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Hypoxia-driven changes in glycolytic and tricarboxylic acid cycle metabolites of two nodulated soybean genotypes

Junior Borella[a,*], Halley Caixeta Oliveira[b], Denise dos Santos Colares de Oliveira[a], Eugenia Jacira Bolacel Braga[a], Ana Claudia Barneche de Oliveira[c], Ladaslav Sodek[d], Luciano do Amarante[a]

[a] Department of Botany, Federal University of Pelotas, C.P. 354, 96160-000, Pelotas, RS, Brazil
[b] Department of Animal and Plant Biology, State University of Londrina, 86050-350, Londrina, PR, Brazil
[c] Brazilian Agricultural Research Corporation/Embrapa Temperate Climate Agricultural Research Center, BR 392, km 78, 96010-971, Pelotas, RS, Brazil
[d] Department of Plant Biology, State University of Campinas, C.P. 6109, 13083-970, Campinas, SP, Brazil

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ABSTRACT

Oxygen deprivation triggers changes at different levels of carbon and nitrogen metabolism, which may differ between plant-genotypes. The aim of this study was to evaluate the hypoxia-induced alterations of carbon and nitrogen metabolites in relation to alanine aminotransferase (AlaAT; EC 2.6.1.2) activity in two genotypes of nodulated soybean (*Glycine max*). Nodulated soybean plants (Fundacep 53 RR and BRS Macota) were grown in vermiculite and transferred to a hydroponic system at the early reproductive stage. The root system was subjected to hypoxia by continuously flushing the solution with N_2 gas for 24 or 72 h. For recovery, after 72 h in hypoxia, plants returned to normoxic conditions after transfer to vermiculite for 24 and 72 h. Root and nodule organic acids and amino acids were analysed by gas chromatography-mass spectrometry and high-performance liquid chromatography, respectively. Relative expression of *AlaAT* and AlaAT activity were also verified in both genotypes. Plants of Fundacep and Macota genotypes responded distinctly to hypoxia. In root tissues, Fundacep presented higher pyruvate and lactate accumulation than Macota, indicating higher glycolytic and fermentation rates. Furthermore, Fundacep responded more effectively on recovery by restoring pre-hypoxic levels of the metabolites. Although the amino acid composition did not differ between the genotypes, there was a clear link between glycolysis and the Krebs-cycle via increased gene expression and activity of AlaAT allied to succinate accumulation in roots of Fundacep. This may represents a metabolic advantage for this genotype over Macota with regard to hypoxia tolerance.

1. Introduction

Waterlogging and flooding are becoming more frequent due to heavy rainfall, a consequence of climate changes. Under these conditions oxygen supply to the roots is impaired, thus inhibiting root respiration and affecting crop growth and productivity of many species worldwide (Limami et al., 2014). Plants have shown wide variations in their ability to tolerate the limitations of oxygen concentration through a series of adaptive mechanisms to ensure their survival as a strategy to avoid or postpone the effects caused by hypoxia (Mommer et al., 2005; Bailey-Serres and Voesenek, 2008; Bailey-Serres et al., 2012; Kreuzwieser and Rennenberg, 2014). Upon hypoxia, inhibition of mitochondrial oxidative phosphorylation triggers the "Pasteur effect" leading to an increase in glycolysis to maintain ATP production and cell viability (Summers et al., 2000). In order to limit energy consumption, plants down-regulate the synthesis of storage products such as starch and protein (Geigenberger, 2003; Gupta et al., 2009). Sucrose (Suc) degradation shifts from the invertase reaction to sucrose synthase (SUS) as well as switching to other enzymes that use PPi (pyrophosphate) instead of ATP (Kumutha et al., 2008; Sairam et al., 2009; Mustroph et al., 2014a).

To keep glycolysis running under hypoxia, fermentative enzymes are rapidly activated to continuously regenerate NAD^+

Abbreviations: Ala, alanine; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; MCW, methanol, chloroform and water; OGDH, 2-oxoglutarate dehydrogenase; PPi, pyrophosphate; RT-PCR, real-time reverse transcription-polymerase chain reaction; TCA-cycle, tricarboxylic acid cycle.

* Corresponding author.
E-mail address: borellaj@gmail.com (J. Borella).

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(Licausi, 2011; van Dongen and Licausi, 2015). Using pyruvate, fermentative reactions produce lactate via lactate dehydrogenase and ethanol via two consecutive reactions catalysed by pyruvate decarboxylase and alcohol dehydrogenase (Tadege et al., 1999; Zabalza et al., 2009). In addition, many plants such as soybean (*Glycine max* L. Merrill), have been shown to accumulate alanine (Ala) under hypoxic conditions, an amino acid produced by the enzyme alanine aminotransferase (AlaAT) (Sousa and Sodek, 2003). Although the synthesis of Ala is not directly associated with the recycling of NAD^+, its production helps regulate the glycolytic flux by preventing the excessive accumulation of pyruvate (Zabalza et al., 2009). Furthermore, Ala can be accumulated in high concentrations even under nitrogen deficiency without causing any cell toxicity (Rocha et al., 2010a).

Hypoxic conditions also affect the activity of nitrogenase in nodules of nitrogen-fixing plants (Justino and Sodek, 2013) and trigger significant changes in amino acid composition (Rocha et al., 2010a), such as a considerable reduction of glutamine (Gln) synthesis (Amarante and Sodek, 2006). In addition to these changes, the increase in Ala content is accompanied by a large increase of γ–aminobutyric acid (GABA), reflecting the metabolism under hypoxia (Puiatti and Sodek, 1999; Thomas et al., 2005; Souza et al., 2016; António et al., 2016). GABA is mainly produced by glutamate decarboxylase (GAD), in a reaction involving proton consumption, thereby avoiding the deleterious effects of cytosolic acidification during hypoxia (Crawford et al., 1994).

AlaAT catalyses the reversible transamination reaction of pyruvate and glutamate into alanine and 2-oxoglutarate, linking carbon and nitrogen metabolism of plants (Rocha et al., 2010a). The connection between glycolysis and tricarboxylic acid cycle (TCA) mediated by alanine aminotransferase activity during hypoxia of *Lotus japonicus* has been proposed as a metabolic model (Rocha et al., 2010a). Under these conditions, 2-oxoglutarate resulting from the AlaAT reaction can further react within mitochondria to form succinate via 2-oxoglutarate dehydrogenase (OGDH) and succinate CoA ligase, leading to ATP production and succinate accumulation under hypoxia (Rocha et al., 2010a; António et al., 2016).

It is important to emphasize that different plant species or even genotypes show significant variation in their level of tolerance to low oxygen stress in order to survive (Shingaki-Wells et al., 2014). Many of these variations are related to changes in carbon metabolism (Rocha et al., 2010a; António et al., 2016). In addition, Borella et al. (2014) reported that the levels of starch, glycolytic substrates (total soluble sugars and sucrose) and fermentation metabolites, in roots and nodules, changed in a different way in soybean genotypes (Fundacep 53 RR and BRS Macota) under hypoxic conditions. However, a similar mechanism described by Rocha et al. (2010a) regarding changes in carbon and nitrogen metabolism linked by AlaAT has not been established as to whether it operates in nodulated soybean genotypes where it could underlie some hypoxic tolerance. Thus, the objective of this study was to investigate changes related to amino acids, TCA-cycle, glycolytic metabolites and AlaAT activity and expression in nodulated plants of two soybean genotypes under oxygen deficiency.

2. Material and methods

2.1. Plant material and growth conditions

Soybean plants (*Glycine max* L. Merril cv. Fundacep 53 RR and BRS Macota) were grown in a greenhouse under natural light (±1.000 μmol photons $m^{-2} s^{-1}$) and temperature conditions (28 ± 5 °C). Three plants were grown in a single plastic pot (3 l) containing vermiculite as substrate and supplied with 250 ml N-free nutrient solution twice per week (Hoagland and Arnon 1938), as described by Lima and Sodek (2003). Plants were inoculated with *Bradyrhizobium elkanii* strain SEMIA 587 (FEPAGRO) at the V0 stage (cotyledon stage) and the hypoxic treatments were carried out in a hydroponic system when plants reached the R2 stage (flowering; early reproductive stage). For soybean growth stages see Fehr et al. (1971). For hydroponic treatment plants were removed from the pots and the entire root system carefully washed in tap water to remove the excess of vermiculite before being transferred to 3 l pots (3 plants per pot) containing N-free nutrient solution at one-third of normal strength. The whole root system (including the nodules) was kept submersed in the nutrient solution. In the experiment, the nodulated root system was subjected to hypoxia by flushing N_2 gas for 24 h and 72 h, respectively. Oxygen concentration of the solution was monitored with an oxygen meter (Handylab OX1), and hypoxia in the solution was rapidly reached after 6 h from 6.30 (control) to 0.50 mg L^{-1} and kept low during the time-course of the experiment.

For recovery, after 72 h of hypoxia, plants were returned to 3 l pots containing vermiculite as substrate under normoxic conditions per 24 h and 72 h. At harvest, four biological replicates of nodules and roots were collected for each treatment and kept frozen (- 80 °C) until analysis.

2.2. Metabolite extraction and analysis

Low molecular weight metabolites were extracted from nodules and roots with 10 ml of methanol:chloroform:water (MCW) (12:5:3 v/v/v) per gram of plant material, following the procedure described by Sousa and Sodek (2003). The aqueous phase resulting from MCW extraction was used for the analysis of organic acids and amino acids. Organic acids were analysed by gas chromatography-mass spectrometry (GC–MS) using a Shimadzu QP2010plus system (Shimadzu Corporation, Tokyo, Japan) under the same conditions described by Oliveira and Sodek (2013). Individual amino acids were determined by reverse-phase high-performance liquid chromatography (HPLC) as their *o*-phthaldialdehyde (OPA) derivates based on the method described by Puiatti and Sodek (1999). The amount of total amino acids was determined by the ninhydrin method using leucine as standard (Yemm and Cocking, 1955).

2.3. Alanine aminotransferase activity assay

AlaAT enzyme activity (EC 2.6.1.2) was determined in root and nodule tissues. Plant material was ground to a powder using a mortar and pestle with 50 mM Tris/HCl (pH 7.5) containing 1 mM mM dithiothreitol. All procedures were carried out at 4 °C. The homogenate was centrifuged at 10 000*g* for 20 min, and an aliquot of the supernatant desalted using a PD10 column (GE Healthcare, Buckinghamshire, UK). Total protein content of the enzyme extract was measured as described by Bradford (1976). The eluted protein fraction was assayed for AlaAT activity as described by Sousa and Sodek (2003). The assay contained, in a final volume of 1.5 ml, 10 mM L-alanine, 5 mM 2-oxoglutarate, 0.1 mM NADH, 50 mM Tris/HCl (pH 7.5) and 5 units of lactate dehydrogenase (Sigma L5132) in a 1.5 ml cuvette. After the addition of extract, the cuvette was maintained in a spectrophotometer (T80 UV/VIS Spectrometer – PG Instruments) with a temperature-controlled cuvette-holder at 30 °C and the absorbance at 340 nm recorded at 10 s intervals.

2.4. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

To determine changes of *AlaAT* gene expression 0.2 g of soybean roots or nodules was ground to a powder in liquid nitrogen using a