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Ana Isabel Oliveira da Silva Dias

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Dissertação de Mestrado
Mestrado em Biologia Molecular, Biotecnologia
e Bioempreendedorismo em Plantas

Trabalho realizado sob orientação da
Profª Doutora Ana Cunha
e do
Doutor Javier Medina

janeiro de 2018

DECLARAÇÃO

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ABSTRACT

Effect of simulated microgravity on the cell cycle in cultured cells of *Arabidopsis thaliana*

Life as we know it on planet Earth always evolved in the presence of the constant gravitational force, both in magnitude and direction. This physical force is responsible for giving the weight to all masses. Altering weight changes many processes in a living being, such fluid regulation, as well as ecological processes on Earth, such rain fall. Thus, it is reasonable to say that gravity shapes life. With this study we pretended to investigate how the gravity modulates cell cycle using the *in vitro* cellular system of *Arabidopsis thaliana* cell suspension line MM2d, characterized to be undifferentiated and highly proliferative. In this system cell growth is strictly correlated with the rate of ribosome biogenesis and protein synthesis by the accurate regulation of cell cycle progression. Such coordination is essential for an optimal production of biomass as well as for the viability of daughter cells after division. We investigated the effects of simulated microgravity on cellular functions by growing MM2d cell line suspensions in a 2D clinostat, a device generating simulated microgravity, for a long-term exposure of 24 hours. In order to study this cellular system, the immobilization of cells was optimized by the use of alginate gelling agent, a crucial step for exposure of cell suspension to 2D clinorotation. Under these conditions, the cell proliferation rate showed significant alterations, accompanied by reduction of cell growth. Analysis of cell cycle by flow cytometry showed increase in the proportion of cells in S phase and a decrease in G1 phase, indicating an increase in progression rate of the cell cycle. With respect to cell growth, the rate of ribosome biogenesis was reduced under simulated microgravity, as shown by variations in the abundance of nucleolar proteins nucleolin and fibrillarin, using immunofluorescence microscopy. These results are in agreement with previous observations in root meristems that have shown a decoupling of the meristematic competence, characterized by a strict coordination between cell growth and cell proliferation. Furthermore, it was shown that undifferentiated plant cells also have the ability to respond to changes in the gravity, independently from their integration into plant tissues and organs.

Key words: Simulated microgravity, *Arabidopsis thaliana*, cell growth, cell proliferation, cultured cells, nucleolin, fibrillarin.

RESUMO

Efeito da microgravidade simulada no ciclo celular de culturas celulares *in vitro* de *Arabidopsis thaliana*

A vida como a conhecemos no planeta Terra sempre evoluiu na presença da constante força gravitacional, tanto em magnitude como direção. Esta força física é responsável por dar peso a todas as massas. Mudança no peso altera vários processos nos seres vivos, como a regulação de fluidos, assim como processos ecológicos na Terra, como a queda de chuva. Então, é sensato dizer que a gravidade molda a vida. Com este estudo pretendemos investigar como a gravidade modela o ciclo celular com o uso de sistema celular *in vitro* de suspensão celular da linha MM2d de *Arabidopsis thaliana*, caracterizada como indiferenciada e altamente proliferativa. Neste sistema o crescimento celular é rigorosamente relacionado com a proporção de biogéneses de ribossomas e com a síntese de proteínas através da regulação precisa na progressão do ciclo celular. Esta coordenação é essencial para uma ideal produção de biomassa para a viabilidade das células filhas após divisão. Os efeitos da microgravidade simulada nas funções celulares pelo crescimento da suspensão celular MM2d foi investigada com o uso do clinostato 2D, um aparelho que gera microgravidade simulada, durante 24 horas. De modo a estudar este sistema celular, a imobilização das células foi otimizada com o uso do agente gelificante alginato, o que é um passo crucial para expor a suspensão celular em clinorrotação neste aparelho. Nestas condições, a proporção da proliferação celular mostrou alterações significativas, acompanhadas pela redução do crescimento celular. Análises do ciclo celular por citometria de fluxo mostraram aumento na proporção das células na fase S e diminuição na fase G1, indicando uma progressão do ciclo celular mais rápida. No que diz respeito ao crescimento celular, a biogénese de ribossomas foi reduzida em microgravidade simulada, como observado pela variação da abundância das proteínas nucleolares nucleolina e fibrilarina, através de microscopia de imunofluorescência. Estes resultados estão em concordância com observações prévias nos meristemas da raiz que mostraram desacoplamento da competência meristemática, caracterizada pela coordenação entre crescimento celular e proliferação celular. Ademais, mostrou-se que células vegetais indiferenciadas também têm aptidão para responder a alterações na gravidade, independentemente da sua integração em tecidos e órgãos vegetais.

Palavras chave: Microgravidade simulada, *Arabidopsis thaliana*, crescimento celular, proliferação celular, culturas celulares, nucleolina, fibrilarina.

ACRONYMS AND ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ions
CDK	Cyclin-dependent kinase
CIB	Centro de Investigationes Biológicas
CK2	Casein kinase 2
CYC	Cyclins
DAPI	4′,6-diamidino-2-phenylindole
DFC	Dense fibrilar componente
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESA	European Space Agency
FCs	Fibrillar centres
g	Gravity
G	Gap
G1	Gap 1 (cell cycle)
G1/S	Gap 1 / Synthesis phase
G2	Gap 2 (cell cycle)
G2/M	Gap 2 / Mitosis
GBFs	Ground based facilities
GC	Granular componente
GDL	Glucono-δ-lactone
h	Hour
HM	Homogenization medium
ISS	International Space Station
KCl	Potassium chloride
kg	Kilogram
l	Liter
M	Molar
min	Minute
MM2d	A line of Arabidopsis cell suspension culture maintained in dark
mRNA	Messenger Ribonucleic acid
MSS	Murashige and Skoog (MS) medium supplemented with Hormones
NAA	α-naphtaleneacetic acid
NASA	National Aeronautics and Space Administration
NSB	Nuclear staining buffer
ºC	Celsius degree
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
pH	Negative log of hydrogen ion concentration
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)

p-value	Statistical test provability value
RNA	Ribonucleic acid
RPM	Random Positioning Machine
rpm	Rotation per minute
rRNA	Ribosomal Ribonucleic acid
RT	Room temperature
s	Seconds
T	Time of samples
w/v	Weight/Volume
µ	Micro

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INTRODUCTION

1. Plants in Gravitational Biology

Space exploration has started since the humankind looked at the sky and started to question about the shining objects overhead. In our days, we have stepped on the Moon, humans orbit our planet in the International Space Station (ISS) and shuttles explore our solar system and beyond. Next, human being will step on the red planet, Mars (Musk, 2017). Certainly, crossing the frontier of our planet and becoming a multi-planetary species will act as a driver on progress of human civilization. To see this, we can become remembered in the history of society as the Age of Exploration. It was opening of the frontier in "new worlds" that catalysed the progress of the entire worldwide.

Future space exploration depends on the development of long-term habitation on space and planetary surfaces and growing plants on these environments is part of this effort. Plants are integrated in bioregenerative life support systems to food production, atmosphere revitalization, and primary water purification (Finger *et al.*, 2015). They together with microorganisms are an essential element of these systems (Ferl *et al.*, 2002; Lasseur *et al.*, 2010) (Figure 1). Since photosynthetic microorganisms, such microalgae *Arthrospira platensis*, also can provide CO_2 fixation, O_2 generation and food production. Besides, non-edible parts from plants and human wastes will need to be degraded and recycled, respectively by thermophilic anaerobes bacteria and for both photo-heterotrophic bacteria and nitrifying bacteria, resulting in production of inorganic nutrients to plants (Lasseur *et al.*, 2010). Plants also can help balance astronauts emotions providing an earthly element to the artificial environment of spacecraft or orbital platforms (Williams, 2002). A set of specific environmental factors on orbital platforms such as lack of gravity, increased radiation and absence of convection affect plant development and individual cells as it was shown in many plant species (Chebli and Geitmann, 2011). The gravitational force is the only constant factor guiding and affecting the evolution of all organisms in a permanent manner, unlike most biotic and abiotic stresses. As consequence all biological functions and mechanisms of terrestrial organisms have been developed under its influence, and they proceed taking into account the presence of this mechanical force. Thus, microgravity is a novel environment for plants, which can also alter the way in which organisms detect and response to other environmental factors (Herranz and Medina, 2014). Plant microgravity research is particularly difficult on Earth due to

ever existing gravity, nevertheless it is crucial to learn about the fundamental processes of plant adaptations to microgravity. Understanding molecular and cellular basis of the plant response to gravity is not only important to grow plants in space, but also to verify the evolutionary value of gravity as an environmental factor and for the general benefit in agriculture on Earth by improving knowledge about plant response to abiotic stresses.

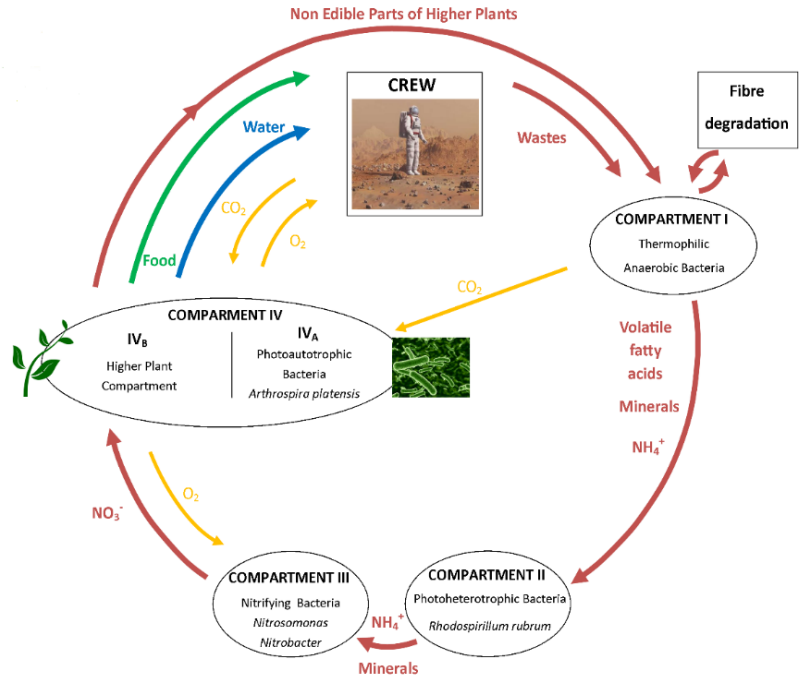

Figure 1: Plant-based Advanced Life support project (Melissa loop), European Space Agency. Melissa (Micro-Ecological Life Support System Alternative) has been conceived as a microorganisms and higher plants based ecosystem intended as a tool to gain understanding of the behaviour of artificial ecosystems, and for the development of the technology for a future regenerative life support system for long term manned space missions. Based on the principle of an "aquatic" ecosystem, Melissa is comprised of 5 compartments colonized respectively by Thermophilic Anaerobic Bacteria (I), Photoheterotrophic Bacteria (*Rhodospirillum rubrum*) (II), Nitrifying Bacteria (*Nitrosomonas/Nitrobacter*) (III), Photoautotrophic Bacteria (*Arthrospira platensis*) (IVA), Higher Plants (IVB), and the crew. Waste products and air pollutants are processed using the natural function of plants which in turn provide food as well as contribute to water purification and supply oxygen for air revitalization. Many other important benefits are being examined for related industrial projects. (From *www.esa.int/spaceinimages/Images/2015/06/MELiSSA_loop_diagram*)

1.1. The impact of gravity in plant biology

Gravity, as any other force, is represented by a vector, meaning that it has magnitude and direction at each point in space. It is responsible for defining the weight of each object which drives many chemical, biological, and ecological processes on Earth. Living organisms have accommodated this force in both their structure and function, developing mechanisms to sense gravity and grow

towards to or against its direction to reach valuable environmental resources such as water and light.

1.1.1. Plants evolution and physiology: the role of gravity

First-ever photosynthetic organisms evolved 3.8 billion years ago in the ancient sea and the first ancestors of land plants evolved from green algae nearly 500 million years ago (Stewart and Mattox, 1975; Zhong *et al.*, 2015). Life on land is under the constant constrain of gravity; the mechanical load on organisms is approximately 1000 times larger on land than in water (Volkmann and Baluška, 2006). Therefore, the development of structural features associated with the support of the mass (a rigid plant body), has been one of the critical evolutionary achievements required for plants to survive under 1 *g* conditions. In fact, during evolution plants have acquired specific organs, tissues, and molecular systems capable of detecting gravity. Root positive gravitropism is essential for water and mineral ion uptake and anchorage of the whole plant. The shoot negative gravitropism, on the other hand, helps the plant to reach sunlight, the energy source for sugar production. Living systems are constantly evolving in response to a wide range of environmental cues. Consequently, all biological functions and mechanisms have developed under the influence of a specific set of environmental conditions. Primitive mechanisms of gravisensing may now coexist with others which arose independently at later stages of evolution (Barlow, 1995).

The study of plants in altered gravitational field is crucial for better understanding the adaptive mechanisms and physiological activities modulated by gravity. Although plants have evolved a set of mechanisms to adapt to extreme environmental conditions, they have never experienced the need to evolve specific mechanisms to respond or adapt to altered gravity.

1.1.2. Perception of gravity in plant cells

Gravity perception in plants plays a key role in their development and acclimation to the environment, from the direction of seed germination to the control of the posture of adult plants. Several models have been proposed to explain plant cellular perception of gravity, based on gravity-sensor organs that drive plant growth according to the direction of the gravity, independently of its magnitude (Perbal *et al.*, 2002; Pouliquen *et al.*, 2017) and on a gravi-resistance mechanism that involves tension on cellular scaffold structures (cytoskeleton, cell wall or membranes), producing an effect that is proportional to the magnitude of the gravity (Soga *et al.*, 2001).

According to the classic *starch-statolith hypothesis* (Haberlandt, 1900; Nemec, 1900), connecting gravity sensing and gravitropism, the perception of gravity starts in specific cells that act as

statocytes. Statocytes are located in different organs. In shoots, they are found in endodermal cells, in roots, within columns of cells located in the central root cap, called columella (Figure 2); and they have recently been localized in the secondary phloem of mature woody stems (Gerttula *et al.*, 2015). These cells contain starch-filled amyloplasts-called statoliths-characterized by a higher density than the surrounding cytosol. When the orientation of the gravity vector changes relative to the orientation of the organism, the statoliths move toward the new downward direction side of the cell and their sedimentation in this new position exert a force, presumably on the plasma membrane and on the endoplasmatic reticulum that triggers the activation of ion (Ca^{2+}) channels (Figure 3A) (Fasano, *et al.*, 2002). The transduction of the gravistimulus leads to the relocation of membrane transport proteins called PIN-FORMED (PIN) proteins (Ottenschläger *et al.*, 2002). Next, the PIN proteins redirect the lateral distribution of the auxin polar transport, the major plant hormone, leading to a differential growth of the organ and ultimately causing bending of the organ to the desired orientation with respect to the gravity vector direction (the *Chodlony-Went hypothesis*) (Cholodny, 1927; Firn and Digby, 1980; Went, 1933). Particularly in roots, this results in a faster growth and/or elongation of the cellular layers on the opposite side, resulting in root curvature in the direction of the gravity vector (Baluška *et al.*, 2010).

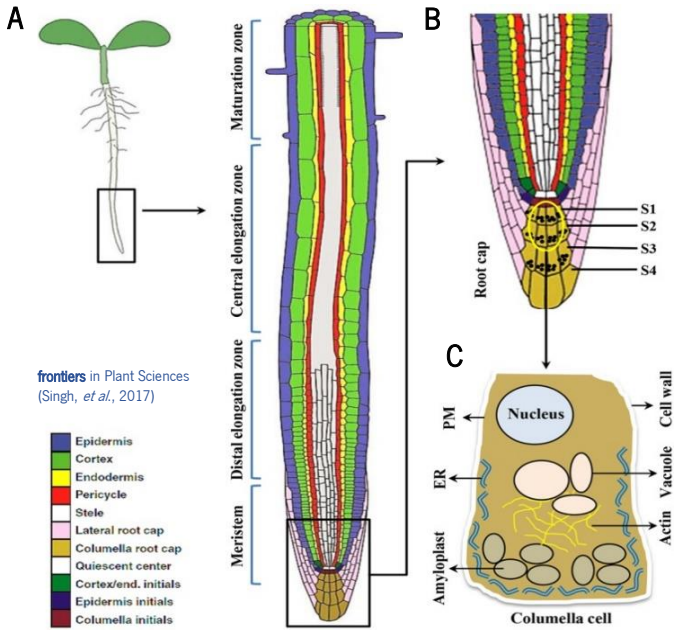

Figure 2: The root cell types responding to gravity stimulus in *Arabidopsis*. A) Longitudinal view of an *Arabidopsis* primary root showing meristem, distal and central elongation zones, maturation zone. **B)** Gravity sensing in roots occurs in the central columella cells of the root cap. The root cap consists of four layers of cells important for root gravitropic response namely S1, S2, S3, S4, respectively. However, the central columella cells from S1 and S2 (the encircled cells) play major role for gravity sensing. **C)** Columella cell (cartoon) contains starch-filled organelles called amyloplasts, nucleus, vacuole, actin filaments, endoplasmic reticulum (ER), plasma membrane (PM), and cell wall (Singh *et al.*, 2017).

At the cellular level, the exact roles of the statoliths, and whether or not they are necessary for a graviperception is still a matter of debate. In experiments using mutants deprived of starch and displaying little if any sedimentation of statoliths, the macroscopic response to a gravistimulation is dramatically diminished compared to the wild-type (Fitzelle and Kiss, 2001; Kiss *et al.*, 1989;

Pickard and Thimann, 1966). However, the response still exists suggesting that other type of gravity perception mechanisms take place causing the cell response to the changes in *g* force. Moreover, most plant cells are not equipped with statoliths (Chebli and Geitmann, 2011). Evidence for the presence of an alternative mechanism comes from studies on mosses, fungi, and algae which show gravity-dependent growth and differentiation without the presence of statoliths (Staves *et al.*, 1997). While at times considered a controversy, it became clear that several mechanisms of gravisensing seem to operate, possibly even in the same cell (Barlow, 1995; Kiss, 2000).

The *gravitational pressure model* provides a possible but not necessary the only explanation for this phenomenon. The model suggests that the entire mass of the protoplast acts as a gravity sensor that behaves like a water-filled balloon that flattens when placed on a surface due to its own weight (Chebli and Geitmann, 2011). In this model, the role of starch-filled amyloplasts would be that of increasing the overall density of the protoplast. It is postulated that membrane proteins located at the top and bottom of the cell may be activated through the action of differential tensile forces as they interact with the lower and upper cells walls, respectively (Figure 3 B) (Wayne and Staves, 1996).

The *cellular tensegrity model* is an alternative, but not exclusive view, on how mechanical forces acting on the cells as a whole could be perceived (Ingber, 1997). It proposes that the whole cell is a pre-stresses tensegrity structure, where tensional forces are borne by cytoskeletal array consisting of elements that resist compressive (microtubules) and tensile stresses (actin filaments). The

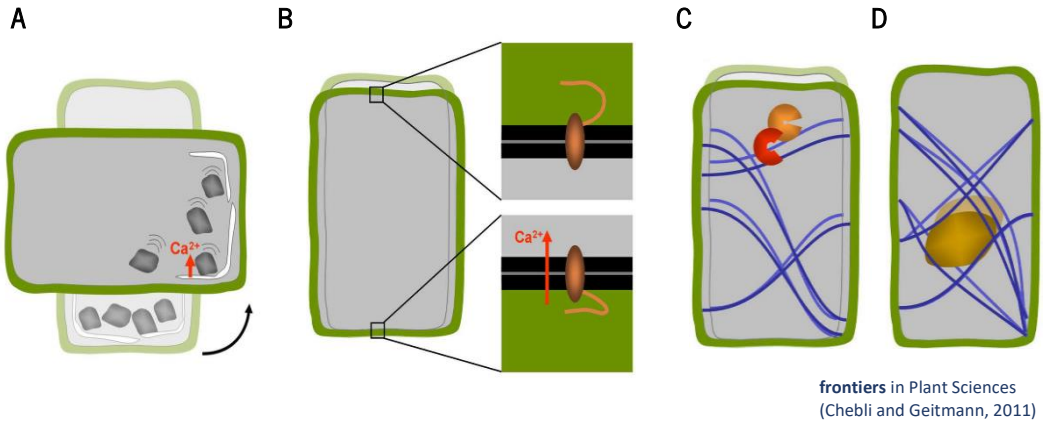

Figure 3: Concepts of cellular gravisensing in plants. A) In **statolith-based gravisensing** the sedimentation or change of position of intracellular organelles with higher density triggers a signal most likely based on a change in trans-membrane ion fluxes. **B)** According to **the gravitational model** the weight of the protoplast causes the forces acting on the membrane-cell wall connections at the upper and lower sides of the cells to be different. **C) The tensegrity model** predicts that cellular distortion due to a change in *g*- forces affects the pre-stress in the cytoskeletal array of the cell which in turn changes biochemical activities. **D)** A variation of **the tensegrity model** is based on a change in cytoskeletal pre-stress being caused by the weight of heavy organelles that are tethered to the cytoskeletal filaments such as the nucleus (Chebli and Geitmann, 2011).

tensional pre-stress that stabilizes the whole cell is generated actively by the contractile actomyosin apparatus and passively from external forces (e.g. gravity, wind, osmotic forces or adhesion to other cells) (Hamant and Haswell, 2017; Ingber, 2003). The distortion induces a change in pre-existing force balance and is supposed to affect local thermodynamics or kinetic parameters and in results biochemical activities (Figure 3 C) (Ingber, 2006; Orr *et al.*, 2006). This change in pre-stress cytoskeletal arrays could be generated by the gravity force acting on organelles attached to this network (Figure 3 D) (Yang *et al.*, 2008). Hence, gravity-induced cytoskeleton polymerization and architecture will likely affect many biological properties (Vogel and Sheetz, 2006).

These proposed mechanisms underline how relevant can be a physical force in the general plant development. The plant detects and responds to the physical stimuli and converts the information into a physiological (chemical) signal, which is then transduced and integrated. As stated by van Loon (2007), "There are no biochemical modifications without prior mechanical change". The fundamental questions concerning the mechanisms of gravity perception, transduction and response remain still unanswered.

1.1.3. Gravity alteration induces plant response to biotic and/or abiotic stress

The evolution of all biological functions and mechanisms in living systems was influenced by the presence of the gravity. They include the strategies and mechanisms of perception, response and adaptation to a wide range of abiotic (such as light, temperature, water, wind, magnetic or electric fields) and biotic (interactions with other living beings) environmental stresses. Plants under stressors respond by altering physiological processes and by modulating the expression of specific genes (Timperio *et al.*, 2008; Zupanska *et al.*, 2013).

Studying plants in altered gravity allows us to understand how plants respond to this environmental change, which may also alter detection and response to other environmental factors. Environmental stresses many times have a synergistic effect on plant, in result promoting a complex environmental stress response (Beckingham, 2010; Herranz *et al.*, 2010). Furthermore, changed in the gravity may also influence other environmental conditions; e.g. the distribution, availability or concentration of nutrients in the atmosphere or in the soil.

Recent studies reveal that plant response to gravity alterations has some common and some unique features compared to other environmental changes (Ferl *et al.*, 2015; Herranz *et al.*, 2013b; Manzano *et al.*, 2016; Paul *et al.*, 2012). Interestingly, a positive red light-based phototropic response in root Arabidopsis has been observed in conditions of microgravity (Millar *et al.*, 2010).

In addition, a decrease in response to red light was gradual and correlated with the increase in gravity (Vandenbrink *et al.*, 2016). A curious fact is that ancient plant lineages (moss and fern) shows this red-light phototropism on Earth, but flowering plants have lost this feature during evolution, that is primarily a response to blue light. It is only removal of the gravity factor that unmasks the capacity for directional red-light sensing for phototropism in higher plants

1.2. Microgravity research in plants

Research on microgravity has contributed greatly to disclose the impact of gravity on biological processes. The real microgravity (see below for definition) is only available in the outer space since the 1 *g* level cannot be avoided on the surface of our planet. However, access to spaceflights and to the ISS is limited by the cost and the labour needed to prepare the flights. In addition, it is difficult to distinguish between the effects of microgravity and changes related to the space and spaceflight conditions (radiation, vibration on shuttle, etc.). To overcome these constraints, ground based facilities (GBFs) are available for preparing spaceflight experiments, and also for developing simulated microgravity experiments on the ground thus, providing additional cost-efficient platforms for gravitational research (Herranz *et al.*, 2013a). In addition, the ground experiments in GBFs enable testing of biological systems and addressing gravity-related issues prior to space experiments.

1.2.1. Definitions used in Gravitational Biology research

Real microgravity (μg). The term "microgravity" is frequently used as a synonym of "weightlessness" and "zero-*g*", but under microgravity there is a remaining *g*-force which is not zero, but just very small. In fact, the term "microgravity" should be applied to *g*-forces equal or lower than 10^{-6} *g*. Real microgravity can only be achieved in a durable and constant way during freefall experiments. Those experiments can be performed on board of the spaceflights (sounding rockets, satellites, space stations), parabolic flights (only for times shorter than 20 s combined with hypergravity periods) or in drop towers (for very short time, providing only 5-10 s of microgravity). Mid- and long-term experiments in real microgravity can only be performed in space (Herranz *et al.*, 2013a).

Simulated microgravity (sim μg) conditions. It has been proposed to use the term "simulated microgravity" to describe the state of acceleration achieved using GBF, as it is perceived by a biological organism (Herranz *et al.*, 2013a). In simulated microgravity experiments the magnitude

of the Earth gravity vector cannot be changed, only the way it is perceived (Briegleb, 1992). In consequence, microgravity cannot be achieved with a simulator. Rather, such simulator may generate functional weightlessness from the perspective of the organism or the cell. In effect, the organism or cell does not perceive the gravity since its value is below the sensitivity of the gravity receptors. The choice of the best GBF to be used for a given research and model system should take into account the sensitivity of the researched biological process and organism used (Herranz *et al.*, 2013a).

1.2.2. Simulated microgravity on Earth: concept, GBFs and limitations

Various GBFs with different physical concepts (essentially mechanical and magnetic) have been constructed to simulate microgravity conditions on ground. None of them is absolutely optimal, and consequently, the final choice will depend on the biological material and the experimental analyses to be performed (Figure 4).

One example of GBF is the clinostat. The clinostats are classical mechanical devices designed for simulation of microgravity, dating from the nineteenth century. In this device samples are rotated to prevent the biological system from perceiving the gravitational force direction (Herranz *et al.*, 2013a). The functionality of clinostats is based on redistributing the gravity in a circle, whereas the sample rotate around a single or multiple axis. The low cost of clinostat and its availability makes it one of the most widely used GBFs.

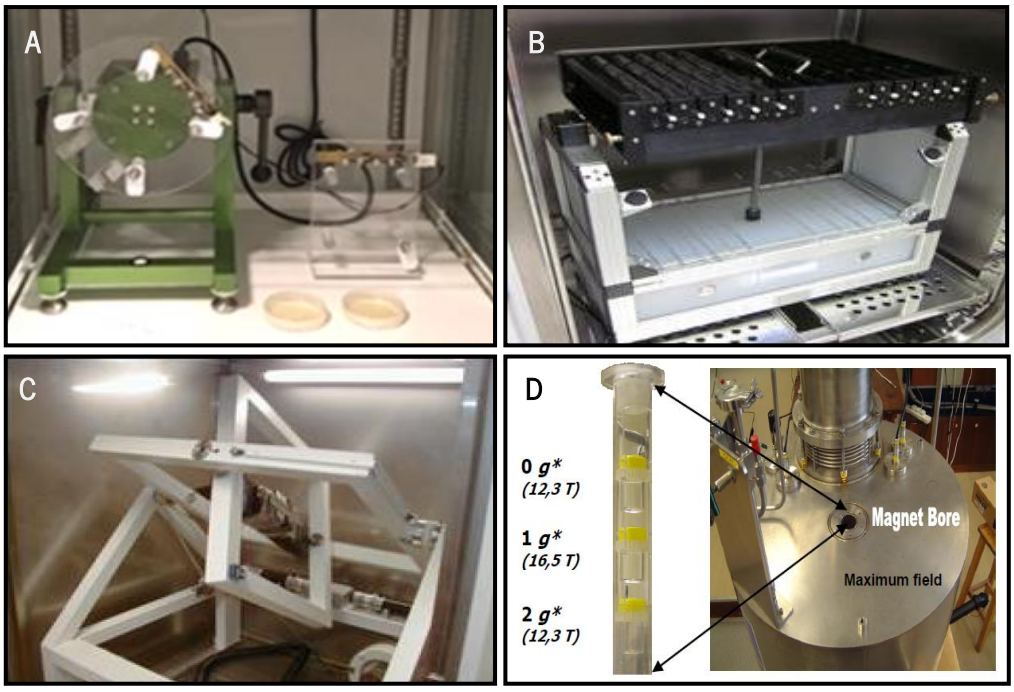

Figure 4: Several ground based facilities (GBFs) used in plant biology research. A) 2D clinostat, located at Centro de Investigations Biológicas (CIB), Madrid, Spain. **B)** Pipette 2D clinostat **C)** 3D RPM **D)** Magnetic levitation facility.

2D clinostat or one-axis clinostat has a single rotational axis, which runs perpendicular to the direction of the gravity vector and rotates at speeds that are matched with the particular time of graviresponse for the sample in question (Figure. 4 A) (Briegleb, 1992; Klaus, 2001). This device has a limitation, when a sample is placed at the rim of the rotational axis the force it experiences increases (Brown *et al.*, 1976; Klaus, 2001). However, this constraint can be avoided by positioning the samples close to the centre of the rotational axis. A rotation on a clinostat is often called clinorotation.

Pipette 2D clinostat. This device is used to achieve functional weightlessness for small objects, mainly single cells (Figure 4 B) (Briegleb, 1992). By fast and constant rotation of a small tube, completely filled with liquid, it is assumed that sedimentation of the cells is prevented physically by a continuous and constant change of the direction of gravity vector (Herranz *et al.*, 2013a; Klaus, 2001). In this scenario, particles are forced to move on circular paths that depend on rotation adjusted to reach a non-sedimentation effect (Klaus, 2001).

3D clinostat (RPM). Clinostat with two axes are called 3D clinostat. The most commonly used is the Random Positioning Machine (RPM) (Figure 4 C), which operates with different speeds and different directions. The quality of simulation is increased by rotating around two axes and randomized speed compared to a classic 2D clinostat. It is optimal especially for larger objects (Kraft *et al.*, 2000; Van Loon, 2007).

Magnetic levitation technology is another physical concept used to simulate microgravity. It uses the diamagnetic properties of water, which is the major component of biological objects.

Magnetic levitation. A magnetic field applied to biological material can produce a diamagnetic force with the same magnitude as gravity and the opposite direction. This force is capable of compensating the weight of the sample, in result producing the levitation phenomenon (Valles *et al.*, 1997). The advantage of magnetic levitation (Figure 4 D) is the stability of the compensated force, together with the possibility of generating partial gravity and even hypergravity in the same controlled environment. The disadvantage are the secondary effects of the strong magnetic field (Herranz *et al.*, 2013a; Manzano *et al.*, 2012b). Also, this technology in terms of power supply is very high and may require, in some cases, the power supply of a small city (Herranz *et al.*, 2015).

2. Cell growth and cell proliferation in *Arabidopsis thaliana*

Arabidopsis thaliana is a model system extensively used for studying plant biology. Its short life cycle, completely sequenced genome, and the existence of a multitude of transgenic plants are just few factors that have contributed to its popularity. These are the reasons why this species has been used to examine fundamental questions on plant growth and proliferation mechanisms under simulated microgravity environment. In meristematic cells, growth and proliferation are strictly coupled and represent so-called "meristematic competence" (Figure 5). Cell growth is required for proliferation which produces daughter cells (Mizukami, 2001).

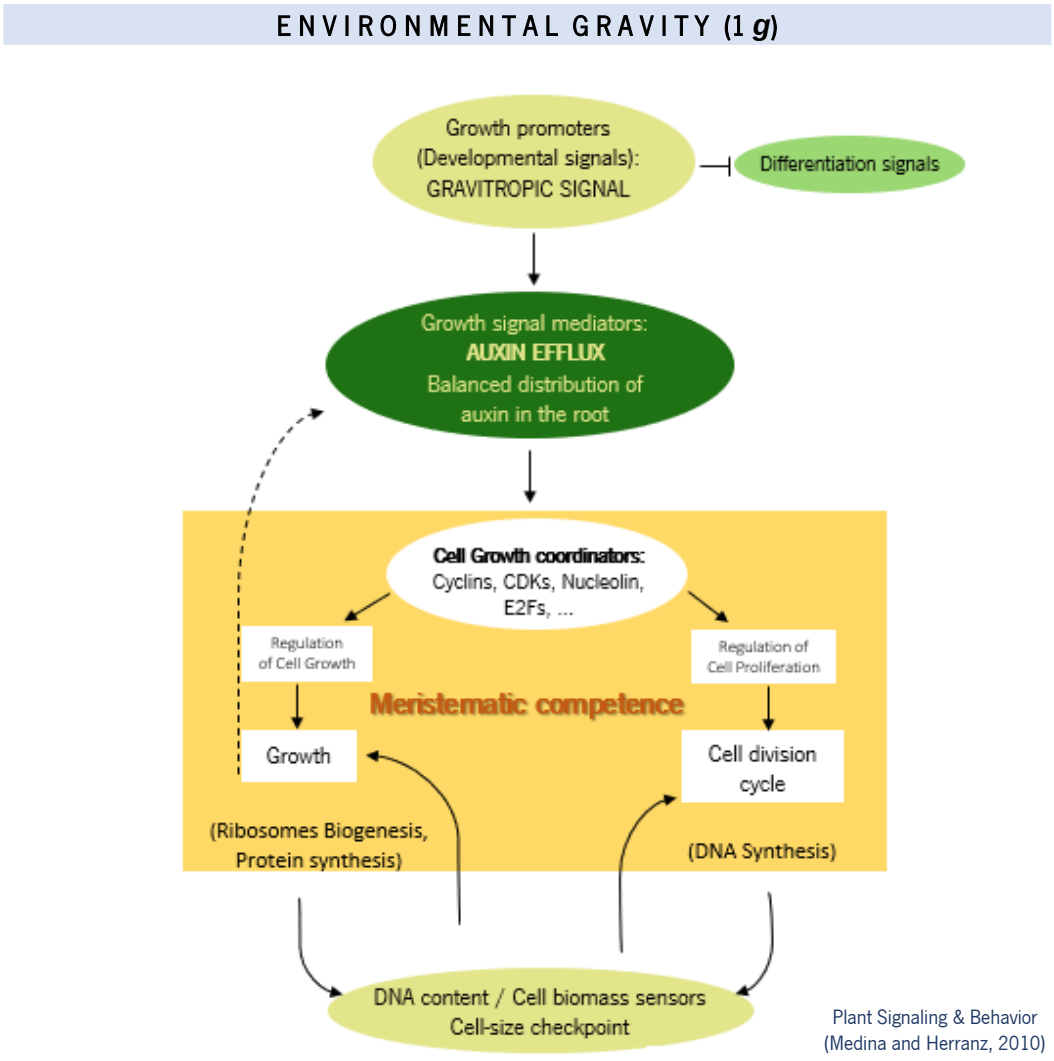

Figure 5: Model of the actions and interactions leading to the maintenance of the meristematic competence (involving the coordination between cell growth and cell proliferation) specifically showing the players involved in the transduction of the gravitropic signal. Under ground 1 *g* conditions the gravitropic signal is a growth promoter, since it stimulates the growth of primary root according the direction of the gravity. The mechanic stimulus is transduced by the mediator auxin that, in the case of meristematic cells, modulates the expression of a variety of molecules that coordinate the regulation of cell growth and cell proliferation and then keep coupled the processes of protein synthesis (cell growth) and cell cycle progression (cell proliferation). (Scheme is adapted and modified by Medina and Herranz, 2010 from Mizukami, 2001.)

2.1. Plant cell cycle

Plant development consists of initiation and growth of new organs throughout the lifespan of the organism. The sessile nature of plants forces them to respond to the changes in the environment adjusting growth and development. Cell division is one of the major processes that contribute to the plant growth. In effect, the control of the cell cycle is essential for understanding plant growth and development. Cell cycle is an ordered and repetitive process that controls spatially and temporally the replication of genetic material and the segregation of duplicated chromosomes into two daughter cells. This cycle is constituted by four successive phases: G1, S, G2 and M (Figure 6). Lag or gap (G) phases separate the replication of the DNA (S phase) and the segregation of the chromosomes (M phase, mitosis) and allow the revision if the previous phase has been accurately and fully completed.

During G1 phase (the first gap) cell growth occurs by synthesis of proteins and RNA, and energy reservoir is stored in form of ATP. The S phase (phase of DNA synthesis) is the phase when the replication of DNA occurs. The G2 phase (gap) is another phase of growth, distinguished from G1 by the fact cells contain a doubled DNA content and by high protein production. This phase is also engaged in the preparation of the cell for division. Finally, during the M phase the mitosis occurs resulting in two daughter cells. The major regulatory points in the cell cycle are the G1/S and G2/M transitions, these points are of potential arrest that may take place after evaluation of external conditions (Dewitte and Murray, 2003; Van't Hof, 1985).

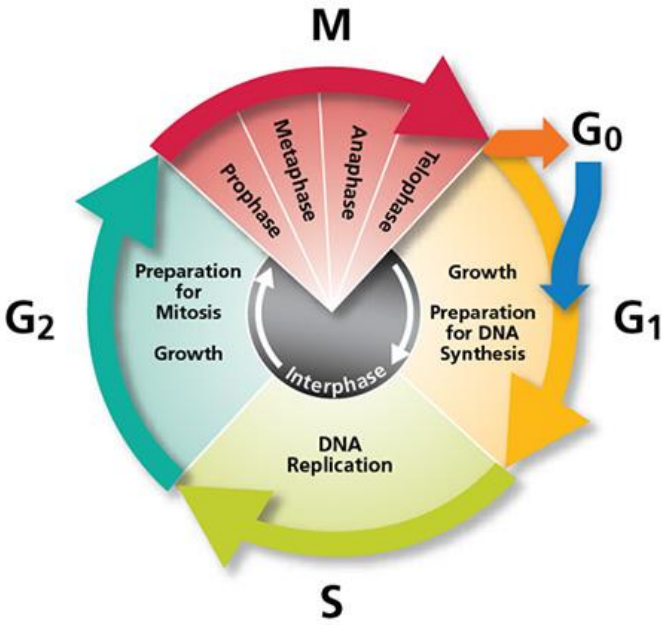

Figure 6: Schematic of the cell (division) cycle. Cell cycle consists of four essential phases, G1 (Gap 1), S (Synthesis), G2 (Gap 2), and M (Mitosis for cell division).

Differentiating plant cells often display an alternative cycle known as endoreduplication, characterized by an increase in the nuclear ploidy level that results from repeated S phases with no intervening mitosis (Joubès and Chevalier, 2000). In all cases, endoreduplication appears to occur only after cells have ceased normal mitotic cycles (De Veylder *et al.*, 2001; Foucher and Kondorosi, 2000).

2.1.1. Cell cycle control

Eukaryotic cell cycle is regulated at multiple points, but all or most of these checkpoints involve the activation of specific class of serine-threonine protein kinases. They require for their activity the binding to regulatory proteins known as cyclins (CYC), and are therefore named cyclin-dependent kinases (CDKs). Cyclins provide the primary mechanisms for the control of CDK activity because the CDK subunit is inactive unless it is bound to an appropriate cyclin (Figure 7) (Dewitte and Murray, 2003; Inzé and De Veylder, 2006).

The cell cycle can be regarded as an oscillator of CDK activity, with low activity in the G1 phase and a peak during mitosis (Coudreuse and Nurse, 2010). This oscillator is driven by highly regulated synthesis and proteolysis of regulatory components through the ubiquitin-mediated selective protein degradation proteasome system at specific points in the cycle (Genschik *et al.*, 2013). Indeed, the exit from mitosis and the return to the ground state in G1 requires the loss of CDK activity through the destruction of cyclins (Scofield *et al.*, 2014).

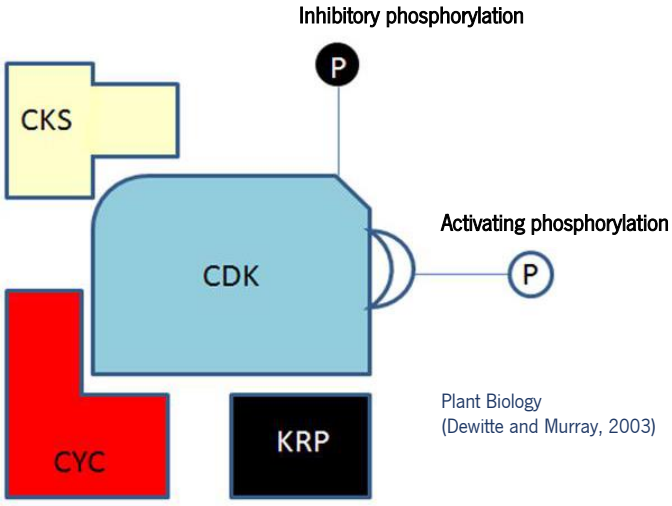

Figure 7: Cyclin-dependent kinase (CDK) activity is regulated at multiple levels. Monomeric CDK lacks activity until it forms a complex with cyclins (CYC) and is activated by phosphorylation by CDK-activating kinase (CAK). In addition, activity can be inhibited by binding of inhibitor proteins such as Kip-related proteins (KRP). Inhibitors may block the assembly of CDK/cyclin complexes or inhibit the kinase activity of assembled dimers. CDK subunit (CKS) proteins scaffold modulate interactions with target substrates. (From Dewitte and Murray, 2003)

Arabidopsis thaliana genome encodes at least 32 cyclins with a putative role in cell cycle progression; 10 A-type, 11 B-type, 10 D-type and 1 H-type cyclins, in addition to 17 other cyclin-related genes which are classified into types C, P, L, and T (Acosta *et al.*, 2004; Vandepoele *et al.*, 2002; Wang *et al.*, 2004). A-type cyclins generally appear at the beginning of S phase, are involved in S phase progression, and are destroyed around the G2/M transition (Dewitte and Murray, 2003). B-type cyclins appear during G2 phase, control G2/M transition and mitosis, and are destroyed as cells enter anaphase. D-type cyclins control progression through G1 and into S phase and differ from A and B types by generally not displaying a cyclical expression or abundance, their presence appears to depend on extracellular signals that stimulate or maintain division. If such signals are removed, levels of D-type cyclins decline rapidly and cells remain blocked in G1. (Dewitte and Murray, 2003; Renaudin *et al.*, 1996; Van Leene *et al.*, 2010). The levels of cyclins are generally determined by their highly regulated transcription as well as by specific protein-turnover mechanisms (Genschik *et al.*, 1998; King *et al.*, 1996).

2.1.2. Cell cycle transitions and progression

Although the cell cycle is regulated at numerous stages, extracellular growth signals seem to act at two main points, G1/S and G2/M. Therefore, different CDK/CYC complexes phosphorylate target proteins whose inhibitory or activatory post-translational modifications are essential for passing these cell cycle checkpoints (De Veylder *et al.*, 2003; Joubès *et al.*, 2000).

G1/S transition

The most outstanding fact in the regulation of the G1/S transition is the induction of the synthesis of D-type cyclins. Plants contain an extensive array of cyclin D genes: genome analysis reveals that *A. thaliana* has at least ten, as opposed to mammals, with only three. D-type cyclins are primary mediators of the G1/S transition and hence have a major responsibility for stimulating the mitotic cell cycle in the presence of growth factors such sucrose, auxin, cytokinin, and brassinosteroids. Their transcription is activated by extracellular signals, and lead to the formation of active CDKA/CYCD complexes (Dewitte and Murray, 2003; Francis, 2007; Inzé and De Veylder, 2006). The major target of CDKA/CYCD kinase activity complexes in G1 is the retinoblastoma-related (RBR) protein, which is phosphorylate by these complexes. (Gutierrez *et al.*, 2002). The retinoblastoma (Rb) protein was identified as a human tumour suppressor protein that controls various stages of cell proliferation through the interaction with members of the E2F family of transcription factors (Desvoyes *et al.*, 2013). It is believed that during the early G1 phase the E2Fs

are mainly involved in the repression of several cell cycle-regulated promotors, whereas during the transition from G1 to S phase the release of transcriptionally active E2Fs, resulting from the phosphorylation of the CDKA/CYCD/RBR pocket proteins, is required to drive the expression of S phase genes (Figure 8 A) (Chabouté *et al.*, 2002; De Veylder *et al.*, 2002; Mariconti *et al.*, 2002)

G2/M transition

Again, in G2/M, CDKA is the major driver of this transition after its association with D-, A- and, particularly, B-type cyclins (Figure 8 B) (Inzé and De Veylder, 2006). In addition to CDKA,, the G2/M transition requires CDKB activity, the expression of which is dependent on transcriptional control of E2F pathway, properly providing a mechanism by which G1/S and G2/M transitions are regulated (Boudolf *et al.*, 2004). Both CDKA and CDKB-cyclin complexes are activated by a CAK activity (likely CDKD and/or CDKF) before they can phosphorylate a variety of targets that contribute to enter mitosis. The activity of the CDK/CYC complexes can be inhibited by their association with CDK inhibitory proteins (ICK/KRP and SIM) that respond to stress stimuli or developmental signals (Verkest *et al.*, 2005; Churchman *et al.*, 2006). In case of replication stress or DNA damage, there is evidence that CDKA is a WEE1 kinase target mediating G2 arrest (De Schutter *et al.*, 2007). In turn, the CDC25 phosphatase is able to dephosphorylate the residues phosphorylated by WEE1, allowing entry into mitosis (Ferreira *et al.*, 1991; Francis, 2007; Inzé and De Veylder, 2006). Once the CDK/CYC complexes are active, they trigger the G2/M transition through the phosphorylation of a plethora of different substrates. Furthermore, the protein casein kinase CK2 shown discrete activity peaks at G1/S and M in tobacco BY-2 cells, and blocking its activity during G1 abolishes the G2/M checkpoint, resulting in premature entry into prophase; this is an evidence of links between G1 processes and G2 controls (Espunya *et al.*, 1999). Finally, exit from mitosis requires the proteolytic destruction of the cyclin subunits. This destruction is initiated by the activation of the Anaphase-Promoting Complex/Cyclosome (APC/C) through its association with CCS52 protein (Heyman and De Veylder, 2012).

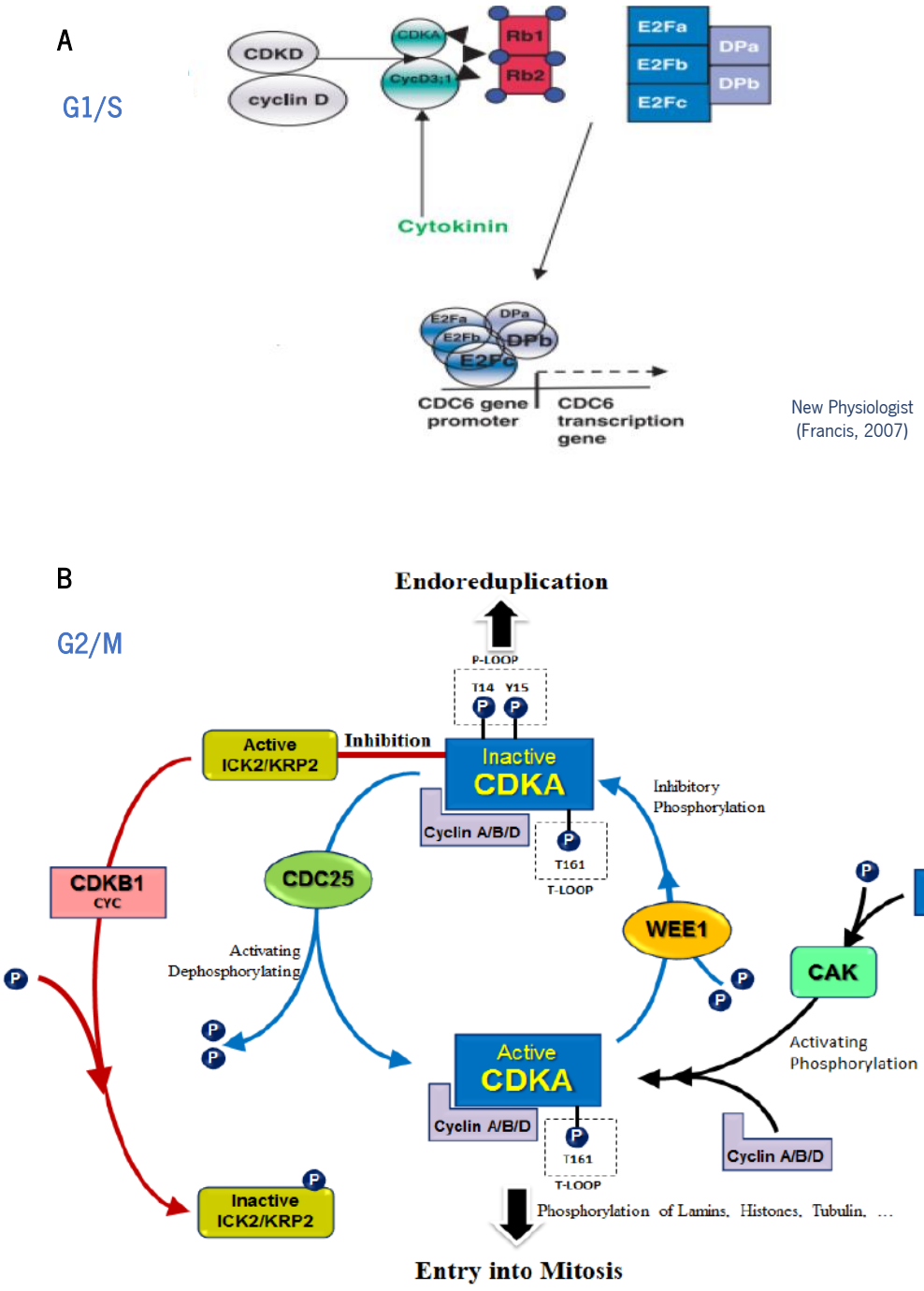

Figure 8: Different cyclin-dependent kinase (CDKs) and cyclins regulate G1/S and G2/M transition points through the cell cycle progression. A) At G1/S transition, CDKD/cyclin D phosphorylates CDKA enabling CycD3;1 to bind. A cytokine-induced transcellular induction culminates in CyCD3;1, which activates CDKA that, in turn, phosphorylates Rb1 and 2. This releases the E2F complex, which then induces the expression of S phase-specific genes (e.g. CDK6) (From Francis, 2007). **B)** G2/M transition is regulated by the activation/inactivation of complex CDKA/cyclins (A, B, D) where several theories of control, is identical to that of animals based on complex regulating CDK-CDC25-WEE1 or by CDKB activity. The activity of the CDKA/CYC complexes can be inhibited by their association with CDK inhibitory proteins (ICK/KRP) that respond to stress stimuli or developmental signals (From De Veylder *et al.*, 2007).

2.1.3. *In vitro* cell cultures: synchronous cell cycle

Root meristem is the most commonly used material to investigate the influence of microgravity on plant growth and proliferation, because it plays a key role in sensing the gravity factor. Although the elongation zone is the known target of the transduced gravitropic signal, the meristematic region has been shown to perceive and react to gravitational changes (Medina and Herranz, 2010). In this biological system, the role of auxin is central in connecting the alterations found within meristematic cells in response to gravity changes. However, it does not provide an explanation on how cells lacking specialized gravity-sensing organelles, as statoliths, perceive and alter biological processes in response to changes in gravity.

Cell suspension culture is a perfect model to investigate this topic since it provides a homogeneous population of nearly identical cells, in the absence of developmental processes (Gould, 1984). In this way, *in vitro* cell cultures are a powerful tool to study cellular response to environmental stresses. Also, they are the most suitable systems for synchronisation, allowing detailed analysis of cell cycle studies (Gould, 1984; Menges *et al.*, 2003; Menges and Murray, 2006). The cell line MM2d of *Arabidopsis thaliana* consists of uniform small clumps of creamy-coloured cells growing fast and at high density, suitable for synchronization. It is composed of a population of undifferentiated actively proliferating cells, similar to the ones in root meristem (Figure 9) (Menges and Murray, 2002).

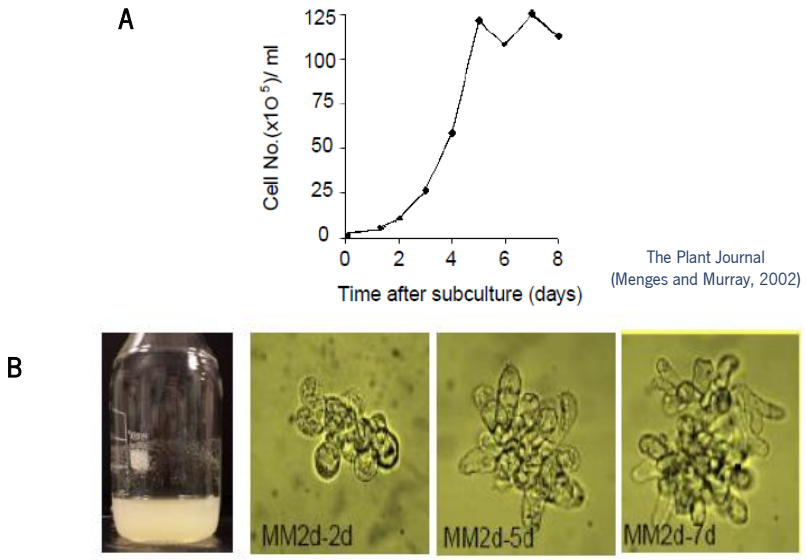

Figure 9: Growth characteristics of *Arabidopsis* cell line MM2d (Menges and Murray, 2002). **A)** Early stationary phase for 7 days, cells were subculture, and cell growth monitored of cell line MM2d. **B)** MM2d cell suspension maintains after 7 days in dark conditions. and cell morphology of MM2d through the cultivation time 2, 5, and 7 days, respectively.

Ideally in a synchronized cell system, all or most cells progress through the cell cycle at the same rate from the same initial starting point. Various synchronization procedures are based on accumulating cells at a specific point in the cell cycle which is followed by the reactivation of cell cycle progression. The most common approaches use reversible inhibitors of a specific cell cycle step or nutrient deficiency to block progression of the cell cycle, followed by the release of the synchronized cells by the removal of the inhibitor or resupply of specific nutrients (Menges and Murray, 2002). Arabidopsis cell suspension cultures synchronized by aphidicolin block/release is a suitable system for following both the re-entry of cells into the cell cycle and progression from the S phase. Aphidicolin is a fungal toxin derived from *Nigrospora sphaerica* that reversibly inhibits DNA polymerase (Dehghan Nayeri, 2014). Synchronization with aphidicolin produced up to 80% S phase cells, which constitutes a clear separation of different cell cycle phases (Menges and Murray, 2002, Menges and Murray, 2006).

2.2. Plant cell growth: the nucleolus and ribosome biogenesis

Whereas the concept of cell proliferation is unambiguous in reference to progression of the cell cycle, by sustained growth to allow cell division, the concept of cell growth may be caused by different phenomena. Growth is the increase in size, however plant cells may grow either via increase in its cytoplasmic mass or via expansion of intracellular vacuoles. The latter is a feature of cell differentiation and accounts for the growth of differentiated organs. On the contrary, cytoplasmic mass increase is a feature of rapidly dividing cells, such as meristematic cells, in which vacuoles are extremely small (Magyar *et al.*, 2005). In this case, cell growth essentially is due to the activity of the protein synthesis (Mizukami, 2001). Thereafter, it is strictly correlated to the rate of biogenesis of ribosomes, since the function of ribosomes is the translation of mRNA into proteins. Ribosome biogenesis occurs in a well-defined nuclear territory called the nucleolus. In actively proliferating cells, nucleolar morphology can serve as a structural marker to state of both cell growth and cell proliferation mechanisms, connecting them in the same framework (Baserga, 2007; Sáez-Vásquez and Medina, 2008). Nucleolar features like size, distribution and accumulation of its components or the level and distribution of certain nucleolar proteins show profound variations throughout cell cycle and are strictly associated to the rate of transcription and processing of ribosomal precursors (Medina and Herranz, 2010).

2.2.1. The plant nucleolus during the cell cycle

Most eukaryotic cells contain a prominent region within the nucleus called nucleolus. The main function of the nucleolus is linked to ribosome biogenesis and closely associated with other important biological processes, including RNA metabolism, regulation of gene expression, cell cycle regulation, DNA repair and cell aging (Sáez-Vásquez and Medina, 2008; Montacié *et al.*, 2017).

As referred above, the nucleolus is the site of ribosome biogenesis, which involves the production of ribosomes subunits by transcription of rRNA genes (in plants 18S, 5.8S and 25S), maturation of the transcripts and their transport to the cytoplasm where they are assembled into functional ribosomes (Sáez-Vásquez and Medina, 2008). Morphological features of the nucleolus can be distinguished for each phase of the ribosome synthesis. In interphase cells, the nucleolus is formed by three basic components: the fibrillar centres (FCs), the dense fibrillar component (DFC) and the granular component (GC), which are sometimes accompanied by other structures, such as vacuoles (Figure 10) (Jordan, 1984). FCs are most likely the sites for anchoring of rDNA where the assembly of transcription complexes takes place. Homogeneous FCs often appear in nucleoli actively producing ribosomes and heterogeneous FCs are associated with low rates of nucleolar transcriptional activity (Risueño and Medina, 1985). In DFC is where the early steps of pre-rRNA processing take place. Finally, in GC, further RNA processing and RNA modification steps occur, together with the formation of pre-ribosomal particles, ready for the export to the cytoplasm after maturation process (Thiry and Lafontaine, 2005).

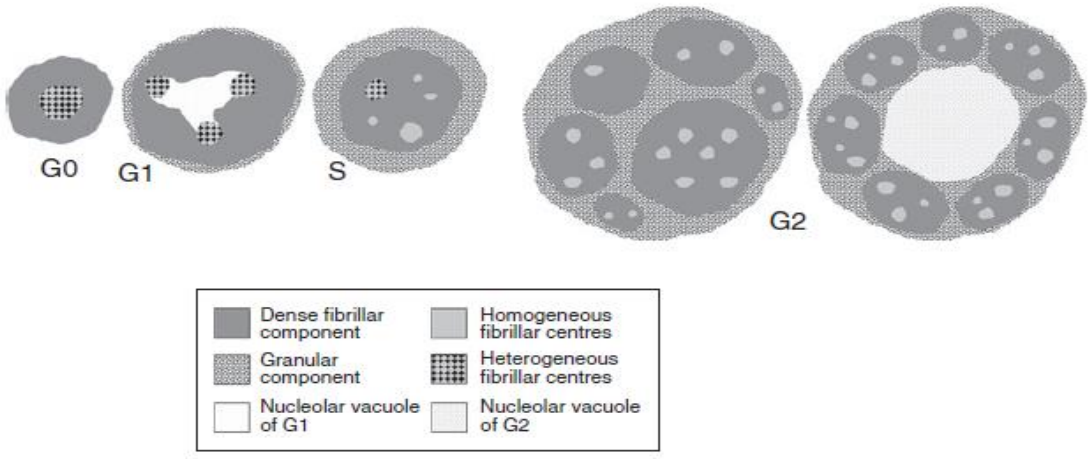

Figure 10: Nucleolar models in the different interphase periods. It is shown the relative nucleolar size, as well as the distribution of the nucleolar structural subcomponents in each period. Morphological and morphometrical features correlate to the rate of nucleolar transcriptional and processing activity. (From González-Camacho and Medina, 2006).

During the cell cycle, the nucleolar size is constant during G1 and S phases and it is doubled in G2 as a consequence of an enhanced nucleolar activity. Morphological features of the FCs also show a clear association with the cell cycle progression: the number of FCs increases and their size decreases. Structurally, heterogeneous FCs are present in G1 phase whereas in G2 phase they are of the homogenous type (González-Camacho and Medina, 2006; Medina, 1983; Risueño, *et al*, 1982). In highly proliferating cells GC enlargement occurs in G2 phase (Figure 11) (Hadjiolov, 1985; Smetana and Busch, 1974). Particularly interesting is the behaviour of the nucleolus during mitosis, when its structure is disorganized and its activity stop, even though the individual transcription and processing complexes are not disassembled. They are carried at the chromosome periphery and then they are organized into discrete entities called prenucleolar bodies, whose fusion, together with the resumption of transcription and processing, originates the new nucleolus (Medina *et al.*, 2000).

2.2.2. Nucleolar proteins and their role in ribosome biogenesis

In *Arabidopsis thaliana* two proteomic studies of the nucleolus have been performed using cell cultures. The first identified around 217 proteins including several non-ribosomal and even "non-nucleolar" proteins (Pendle *et al.*, 2005). The actualized proteome extended the initial list up to 1602 proteins in the nucleolar fraction (Palm *et al.*, 2016). The nucleolus contains many proteins specific for the nucleolus, but some are also present in other cellular locations. The changes in nucleolus during cell cycle are associated with the changes in the abundance and distribution of certain nucleolar proteins. Two of the most abundant nucleolar proteins are nucleolin and fibrillarin (Shaw, 2005).

Nucleolin is the major non-ribosomal nucleolar protein in all eukaryotic proliferating cells. It plays an important role in growth and development, not only in plants but also in other higher eukaryotic organisms (Tajrishi *et al.*, 2011). It is involved in different steps of ribosome biogenesis, including RNA polymerase I transcription, processing of pre-rRNA, as well as assembly and nucleocytoplasmic transport of ribosome particles (Montacié *et al.*, 2017; Pontvianne *et al.*, 2007; Roger *et al.*, 2002). Additionally, nucleolin has been involved in different cellular aspects that take place in the nucleus and cytoplasm, including chromatin organization and stability, cytokinesis, cell proliferation and stress response (Durut and Sáez-Vásquez, 2014).

In plants, nucleolin can be considered as nucleolin-like protein (Tong *et al.*, 1997). *Arabidopsis thaliana* encodes two nucleolin genes *AtNUC-L1* and *AtNUC-L2*, in contrast to animals and yeasts,

in which a single nucleolin gene is present. However, only the *AtNUC-L1* gene is constitutively expressed in normal growth conditions (Sáez-Vásquez and Medina, 2008). The localization and distribution of nucleolin-like protein in each nucleolar compartment is linked to the different processes (Ginisty *et al.*, 1999; Tajrishi *et al.*, 2011). In plant nucleolus, nucleolin-like protein is present in DFC around FCs, suggesting it is involved in controlling pre-rRNA transcription and processing (De Cárcer and Medina, 1999). Indeed, increased nucleolin-like protein expression is correlated with the cell cycle progression and cell proliferation, and reaches maximum values in G2 phase, the period characterized by the highest rate of pre-rRNA synthesis and processing (González-Camacho and Medina, 2006; Medina *et al.*, 2000). Additionally, Arabidopsis' nucleolin genes disruption resulted in a reduced growth rate and a prolonged life cycle and a defective vascular system pattern (Pontvianne *et al.*, 2007). It was suggested that nucleolin in Arabidopsis might be involved in auxin-dependent organ growth and patterning, potentially indicating the way by which nucleolin may be controlling plant development (Petricka and Nelson, 2007).

Fibrilarin is an essential nucleolar protein that has been conserved in its sequence and function throughout evolution from archaebacteria to higher Eucaryota (Ochs *et al.*, 1985; Rodriguez-Corona *et al.*, 2015). In *A. thaliana* two genes encoding fibrillarin were found, namely *AtFib1* and *AtFib2* (Barneche *et al.*, 2000). Fibrillarin has been shown to be involved in rRNA maturation and RNA methylation by various approaches (Barneche *et al.*, 2000; Cerdido and Medina, 1995). Therefore, it is commonly used as a marker of active nucleoli. With regards to sub-nucleolar localization of fibrillarin and levels during the cell cycle, fibrillarin can be detected in the transition zone between FC and DFC in interphase nucleolus, where rDNA transcription and the pre-rRNA processing takes place (Cerdido and Medina, 1995; Ochs *et al.*, 1985; Staněk *et al.*, 2000). In G2 phase, when activity of nucleolus increase, its levels are doubled, reaching the maximum level just before mitosis (Cerdido and Medina, 1995).

2.2.3. Nucleolar dynamics under stress conditions

The nucleolus is a very dynamic structure that may vary both in size and morphology, from one cell type to another, and also depending on transcriptional activity. There are several lines of evidence suggesting that the nucleolus has a role in sensing and responding to environmental stresses (Boulon *et al.*, 2010). Environmental stresses may have varied effects on ribosome subunit production and cell growth and are often accompanied by dramatic changes in the organization and the composition of the nucleolus (Mayer and Grummt, 2005; Shaw and Brown, 2012). Several

stresses induce the nucleolar segregation in which the DFC and GC are subsequently separated (Shav-Tal *et al.*, 2005). This disruption of the nucleolar components is the consequence of alterations in the nucleolar activity and ribosome biogenesis. Microgravity conditions, understood as a stress, alter plant nucleolus structure and disrupt the nucleolar components. It was found that the nucleolar ultrastructure in microgravity-exposed cells, corresponded to that of a proliferating cell actively engaged in the production of ribosomes, but showing a smaller nucleolus with abnormal distributions of the nucleolar components (Matía *et al.*, 2010).

3. *Arabidopsis thaliana* cell growth and cell proliferation are affected by gravity alteration

Microgravity alteration had been investigated in *A. thaliana* in various laboratories in the world. Different materials originated from Arabidopsis plants have been used in microgravity research, including seedlings (Ferl *et al.*, 2015; Millar *et al.*, 2010), root meristematic tissues (Boucheron-Dubuisson *et al.*, 2016; Haubold, 2013), *callus* (Manzano *et al.*, 2016; Zupanska *et al.*, 2013) and, recently, cell suspension cultures (Kamal *et al.*, 2016). The research team under the leadership of Dr. Francisco Javier Medina has been investigating the response of *A. thaliana* root to microgravity for many years. Experiments have been performed in the real microgravity conditions in the ISS during the spaceflight supported by European Space Agency (ESA) and National Aeronautics and Space Administration (NASA) projects. Complementary experiments in ground-based facilities were performed, to support the real microgravity results or to study additional questions not addressed in space-based experiments yet.

Gravity has a key role in plant developmental programme and its alterations produce substantial changes in basic and essential functions like cell growth and proliferation (Perbal, 2001; Ferl *et al.*, 2002). The change of gravity has been shown to result in the uncoupling of cell growth and cell proliferation in high proliferating cells, which are strictly coordinated under normal ground gravity conditions (Herranz *et al.*, 2014; Manzano *et al.*, 2012a; Manzano *et al.*, 2013; Matía *et al.*, 2010).

3.1. Plant cell growth and proliferation in Arabidopsis seedlings were altered by real microgravity

Experiments performed on orbit ("Root" experiment - Cervantes Soyuz mission, in 2003, on ISS) have evidenced that, in root meristematic cells, the absence of gravity results in the uncoupling of cell growth and cell proliferation (Matía *et al.*, 2007; Matía *et al.*, 2010). Proliferation rate, or cell

division, measured by counting the number of cells per unit of length (mm) in the different cellular rows of the root meristem was found to be higher in microgravity conditions. Cell growth, estimated by means of ribosome biogenesis rate, using the level of the nucleolar protein nucleolin, was reduced under microgravity conditions compared with the 1 *g* control. Furthermore, the nucleolar size was significantly smaller in seedlings grown in space under microgravity conditions compared with the 1 *g* ground control. Lastly, cell growth and proliferation, which are strictly associated functions under normal 1 *g* conditions, appeared decoupled under gravity absence: cell proliferation was enhanced, but cell growth was reduced (Matía *et al.*, 2005; Matía *et al.*, 2010).

3.2. Plant cell growth and proliferation in Arabidopsis seedlings were altered by simulated microgravity

Experiments performed using different ground-based facilities, such as the clinostat, the random position machine (RPM) and magnetic levitation instruments, confirmed the general trends observed during the spaceflight experiments: The cell proliferation rate and the cell cycle progression showed significant alterations, accompanied by a reduction of cell growth. In addition to the regulation of cell cycle progression, determined by flow cytometry and the expression of the cyclin B1 gene, a decrease in a marker of entry into mitosis was observed in simulated microgravity. With respect to the cell growth, the rate of ribosome biogenesis was reduced under simulated microgravity, as shown by morphological and morphometric nucleolar changes. Also, lower levels of nucleolin indicated decrease of the nucleolar activity and ribosome biogenesis (Boucheron-Dubuisson *et al.*, 2016; Matía *et al.*, 2010; Manzano *et al.*, 2009; Manzano *et al.,* 2013).

3.3. Plant cell growth and proliferation in Arabidopsis undifferentiated cell cultures were altered by simulated microgravity

The effect of microgravity on cell growth and cell proliferation observed in *A. thaliana* seedlings was validated in experiments using *A. thaliana in vitro callus* cultures exposed to simulated microgravity using RPM and magnetic levitation (Manzano *et al.*, 2012b; Manzano *et al.*, 2016). Increment in the cell proliferation rate and decrease in cell growth was observed in the samples exposed to microgravity, meaning that, as observed before, cell proliferation and growth are decoupled. Moreover, a morphofunctional nucleolar model was defined, as an easy and reliable indicator of the ribosome biogenesis activity and, consequently, of the protein biosynthesis. The method consisted of defining three structural nucleolar types, which can be called as vacuolated, compact and fibrillar. Functionally, vacuolated nucleoli appear in actively proliferating cells with high rate of

production of ribosomal precursors (Risueño and Medina, 1985). Typically, they are cells found in the G2 cell cycle period. The compact nucleoli appear in proliferating cells with a variable activity of ribosome production, corresponding to the cell cycle periods G1 and S. Fibrillar or inactive nucleoli are cells found in G0 phase, a temporary or permanent period originated either from G1 or G2 (Risueño and Medina, 1985). In experiments after a short-term exposure (200 min) to microgravity conditions (RPM), it was observed an increased frequency of the inactive nucleoli model compared with 1 *g* control. Flow cytometry analysis has shown that simulated microgravity alters the cell cycle progression in *callus*, where an increment in cells in S and G2/M phases and a decrease in G1 cell population was observed (Manzano *et al.*, 2016). Moreover, the cell cycle regulated genes expression level (CdkB1 and cyclin B1) were decreased in simulated microgravity conditions. Genetic analyses using qPCR analysis revealed that the levels of nucleolar activity related genes, nucleolin and fibrillarin were increased in simulated microgravity. However, these results are relatively uncertain and previous data obtained from microarrays experiments were different and more consistent with the results obtained from structural study of nucleoli (decreased in simulated microgravity) (Manzano *et al.*, 2012b). Microarray analyses also shown changes in the overall gene expression of cultured cells exposed to altered gravity. It was observed a significant effect, mainly on structural, abiotic stress genes and secondary metabolism-related genes (Manzano *et al.*, 2012b). Moreover, 2D Western-blots analysis, nucleolin-L1 isoforms showed a reduced span of isoelectric point in the RPM, the same as it was found in root meristematic cells (Manzano *et al.*, 2009; Matía *et al.*, 2010). This suggests that the changes observed after a short altered gravity treatment were mainly caused by post-translational modifications of the protein (phosphorylation/dephosphorylation), and not by changes in gene expression (Manzano *et al.*, 2016). Interestingly, these results suggest that, in this case (short altered gravity exposure), it is easier and quicker to respond to the environmental changes by modifying a component already present in the cell than to re-arrange the complex machinery of the regulation of the gene.

OBJECTIVES

The main objective of this work was to investigate the influence of simulated microgravity conditions on the cell proliferation and cell growth in cell suspension cultures of *Arabidopsis thaliana*.

For this, the following specific objectives were proposed:

1. To optimize the conditions of embedding *in vitro* plant cell cultures minimizing the interference of external factors other than gravity.

2. To investigate suspension cultures' cell cycle in simulated microgravity by flow cytometry. For this the adaptation of the nuclei extraction protocol and nuclear staining was needed.

3. To investigate changes in nucleolar size in cell suspension cultures under simulated microgravity by confocal fluorescent microscopy as an indirect indicator of cell growth. For this the localization and abundance of two nucleolar proteins, fibrilarin and nucleolin, were investigated.

4. To determine the status of cell proliferation in the suspension cell cultures, changes in cell cycle progression and the relative distribution of phases will be evaluated by flow cytometry.

MATERIAL AND METHODS

1. Fast-growing Arabidopsis thaliana cell suspension culture (MM2d)

2. Immobilization and recovery of cell cultures

3. Flow cytometry technique

4. Confocal fluorescence microscopic techniques

5. Clinostat ground-based facility mode operation, hardware and experimental design

6. Statistical analysis

7. Synchronization of cell suspension cultures

MATERIAL AND METHODS

1. Fast-growing *Arabidopsis thaliana* cell suspension culture (MM2d)

The fast-growing *A. thaliana* cell line MM2d was the biological material used for all the experiments described in this work and was kindly supplied by Dr. Crisanto Gutierres at CBM (UAM-CSIC). This MM2d cell line was derived from the cell suspension culture MM1 by continuous subculture for more than two years in dark-growing conditions (Menges and Murray, 2002). Originally, it was derived from a cell suspension obtained from Landsberg *erecta* stem explants (May and Leaver, 1993).

The suspension culture was maintained in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with hormones as described below, with shaking and in darkness (120 rpm in an Excella™ E24 shaker incubator, New Brunswick product by Eppendorf, USA) at 27 °C and subcultured every 7 days (1:20 dilution in 250 ml Pyrex flask). The MS basal medium at pH 5.8 (adjusted with 1 M NaOH), containing 3% (w/v) sucrose was autoclaved at 110 °C and stored at 4 °C no more than for two weeks before use. before subculture The medium was supplemented with MS vitamins, 0.5 mg/L NAA (α-naphtaleneacetic acid) and 0.05 mg/L kinetin-medium MSS (Menges and Murray, 2006). All these products were purchase to Duchefa.

2. Immobilization and recovery of cell cultures

2.1. Immobilization and recovery of cell suspension culture by embedding in low melting agarose

Sieberer and coworkers (2009) published a procedure to immobilize cell suspension cultures in agarose, to be used in spaceflight, which was adapted by us. For the immobilization, 7-day old high proliferative Arabidopsis cell suspension MM2d culture was diluted (1:5) with fresh MSS medium and gently mixed with an equal volume of 1% (w/v) SeaPlaque low melting temperature agarose (Lonza), resulting in a final concentration of 0,5% agarose and a 1:10 cell dilution. Agarose was previously dissolved in MSS medium in a sterile glass flask by boiling for 10 seconds in a microwave and cold down to 28-27 °C, proper temperature to mix with the prepared cell suspension.

After embedding the cell suspension in agarose, 10 ml of agarose-cell mixture was poured into 9 cm Petri dishes and allowed to solidify for 30 minutes. Thereafter, plates were sealed with Micropore™ tap and kept at 27 °C in clinorotation during 24 h, in darkness.

Embedded cells, to be subsequently analysed by flow cytometry, were fixed with 1 ml 1% (w/v) PFA (paraformaldehyde, Electron Microscopy Science) in PBS buffer for 15 min, the fixative arriving to the cells by free diffusion through the agarose matrix. Fixed samples were washed quickly with PBS buffer. For posterior immunofluorescent microscopy analysis, the embedded cells were fixed with 1 ml 4% (w/v) PFA in PBS buffer for 1 hour and washed for 15 min with PBS buffer.

After chemical fixation, the agarose-fixed cell mixture was transferred to a 50 ml Falcon tube and the matrix dissolved by immersion in a water bath at 63 °C for 30 min and centrifuged at 800 *g* for 5 min to recover the pellet of fixed cells. At this point, the cells were frozen by immersion in liquid nitrogen and kept in -80 °C for flow cytometry analysis and in glycerol, at -20 °C, for microscopy analysis.

2.2. Immobilization and recovery of cell suspension culture by embedding in alginate and gelatine

Embedding of cell suspension involving the immobilization in a mixture of alginate and gelatine was developed in our lab. Alginate allows heat-independent gelation easily induced by addition of Ca^{2+} ions and liquefaction by addition of a chelating agent, as EDTA. These properties allow us to grow and recover cell cultures without affecting their growth and the quality of recovered cells (Brodelius and Nilsson, 1980; Draget *et al.*, 1989; Lopez *et al.*, 2017). Gelatine upon cooling allows the solid gel formation and enables the attachment of the gel layer to the base of Petri dish (Nussinovitch, 2010).

Alginate (1.25% (w/v); Sigma-Aldrich) was dissolved in warm MSS medium in a sterile glass flask by shaking for 10 minutes and the gelatine (12.5% (w/v)) was dissolved by the same procedure and filtered. Equal volume of each solution was gently mixed with Arabidopsis MM2d culture at 7th day of growth in a 15 ml Falcon tube (10 ml in total), resulting in a final concentration at 0.5% alginate, 5% gelatine and a 1:10 dilution of cells in MSS medium.

The prepared mixture was poured into 9 cm Petri dishes, which contained a thin layer of agar with 5 mM $CaCl_2$ (Sigma-Aldrich), and was let setting for 20 min at room temperature to give time for Ca^{2+} diffusion. After solidifying, the Petri dishes were sealed with Micropore™ tap then kept at 27 °C

in the clinostat, in darkness, for 24 h. Control samples were prepared in the same conditions but leaving the Petri dishes at the upright position instead of in the clinostat.

After clinorotation experiment, cells to be analysed by flow cytometry were fixed with 4 ml 1% (w/v) PFA in PBS buffer for 15 min and washed quickly with 10 ml PBS buffer. The recovery of the cells was made by adding 5 ml of Recovery buffer pH 8 (55 mM citric acid monohydrated (Sigma-Aldrich C7129); 30 mM EDTA (Serva 11280); 150 mM NaCl (Merck)), mixing gently until the gel started to liquify and then transferred to a 50 ml Falcon tube. The alginate/gelatine-cell suspension was centrifuged at 800 *g* for 5 min to recover the cell pellet and washed one time with Recovery buffer to dissolve remaining alginate. At this point, the fixed cells were submitted to analysis or, if necessary, frozen at -80 °C until use.

2.3. Immobilization and recovery of cell suspension culture by embedding in alginate

Immobilization technique only using alginate as gelling agent was based on the work of Draget and coworkers (1989). Here, we used dispersed $CaCO_3$ and the slowly hydrolysing acid glucono-δ-lactone (GDL) for the gelation. This method passes by gradually releasing the crosslinking ion from a less soluble form of calcium triggered by the reaction with protons from the hydrolysis of GDL.

The $CaCO_3$ (Sigma-Aldrich 795445) was dissolved in MS basal medium to a final concentration of 16.5 mM and alginate (1% (w/v); Sigma-Aldrich 71238) was added slowly. This solution was autoclaved and cooled at room temperature. In a 15 ml Falcon tube, 0.7 ml of 7-days cell suspension was mixed with 9 ml of 1% (w/v) alginate containing 16.5 mM $CaCO_3$, then vitamins, NAA and kinetin were added. A freshly prepared 0.33 ml of 1 M GDL (Sigma-Aldrich GA750) was added to the mixture, resulting in 33 mM final concentration. All the compound in the tube were mixed and poured on 6 cm Petri dishes. After immobilization of the cell suspension, the Petri dishes were placed on clinorotation for 24 h, at 27 °C and under dark conditions.

Immobilized cell cultures were fixed with 4 ml of 4% PFA for 1 h at room temperature (RT) for immunofluorescence analysis and with 4 ml of 1% PFA for 30 min at RT for flow cytometry analysis, both were washed with PBS. The fixed cells were recovered by adding 2 ml of Recovery buffer. The content was transferred to Falcon tube and mixed with vortex until completely liquified. Sample was washed twice in Recovery buffer after centrifugation for 5 min at 2050 *g* at RT.

2.4. *In vitro* plated cell culture derived from cell suspension culture

Another method to immobilize MM2d undifferentiated cell suspension is to plate the cell culture, a procedure used previously in our laboratory (Manzano *et al.*, 2016). For that, an aliquot of 7-day-old cell suspension was added into a 50 ml Falcon and let to settle down, posteriorly the supernatant was discarded and the cell pellet was added to a 9 cm Petri dishes as a 1-2 mm thick layer on the surface of a 1 cm layer of 1% (w/v) agar with MSS medium. The cell cultures were grown for 7 days, at 22 °C, in dark conditions to obtain microcalli formation. Afterwards, the plated cell culture was exposed to clinorotation for 24 h in the same conditions above. The samples were then harvested and preserved immediately (less than 2 min) by freezing in liquid nitrogen and subsequently stored at -80 °C for posterior flow cytometry analysis.

3. Flow cytometry technique

Flow cytometry (FC) is an important tool to study the cell cycle. It permits rapid measurements of cellular DNA contents, and thus to determine the distribution of cell cycle phases and its progression with high precision. Furthermore, the accuracy, speed and available software packages to analyse the data (e.g. CXP, ModFit, Verify, Flomax) turned FC the method of choice for monitoring cell cycle for almost all researchers working in the field (González-Camacho and Medina, 2006; Manzano *et al.*, 2016; Menges and Murray, 2002). However, unlike animal cells, plant cells contain rigid cellulose cell wall which interferes with FC analysis. For this reason, the use of intact cells is not recommended. Additionally, the big size and irregular shape of plant cells can disturb the flow and therefore interact with the excitation light beam differently. As a result, single particle suspension is required for FC analysis and mechanical disruption and subsequent nuclei extraction has become the universal method of sample preparation for FC analysis of plant cell cycle (Galbraith *et al.*, 1983).

3.1. Samples preparation

3.1.1. Extraction and staining of the nuclei with Kit Cystain UV precise P

The extraction and staining of the nuclei from *A. thaliana* MM2d cell suspensions was performed on frozen samples (500 mg cell pellet) using Kit Cystain UV precise P (Partec GmbH) containing Nuclei extraction (NE) buffer and DAPI Nuclei staining (NS) buffer, which enables to determine the DNA content for each individual cell using flow cytometry approach.

Cells were mixed with 300 µl of NE buffer, chopped using a sharp razor blade to release the nuclei. The mixture was incubated for 2 min at 4 °C and filtered through a 50 µm nylon mesh for cleaning the samples from debris and whole cells. Next, 600 µl of NS buffer was added and 10 µl DAPI solution (1 µg/µl), to increase the efficiency of the staining buffer due to the small *A. thaliana* genome (Menges and Murray, 2006). The sample was incubated at 4 °C in darkness. Samples could be stored in these conditions overnight before the FC analysis.

3.1.2. Extraction of the nuclei with optimized homogenization medium

The extraction of cell nuclei to be submitted to flow cytometry analysis was performed using the homogenization medium (HM) adapted from (Komatsu, 2007). However, other buffers had also been tested, such as Galbraith (Galbraith *et al.*, 1983), LB01 (Doležel *et al.*, 1989), Tris.$MgCl_2$ (Pfosser *et al.*, 1995) (Table 1). The fixed or frozen sample was mixed with the HM (Table 1) in a ceramic mortar and homogenized with a pestle for 3 min. The homogenates were filtered twice through a nylon mesh, firstly by 50 µm and then by 30 µm. Next, 1 ml of NS buffer with 10 µl DAPI solution (1 µg/µl) was added and incubated in darkness. All the steps were performed at 4 °C. The samples could be stored in staining solution overnight.

Table 1: Constituents of the extraction buffers.

Homogenization medium (HM)	50 mM	PIPES (Piperazine-N,N′-bis(2-ethanesulfonic acid), pH 7.4; Sigma-Aldrich),
	10 mM	KCl (Potassium chloride; MERCK),
	1 mM	EDTA (Ethylenediaminetetraacetic acid; MERCK),
	10 mM	Ascorbate (Sigma-Aldrich),
	0.1%	BSA (Bovine serum albumin; Sigma-Aldrich),
	400 mM	Sucrose (MERCK),
	10 mM	PMSF (Phenylmethane sulfonyl fluoride; Sigma-Aldrich);
	20 mM	DTT (Dithiothreitol; Sigma-Aldrich)
Galbraith	20 mM	MOPS (3-(N-morpholino) propanesulfonic acid), pH 7.0;
	45 mM	$MgCl_2$;
	30 mM	Sodium citrate;
	0.1%	Triton X-100
LB01	15 mM	Tris (Tris-(hydroxymethyl)-aminomethane), pH 7.5,
	80 mM	KCl;
	2 mM	Na_2EDTA (Disodium ethylenediaminetetraacetate);
	0.5 mM	spermine tetrahydrochloride;
	20 mM	NaCl;
	0.1%	Triton X-100
	15 mM	β-mercaptoethanol
Tris.$MgCl_2$	200 mM	Tris, pH 7.5;
	4 mM	$MgCl_2 \cdot 6H_2O$;
	0.5%	Triton X-100

3.2. Determination of individual Cell DNA content (phases and duration)

Flow cytometry is an excellent method to analyse fluorescent microscopic particles in liquid suspension. As the nuclear DNA content undergoes characteristic changes during the cell cycle, it is possible to determine the phase of the cell cycle based on the DNA content (Pfosser *et al.*, 2007). To measure the percentage of cells in each phase of cell cycle, 10,000 particles were counted by analysing the nuclear extracts of each sample in a Cell sorter FACS (Vantage, Becton-Dickinson, San Diego, California), using an argon ion laser tuned at 360 nm and detecting emission with a blue fluorescence emission filter (band pass filter of 424/44 nm Ban Pass).

The results were analysed with CXP analysis software for Windows XP to determine the ratio of each cell cycle phase according to the DNA content of individual cells (2C for phase G1, between 2C and 4C for S phase, 4C for G2/M phases) (Figure 11).

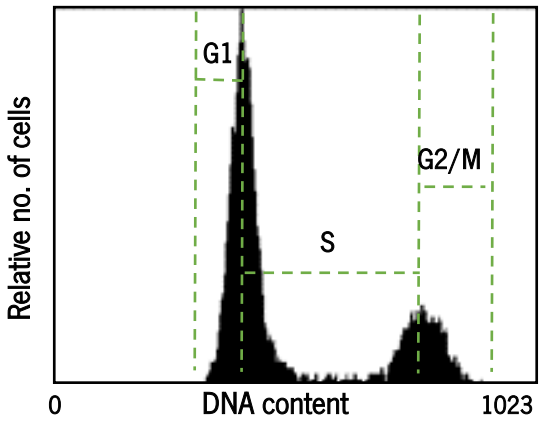

Figure 11: Cell cycle phases determination according to the DNA content in each cell. Each panel represents the relative number of cells according to the DNA content in each cell. First peak (2C) corresponds to G1 phase and the second peak (4C) corresponds to G2/M phase, the DNA content between the two peaks corresponds to the cells in S phase (which is manually estimated).

4. Confocal fluorescence microscopic techniques

In the present study, we used confocal fluorescence microscopy techniques that provide 3D-dimensional optical resolution. In order to measure nucleolus area, immunofluorescence confocal microscopy approach was used. The method is based on the use of specific antibodies in combination with secondary antibodies chemically conjugated to fluorescent dyes (Coons and Kaplan, 1950) for subcellular localization and quantification of specific proteins in cell suspensions, cultured cells or tissues.

For the determination of the mitotic index and the analysis of cellular area, two methods of different fluorescent staining were used. To determine the mitotic index, nuclear DNA was stained with the fluorescent stain DAPI (4′,6-diamidino-2-phenylindole), that allows easy visualization of the nucleus (Chazotte, 2011). Cells must be permeabilized and/or fixed for DAPI to enter the cell and bind to the DNA.

4.1. Confocal immunofluorescence microscopy

The immunofluorescence protocol can be divided into four steps: cell fixation, specific hybridization with primary and secondary antibodies, confocal laser scanning microscopy and images evaluation. Cells were previously fixed using 4% PFA as described before. Cell wall was digested with enzyme digestive cocktail (2% cellulase + 1% Pectinase + 0.05% Macerozime + 0.4% D-Manitol; 10% glycerol and 0.2% Triton X-100), for 30 min at 37 °C. Next, cells were washed twice with PBS containing 10% glycerol and 0.2% Triton X-100 and centrifugated at RT (5 min, 380 *g*). The pellets were resuspended in PBS and set onto 0.1% poly-L-lysine coated multi-well slides (Teflon printed slides, ES-139W, Electron Microscopy Science). Cells were dehydrated with 100% methanol and stored at -20 °C until immunofluorescence assay.

During our experiment two specific antibodies were used; anti-nucleolin L1 and anti-fibrillarin to study changes under the experimental conditions. The primary antibodies were used at a dilution 1:1000, while the secondary antibodies were used at a dilution 1:100 as shown in Table 2.

Table 2: **List of the specific antibodies used in the immunofluorescences bases-analysis.** Its references and dilutions in the blocking solutions.

Protein of interest	Specific antibodies	
	Primary (1:1000)	Secondary (1:100)
Nucleolin L1	Rabbit polyclonal Anti-AtNUC-L1 (Provided by Dr. Julio Sáez Vásquez)	Anti-Rabbit (Alexa 488-Green)
Fibrillarin	Mouse monoclonal Anti-Fibrillarin (Abcam, ab4566)	Anti-Mouse (Alexa 488-Green)

Immunofluorescent labelling involves incubation of cell preparations with primary antibody, removal of unbound primary antibody by washing, and the binding of fluorochrome-labelled secondary antibody. The samples were placed in wet chambers to avoid drying during the procedure, and were washed (2x5 min) with PBS + 1% IGEPAL CA-630 (Sigma-Aldrich) + 0.5%

DOC at RT. Cells were incubated with blocking solution (PBS + 0.05% Tween + 2% BSA) for 30 min at RT followed by the incubation with the primary antibody overnight at 37 °C. Next, cells were washed (3x5 min) with PBS + 1% IGEPAL CA-630 + 0.5% DOC at RT, to remove the unbound primary antibody.

Cells were incubated with the secondary antibody for 3 hours at 37 °C, and then washed (3x5 min) with PBS + 1% IGEPAL CA-630 + 0.5% DOC at RT, to remove the unbound antibody. DNA was labelled with 10 µl DAPI staining for 5 min at RT. Samples were washed (2x5 min) with PBS + 1% IGEPAL CA-630 + 0.5% DOC and double distilled water (H_2O dd) (2x5 min) at RT. The slide wells were filled with PVA-DABCO™ (a glycerol based mounting medium containing an anti-fading reagent for use with immunofluorescence preparations), coverslips were placed and the excess of medium was adsorbed. The preparations can be stored in darkness at 4 °C until they are used for confocal microscopy. Confocal laser scanning microscopy was performed using Leica TCS SP5 with AOBS (Acousto Optical Beam Splitter) with 63X oil immersion optics. Laser lines at 488 nm (Green channel) were provided by an Ar laser and a DPSS laser. Images were analysed using image analysis software ImageJ.

4.2. Confocal fluorescence microscopy

The mitotic index, the percentage of cells in M phase, was determined through microscopic examination and counting of the proportion of DAPI-stained cells in M phase compared to the total number of cells. The small size of the *A. thaliana* nucleus and the late condensation of the chromosomes in prophase make it difficult to observe prophase and telophase stages of mitosis and leads to underestimation of the number of mitotic cells (Menges *et al.*, 2003). In order to compensate for this underestimation, the metaphase/anaphase index (M/AI) was only taken into account.

For that, fixed dehydrated samples were recovered from -20 °C, thawed and hydrated (2x5 min) with PBS + 1% NP40 + 0.5% DOC at RT. DNA was labelled with DAPI staining for 5 min at RT. Next, that samples were washed (2x5 min) with PBS + 1% IGEPAL CA-630 + 0.5% DOC and with H_2Odd (2x5 min) at RT. Samples were then mounted between glass slide with PVA-DABCO™ medium and cover slip for microscopic observation, the preparations can be stored in darkness at 4 °C.

5. Clinostat ground-based facility mode of operation, hardware and experimental design

The experiments in simulated microgravity performed in this work were only possible by the achievement of the one-axis clinostat (2D clinostat), supplied to our lab by the Zero-Gravity Instrument Project. The clinostat used in this thesis enables the rotation of the samples around a single rotational axis, the inclination of which can be varied from 0° (parallel to the ground) to 90° (perpendicular to the ground). The clinostat provides a rotational speed in a range of 0 to 90 revolutions per minute (rpm). The specifications of the clinostat are show in Appendices 1, while Figure 12 shows the 2D clinostat used.

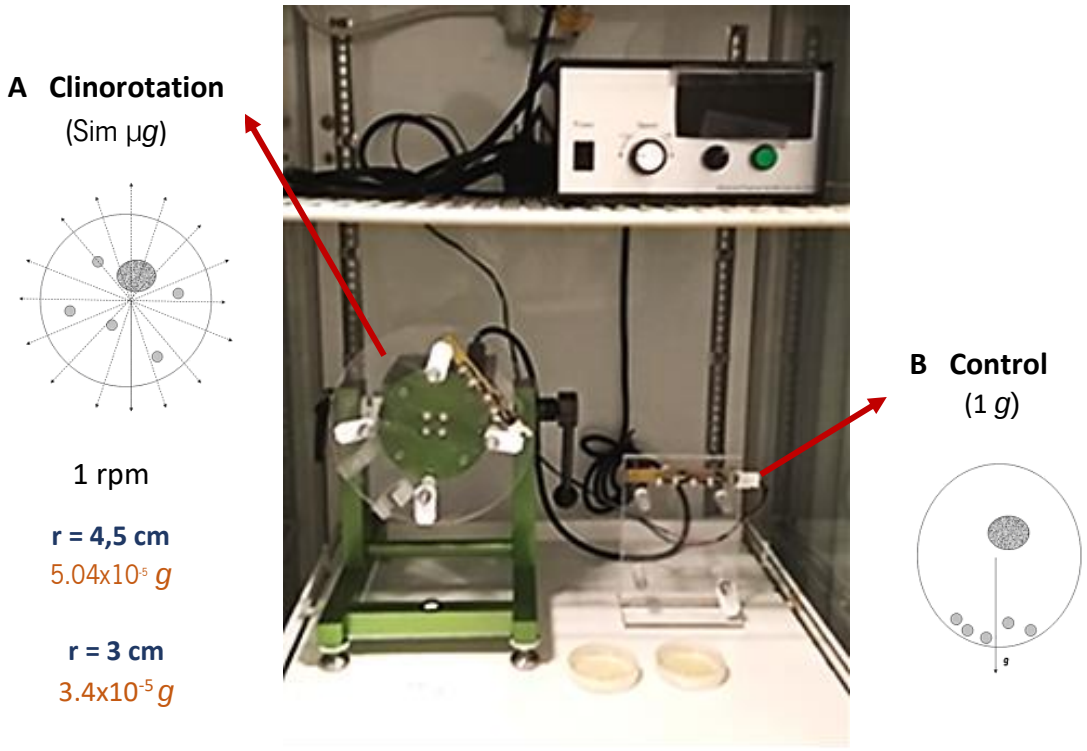

Figure 12: 2D clinostat used in the project. A) Clinorotation (Sim μ*g*) samples, rotated inside a 9 or 6 cm Petri dishes at 1 rpm; under clinorotation, the cell rotated around an axis passing through its centre. **B)** 1 *g* control; a stationary cell.

There are essential parameters which determine the effectiveness of the microgravity simulation when using a clinostat, the speed of rotation, the size (the distance from the centre of the clinostat to the external edge of the sample container), the stability of the sample, as well as the horizontal placement of the clinostat. In all the experiments, the rotational speed was set up at 1 rpm and the biological samples were placed in clinorotation in Petri dishes with a radius of 4.5 cm or 3 cm,

producing a residual acceleration of 5.04x10^{-5} *g* and 3.4x10^{-5} *g*, respectively. The calculation of the acceleration during clinorotation was done as described below:

Centrifugal force calculation:

$$\frac{Fc}{g} = \frac{v^2}{r} * \frac{1}{g}$$

Fc: *acceleration due to centrifugation* $[m/s^2]$
g: *gravitational acceleration* $= 9.81\ [m/s^2]$
v: *rotational velocity* $[m/s]$
r: *radius* $[m]$

Set ω as the rotational speed (revolutions per minute)

$$v = 2\pi r(\frac{\omega}{60}) = (\frac{\pi}{30})r\omega \qquad \frac{Fc}{g} = \frac{(\frac{\pi}{30})^2 r^2}{r} * \frac{\omega^2}{9.81} = 1.12 * 10^{-3} r\omega^2$$

The horizontal placement of the rotational axis of the clinostat was considered and set within an error of 0.1 degree, since an error of 0.5 degrees could create an axial acceleration in the order of 10^{-2} *g* (Beysens and van Loon, 2015; United Nations, 2013).

To investigate the quality of the different immobilization techniques and the impact of simulated microgravity using the clinostat, 7-days-old cell suspensions immobilized in Petri dishes with gelifying agents, or plated cell culture were prepared. Both were submitted to clinorotation at 1 rpm for 24 h under dark conditions at 27 °C (suspensions) or 23 °C (plated cell cultures). The impact of simulated microgravity on cell growth was analysed by immunofluorescence confocal microscopy and flow cytometry was used to investigate the effects of simulated microgravity on cell proliferation.

6. Statistical analysis

Data from each experiment were organized in an Excel datasheet (Microsoft office 2016), where the average and standard deviation were estimated to represent graphically the main results. Data were also statistically analysed using SPSS v.22 program, testing the normality of the samples with Kolmogorov-Smirnov (KS) test, the homoscedasticity using the Levene test and the comparison between mean values was performed using student t-test bilateral. Statistical significant differences ($p \leq 0.05$) were denoted by the asterisk notation (*).

7. Synchronization of cell suspension cultures

The synchronization of MM2d cells was obtained by reversibly blocking the cell cycle in late G1/ early S phase with aphidicolin (Pedrali-Noy *et al.*, 1980; Menges and Murray, 2006). The MM2d cell suspension was subcultured weekly by adding an aliquot of 5 ml of MM2d cell suspension in early stationary phase (7 days after last subculture) into 95 ml of fresh MSS medium (1:10 dillution). It was incubated at 27 °C, 120 rpm in the dark for 7 days to obtain a cell suspension at the stationary phase.

To obtain synchronized population of cells the 7-day-old cell suspension was diluted by transferring 20 ml of the cell suspension into 100 ml of fresh MSS medium (dilution 1:5). Aphidicolin from *Nigrospora sphaerica* (Sigma-Aldrich) was added at the final concentration of 4.16 µg/ml to 120 ml of diluted cell suspension and incubated at 27 °C, 120 rpm in the dark, for 24 h.

The cell suspension was washed to remove the aphidicolin by filtering through a home-made sterile filtration unit (using nylon mesh 50 µm). The filtered cells were resuspended in 100 ml fresh MSS medium by carefully swirling the glass beaker to wash the cells. The medium was changed several times (a total of 1 L of MSS medium was used), to wash the cells, by simply lifting the filtration unit out of the glass beaker and discarding the washing solution. In the last wash, the cell suspension was rinsed in 100 ml of MSS medium and kept in a sterile glass beaker. The washing procedure should not take more than 15 min in total and it is important to minimize physical disturbance during the procedure.

A volume of 10 ml of the synchronized cell culture was taken after the removal of the aphidicolin block as the T0 sample, and filtered with the filtration unit. The pellet was frozen with liquid nitrogen and kept in -80 °C until the flow cytometry analysis. After washing, the cell cycle is resumed. Samples were collected at different times (T0, T2, T6, T10, T16, T20 and T24) counting from the aphidicolin block release and typical flow cytometry analysis was used to check synchrony.

RESULTS

1. Optimization of a protocol for immobilization and recovery of plant cell suspension for clinostat experiments

2. Effect of simulated microgravity on the cell cycle of cell suspensions cultures

3. Effect of simulated microgravity on the cell growth of cell cultures

4. Cell cycle phases subpopulations progression

RESULTS

1. Optimization of a protocol for immobilization/embedding and recovery of plant cell suspensions for clinostat experiments

In vitro undifferentiated cell suspension culture is a suitable system to investigate the effects of altered gravity on plant cell proliferation and growth. In microgravity research, the 2D pipette clinostat had been considered a suitable tool to simulate microgravity in cell suspensions. However, studies using plant cell suspensions raised some concerns about the standard requirement for suspension agitation assuring its viability and active proliferation status in the control samples. For this reason, an alternative culture method was proposed using the 2D clinostat. The method is based on the immobilization of the cell culture before the altered gravity treatment. In this way, the cell culture is treated as a solid object allowing to overcome the mentioned constraints without compromising basic cell properties and enabling the use of 2D clinostat as a proper facility to study cell suspension cultures.

1.1. Immobilization and recovery of plant cell suspension culture in low-melting agarose: heat-dependent recovery of the cells affects the quality of DNA as shown in flow cytometry assay (of DAPI-labbeling)

The procedure developed in our lab is an adaptation of the method described previously for tobacco BY-2 cells (Sieberer *et al.*, 2009). We have introduced a cell recovery step from low-melting agarose. 7-day-old Arabidopsis cell suspension culture was immobilized in agarose at final dilution 1:10 (w/v). The cells were then recovered by melting the agarose at 63 °C for 30 min, washed and centrifuged and the cell pellet used for analysis.

To assess the effectiveness of the recovery of the cells from agarose, the effect of the heating necessary to melt the agarose on the cells was tested. For that cell suspensions (7-day-old) were exposed to **63 °C** for 30 min (temperature and time necessary to melt the agarose) and let at **RT** for 30 min as control. Next, cell nuclei were extracted from fixed cells of both treatments and analysed by flow cytometry. Figure 13 shows that samples exposed to 63 °C (Figure 13 A and B) did not present standard cell cycle phases, while the control (Figure 13 C) present a normal distribution of the cell phases.

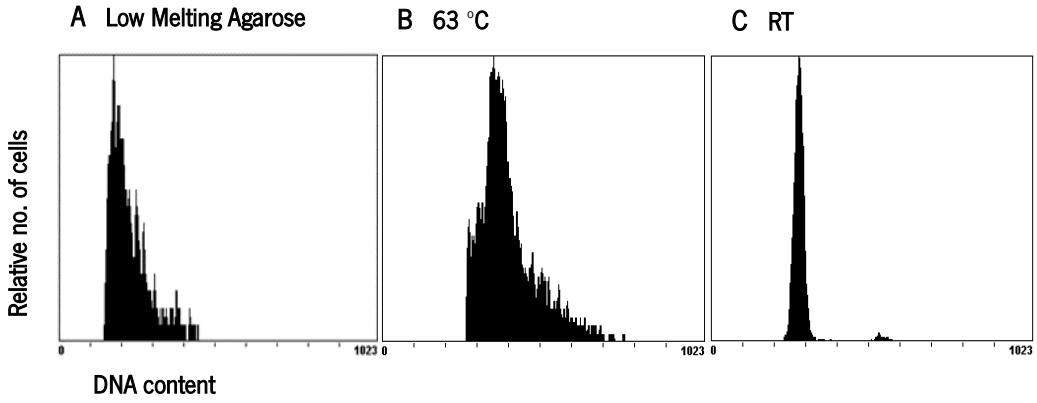

Figure 13: DNA content in *A. thaliana* cell suspensions after exposure to 63 °C for 30 min. Each panel represent the relative number of cells according to the DNA content in each cell. **A)** Cells recovered from the low-melting agarose at 63 °C. **B)** Fresh cell culture exposed to 63 °C. **C**) Fresh culture without treatment kept at RT. In this panel it is possible to distinguish the typical cell cycle peaks: first peak (2C) that reflects G1 phase, and the second peak (4C) that reflects G2/M phase. In panels A and B, of the cells exposed to 63 °C, the peaks are not observed.

1.2. Immobilization and heat-independent recovery of plant cell suspension culture in alginate/gelatine and in alginate as a suitable technique used for simulated microgravity by clinorotation

Embedding Arabidopsis cell suspension in alginate was encouraged by the fact it provides the required support for the cells and an recovery efficiency comparable to that made in agarose without the need of high temperature exposure in this way avoiding possible DNA damage (Adaoha *et al.*, 1982; Brodelius and Nilsson, 1980; Draget *et al*, 1989).

To identify the optimal concentrations of the compounds to obtain the desired gel consistency, MM2d cells were immobilized in different alginate/gelatine or alginate formulations, and the Petri dishes were placed upright for 24 hours, at 27 °C, under dark conditions.

The best alginate/gelatine gel consistency was achieved with 5 mM $CaCl_2$ and final concentrations of 0.5% alginate and 5% gelatine.

For the immobilization technique with alginate the best conditions to obtain optimal consistency of the gel were obtained using 16.5 mM $CaCO_3$, 1% (w/v) alginate and 33 mM GDL.

Data from cytometry analysis revealed that, between Arabidopsis cell suspensions immobilized in alginate/gelatine (Figure 14 B) and those immobilized in alginate (Figure 14 C), there were no significant differences in the distribution of the cell cycle phases compared to 7-day-old cell suspension control (Figure 14 A).

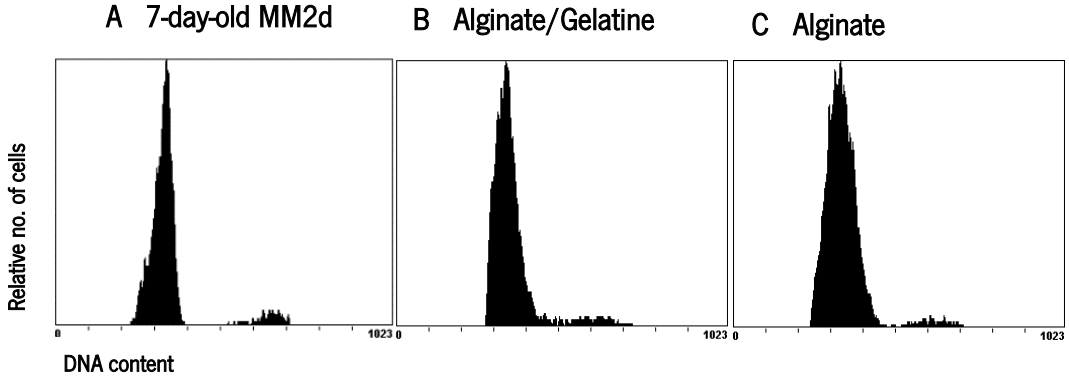

Figure 14: Arabidopsis cell cycle phases of cell suspension immobilized in alginate/gelatine and alginate. Flow cytometry analysis, each panel represents the relative number of cells according to the DNA content in each cell. First peak 2C reflects G1 phase and the second peak reflects G2/M phases.

2. Effect of simulated microgravity on the cell cycle of cell suspensions cultures

2.1. Optimized Homogenization medium as the best homogenization buffer for the extraction of nuclei

The extraction of the plant nuclei is a decisive step for analysing the nuclear DNA content by flow cytometry. The nuclei were released from cells using different homogenization media, which have the functions of allows the preservation of their integrity. Filtration and washes help in obtaining good quality samples and after addition of a DNA fluorochrome they are ready for analysis. To test the effect of the homogenization medium, nuclei were extracted from previously fixed 7-day-old Arabidopsis cell suspensions, using the Kit Cystain UV precise P, Galbraith, LBO1, Tris.$Mgcl_2$ and Homogenization medium (HM). After microscopic observation HM proved to be the best method (results not shown). Indeed, by flow cytometry analysis it was observed that with HM a normal distribution of the cell cycle phases was obtained when compared to the Kit Cystain revealing that this method gave a high level of background due to debris and the cell cycle phases' distribution did not present distinguished peaks (Figure 15).

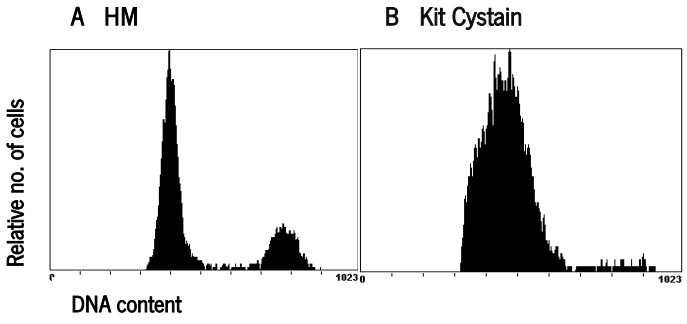

Figure 15: Extraction of the cell nuclei from MM2d cell suspension cultures. Cell cycle analysis by flow cytometry in a comparison of the two methods.

2.2. Simulated microgravity changes cell cycle phases

The optimized immobilization method was used to embed 7-day-old MM2d suspension cultures in alginate and the Petri dishes were exposed to simulated microgravity (clinostat) and 1 *g* control gravity for 24 h. By flow cytometry analysis it was observed that in the sample exposed to clinorotation the percentage of cells that entered S phase increased in comparison to the 1 *g* control (Figure 16).

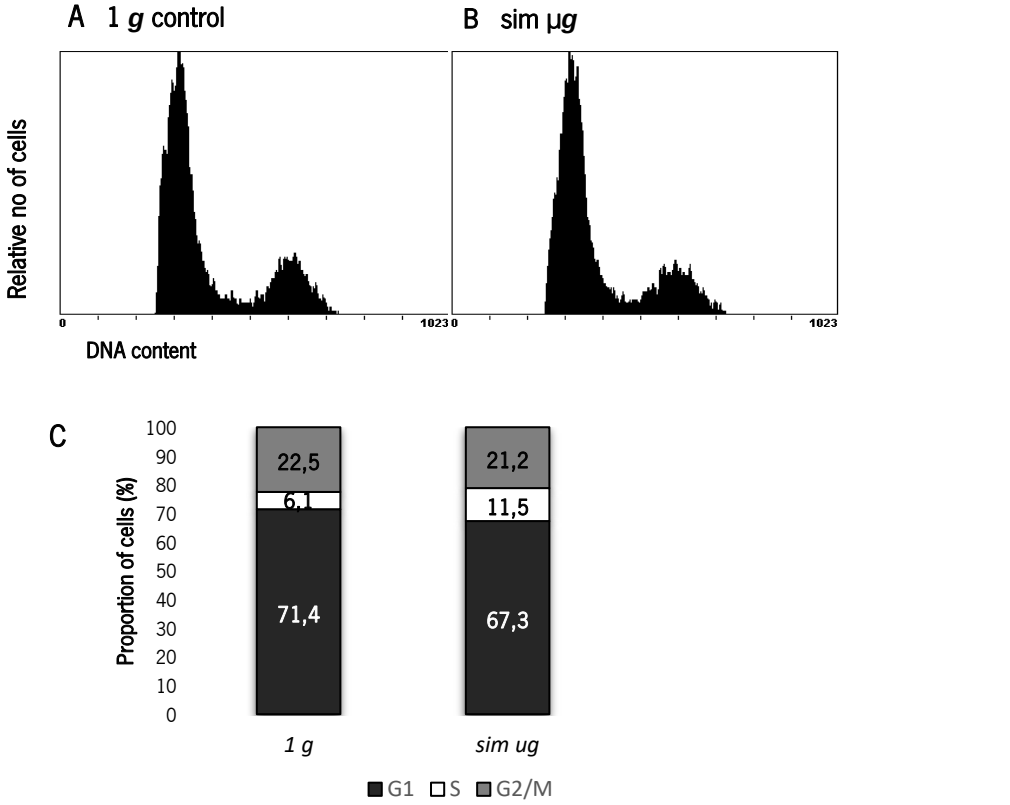

Figure 16: Cell cycle progression of Arabidopsis cell line MM2d for 24 h after clinorotation assessed by flow cytometry (representative essay). Panels **A** and **B** represent respectively the relative number of cells according to the DNA content in 1 *g* control and simulated microgravity. **C**) Relative proportion of cells in the G1, S and G2/M phases.

Cells in mitosis, particularly in metaphase (M) and anaphase (A), were counted from microscopic images using DAPI (4', 6-diamino-2-phenylindole) staining assay (Figure 17 A). Mitotic index, determined as the proportion mitotic cells per rest of cell population gives an idea about the cell proliferation rate in a given cell population and was used here to evaluate the impact of microgravity on Arabidopsis cell proliferation. Cells used in this analysis were immobilized in alginate and after recovery stained with DAPI. Figure 17 B show an increase in the mitotic index under simulated microgravity conditions compared with the 1 *g* control.

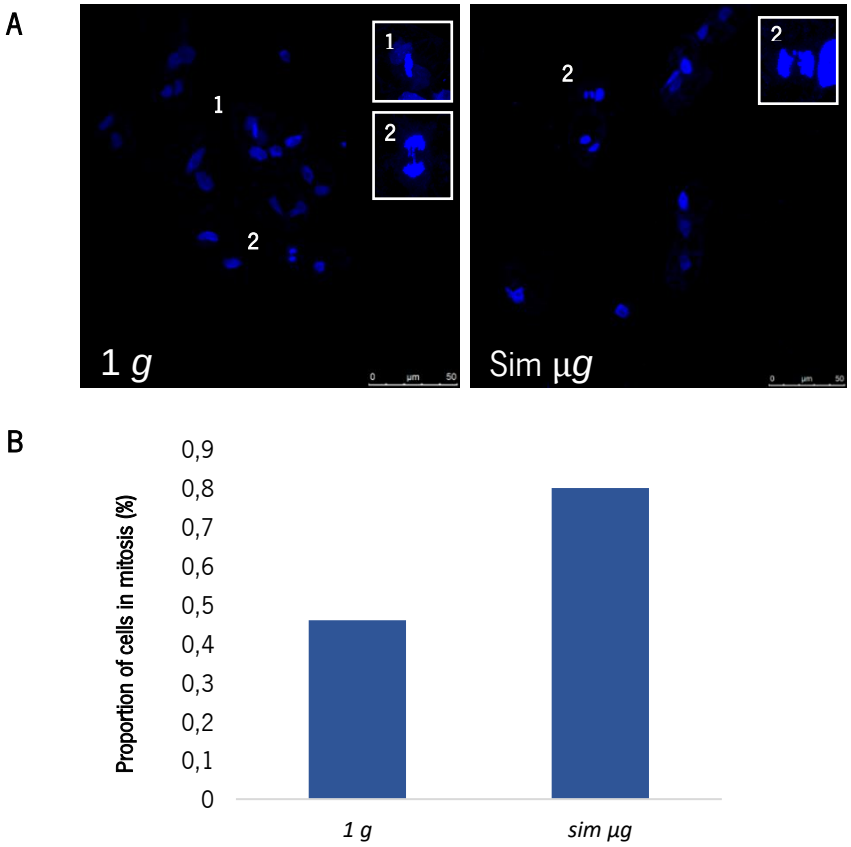

Figure 17: Cells of Arabidopsis in mitosis under simulated microgravity for 24 h experiment. More than 1000 cells were counted A) Confocal images of DAPI staining cells showing metaphase (1) and anaphase (2) phases. **B)** Cell division bar chart represented by the mitotic index.

2.3. The proliferation of plated plant cell culture (microcalli) is not changed by simulated microgravity

Arabidopsis plated cell cultures were successfully induced from Arabidopsis cell suspension MM2d culture before (Manzano *et al.*, 2016). Plated cell culture was exposed to simulated microgravity in clinostat for 24 h. Parallelly, 1 *g* control was also performed. No significant changes at cell proliferation level were observed between the samples when analysed by flow cytometry (Figure 18).

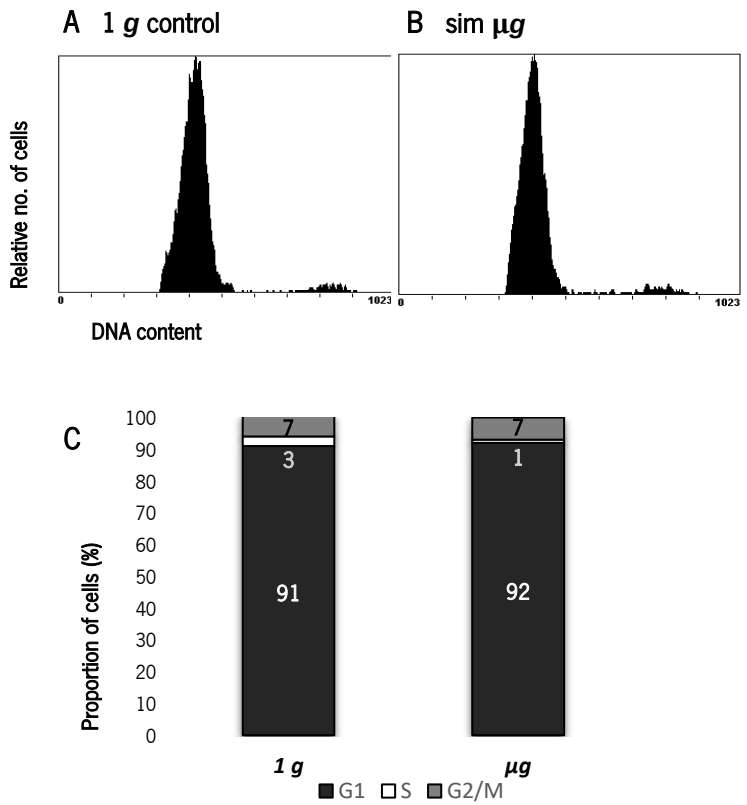

Figure 18: Cell cycle progression of microcalli film Arabidopsis cell line MM2d for 24 h after clinorotation (representative essay). Cell cycle analysis by flow cytometry. **A)** 1 *g* control. **B)** Simulated microgravity. **C)** Relative proportion of cells in the G1, S and G2/M phases.

3. Effect of simulated microgravity on the cell growth of cell cultures

3.1. Area of localization of proteins involved in ribosome biogenesis is reduced by simulated microgravity

The nucleolus is well known as a reliable indicator of the cell growth in proliferating cells (Medina *et al.*, 2000). We used two nucleolar proteins, AtNUCL1 and AtFIB, to quantify the nucleolar area in cells recovered both from agarose and alginate immobilization techniques. Statistical analysis revealed that the area of nucleolin decreased significantly after a 24 h-exposure to simulated microgravity compared to the control, when embedded in agarose (Figure 19 A, B) or alginate (Figure 19 C, D). In the case of fibrillarin, the nucleolar area decreased significantly compared to the 1 *g* control in cell culture embedded in alginate, but the decrease was not statistically significant in case of embedding in agarose.

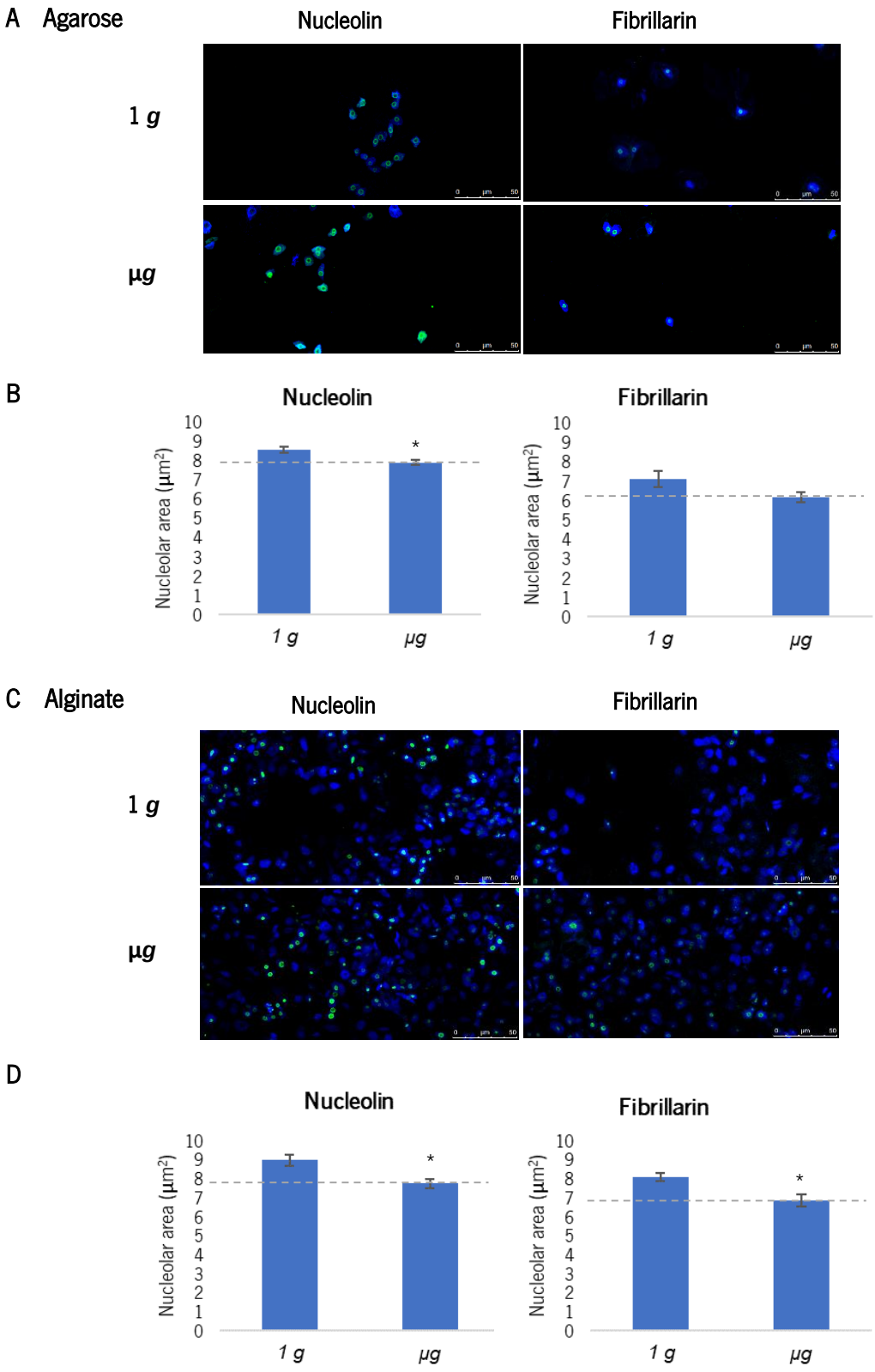

Figure 19: Nucleolar area in 24 h clinorotation experiment in Arabidopsis cells embedded in agarose and alginate. More than 300 nucleolus areas (for each, nucleolin and fibrillarin staining) were measured. Nucleolar nucleolin and fibrillarin staining was observed using the confocal microscope. **A)** Confocal images from cells recovered from agarose. **B)** Nucleolar mean areas estimated by nucleolin and fibrillarin fluorescence areas (agarose). **C)** Confocal images from cells recovered from alginate. **D)** Nucleolar mean areas estimated by the abundance of nucleolin and fibrillarin using immunofluorescence microscopy (alginate). Significant differences between mean values ($p \leq 0.05$) are denoted by *.

4. Cell cycle progression

Cell cycle synchronization is a powerful tool to investigate cell cycle and proliferation, it consists of arresting cells in late G1/S phase allowing to evaluate with precision cell cycle progression rates. In future experiments, after synchronization, the cells will be immobilized during clinorotation. Sampling at different times allows the comparison of the rate at which cells undergo the cell cycle in the simulated microgravity and in the 1 *g* control.

4.1. Using Arabidopsis cell cycle synchronization with aphidicolin block/release to localize the cell cycle phases

The distribution of cell cycle phases was determined by DAPI staining of the DNA using flow cytometry. After the release from the aphidicolin block (T0), cell cycle progressed synchronously for 24 hours as shown in the Figure 20 A. Aphidicolin synchronized the cell cycle by arresting 90% of the cells in the late G1/S phase at T0. The peak of synchronized cells corresponding to S phase is obtained after 2 hours (T2=34%). Through 6 hours, T6 sample shows the maximum peak for G2/M subpopulation of cells reaching 54%. These cells need 10 hours (T16) more to divide through G2/M and enter a new cycle, with G1 subpopulation peak of 30% and increasing at the T20 and T24 after the block release (Figure 20 B).

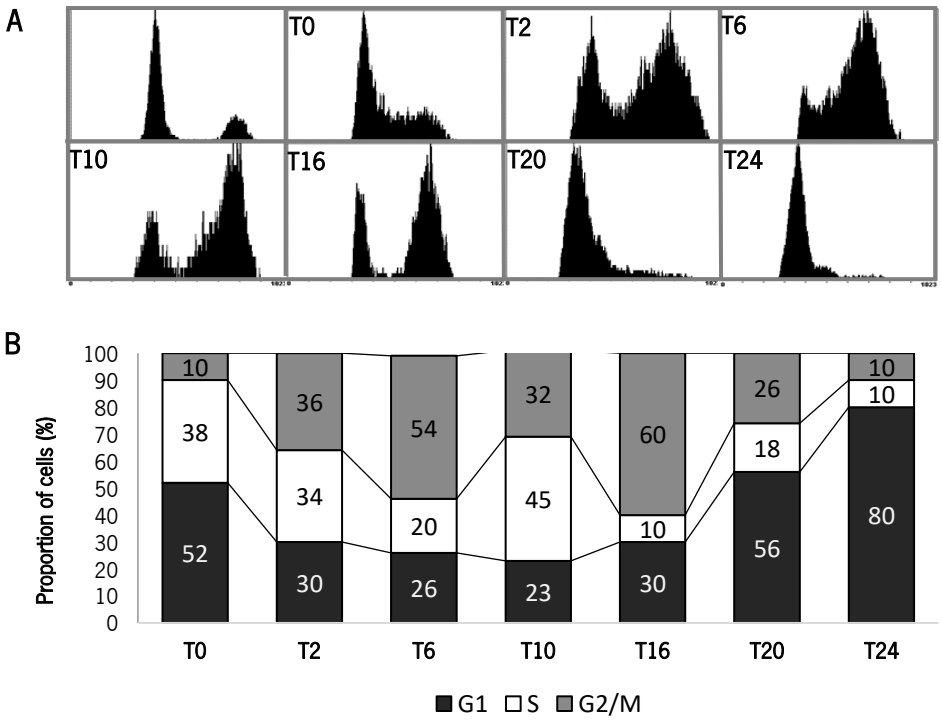

Figure 20: Cell cycle progression for 24 h after aphidicolin block/release of Arabidopsis cell line MM2d. A) Distribution of cell cycle phases by flow cytometry analysis (DAPI) regarding to the DNA content corresponding to the relative number of cells. Samples taken at different times after the release of aphidicolin block in late G1/early S phase (T0). **B)** Relative proportion of cells in the G1, S and G2/M phases.

DISCUSSION

1. Optimized techniques for plant cell suspension immobilization and recovery are suitable for microgravity studies using clinorotation

2. Simulated microgravity causes changes in *Arabidopsis thaliana* cell proliferation and cell growth

3. Implications of alterations in Arabidopsis cell development processes for agriculture on Earth and space exploration

4. Suggestion of new technique to be used in future for simulated microgravity experiments using 2D clinostat

DISCUSSION

1. Optimized techniques for plant cell suspension immobilization and recovery are suitable for microgravity studies using clinorotation

Clinostats are the most extensively used GBFs for exposing biological material to simulated microgravity. Specifically, these are considered suitable facilities for cell culture model systems (Herranz *et al.*, 2013a). The pipette 2D clinostat is a well-known facility used to expose cell suspension cultures to simulated microgravity (Herranz *et al.*, 2013a). However, the use of a proper 1 *g* plant cell suspension control provides some constrains, since these rely on shaking for survival. Consequently, it is mandatory a proper 1 *g* control setup different from a static one (Kamal *et al.*, 2015). In this way, the use of Petri dishes for 2D clinostat eliminates this problem by the use of immobilized cell culture systems. Here, we discuss different techniques used for the immobilization of Arabidopsis cell suspensions. These techniques could serve as a valuable tool for preparing spaceflight experiments using clinostat and also allow stand-alone studies, thus providing additional and cost-efficient platforms for gravitational research. We will discuss which immobilization agents might be most appropriate for cell suspension clinorotation and focus on a proper recovery required for posterior cell cycle studies using the flow cytometry approach.

1.1. Optimized technique for immobilizing cell suspension to expose to clinorotation: a heat-independent recovery of plant cells suitable for flow cytometry analysis

We have successfully used a new method for cell suspension immobilization in order to expose it to simulated microgravity in 2D clinostat, as well in future experiments using the RPM. These devices cannot be used for exposing to simulated microgravity a liquid cell suspension, since the fluid movements will greatly demise the quality of the microgravity simulation (Van Loon, 2007). The solution involves the immobilization of the cells in a gelling agent to protect the flooding of the cells. Here, we demonstrated that embedding the cells in alginate is a more suitable technique to observe the effects of simulated microgravity in cells than the embedment in agarose.

A procedure of immobilizing cells in agarose to be used in spaceflight experiments was already published (Sieberer *et al.*, 2009). However, in that case the recovery of the cells was not necessary in subsequent phases of the experiment, which was an indispensable step in our studies for posterior analyses. Thus, a modified version was assessed by our laboratory, where the recovery

of the cells from agarose required a melting temperature of 63 °C and previous formaldehyde fixation. However, the heat-dependent recovery of the cells proved to affect DNA quality, making it inviable for flow cytometry analysis (Figure 13). A plausible justification for this could reside in formaldehyde fixation after exposure to recovery temperature. It is known that formaldehyde fixation causes covalent cross-links between molecules, which preserve the structure of nuclei scaffold (Fox *et al.*, 1985). However, formaldehyde cross-links can be reversed by diverse ways (Hoffman *et al.*, 2015; Kennedy-Darling and Smith, 2014), particularly by temperature (Jackson, 1978). This could present a problem if undesired dissociation occurs in the course of the experiment. The rate cross-linking reverse as a function of temperature is exponential, as shown by reverse protein-DNA cross-linking (Kennedy-Darling and Smith, 2014). However, in this experiment the formaldehyde reverse cross-links only were tested at 47 °C. A total breakage of formaldehyde cross-linked protein was observed at 100 °C (Jackson, 1978). Taking into account these results it is reasonable to say that the temperature necessary to recover the cells from agarose had reverse effect on the fixation by formaldehyde and interfered with the quality of the nuclei used for flow cytometry analysis, at least in part. It is noteworthy, that fixation of the immobilized cells for flow cytometry was at lower concentration and at shorter period of time than the conditions for fixation of immunofluorescence microscopy samples, which could explain why the last ones conserved the nucleus structure and revealed the effects of simulated microgravity by nucleolar proteins areas. Additionally, the preparation of the samples when comparing the two techniques is much more mechanically intrusive in the case of flow cytometry.

Therefore, the best choice of a gelling agent for immobilization of the cell suspension culture should be heat-independent to release the cells. In this way, our choice was the alginate method based on the work of Draget and coworkers (1989), as an alternative to agarose-based immobilization. Alginate was previously used to plant seeds and microcalli. Here, we adapted this method to immobilize plant cell suspensions by the use of dispersed $CaCO_3$ and the slowly hydrolysing acid glucono-δ-lactone (GDL). This method is advantageous when compared with alginate/gelatin method since gelatin has low gel stability (Krikorian, 1996). Additionally, alginate has the advantage of being cheaper than agarose. In previous spaceflight experiments, alginate beads had already been used for plant protoplasts immobilization (Iversen *et al.*, 1999), however, they could not recover a single protoplast. In literature, as far as we know, there was no reported procedure to immobilize plant cell suspensions in an alginate matrix to be exposed to altered gravity.

2. Simulated microgravity causes changes in *Arabidopsis thaliana* cell proliferation and cell growth

The use of immobilized cell cultures in clinostat-based altered gravity conditions, combined with flow cytometry and confocal microscopy techniques, have allowed us to confirm previously described alterations in cell cycle and cell growth, crucial processes in plant physiology in space. Alterations in these processes caused by changes in the direction of the gravity vector, as perceived by the plant, rely on mechanisms that take place at the cellular level.

2.1. Optimized method of plant nuclei extraction from cell cultures for flow cytometry analysis

Flow cytometry was the method of choice as it is a rapid and sensitive method for analysing the nuclear DNA content of *in vitro* plant cell cultures (Doležel *et al.*, 2007). As the nuclear DNA content undergoes characteristic changes during passage through the cell cycle, it was possible to determine the phase of the cell cycle the cells are in (Pfosser *et al.*, 2007). It is well known that for flow cytometry analysis, the sample have to be a suspension of single particles. Thus, the method for investigating the plant cell cycle includes the isolation of the nuclei in an appropriate buffer solution, filtration of the homogenate and addition of a DNA fluorochrome (Galbraith *et al.*, 1983). A proper nuclei extraction buffer should facilitate isolation of intact nuclei free of adhering cytoplasmic debris, maintain nuclei stability in liquid suspension and prevent their aggregation. It also ought to protect nuclear DNA from degradation and provide an appropriate environment for staining DNA, including the minimization of negative effects of some cytosolic compounds on DNA staining. The choice of the buffers is a decisive step, since they vary in their efficiency for extraction and staining depending on species and biologic model system. Thus, there is no *a priori* rule for the selection of the buffer and staining, and a trial-and-error approach was necessary to achieve the best results. Therefore, we tested several buffers as well as two approaches for mechanical homogenization of the samples to plant nuclei extraction from Arabidopsis cell cultures. The starting point was to compare two methods for mechanical homogenization by cutting with sharp razors and homogenization on ice with a pestle. Homogenization is needed for eliminating cell clumps and breaking the cell wall and membranes releasing the nuclei. The results obtained with both methods showed that the homogenization with a pestle is the most suitable for *Arabidopsis thaliana* cell suspensions and microcalli used in our experiments. Perhaps, the razor is more suitable for plant tissues (Galbraith *et al.*, 1983). This could be related with the fact that single cells

and small cell clusters are more difficult to homogenize when in suspension, which is a bigger challenge than the homogenization of compact tissues (Doležel *et al.*, 2007).

To find an appropriate buffer, tests were performed comparing five buffers (Table 1), such as, the Kit Cystain UV precise P (Partec GmbH), Galbraith (Galbraith *et al.*, 1983), LB01 (Doležel *et al.*, 1989), Tris.$MgCl_2$ (Pfosser *et al.*, 1995) and HM (Komatsu, 2007). The most adequate for nuclei extraction in our case was the latter. Its suitability is conferred by the proprieties of its ingredients. Homogenization buffer is composed of PIPES (50 mM), an organic buffer required to stabilize pH in the solution, a crucial role in recovery of stable nuclei (at neutral pH 7.4); KCl (10 mM) is an inorganic salt proper to maintain ionic strength of the solution (Doležel *et al.*, 1989; Marie and Brown, 1993); chelating agent EDTA (1 mM) added to bind divalent cations, which serve as cofactors of DNases, in effect suppressing damage to DNA (Doležel and Bartoš, 2005); ascorbate (10 mM) avoids oxidation of polyphenols and their interference with DNA staining (Borse *et al.*, 2011); BSA stabilize the nuclei during the isolation procedure by preventing nuclear clumping and aggregation of cytoplasmic components; sucrose (400 mM) helps to maintain nuclear integrity as it provides the stable osmotic environment, and prevents their clumping (Saxena *et al.*, 1985; Sikorskaite *et al.*, 2013; Willmitzer and Wagner, 1981). The buffer was also supplemented with PMSF (10 mM), a protease inhibitor, and DTT (20 mM), a reducing agent, to preserve chromatin proteins and also to counteract the interference of phenolic compounds with DNA staining (Loureiro *et al.*, 2007). It is important to note that commercial buffers have the disadvantage that frequently the exact composition is not provided, that is why we have chosen a homogenization buffer recipe provided in scientific publication.

After the extraction of the nuclei the remains of the cell wall, cytoplasm, remaining organelles and unbroken cells should be washed out and discarded. For this, a two-filtration step was carried out, firstly by a 50-μm-nylon mesh followed by a 30 μm. Since Arabidopsis nuclei is small in diameter (5-10 μm) this double filtration allows a good separation of the nuclei from debris particles. Lastly, the purified nuclei suspension was suspended in staining buffer (NSB) and the nuclei dye DAPI, which permits the detection of nuclei in flow cytometry.

2.2. Plant cell cycle progression rate is increased under simulated microgravity conditions

Previous results showed that cell growth and proliferation was uncoupled in the root meristem under spaceflight conditions (Matía *et al.*, 2010), but further analysis of the possible changes in cell cycle was needed. The best system for studying the cell cycle is a large and homogeneous

population of actively proliferating cells. Here, we have used flow cytometry technique to study the cell cycle status on *Arabidopsis thaliana* cell cultures in the absence of the developmental and differentiation programs of seedlings. Cell cultures are a useful system to investigate the cell cycle and its regulation under environmental cues (Gould, 1984). Synchronization of such cultures can provide material representative of specific cell cycle phases. However, few plant cell suspensions can be synchronized to a high degree, such as occurs in cell suspension of *A. thaliana* cell line MM2d (Menges and Murray, 2002; Menges and Murray, 2006).

Our experiment, on immobilized cell suspension of Arabidopsis, showed changes in the distribution of cell cycle phases after exposure to simulated microgravity (Figure 16). However, microcalli did not show alterations in the cell cycle status (Figure 18), even though changes had been observed in previous experiments performed in the laboratory (Manzano, 2011; Manzano *et al.*, 2016; Moustafa, 2014). Our results might be related with the lower proliferation rate of microcalli when compared to cells in suspension. Additionally, previous experiments were performed using RPM, a device with superior quality simulation of microgravity than 2D clinostat (Herranz *et al.*, 2015). On the other hand, our results revealed an increase of the population of cells in S phase under simulated microgravity, while in G1 slightly decreased (Figure 16). The same effects has been shown in previous works using cell suspension and microcalli cultures (Manzano *et al.*, 2016; Moustafa, 2014). Our data suggests a faster entry into the S phase of the cell cycle. It is worth to note that G1 is a period much longer than S and G2 in proliferating cells of Arabidopsis (Manzano *et al.*, 2016). Thus, a reduction in the proportion of cells in G1 phase could be interpreted as a shortening of this period and, consequently, as a shortening of the overall duration of the cell cycle. A shorter cell cycle would mean a higher frequency of cell divisions, i.e., an increase of the cell proliferation rate. Therefore, our observations are compatible with the acceleration of cell cycle, resulting in the enhancement of the cell proliferation rate. Moreover, G2/M content of cells did not change, which could mean that the S phase is prolonged in simulated microgravity conditions. However, the count of cells that entered mitosis was higher in case of cell culture grown under clinorotation (Figure 17). These observations, additionally, suggest that the cause for this could be found in a failure or malfunction of the cell size checkpoint that immediately precedes mitosis (De Schutter *et al.*, 2007; Gonzalez *et al.*, 2007). It is important to remark that the observed changes in cell cultures exposed to microgravity are not as visible as in synchronized samples. However, our results are in concordance with previous ones using synchronized cell suspension of Arabidopsis cell line MM2d (Moustafa, 2014). Here an increase of S phase and decrease of G1

phase were also observed, even though in this works RPM was used instead of 2D clinostat. Subsequently, the enhancement of the cell proliferation rate was observed. A previous microarray study of core cell cycle regulators showed a general upregulation of the D-type cyclins (CYCD), which are associated with the CDKA activity through the G1/S transition. The CDKA/CYCD complex activity request phosphorylation by the CYCD (Inzé and De Veylder, 2006; Menges and Murray, 2002; Vandepoele *et al.*, 2002), in contrast to the down-regulation observed in the G2/M subpopulation (Moustafa, 2014). These observations suggest a reinforcement in G1/S transition checkpoint supported by the upregulation of the E2F/DEL (DEL stands for DP-E2F-like) protein families (Moustafa, 2014), which are associated with the S phase (Del Pozo *et al.*, 2002; Menges and Murray, 2006; Vandepoele *et al.*, 2002). Interestingly, KRPs which can inhibit the activated CDK/CYCD complexes under stress (De Veylder *et al.*, 2001; Verkest *et al.*, 2005; Zhou *et al.*, 2003) are not altered under simulated microgravity (Moustafa, 2014), in contrast with what happen with other types of abiotic stresses such as abscisic acid (ABA) and cold (Wang *et al.*, 1997). On the other hand, in G2/M regulatory mechanism, cell proliferation gene markers showed a decreased expression level in simulated microgravity (Moustafa, 2014). Particularly, the cyclin B1 gene, which is a marker of G2/M transition, is expressed throughout the G2 period and is specifically destroyed in anaphase (Colón-Carmona *et al.*, 1999). Thus, the expression of the cyclin B1 gene is considered a good marker of cell division, indirectly indicating the rate of entry into mitosis. Although cyclin B expression decrease is normally associated with a lower proliferation rate, other works have shown enhanced proliferation rate with reduced levels of cyclin B (Boucheron-Dubuisson *et al.*, 2016; Manzano *et al.*, 2016). This can be interpreted by assuming that a shorter G2/M period will lead to lesser accumulation of the cyclin B messenger. Downregulation of elements of the CDK-activating kinase pathway also produces effects in controlling the activity of the distinct CDK/CYC complexes (Shimotohno *et al.*, 2004; Umeda *et al*, 2000; Umeda *et al*, 2005). Therefore, downregulation of the WEE1 kinase was observed (Moustafa, 2014), and being it putatively involved in the inhibitory phosphorylation of CDKs (Sorrell *et al.*, 2002; Vandepoele *et al.*, 2002), this effect promotes the acceleration of the G2/M transition; alternatively, the overexpression of WEE1 genes causes cell cycle arrest (Sorrell *et al.*, 2002; Sun *et al.*, 1999). These data are supporting an "early" entry into M phase, with the observed reduction in the cell growth discussed below.

2.3. Plant cell growth is reduced by simulated microgravity conditions: nucleolar area measured by labelling of proteins involved in ribosome biogenesis is reduced by simulated microgravity

Nucleolin and fibrillarin are proteins involved in the regulation of ribosome biogenesis, which in turn is linked to the factors controlling cell growth and cell proliferation. In nucleolus, these two proteins show slightly different localizations, which has a physiological significance since there is correlation of these structural subnucleolar domains with the different steps of pre-rRNA synthesis and processing (Sáez-Vásquez and Medina, 2008). Nucleolin is localized preferentially in the DFC near FC, with a vectoral distribution from the border of FCs outwards (De Cárcer *et al.*, 1997; Martin *et al.*, 1992; Medina *et al.*, 2010). However, this protein is also localized in GC (Biggiogera *et al.*, 1990). This is consistent with the implication of nucleolin in the transcription of the ribosomal genes and also in the early processing of the pre-rRNA transcripts. Transcription was shown to take place around FCs and in the transition zone between FCs and the DFC (De Cárcer and Medina, 1999; Martin and Medina, 1991; Shaw and Jordan, 1995), while early pre-rRNA processing has been described as taking place in the region of the DFC closer to FCs (De Cárcer and Medina, 1999; Shaw and Jordan, 1995). Additionally, in GC, further RNA processing and RNA modifications steps occur, together with formation of the pre-ribosomal particles for export to the cytoplasm (Thiry and Lafontaine, 2005). In case of fibrillarin, it is localized in the transition zone between FCs and DFC and is more concentrated in proximal part of the DFC rather than in the zones of DFC more distant from FCs (Cerdido and Medina, 1995).

In our experiments, analyses were performed to determine the nucleolar area by labelling the proteins nucleolin and fibrillarin for immunofluorescence confocal microscopy. In connection with the alteration of cell cycle phases discussed above, Arabidopsis cell suspension revealed a reduction in proteins area labelled under simulated μ*g*. The values of the area labelled with nucleolin are higher than for fibrillarin with same magnitude for both (Figure 19). This could be explained by additional localization of nucleolin in outer part of the nucleolus (in GC), compared with a more specific zone for fibrillarin (between FCs and DFC) (Figure 10). These observations are related with a reduction in the nucleolus size and implicate decrease of ribosome biogenesis under simulated microgravity conditions. Table 3 summarizes the results obtained from the cell proliferation and cell growth studies.

Table 3: Summary of the results obtained in exposing *Arabidopsis thaliana* cell suspension to simulated microgravity on 2D clinostat.

Cell Proliferation (Enhanced)	Cell cycle	altered
	G1 phase	-
	S phase	++
	G2 phase	-
	Mitotic index	+
Cell Growth (Ribosome Biogenesis) (Reduced)	Nucleolin nucleolar area	- -
	Fibrillarin nucleolar area	-

Our observations are in concordance with other experiments reporting a decrease in the number of active nucleoli and smaller nucleolus compared to the 1 *g* control in Arabidopsis cell cultures grown in RPM (Manzano, 2011; Manzano *et al.*, 2016; Moustafa, 2014). The same was observed in root meristems under real microgravity (Manzano *et al.*, 2009; Matía *et al.*, 2005; Matía *et al.*, 2010) and simulated microgravity (Boucheron-Dubuisson *et al.*, 2016; Manzano *et al.*, 2013; Matía *et al.*, 2010). It is well known that morphological features in the nucleolus are correlated to the rate of ribosome biogenesis (Sáez-Vásquez and Medina, 2008). Consequently, these nucleolar changes reveal reduction of ribosome biogenesis. In general, in dividing cells a decrease in the rate of ribosome biogenesis is closely correlated with a decrease in the synthesis of proteins, whose factories are cytoplasmic ribosomes, and, consequently, to cell growth arrest (Baserga, 2007). Also, it is known that in these cells the peak of nucleolar activity/ribosome biogenesis occurs in G2 phase, immediately preceding mitosis, corresponding to the peak of nucleolin and fibrillarin quantity, taken as cell growth markers (Sáez-Vásquez and Medina, 2008). Indeed, quantitative analyses in Arabidopsis cell cultures under simulated microgravity, for a long-term exposure (24 h), showed a decreased quantity of nucleolin and fibrillarin proteins and less gene expression compared to 1 *g* control (Moustafa, 2014). Interestingly, in a shorter exposure (200 min) to simulated microgravity, Arabidopsis cell cultures showed non-significant variations in nucleolin and fibrillarin gene expression (Manzano *et al.*, 2016). However, results at the protein level indicated that nucleolin-L1 isoforms showed a reduced span of isoelectric point (Manzano *et al.*, 2016), the

same as was found in root meristematic cells (Matía *et al.*, 2007), corresponding to differences in the phosphorylation state of the protein (González-Camacho and Medina, 2005; González-Camacho and Medina, 2006). It is known the importance of the phosphorylative state for functions in the regulation of ribosome biogenesis (Warrener and Petryshyn, 1991). In addition, it was observed in a mutant defective on the major gene of nucleolin (*AtNucL1*) that different elements of the machinery of pre-rRNA synthesis and processing in the nucleolus are affected by this environmental change (Boucheron-Dubuisson *et al.*, 2016). Although the mutant is characterized by disorganized nucleolar structure, microgravity treatment intensified disorganization. Additionally, wild-type seedlings grown in RPM showed smaller nucleoli and less GC (Boucheron-Dubuisson *et al.*, 2016). This indicates that the decrease in the nucleolin resulted from gravitational stress has effects on nucleolar assembly, in a synergistic mode, where altered gravity contributes to the enhancement of disruptive effects originated by other causes.

Our observations, in major nucleolar proteins (nucleolin and fibrillarin), incorporated with previous ones are indicative of decreased ribosome biogenesis, and, consequently, reduced cell growth. In addition, their gene expression is in part regulated during cell cycle (González-Camacho and Medina, 2004; González-Camacho and Medina, 2005), which are indicative of coordination between the regulation of ribosome biogenesis and cell cycle progression, as discussed next.

2.4. Simulated microgravity disrupts the coordination between cell proliferation and ribosome biogenesis in proliferating cell systems in both root meristems and undifferentiated cell culture

In previous topics, we discussed the effects of microgravity on cell proliferation and cell growth. Here, we relate our data to the uncoupling of these two processes. Cell growth and cell proliferation are closely interconnected in actively proliferating cells (Mizukami, 2001). In these systems, all the basic activities are affected in different degrees by the mechanism driving cell growth, in order to reach the critical size capable of allowing cell division. A precisely organized cell cycle takes place throughout this functional process, whose progression is regulated at several checkpoints and depends on many different signals and inputs (Inzé and De Veylder, 2006; Mizukami, 2001). For it, cell growth requires a continuous supply of proteins, which are necessary for building new cellular materials. Since ribosomes are the cellular factories of proteins, the control of ribosome biogenesis is necessarily a key element in the control of cell cycle proliferation (Medina *et al.*, 2010).

In this context, microgravity has been demonstrated to disrupt coordination between cell growth and cell proliferation in proliferating Arabidopsis cell cultures. Here, the cell proliferation rate was enhanced, while the cell growth was reduced. The use of *in vitro* cell culture has several advantages in these studies since they provide a system of highly proliferating undifferentiated cells in quantity needed for the analysis. In case of root meristems, in opposition, the number of proliferating cells is very limited (especially in Arabidopsis). Similar observations, uncoupling of cell growth and cell proliferation, has been reported in seedlings' root meristems, in both simulated and real microgravity conditions, resulting in disruption of the meristematic competence (Manzano *et al.*, 2013; Matía *et al.*, 2010; Medina and Herranz, 2010; Boucheron-Dubuisson *et al.*, 2016; Herranz and Medina, 2014).

The uncoupling of these processes in microgravity conditions could be explained in part by factors regulating cell proliferation and cell cycle progression, on which ribosome biogenesis depends (González-Camacho and Medina, 2006). Nucleolar proteins, such nucleolin and fibrillarin studied in this work, apart from acting as regulators of the ribosome biogenesis process, are targets of factors controlling cell cycle progression and proliferation (Cerdido and Medina, 1995; González-Camacho and Medina, 2004; González-Camacho and Medina, 2006; Sobol *et al.*, 2006). This double involvement makes them candidates to be the bridge connecting cell cycle progression events and the regulation of ribosome biogenesis at the molecular level (Hemleben *et al.*, 2004; González-Camacho and Medina, 2006; Medina and González-Camacho, 2003; Olson, 1990). Therefore, in ribosome biogenesis it is known that nucleolin and fibrillarin together with several small nucleolar RNAs (snoRNAs) play an important role in the formation of small nucleolar ribonucleoprotein particle (snoRNP) complex, required for the processing of rRNA (Sáez-Vasquez *et al.*, 2004). This complex interacts in the early processing of pre-rRNA, in the primary cleavage site (Sáez-Vasquez *et al.*, 2004). At least in part, the function of this complex is determined by the level of phosphorylation of nucleolin by casein kinase 2 (CK2) (Medina *et al.*, 2010), being highly phosphorylated by this kinase (Bögre *et al.*, 1996; De Cárcer and Medina, 1997). Moreover, it has been speculated that unphosphorylated nucleolin binds to rDNA and represses transcription; thus, phosphorylation induces proteolysis, and consequently releases nucleolin from DNA, resulting in transcriptional activation (Bourbon *et al.*, 1983; Lapeyre *et al.*, 1987; Warrener and Petryshyn, 1991). CK2 is an essential and well-conserved Ser/Thr kinase that regulates proteins in a post-translational manner (Mulekar and Huq, 2015). Its function has been shown to affect a large number of developmental processes in plants (Mulekar and Huq, 2015; Riera *et al.*, 2001),

including regulation and co-ordination of cellular events involved in cell cycle, cell proliferation and cell growth (Espunya *et al.*, 1999; Moreno-Romero *et al.*, 2011). The role of CK2 in nucleolin phosporylation to transcription and processing of ribosomes, and hence protein synthesis, could explain, at least in part, the reduced cell growth observed under microgravity.

On other hand, additional to involvement of nucleolin in ribosome biogenesis, its levels have been shown highly dependent on cell proliferation, since its expression is restricted to proliferating tissues of the plant (Medina *et al.*, 2001; Srivastava and Pollard, 1999), and its phosphorylation activity is correlated with increase in cell proliferation (González-Camacho and Medina, 2004). Along the cell cycle phases nucleolin levels increase, reaching highest levels in G2 (González-Camacho and Medina, 2004; González-Camacho and Medina, 2007). This relation of the nucleolin protein with cell proliferation can be explained in part through the known phosphorylate modulation of its function by CK2 and CDKA kinases (De Cárcer *et al.*, 1997; Medina *et al.*, 2001). It is speculated that successive phosphorylation of nucleolin by CK2 and CDKA could be a mechanism for nucleolin to regulate the cell cycle and cell division. Also, these two protein kinases are known to phosphorylate a battery of different targets as a response to proliferation signals, and as a modulators of different cell cycle phases (Dorée and Galas, 1994; Pinna, 1990). In fact, if CK2 activity is blocked during G1 it abolishes the G2/M checkpoint, resulting in premature entry into mitosis, just showing discrete activity peaks at G1/S and M phases in proliferating cells (Espunya *et al.*, 1999). Moreover, experiments with an Arabidopsis dominant negative mutant of CK2 on its effect on expression of the core cell cycle regulators have shown a downregulation of most genes known to be up-regulated at the G2/M and S phases and consequent alterations on cell cycle phases were observed (Moreno-Romero *et al.*, 2011). CDKA kinase is a major driver of the plant cell cycle, which is constitutively present during cell cycle and demonstrates high kinase activity at G1/S and G2/M phases in the cell cycle of Arabidopsis (Joubès *et al.*, 2000), controlling both transition points (De Veylder *et al.*, 2007). In early cell cycle re-entry it was shown a strict coordination between nucleolin and cyclin gene expression, indicatory that the same factors could regulate expression of both genes (Bögre *et al.*, 1996). Therefore, these factors are involved in regulatory mechanisms of ribosome biogenesis and cell cycle, by modulation of nucleolin activity. In this way, CK2 and CDKA could be signal mediators for gravity sensing (Figure 21), relating coordination of the cell growth and cell proliferation under microgravity conditions.

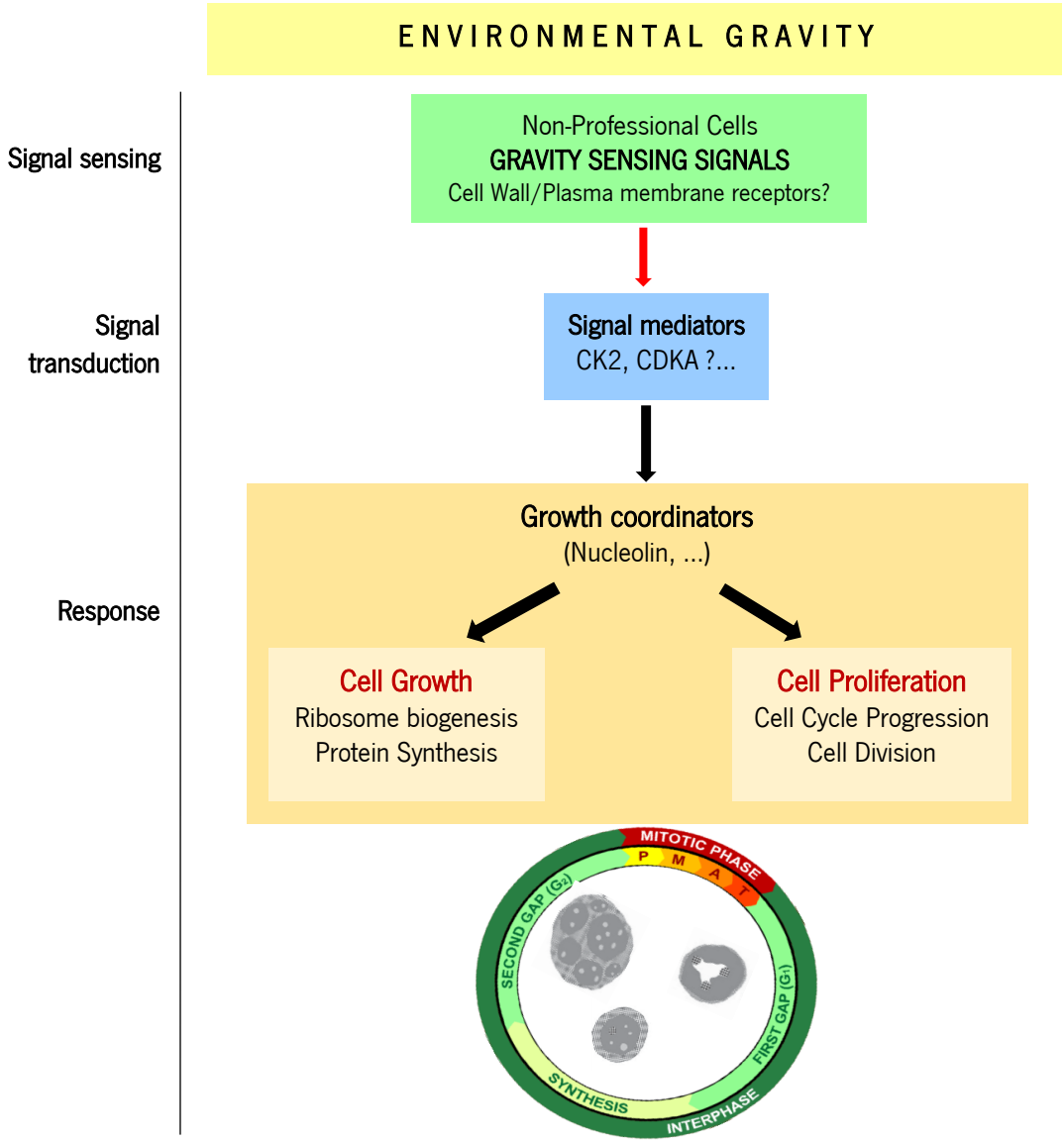

Figure 21: Schematic model of the main factors and functional processes playing a role in the regulation of the coordination between cell growth and cell proliferation *in vitro* undifferentiated high proliferative cultured cells by environmental gravity. Black arrows represent experimentally supported connections, whereas red arrows indicated suitable processes, compatible with experimental data, but still pending of further investigations for their demonstrations. Sensing of the parameters of the gravity (magnitude, direction) *in vitro* cultured cells may occurs by receptors of the cell wall (Hoson et al., 2005). Gravitropic signals are transduced, resulting in alterations of growth coordinators such as protein nucleolin. Mediators of the transduction of gravity mechano-signal sensed in this way are experimentally unknown. The proteins kinase CK2 and CDKA are proposed as candidates to be part of this scheme in view of the experimental findings that put it in close relationship with some cellular processes involved, such as ribosome biogenesis and cell cycle. (Adapted from Medina and Herranz, 2010).

Although it was not a topic experimentally supported in our work, it is interesting to note that CK2 has been related with the mechanism of gravity sensing in roots, in the regulation of the signal mediator auxin (Marquès-Bueno *et al.*, 2011). In this study, mutant plants depleted of CK2 activity resulted in an enhanced gravitropic response. Moreover, it has been observed that gravitational alterations induce inhibition of auxin polar transport and depletion of nucleolin levels in root

meristem, with the consequent inhibition of growth and development (Matía *et al.*, 2010). On the other hand, nucleolin loss of function in plants alters auxin transport and auxin response (Petricka and Nelson, 2007). This observation strongly suggests the role of nucleolin and auxin in the uncoupling of the cell growth and proliferation in root meristematic cells under microgravity, possibly by the involvement of CK2 functions. However, *in vitro* cell cultures are not integrated in an organism possessing specialized mechanisms for gravity sensing. Therefore, the response to gravistimulus in cell cultures cannot be the result of the transduction of a signal from a more-or-less distant receptor organ (Herranz and Medina, 2014). Thus, the gravity response in this system can only rely on mechanisms compatible with the general properties of all (or almost) cell types (Hoson *et al.*, 2005). Therefore, although there is no direct evidence of the involvement of CK2 in the response to gravity alteration, the known function of this essential protein kinase appoints it as a suitable candidate for this functional role. The use of the CK2 negative mutant in experiments of real or simulated microgravity could help in discerning this problem. In any case, the functions of CK2 in cell cycle and ribosome biogenesis are a good example of the suitability of a model involving different mechanism of gravity sensing and different signal transduction pathways to produce the same final, namely the disruption in the cell proliferation and cell growth.

3. Implications of alterations in Arabidopsis cell development processes for agriculture on Earth and space exploration

In this chapter possible implications of our findings in others research fields, such as sustainable agriculture under suboptimal conditions, will be discussed. The study of plant cells in microgravity, a completely new suboptimal environment, can reveal how plants can adapt to an environment with decreased gravity, an essential clue for their existence and survival on our planet. The knowledge of molecular mechanisms of perception, response and adaptation to abiotic environmental stresses is important for sustainable agriculture and food production on Earth in suboptimal environments. This is an important aspect for scientific community but also for the society, as it may help to maximize the (re)use of available resources. The change in gravity not only is an abiotic stress factor for the plant but also alters the way in which it detects and responds to other environmental factors, suggesting a synergistic effect between gravity response mechanism and other stresses' elements (Beckingham, 2010; Herranz *et al.*, 2010).

Studies suggest that, as in case of many other environmental stresses, after the rapid initial response the organism undertakes adaptive strategies after exposure to microgravity, both in cell

cultures (Moustafa, 2014), as in seedlings (Boucheron-Dubuisson *et al.*, 2016). The most affected response observed in cell cultures to the spaceflight was upregulation of UV-related and heat shock genes (Moustafa, 2014; Paul *et al.*, 2012; Zupanska *et al.*, 2013). Moreover, heat shock proteins, which are known to take part in response to multiple environmental stresses, were also affected (Swindell *et al.*, 2007). These observations confirm signal transduction pathway overlapping in mechanisms of response to microgravity and other environmental factors.

Previous studies have been shown conspicuous interactions between light and gravity mediated growth responses (Vandenbrink *et al.*, 2016). Plants utilize both gravity and light cues to direct plant growth and development. Plants evolve blue-light receptors located near the upper portion of roots and red-light receptors near the apices (Mo *et al.*, 2015). The pattern of light penetration in the soil has led plants to evolve/select these features; the red and far-red part of the light spectrum can penetrate to greater depths than blue light (Mandoli and Briggs, 1984). Plants utilize blue-light cues to control root architectural features, such as lateral root growth, and light signals sensed by root apices (Moni *et al.*, 2015). Light perception by the roots may also impact plant growth and development elsewhere, as light signals sensed by root apices have also been shown to have an effect on shoot gravitropic response (Hopkins and Kiss, 2012). Specifically, an effect of light on shortening the root length has been reported (Silva-Navas *et al.*, 2015). Modulation of cell cycle progression by photoreceptors, the light receptors, in shoot meristems is well known, but there are data indicating that action of light is different in the root and in the shoot meristems (López-Juez *et al.*, 2008; Silva-Navas *et al.*, 2015). Additionally, to regulate phototropic growth, phytochromes have been implicated in a multitude of biological processes, from seed germination to plant senescence. Typically, in ground studies, root plants respond to light with negative tropism (growth away from the light source) (Briggs, 2014; Kutschera and Briggs, 2012; Liscum *et al.*, 2014). On other hand, plants response to gravity with positive gravitropism, by growing roots along gravity direction. In this context, a novel positive blue-light phototropic response in Arabidopsis roots was observed during conditions of microgravity (Vandenbrink *et al.*, 2016). Additionally, a positive phototropic response in roots when exposed to red light in condition of microgravity was observed (Kiss *et al.*, 2003; Vandenbrink *et al.*, 2016). A decrease in blue- and red-light responses was observed to be gradual and correlated with the increase in gravity. However, the magnitude of phototropic response was less during red-light stimulation compared with the robust response to blue light. Also, different threshold value between the two light treatments was observed, where 0.1-0.3 *g* is enough to cease phototropic curvature illuminated with blue-light, while red-light

exhibits a gradual reduction in the magnitude of curvature with increasing levels of gravity (Vandenbrink *et al.*, 2016). This suggests that the mechanisms controlling the interaction between gravitropic response and phototropic response are different for the two lights. Additionally, enhancement of the blue-light phototropic response in roots after a short red-light pretreatment, suggests that while the two responses differ in pattern and, potentially, mechanisms, there is still an additive effect that causes an enhancement to total phototropic curvature. The relationship between phototropism and gravitropism is a complex, poorly characterized interaction, where the direction of growth is an amalgamation of the two processes, contributing in the overall direction and form of plant growth (Correll and Kiss, 2002). It is possible that the near-linear relationship between red-light phototropic response and gravity in roots is a result of cytosolic Ca^{2+} (Toyota *et al.*, 2013), which have been thought to play a key role in the transmission of gravity sensing (Correll *et al.*, 2013; Gilroy *et al.*, 1993; Poovaiah *et al.*, 1987), potentially through regulating the distribution of auxin and auxin transporters within the root cells (Zhang *et al.*, 2011). Furthermore, cytosolic calcium levels in Arabidopsis cell cultures, also reported an inverse linear relationship between changes in levels of several known Ca^{2+} inhibitors/antagonists (Neef *et al.*, 2015) and the observed red-light-induced phototropic curvature in roots, suggesting a potential antagonistic relationship with the root red-light phototropic response mechanisms (Vandenbrink *et al.*, 2016). It is possible that a gradual reduction in cytosolic calcium levels is responsible for the decreased positive phototropic response associated with increasing gravity. Also, it is evident the involvement of CK2 in light signalling pathway in Arabidopsis. In spite of not being a light-regulated kinase, CK2 promotes plant development under light not only by stabilizing the positive regulators but also promoting degradation of negative regulators (Lee *et al.*, 1999). It is important to know detailed mechanisms between microgravity and light perception to be able to improve sustainable agriculture strategies both on Earth and beyond. As example, to plan optimal plant development in the conditions of microgravity, the lack of gravity could be compensated with red light. Indeed, to improve "soiless agriculture", such as hydroponics, by know which exactly proteins might be modified to avoid negative effect of light exposure to the root without changing its gravitropic response. Elaborate these mechanisms in crop plants by studying the behaviour of the plant response to biotic/abiotic stresses under the space conditions, particularly the synergies that could exist among them, could be very useful to improve agriculture.

4. Suggestion of new technique to be used in future for simulated microgravity experiments using 2D clinostat

With the increasing interest of the scientific community to return man to the Moon and to go further in the exploration of Mars, studies performed with their gravity fields (0.16 *g* and 0.37 *g*, respectively) are becoming increasingly valuable (Figure 22). In fact, this year it will be sending to germinate the first seeds on Mars, which will be carried out by the project Seed of the Mars One mission (https://community.mars-one.com/projects/seed1). The possible scientific outcomes of this experiment could contribute to a better understanding of plant growth on Mars. Therefore, it will become increasingly demanded to provide sustainable life support systems.

Experiments in real altered gravity are of extreme value, but also rare. Thus, experiments using simulated partial microgravity on Earth are indispensable. The 2D clinostat rotates around one axis, which gives an advantage of easy establishing simulated partial microgravity conditions, since the orientation of this axis determines its properties (Hasenstein and Van Loon, 2015). Under the correct conditions (distance from the rotational axis and rotational speed), it compensates or averages the vectorial character of gravity (United Nations, 2013). In a vertical position, it moves an object, but the gravity vector is consistently experienced toward the basal (Earth-facing) side of the object. Positioning the axis at an angle in between the horizontal and vertical position results in a net proportion of Earth's gravity such that the simulated microgravity is equal to the sin α (the angle from the horizontal) times g. A simulation of Moon's gravity (0.16 *g*) therefore requires an angle of about 10 degrees. Mars' gravity (0.37 *g*) could be simulated by tilting the axis by 22 degrees (Hasenstein and Van Loon, 2015).

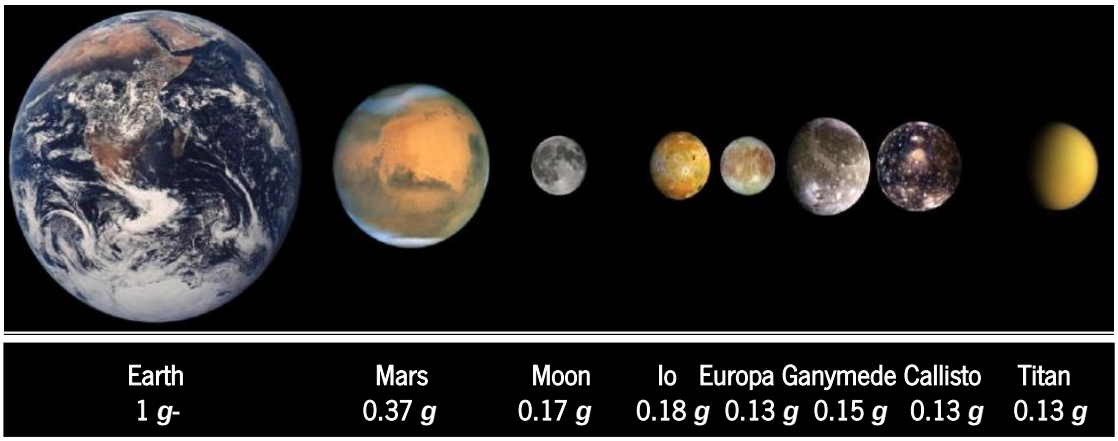

FIGURE 22: Gravity levels in potentially life-compatible solar system celestial bodies. Gravity level of some solar system celestial bodies in a comparison with Earth gravity reveals that the importance of the partial gravity studies. (Adapted from doctoral thesis Moustafa, 2014).

CONCLUSIONS AND FUTURE PERSPECTIVES

1. Alginate- $CaCO_3$-GDL system is an adequate immobilization method to investigate the effect of simulated microgravity on suspension cell culture. Its advantage over the agarose system is the fact that the cell recovery is not temperature dependent. Its advantage over the alginate-gelatine-$CaCl_2$ system is the stability of the gel. Its advantage over the use of *callus* is that it doesn't need the long period of "setting" on agar medium and actively proliferating cells can be investigated in contrast to the cells in stationary phase in case of *callus*.

2. Extraction of plant nuclei according to homogenization method (Komatsu, 2007) yields a higher number of nuclei than the Cystain Kit that is optimized for fresh tissues but not for the cell cultures.

3. Long term simulated microgravity (24 h) caused the increase in the number of cell population entering S phase in immobilized cell suspension culture MMd2 in comparison the control cells under 1 *g*. This increment was on the cost of cell population in G1 but not G2 phase which could mean the S phase is prolonged in microgravity conditions.

4. The exposure to simulated microgravity conditions results in reduction of the ribosome production rate, and in consequence the decrease of cell growth, as assessed by the abundance of two nucleolar proteins, nucleolin and fibrillarin, in comparison to the 1 *g* control.

The reduction in nucleolar size is in agreement with the results from cell cycle. The nuclei in cells in G1 and S phase are similar, the nucleolar size increases in G2.

5. After the cell cycle arrest in G1/early S phase cells quickly enter S phase and the S peak joins with the G2/M peak 2 h after the cell cycle release. After 6 hours the G2/M peak reaches maximum value and after the 10 h the cells divide (increase of the G1 peak at the cost of G2/M).

To better understand the effects of microgravity in cell proliferation and cell growth of Arabidopsis cell suspensions future studies should consist, firstly, in the confirmation of these results and, then, in the validation/analysis of these changes by other methods, like proteomics methods, to

determine quantitatively variation in levels of nucleolin and fibrillarin, or qPCR to evaluate the rate of expression of the related genes. Also, other proteins, which have a key role in cell cycle regulation and in the control of ribosome biogenesis, e.g., cyclin B1, CDKA-1 and CK2, would be evaluated. Additionally, the study of a CK2 negative mutant would complement our findings allowing to probe the effect in nucleolin and, consequently, in cell cycle and ribosome biogenesis under microgravity.

Futures studies using the 2D clinostat would be perform in order to test different durations of exposure to study cell cultures adaption to simulated microgravity. Exposure to simulated partial microgravity conditions would also be very interesting to assess the behavior of plant cell suspension cultures under gravity fields of others celestial bodies, such Mars and Moon. Moreover, in further experiments, synchronized cell cultures will be used to focus on a more detailed influence of microgravity treatment in cellular activities taking place at each cell cycle phases.

Lastly, studies using suspension cells of Arabidopsis in real microgravity conditions will be necessary to validate the observations on GBF, since, other spaceflight-related phenomena linked to microgravity are not present in Earth-based experiments, e.g., hydrostatic pressure and cell surface binding forces. Here, the optimal immobilization of Arabidopsis cell suspension cultures in alginate will be a method compatible with the realization of these experiments in space.

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ANNEXES

ANNEXE 1: Specification of the one-axis clinostat provided in the Project.

Equipment size (cm):	Main body: 25 x 25 x 25 Control box: 23 x 20 x 11
No. of rotational axes:	One
Rotational speed:	0-90 rpm Accuracy: 1 per cent
Rotational axis angle:	0° to 90°
Rotation direction:	Clockwise or counterclockwise
Input voltage:	100V-240V
Building material:	Aluminium
Experiment conditions:	Max. weight of samples: 500 g Max. diameter of a sample container: 10 cm