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Waterlogging tolerance of five soybean genotypes through different physiological and biochemical mechanisms

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ABSTRACT

Waterlogging is a serious environmental threat that limits crop growth and yield in low-lying, rainfed areas in many regions across the globe. Here we investigated the effects of waterlogging and subsequent re-oxygenation on the physiology and biochemistry of three soybean [*Glycine max* (L.) Merrill] genotypes (PELBR10-6000, PELBR11-6028, and PELBR11-6042) and two cultivars (TEC IRGA 6070 and BMX Potência). Plants were grown under greenhouse conditions until the V4 stage when they were subjected to waterlogging for seven days. The water was then drained and plants were allowed to recover for another seven days. Overall, all genotypes suppressed waterlogging stress with distinct mechanisms. Waterlogged PELBR10-6000 surpassed control plant levels of CO_2 assimilation rate and readily responded to the energy lack induced by hypoxia by activating the fermentative enzymes and alanine aminotransferase. Similar mechanisms were observed in BMX Potência, which restored metabolism to control levels at the end of the recovery. PELBR11-6028 and PELBR11-6042 activated the antioxidant defenses, and TEC IRGA 6070 did not delay flowering.

1. Introduction

Soybean [*Glycine max* (L.) Merrill] plants comprise a major source of protein and oil for humans, livestock and various industrial products, with a total grain production of approximately 341 million tons worldwide (United States Department of Agriculture, 2019). Waterlogging is a widespread phenomenon drastically reducing the growth and production of soybean in many regions of the world (Van Nguyen et al., 2017), mostly due to the occurrence of flat topography (Collaku and Harrison, 2002), high water tables and poor drainage of clay-like soils (Jitsuyama, 2017). In Brazil, ca. 28 million hectares are flood-prone (alluvial and hydromorphic soils), in which 13 million hectares are located in the lowlands of the Rio Grande do Sul state (Empresa Brasileira de Pesquisa Agropecuária, 2018). These areas are frequently used for rice farming, with manipulated flooding regimes (paddy rice) (Jackson and Colmer, 2005), and soybean has been introduced as crop rotation to enhance the efficiency of the rice cropping system by improving the soil conditions and breaking pests, diseases and weed cycles.

The negative impacts of waterlogging on crops is mostly a consequence of the slow diffusion rates of gases and the relatively low solubility of O_2 in water (Bailey-Serres and Voesenek, 2008; Limami et al., 2014). One of the most severe problems encountered by plants subjected to waterlogging is the energy deficit as a result of root respiration inhibition caused by a shortage of O_2 (van Dongen and Licausi, 2015). As a consequence, the increased levels of pyruvate accumulated due to glycolysis enhanced the fermentative enzymes (Borella et al., 2019). Two important pathways induced under anaerobic conditions are lactic and ethanolic fermentation. The first is a one-step reaction from pyruvate to lactate, catalyzed by lactate dehydrogenase (LDH) using NADH. The latter is a two-step process regenerating NAD^+, in which pyruvate is first decarboxylated to acetaldehyde by pyruvate decarboxylase (PDC), and acetaldehyde is then converted to ethanol by alcohol dehydrogenase (ADH) (Zabalza et al., 2009; Zhang et al., 2017). Therefore, waterlogging and, ultimately, anaerobic metabolism potentially result in acute growth inhibition and even death in most crops due to energy limitation, accumulation of toxic products (such as lactate) and carbon loss (via ethanol loss from roots) (Tamang et al., 2014).

Several species such as soybean (Sousa and Sodek, 2003; Rocha

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et al., 2010a; Borella et al., 2017), *Lotus japonicas* L. (Rocha et al., 2010b), and *Arabidopsis thaliana* L. (Miyashita et al., 2007) accumulate alanine, an amino acid produced by the enzyme alanine aminotransferase (AlaAT), under hypoxia. The synthesis of alanine plays an important role in regulating the glycolytic flux by preventing the excessive accumulation of pyruvate (Zabalza et al., 2009) while retaining carbon and nitrogen resources within the cell (Rocha et al., 2010b). Unlike the production of lactate and ethanol, alanine accumulation does not have detrimental side effects on the cell.

Under waterlogging conditions, plants also exhibit reduced stomatal conductance (g_s) (Posso et al., 2018; Barickman et al., 2019), which is often accompanied by reduced net CO_2 assimilation and leaf chlorosis (de Souza et al., 2013; Posso et al., 2018). Impaired net CO_2 accumulation accompanied by the limited capacity to absorb water and nutrients represent additional factors reducing plant growth and biomass accumulation (Marashi, 2018; Ploschuk et al., 2018; Ye et al., 2018).

In addition to the various negative effects of waterlogging, as water level descends due to soil drainage, roots are suddenly exposed to elevated oxygen levels resulting in redox imbalance and overproduction of reactive oxygen species (ROS) in mitochondria and chloroplasts (Halliwell, 2006). To counteract the hazardous effects of ROS, plants have evolved a complex antioxidant system comprised by enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Fukao et al., 2019), and non-enzymatic scavengers, such as carotenoids (Doupis et al., 2017).

Previous studies using wheat (*Triticum aestivum L.*) (Malik et al., 2002), barley (*Hordeum vulgare* L.) (de San Celedonio et al., 2014), and rapeseed (*Brassica napus* L.) (Wollmer et al., 2017) observed that plants exposed to waterlogging at the vegetative phase exhibit reduced growth rate which demonstrates their sensitivity in early vegetative stages (Xu et al., 2015). Given that few studies addressed the effects of waterlogging on soybean plants at the vegetative stage (Mutava et al., 2015; Andrade et al., 2018), we aimed to describe the influence of waterlogging on the physiology and metabolism of leaves and roots of five soybean genotypes during and after waterlogging stress.

2. Material and methods

2.1. Plant material and growth conditions

To perform this study, we selected two commercial cultivars, BMX Potência (POT) and TEC IRGA 6070 (6070), often cultivated in lowland areas in Southern Brazil, and three lineages from Embrapa Soybean Breeding Program PELBR10-6000 (6000), PELBR11-6028 (6028) and PELBR11-6042 (6042). The experiment was carried out in a greenhouse at Embrapa Lowlands Experimental Station (see environmental conditions in Fig. S1 and Table S1), Capão do Leão (31°80′36″S and 52°41′12″W), Brazil, from December 2016 to January 2017.

Seeds were inoculated on sowing with *Bradyrhizobium japonicum,* strain SEMIA 5079 (BIOAGRO). Plants were cultivated from seeds in 3 L pots (two plants per pot) containing vermiculite and watered daily. Twice a week, they received 200 mL of N-free nutrient solution at full strength (Hoagland and Arnon, 1950). Plants were subjected to waterlogging 30 d after sowing at the V4 stage (Fehr et al., 1971). Waterlogging was imposed during seven days by placing each pot (with drainage holes) into non-punctured pots of the same dimension and filling the pots with $^1/_3$ full strength N-free nutrient solution until the water level reached 2 cm above the vermiculite surface. The nutrient solution was monitored daily and refilled when necessary. The O_2 concentration in solution was monitored with an O_2 meter (Handylab OX1, Hofheim, Germany) (Fig. S2). After seven days of waterlogging, the pots were drained and plants were allowed to recover for additional seven days. Control plants were not subjected to waterlogging.

Plants were sampled during waterlogging at days two and seven, and during the recovery at days two and seven. During harvesting, roots were carefully washed and homogeneous healthy leaves of the same size and age (first fully expanded trifoliate) were harvested, weighed and stored frozen (-80 °C) until biochemical analysis.

2.2. Plant growth, leaf gas exchange and water potential

Stem diameter (SD) was assessed using a caliper rule, while root dry matter (RDM) and shoot dry matter (SDM) were obtained placing samples in an oven at 65 °C for 72 h. Pre-dawn leaf water potential (WP) was assessed prior to sunrise using a pressure pump (SEC-3115-P40G4V, Soil moisture, Santa Barbara, USA).

Net CO_2 assimilation rate (A), stomatal conductance (g_s) and transpiration rate (E) were performed from 11:00 to 12:00 h, using a portable open-flow gas exchange system (LI-6400XT, LI-COR, Lincoln, USA). Conditions in the leaf cuvette were set at relative humidity of ca. 50%, light intensity of 1000 μmol m^{-2} s^{-1}, temperature of 25 °C and CO_2 of 400 ppm.

2.3. Antioxidant enzymes

Leaves and roots (0.250 g) were ground into powder using 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ascorbic acid (AsA) and 10% (w/w) polyvinylpyrrolidone (PVPP). Then, the homogenate was centrifuged at 12.000 × g at 4 °C for 20 min and the supernatant was used for enzyme activity measurements.

SOD (EC 1.15.1.1) activity was estimated by the ability of the enzyme to inhibit the nitroblue-tetrazolium (NBT) reduction (Giannopolitis and Ries, 1977) in a reaction medium consisting of enzyme extract, 86.5 mM potassium phosphate buffer (pH 7.8), 14 mM methionine, 0.1 μM EDTA, 5.6 μM NBT and 0.2 μM riboflavin. One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of the reduction of NBT. CAT (EC 1.11.1.6) activity was assayed by measuring the initial rate of disappearance of hydrogen peroxide (H_2O_2) (Havir and McHale, 1987). The incubation mixture contained crude enzyme extract, 100 mM potassium phosphate buffer (pH 7.0) and 12.5 mM H_2O_2. The measurements were recorded at 240 nm and the enzyme activity was calculated using the molar extinction coefficient (ε = 36 μM^{-1} cm^{-1}). APX (EC 1.11.1.11) activity was measured following the method of Nakano and Asada (1981). The assay mixture contained crude enzyme extract, 37.5 mM potassium phosphate buffer (pH 7.0), 0.25 mM AsA and 5 mM H_2O_2. The absorbance was recorded at 290 nm and the activity of APX was calculated using ε = 2.8 mM^{-1} cm^{-1}. The protein content was determined according to Bradford (1976) using bovine serum albumin as the standard.

2.4. H_2O_2 content

Leaves and roots (0.250 g) were ground with 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged (12,000 × g; 4 °C; 20 min) and the supernatant was used for the analyses according to Velikova et al. (2000). The samples were incubated in a reaction medium containing 10 mM potassium phosphate buffer (pH 7.0) and 1.0 M potassium iodide at 30 °C for 10 min. The absorbance was measured at 390 nm. A calibration curve was obtained with H_2O_2 standard.

2.5. Fermentative enzymes and AlaAT

Frozen roots (0.250 g) were ground into powder using an extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol and 3% (w/w) PVPP). The homogenate was centrifuged at 12,000 × g at 4 °C for 20 min, and an aliquot of the supernatant was desalted using a PD-10 column (GE Healthcare, Buckinghamshire, UK). The eluted protein fraction was used to determine ADH (EC 1.1.1.1), PDC (EC 4.1.1.17), LDH (EC 1.1.1.17) and AlaAT (EC 2.6.1.2) activities. The enzymatic assays were performed by monitoring the oxidation of NADH at 340

nm.

ADH and PDC activities were assayed following the method proposed by Hanson et al., 1984 with some modifications. For ADH, the reaction mix contained 50 mM phosphate buffer (pH 7.0), 0.6 mM NADH and 5.0 mM acetaldehyde. The PDC assay was monitored in a reaction mix containing 50 mM MES buffer (pH 6.0), 0.2 mM NADH, 0.5 mM thiamine pyrophosphate, 1 mM magnesium chloride, 20 mM oxamic acid, 5 units of ADH and 10 mM Na-pyruvate. The LDH assay followed the method proposed by Hanson and Jacobsen (1984) with some modifications: the reaction was monitored in a reaction mix containing 50 mM buffer Tris-HCl (pH 7.5), 0.6 mM NADH, 3.0 μM KCN, 0.2 mM 4-methylpyrazole and 10 mM Na-pyruvate. The AlaAT assay contained 10 mM L-alanine, 5 mM 2-oxoglutarate, 0.6 mM NADH, 50 mM Tris/HCl (pH 7.5) and 5 units of lactate dehydrogenase, as described by Good and Muench (1992).

2.6. Photosynthetic pigments

Chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoids were extracted from leaves using dimethyl sulfoxide as described by Wellburn (1994).

2.7. Experimental design and statistics

Pots were arranged in a complete randomized design with five replicates. The two plants in each pot were pooled together to form a single replicate. For each treatment group (2 W, 7 W, 2 R, and 7 R), there was a control group of plants (2 WC, 7 WC, 2 RC, and 7 RC). Each treatment was compared to its corresponding control (*e.g.* 2 W *vs* 2 WC).

The data were analyzed by two-way ANOVA and the means were compared by Student's *t*-test ($P \leq 0.05$). Statistical analyses were performed using SigmaPlot 11.0 (Systat Software Inc., San Jose, California). Results are shown as treatment/control (T/C) ratio resulting in the waterlogging tolerance index proposed by Jitsuyama (2017). This ratio was transformed by log10 according to Goufo et al. (2017). T/C ratios greater than zero indicate that the means were higher in the treatment than in the control group, while T/C ratios lower than zero showed that control plants presented higher values than those treated (waterlogging or recovery). For water potentials, which present negative values, the contrary is applied. Ratios close to zero indicate similar values for control and treatment. The absolute value indicates the magnitude of the difference.

3. Results

During the first two days of waterlogging, O_2 levels decreased markedly in the rooting medium, while from day two to seven, decreases in the O_2 level were much less sharp (Fig. S2).

Waterlogging limited root biomass accumulation in a genotype-dependent manner (Fig. 1A; Table S2). Genotype 6028 presented the greatest difference in root biomass under waterlogging compared to control, while genotype 6000 presented the least difference. Draining allowed an increase in the accumulation of root biomass, resulting in a lower difference in root biomass between recovered and control plants. After seven days of recovery, genotypes 6070 and POT exhibited similar root dry mass to control plants. Waterlogging and recovery did not affect shoot biomass production of all genotypes in a similar fashion (Fig. 1B). While POT plants increased shoot biomass during waterlogging, the other genotypes presented similar shoot biomass to control plants. During recovery, genotype 6000 increased and genotypes 6070 and POT decreased shoot biomass production compared to control. The stem diameter of all genotypes consistently increased during waterlogging (Fig. 1C). Seven days of recovery resulted in a similar stem diameter between control and recovery plants due to decreases in the stem diameter of waterlogged plants (Table S2). Most genotypes subjected to waterlogging followed by recovery, except for 6070, exhibited

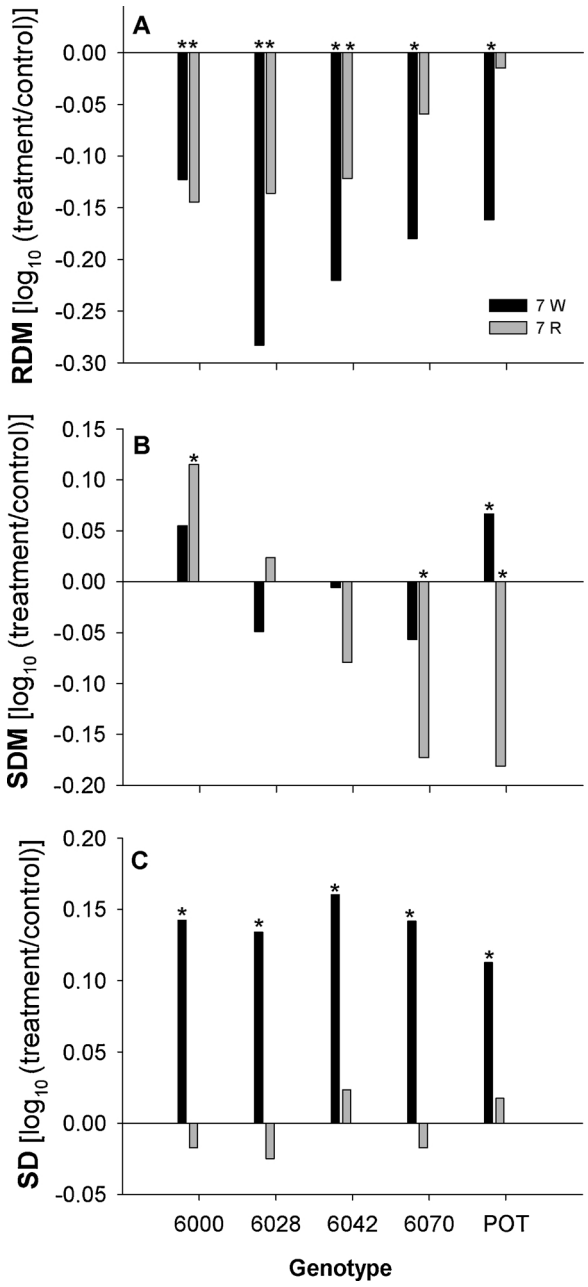

Fig. 1. Root dry matter (RDM) (A), shoot dry matter (SDM) (B) and stem diameter (SD) (C) for the different soybean genotypes at seven days of waterlogging (7 W) and seven days of recovery (7R). Values are the ratio between treatment and control performed on log10-transformed data and represented the mean of five independent measurements. Asterisk (*) indicates a significant difference from the control (Student's *t*-test; $P \leq 0.05$).

a delayed development in terms of flowering at the end of the experiment (Table S3).

Overall, waterlogging significantly decreased the leaf gas exchange of soybean plants (Fig. 2, Table S4). The *A* was lower for almost all genotypes when plants were subjected to waterlogging for two days when compared to control, and consistently lower for all genotypes subjected to waterlogging for seven days (Fig. 2A). Plants subjected to waterlogging presented *A* lower than control at two days of recovery, but similar or even higher *A* (genotype 6000) at seven days of recovery. Two days of waterlogging resulted in stomatal closure only for the genotype 6028, consistent with the largest reduction in *E* (Fig. 2B–C). Waterlogging for seven days resulted in stomatal closure and decreased

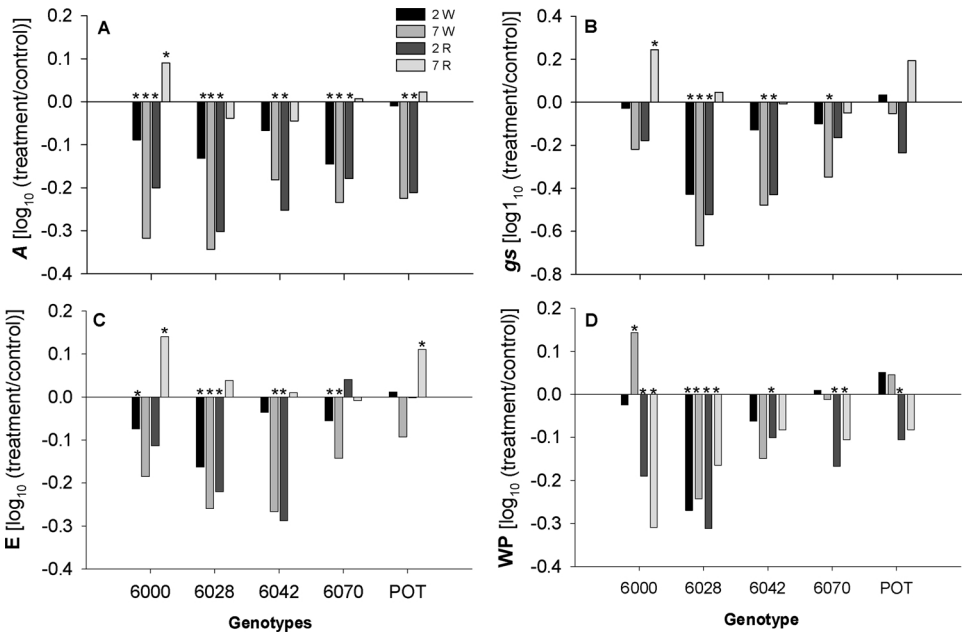

Fig. 2. Net CO_2 assimilation rate (*A*) (A), stomatal conductance (g_s) (B), transpiration rate (*E*) (C) and water potential (WP) (D) at two (2 W) and seven days of waterlogging (7 W), two (2R) and seven days of recovery (7R). Values are the ratio between treatment and control performed on log10-transformed data and represented the mean of five independent measurements. Asterisk (*) indicates a significant difference from the control (Student's *t*-test; $P \leq 0.05$).

E for most genotypes (genotypes 6028, 6042, 6070). On day seven of recovery, all genotypes presented similar or higher g_s and *E* than control. Regarding WP, only genotype 6028 presented WP different (higher) to control at day two of waterlogging (Fig. 2D). On day seven of waterlogging, genotype 6028 was maintained at higher WP than control. On day two of recovery, all genotypes exhibited higher WP than control, and on day seven genotypes 6000, 6028 and 6070 remained more hydrated than control.

Waterlogging resulted in lower amounts of photosynthetic pigments in a genotype-dependent manner (Fig. 3, Table S5). The Chl *a* level decreased with waterlogging and seven days of recovery allowed plants to restore it to control levels (Fig. 3A). Plants of genotypes 6000, 6070 and POT subjected to waterlogging also presented lower amounts of Chl *b* on day two of recovery, with plants of genotype 6000 also presenting lower levels of this molecule on day seven of recovery (Fig. 3B). Carotenoid levels were not reduced by two days of waterlogging (Fig. 3C). Seven days of waterlogging, on the other hand, resulted in decreased carotenoid levels for genotypes 6000, 6028 and 6042. Seven days of recovery resulted in similar or even higher (POT) levels of carotenoids for all genotypes.

In leaves, SOD and CAT were the most affected antioxidant enzymes during waterlogging and recovery (Fig. 4, Table S6). The activity of SOD increased in leaves at seven days of waterlogging for all genotypes, but POT (Fig. 4A). All genotypes presented similar or higher (genotypes 6028 and 6042) SOD activity at seven days of recovery. Waterlogging and recovery did not affect the activity of APX in a consistent and significant fashion for leaves (Fig. 4B). The CAT activity in leaves was modified in a genotype- and condition-manner (Fig. 4C). Genotype 6000 increased CAT activity when compared to control at two days of recovery and decreased at seven days of recovery.

Genotype 6028 decreased CAT activity within seven days of waterlogging and increased on day two of recovery. Genotype 6042 increased CAT activity within two days of waterlogging and at seven days of recovery. The CAT activity was slightly reduced at two days of waterlogging in leaves of the genotype 6070 and remained similar to control during the rest of the experiment. POT plants reduced CAT activity at seven days of waterlogging and recovery. Waterlogging and recovery did not significantly affect the activity of SOD and APX in roots (Figs. 4D–E). The activity of CAT in roots increased at the beginning of waterlogging (2 d) for genotypes 6000, 6028 and POT, and remained unaltered and lower during the rest of the experiment (Fig. 4F).

The H_2O_2 content in leaves did not change within two days of waterlogging and increased at seven days of waterlogging and two days of recovery for all genotypes (Fig. 5A and Table S7). Only genotype 6042 maintained high levels of H_2O_2 at the end of the experiment. The H_2O_2 content in roots increased only on day two of waterlogging for genotype 6000 and day two of recovery for genotype 6070 (Fig. 5B). The remaining genotypes exhibited similar or lower levels of H_2O_2 over the course of the experiment.

Increases in LDH activity were observed at two days of waterlogging for genotypes 6000, 6028 and 6042, at two days of recovery for POT, and at seven days of recovery for 6070 (Fig. 6A, Table S8). The activity of ADH and PDC increased at seven days of waterlogging (Fig. 6B–C), except for genotype 6028, which increased PDC, but not ADH. At the end of the recovery, the activity of these enzymes was similar or lower than the control for almost all genotypes. The AlaAT activity increased only at day seven of waterlogging and two of recovery for 6000 and day two of waterlogging for POT (Fig. 6D).

4. Discussion

Waterlogging for seven days did not result in plant mortality or in severe limitation in plant biomass accumulation for any genotype. Overall, all genotypes exhibited moderate tolerance to waterlogging through distinct mechanisms to overcome the waterlogging-induced hypoxia stress. While some genotypes used several tolerance mechanisms during waterlogging and recovery (e.g. 6000 and 6028), others overcame the stress by exhibiting less alteration regarding such mechanisms (e.g. 6070 and POT).

Waterlogging negatively affected root biomass accumulation of almost all genotypes. Regarding shoot biomass, most genotypes presented biomass similar to the control, and POT waterlogged plants accumulated more shoot biomass. The investment in shoot growth is an interesting mechanism of waterlogging tolerance, possibly related to metabolites sent to the shoot via the xylem, permitting the removal of electrons from the hypoxic root (Vitor and Sodek, 2019).

Lower water uptake by the roots and lower g_s are the main factors limiting plant growth (Barickman et al., 2019). The higher WP for POT plants during waterlogging may have contributed to maintaining g_s and *E* similar to the control, as well as may have promoted higher shoot growth. In the meantime, the lower WP found in 6028 plants likely

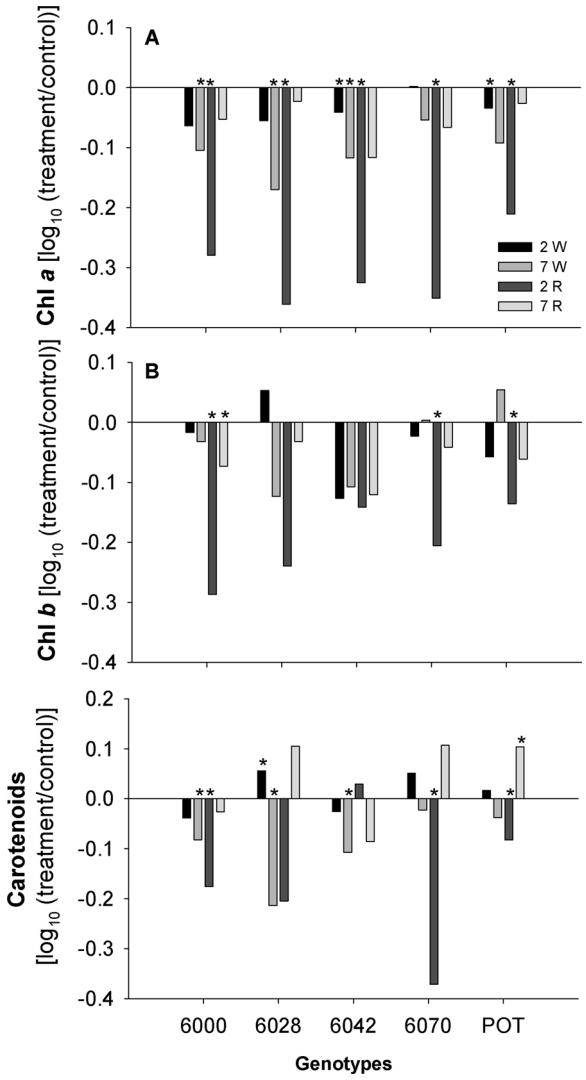

Fig. 3. Level of chlorophyll *a* (Chl *a*) (A), chlorophyll *b* (Chl *b*) (B) and carotenoids (C) at two (2 W) and seven days of waterlogging (7 W), two (2R) and seven days of recovery (7R). Values are the ratio between treatment and control performed on log10-transformed data and represented the mean of five independent measurements. Asterisk (*) indicates a significant difference from the control (Student's *t*-test; $P \leq 0.05$).

resulted in stomatal closure and proportionally higher inhibition of root biomass accumulation. Ren et al., 2016 suggest that reductions in photosynthesis after waterlogging are mainly due to stomatal factors, which can be confirmed by our data. Most genotypes presented decreases in *A* accompanied by stomatal closure during waterlogging, however, decreases in *A* in POT waterlogged plants were not driven by stomatal limitations, since g_s did not decrease upon waterlogging. For this genotype, decreases in *A* were probably due to decreases in chl *a* content. Indeed, in this genotype visible yellowing of leaves was noticed during waterlogging indicating chlorophyll degradation.

Reductions in chlorophyll content may be related to nitrogen deficiency. According to Amarante et al., 2006, nitrogen fixation in soybean plants under waterlogging is inhibited almost immediately due to the decrease in O_2 supply. Limited nitrogen content in plants results in the remobilization of nitrogen from older to younger leaves. Decreases in chl *a* were also observed to the genotypes 6000, 6028 and 6042. In addition, genotypes 6000, 6028 and 6042 also decreased carotenoid content. Genotype 6070 presented normal levels of chl *a*, chl *b* and carotenoids under waterlogging, as well as genotype POT for carotenoid content. On the other hand, genotype 6028 presented high levels of carotenoids. Carotenoids probably acted as an antioxidant defense protecting photosynthetic apparatus from ROS (Li et al., 2012) and may have been an important antioxidant defense for genotype 6028.

The lower content of photosynthetic pigments, as well as the decreases in *A*, can be caused by ROS accumulation. Indeed, the content of H_2O_2 increased in leaves of all genotypes under waterlogging. The H_2O_2 accumulation can be related to the increase in SOD activity, which in turn may have activated CAT (for genotype 6042) and APX (for genotype 6028). In roots, the content of H_2O_2 increased during waterlogging just for genotype 6000, however, the H_2O_2 level was not enough to activate scavenger enzymes. Regarding re-oxygenation, soybean plants did not present ROS accumulation and oxidative damage in roots. On the other hand, the levels of H_2O_2 in leaves increased in all genotypes. Similar to leaves of waterlogged plants, the overproduction of ROS in leaves of plants under re-oxygenation may be a result of impared photosynthesis, resulting from reduced stomatal opening and pigment damage. Light-harvesting under these conditions leads to an overload of the electron transport chain in chloroplasts, leading to electron leakage and ROS accumulation (Gill and Tuteja, 2010).

At the end of the recovery, only genotype 6042 still had high levels of H_2O_2. In all other genotypes, H_2O_2 levels were restored due to the high activity of the enzymes APX (for genotype 6070) and CAT (for genotypes 6000 and 6028) and the high levels of carotenoids (for genotype POT). Oxidative control was efficient in leaves, given that at the end of recovery, chl *a* and *b* and carotenoid levels were restored (except genotype 6000 which still had low chl *b* levels). Consequently, *A*, g_s, *E* and WP were also restored to control levels.

During hypoxia, fermentative enzymes prevent pyruvate accumulation, contribute to NADH cycling, and produce substrate-level ATP (Borella et al., 2013; Bui et al., 2019). However, the first pathway enzyme, LDH, produces lactate, which acidifies the cytosol of cells (Banti et al., 2013). The genotypes 6000, 6028 and 6042 increased LDH under waterlogging. Lactate induces PDC activity, which converts pyruvate in acetaldehyde, resulting in ethanol production by the subsequent activity of ADH. Both enzymes were activated in genotypes 6000, 6042, 6070 and POT. Komatsu et al. (2009) detected an up-regulation of ADH and PDC genes in soybean under waterlogging, suggesting that these genes are involved in the perception of O_2 deprivation and exhibit a similar enzyme cascade as other plant species under hypoxia. PDC activity in genotype 6028 increased after two and seven days of waterlogging. However, increases in ADH activity were not observed during waterlogging. AlaAT increased only in genotypes 6000 and POT, likely as a tolerance mechanism since alanine metabolism can prevent pyruvate accumulation, facilitating the continued operation of glycolysis during waterlogging (Rocha et al., 2010b; Borella et al., 2017).

Regarding the activity of the fermentative enzymes in the recovery period, genotypes 6028 and 6070 remained with higher LDH activity than control. In addition, genotypes 6070 and 6000 still maintained high activities of PDC and AlaAT, respectively. ADH activity was completely restored at the end of the recovery period. At the beginning of the recovery period and the end of the experiment, LDH, PDC, and AlaAT activities were maintained guaranteeing ATP production and preventing future hypoxic stress (Zhang et al., 2017).

Facilitating the O_2 entry into the roots is an important way to prevent larger cell damage. Soybean plants exhibited thickened stems presumably because of the aerenchymatous tissue induced by waterlogging as reported by Thomas et al. (2005), and Shimamura et al. (2010). In nodulated legumes, a large number of studies have shown the beneficial effects of aerenchyma towards nitrogen fixation under waterlogging conditions (Minchin and Summerfield, 1976; James and Sprent, 1999; Shimamura et al., 2002), by providing a pathway for the diffusion of gases to the submerged nodules.

At the end of the experiment, all genotypes were able to overcome waterlogging stress, presenting most of the parameters analyzed at control levels. However, only genotype 6070 showed no developmental delay in terms of flowering at the end of the stress period. The 6000

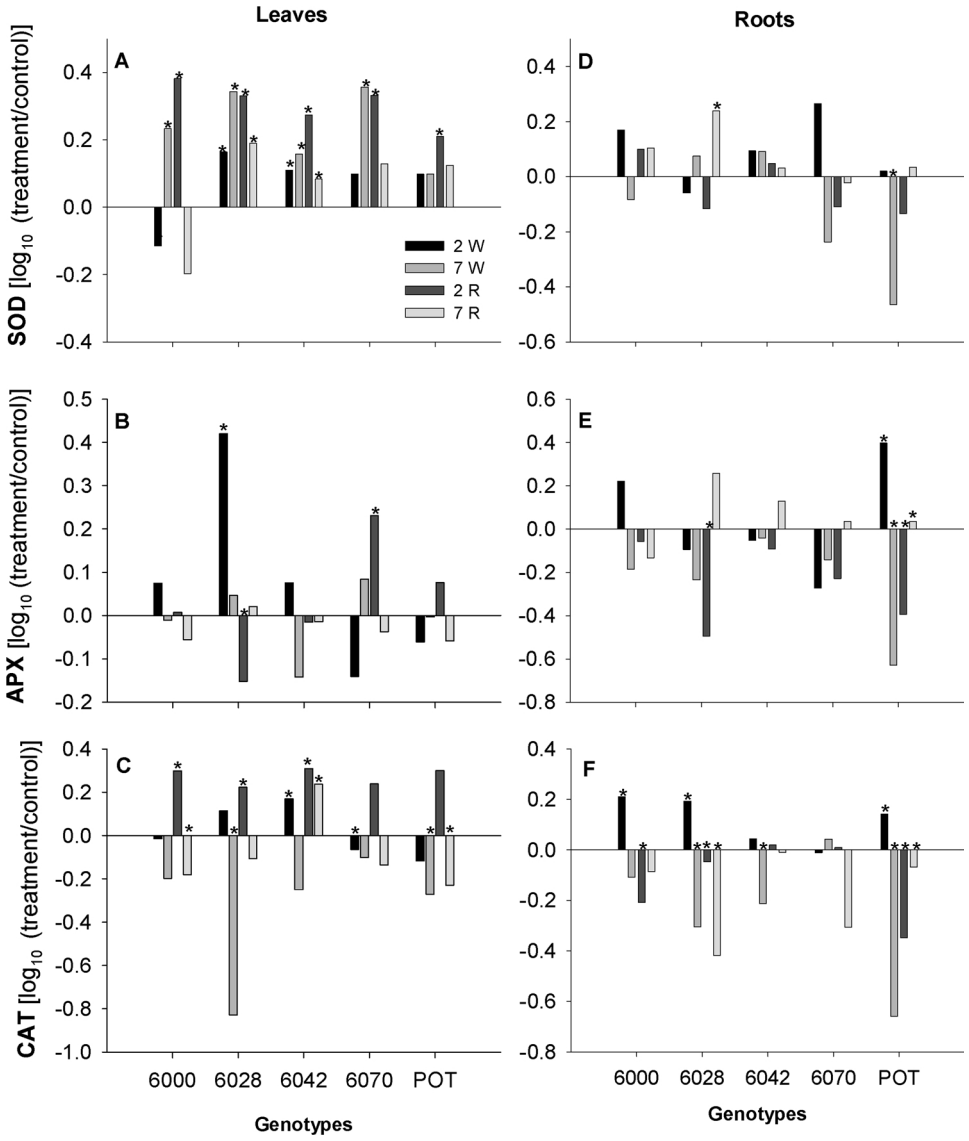

Fig. 4. Activity of superoxide dismutase (SOD) (A, D); ascorbate peroxidase (APX) (B, E); catalase (CAT) (C, F) in leaves (A, B, C) and roots (D, E, F) at two (2 W) and seven days of waterlogging (7 W), two (2R) and seven days of recovery (7R). Values are the ratio between treatment and control performed on log10-transformed data and represented the means of five independent measurements. Asterisk (*) indicates a significant difference from the control (Student's *t*-test; $P \leq 0.05$).

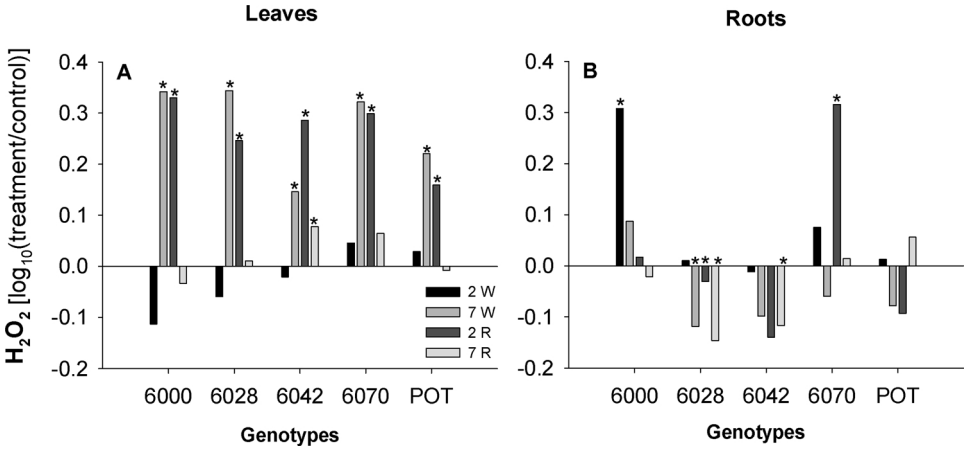

Fig. 5. Level of hydrogen peroxide (H_2O_2) in leaves (A) and roots (B) at two (2 W) and seven days of waterlogging (7 W), two (2R) and seven days of recovery (7R). Values are the ratio between treatment and control performed on log10-transformed data and represented the mean of four independent measurements. The asterisk indicates a significant difference from the control (Student's *t*-test; $P \leq 0.05$).

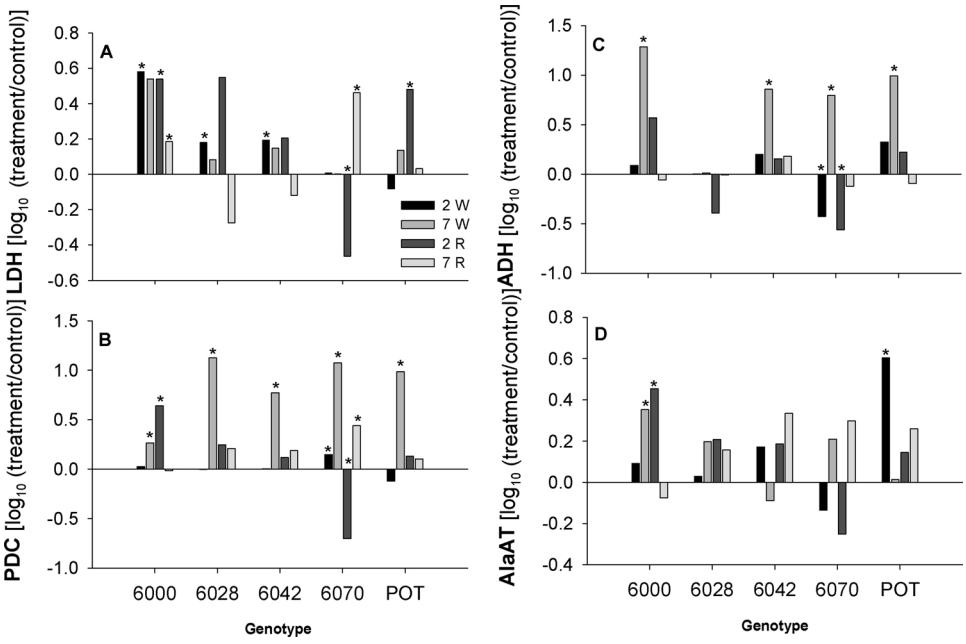

Fig. 6. Activity of lactate dehydrogenase (LDH) (A), pyruvate decarboxylase (PDC) (B), alcohol dehydrogenase (ADH) (C) and alanine aminotransferase (AlaAT) (D) in roots at two (2 W) and seven days of waterlogging (7 W), two (2R) and seven days of recovery (7R). Values are the ratio between treatment and control performed on log10-transformed data and represented the mean of four independent measurements. The asterisk indicates a significant difference from the control (Student's *t*-test; $P \leq 0.05$).

genotype was the one that delayed flowering finishing the evaluation in V10. The other genotypes were in transition stages (V10/R1). These data reveal the damaging effect of waterlogging on soybean plant growth and development and demonstrate the importance of evaluating the developmental stage of plants upon waterlogging stress, as developmental delays may compromise grain harvesting (Ploschuk et al., 2018).

Author Statement

N.G., K.L.T.C., and J.V.L.S. performed the greenhouse experiment, as well as the samplings and analyses of growth. N.G., K.L.T.C., D.P., and F.K.O. performed biochemistry analyses. L.A. and A.C.B.O. supervised the project. L.A. and C.J.S. provided critical feedback and helped shape the research. N.G. and C.J.S. wrote the manuscript with help from L.A.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2020.103975.

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