Ameliorating Drought Stress Effects on Soybean Physiology and Yield by Hydrogen Peroxide

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Summary

Soybean is considered as an important legume because of its high content of protein and oil. However, it is sensitive to drought stress that was increasingly recorded recently. Plants respond to drought by several changes on the physiological and molecular levels and by endogenously changing the concentrations of certain substances, sauch as reactive oxygen species (ROS). An experiment was conducted in 2018 and 2019 to investigate the effects of drought stress and the possible effects of exogenously sprayed hydrogen peroxide (H_2O_2) at R1 stage on two soybean cultivars. Significant decreases in all studied traits were recorded as a result of drought stress. Noticeable enhancements in stomatal conductance, relative chlorophyll content, relative water content, leaf area index, plant height and the final seed yield of both cultivars when treated with H_2O_2 were also recorded. It could be concluded that H_2O_2 spraying can be a good strategy to alleviate presumable negative influence of drought.

Key words

g_s, height, LAI, RWC, SPAD, yield

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Introduction

Among seed legumes, soybean (*Glycine max* (L.) Merrill) has the greatest global area harvested representing the main source of relatively-cheap protein and vegetable oil (Mutava et al., 2015). Soybean is highly affected by many abiotic stresses, majorly drought stress (Fan et al., 2013) that intensively increased over the past decades, affecting the world's food security (Vurukonda et al., 2016). Soybean is considered highly-sensitive to drought stress compared to other crops (Maleki et al., 2013) especially during certain periods of plant lifecycle (Liu et al., 2004). Drought stress decreases stomatal conductance, biomass, grain yield and its components (Ruppenthal et al., 2016). Drought also affects chlorophyll pigments and photosynthetic electron transport system. As such, production of reactive oxygen species (ROS) is induced (Zgallai et al., 2005) in higher concentrations (Shigeoka et al., 2002) resulting in cellular damage as a result of gene alteration, protein degradation and enzyme inactivation (Mahajan and Tuteja, 2005). High concentrations of ROS cause damages to the cells, yet low concentrations play the role of signaling molecules that can ease several processes like germination and growth (Dowling and Simmons, 2009). For example, it was reported that ROS play noticeable role in regulating stomatal closure in order to optimize water use efficiency (Wang and Song, 2008; Huang et al., 2009). Hydrogen peroxide (H_2O_2) is a compound that belongs to non-radical ROS (Matilla-Vázquez and Matilla, 2012). It regulates many physiological mechanisms, such as growth and development, under both normal and stressed conditions, playing a major role in activating various signal molecules in plants leading to induction of different mechanisms of tolerance (Wendehenne et al., 2004; Bright et al., 2006; Foyer and Noctor, 2009). Many reports have demonstrated that treating plants with suitable concentrations of H_2O_2 increases tolerance to abiotic stresses (Chao et al., 2009; He et al., 2009; Xu et al., 2010; Gondim et al., 2013; Hossain and Fujita, 2013). It was previously reported that treating seeds before sowing with H_2O_2 or applying it as a foliar spray can enhance abiotic stress tolerance in plants. For example, a stimulation in the germination was reported when seeds of *Pseudotsuga menziesii, Sorghum nutum, Andropogon gerardii, Panicum virgatum* (Sarath et al., 2007) and *Zinnia elegans* (Ogawa and Iwabuchi, 2001) were pre-treated with H_2O_2. Similarly, enhanced germination rates were recorded in maize seeds pre-soaked in 140 mM of H_2O_2 (Ashraf et al., 2015). Jubany-Marí et al. (2009) reported that H_2O_2 is involved in the acclimation of *Cistus albidus* to summer drought. Ishibashi et al. (2011) concluded that spraying soybean plants with H_2O_2 resulted in better net photosynthesis (P_n) and that this application made the plants more tolerant to drought stress. Similar conclusion was also reported for melon plants (Ozaki et al., 2009) and for cucumber seedlings (Sun et al., 2016). Liu et al. (2010) reported improved osmotic stress resistance when two cucumber varieties were pre-treated with H_2O_2 as a result of the activation of antioxidant system. It was suggested that the mechanism by which plants exogenously-sprayed with H_2O_2 better tolerate stress could be by ROS-detoxification modification or by regulation of multi-pathways that respond to stress (Terzi et al., 2014; Hossain et al., 2015). Other authors reported that H_2O_2 alleviated the negative stress effects by either a regulated stomatal closure (Kolla et al., 2007; Quan et al., 2008; Wang and Song, 2008) or by promoting the biosynthesis of the oligosaccharide and, accordingly, maintaining the leaf water content (Ishibashi et al., 2011). Many efforts were made to exploit this characteristic in enhancing stress tolerance in plants (Jubany-Marí et al., 2009; Liu et al., 2010).

Although some experiments were conducted to investigate the effects of early exogenous H2O2 spraying on some physiological and molecular traits of some plant species, yet, to our knowledge, no report was introduced on the influence of the application of H2O2 at reproductive stages on soybean physiology and yield, especially in the study area where the soybean production is newly introduced. Therefore, our study aimed at revealing the probable positive effects of exogenously spraying H_2O_2 at R1 stage on both the physiology and the seed yield of two soybean cultivars.

Materials and Methods

Two soybean cultivars, 'Pannonia Kincse' and 'Boglár', were sown in Debrecen University's experimental site (Látókép) (N. latitude 47° 33', E. longitude 21° 27') on April 23rd and 26th and were harvested on September 15th and 16th in 2018 and 2019, respectively. The soil type is calcareous chernozem. The precipitation and the average temperature during the vegetative period of the soybean plants are presented in Figure 1. The experimental design was split-plot with cultivars representing the main plots and irrigation treatments being the sub-plots. Three treatments were applied in three replications: fully-irrigated (FI) treatment, where irrigation was conveniently applied (100 mm in total) to reach water demands as recommended by the experimental site's management (taking into account the precipitation amounts which were recorded as 271.2 mm and 275.9 mm in 2018 and 2019, respectively); drought-stressed treatment (counting only on precipitation) with the application of 1mM of hydrogen peroxide (H_2O_2) as a foliar spray at R1 stage (Fehr and Caviness, 1977) (HP) and drought-stressed treatment with distilled water foliar spray at R1 stage (DW). Hydrogen peroxide and DW treatments received 1.5 liters of H_2O_2 and distilled water per plot, respectively, ensuring that the whole plant parts were completely covered by the sprayed liquid. The final number of plots was 18 (2 cultivars * 3 treatments * 3 replications), with a plot area of 9 m² (3 * 3 m). Each plot consisted of six rows.

The relative chlorophyll content (SPAD) was measured using SPAD-502Plus (Konica Minolta, Japan).

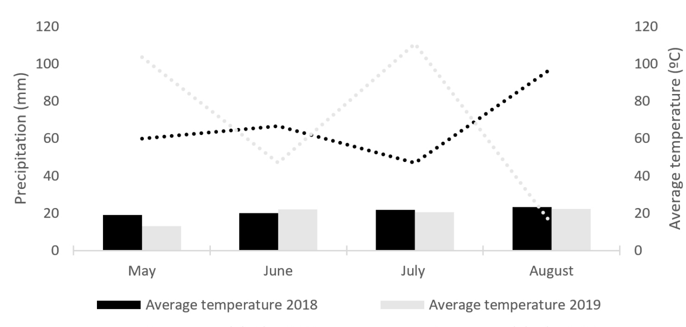

Figure 1. Precipitation (mm) and average temperature (°C) during the vegetative period of the soybean plants in 2018 and 2019

The stomatal conductance (g_s) was measured using AP4 porometer (Delta- T Devices, UK). Leaf area index (LAI) values were recorded using SS1 – SunScan canopy analysis system (Delta-

T Devices, UK). Plant height was measured using a standard ruler. For SPAD, LAI and gs measurements, 10 randomly-selected plants from the middle rows of each plot were used for the mentioned traits, and three measurements from the second most developed trifoliate (one measurement for each leaflet) were taken and then averaged.

To calculate the relative water content (RWC), ten fully-matured leaves (homogenous in size) were collected at 6.30 am and fresh weight (FW) of the leaf was measured immediately. Dry weight (DW) was determined via drying the sample at 80°C to constant weight (after 48 h), and turgid weight (TW) was obtained after floating the leaf in distilled water in a tray at 4°C for 48 h. RWC was calculated as $RWC(\%) = (FW - DW)/(TW - DW) \times 100\%$ (Weatherley, 1950). All traits were measured at R2 stage (1 - 2 weeks after H_2O_2 application).

Yield was estimated by manually harvesting the middle two rows of every plot at full maturity (R8) stage. The analysis of variance (ANOVA) was conducted to compare the means and to indicate the effect size of each treatment, and then Tukey post-hoc test was conducted to indicate the statistically-different means (IBM SPSS ver.25, USA Software).

Results

Stomatal Conductance (g_s)

In both cultivars, g_s was significantly higher when irrigation (FI) was applied. However, H_2O_2-sprayed plots were significantly higher than drought-stressed counterparts in terms of g_s value. Drought application reduced g_s by 51.6 and 47.7% compared to irrigated counterparts, whereas H_2O_2 spraying decreased the reduction ratio to 21.6 and 19.5% in 'Boglár' and 'Pannonia Kincse', respectively (Table 1). Correlation between g_s and irrigation treatment was highly significant (< 0.01) (Table 3), and the effect size of H_2O_2 application (calculated as partial Eta squared) was 81.6 and 90.5% in 'Boglár' and 'Pannonia Kincse', respectively. In other words, H_2O_2 application was responsible for 81.6 and 90.5% of gs changes in 'Boglár' and 'Pannonia Kincse', respectively.

Relative Chlorophyll Content (SPAD)

In 'Boglár', drought significantly decreased SPAD value by 26.7% compared to the irrigated counterpart, whereas H_2O_2 spraying resulted in better SPAD than both FI and DW counterparts. In 'Pannonia Kincse', on the other hand, irrigation resulted in the highest SPAD value; it was significantly higher (by 22.4%) than drought-stressed counterpart. However, HP treatment enhanced this value (by 13.1%) compared to DW, without reaching the same level of FI treatment as in 'Boglár' (Table 1). The correlation with irrigation was significant (< 0.05) and highly significant (< 0.01) in 'Boglár' and 'Pannonia Kincse' cultivars, respectively (Table 3), and the effect size of H_2O_2 application was noticeably higher (85.7%) in 'Boglár' compared to 'Pannonia Kincse' (59.1%).

Relative Water Content (RWC)

In 'Boglár', drought significantly decreased RWC by 21.2% compared to the irrigated counterpart. However, H_2O_2-sprayed treatment significantly increased RWC (by 17.3%) compared to drought-stressed treatment and had very close value to FI treatment. In 'Pannonia Kincse', applying H_2O_2 significantly increased RWC by 29.2% compared to the drought-stressed treatment; however, irrigation treatment had significantly higher RWC (by 8.3 and 39.9%) compared to HP and DW treatments, respectively (Table 1).

Table 1. Stomatal conductance (g_s), relative chlorophyll content (SPAD) and relative water content (RWC) of soybean cultivars 'Boglár' and 'Pannonia Kincse' under three irrigation treatments; fully-irrigated (FI), drought-stressed with H_2O_2 foliar spray (HP) and drought-stressed (DW)

Trait	Treatment	Boglár	Pannonia Kincse
g_s (mmol m^{-2} s^{-1})	DW	190.0^c	218.3^c
	HP	307.7^b	336.3^b
	FI	392.7^a	417.7^a
SPAD	DW	26.4^b	33.6^b
	HP	37.2^a	38.0^b
	FI	36.0^a	43.3^a
RWC (%)	DW	57.333^b	61.713^c
	HP	69.331^a	79.707^b
	FI	72.701^a	86.302^a

In each trait, the same letter indicates no significant differences at $p \leq 0.05$ level among the treatments within the same cultivar

Application of H_2O_2 had a significant effect on RWC (by 95.9 and 97.3% in 'Boglár' and 'Pannonia Kincse', respectively) with a highly significant correlation coefficient (Table 3).

Leaf Area Index (LAI)

Both cultivars followed the same trend; LAI was significantly lower in DW treatment (by 13.0 and 17.5% in 'Boglár' and 'Pannonia Kincse', respectively) compared to FI counterpart. Hydrogen peroxide treatment resulted in the highest LAI in both cultivars; LAI was 21.3 and 28.8% higher compared to DW counterparts in 'Boglár' and 'Pannonia Kincse', respectively (Table 2).

Significant correlation was recorded between irrigation and LAI (Table 3). The effect of H_2O_2 application was also significant in both cultivars as well (with ratios of 92.8 and 95.1% in 'Boglár' and 'Pannonia Kincse', respectively).

Plant Height

Drought significantly reduced plant height in both cultivars compared to irrigated counterpart; the reduction ratio was 13.2 and 7.1% in 'Boglár' and 'Pannonia Kincse', respectively. Applying H_2O_2 significantly increased plant height in both cultivars; plant height was 3.6% less in 'Boglár', whereas it was only 0.2% less in 'Pannonia Kincse' compared to irrigated counterparts (Table 2). Application of H_2O_2 had an effect size of 84.2 and 82.8% in 'Boglár' and 'Pannonia Kincse', respectively.

Table 2. Leaf area index (LAI), plant height and yield of soybean cultivars 'Boglár' and 'Pannonia Kincse' under three irrigation treatments: fully-irrigated (FI), drought-stressed with H_2O_2 foliar spray (HP) and drought-stressed (DW)

Trait	Treatment	Boglár	Pannonia Kincse
LAI	DW	4.7^{b}	5.2^{b}
	HP	5.7^{a}	6.7^{a}
	FI	5.4^{a}	6.3^{a}
Plant Height (cm)	DW	86.9^{b}	94.1^{b}
	HP	96.5^{a}	101.1^{a}
	FI	100.1^{a}	101.3^{a}
Yield ($t\ ha^{-1}$)	DW	3.3^{b}	3.7^{b}
	HP	4.0^{a}	4.2^{ab}
		4.2^{a}	4.6^{a}

In each trait, the same letter indicates no significant differences at $p \leq 0.05$ level among the treatments within the same cultivar

Yield

Irrigation resulted in the best yield in both cultivars; the yield significantly increased by 27.3 and 24.3% in irrigated treatment compared to drought-stressed counterpart in 'Boglár' and 'Pannonia Kincse', respectively. Spraying with H_2O2 also increased the yield of both cultivars compared to drought-stressed treatment; the increase ratio was 21.2 and 13.5% in 'Boglár' and 'Pannonia Kincse', respectively (Table 2). The effect of H_2O_2 application was bigger (78.9%) in 'Pannonia Kincse' compared to 'Boglár' (72.1%). Correlation of irrigation with yield was highly significant in both cultivars (Table 3).

Table 3. Correlation coefficient of irrigation treatments with the studied traits

Trait	Boglár	Pannonia Kincse
g_s	.959**	.975**
SPAD	.758*	.926**
RWC	.929**	.948**
LAI	.668*	.720*
Plant Height	.908**	.755*
Yield	.861**	.912**

* Correlation is significant at $p \leq 0.05$ level (2-tailed).

** Correlation is significant at $p \leq 0.01$ level (2-tailed).

Discussion

Drought reduced g_s in both cultivars as compared to irrigated counterparts, whereas H_2O_2 application could measurably alleviate g_s. Drought stress induces stomatal closure, limits gas exchange and photosynthesis (Yordanov et al., 2000). Ohashi et al. (2006) reported that stomatal conductance of soybean plants significantly decreased under drought stress conditions; similar result was reported by Zhang et al. (2016); a 98.8% decrease in g_s under drought. They concluded that this reduction in g_s was a result of the reduced ratio of open stomata and stomatal aperture size in the plants subjected to drought stress. Hao et al. (2013) reported a significant reduction in stomatal conductance from 0.25 to 0.10 mol $H_2O\ m^{-1}\ s^{-1}$ as a result of drought applied on soybean plants. Mathobo et al. (2017) justified the reduction in g_s in their experiment on dry beans (*Phaseolus vulgaris*) by the prevention of CO_2 from entering the leaf by stomatal closure. Similarly, Rosales et al. (2012) reported a 70% reduction of g_s after 22 days of drought stress application. Tang et al. (2017) concluded that polyethylene glycol (PEG) induced water stress on soybean significantly reduced g_s by 73%. Ishibashi et al. (2011) compared g_s of two groups of soybean seedlings under drought stress conditions. One group was sprayed with H_2O_2 and the other group with distilled water (DW). They reported that gs was significantly higher in H_2O_2-treated plants than in DW-treated plants. After two days of spraying, g_s levels in H_2O_2-treated and DW-treated plants were 508 and 323 mmol $m^{-2}\ s^{-1}$, respectively. They concluded that H_2O_2 spraying reduced stomatal closure caused by drought stress; i.e. H_2O_2 treatment reduced soybean sensitivity to drought stress. In experiment of Terzi et al. (2014) maize leaves pretreated with 10 mM H_2O_2 significantly enhanced g_s (by approximately 50%) as compared to drought-stressed leaves. They concluded that spraying leaves with H_2O_2 can reduce water loss under drought stress conditions by increasing the concentrations of metabolites that are involved in osmotic adjustment (such as proline, polyamines and soluble sugars). Other ROS species were also reported to have a role in alleviating drought stress. Razmi et al. (2017) reported that water stress reduced stomatal conductance of three soybean leaves compared to well-watered counterparts, and foliar spray of 0.4 mM salicylic acid (SA) significantly reversed drought induced stomatal closure and increased it.

Drought resulted in lower SPAD values compared to irrigated counterparts of both cultivars, whereas H_2O_2 application enhanced this value; it even increased SPAD to a level more than did irrigation in 'Boglár', but not in 'Pannonia Kincse'. Similarly, Ergo et al. (2018) reported that SPAD values significantly decreased from 35.5 to 22.4 under drought stress applied 30 days after R5.5 stage. Chlorophylls are the main pigments of absorbing, transporting and converting light energy, and their content is a major parameter that indicates photosynthetic performance (Liu et al., 2007). Subjecting plants to drought stress resulted in a significant decline in chl_{a+b} (from 19.5 to 13.0 mg g^{-1} DW), indicating a reduced capacity of absorbing and converting light energy (Tang et al., 2017). Similarly, Dong et al. (2015) concluded that light absorption was reduced by drought stress that resulted in changing both leaf area index and leaf chlorophyll content. Both chlorophylls *a* and *b* were reduced under drought stress (Farooq et al., 2010). Other papers also reported that chlorophyll content was decreased because of drought in soybean (Makbul et al., 2011), chickpea (Mafakheri et al., 2010) and pea (Inaki-Iturbe et al., 1998). That reduction was attributed to induced destruction of the chloroplasts and to the instability of the chlorophyll protein complex (Khan et al., 2015). Sun et al. (2016) reported that the application of H_2O_2 significantly increased the leaf chlorophyll content of cucumber plants exposed to medium drought conditions. An evaluation of the effects of H_2O_2 on leaf chlorophyll content during adventitious rooting under drought conditions showed that drought stress resulted in a decline in chlorophyll content after 72 h of its application, producing a 39.1% decrease in the chlorophyll *a* content compared to control. However, applying exogenous H_2O_2 retarded chlorophyll degradation, especially chlorophyll *a* (Liao et al., 2012). Maize leaves had higher levels of both chlorophylls *a* and *b* when seeds were soaked in 140 mM H_2O_2 before sowing (Ashraf et al., 2015). Enhanced chlorophyll levels induced by hydrogen peroxide treatment were justified by H_2O_2-stimulated antioxidant enzyme activities (Azevedo Neto et al., 2005; Gao et al., 2010). Razmi et al. (2017) reported that drought significantly reduced both chlorophyll *a* and *b* contents in soybean leaves. However, significant increases (by 15% in chlorophyll *a* and 19% in chlorophyll *b*) resulted from foliar application of 0.4 mM of salicylic acid (SA) compared to control treatment (no SA).

Application of H_2O_2 significantly increased RWC in both cultivars compared to drought-stressed counterparts, and irrigation further enhanced this trait. It was previously reported that drought stress reduced RWC of soybean leaves (Razmi et al., 2017). Ishibashi et al. (2011) reported that RWC in H_2O_2-treated and DW-treated (control, treated with distilled water only) plants was 60 and 40%, respectively after four days of drought stress application, and RWC was also higher in H_2O_2-treated plants than in DW-treated plants after six days of drought stress imposition. Authors concluded that H_2O_2 spraying enabled the leaves to maintain high levels of RWC by regulating the osmolality in the leaves, consequently ameliorating the negative effects of drought stress. Similar results on cucumber seedlings were reported later by Sun et al. (2016). Application of SA on common bean improved RWC under drought stress conditions (Sadeghipour and Aghaei, 2012).

Irrigation significantly increased LAI in both cultivars compared to drought-stressed counterparts. Furthermore, H_2O_2 application could further increase LAI in both cultivars. drought stress decreases LAI (Liu et al., 2008). Ashraf et al. (2015) reported that seeds soaked in 20, 80, 100 and 140 mM of H_2O_2 later formed plants with higher leaf area under drought stress conditions compared to non-treated seeds. Using (SA), Other authors concluded that treatments with SA could improve LAI in different plants including soybean (Kuchlan et al., 2017; Razmi et al., 2017), strawberry (Ghaderi et al., 2015) and lemongrass (Idrees et al., 2010). This was attributed to increased accumulation of certain proteins (such as proline) and soluble sugars that, in part, enhances cell turgor pressure (Razmi et al., 2017).

Drought decreased plant height in both cultivars, and H_2O_2 application could ameliorate drought's effects and result in enhanced plant height, reaching a very close level of irrigated counterparts, especially in 'Pannonia Kincse' cultivar. Many previous papers reported a reduction in plant height under drought stress conditions (e.g. Atti et al., 2004; Demirtas et al., 2010; Hao et al., 2013; Mak et al., 2014). Moreover, Garcia et al. (2010) reported a significant difference in plant height of drought-stressed soybean genotypes compared to control counterparts. They also reported the different examined genotypes to be significantly different in plant height, which was demonstrated later by Hossain et al. (2014) who studied the effect of drought stress on the plant height of three soybean genotypes, one drought-susceptible and two drought-tolerant genotypes; they reported plant height to be shortened as a result of drought stress for the three genotypes. However, the drought-susceptible genotype had a 44.3% of height of the control plants, whereas it was 56.7% and 59.1% for the two drought-tolerant genotypes. The authors attributed this reduction to a drought tolerance mechanism, as cell swells, cell wall and synthesis enzymes reduces, consequently, growth and plant height e decreases (Levitt, 1980; Austin, 1989). Banon et al. (2006) justified this decrease by a reduction in cell elongation caused by inhibited growth promoting hormones that, in part, led to decrease of cell turgor, cell volume and eventually cell growth and/or by a restriction of xylem and phloem vessels (Lavisalo and Schuber, 1998). Another possible explanation is that drought results in a decrease in the rate at which the stem nodes are produced (Frederick et al., 1989). Abbas and Mohamed (2011) conducted an experiment on common bean (*Phaseolus vulgaris*) seeds where half of the seeds were soaked in hydrogen peroxide (2%) for four hours and then air dried, and the other half of the seeds were soaked in distilled water for four hours and then air dried. Their results showed an increase by 43.6% in the H_2O_2-treated seedling height under a drought level of 60% of field capacity. Increasing the drought severity (to reach only 40% of field capacity) decreased the seedling height of both treatments. However, H_2O_2-treated seedlings showed better height by 38.4%.

Yield was significantly reduced by drought in both cultivars, whereas H_2O_2 application measurably recovered yield. The recovery was significant in 'Boglár' cultivar. Soybean seed yield decreases under drought stress conditions (Karam et al., 2005; Dogan et al., 2007; Bajaj et al., 2008; Sincik et al., 2008; Gercek et al., 2009; Sadeghipour and Abbasi, 2012; Li et al., 2013). It was also reported that genotypes significantly differ in yield production under drought stress conditions (Bellaloui and Mengistu, 2008; Maleki et al., 2013; He et al., 2017). Explanations for under drought stress conditions were that drought stress shortens the seed-filling period what explains yield decrease (Smiciklas et al., 1992). Other authors suggested that it is due to the reduction of seeds number (Dornbos et al., 1989), seeds weight (Samarah et al., 2006; Demirtas et al., 2010) and pod number per plant (Atti et al., 2004; Khatun et al., 2016). However, exogenous application of H_2O_2 had improved plant biomass in wheat under drought stress (He et al., 2009), and SA application improved the grain yield of common bean under drought stress conditions (Sadeghipour and Aghaei, 2012). Horvath et al. (2007) reported that SA can enhance metabolite stream to the developing grains which reduces the abortion rate that, in part, can significantly increase pod number per plant and seed number pod^{-1} in soybean (Khatun et al., 2016). Not only yield, but also yield components (number of grains m^{-2}, pods plant^{-1}) were enhanced with the application of SA on soybean leaves under drought stress conditions (Razmi et al., 2017). The authors attributed the increase of grain yield due to SA application, improved RWC, reduced restrictions of stomatal conductance and the enhanced biosynthesis of photosynthetic pigments in the leaves.

Conclusions

Drought stress negatively affects soybean morphology, physiology and, consequently, the final seed yield; it resulted in significant reductions in all studied traits. Treating drought-stressed soybean plants with 1 mM of H_2O_2 could alleviate that negative influence and enhance all studied traits. Its effect was more noticeable on the leaf area index LAI as H_2O_2-sprayed plants had higher LAI values than both drought-stressed and fully-irrigated counterparts. The effect was also noticeable on the relative water content RWC of both cultivars. The correlation of irrigation with the studied traits was also significant.

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