Plant Physiology and Biochemistry 139 (2019) 389–394

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Glyoxylate cycle activity in *Pinus pinea* seeds during germination in altered gravity conditions

Paola Faraoni[a,*,1], Elettra Sereni[b,1], Alessio Gnerucci[a,1], Francesca Cialdai[b], Monica Monici[b], Francesco Ranaldi[a]

[a] Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Viale Pieraccini 6, I-50139, Florence, Italy
[b] ASAcampus Joint Laboratory, ASA Research Division & Department of Experimental and Clinical Biomedical Sciences "Mario Serio", University of Florence, Viale G. Pieraccini 6, I-50139, Florence, Italy

ARTICLE INFO

Keywords:
Microgravity
Hypergravity
Random position machine
glyoxylate cycle
isocitrate lyase
Seed germination

ABSTRACT

This work inserts in the research field regarding the effects of altered gravity conditions on biological plant processes. *Pinus pinea* seeds germination was studied in simulated microgravity ($2x10^{-3}g$) and hypergravity ($20g$) conditions.

The effects of simulated gravity were evaluated monitoring the levels of the key enzymes, involved in the main metabolic pathway during germination process of lipid–rich seeds (oilseeds): isocitrate lyase and malate synthase for glyoxylate cycle, 3-hydroxyacyl-CoA dehydrogenase for beta-oxidation, isocitrate dehydrogenase for Krebs cycle, pyruvate kinase for glycolysis and glucose 6 phosphate dehydrogenase for pentose phosphate shunt. The simulated micro and hypergravity conditions were obtained by a Random Position Machine and a Hyperfuge, respectively.

Results show that the levels of some tested enzymes, at different lag times of the germination process, have the same trend of controls ($g = 1$), but with significant differences from quantitative point of view. They are higher in microgravity conditions and lower in hypergravity ones, suggesting that, from a biochemical point of view, the germination process results accelerated in microgravity conditions and delayed in hypergravity ones. These biochemical results show a good correlation with morphological ones, obtained with the measurement of the length of the seeds sprouting radicle.

These results give promising indications regarding the possibility to grow plant with lipid-rich seeds in spatial environment, to obtain food sources for astronauts during long term space missions and to reconstitute new atmosphere.

1. Introduction

Gravity has nearly been unchanged during life evolution on Earth. It exerted thus a crucial influence on biological processes and can markedly affect many different biological and biochemical functions (Claassen and Spooner, 1994).

Gravity is a very important factor for plant life, since it orients and coordinates the growth in order to optimize access to light, water and nutrients by means of the mechanism called gravitropism (Morita and Tasaka, 2004).

Many studies investigated the effects of altered gravity conditions on several biological events in plants: seed germination and root anatomy (Manzano et al. 2009; Busse and Stankovic, 2014; Aronne et al. 2002; Levine et al. 2003), pollen tube development (De Micco et al. 2006), expression of genes involved in seed storage (Tamaoki et al. 2014), distribution and activation of ion channels in the plant cell plasmalemma (Sytnik et al. 1989), duration and propagation rate of action potentials (Masi et al. 2015) and phototropism (Millar et al. 2010).

Although significant morphological differences between the plants grown in microgravity and those grown on the Earth have been not observed, some metabolic alterations have been reported at cellular and

Abbreviations: ICL, isocitrate lyase; MS, malate synthase; 3-HADH, 3-hydroxyacyl-CoA dehydrogenase; ICDH, isocitrate dehydrogenase; G6PDH, glucose 6 phosphate dehydrogenase; PK, pyruvate kinase; RPM, Random Position Machine

* Corresponding author.
E-mail address: paola.faraoni@unifi.it (P. Faraoni).
[1] These authors contributed equally to this paper.

https://doi.org/10.1016/j.plaphy.2019.03.042
Received 28 February 2019; Received in revised form 28 March 2019; Accepted 28 March 2019
Available online 03 April 2019
0981-9428/

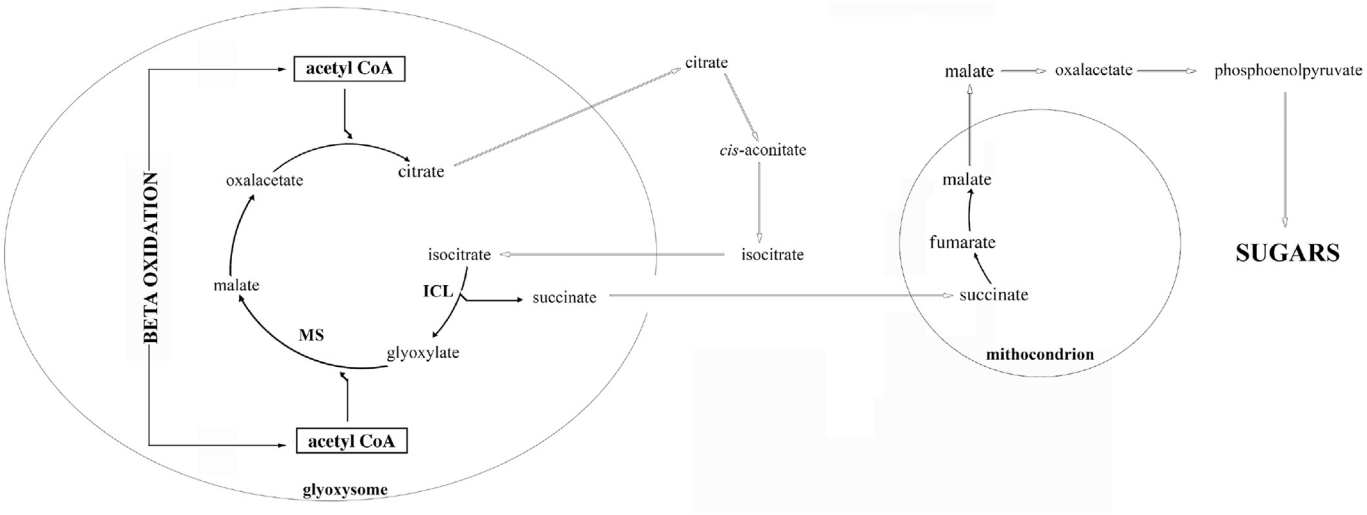

Fig. 1. Glyoxylate cycle in endosperme of germinating oilseeds.

subcellular levels (Kordyum, 2014; Moore and Cogoli, 1996; Zhao et al. 2003).

In this work *Pinus pinea* seeds germination has been studied in simulated microgravity and hypergravity conditions. *Pinus pinea* seeds are lipid-rich ones (oilseeds) and present a ligneous shield that protects them against mechanical stress. As a result, the seed is able to germinate after many years of storage, simply by imbibition with water. Therefore, it represents a suitable model to perform experiments during long term space travels.

In these lipid rich-seeds, the glyoxylate cycle plays a crucial role because it allows the net synthesis of sugars, by using the pool of Acetyl-CoA produced by beta-oxidation of non-esterified fatty acids, during the germination phase, when the plant is not yet able to perform the photosynthesis process (Fig. 1).

The breakdown of fatty acid to acetyl-CoA takes place into the glyoxysome, as well as the glyoxylate cycle, except the isomerization reaction of citrate in isocitrate, that occurs in cytosol where is operating a cytosolic isoform of *cis*-aconitase (Courtois-Verniquet and Douce, 1993; De Bellis et al. 1994, 1995). So, from the cytosol, the neo-formed isocitrate comes back into the glyoxysome where ICL catalyses its cleavage in glyoxylate and succinate. Glyoxylate by a molecule of acetil-CoA continues along the glyoxylate cycle, while succinate moves into the mitochondrion and, as intermediate of Krebs cycle, generates malate that in cytosol, is transformed into oxaloacetate and thereafter in phosphoenolpyruvate triggering the gluconeogenesis. It should be noted that the glyoxylate cycle supplies intermediate molecules to Krebs cycle (anaplerotic function).

Isocitrate lyase (ICL) and malate synthase (MS) are the key enzymes of glyoxylate cycle and represent excellent molecular markers of the germination state of lipid-rich seeds. They increase during the seed germination in glyoxysome, reach the maximum level at about the fifteenth day of the process, thereafter decrease and disappear at the end of germination (Cioni et al. 1981). These enzymes are related to the stage of germination, the growth of the embryo, the activation and progress of protein synthesis and the depletion of lipidic supplies (Firenzuoli et al. 1968a).

3-hydroxyacyl-CoA dehydrogenase (3-HADH) in glyoxysome is strictly correlated to the glyoxylate cycle, because it is involved in the oxidation of fatty acids and then in the production of acetylCoA, the substrate of the glyoxylate cycle (Firenzuoli et al. 1968b). The enzyme catalyses the oxidation of 3-hydroxyacylCoA to 3-oxoacylCoA and its trend is comparable to those of isocitrate lyase and of malate synthase.

In this work the germination process under altered gravity conditions was investigated from a molecular-biochemical point of view, monitoring at different times the levels of enzymes representative of the main metabolic pathways: 3-HADH (fatty acids oxidation), ICL and MS (glyoxylate cycle), isocitrate dehydrogenase, ICDH, (Krebs cycle), glucose 6 phosphate dehydrogenase, G6PDH, (pentose phosphates shunt) and pyruvate kinase, PK, (glycolysis).

In a previous work, *Pinus pinea* seeds exposed to extreme hypergravity conditions (1000*g*) at the beginning of the germination phase (for 64 h at 4 °C), showed a delay in germination and a slowdown in ICL level and other correlated enzymes (Ranaldi F. et al. 2003a). The gravitational condition applied to the seeds was much higher than that implied in in-flight experiments. In the present work, the investigation has been extended to the effects of a lower hypergravity condition and also of microgravity one during the whole time of the germination process (24 days).

2. Materials and methods

2.1. Gravitational conditions

The experiments were performed using a Random Position Machine (hereafter RPM), a device known to simulate microgravity, and an Hyperfuge for hypergravity.

The RPM (developed by Fokker Space Leiden, The Netherlands) consisted of two frames, one positioned inside the other, each rotating independently with the same constant velocity and verse (in this operation mode the RPM is equivalently called 3D-clinostat). In the center of the frame(s) (i.e. the center of rotation) is positioned a plate where the samples are located. Microgravitational condition simulated by the RPM is based on the principle of gravity vector average and depends on the speed of rotation and distance of the sample from the center of rotation (see for example van Loon, 2007; Borst and van Loon, 2009; Maccarrone et al. 2003; Huijser, 2000; Hoson et al.1996).

The RPM, with the angular velocity set to $60°s^{-1}$, provided to the batch of seeds an average gravity vector of $2x10^{-3}g$ (Maccarrone et al. 2003). As controls, two batch of seeds were placed to germinate at 1 *g*, on the basement of the RPM (outside the rotating frames) to provide the same conditions regarding the device vibration of the treated ones.The Hyperfuge (Diport AG) is a centrifuge specially constructed to generate hypergravity conditions, where speed and inclination angle of samples can be finely tuned to obtain the desired value of *g* (ranging from 2 to 150*g*). The Hyperfuge was set to obtain 20*g* and control sample were placed near to the device to obtain the same vibration conditions.

Seeds were placed to germinate in Petri dishes on RPM or in cell culture flasks on Hyperfuge (supports chosen to match the operation

indications of the two devices), in cotton wool imbibed with distilled water (30 seeds/dish on RPM and 20 seeds/flask for Hyperfuge).

Hyperfuge and the RPM were kept in a temperature-controlled room at 20 °C, for the whole period of the germination (24 days).

2.2. Preparation of the extracts

At different times from the start of germination (5, 8, 12, 14, 18, 24 days for the RPM and 6, 11, 15, 19, 24 days for Hyperfuge), three samples (petri dishes or flasks) for each of the three different experimental gravity conditions ($2x10^{-3}g$, 20*g* and 1*g*) were used to perform the enzymatic assays. The imbibition of seeds by water was considered the first day of germination.

The endosperm was removed from the germinated seeds and it was homogenised by ultraturrax in 150 mM triethanolamine, pH 7.5, 9 mM $MgCl_2$, 1.5 mM EDTA in ratio 1:2 (w/v). The homogenate was filtered using a gauze, centrifuged (10000*g*, 30 min) and the supernatant was filtered. The enzymatic activities and the total protein content of the filtered supernatant were determined.

2.3. Determination of enzymatic activities

All the enzymatic assays were performed at 30 °C spectrophotometrically using an UV-2100 spectrophotometer (Shimadzu, Columbia, MD).

The rate of the enzymatic reactions was continuously determined, following the formation of product molecules (NADH, NADPH, glyoxylate-phenylhydrazone) by their absorption peaks.

The values of 6.22 $mM^{-1}\,cm^{-1}$ and 17 $mM^{-1}cm^{-1}$ are considered to be the NADH (or NADPH) and glyoxylate-phenylhydrazone molar extinction coefficients, respectively.

One unit of activity is defined as that quantity of enzyme which transforms one μmole of substrate in 1 min, at 30 °C.

The content of total proteins was determined according to Bradford, (1976).

2.3.1. 3-Hydroxyacyl-coa dehydrogenase (EC 1.1.1.35) (3-HADH)

The assay mixture (1 mL final volume) consisted of 90 mM phosphate buffer (pH 7.3), 0.15 mM NADH.

3-Hydroxyacyl-CoA dehydrogenase activity was determined following the NADH oxidation at 340 nm.

The reaction was started by adding the substrate (0.12 mM Acetoacetyl-CoA).

2.3.2. Isocitrate lyase (EC 4.1.3.1) (ICL)

Isocitrate lyase activity was determined by the method of Dixon and Kornberg (Dixon et al. 1959) as described in (Ranaldi F. et al., 2003b).

The reaction of isocitrate cleavage was chemically coupled with phenylhydrazine which reacts with glyoxylate to form glyoxylate-phenylhydrazone, that can be quantified spectrophotometrically at 324 nm.

The reaction was started by adding the substrate (4 mM D,L isocitrate).

2.3.3. Malate synthase (EC 2.3.3.9) (MS)

Malate synthase activity was measured according to Silverstein, R.M. (1975) following the rate of formation of 5-mercapto-2-nitrobenzoic acid in the presence of glyoxylate, and acetyl-CoA. at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$).

The reaction mixture contained: 20 mM Tris buffer, pH 8.0; 2 mM EDTA; 3 mM KCl; 5 mM $MgCl_2$; 0.1 mg/ml DTNB; 0.5 mM glyoxylate and 0.2 mM acetyl-CoA. The reaction was started by the addition of acetyl-CoA.

2.3.4. Isocitrate dehydrogenase (NADP) (EC 1.1.1.42) (ICDH)

ICDH activity was determined according to Bernt and Bergmeyer (Bernt et al. 1974) with slight modifications, monitoring the formation of NADPH at 340 nm.

The reaction was started by adding the substrate (4 mM D,L isocitrate).

2.3.5. Glucose 6 phosphate dehydrogenase (EC 1.1.1.49) (G6PDH)

G6PDH activity was determined according to Löhr and Waller (Löhr et al. 1974), with slight modifications, monitoring the formation of NADPH at 340 nm.

The reaction was started by adding the substrate (3.3 mM glucose-6P).

2.3.6. Pyruvate kinase (EC 2.7.1.40) (PK)

PK activity was determined according to Hess and Wieker (Hess et al. 1974), with slight modifications, monitoring the NADH oxidation at 340 nm.

The reaction was started by adding the substrate (0.8 mM phosphoenolpyruvate).

The measured activities refer to *in vitro* determinations on seeds homogenates, in conditions of a saturating substrate. So these values are not indicative of the *in vivo* physiological activity, but of the amount of the enzymes.

2.4. Morphological analysis of germination

Seed germination was evaluated observing the sprouting of the radicle, the early young root. The average length of radicle in 30 seeds was measured. These morphological data were compared with the biochemical-molecular ones.

2.5. Statistical analysis

The differences in the levels of the studied enzymes were analyzed by means of Student's t-test.

The data here shown are averages and standard deviations of independent experiments performed in triplicate. Student's t-test was performed (two-tailed unpaired test) to evaluate pairwise differences, with $p < 0.05$ being considered significant.

3. Results

The simulated micro and hypergravitational conditions were applied during the whole time of germination. 3-HADH and ICL are two enzymes strongly involved in the germination process: they reach a maximum activity level between the fourteenth-eighteenth day of the germination, thereafter they decrease and disappear at the end of germination. Interesting variations of their levels were observed during the whole time of the investigation. In microgravity and hypergravity conditions opposite results were observed.

3.1. Germination in simulated microgravity conditions

Fig. 2A shows the effect of microgravity condition on 3-HADH. In the seeds germinating at $2x10^{-3}g$, 3-HADH level reaches a maximum 18 days after imbibition, as occurred in the controls (1*g*) but with higher values, ($p < 0,05$). In Fig. 2B the ICL activity is shown: its trend is similar to that of 3-HADH, with a peak value at 18 days after imbibition.

Fig. 2C shows MS enzyme activity. Likewise, the $2x10^{-3}g$ microgravity stimulates the activity of the enzyme that shows a maximum at 18 days after imbibition, similarly to what observed for 3-HADH and ICL.

Fig. 3 shows the level of ICDH. The levels of this enzyme always increase during the germination time, as observed in other experimental studies conducted in normal gravity conditions (Firenzuoli et al. 1968a, 1968b). Also our data showed an increase in the level of this enzyme between the 14th and the 24th days, differently from the 3-

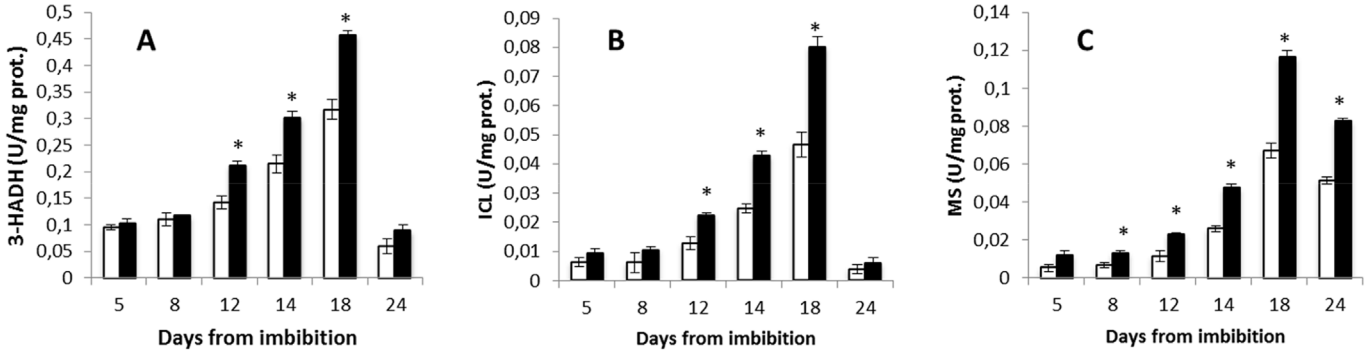

Fig. 2. Levels of 3-HADH (A), ICL (B) and MS (C) during *Pinus pinea* seed germination in simulated microgravity environment. □ 1*g* (control); ■ $2x10^{-3}g$.; * indicates a statistically significant difference, $p < 0,05$, between control and microgravity condition.

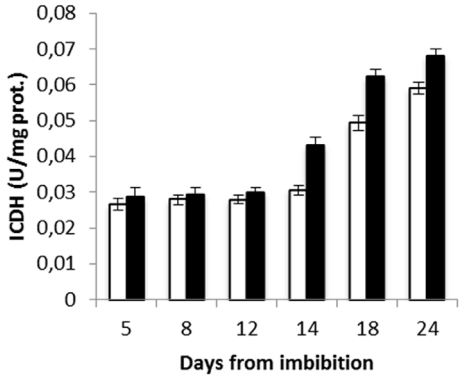

Fig. 3. Levels of ICDH during *Pinus pinea* seeds germination in microgravity. □ 1 g (control), ■ $2x10^{-3}g$.; * indicates a statistically significant difference, $p < 0,05$, between control and microgravity.

HADH, ICL and MS ones, which at day 24 (end of germination) had already fallen.

In microgravity condition, during the whole time of germination, the levels of ICDH showed the same trend (they increased with time) observed in 1*g* controls, but with higher values.

The levels of G6PDH and PK did not show significant differences comparing samples exposed to microgravity conditions and controls (data not shown).

3.2. Germination in hypergravity conditions

Fig. 4A reports the effect of hypergravity condition (20*g*) on the levels of 3-HADH. A statistically significant lowering of the enzymatic level, compared to the controls, is evident at each time considered ($p < 0,05$).

In Fig. 4B and C a tightly analogous behaviour can be observed for ICL and MS, respectively ($p < 0,05$).

ICDH, G6PDH and PK did not show significantly different levels in 20 g-treated and untreated control samples (data not shown).

3.3. Morphological observations

As shown in Fig. 5, at the same time of germination (14th day), seeds grown in simulated microgravity and hypergravity conditions showed roots longer (~2 ± 0.32 cm) and shorter (~0.1 ± 0.02 cm) than the ones maintained at 1 g (~0.6 ± 0.13 cm), respectively. This indicates that in microgravity conditions seed germination is faster than at 1*g*. The hypergravity condition, on the contrary, induced a delay in germination.

4. Discussion

Several studies have been carried out about the effects of altered gravity conditions on seed germination process, taking advantage of different morphological and molecular criteria to discuss it. In this work, indeed, these effects are evaluated on a purely metabolic basis, following the levels of enzymes involved in the production processes of energy and sugars from seed lipid reserves. The germination process under altered gravitational conditions was studied from a morphological and biochemical point of view; in particular, the levels of some enzymes representative of the principal metabolic pathways involved in the germination process were evaluated.

The data obtained in the present study confirms that gravity and, in particular, its modulation influences physiological processes of living organism by means of key-enzymes levels alterations.

The levels of three of the six enzymes assayed, 3-HADH, ICL and MS, have been revealed to be sensitive to alterations of gravitational

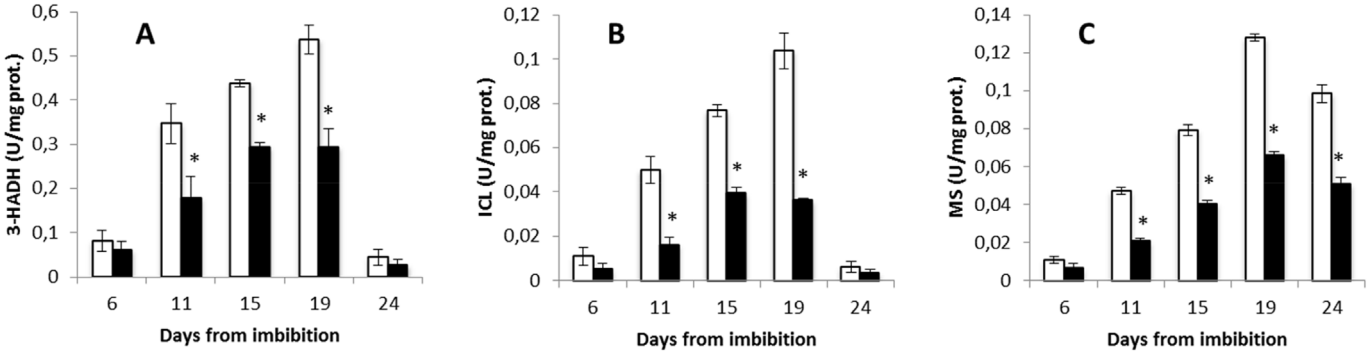

Fig. 4. Levels of 3-HADH (A), ICL (B) and MS (C) during *Pinus pinea* seeds germination in simulated hypergravity environment. (20*g*). □ 1*g* (control); ■ 20*g*; * indicates a statistically significant difference, $p < 0,05$.

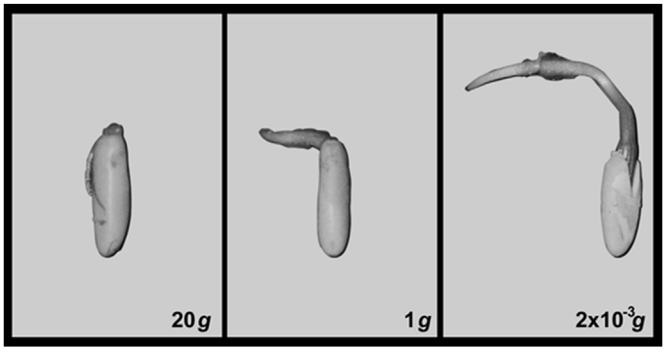

Fig. 5. *Pinus pinea* seed germination (14th day) at different simulated gravity conditions. Left panel: hypergravity (20*g*). Central panel: control (1*g*). Right panel: microgravity ($2x10^{-3}g$).

conditions. In fact, the results clearly show that their levels increase in microgravity (Fig. 2), whereas decrease in hypergravity (Fig. 4). Because these enzymes are key markers of glyoxysome activity from which depends the germination process, we suppose that microgravity could stimulate this process and conversely hypergravity could delay it.

The level trend of ICDH (a key enzyme of mitochondrial activity at the level of the Krebs cycle) is similar to that of glyoxysome enzymes. Moreover the glyoxylate cycle supports the functionality of the Krebs cycle: the succinate produced by ICL exits from glyoxysome to enter in the mitochondrion, where is transformed to malate by Krebs cycle enzymes. In the cytosol, malate is converted into oxaloacetate and thereafter in phosphoenolpyruvate, that triggers the gluconeogenesis pathway (Fig. 1).

G6PDH and PK levels seem not to change between hypergravity and microgravity conditions and this confirms that the observed effects are not general metabolic effects, but specific at the level of beta-oxidation and glyoxylate cycle.The morphological aspect of germinating seeds confirms the results deriving from enzymatic activity assays in micro and hypergravity conditions (Fig. 5).

Microgravity, in fact, increases the levels of 3-HADH, ICL, MS and ICDH and therefore shortened the germination time in treated seeds, compared with the control ones.

On the contrary, hypergravity causes a decrease in 3-HADH, ICL and MS levels and a delayed seed germination.

On the other hand, the EMEC project (Effects of Microgravity on Enzyme Catalysis, Tacconi et al. 1997) with its two experimental replications (Ranaldi et al., 1999, 2003b; Giachetti et al., 2001) demonstrated that the enzyme ICL, *in vitro*, from the point of view of its catalysis mechanism, is not altered by a condition of microgravity.

Therefore, the observed effects on ICL level during seed germination may be related to a possible role of gravity condition not directly on the molecule, but on the mechanisms of transcription, translation and gene activation, or perhaps directly on the cellular organelles involved in the studied process.

Additional studies will be necessary to investigate how gravity affects the enzymatic levels. It is important to clarify if this influence originates from transcriptional or translational events or from enzymatic activations.

Probably the variations of 3-HADH, ICL, MS and ICDH levels could also be determined by transcriptional or translational events.

It is known that seed imbibition is the germination starting event that promotes the mobilization of the reserves and the expression of the genes involved in this process. Therefore it is possible to suppose that reduced gravity conditions cause a faster imbibition of a porous matrix and consequently a faster activation of the genes involved in the expression of proteins functional to germination, including 3-HADH, ICL and MS. The opposite situation could take place in hypergravity conditions.

In the light of long-term human space missions and future attempts to colonize the Lunar or Martian environment, it will be important to cultivate plant species relate both to the nutrition of the astronauts and to reconstitute new atmosphere conditions. Therefore, assessing the physiological process of germination in gravitational environments different from Earth's one becomes paramount.

In tested conditions of $g < 1$ and $g > 1$, the levels of the key enzymes of the glyoxylate cycle vary, respect to $g = 1$, not as temporal trend, but simply in quantitative terms. The peaks of enzymatic levels are always in good correspondence with those of the controls, but they reach levels significantly higher in the case of $g < 1$ and lower for $g > 1$, respectively, compared to controls. A higher activity of the glyoxylate cycle leads to an increased synthesis of glucose (by which it is possible to obtain sucrose that through phloem reaches all tissues of growing embryos) and then to an anticipation of the germination process.

Many food plants are propagated by seeds (Oaks et al. 1964; Laidman and Tavener, 1971; Landolt and Matile, 1990; Pistelli et al. 1996; Gonzalez et al. 2007, Ma et al. 2016) and this feature offers them as optimal for long space missions where the seeds can be stored for a long time before germination by simple imbibition at the desired time.

The seeds of many of these plants of interest germinate thanks to the activity of the enzymes of the glyoxylate cycle. Many evidences on the germination and growth of plants in space environment support our study, in which for the first time it is highlighted the effect of gravity on the germination process from the point of view of enzyme activities of the beta-oxidation and glyoxylate cycle, proving that the experimental morphological evidences are in agreement with the biochemical ones.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors

Author contributions

PF, ES, AG and FR designed the study, obtained data and analyzed data. All Authors prepared the manuscript.

References

Aronne, G., De Micco, V., De Pascale, S., 2002. The effect of simulated microgravity on seed germination and seedling anatomy of *Phaseolus vulgaris*. L. J. Gravitation. Physiol. 9, 233–234.

Bernt, E., Bergmeyer, H.U., 1974. Methods for determination of enzyme activity: isocitrate dehydrogenase. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, second ed. vol. 2. Academic Press, Inc, New York, USA, pp. 624–627.

Borst, A.G., van Loon, J.J.W.A., 2009. Technology and developments for the random positioning machine. RPM. Microgravity Sci. Tech. 21, 287–292. https://doi.org/10.1007/s12217-008-9043-2.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 7, 248–254.

Busse, J.S., Stankovic, B., 2014. Seed-to-seed-to-seed growth and development of Arabidopsis in microgravity. Astrobiology 14, 866–875.

Cioni, M., Pinzauti, G., Vanni, P., 1981. Comparative biochemistry of the glyoxylate cycle. Comp. Biochem. Physiol. 70 B, 1–26.

Claassen, D.E., Spooner, B.S., 1994. Impact of altered gravity on aspects of cell biology. Int. Rev. Cytol. 156, 301–373.

Courtois-Verniquet, F., Douce, R., 1993. Lack of aconitase in glyoxysomes. and peroxisomes. Biochem. J. 294 (1), 103–107. https://doi.org/10.1042/bj2940103.

De Bellis, L., Hayashi, M., Biagi, P.P., Hara-Nishimura, I., Alpi, A., Nishimura, M., 1994. Immunological analysis of aconitase in pumpkin cotyledons: the absence of aconitase in glyoxysomes. Physiol. Plantarum 90 (4), 757–762. https://doi.org/10.1111/j.1399-3054.1994.tb02534.x.

De Bellis, L., Hayashi, M., Nishimura, M., Alpi, A., 1995. Subcellular and developmental changes in distribution of aconitase isoforms in pumpkin cotyledons. Planta 195 (3), 464–468. https://doi.org/10.1007/BF00202606.

De Micco, V., Scala, M., Aronne, G., 2006. Effects of simulated microgravity on male gametophyte of *Prunus, Pyrus*, and *Brassica* species. Protoplasma 228, 121–126.

Dixon, G.H., Kornberg, H.L., 1959. Assay methods for key enzymes of the glyoxylate. Biochem. J. 72, 3P.

Firenzuoli, A.M., Vanni, P., Mastronuzzi, E., Zanobini, A., Baccari, V., 1968a. Enzymes of

glyoxylate cycle in conifers. Plant Physiol. 43, 1125–1128.
Firenzuoli, A.M., Vanni, P., Mastronuzzi, E., Zanobini, A., Baccari, V., 1968b. Participation of the glyoxylate cycle in the metabolism of germinating seeds of Pinus pinea. Life Sci. 7, 1251–1258.
Giachetti, E., Ranaldi, F., Vanni, P., 2001. Enzyme catalysis in microgravity: an intricate problem to be solved. FEBS (Fed. Eur. Biochem. Soc.) Lett. 504, 78–79.
Gonzalez, D.O., Vodkin, L.O., 2007. Specific elements of the glyoxylate pathway play a significant role in the functional transition of the soybean cotyledon during seedling development. BMC Genomics 8, 468. https://doi.org/10.1186/1471-2164-8-468.
Hess, B., Wieker, H.J., 1974. Methods for determination of enzyme activity: pyruvate kinase. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, second ed. vol. 2. Academic Press, Inc, New York, USA, pp. 778–783.
Hoson, T., Kamisaka, S., Buchen, B., Sievers, A., Yamashita, M., Masuda, Y., 1996. Possible use of 3-D clinostat to analyze plant growth processes under microgravity conditions. Adv. Space Res. 17, 47–53.
Huijser, R.H., 2000. Desktop RPM: New Small Size Microgravity Simulator for the Bioscience Laboratory. Fokker Space B.V., P.O. Box 32070, 2303. DB, Leiden, The Netherlands. http://www.desc.med.vu.nl.
Kordyum, E.L., 2014. Plant cell gravisensitivity and adaptation to microgravity. Plant Biology Journal 16, 79–90.
Laidman, D.L., Tavener, R.J., 1971. Triglyceride mobilization in the germinating wheat grain. Biochem. J. 124, 4P–5P.
Landolt, R., Matile, P., 1990. Glyoxisome-like microbodies in senescent spinach leaves. Plant Sci. 72, 159–163.
Levine, H.G., Anderson, K., Boody, A., Cox, D., Kuznetsov, O.A., Hasenstein, K.H., 2003. Germination and elongation of flax in microgravity. Adv. Space Res. 31, 2261–2268.
Löhr, G.W., Waller, H.D., 1974. Methods for determination of enzyme activity: glucose6-phosphate dehydrogenase. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, second ed. vol. 2. Academic Press, Inc, New York, USA, pp. 636–643.
Ma, Z., Marsolais, F., Bernards, M.A., Sumarah, M.W., Bykova, N.V., Igamberdiev, A.U., 2016. Glyoxylate cycle and metabolism of organic acids in the scutellum of barley seeds during germination. Plant Sci. 248, 37–44. https://doi.org/10.1016/j.plantsci.2016.04.007.
Maccarrone, M., Battista, N., Meloni, M., Bari, M., Galleri, G., Pippia, P., Cogoli, A., Finazzi-Agrò, A., 2003. Creating conditions similar to those that occur during exposure of cells to microgravity induces apoptosis in human lymphocytes by 5-lipoxygenase-mediated mitochondrial uncoupling and cytochrome c release. J. Leukoc. Biol. 73, 472–481.
Manzano, A.I., Matía, I., González-Camacho, F., Carnero-Díaz, E., van Loon, J.J.W.A., Dijkstra, C., Larkin, O., Anthony, P., Davey, M.R., Marco, R., Medina, F.J., 2009. Germination of *arabidopsis* seed in space and in simulated microgravity: alterations in root cell growth and proliferation. Microgravity Sci. Technol. 21, 293–297.
Masi, E., Ciszak, M., Comparini, D., Monetti, E., Pandolfi, C., Azzarello, E., Mugnai, S., Baluška, F., Mancuso, S., 2015. The electrical network of maize root apex is gravity dependent. Sci. Rep. 5, 7730. https://doi.org/10.1038/srep07730.
Millar, K.D.L., Kumar, P., Correll, M.J., Mullen, J.L., Hangarter, R.P., Edelmann, R.E., Kiss, J.Z., 2010. A novel phototropic response to red light is revealed in microgravity. New Phytol. 186, 648–656.
Moore, D., Cogoli, A., 1996. Gravitational and space biology at the cellular level. In: Moore, D., Bie, P., Oser, H. (Eds.), Biological and Medical Research in Space. Springer-Verlag, Heidelberg, Germany 1-106.
Morita, M.D., Tasaka, M., 2004. Gravity sensing and signaling. Curr. Opin. Plant Biol. 7, 712–718.
Oaks, A., Beevers, H., 1964. The glyoxylate cycle in maize scutellum. Plant Physiol. 39, 431–434.
Pistelli, L., Nieri, B., Smith, S.M., Alpi, A., De Bellis, L., 1996. Glycoxylate cycle enzyme activities are induced in senescent pumpkin fruits. Plant Sci. 119, 23–29.
Ranaldi, F., Fiusco, A.R., Tacconi, M., Varatti, R., Falciani, P., Vanni, P., 1999. Enzyme kinetics parameters are not altered by microgravity. Microgravity Sci. Technol. 12, 36–40.
Ranaldi, F., Giachetti, E., Guerin, E., Bacci, S., Paoletti, E., Boddi, V., Vanni, P., 2003a. Gravitational stress on germinating Pinus pinea seeds. Comptes Rendus Biol. 326, 553–554.
Ranaldi, F., Vanni, P., Giachetti, E., 2003b. Enzyme catalysis in microgravity: steady-state kinetic analysis of the isocitrate lyase reaction. Biophys. Chem. 103, 169–177.
Silverstein, R.M., 1975. The determination of the molar extinction coefficient of reduced DTNB. Anal. Biochem. 63, 281–282.
Sytnik, K.M., Demkiv, O.T., Kordyum, E.L., Nedukha, E.M., Danevich, L.A., 1989. Calcium gradient in plant cells with polarized growth in simulated microgravity. Adv. Space Res. 9, 41–44.
Tacconi, M., Veratti, R., Falciani, P., Giachetti, E., Ranaldi, F., Vanni, P., 1997. Enzyme catalysis under low gravity conditions. Europ. Low Gravity Assoc. (ELGRA) Biennal Meeting Paris 17–19 March: 19.
Tamaoki, D., Karahara, I., Nishiuchi, T., Wakasugi, T., Yamada, K., Kamisaka, S., 2014. Effects of hypergravity stimulus on global gene expression during reproductive growth in Arabidopsis. Plant Biol. 1, 178–186.
van Loon, J.J.W.A., 2007. Some history and use of the random positioning machine, RPM, in gravity related research. Adv. Space Res. 39 (7), 1161–1165. https://doi.org/10.1016/j.asr.2007.02.016.
Zhao, H.C., Zhu, T., Wu, J., Xi, B.S., 2003. Effect of simulated microgravity on aged pea seed vigour and related physiological properties. Colloids Surfaces B Biointerfaces 27, 311–314.