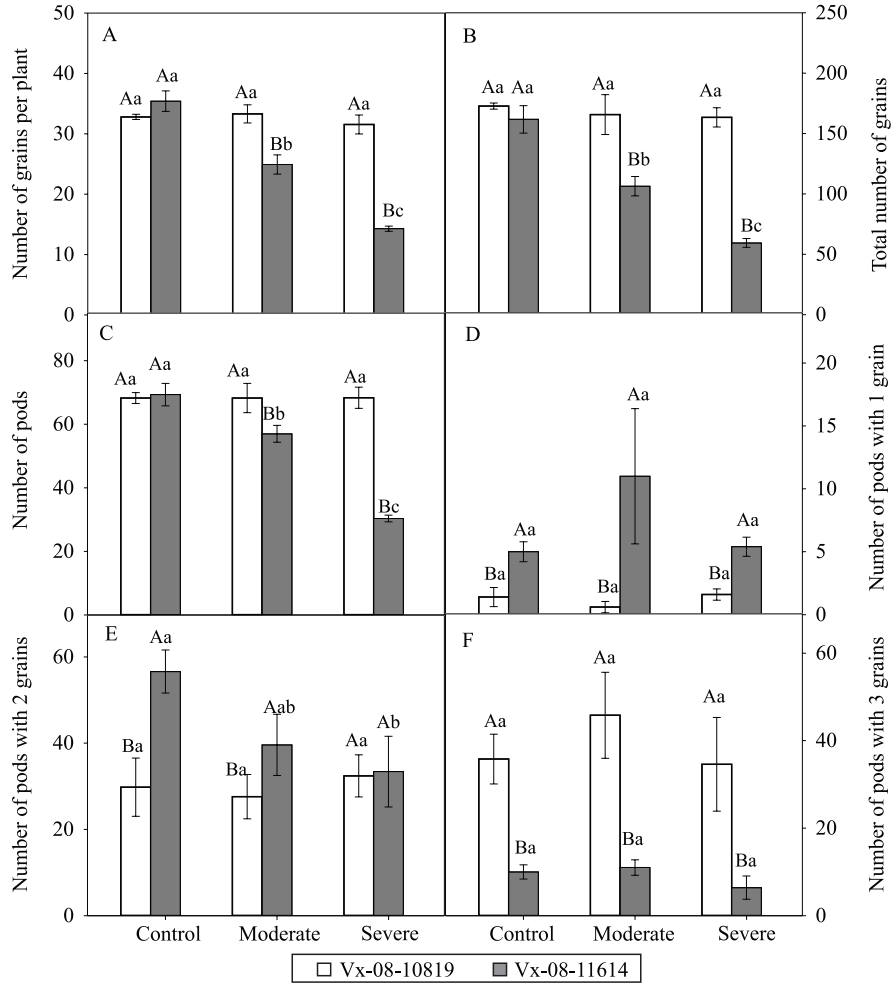

Figure 6. Grain yield per plant (A), total number of grains (B), total number of pods (C), and number of pods with one (D), two (E), or three grains (F) of the Vx-08-10819 and Vx-08-11614 soybean (*Glycine max*) lines at the reproductive stage after moderate (60% field capacity) and severe (40% field capacity) water deficit. The control treatment was maintained at 100% field capacity. Means followed by equal letters, uppercase between the lines for the same treatment and lowercase among treatments for the same line, do not differ by Tukey's test, at 5% probability. Bars represent standard deviation (n = 5).

more efficiently withstand drought periods during the reproductive stage, with no interference on final productivity, being considered more tolerant to water stress than Vx-08-11614.

Conclusions

1. The Vx-08-10819 soybean (*Glycine max*) line shows traits that contribute to drought tolerance, such as a better water-use efficiency, modulation of leaf area, more efficient photosynthetic apparatus, efficient enzymatic activity, and lower lipid peroxidation rate, compared with Vx-08-11614.

2. The Vx-08-10819 line can more efficiently withstand drought periods during the reproductive stage, without any negative effects on final productivity, and, therefore, can be considered more tolerant to drought than Vx-08-11614.

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