JONAS RAFAEL VARGAS

THE ROLE OF 2-OXOGLUTARATE DEHYDROGENASE DURING WATER DEFICIT AND RECOVERY IN *Arabidopsis thaliana*

Dissertation submitted to the Universidade Federal de Viçosa, as part of the requirements of the Graduate Program in Plant Physiology, to obtain the *Magister Scientiae* degree.

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ABSTRACT

To deal with periods of water limitation, plants use different mechanisms. Among them, stomatal closure and proline accumulation are the major ones during stress exposure. After stress, proline is degraded releasing glutamate in the mitochondrial matrix. The glutamate is metabolized by different metabolic pathways and may: (i) participate in nitrogen metabolism, being used for ammonia assimilation under the action of enzymes like Glutamine synthetase (GS) and Glutamine 2-oxoglutarate aminotransferase (GOGAT), (ii) being reintroduced in the tricarboxylic acid cycle (TCA cycle) as succinate via GABA shunt activity or (iii) enter the TCA cycle as 2-oxoglutarate (2-OG) when undergoing the action of the enzyme glutamate dehydrogenase. 2-OG is converted into succinyl-CoA by the action of the enzyme 2-oxoglutarate dehydrogenase (2-OGDH). In order to understand the importance of 2-OGDH during water deficit and stress release, two T-DNA insertion lines with low expression of the E1 subunit of 2-OGDH encoding gene, e1-ogdh1.1 and 1.2 were submitted to drought conditions for 22 days and then, after rewatering, three days of recovery period. This work revealed that the TCA cycle goes through downregulation in such a way that allows plants to cope with water deficit and that following recovery period its activity is reestablished. Furthermore, it was observed that the reduction in the activity of 2-OGDH did not demonstrate great impact in the metabolism during the implementation and recovery of water deficit.

Keywords: abiotic stress; metabolism; proline; TCA cycle; 2-OGDH.

RESUMO

Quando expostas a limitação de água, as plantas lançam mão de diferentes mecanismos; entre os quais podem ser citados, o fechamento estomático e o acúmulo de prolina. Após o estresse, a prolina é degradada liberando glutamato na matriz mitocondrial. O glutamato é metabolizado por diferentes vias metabólicas e pode: (i) participar do metabolismo de nitrogênio, sendo utilizado para a assimilação de amônia sob ação de enzimas como Glumamine synthetaze (GS) e Glutamina 2-oxoglutarato aminotransferase (GOGAT), (ii) sendo reintroduzido no ciclo de ácido tricarboxílico (ciclo TCA) como succinato através da via do GABA *shunt* ou (iii) entrar no ciclo do TCA como 2 oxoglutarato (2-OG) após sofrer ação da enzima glutamato desidrogenase. No ciclo TCA o 2-OG é convertido em succinil-CoA pela ação da enzima 2-oxoglutarato desidrogenase (2-OGDH). Com o objetivo de compreender a importância do 2-OGDH durante a implementação e recuperação de déficit hídrico, duas linhas de inserção de T-DNA com baixa expressão do gene codificador da subunidade E1 da enzima 2-OGDH (e1-ogdh1.1 e 1.2) foram submetidas a condições de seca por 22 dias e depois, a três dias de recuperação. Este trabalho revelou que o ciclo TCA passa por uma regulação negativa quando exposto a períodos de déficit hídrico e que, após o período de recuperação, sua atividade é restabelecida. Além disso, observou-se que a redução na atividade da 2-OGDH não demonstrou grande impacto no metabolismo durante a implementação e recuperação do déficit hídrico.

.**Keywords:** estresse abiótico; metabolismo; prolina; ciclo do TCA; 2-OGDH.

INTRODUCTION

The tricarboxylic acid (TCA) cycle, composed of a set of eight enzymes is described as a central pathway in plant respiration, since it is responsible for the production of organic acids, ATP and coenzymes (NADH and $FADH_2$) in the mitochondrial matrix (Millar et al., 2011)The TCA cycle is also fundamental in plant metabolism once it plays a role in the regulation of carbon and nitrogen metabolism, and even though it is described as a cycle, in several situations it can operate in a non-cyclic mode (Sweetlove et al., 2010). This possibility occurs because one of its functions is to provide intermediates as carbon skeletons for the assimilation of nitrogen as well as for other metabolic pathways, thus acting as a regulator of carbon and nitrogen metabolisms (Sweetlove et al., 2010).

Among the TCA cycle intermediates that can be used in other routes, acetyl-CoA, fumarate, malate, and 2-oxoglutarate (2-OG) are the most studied. Acetyl-CoA can be used in the metabolism of flavonoids, alkaloids, fatty acids, isoprenoids and pigments (Araújo et al., 2012a). Fumarate and malate can participate in the synthesis of glucose during the gluconeogenesis as well as in the photosynthesis and fruit ripening (Araújo et al., 2012a). The 2-OG can be exported from the mitochondrial matrix to the cytosol and be used in other major metabolic pathways such as ammonium assimilation and amino acid biosynthesis by the action of glutamine synthetase (GS) and glutamine 2-oxoglutarate-aminotransferase (GOGAT) (Araújo et al., 2012a).

2-OG can be produced in the TCA cycle by the action of the NAD-dependent isocitrate dehydrogenase (IDH) which, through an oxidative decarboxylation of isocitrate, produces 2-OG in a reaction that also results in NADH, water and CO_2. 2-OG is then used as a substrate for the multienzymatic complex 2-oxoglutarate dehydrogenase (2-OGDH) that converts it into succinyl-CoA through another oxidative decarboxylation that results in the release of water, CO_2 and NADH (Bunik and Fernie, 2009).

The 2-OGDH complex, responsible for the metabolization of 2-OG, is composed of three subunits, namely 2-oxoglutarate dehydrogenase (E1), dihydrolipoyl succinyltransferase (E2) and dihydrolipoyl dehydrogenase (E3) (Millar et al., 1999). This complex has a molecular mass of approximately 2000 kDa, and it is formed by the union of a core with about 24 E2 subunits to which E3 and E1 homodimers join (Millar et al., 1999). The E1 subunit requires the presence of the cofactor thiamine pyrophosphate (TPP) and is responsible for the

initial decarboxylation of 2-OG in which it releases CO_2 and forms a succinyl-TPP molecule. The E2 subunit transfers the succinyl to lipoic acid and then to a CoA releasing succinyl-CoA and forming a dihydrolipoic acid that is oxidized by E3 using FAD^+ as a cofactor to form lipoic acid with consequent reduction of NAD^+ and production of NADH (Millar et al., 1999).

The TCA cycle had basically all of its enzymes and theirs importance described under optimal conditions (Carrari et al., 2003; Nunes-nesi et al., 2005; Fait et al., 2007b; Lemaitre et al., 2007; Nunes-nesi et al., 2007; Araujo et al., 2008; Sienkiewicz-porzucek et al., 2008; Araújo et al., 2012b; Sienkiewicz-porzucek et al., 2012; Yue et al., 2018). Recently, two mutants lines of *Arabidopsis thaliana* with low expression for the gene encoding the E1 subunit of the 2-OGDH complex (*At*3g55410), (*e1-ogdh1.1* and *e1-ogdh1.2*) were isolated and characterized (Condori-Apfata, 2016). This characterization revealed that, under optimal conditions, these mutant lines have significantly reduced 2-OGDH activity coupled with a reduction in respiration, photosynthesis, plant growth, seed production, and also displayed higher levels of starch and organic acids in the middle of the light period.

Under most stress conditions, plants suffer some form of oxidative stress (Hayat et al., 2012). These conditions may lead to a reduction of the 2-OGDH activity by the presence of lipid catabolism intermediates such as 4-hydroxy-2-nonena (HNE), that act as inhibitors of the E2 subunit (Taylor et al., 2002; Taylor et al., 2004; Taylor et al., 2009). During abiotic stress such as water deficit, the non-cyclic flow of the TCA cycle is increased in order to meet the demand for 2-OG in the assimilation of ammonia resulted from the photorespiration or protein degradation (Sweetlove et al., 2002; Atkin and Macherel, 2009; Jacoby et al., 2011). This condition can also activate certain alternative pathways such as GABA shunt (Fait et al., 2007a). This pathway is responsible for supplying the demand of the TCA cycle for succinate through the action of the enzymes glutamate decarboxylase (GAD), GABA transaminase (GABA-T) and succinate-semialdehyde dehydrogenase (SSDH) which are able to establish an alternative pathway to the reactions performed by 2-OGDH and Succinyl-CoA ligase within the TCA cycle (Fait et al., 2007a).

One of the most described plant responses to water deficit is an accumulation of compatible solutes, a group of molecules known by their capacity

of being accumulated without altering cellular homeostasis (Hayat et al., 2012; Obata and Fernie, 2012; Liang et al., 2013). These metabolites are involved in protein stabilization and can be classified into three categories: polyalcohols (e.g. glycerol and sorbitol), sugars (e.g. sucrose and trehalose) and amino acids and their derivatives (e.g. proline, glycine, and betaines) (Viana et al., 2005).

Proline is an amino acid that accumulates in *Arabidopsis thaliana* when exposed to abiotic stresses such as water deficit (Hummel et al., 2010; Obata and Fernie, 2012). This osmolyte can act in water deficit resistance by reducing the tissues water potential where it accumulates in order to avoid water loss by the plant (Hayat et al., 2012; Obata and Fernie, 2012; Liang et al., 2013). Proline can be produced by proteins degradation, due to abiotic stress conditions as well as *de novo* synthesis (Hayat et al., 2012). During the *de novo* synthesis proline is synthesized by two major enzymes pyrroline-5-carboxylate synthase (P5CS) and pyrroline-5-carboxylate reductase (P5CR). P5CS has two isoforms being one found in the chloroplast and another in the cytosol. These enzymes are responsible for converting glutamate to glutamate-semialdehyde, which spontaneously releases a H_2O molecule and is converted to delta1-pyrroline-5-carboxylate. This intermediate is then converted by P5CR into proline. Proline synthesis can occur in the cytosol or chloroplasts and results in the oxidation of NADH and NADPH molecules. In addition, proline can be synthesized by another pathway by the action of ornithine aminotransferase (OAT) in the mitochondria matrix (Liang et al., 2013; Cabassa-Hourton et al., 2016).

Independent of its biosynthetic origin, proline may act in different ways during stress establishment, such as chemical chaperone (Arakawa and Timasheff, 1985), heavy metal chelator (Kaul et al., 2008), and ROS quenching (Kaul et al., 2008). Since it is synthesized in chloroplasts and degraded in mitochondria, proline metabolism may be important to maintain the NAD(P)/NAD(P)H ratio within these organelles maintaining the redox homeostasis of the cell (Liang et al., 2013). Degradation of proline occurs in the mitochondrial matrix through the enzymes proline dehydrogenase (ProDH) and pyrroline-5-carboxylate dehydrogenase (P5CDH). ProDH presents two isoforms to ProDH1 and 2 (Schertl et al., 2014). While ProDH 1 is mitochondrial and more abundant, ProDH 2 is almost non-expressed and found most frequently in vascular tissues (Schertl et al., 2014). ProDH converts proline to delta1-pyrroline-

5-carboxylate, which is spontaneously converted to semialdehyde glutamate which is converted by P5CDH into glutamate (Schertl et al., 2014). The glutamate formed from the proline degradation can be used by different routes (i) it can be transported to the chloroplast where it is used in nitrogen assimilation; (ii) remains in the mitochondrial matrix and then is metabolized via GABA shunt returning to the TCA cycle as succinate; (iii) additionally, still in the matrix, glutamate can be converted to 2-OG by the action of glutamate dehydrogenase (GDH-NADH) and thus return to the TCA cycle to be converted into succinyl-CoA by 2-OGDH (Fait et al., 2007a; Araújo et al., 2012b; Schertl et al., 2014).

During proline degradation, reduced coenzymes are produced in the mitochondrial matrix in the form of NADH and $FADH_2$. This indicates that proline serves as both as an electron source for the electron transport chain during the recovery of abiotic stresses, and as a source of intermediates for the maintenance of the TCA cycle (Liang et al., 2013; Schertl and Braun, 2014; Hildebrandt et al., 2015; Cabassa-Hourton et al., 2016).

Based on the above, the objectives of this work were: (i) to evaluate the role of TCA cycle enzymes during the establishment and recovery of drought stress; (ii) understand the role of the subunit E1 of 2-OGDH during the stress response; and (iii) characterize the role of 2-OGDH in proline degradation and stress release in *A. thaliana*.

MATERIALS AND METHODS

Plant material

In this study, two T-DNA-insertion lines for *E1-OGDH1 gene* (At3g55410) encoding the E_1 subunit of the 2-OGDH complex were used. These lines were obtained from the Salk collection (Salk Institute for Biological Studies, La Jolla, USA). For the *E1-OGDH1* gene, the lines *e1-ogdh1.1* (Salk_088518) and *e1-ogdh1.2* (Salk_072343) were selected. After screening, knockout lines were isolated and homozygous plants were selected as previously described (Condori-Apfata, 2016).

These two lines for the gene At3g55410 (*e1-ogdh1.1* and *1.2*) were previously characterized under optimal environmental conditions, and they were

described as presenting significantly reduced activity of the 2-OGDH complex when compared to plants WT (condori-Apfata, 2016

Growth conditions

Seeds from *A. thaliana* (ecotype Columbia 0) and homozygous mutant lines were germinated on commercial substrate Tropstrato® under short day conditions (8 h light / 16 h dark) with an irradiance of 150 µmol photons m^{-2} s^{-1} 22°C in light and 20°C in dark, with a relative humidity of 60%. Ten days after sowing, the seedlings were transplanted into 500 g pots containing 400 g of substrate and were grown for four weeks under the same conditions where seeds were germinated. Three seedlings were transplanted per pot, one of each genotype (WT, e1-ogdh1.1, and e1-ogdh1.2). Each pot constituted an experimental unit.

Irrigation management was carried out via soil, following a method of moisture control by weight as described for Marouelli et al. (2011). In this method the substrate humidity is analyzed by the following formula:

$$Ua(\%) = ((M2-M1)/M1)*100$$

Where: Ua% is the current substrate moisture in percentage (%mass). M2 is the current mass of the substrate, and M1 is the dry mass of the substrate and it was obtained before the experiment by oven drying 400 g of the substrate for 72 hours at 100 °C before the experiment installation.

Following this method, the humidity of the pots was checked daily in the predawn throughout the experiment. During four weeks after transplanting, the moisture of the pots was maintained at 90% of the water retention capacity of the substrate. After this period, the plants were submitted to stress due to water deficit through the suspension of irrigation. Also in the predawn, during the whole experiment, chlorophyll *a* fluorescence parameters were monitored (F_v/F_m, F_o, and F_m). This analysis was performed using the portable chlorophyll fluorometer MINI-PAM (WALZ).

The period of water deficit lasted a total of 22 days, which were followed by three days of recovery. During the experiment, five harvests were carried out in the middle of the light period, being three during the stress period (0, 21 and 22 days of stress) and two within the recovery period (24 and 72 hours of

recovery). The whole *Arabidopsis* rosettes were collected and snapped frozen in liquid nitrogen. Afterward, they were ground and aliquoted for further analysis. The harvest points were determined regarding the results of a pilot experiment where we observed how many days would be necessary to cause a reduction and recovery of the F_v/F_m in our plants.

Determination of relative water contents

At the five harvesting points, the relative water content (RWC) of plants was determined. For this, a fully expanded leaf of each plant was detached from the rosette and immediately weighed to determine the fresh weight (FW). This leaf was then deposited on petri dish with deionized water for 24 hours and then weighed to determine the turgid weight (TW). Thereafter, this leaf was oven dried at 70 °C for 72 hours and then weighed to determine the dry weight (DW). Having these data the RWC was determined using the following formula: RWC (%) = [(FW-DW) / (TW-DW)] * 100.

Sample extraction

The whole rosette was collected in the middle of the light period, and for the biochemical analysis an ethanolic extraction was performed as described by Cross et al. (2006)Briefly the plant material was ground in liquid Nitrogen, and aliquoted in 20 mg into 1.5 mL tube. Subsequently, it was added 250 µL of 98% ethanol and the tubes were heated at 80°C for 20 minutes.

After heating, all tubes were centrifuged for five minutes at 14000g/5ºC and then the supernatant (S1) was transferred to a new two ml tube. The pellet was resuspended in 250 µL of 80% ethanol and then heated for an additional 20 minutes at 80°C. After cooling, the material was centrifuged for five minutes at 14000g/5ºC. The supernatant (S2) was then deposited together with the S1. The pellet was then resuspended in 250 µl of 50% ethanol, again heated at 80°C for 20 minutes and centrifuged for five minutes at 14000g/5ºC to obtain supernatant S3. Together the three supernatants were kept on ice to determine the chlorophyll content and then stored in a freezer at -20°C. The remaining pellet was also stored in a freezer at -20ºC for further analysis.

Metabolite analysis

The levels of glucose, fructose and sucrose were determined in the ethanol soluble fraction, and starch in the insoluble fraction exactly as described by Fernie

et al. (2001). The levels of protein and amino acids were determined exactly as Bradford, (1976). Malate and fumarate contents were determined following the protocol described by Nunes-nesi et al. (2007). Proline determination was performed according to the protocol described by Carillo et al. (2011). The chlorophyll levels were determined as described by Porra (1989). All metabolite levels were expressed in a dry weight base.

Analysis of enzymatic activities

Aliquots of about 50 mg of ground material were used for the protein extraction with 1 mL of the extraction buffer containing 50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 1mM aminocaproic acid, 1 mM EGTA, 0.25% (w/v) Bovine serum albumine, 1% (v/v) Triton-X100, 0.25 mM DTT, 1 mM phenylmethylsulfonylfluoride, 17.4% (v/v) glycerol. The last three components were added just prior to extraction (Gibon et al., 2004).

The total enzymatic activity of glucokinase, fructokinase, and NADH dependent glutamate dehydrogenase were analyzed as described by Gibon et al. (2004). The fumarase activity was assayed in the malate-forming direction as described previously (Omena-garcia et al. (2017). The maximum enzymatic activity of phosphoglucomutase, NADP-dependent malate dehydrogenase, citrate synthase, NAD-dependent malate dehydrogenase, and NAD-dependent isocitrate dehydrogenase were obtained as described by (Hummel et al., 2010)

Germination assay

To analyze the effect of proline and mannitol in the establishment of seedlings of WT and mutant lines, an experiment was set out in which the treatments were MS medium, MS medium + 50 mM of proline, as described for Schertl et al. (2014) and MS medium + 50 mM of mannitol to confirm that the effect of 50 mM of proline was not being caused as a response to the osmotic stress caused by the 50 mM concentration. Seeds from *A. thaliana* (ecotype Columbia 0) and homozygous mutant lines were surface-sterilized and incubated for four days at 4°C in the dark on agar plates containing half-strength MS medium (Murashige and Skoog, 1962) supplemented with sucrose 1%, proline, and mannitol. Seeds were then germinated and grown under short-day conditions (8 h light/16 h dark) with an irradiance of 150 µmol photons m^{-2} s^{-1}, 22°C and 20°C in the light and dark respectively, and 60% relative humidity. After 15 days

under these conditions, we analyzed the percentage of chlorotic seedlings. Each plate contained 40 seed of each genotype and represented one experimental unit.

Statistical analysis

The experiment was set up in a completely randomized design where each pot containing three plants, one of each genotype, constituted an experimental unit. At each harvest point, six pots were collected totaling six replicates of each genotype by collection. In the germination assay, five independent plates of each treatment were analyzed. Statistical analysis was performed with Student's *t*-test through the Excel-2013 software (Microsoft, Seattle). The term 'significant' was applied only when the *t*-test confirmed a value of $p \leq 0.05$ between the samples compared.

RESULTS

Effect of proline on the establishment of WT and mutant seedlings

The percentage of chlorotic seedlings after germination in medium supplemented with 50 mM of proline revealed that high concentrations of proline have negative effects on the establishment of plants from all genotypes when compared to the other treatments (50 mM mannitol and half strength MS medium). However, when germinated in medium with 50 mM proline, mutant plants with low expression of 2-OGDH enzyme present significantly higher values of chlorotic seedlings, about 30%, when compared to WT plants (Figure 1).

The percentage of chlorotic seedlings in the mannitol treatment (Figure 1) did not exceed 6% in WT plants being less than 2% in the two mutant lines. In this treatment, as in the control treatment, there was no significant difference between the genotypes.

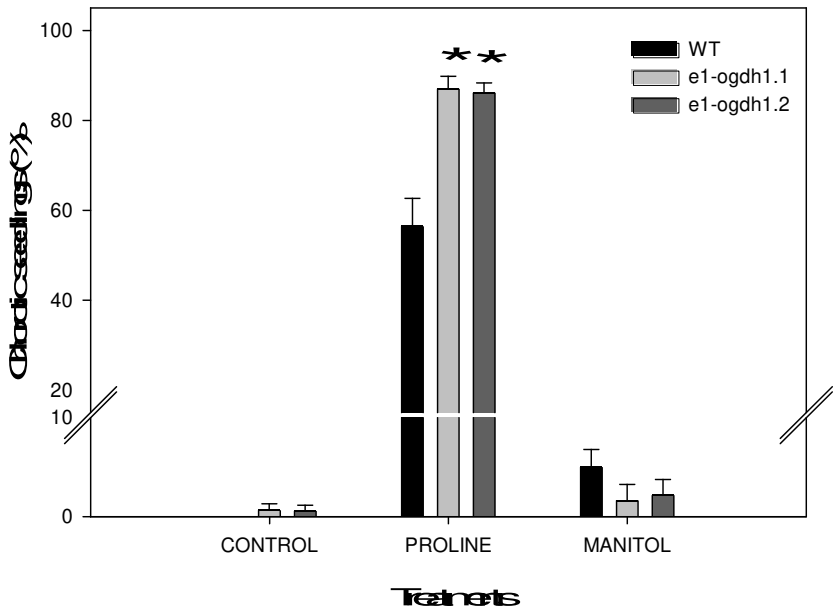

Figure *1*: Effect of 50 mM of proline and mannitol on the establishment of seedlings of WT plants and plants with reduced expression of 2-OGDH. Values are mean ± SE of 5 independent samples. (*) indicate a significant difference in relation to WT as determined by Students *t*-Test ($p < 0.05$).

Monitoring and control of soil moisture during the experiment

The method used to determine the soil moisture was the method of control by weight. The pots started the experiment with a humidity corresponding to 97% of their water retention capacity. After 22 days without water supply, the pots displayed a reduction of their humidity reaching 16% (Figure 2). During the recovery period, it was possible to observe a fast recovery of the initial substrate moisture, reaching 80% of humidity 12 hours after irrigation, with humidity of 91% at 72 hours after rehydration.

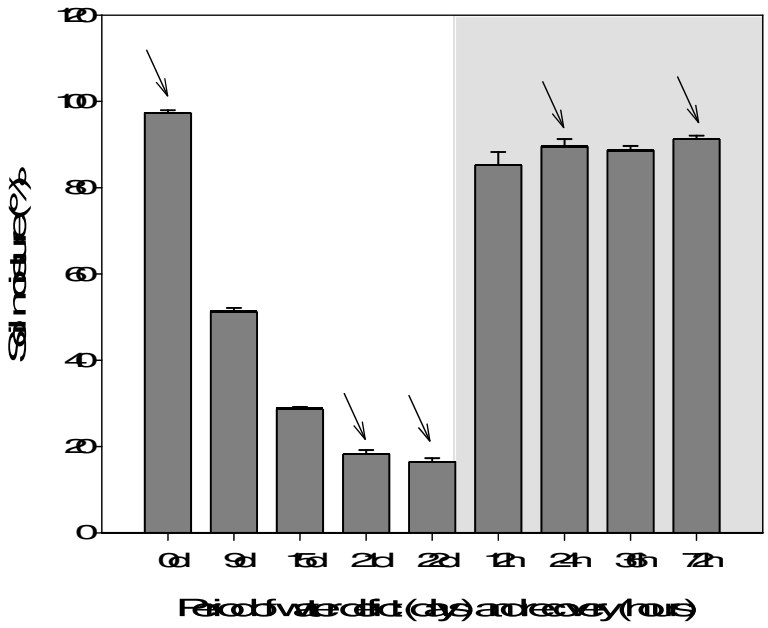

Figure 2. Soil moisture during the water deficit and recovery period. Values are mean ± SE of 12 independent pots. Arrows indicate the dates that the harvests were made. The shaded area represents the recovery period.

RWC, chlorophyll *a* and chlorophyll levels during drought and recovery period

When exposed to water deficit conditions there was a significant reduction of the RWC of both WT and the mutant plants (Figure 3A). No difference was observed between mutant and WT genotypes except after 72 hours of rehydration. After 22 days without water, and soil moisture of 17%, there was a significant reduction in the RWC of both e1-ogdh1.1 and e1-ogdh1.2 mutants (16 and 22% respectively) and of WT (25%) when compared with the beginning of the experiment.

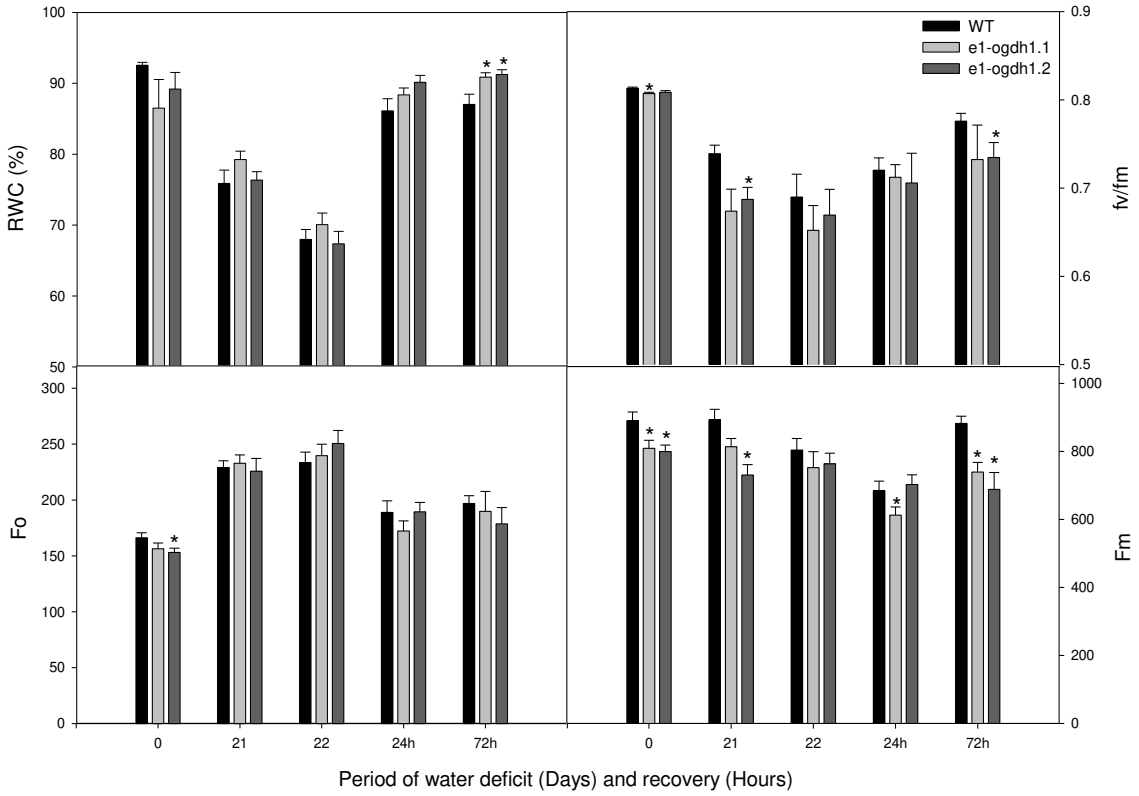

Figure 3. Effect of water deficit and its recovery period on relative water content (RWC; A), F_v/F_m (B), F_o (C) and F_m (D) in WT plants and plants with reduced expression of 2-OGDH. Values are means ± SE of 6 independent plants. (*) indicate a significant difference in relation to WT as determined by Students *t*-Test ($p < 0.05$). The shaded area represents the recovery period.

Similarly to the RWC, the values of F_v/F_m decreased during the water deficit period for all genotypes (Figure 3B). After 22 days without irrigation the e1-ogdh 1.1, 1.2 mutants and WT Plants displayed a reduction in F_v/F_m of 19.2%, 17.2%, and 15.2%). Respectively. During the recovery period, after 24 hours, it was possible to observe an increase in F_v/F_m values in all genotypes. although after 72 hours of recovery the F_v/F_m values had not returned to the initial standards.

F_o and F_m analysis allow the evaluation of the PSII integrity (F_o) as well as the intensity of the non-photochemical quenching of the plants (F_m). During the water deficit period, the reduction of the F_v/F_m ratio, observed for all genotypes, was a major consequence of the increase in F_o (around of 54%, Figure 3C) instead of the decrease of F_m (around of 7.0%, Figure 3D). During the recovery period, F_v/F_m was altered more by the change in F_m (Figure 3D).

After 24 hours of recovery, the values of F_o had already returned to values near to the ones found before of the stress application, while the F_m values start to show signs of recovery only with 72 hours of recovery.

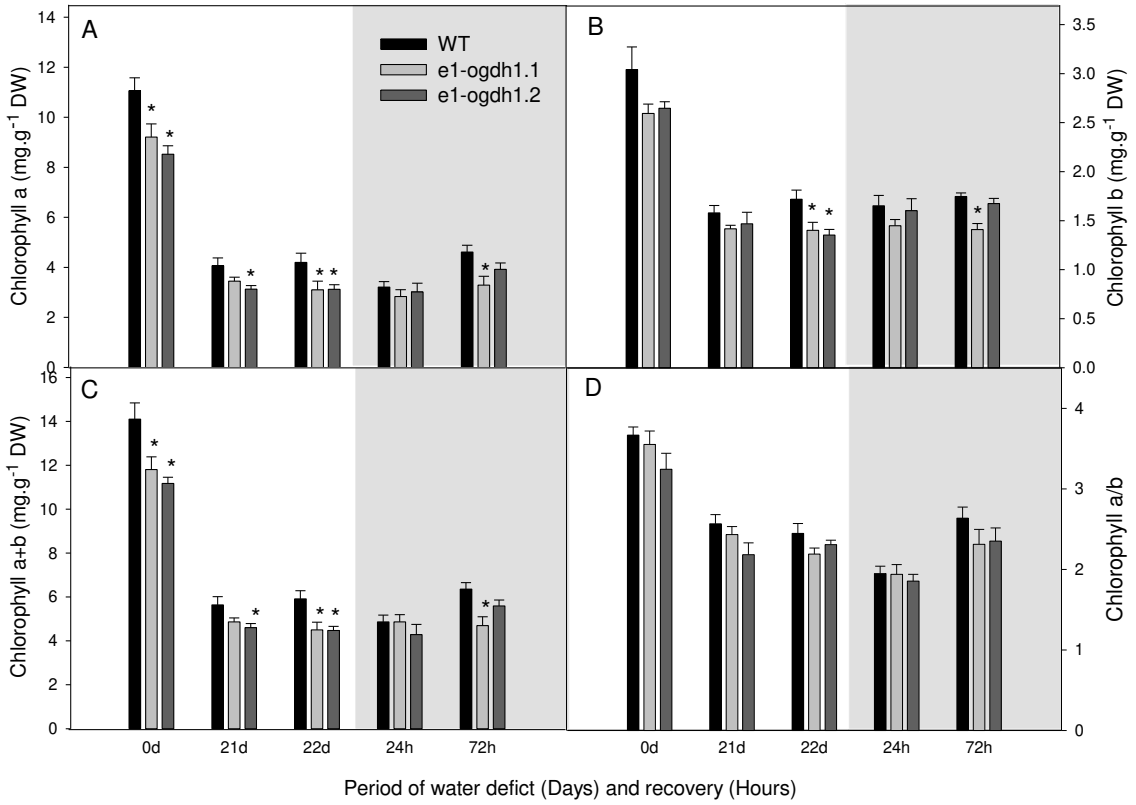

Figure 4. Effect of water deficit and its recovery on the levels of: Chlorophyll *a* (A), chlorophyll *b* (B), chlorophyll *a + b* (C) and chlorophyll *a/b* ratio (D) in WT plants and plants with reduced expression of 2-OGDH. Values are mean ± SE of 6 independent samples. (*) indicate a significant difference in relation to WT as determined by Students t-Test ($p < 0.05$). The shaded area represents the recovery period.

The quantification of chlorophyll *a* levels (Figure 4A) revealed that its content before the application of water deficit were significantly lower in the mutant plants, as well as the levels of total chlorophyll (Figure 4C). With 22 days without water, a great reduction of total chlorophyll levels (60%), as well as the chlorophyll *a/b* ratio (33%), was observed for all genotypes (Figure 4C and D). The reduction in both parameters over time occurred because of the more intense decrease found for chlorophyll *a* (60%) (Figure 4A) than for chlorophyll *b* (43%; Figure 4B).

At 24 hours of recovery, it was observed the lowest chlorophyll *a*/*b* ratio (Figure 4D). At this time point, the plants had the lowest chlorophyll *a* levels observed during the experiment with falls of 66% for WT plants; 59% for the line e1-ogdh1.1 and 62% for the line e1-ogdh1.2.

Effect of water deficit on carbon metabolism

During water deficiency, a reduction in the levels of starch, sucrose, and fructose was observed in all genotypes (Figure 5A, 5C and 5D). For the glucose level (Figure 5B) only minor changes were observed during the whole stress period. After 22 days of water deficit, starch levels were more reduced in the mutants lines (43.3 and 42.7% for e1-ogdh1.1 and e1-ogdh1.2 respectively) than in WT plants (25.1%; Figure 5A). The lowest level of starch, however, occurred after 24 hours of recovery.

The water deficit resulted in a drastic reduction on sucrose levels (Figure 5C). After 22 days without water supply, decreases of 85.6%, 87.7%, and 89.9% were observed for WT plants, and e1-ogdh1.1 and e1-ogdh1.2 lines, respectively. At this time point, the levels of fructose (Figure 5D) were below the detection limit.

Before water deficit, sucrose and fructose levels were lower in the mutant lines than in the WT plants. After 72h of recovery, the pattern was reestablished for sucrose and reverted for fructose. Differently from starch, sucrose and fructose levels showed signs of recovery after 24 hours of re-irrigation.

The quantification of organic acids during stress showed an increase in malate levels (Figure 5E) and a reduction in fumarate levels (Figure 5E). After 22 days of water deficit, a significant increase in the levels of malate was observed in the WT plants and mutant lines. The fumarate levels, at the first harvesting point, were higher in the mutant lines when compared to the WT plants. During stress and recovery period, the fumarate levels were below the detection limit.

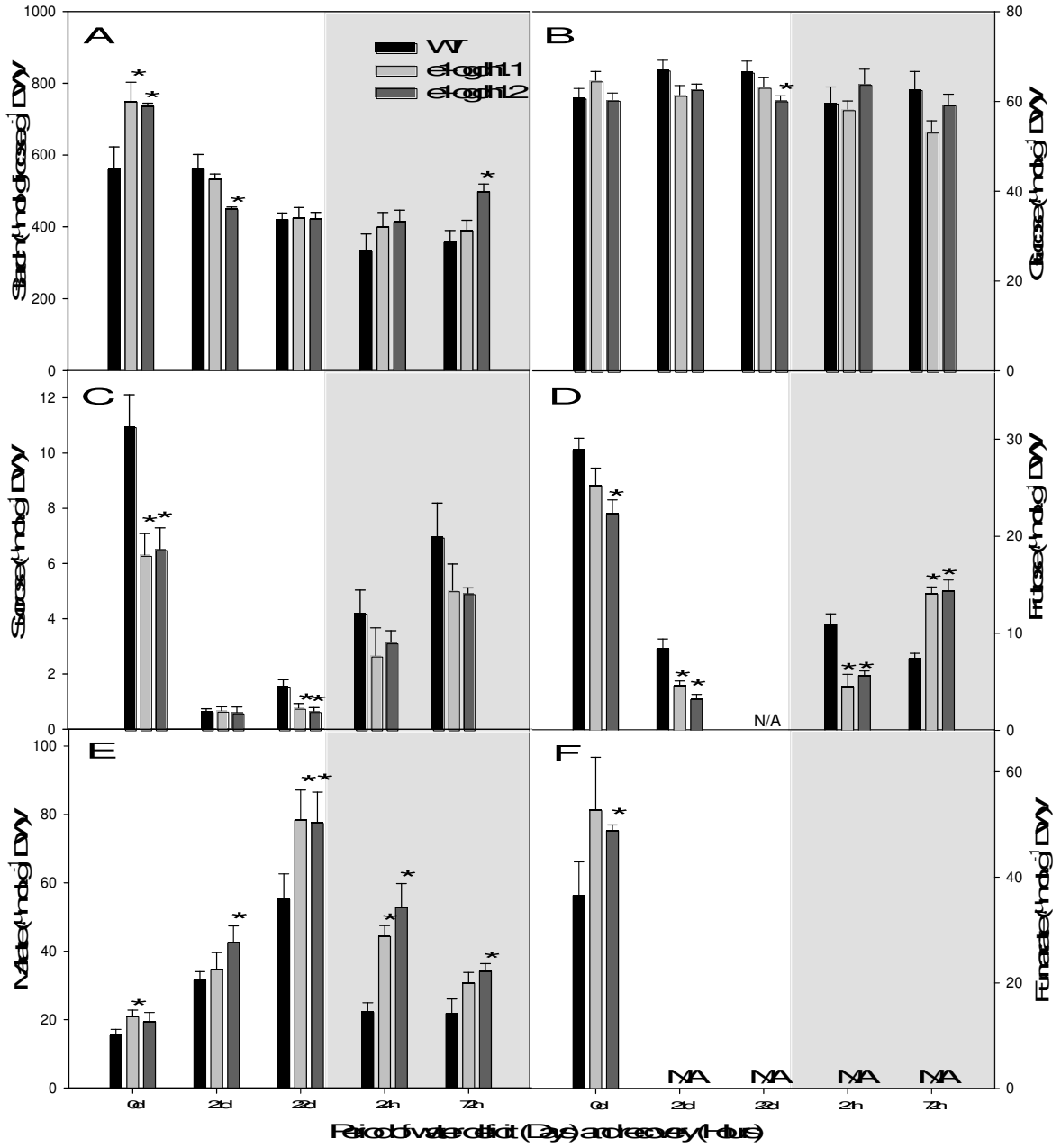

Figure 5. Effect of water deficit and its recovery on the levels of: Starch (A); Glucose (B); Sucrose (C); Fructose (D); malate (E) and fumarate (F) in WT plants and plants with reduced expression of 2-OGDH. Values are mean ± SE of 6 independent samples. (*) indicate a significant difference in relation to WT as determined by Students t-Test ($p < 0.05$). (N/A) indicates points where the metabolite levels were bellow the detection limit. The shaded area represents the recovery period.

Effect of water deficit on protein and amino acid levels

During water deficit, the protein level (Figure 6A) decreased in a range of 46.2% for WT, 51.5 and 53.7% for the mutant lines e1-ogdh1.1 and *e1-ogdh1.2* respectively. Despite the difference in the values, there were no significant differences between the genotypes in any of the analyzed points.

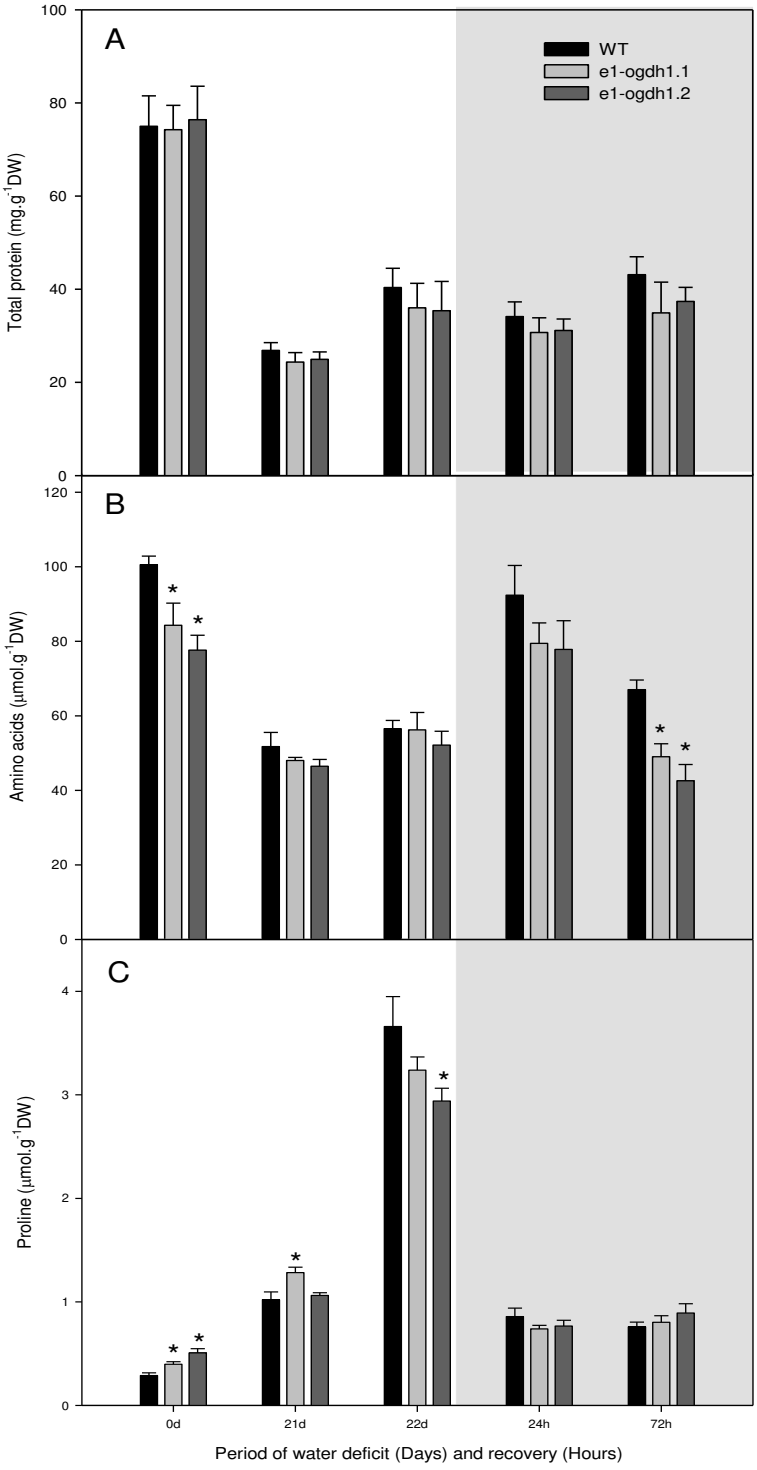

Figure *6*: Effect of water deficit and its recovery on levels of: total protein (A), amino acids (B) and proline (C) in WT plants and plants with reduced expression of 2-OGDH. Values are mean ± SE of 6 independent samples. (*) indicate a significant difference in relation to WT as determined by Students t-Test ($p < 0.05$). The shaded area represents the recovery period.

During the recovery period, it was observed that 72 hours were insufficient to reestablish the protein levels observed before the stress application. However, after 72 hours there was an increase of 26.4%; 13.7% and 20% in protein levels

of WT, e1-ogdh1.1 and *e1-ogdh1.2* mutants lines respectively when compared to 24 hours of recovery.

Analysis of total amino acid levels (Figure 6B) revealed that when exposed to water deficit both WT plants and mutant lines showed a decrease in their amino acid values. This drop was more accentuated in WT plants (43.7% of reduction) whereas the mutant lines showed reductions of 33.3 and 32.8%. It was also observed, that under optimal conditions, the amino acid levels in the mutants, e1-ogdh1.1 and 1.2 were significantly lower than those observed in WT plants. Once the plants were re-irrigated, they exhibited a fast recovery of the amino acid contents, and within 24 hours, both mutant lines and WT plants showed the same values observed before the application of stress. At 72 hours of recovery however, the amino acid levels diminished again in all genotypes.

The quantification of proline levels during the water deficit and recovery period (Figure 6C) showed that initially, the proline levels in the e1-ogdh1.1 and e1-ogdh1.2 plants were significantly higher than that observed in WT plants. After 22 days without water, the proline levels of the WT plants increased by 20.3 fold while in the e1-ogdh1.1 and e1-ogdh1.2 lines underwent a more discrete increase of 8.1 and 5.3 fold respectively. During the recovery period, after 24 hours proline levels showed a large reduction in all genotypes

Effect of water deficit on the activity of mitochondrial enzymes

Analysis of the maximal activity of TCA cycle enzymes during the deficit period (Figure 7), revealed a reduction in the activity of Citrate synthase (CS), NAD-dependent Isocitrate dehydrogenase (IDH) and Fumarase (FUM) when compared with the first time point of analysis. During the recovery, the activities of CS and IDH were reestablished to the levels observed before the beginning of the stress period. The WT plants and the mutant genotypes showed an increase in FUM activity but did not reach the values observed before the application of the water deficit. The NAD-dependent Malate dehydrogenase (MDH) (Figure 7D) maintained its activity stable during the period of water deficit and showed an increase in the mutant lines after 72 hours of recovery. In general, the activities of MDH and FUM in the control treatment, before the application of stress, were

much higher than those observed for the other TCA cycle enzymes that were analyzed.

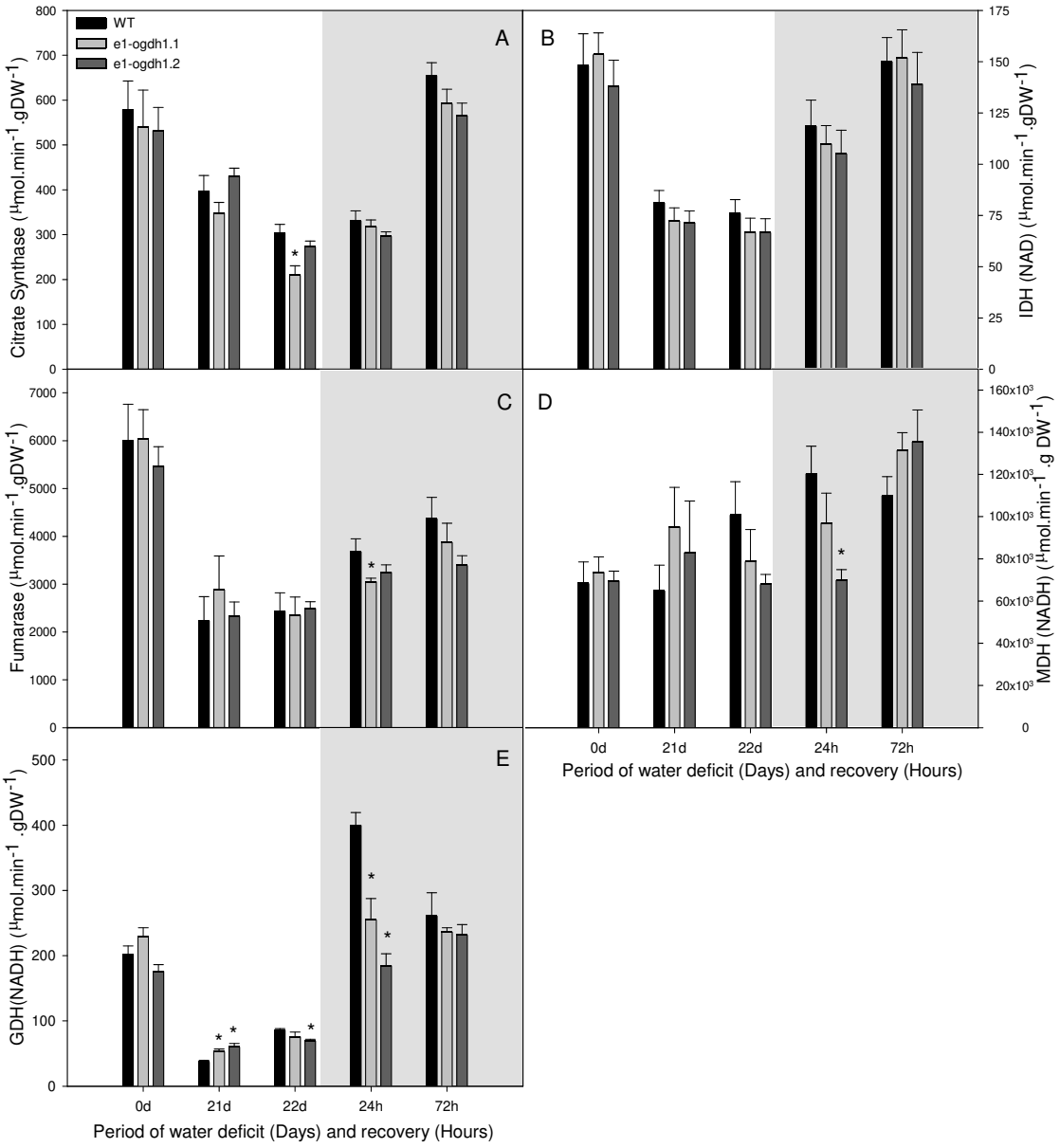

Figure 7: Effect of water deficit and its recovery on maximum activity of: Citrate synthase (A), Isocitrate dehydrogenase-NADH (IDH-NADH; B), Fumarase (C) NADH dependent Malate dehydrogenase (MDH-NAH; D) and NADH dependent Glutamate dehydrogenase (GDH-NADH; E) in WT plants and plants with reduced expression of 2-OGDH. Values are mean ± SE of 5 independent samples. (*) indicate a significant difference in relation to WT as determined by Students t-Test ($p < 0.05$). The shaded area represents the recovery period.

The effect of water deficit and recovery on the activity NADH dependent-Glutamate dehydrogenase (GDH) was also evaluated (Figure 7E). GDH showed

a reduction of its total activity in all analyzed genotypes during the water deficit period, showing the lowest values at 21 days, followed by an increase after 22 days. During the recovery period, after 24 hours there was an increase in the activity of this enzyme in all genotypes, however, the e1-ogdh1.1 and 1.2 mutant plants displayed a significantly lower activity of GDH in comparison with WT. After 72 hours of recovery, it was possible to observe a return to the initial values of this enzyme for all analyzed genotypes.

Effect of water deficit on the activity of chloroplast and cytosolic enzymes

The activities of glucokinase (GK) and fructokinase (FK) were also evaluated during our experiment. The mutant lines displayed lower GK activity when compared to the WT plants (Figure 8A). With the implementation of stress, it was possible to observe a reduction in the maximum activity of GK in the WT plants as in the e1-ogdh1.1 and 1.2 mutants. After suffering reduction during the stress period, it was observed an increase in the activity of GK during the recovery.

The FK (Figure 8B) sustained its activity until 21 days of stress and showed a reduction of its activity after 22 days of water deficit. At this time point, the e1-ogdh1.1 and 1.2 lines displayed a major reduction in the activities.

The PGM activity initially was higher in the mutants when compared to WT plants (Figure 8C). In the course of stress, there was a reduction in the activity of about 50% for all genotypes studied. During the recovery period, after 72 hours, the plants showed a slight increase in their activity for all genotypes studied but did not reestablish the values observed before the water deficit application.

The total NADP-MDH activity (Figure 8D) was significantly lower for e1-ogdh1.1 and 1.2 mutant lines when compared to WT plants at the beginning of the experiment. In the course of stress, a drop in maximum activity was observed at 21 days, followed by an increase for all genotypes after 22 days. The increase was much more expressive for the WT plants. With 22 days of water deficit, a significant difference between mutant lines and the WT plants was observed. The lowest value of total NADP-MDH activity was verified during the recovery period. 72 hours after rehydration, the total NADP-MDH activity showed a decrease in its

maximum activity of 64, 65 and 63% for the WT and e1-ogdh1.1 and 1.2 mutant plants respectively

The determination of the initial activity of NADP-MDH revealed a significantly lower activity in the mutant lines and a tendency of drop during the stress period for all genotypes analyzed (Figure 8E). After 22 days without water supply, the plants showed a decrease of 56%, 19% and 27% for WT, e1-ogdh1.1, and e1-ogdh1.2 lines, respectively. During the recovery period, at 24 hours it was possible to observe an increase of the activity, which was significantly higher for the mutant lines when compared to the WT plants. This increase was followed by a reduction reaching values lower than those previously observed in the control period.

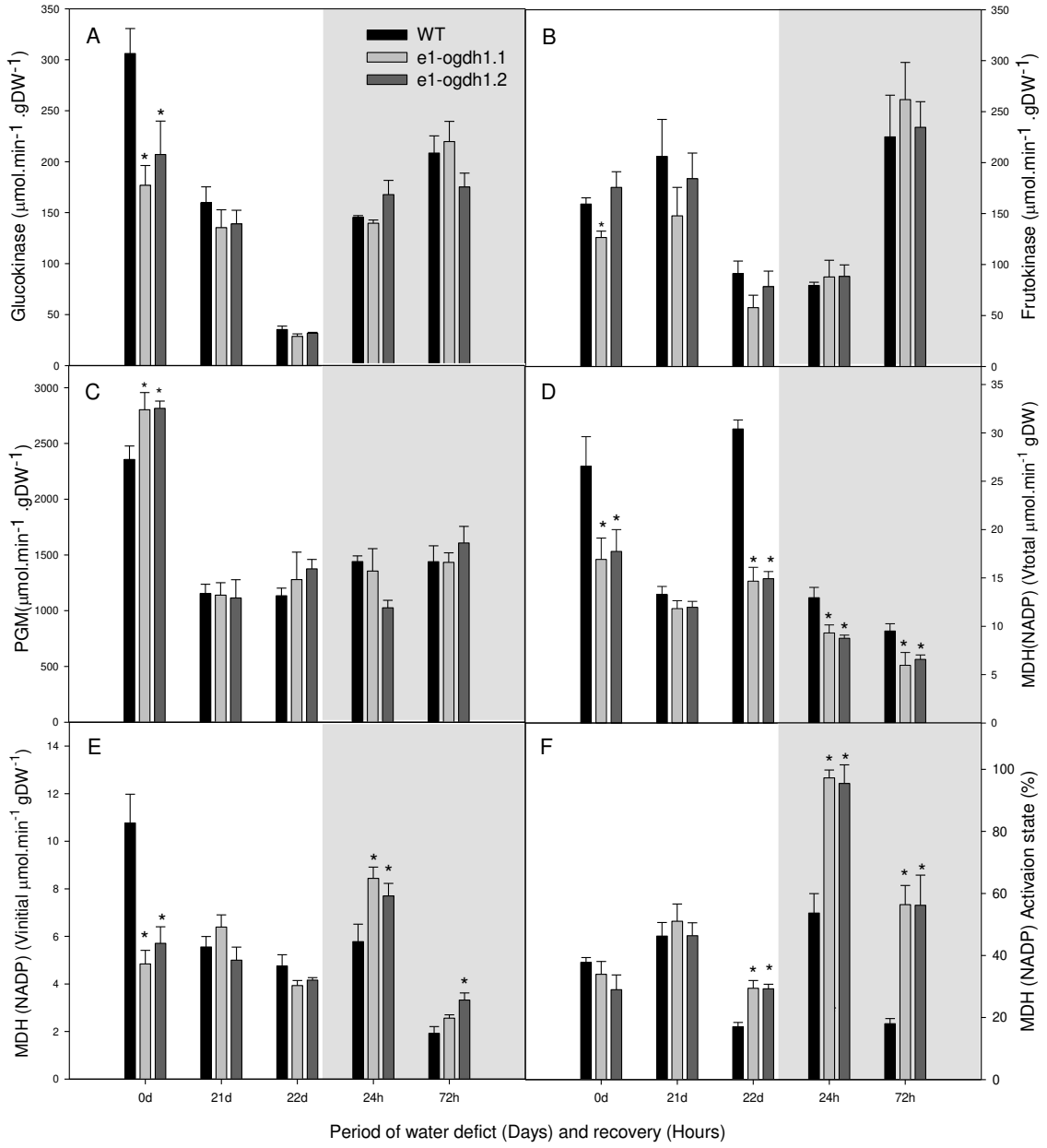

Figure *8:* Effect of water deficit and its recovery on maximal activity of the enzymes: Phosphoglucomutase (PGM; A), Glucokinase (GK; B), Fructokinase (FK; C), on the total activity of NADP dependent malate dehydrogenase (MDH-NADP; D), on the initial activity of NADP dependent malate dehydrogenase (MDH-NADP; E), and on the MDH-NADP activation state (F)) in WT plants and plants with reduced expression of 2-OGDH. Values are mean ± SE of 5 independent samples. (*) indicate a significant difference in relation to WT as determined by Students t-Test ($p < 0.05$). The shaded area represents the recovery period

The activation state of MDH-NADP was lower for the mutant genotypes when compared with the WT plants (10% for e1-ogdh1.1 and 23% for e1-ogdh1.2) (Figure 8F). After 21 days of water deficit, the activation state was increased for both mutant genotypes (50% e1-ogdh1.1 and 60% e1-ogdh1.2), without changes for WT plants when compared to the beginning of the experiment.

After 22 days without water supply, an expressive decrease of the activation state for the WT plants was observed which was significantly lower than the activation state found for our mutant lines that had the same activity as at the beginning of the experiment.

During the recovery period, we observed an increase in the activation state in all genotypes after 24 hours, followed by a decrease after 72 hours. At this both points, the mutant lines showed an activation state about 40% higher than the WT plants. After 72 hours of recovery, neither the activities nor the reasons had returned to the values found before the application of the stress.

DISCUSSION

The reduction of 2-OGDH activity cause changes in the nitrogen metabolism in plants during water deficit periods

Considered as an osmoprotective molecule, proline is known for its accumulation in several plants exposed to numerous stress conditions (Hayat et al., 2012; Obata and Fernie, 2012; Liang et al., 2013). The lower accumulation of proline in the mutant lines (Figure 6C), together with the significantly higher activity of GDH-NADH during the stress period presented in our mutants (Figure 7E), and with the significantly higher percentage of dead seedlings observed in the mutants when germinated in medium with 50 mM proline (Figure 1) make possible infer, that the enzyme 2-OGDH participate in the metabolism of degradation of proline and that the reduction in the activity of 2-OGDH decreases the tolerance of the plants to high levels of proline during the stress implementation.

During the recovery period, plants from all studied lines reestablished the initial values of proline, close to those found before the application of stress (Figure 6C). Also during the recovery, it was observed a large increase in the GDH activity, which was significantly lower in the mutant lines, e1-ogdh1.1 and 1.2. Together with the amino acid level at 24 hours of recovery (Figure 6B) and the tendency of higher activities found in the control plants for the FUM and MDH enzymes in that same period (Figure 7C and D) allowed to confirm that while in control plants, the recovery from periods of water deficit probably resulted in the reinsertion of proline into the TCA cycle directly at the 2-oxoglutarate level, in the

mutant lines the final step of proline degradation may have been transferred out of mitochondria and possibly favored the amino acids synthesis.

The reduction of protein levels observed (Figure 6A) has been described for several types of abiotic stress (Bray, 1993; Bray, 1997) , such as water deficit (Pires et al., 2016), nutritional deficiency, shading, osmotic stress, and high temperature (Ishihara et al., 2015). The reduction of protein can happens both by the reduction of protein synthesis, since it requires energy (Vries, 1975), and by the activation of its degradation in order to remobilize amino acids and nitrogen, as well as maintaining respiratory processes once they can be used as an alternative source of carbon (Araújo et al., 2011). The degradation of proteins can also be correlated with the increase of proline levels. After 22 days of stress, an increase in protein content was observed and this level was maintained during the recovery period in all analyzed genotypes.

Following the same pattern observed for proteins, a reduction was also observed for the amino acid levels (Figure 6B). Surprisingly the accumulation of total amino acids is described as an important mechanism to water deficit tolerance (Planchet et al., 2011; Pires et al., 2016) However, according with Hummel et al. (2010), water deficit experiments are generally performed with seedlings or fully grown plants, using fast and intensive osmotic stresses or even using shorter periods of time under stress than the one analyzed in this study. Thus, given the long time of the evaluation, the reduction in amino acid levels found in here, may be related to its degradation for use as intermediates in other important pathways such as the TCA cycle (Berg et al., 2002; Araújo et al., 2011; Hildebrandt et al., 2015).

The effect of reduced 2-OGDH activity during stress establishment and stress release

In addition to serving as substrates for respiration reactions and energy production, sugars (sucrose, glucose, and fructose) play a role in the regulation of metabolism. They can act as signaling molecules of environmental conditions in order to prevent the plant from going through periods of intense carbon starvation when exposed to different environments including water deficit, by regulating gene expression and posttranslational alterations (Chaves et al., 2009; Muller et al., 2011). The decrease in sucrose contents (Figure 5C) observed

during the stress period may be related to the maintenance of glucose levels that remained unchanged during the stress period (Figure 5B) (Pereira et al., 2018). Glucose may have been used as a plant response mechanism to reduce osmotic potential in order to avoid water loss to the environment (Thalmann and Santelia, 2017)

The decrease in fructose levels (Figure 5D) is probably related to the maintenance of glycolysis during the stress period. By analyzing the activities of FK (Figure 8C) and GK (Figure 8B), it was observed that GK activity showed a continuous decrease during the period of water deficit correlating with the sustained levels of glucose that was probably coming from starch and sucrose degradation (Figure 5A and C). On the other hand, the FK had its activity maintained after 21 days without water. In this period of time, the degradation of fructose occurred with the same intensity for all genotypes (Figure 5D). FK showed reduction only with 22 days of deficit when the levels of fructose were so reduced that they could not be quantified. Perhaps 22 days under water deficit were not sufficient for our plants to start glucose degradation and instead, managed to keep its levels as an osmolyte (Chaves et al., 2002).

In addition to integrating the TCA cycle, organic acids are described as having great importance for plant metabolism. They are involved in the process of opening and closing the stomata (Nunes-Nesi et al., 2007; Medeiros et al., 2016), osmotic balance and maintenance of cellular homeostasis (Hummel et al., 2010) besides being related to the absorption of different nutrients (Schulze et al., 2002). In this study, malate levels were higher for the mutant lines compared to the WT plants throughout the experiment. This fact may indicate that plants accumulated malate as an osmolyte to cope with the water deficit period. This can also be related to the greater stomatal control that mutant lines presented during a water loss curve analysis (Supplementary figure 1). The greater stomatal control may also help to explain the trend of higher photosynthetic stress observed in our experiment through the lower F_v/F_m values found in the course of stress for mutant lines.

The pool of malate quantified in our experiment does not take into account the different organelles in which this metabolite can be synthesized. Thus our results allowed us to infer that, initially, during day 0 and 21, FUM (Figure 7C)

present in the mitochondria could be responsible for the malate synthesis. In this study, this enzyme displayed a decrease of more than 50% in the course of the deficit associated with a large reduction in the levels of fumarate.

Analysis of the activity of enzymes involved in the metabolism of malate in different compartments revealed that malate synthesis during the stress period, probably occurred more intensively through NAD-dependent MDH (Figure 7D) present in mitochondria, peroxisome, and cytosol (Hebbelmann et al., 2012), than by the action of the NADP dependent MDH present in the chloroplasts (Figure 8E). Since the NADP dependent MDH suffered a reduction of its initial activity during the stress, differing from the NAD-dependent MDH, which showed an increase of its maximum activity with the imposition of the water deficit. The importance of the NAD-dependent MDH in the synthesis of malate is also based on three facts. (i) The conversion catalyzed by MDH is a reversible reaction. (ii) This enzyme shows a preference for the reduction of oxaloacetate (Tomaz et al., 2010). (iii) When analyzed, the activity of subsequent enzymes in the TCA cycle, CS and IDH (Figure 7A and 7B), it was observed a general reduction of its activities, and the reduction of amino acids (Figure 6B) can lead to increase of oxaloacetate levels. All this together would lead to the accumulation of oxaloacetate at the mitochondrial level and a possible reduction of the NAD/NADH ratio that would favor the reaction of MDH in the sense of malate formation in order to normalize the redox state of this organelle.

During the recovery period, the levels of malate returned to those found before the application of the water deficit, however, the activity of NAD-dependent MDH continued to increase. The other enzymes of the TCA cycle that we analyzed during the recovery period were able to return to the activity values observed before the stress period. This recuperation of the TCA cycle enzymes may explain the high activities of NAD-dependent MDH found during the recovery period.

Severe water deficit leads to photoinhibition and oxidative stress

When exposed to severe water deficit, our plants displayed a negative response in terms of F_v/F_m values (Figure 3B), and a reduction in total chlorophyll levels. This responses may be related to oxidative stress and photoinhibition processes caused by the increase in formation of ROS that leads to degradation of membranes, the release of chlorophyll from protein-pigment complexes (Urban

et al., 2017) and degradation of chlorophyll (Streit et al., 2005; Chaves et al., 2009; Gill and Tuteja, 2010; Muller et al., 2011; Murchie and Lawson, 2013; Pereira et al., 2018) what can be related with the elevation of Fo values found in our study (Figure 3C).

During the water deficit, a higher activation state of the NADP-MDH found after 22 days of water deficit in the mutant plants suggests an increase in the oxidative state of the chloroplast (Figure 8F). In addition, the water deficit probably activated photoprotection mechanisms which could be inferred by the reduction of the F_m values (Figure 3D). This pattern can be correlated with the upregulation of the xanthophyll metabolism to dissipate the excess of energy as heat, reducing damage to the photosystems (Urban et al., 2017).

During recovery, the increase in the activation state of the NADP-MDH at 24 hours of recovery suggests an increase in the redox state inside the chloroplast of E1-OGDH mutant plants. Once the maximum activity of NADP-MDH at this point suffered a great reduction for all genotypes (Figure 8D), and the fact that the mutants showed a lower water loss, indicating better control of stomatal aperture (Supplementary figure 1) we can infer that the increase in the activation state found 24 hours after rehydration can be a response to the maintenance of limitations in the biochemical phase of photosynthesis that eventually leads to a decrease in NADP/NADPH.

Much is still discussed about what happens to carbon metabolism during stress. Some studies describe that when exposed to a short or moderate water deficit, the reduction of growth occurs much faster than the inhibition of photosynthesis leading to an accumulation of metabolite of the carbon metabolism as sucrose, fructose and glucose (Hummell et al., 2010; Muller et al., 2011; Pantin et al., 2013). Additionally, it has been also described that periods of water deficit culminates with reduction of photosynthetic processes that lead to a decrease in the levels of reserve metabolites such as starch (Chaves et al., 2002). The slow imposition of severe water deficit conditions resulted in a drop for almost all analyzed carbon compounds (Figure 5). With the exception of glucose (Figure 5B) and malate (Figure 5E), 22-day water deficit led to a drastic reduction in the levels of starch, sucrose, fructose and fumarate (Figure 5A, 5C, 5D, and 5F). The water deficit also had a negative effect on the maximum activity

of enzymes involved in the metabolism of these molecules, such as PGM, GK, FK (Figure 8A, B and C) and FUM (Figure 7C).

The observed reduction in the starch levels suggests that the water deficit led to osmotic stress and a moderate carbon starvation conditions. In such conditions, the degradation of starch contributes to the maintenance of sugar levels that are important both for use as energy source as well as osmoprotection through maltose and their derived sugars (glucose and fructose) (Thalmann et al., 2016). The reduction of 50% found for all genotypes in the PGM activity during the drought period and its slight increase after 72 hours of recovery (Figure 8A) suggests that starch synthesis is impaired during the stress period and that 72 hours of recovery period is not enough to properly recover its metabolism.

CONCLUSIONS

Taken together the results obtained in this study suggest that despite the lack of 2-OGDH activity, the mutant plants are able to respond the water limitation, survive and recover from this stress as do control plants, but undergoing distinct metabolic and physiological adjustments. This work also demonstrated that some of the TCA cycle enzymes are downregulated in leaves during drought stress period and quickly reestablish its activity during the stress release. However further studies are necessary to fully understand the role of this pathway during stress implementation and release.

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