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Nicotiana benthamiana seeds tolerate hyperaccelerations up to 400,000 x g

Sementes de *Nicotiana benthamiana* toleram hiperacelerações de até 400.000 x g

Las semillas de *Nicotiana benthamiana* toleran hiperacceleraciones de hasta 400.000 x g

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Abstract

Exposure to hypergravity can alter the viability, morphology, development and behavior of living beings. Thus, the analysis of these factors is essential when considering life on supermassive planets, as well as in 'ballistic panspermia' scenarios related to the ejection of rocks from the surface of a planet, which could serve as transfer vehicles to spread the life between planets within a solar system. Studies analyzing the effects of hypergravity regimes are abundant in the literature, however, only a few researches carried out experiments using conditions of the order of 10^5 x *g*. In addition, the only plant species tested so far, as an entire structure instead of detached parts, exposed to gravity stress of this order of magnitude in its entirety was *Oryza sativa*, whose seeds were able to germinate after being exposed to 450,000 x *g*. Recently, our research group demonstrated that some free-living nematode species can support 400,000 x g. In the present study, we report that seeds of the plant model *Nicotiana benthamiana* exposed to 400,000 x *g* for 1h are able to germinate into fully normal young seedlings, with no apparent morphological alterations. Since *N. benthamiana* is used in laboratories worldwide and an easy to cultivate plant model, theoretical and experimental models of lithopanspermia and life in supermassive planets may benefit from it.

Keywords: Hypergravity; Ultracentrifugation; Acceleration; *g*-force; *Nicotiana benthamiana*.

Resumo

A exposição à hipergravidade pode alterar a viabilidade, morfologia, fisiologia, desenvolvimento e comportamento de seres vivos. Dessa forma, a análise desses fatores é essencial quando se considera a vida em planetas supermassivos, bem como em cenários de 'panspermia balística' relacionados à ejeção de rochas a partir da superfície de um planeta, que poderiam servir como veículos de transferência para espalhar a vida entre os planetas dentro de um sistema solar. Estudos analisando os efeitos de regimes de hipergravidade são abundantes na literatura, entretanto, apenas alguns realizaram experimentos utilizando condições da ordem de 10^5 x *g*. Ademais, a única espécie de planta testada até agora, como uma estrutura inteira em vez de partes separadas exposta, ao estresse gravitacional dessa ordem de grandeza na sua totalidade foi *Oryza sativa*, cujas sementes foram capazes de germinar após terem sido expostas a 450.000 x *g*. Recentemente, o nosso grupo de pesquisa demonstrou que algumas espécies de nematóides de vida livre podem suportar 400.000 x *g*. No presente estudo, relatamos que sementes da planta modelo *Nicotiana benthamiana* expostas a 400.000 x g por 1h são capazes de germinar em plântulas jovens totalmente normais, sem alterações morfológicas aparentes. Uma vez que *N. benthamiana* é usada em laboratórios em todo o mundo e é uma planta modelo de fácil cultivo, modelos teóricos e experimentais de litopanspermia e vida em planetas supermassivos podem se beneficiar disso.

Palavras-chave: Hipergravidade; Ultracentrifugação; Aceleração; Força *g*; *Nicotiana benthamiana*.

Resumen

La exposición a la hipergravedad puede alterar la viabilidad, morfología, fisiología, desarrollo y comportamiento de los seres vivos. Por tanto, el análisis de estos factores es fundamental a la hora de considerar la vida en planetas supermasivos, así como en escenarios de 'panspermia balística' relacionados con la expulsión de rocas de la superficie

de un planeta, que podrían servir como vehículos de transferencia para esparcir la vida entre planetas dentro de un sistema solar. Los estudios que analizan los efectos de los regímenes de hipergravedad son abundantes en la literatura, sin embargo, solo unos pocos realizaron experimentos utilizando condiciones del orden de 10^5 x *g*. Además, la única especie de plantas probada hasta ahora, como una estructura completa en lugar de partes separadas, a un estrés gravitacional de este orden de magnitud en su totalidad, fue *Oryza sativa*, cuyas semillas pudieron germinar tras ser expuestas a 450.000 x *g*. Recientemente, nuestro grupo de investigación ha demostrado que algunas especies de nematodos de vida libre pueden soportar 400.000 x *g*. En el presente estudio, reportamos que semillas de la planta modelo Nicotiana benthamiana expuestas a 400.000 x *g* durante 1h pueden germinar en plántulas jóvenes completamente normales, sin alteraciones morfológicas aparentes. Dado que *N. benthamiana* se utiliza en laboratorios de todo el mundo y es un modelo de planta fácil de cultivar, los modelos teóricos y experimentales de litopanspermia y vida en planetas supermasivos pueden beneficiarse de ella.
Palabras clave: Hipergravedad; Ultracentrifugación; Aceleración; Fuerza g; *Nicotiana benthamiana*.

1. Introduction

The study of life's resilience to abiotic stresses is of great interest to astrobiology. Among these stresses, hypergravity, or hyperacceleration, defined as a regime of acceleration higher than 9.8 m/s2 (g-force >1) is probably the less studied one. The effects of hypergravity on the viability, development, morphology, physiology and behavior are essential when considering life in supermassive planets, as well as in scenarios of 'ballistic panspermia'. Mastrapa (2001; based on Melosh, 1993) estimated that the hyperacceleration conditions to which an organism would be exposed in a ballistic panspermia scenario would be on the order of 10^5 x g. Notoriously, distinct research groups have demonstrated the survival and proliferation microorganisms under such order of hypergravity (Deguchi et al., 2011; Gao et al., 2013; Montgomery et al., 1963; Yoshida et al., 1999). Taken together, all these works and others have opened a new avenue of investigation: life's resilience to 'extreme hyperacceleration' regimes ($\geq 10^5$ x g). However, these reports have focused on the analyses of unicellular microorganisms or cells/tissues/parts of multicellular species (*e.g.*, isolated cells, callus or root tips).

To our knowledge, the only plant species tested so far, as an entire structure instead of detached parts, is *Oryza sativa* (rice) (Kwon et al., 1992; Kwon and Oono, 1992), whose seeds (i.e., the embryo and accessory tissues) were able to germinate after 450,000 x *g*. More recently, we have revealed that two free-living nematode species are able to withstand 400,000 x *g* (Souza et al., 2017; Souza and Pereira, 2018). Here, we decided to evaluate the effects of exposing seeds of the plant model *Nicotiana benthamiana* to 400,000 x *g*, followed by analyses on their capabilities to germinate. This specie is phylogenetically close related with *Nicotiana tabacum*, which is also a widely used plant model (Micheten & Pessenti, 2021).

2. Methodology

The present study can be characterized as a laboratory research with qualitative and quantitative approaches (Pereira et al., 2018).

2.1 Nicotiana benthamiana

Seeds of *N. benthamiana* were kindly provided by Prof. Maria Helena de Souza Goldman (Dept. of Biology, FFCLRP, University of São Paulo), and stored at room temperature before experimental procedures.

2.2 Centrifugation at 400,000 x g

Extreme hypergravity regime was achieved by centrifuging samples at 4°C for 1 hour, using the Optima MAX-XP high-performance ultracentrifuge - Beckman Coulter (Department of Biochemistry, FMRP, University of São Paulo). After placing samples into the ultracentrifuge, extreme hypercentrifugations started only when ideal conditions were reached (1.34 Pascal inside the centrifuge chamber). *N. benthamiana* seeds were divided into the three groups, as follows: (i) Negative

Control 1 (NC1) - seeds kept at room temperature (20 °C) for 1 hour, not centrifuged; (ii) Negative Control 2 (NC2) - seeds kept at 4 °C, for 1 hour, not centrifuged; (iii) Experimental Group (EG) - seeds centrifuged at 400,000 x *g*, 4 °C, for 1h. After treatments, seeds were sown in substrate for plants (Bioplant Prata) and the recipients were kept at 4 °C for two days, in order to synchronize plant growth (Tada et al., 2014). Subsequently, they were transferred to the incubator at 22 °C (under a light regime of 12h light, 12h dark; 55% humidity). The number of seedlings in each recipient was determined at the end of the second week.

2.3 Morphological analyses

Integrity, size and general morphology of all seeds were analysed using a stereomicroscope (ZEISS, Discovery V20); seedlings were submitted to directed observation.

2.4 Statistical analysis

Each experiment was composed of biological triplicates, each biological replicate was composed of 30 seeds in each treatment. Biological replicates were performed in different weeks. The data were analysed via 'One Way ANOVA' followed by Student Newman, with a cut-off of $p < 0.05$.

3. Results

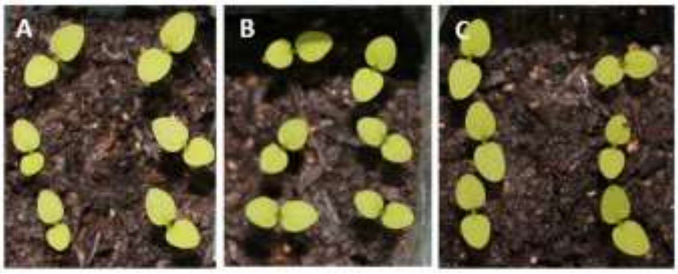
Interestingly, extreme hyperacceleration of 400.000 x *g* did not promote any drastic alteration in seeds' morphologies from any groups (Figure 1), all of which were able to germinate within the regular period of time, into morphologically normal young seedlings (Figure 2). Only a small decrease in the viability of the centrifuged group was observed (12% compared to NC2 control group) (Figure 3).

Figure 1. Morphology of *Nicotiana benthamiana* seeds.

(A) NC1: non-centrifuged seeds kept at room temperature (~20 °C). (B) NC2: seeds kept at 4 °C, not centrifuged. (C) EG: seeds centrifuged at 4 °C (400,000 x *g*). Twelve seeds were analyzed in each one groups. No obvious alterations could be observed among different groups. Source: Authors.

In Fgure 1, it is possible to observe seeds from different groups aligned in a similar way, with the same amplification (observation under the stereomicroscope), exposed to different experimental conditions. Through a detailed morphological analysis of the seeds, it was possible to observe that there were no changes in seeds morphology in the experimental group compared to the controls groups (NC1, NC2).

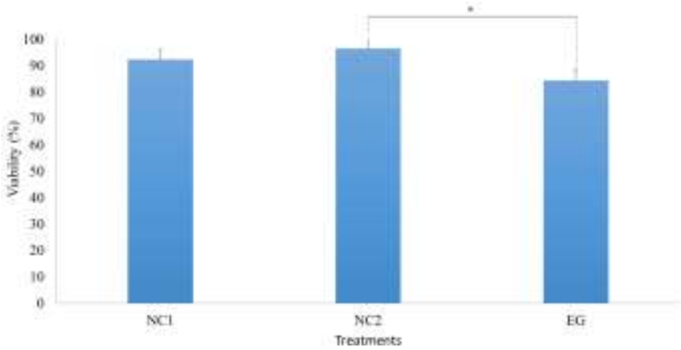
Figure 2. *Nicotiana benthamiana* young seedlings, two weeks after exposure to different treatments.



(A) NC1: non-centrifuged seeds kept at room temperature (~20 °C). (B) NC2: seeds kept at 4 °C, not centrifuged. (C) EG: seeds centrifuged at 4 °C (400,000 x *g*). No obvious alterations could be observed among different groups. Source: Authors.

The similarity in seedlings development from different groups can be seen in Figure 2, which presents photos of 06 seedlings of each one of the experimental groups. Therefore, it is evident that there was no delay in the development of *Nicotiana benthamiana* seeds in the group exposed to 400, 000 x *g* (EG) compared to the control groups (NC1, NC2).

Figure 3. Percentage of seeds that germinated after exposure to different treatments.



NC1: non-centrifuged seeds kept at room temperature (20 °C). NC2: seeds kept at 4 °C, not centrifuged. EG: seeds centrifuged at 4 °C (400,000 x *g*). (*) statistical difference (p <0.05). Source: Authors.

The graph depicted in Figure 3 presents a comparative analysis of viability expressed in terms of percentage of the three groups used in the experimental design. In the analysis of the total number of seeds exposed in each treatment, it is possible to verify that there was a significant decrease in viability (p<0.05) in the experimental group (EG) compared to the control group formed by seeds kept at 4 °C and not centrifuged (NC2).

4. Discussion

Plant parts have been submitted to extreme hypergravity and recovered alive at least since 1930. For example, Beams and King (1935) ultracentrifuged cells of root-tips of *Phaseolus vulgaris* (bean), at 400,000 x *g* for 15-20 minutes, thus reporting a redistribution of the cytoplasmic components and inclusions into layers, distinctly stratified. Notably, since then, microphotographs clearly showed that extreme hyperacceleration regimes do not promote the catastrophic, permanent structural and cellular damages one might intuitively speculate. Beams (1949) submitted tips of actively growing stems from the aquatic plant *Elodea canadensis* to 350,000 x *g* for 30 minutes, also reporting the organellar stratification.

Although several groups worldwide have worked with hypergravity regimes (>1 x g) (Bouck 1963a,b; Waldron and Brett, 1990), only a few have performed experiments at 10^5 x g or beyond. For example, Kwon and Oono (1992a,b) reported the survival of rice (*Oryza sativa* L. var. Nipponbare) suspension callus (±95% and 32%), dehulled seeds (~95% and 15%) and 2-days-old seedlings (~15% and 0%) after 450,000 x *g* for 1 and 6 hours, respectively. Survival of seeds and seedlings was determined by counting the number of germinating seeds and growing seedlings five days after ultracentrifugation. Cell viability of suspension callus was determined by TTC staining method. However, in all cases (callus, seeds, and seedlings) survival was limited to five days after ultracentrifugation.

The findings of Kwon and Oono (1992 a,b), along with others, reporting survival of organisms submitted to g-forces ≥105 x g (Deguchi et al., 2011; Gao et al., 2013; Montgomery et al., 1963; Souza and Pereira, 2018; Yoshida et al., 1999), call into attention that a special type of tolerance, to extreme hyperaccelerations, may have been largely ignored until now. Importantly, although all the species tested so far revealed to be tolerant, one should not expect that all life forms are, since they vary in size, composition and structure. In order to probe this open question, we decided to ultracentrifuge seeds of *N. benthamiana*. Remarkably, they were able to germinate into fully anatomically normal young seedlings (Figures 2 and 3), probably due to their small sizes, low water content, compactness and physical resistance of their external structures (Figure 1).

However, it is important to highlight that in Kwon and Oono's (1992b) experiment, rice seedlings died within 18 hours post-ultracentrifugation. Therefore, although *N. benthamiana* young seedlings displayed no obvious alterations throughout the two weeks under monitoring, the question whether they are able to develop into normal and fertile adult plants must be investigated. Also relevant, Kostoff (1937; 1938) evidenced that repetitive cycles of centrifugation (<2,500 x g), for several days, in germinated seeds of diverse species, including *Nicotiana langsdorffui* and a hybrid (*Nicotiana rustica* x *N. tabacum*), resulted in cellular lethality, chromosomal aberrations and/or morphological anomalies observed latter in development. Our experiments did not involve germinated seeds, which display elevated mitotic activity and, thus, are prone for such aberrations. Nevertheless, it is still relevant to investigate whether such genetic alterations occur in our experimental set up, which might result in posterior abnormalities or lethality. Finally, our data evidence that the tolerance of organisms to extreme hyperagravity regimes is broader than originally reported (now including *N. bethamiana*). *N. benthamiana* is an extremophile plant from central Australia (Bally et al., 2015) and it has been an important model in diverse research fields. Now, our data expands its applications for the study of extreme hyperaccelerations ($\geq 10^5$ x g).

It is also important to highlight that Mastrapa and colleagues (2001), based on Melosh (1984; 1993), calculated the *g*-force experienced by a hypothetical organism present in a volcanic fragment from Mars, during its acceleration towards space (300,000 x *g*). Interestingly, our results reveal that *N. tabacum* withstand a hyperacceleration superior to that, with only a minor decrease in viability, and no alterations in the period of germination or morphology of the young seedlings. However, some differences between Mastrapa's study and the present work must be considered. First, our acceleration time (300 s) is 6×10^5 times longer than Mastrapa's (0.5 ms), while the jerk (i.e., the rate of change of acceleration) in our set up (1.3×10^4 m/s^3) is 4.6×10^5 lower the Mastrapa's (6×10^9 m/s^3). Additionally, once a rock ejected from a planet achieves top speed, the g-force is no longer present, differently from our experiment, in which the seeds were subjected to 400,000 x *g* for one hour, continuously. Although these differences are pertinent, we believe our experimental set up is an interesting approach to be compared to Mastrapa's calculations. Finally, since *N. benthamina* is a very important plant model used worldwide, whose biology is well characterized, its potential for studies in the field of hypergravity is enormous.

5. Conclusion

Our data evidences that seeds of *N. tabacum* may be used to further characterize the tolerance of multicellular organisms to extreme hyperacceleration regimes. To our knowledge, this is the second only reported plant species able to withstand and germinate after exposure to $\geq 10^5$ x *g*, thus expanding the number of the extreme hypergravity-tolerant species. Since *N. benthamiana* is a widely used and easy to cultivate plant model, its seeds are rigid, small and light, theoretical and experimental models of lithopanspermia and of life in supermassive planets may benefit from it.

Further *Nicotiana benthamiana* studies, analyzed in the light of literature data on the hypergravity effects in plant models (Dos Santos et al., 2012; Faraoni et al., 2019; Tamaoki et al., 2014; Takemura et al., 2017), will contribute to the understanding of extreme hypergravity effects on multicellular organisms, as physiological adaptations and metabolic pathways activated in response to this type of stress, as well as evaluate the effect of hypergravity in germinated seeds.

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