Life Sciences in Space Research 8 (2016) 30–37

Contents lists available at ScienceDirect

Life Sciences in Space Research

www.elsevier.com/locate/lssr

Oxygen dependency of germinating Brassica seeds

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ARTICLE INFO

Article history:
Received 16 October 2015
Received in revised form 7 January 2016
Accepted 15 January 2016

Keywords:
Germination
Oxygen partial pressure
Gene transcription profiling
Hypoxia
Fermentation

ABSTRACT

Establishing plants in space, Moon or Mars requires adaptation to altered conditions, including reduced pressure and composition of atmospheres. To determine the oxygen requirements for seed germination, we imbibed *Brassica rapa* seeds under varying oxygen concentrations and profiled the transcription patterns of genes related to early metabolism such as starch degradation, glycolysis, and fermentation. We also analyzed the activity of lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH), and measured starch degradation. Partial oxygen pressure (pO_2) greater than 10% resulted in normal germination (i.e., protrusion of radicle about 18 hours after imbibition) but lower pO_2 delayed and reduced germination. Imbibition in an oxygen-free atmosphere for three days resulted in no germination but subsequent transfer to air initiated germination in 75% of the seeds and the root growth rate was transiently greater than in roots germinated under ambient pO_2. In hypoxic seeds soluble sugars degraded faster but the content of starch after 24 h was higher than at ambient oxygen. Transcription of genes related to starch degradation, *α-amylase* (*AMY*) and *Sucrose Synthase* (*SUS*), was higher under ambient O_2 than under hypoxia. Glycolysis and fermentation pathway-related genes, *glucose phosphate isomerase* (*GPI*), *6-phosphofructokinase* (*PFK*), *fructose 1,6-bisphosphate aldolase* (*ALD*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *pyruvate decarboxylase* (*PDC*), *LDH*, and *ADH*, were induced by low pO_2. The activity of LDH and ADH was the highest in anoxic seeds. Germination under low O_2 conditions initiated ethanolic fermentation. Therefore, sufficient oxygen availability is important for germination before photosynthesis provides necessary oxygen and the determination of an oxygen carrying capacity is important for uniform growth in space conditions.

1. Introduction

Establishing human presence in space or eventually on other worlds requires cultivating plants under conditions that most probably do not resemble the conditions on Earth. Because of cost and mass considerations, plants will have to be grown under hypobaric conditions (Richards et al., 2006), different atmospheres, and less than optimal temperatures or different gravity levels. Changes in gravitational acceleration, in the extreme case weightlessness, and fluctuations of contaminants because of human interference in closed systems (International Space Station, future outposts on Moon or Mars) will also affect plant performance.

Because access to space is rare and expensive, and expectations for space experiments are high, one might be tempted to grow plants in tight places at high density, and neglect that the planting density affects plant performance. Large numbers of seeds require more oxygen and oxygen delivery may be limited because of space conditions. For example, weightlessness prevents water run-off and water may form a diffusive barrier that reduces gas exchange compared to ground conditions and leads to hypoxia (Musgrave, 2002). The lack of buoyancy leads to hypoxia in the root zone (Stout et al., 2001) and differences in seed development between weightlessness one-g and hypergravity indicate that developmental processes are sensitive to gravity (Kuang et al., 2005; Musgrave et al., 2009). Therefore, it is necessary to understand the effect of hypoxia on plants in conjunction with space requirements and related growth conditions such as elevated levels of ethylene that are routinely observed in closed (space) systems (Kiss et al., 2000). Ethylene in turn reduces gas exchange (He and Davies, 2012); in addition, ethylene responsive transcriptional activators are involved in oxygen sensing (Voesenek and Bailey-Serres, 2013).

Although some plants can germinate under anoxic conditions, such as rice (Alpi and Beevers, 1983; Horton, 1991), barnyard grass

Abbreviations: ADH, alcohol dehydrogenase; ALD, fructose 1,6-bisphosphate aldolase; AMY, α-amylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glucose phosphate isomerase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; PFK, 6-phosphofructokinase; pO_2, partial pressure of oxygen; SUS, sucrose synthase.

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http://dx.doi.org/10.1016/j.lssr.2016.01.002
2214-5524/

(Rumpho and Kennedy, 1981), and the coral tree (Small et al., 1989), oxygen demand is high for germination of most seeds and the oxygen requirement for seeds is much higher than for embryos, suggesting that the embryo-surrounding tissue represents a barrier for oxygen diffusion (Bradford et al., 2008). The rate of germination of fatty (dicot) seeds is linearly dependent on partial pressure of oxygen (pO_2) between two and 21 kPa (Alani et al., 1985). The germination in starchy (monocot) seeds is possible at lower pO_2 (Alani et al., 1985; Heichel and Day, 1972).

During seed germination, O_2 deficiency inhibits mitochondrial respiration (Alani et al., 1985) and fermentation becomes the cell's primary means of ATP production for growth (Good and Muench, 1993; Kennedy et al., 1992). Fermentation depends on three metabolic pathways in plant starch degradation, glycolysis, and pyruvate fermentation (PMN, http://plantcyc.org/), (Magneschi and Perata, 2009; Perata et al., 1997; Ricard et al., 1994; Tadege et al., 1999). The enzymes in this pathway include sucrose synthase (SUS, EC 2.4.1.13) α-amylase (AMY, EC 3.2.1.1), glucose phosphate isomerase (GPI, EC 5.3.1.9), 6-phosphofructokinase (PFK, EC 2.7.1.11), fructose 1,6-bisphosphate aldolase (ALD, EC 4.1.2.13), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), lactate dehydrogenase (LDH, EC 1.1.1.27), pyruvate decarboxylase (PDC, EC 4.1.1.1), and alcohol dehydrogenase (ADH, EC 1.1.1.1). The effect of O_2 deficiency on these catalysts have been described in the maize root (Gerlach et al., 1982; Kelley, 1989; Sachs et al., 1980), rice embryos (Ricard and Pradet, 1989) and seeds (Perata et al., 1992), soybean roots (Russell et al., 1990), and seedling of barley, wheat, rye, and triticale (Park et al., 2005), and these enzymes are involved in pathways that mobilize sucrose or starch to maintain energy production under anaerobic conditions (Kennedy et al., 1992; Umeda and Uchimiya, 1994).

Pyruvate is converted by the catalytic action of Lactate dehydrogenase (LDH) whereas ethanol synthesis is the consequence of the decarboxylation of pyruvate to acetaldehyde by PDC, followed by the reduction of acetaldehyde to ethanol, which is catalyzed by ADH (Magneschi and Perata, 2009; Tadege et al., 1999). The anaerobic induction of the enzyme activities is accompanied by a large increase in the transcripts of *LDH, PDC* and *ADH* (Gerlach et al., 1982; Kelley, 1989; Sachs et al., 1980). Lactate and ethanol are prominent end products during anaerobic metabolism (Roberts et al., 1985; Smith and Rees, 1979) and lactate accumulation contributed to cytosolic acidification under anaerobic condition (Roberts et al., 1985).

Although fermentation in roots and seeds of diverse species of monocot plants is well documented, little is known on response of dicot seeds to hypoxia. Brassica spec. are representative for this group are not only economically important but as a result of their enormous diversity likely to provide nutrition for space applications. Recent attempts to characterize the transcriptome under waterlogging (Zou et al., 2013) indicate that studies on hypoxia are not limited for space applications but have commercial significance as well.

We studied the response of *Brassica* seed under hypoxic conditions and correlated the rate of germination with pO_2. Transcription profiling of genes related to fermentation and measurement of essential components in seeds imbibed under diverse pO_2 describe a remarkably adaptable metabolism of germinating *Brassica* seeds.

2. Materials and methods

2.1. Plant material and RNA extraction

Batches of about 100 *Brassica rapa* seeds were incubated with degassed-water in a dark desiccator under air or nitrogen. Oxygen was added to nitrogen to establish pO_2 of 2.5, 5, and 10% O_2 at 25 °C. The incubator in turn was kept in a sealed plastic glove bag with the same pO_2. After predetermined times, seeds were removed and immediately frozen in liquid N_2 and then stored at −80 °C. Seeds incubated in anoxic conditions for 72 h were transferred to ambient O_2 condition and germination was observed for the following 48 h.

Total RNA was extracted from seeds of *Brassica* using the innuPREP RNA mini kit (Analytik Jena AG, Germany) based on the manufacturer's protocol.

2.2. Measurement of water, total soluble sugars, and starch

Seeds were weighed before and after drying at 80 °C for 72 h to determine fresh and dry weight. Water fraction is reported as WC% = (FW − DW)/FW × 100. Total soluble sugars were extracted according to (Lunn and Hatch, 1995). Dried seeds were ground in liquid N_2 with mortar and pestle and 0.5 g was extracted with 20 ml of 80% (v/v) ethanol, boiled for 1 min and vortexed for 1 min. The homogenate was centrifuged (13,200 g, 10 min) and the supernatant collected in a flask. The pellet was re-extracted in 15 ml of 50% (v/v) ethanol, kept for 10 min (RT), and centrifuged as above. This step was repeated until the extract was colorless. All extracts were combined and dried using a rotary evaporator (Rotavapor RE-111, Büchi, Switzerland). The dried sample was dissolved in 5 ml water and 5 ml perchloric acid (35%, v/v).

Starch was extracted from 0.5 g ground seeds with 10 mL acetone (Rose et al., 1991) and filtered through Whatman No. 1 filter paper. The acetone-extracted sample was mixed with 20 ml 80% ethanol and boiled in a water bath for 1 h. The sample was cooled (RT) and centrifuged (13,200 g, 10 min) and the supernatant discarded. This step was repeated until the extract was colorless. The pellet was suspended in 5 ml of 35% perchloric acid and shaken for 0.5 h. After centrifugation the supernatant was collected. This step was conducted three additional times. The combined supernatants were diluted to 25 ml with water. 0.5 ml of each sample and glucose standards (0 to 50 mg/100 ml) and 5 ml of anthrone solution (1.146 g of anthrone in 655 mL of 72.5% sulfuric acid) were mixed in 15 ml test tubes. The test tubes were boiled for 12 min and then transferred to ice water. The solution was read at 625 nm on a UV/VIS spectrophotometer. Sugars or starch content was calculated based on the glucose standards (Volenec, 1986).

2.3. LDH and ADH activity

Samples of 0.5 g were ground in 4 mL of ice-cold extraction buffer (50 mM Tris/HCl, 10 mM sodium borate, 5 mM DTT, 15% glycerol, and 5 mg ml^{-1} BSA, pH 7.5, no BSA for protein determination), centrifuged (13,000 rpm, 5 min), and the supernatant was immediately desalted using PD SpinTrap™ G-25 column (GE Healthcare, USA) equilibrated with the extraction buffer as per manufacturer's protocol. Protein in the extracts was determined (Bradford, 1976) as modified by Bouny and Saglio (1996). Enzyme activities were assayed at 25°C in 1 mL Tricine buffer (100 mM, pH 7.5). NAD reduction (for ADH) or NADH oxidation (for LDH) was recorded at 340 nm before and after the reaction to correct for nonspecific activity. For ADH, the reaction was measured in the ethanol-to-acetaldehyde direction and was initiated by the addition of 0.4 mM NAD and 100 mM ethanol. LDH activity was measured in a solution containing 100 mM pyrazole, 10 mM KCN, 0.2 mM NADH. The activity was measured in the pyruvate-to-lactate direction; the presence of pyrazole inhibited the reaction of PDC and ADH. The reaction was initiated by the addition of 12 mM pyruvate.