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Alterations in endogenous hormone levels and energy metabolism promoted the induction, differentiation and maturation of Begonia somatic embryos under clinorotation

Hoang Dac Khai [a], Le The Bien [a,d], Nguyen Quang Vinh [b], Doan Manh Dung [b], Ngo Dai Nghiep [c], Nguyen Thi Nhu Mai [a], Hoang Thanh Tung [a], Vu Quoc Luan [a], Do Manh Cuong [a], Duong Tan Nhut [a,*]

[a] Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology, Viet Nam
[b] Tay Nguyen University, Viet Nam
[c] University of Science, Vietnam National University, Ho Chi Minh City, Viet Nam
[d] Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Viet Nam

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ABSTRACT

The present study provides a visual insight into the effects of simulated microgravity (MG) on somatic embryogenesis (SE) in Begonia through the analysis of phytohormone fluctuations and energy metabolism. To investigate this relationship, thin cell layer culture model was first used. The results showed that MG changed the phytohormone content and stimulated starch biosynthesis to convert into sugar to release energy needed for regeneration and proliferation. Moreover, from the results it is likely that MG accelerated the initiation and subsequently maturation and aging of SE via decrease of AUX and increase of ABA. High content of GA, CKs, starch, sugar and low ABA as well as high CKs/ABA ratio were responsible for the increase in the number of embryos under clinorotation which was 1.57-fold higher than control after 90 days. The increase in fresh and dry weight of somatic embryos and chlorophyll content under MG were confirmed as their adaptive responses to gravitational stress. However, long-term exposure to MG (120 days) stimulated biosynthesis of ABA levels 1.85-fold higher than controls, which resulted in a decrease in chlorophyll content, increase in number of mature embryos and stomata length. These results revealed that MG regulated the induction, differentiation and senescence of somatic embryos via a biochemical interaction pathway.

1. Introduction

Conservancy in the magnitude and direction of gravity has been confirmed during millions of years of adaptation and life's evolution on earth. Based on this stability, plants and many other organisms use gravity as the most reliable signal source for their growth, development and reproduction [1,2]. The change of gravity causes abiotic stress on plants. Under direct or indirect pathways, this stress can induce positive or negative controls on the physiology, biochemistry, and morphology of plants [3,4] or more seriously, plant reproduction [5].

From the outset of space programs, plants have been identified as an integral element of the human mission to conquer space. Based on photosynthetic capacity, plants can provide food, recycle water, oxygen and waste in long-term flights. Therefore, plants are considered the centerpiece of the Biological Life Support System/Controlled Ecological Life Support System. In space, however, plants face challenges that they have never known before; e.g., cosmic radiation, changing magnetic fields and especially extreme microgravity environments (MG) [5]. Consequently, plants have shown failure to reproduce in space [6–8].

In space conquests, plant seeds play an ever more important role. Indeed, in long-term space missions seed acts as an important food reserve or can either be consumed directly or planted for the next crop [9]. Therefore, investigating the reproduction and maintenance of plant and animal generations under MG is the goal of NASA's space life science program [9,10]. However, the effect of MG on plant embryogenesis has been limited by research. *Arabidopsis thaliana* was the first plant to

Abbreviations: ABA, abscisic acid; AUX, auxin; CKs, cytokinins; GA, gibberellin; MG, microgravity condition; SE, somatic embryogenesis; TCL, thin cell layer.

* Corresponding author.
E-mail address: duongtannhut@gmail.com (D.T. Nhut).

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complete its seed-to-seed cycle in MG; the results showed that most of seeds produced were nonviable embryos [11]. According to [6] wheat also completed its life cycle in the Russian Space Station MIR but did not produce any seeds. In 2000, two independent groups of [12] and [13] reported two similar results that the *Brassica rapa* in MG produced seeds could germinate; however, there were significant physiological and biochemical changes in MG-reproduced embryos. More satisfactory results were reported later on dwarf tomatoes [14] and peas [15]. Nevertheless, more research is needed to contribute to the database clarifying the question of MG effects on plant embryogenesis. Previous studies on plant embryogenesis under MG conditions have not only investigated sexual reproduction but also asexual reproduction [9] i.e., somatic embryogenesis.

Plant embryos are formed without fusion of gametes instead from somatic cells called somatic embryogenesis (SE). Somatic embryogenesis offers important opportunities for understanding the morphology, physiology and genetic mechanisms of embryonic development stages [16]. It is well known that phytohormones are directly involved in the initiation, formation and maturation of SE via transmitting a variety of molecular signals to express related genes [16,17]. Although SE has been described in MG several decades ago [9,18,19]; however, information on the fluctuations of endogenous hormones and energy substances during the development of somatic embryos under MG is still very limited. Therefore, in this study the dynamics of changes in endogenous hormones and energy substances during SE were reported in Begonia.

In the present work, in order to effectively and accurately study the effects of MG on different stages of embryo development, thin cell layer culture (TCL) has been used. The TCL system was first described by [20], who used it in tobacco. TCL systems are conceptualized as small explants cut from different plant organs [21]. The thin profile is what makes TCL such a preeminent technology. In fact, in this way it is easy to locate the responsive cell by a small number of cells present in a TCL. In addition, the biological events that take place during the generation process are easily recorded. TCL contains low levels of endogenous hormones, making it easy to assess endogenous hormone fluctuations during regeneration. All these proved that TCL is considered as an ideal model for studying the mechanisms controlling plant morphology and formation [20,22].

This study was conducted to evaluate the potential effects of MG on SE induction, differentiation, growth of somatic embryos and ultimately their maturation under slow clinorotation. For that purpose, the Begonia petiole TCL culture model was applied for direct SE under clinorotation. The accumulation and changes in carbohydrate concentrations were measured over all SE stages; furthermore, endogenous hormone content and balance throughout SE were also evaluated to provide a visual insight into the effect of MG on SE during long-term exposure to clinorotating. Besides, the effect of MG at the cellular level was evaluated through the change in stomata morphology. Finally, in the discussion, we explored the causes of SE changes under MG.

2. Materials and methods

2.1. Plant material and culture medium

The petioles of uniform size (0.3 cm in diameter and 5 cm in length) were collected from 4-month-old *Begonia tuberous* plants (Dalat Hasfarm Co., Ltd., Vietnam). The petioles were treated with detergent and washed under running water for 30 min. Next, the petioles were treated with 70 % alcohol for 10 s and rinsed with sterile distilled water for 3–4 times and then disinfected with 0.1 % $HgCl_2$ solution and 2–3 drops of tween 80 for 6 min, followed by 4–5 rinses with sterile distilled water. Finally, the petioles were cut into transverse thin cell layer (p-tTCL) (0.8−1 mm in thick), and it was used as the explant for SE induction (Fig. 1).

The p-tTCL explants were cultured on half-strength MS medium [23]

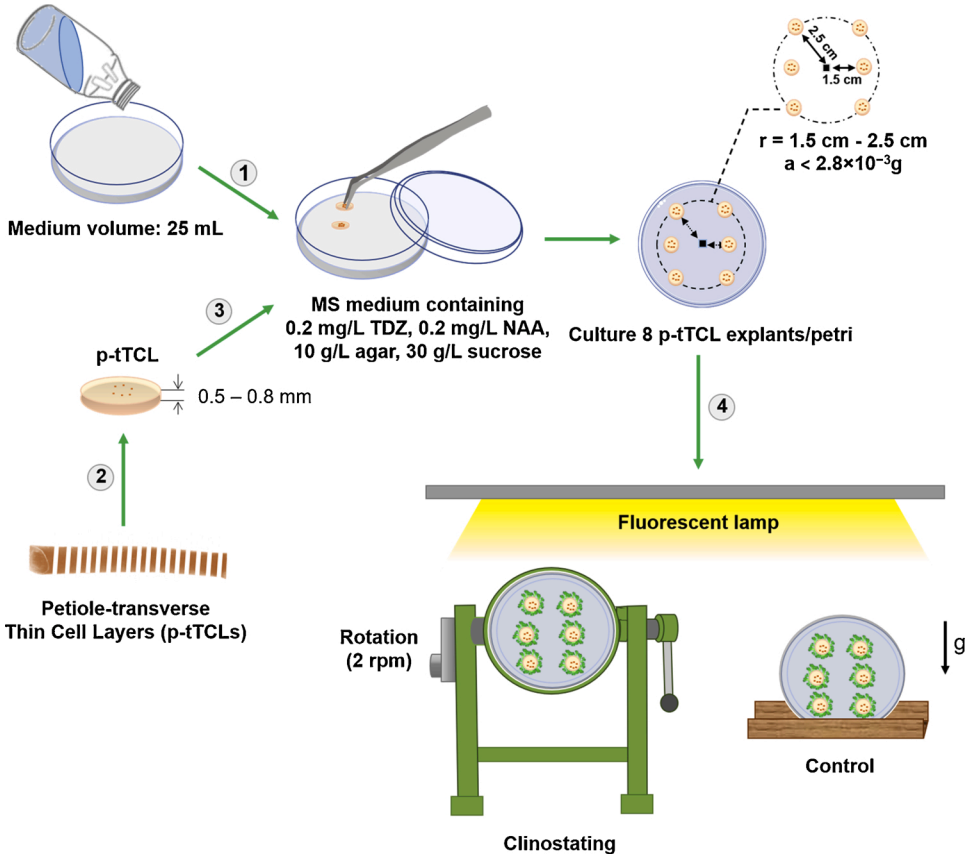

Fig. 1. Schematic illustration of experimental setup. (**1**) Culture media were prepared in petri dishes. (**2**) Surface-sterilized *B. tuberous* petioles were cut into transverse thin cell layer (p-tTCL) (0.8-1 mm in thick). (**3**) Six explants were cultured at a position 1.5 cm - 2.5 cm from the center of the axis of rotation (corresponding to the acceleration acting on the explant always less than 2.8×10^{-3}g). (**4**) The explants were grown under a stationary condition for the control and the explants were rotated horizontally on clinostat at 2 rpm. Both treatments were incubated under the same conditions and using top-down fluorescent light to ensure that the explants received similar light intensities.

supplemented with 30 g/L sucrose, 10 g/L agar, 0.2 mg/L 1-Naphthaleneacetic acid (NAA) and 0.2 mg/L Thidiazuron (TDZ) for somatic embryo induction [22] (Fig. 1). The pH value of the culture medium was adjusted to 5.8 and autoclaved at 121 °C, 15 psi for 20 min.

The chemicals include mineral salts to prepare the medium; hormones, standards, enzymes and reagents for biochemical experiments; histological dyes were purchased from Sigma-Aldrich®, USA.

2.2. Simulated MG and growth conditions

To simulate MG conditions, a clinostat 2D machine (Advanced Engineering Services Co., Ltd. Japan) was used. The machine has a horizontal axis of rotation that is perpendicular to the gravity vector and an amplifier that regulates the rotational speed. Six explants were cultured at a position 1.5 cm–2.5 cm from the center of the axis of rotation (corresponding to the acceleration acting on the explant always less than 2.8×10^{-3}g) [3]. For the control, explants were grown under a stationary condition and other explants were fixed on the rotating part of clinostat and set to a rotational speed of 2 rpm. Both treatments were incubated under the same conditions and using top-down fluorescent light to ensure that the explants received similar light intensities. The explants of both treatments were equally cultured under laboratory conditions. The temperature was set at 25 ± 2 °C, the relative humidity was 50–60 %, and the photoperiod was 8 h light and 16 h dark, illumination by fluorescent lamp (36 W) with light intensity 40-45 μmol m^{-2} s^{-1}. Light was shone from above to ensure that the explants received the same light intensity (Fig. 1).

2.3. Experimental designs

To investigate the effect of MG on SE induction of p-tTCL explants, during the first 30 days of culture, corresponding to the induction phase of SE, the initiation of somatic embryos was evaluated histologically at each collection date. In addition, explants were collected every 5 days to assess the dynamics of carbohydrate level changes during this period.

Effects of MG conditions on different SE stages were evaluated, including induction (30 days), differentiation and growth (60–90 days) and maturation (120 days). Accordingly, the explants in the two treatments were obtained monthly (30, 60, 90 and 120 days) to obtain growth data including: total number of embryos; fresh weight (FW) (mg) and dry weight (DW) (mg) of somatic embryos; total chlorophyll content in leaves of mature embryos (nmol/cm^2) was measured with a chlorophyll meter SPAD-502 (Minolta, Japan). The ratio of embryo stages at the same time was calculated using the formula: number of each embryo stage/total number of embryos; in which, the SE stages include: globular, heart, torpedo, mature (embryos have formed roots and cotyledons or even leaves), multiple embryos (somatic embryos in a fused form that form an inseparable cluster) were isolated and counted under a SMZ800 N stereo microscope (Nikon, Japan).

After 30, 60, 90 and 120 days of culture, the explants were collected and washed with distilled water for biochemical analyses. For the qualitative and quantitative determination of endogenous hormones, fresh samples were used. To measure the carbohydrate content, the samples were lyophilized and digested with a mill; that lyophilized biomass was stored in a plastic bag at −18 °C for starch and reducing sugar analysis.

2.4. Determination of endogenous hormone content by High performance liquid chromatography (HPLC) analysis

The endogenous hormones measured in this study were Indole-3-acetic acid (IAA), three cytokinins (2ip [N6-isopentenyladenine], KIN [Kinetin], Zeatin [trans-Zeatin]), Gibberellin (GA_3) and Abscisic acid (ABA).

Fresh samples were ground in Bieleski solution of $CHCl_3$:MeOH: HCOOH:H_2O (25:60:5:10, v/v/v) at a ratio 0.1 g plant material per 1 mL solution. The mixture was then extracted at −30 °C in the dark for 4 h (the sample was divided into 2 parts; one part was kept unchanged and the other part was added with internal standard at known concentrations). Each sample was then centrifuged at 10,000*g* for 10 min at 4 °C and the supernatant was transferred to a clean tube. The residue was re-extracted in 4 mL of 80 % methanol for 1 h at 4 °C on rotary shaker, and re-centrifuged. The supernatants were pooled and loaded on Sep-Pak C18 cartridges, which had been equilibrated with 1 mL of 100 % methanol followed by 1 mL of 80 % methanol. The cartridge was rinsed with 500 μL of 80 % methanol. The purified extract was dried in a vacuum evaporator at 50 °C to remove the solvent and reconstituted with 1−2 mL of water with pH = 2 (adjusted with formic acid). The solution was filtered through a 0.45 μm membrane before injecting into the HPLC system. The hormones were separated by Thermo-Ultimate 3000 HPLC system (Thermi Scientific, USA) equipped with a 25 mm x 4.6 mn, particle size 0.5 μm (BDS Hypersil C18 column) and connected to a UV detector monitored at 280 nm. A binary solvent system was used; it was comprised of (A) acetonitrile (B) Milli-Q water acidified with 0.5 % formic acid. Separations were performed using segmented gradients of 0−10 min A from 100 % to 75 %, followed by 11−17 min A from 75 % to 50 % and finally 18−25 min A from 50 % to 75 % with the flow rate of 0.7 mL/min. Calibration curves were made from the signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyze to that of the corresponding internal standards. Quantification of the hormones was calculated from calibration curve of specific standards.

2.5. Determination of carbohydrate content by ultraviolet-visible spectroscopy (UV–vis) analysis

2.5.1. Determination of starch content

The starch content was quantified as described by [24]. Accordingly, the starch in the sample was hydrolyzed to maltodextrin by and α-amylase (3000 U m/L) and then hydrolyzed to glucose by amyloglucosidase (3300 U m/L). The glucose content in the sample was determined using GOPOD (glucose oxidase-peroxidase) reagent which gave the product with the maximum absorbance at 510 and D-glucose was used as a control. Reagent blank solution prepared with 0.1 mL distilled water supplemented with 3.0 mL GOPOD. The total starch content (*I*) (% w/w DW) was determined by the formula: $I = A \times F/W \times 90$.

In which: *A* is the absorption coefficient of the test solution compared to the blank sample; *F* is the conversion factor from the absorbance value to the glucose value; *W* is the test sample mass (mg).

2.5.2. Determination of reducing sugar content

The reducing sugar content was determined as described by [25]. Briefly, the reducing sugar content of the sample was determined using 3,5-dinitrosalicylic acid (DNS) reagent which gave the product with the maximum absorbance at 540 nm, while distilled water was used as a control. D-glucose was used for the preparation of the calibration curve. Sugar content (*S*) (mg/g DW) was determined by the formula: $S = A \times n \times V/m$.

In which: *A* is the content of reducing sugar in the diluted sample solution (mg/g); *n* is the dilution factor; *V* is the volume of standard solution (mL); is the test sample mass (g).

2.6. Anatomy and histological analysis

To observe SE stages, every 15 days of culture, explants were obtained and anatomized. Anatomical method was performed according to [26]. Samples were sliced with a razor blade and bleached with 10 % javel, rinsed with distilled water at least 3 times; then neutralized with 10 % acetic acid, rinsed with distilled water 3 times. Samples were stained with carmine (Sigma-Aldrich, USA) for 3 min and rinsed with distilled water 3 times. Finally, the samples were observed and photographed under an OLYMPUS CH30 optical microscope (OLYMPUS,

Japan) at different magnifications (×10, ×40, ×100).

To evaluate stomata morphology, the leaves of the mature somatic embryos after 120 days of culture were collected for stomata observations. Epidermal tissues were separated from the underside of leaves and placed on a slide containing a drop of distilled water. The stomata were observed and imaged with different objectives using an optical microscope. The size of the stomata was determined using imageJ software version 1.49.

2.7. Statistical analysis

All experiments were arranged in a completely randomized design with 6 replicates per treatment and 12 samples/replication. The data were statistically analyzed using SPSS software version 20.0. One-way or two-way analysis of variance (ANOVA) was used to compare the significant differences between the mean values (depending on the experiment) by Tukey's test. The results were expressed as mean ± standard error at the significance level $P \leq 0.05$. The tables and histograms were created by Excel 2016.

3. Results

3.1. Early effects of MG on SE induction

The results showed that MG did not affect the regeneration ability of the explants. The regeneration rate reached 100 % in both the MG and control treatments after 30 days of culture (Fig. 2A–D). However, on the 14th day of culture, none of the control explants' epidermal cells had yet divided (Fig. 2A); while the explants of the MG treatment started to undergo mitotic reactions (Fig. 2B). Furthermore, morphological observations showed that differentiation of somatic embryos from embryogenic callus under gravity conditions (Fig. 2C) occurred at a slower rate than under MG (Fig. 2D) after 30 days of culture. As shown in Fig. 2E, clinorotation significantly shortened the SE induction time compared with the control (approximately 18 days).

Starch accumulation plays a pivotal role in cell division and meristem formation during organogenesis/SE [3,27]. During the first 30 days of culture, which corresponds to the induction phase of SE, changes in starch and reducing sugar level under clinorotation and control were recorded and shown in Fig. 1F. The results showed that total starch and sugar levels were significantly increased in both MG and control treatments. However, over a period of 15–25 days, the total sugar level gradually decreased and the total starch level gradually increased in both treatments. The decrease in sugar levels over a period of 15–25 days was possible because they were consumed to activate the energy metabolism required for SE. Interestingly, the total sugar level of the MG treatment was significantly higher than that of the control at 15 and 25 days of culture i.e., it could provided more energy for SE. In addition, the total starch levels in the p-tTCL explants under clinorotation were significantly higher than in the control at 5, 10, 25 and 30 days of culture; meanwhile, the total sugar level of the MG treatment was lower than that of the control at 20 and 30 days of culture was observed. These

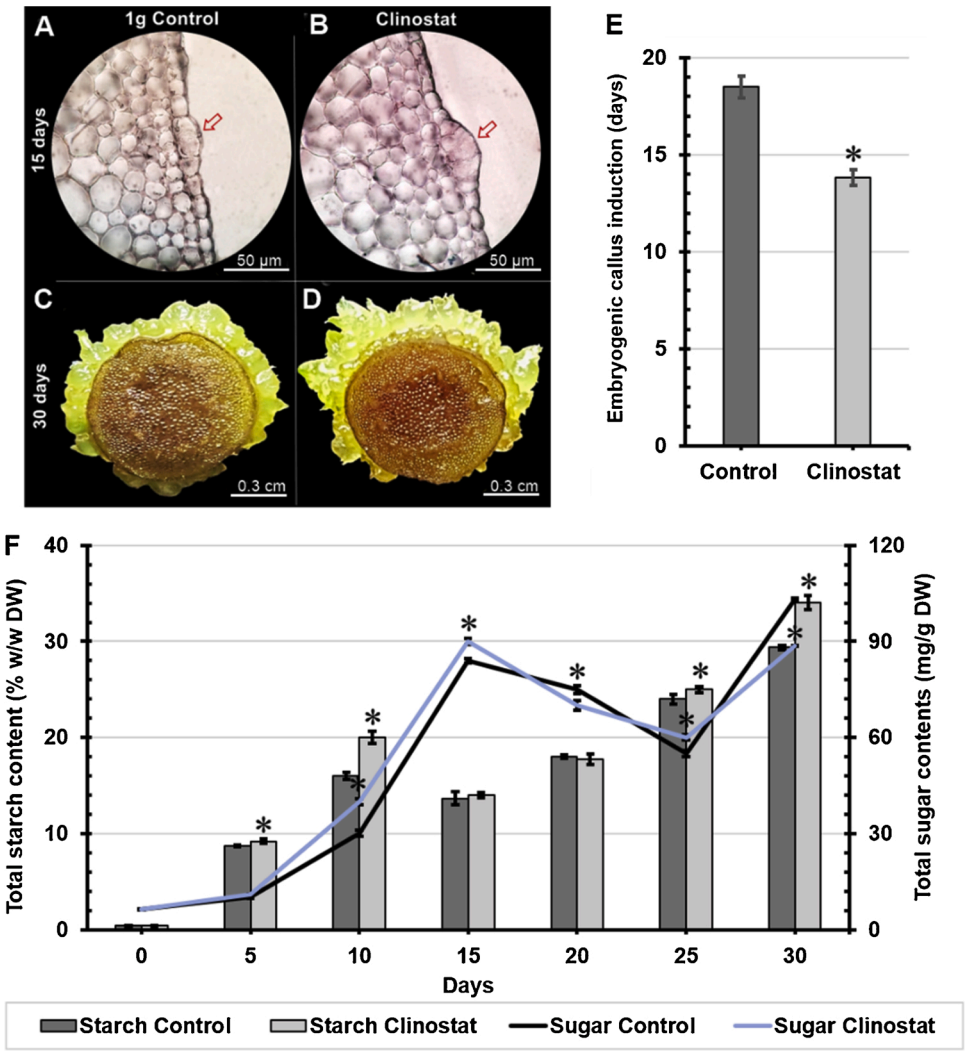

Fig. 2. Effect of MG on SE induction in *B. tuberous* during the first 30 days of culture. **A, B** Embryogenesis was initiated from the epidermis of petiole-transverse thin cell layer explants after 15 days of culture. Arrows point to dividing cells. **C, D** Differentiation of somatic embryos from embryogenic callus after 30 days of culture. **E** SE induction time. **F** Total starch and sugar levels during the induction phase of B. tuberous SE. Data represent the mean ± standard error of six replicates. *Indicates that the values are significantly different according to Tukey's test ($p < 0.05$).

results indicated early effects of MG on SE induction in *B. tuberous* despite short-term exposure to clinostating.

3.2. Somatic embryos formation under MG

After 60 days of culture, two differentiation programs took place in both MG and 1 g control conditions, namely somatic embryos (Fig. 3A–C) and adventitious roots (Fig. 2D). In which, SE was predominant while the indeterminate root organ was formed with negligible quanlity (data not shown). These results revealed that MG did not interfere with the morphogenesis program from *B. tuberous* p-tTCL explants. Furthermore, histological observations showed that under clinostating, SE still had to go through the typical developmental stages of somatic embryos similar to controls (Fig. 2E).

The developmental stages of somatic embryos under clinostating were observed in detail, which included globular; heart (Fig. 3A); another form has been observed called multiple embryos (somatic embryos in a fused form that form an inseparable cluster) (Fig. 3B); torpedo (Fig. 3C) and mature (embryos have formed roots and cotyledons or even leaves). In addition, the developmental stages of somatic embryos were pretty simultaneous under MG and 1 g control (Fig. 3E). Specifically, after 30 days of culture, globular somatic embryos were dominant (100 % of all embryos) in both treatments. On the 60th day of culture, the frequency of somatic globular embryos decreased sharply and was replaced by the appearance of embryos at the heart and torpedo stage, as this was the only time when heart embryos were present. During the period of 90–120 days of culture, the embryos at the globular and heart stages were completely terminated; while this was the transition to torpedo, mature and multiple embryos in both treatments (Fig. 3E). Overall, MG did not affect the developmental stages of *B. tuberous* somatic embryos; however, MG significantly affected embryogenesis potential as demonstrated by the number of somatic embryos (Fig. 3F).

The number of embryos at 30, 60, 90 and 120 days was recorded and shown in Fig. 3F. The results showed that after 30 and 60 days of culture, the number of embryos of the MG treatment and the control did not show a statistically significant difference ($p < 0.05$). However, after 90 days of culture under clinostating, the number of embryos was 1.57-fold higher than that of the control (62.33 and 39.67 embryos, respectively). When the MG exposure time was extended from 90 to 120 days, the number of embryos of the MG treatment increased indistinctly; meanwhile, the number of embryos of the control treatment continued to increase linearly (Fig. 3F). The data demonstrated that MG stimulates SE; in other words, the presence of gravity negatively controls the SE of *B. tuberous*.

3.3. Somatic embryos development under MG

The effects of MG on the development of somatic embryos were evaluated through fresh weight and dry weight and total chlorophyll content (Fig. 4). The results showed that no significant difference was found in biomass between the MG treatment and the control after 30 and 60 days of culture. However, the development of somatic embryos was markedly promoted by MG exposure after 90 days (Fig. 4A); when the fresh and dry weight of embryos was significantly increased under clinostating (4485.00 mg and 615.00 mg respectively) compared with 1 g control (3776.67 mg and 476.67 mg respectively) (Fig. 4B). However, the fresh and dry weights of the embryos were not significantly different between the two treatments after 120 days of culture (Fig. 4B). After 90 days of culture, the chlorophyll content of the clinorotation treatment (35.00 nmol/cm^2) was significantly higher than that of the control (26.55 nmol/cm^2); while the chlorophyll content under MG was decreased with increasing the culture time to 120 days and the opposite result was observed in the case of the control (Fig. 4C). These results indicated the potential effects of long-term exposure to MG on plant growth, which are discussed below.

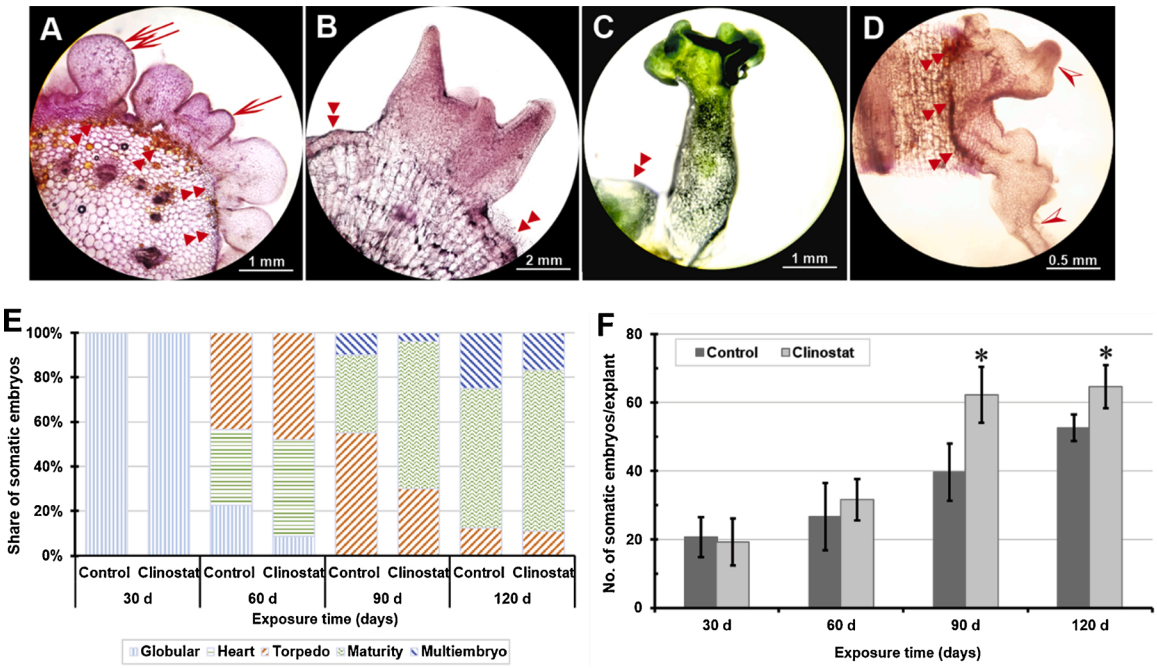

Fig. 3. Effect of MG on developmental stages and proliferation of somatic embryos in *B. tuberous* after 30, 60, 90 and 120 days of culture. (**A - C**) Developmental stages of somatic embryos stained with carmine red, the double triangle points to the epidermal cell layer of petiole-transverse thin cell layer explant. **A** Formation of globular embryos (double arrows) and heart embryos (arrows) under MG after 60 days of culture. **B** Formation of multiple embryos (somatic embryos in a fused form that form an inseparable cluster) under MG after 90 days of culture. **C** Formation of torpedo embryos under MG after 60 days of culture. **D** Formation of adventitious roots (arrows) from the epidermis of petiole-transverse thin cell layer explants under MG after 60 days of culture. **E** Ratio of embryonic stages. **F** Total number of embryos. Data represent the mean ± standard error of six replicates. *Indicates that the values are significantly different according to Tukey's test ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

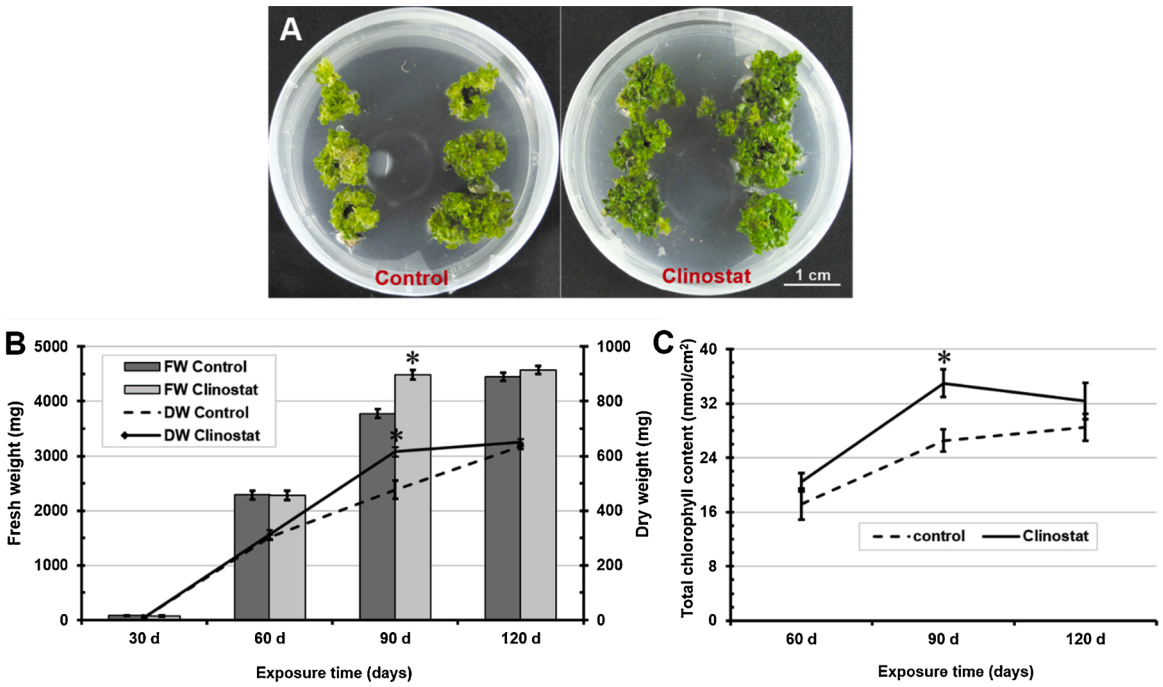

Fig. 4. Effect of MG on growth of *B. tuberous* somatic embryos after 30, 60, 90 and 120 days of culture. **A** Somatic embryogenesis h under clinorotation and 1 g control after 90 days of culture. **B** Fresh and dry weight of somatic embryos. **C** Total chlorophyll content in leaves of mature somatic embryos. Data represent the mean ± standard error of six replicates. *Indicates that the values are significantly different according to Tukey's test ($p < 0.05$).

3.4. Changes in endogenous hormones content during SE under MG

The results of AUX, CKs, GA and ABA content showed significant differences at different SE stages (Fig. 5). In both treatments, the total GA and ABA concentrations showed an increasing and opposite trend for AUX and CKs with increasing culture time.

The AUX content in somatic embryos has been shown in Fig. 5A. The results showed that in both MG and 1 g control treatments, AUX was detected and ABA was not detected after 30 days of culture - SE induction stage. However, the total AUX content in the MG treatment was significantly higher than in the control (Fig. 5A). Subsequent culture periods of 60, 90 and 120 days, no AUX was detected but ABA was

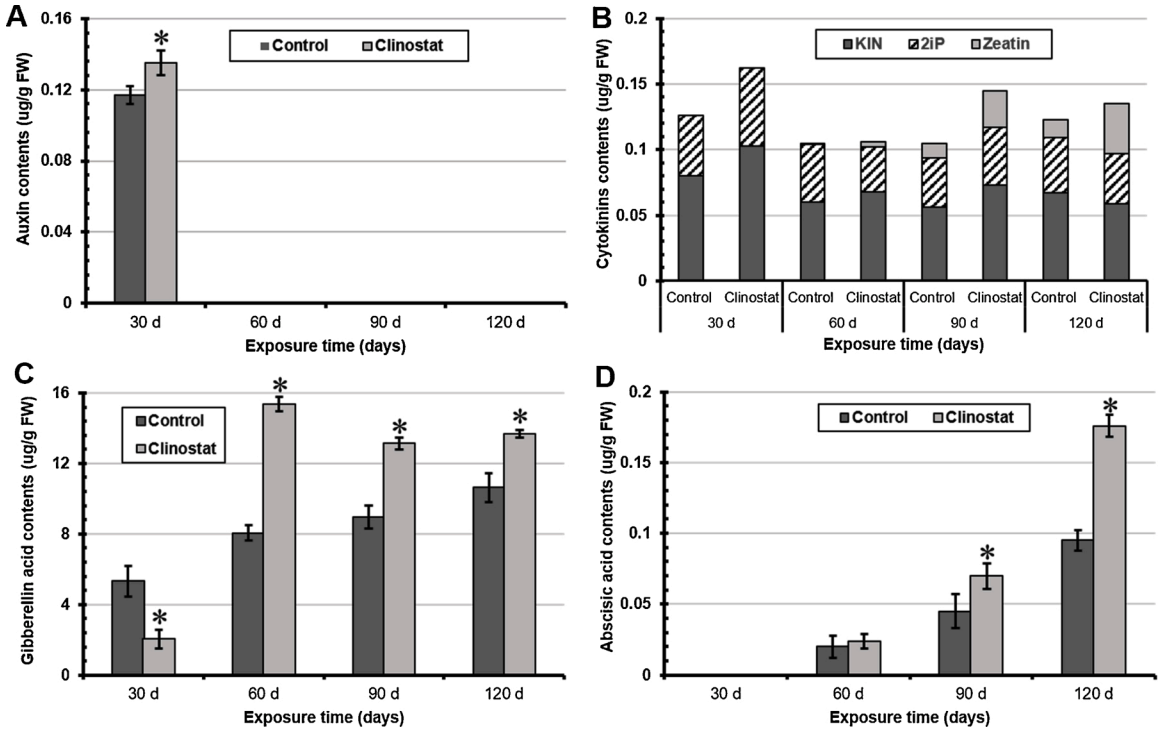

Fig. 5. Effect of MG on endogenous hormones concentrations during SE in *B. tuberous*. **A** oncentrations of IAA. **B** Concentrations of KIN, 2ip, Zeatin. **C** Concentrations of GA_3. **D** Concentrations of abscisic acid. Data represent the mean ± standard error of six replicates. *Indicates that the values are significantly different according to Tukey's test ($p < 0.05$).

detected. This result demonstrated an early influence of MG on changes in AUX content, which could have led to significant changes in the subsequent SE period.

Quantitative results of CKs showed that KIN and 2iP were synthesized throughout the whole SE process, as well as their concentrations did not change significantly over time in both treatments. Zeatin was detected after 60, 90 and 120 days of culture at significantly higher concentrations in the MG treatment than in the control (Fig. 5B).

Except for the lowest total GA content at the SE induction stage (30d), the content of GA in the MG treatment was always significantly higher than the control. GA content increased linearly with culture time under gravity conditions; meanwhile, under clinostating, the GA content was highest after 60 days of culture (embryonic development stage) and decreased significantly after that (Fig. 5C).

In contrast to the appearance of AUX only in the early stages of SE (30d), ABA appeared at later stages. In particular, after 120 days of culture, the ABA content under clinorotation was 1.85-fold higher than that of the control (Fig. 5D). The results suggested that the absence of ABA in early stages favored SE induction, and that high ABA levels stimulated somatic embryo maturation.

3.5. Changes in endogenous hormones ratio during SE under MG

The endogenous hormone balance plays an important role in the control of SE and it is represented by the endogenous hormone ratio. As shown in Table 1, endogenous hormone ratios in different SE stages were evaluated, which included induction (30 days), differentiation and growth (60–90 days), maturation stages (120 days). The ratio of AUX / CKs, AUX / GA, CKs / GA, CKs / ABA gradually decreased with increasing culture time; in contrast, the ABA/GA ratio increased with the maturation of somatic embryos in both treatments. At the SE induction stage, the ratio of AUX / GA and CKs / GA under clinostating was significantly higher than that of the control; while the opposite was observed in the AUX / CKs ratio. During the differentiation phase, CKs / ABA decreased in both treatments. At the maturation stage, the CKs / ABA ratio of the MG treatment was significantly lower than that of the control (Table 1).

3.6. Energy metabolism during SE under MG

The differentiation and development of somatic embryos is associated with changes in carbohydrate synthesis and mobilization [3,27]. In this study, total starch levels under MG peaked at the 60th day of culture (Table 2) when globular somatic embryos were differentiating into heart and torpedo embryos. Thereafter, total starch levels under MG decreased after 90 and 120 days of culture which coincided with the maturation of somatic embryos. Interestingly, the total starch level under MG was consistently significantly higher than that of the control throughout the development and maturation of the somatic embryos (Table 2). The decrease in starch levels over time maybe because they are consumed to activate the cellular metabolism required for SE [3,27]. Indeed, reducing sugar levels under MG and control both increased rapidly and peaked at the 90th day of culture and decreased thereafter; however, the reducing sugar content under MG was significantly higher than that of the control during this period (153.61 mg/g DW, 124.43 mg/g DW respectively). The results indicated that the MG condition promoted energy metabolism during SE in *B. tuberous*; therefore, it was possible to have a role in SE regulation under MG.

3.7. Changes in stomata morphology under MG

Stomata have an important role in gas exchange between the inside of the plant body and the environment; thus, it determines the yield of crops and the efficiency of gas exchange to recycle oxygen in space. As shown in Fig. 6, MG caused abnormal stomatal morphology. Accordingly, morphological observations showed that the stomata in the control treatment had a characteristic oval shape (Fig. 6A); whereas the stomata under clinorotation were elliptical (elongated), and a larger stomata opening was observed (Fig. 6B). Under clinostating, the stomata length was 4-fold higher than that of the control; meanwhile, the width of the stomata did not change under MG (Fig. 6C). This evidence revealed that long-term exposure to MG caused cell-level changes of concern.

4. Discussion

In the present case, MG has been identified as the cause of early SE induction in *B. tuberous*. The available studies have confirmed that MG induces significantly accelerated plant cell division [9,31]. These results suggest that MG stimulation is beneficial for SE initiation. It is well known that carbohydrate as metabolic energy is closely related to the induction, differentiation, and development of somatic embryos [28,29]. Further study showed that a higher starch and sugar content was accumulated in the p-tTCL explants under clinostating compared with the control, and it was highly correlated with early SE induction. As reported by [32], starch accumulation is the first metabolic response of explants; in which the explants convert sucrose contained in the culture medium to starch for storage in tissues, where meristems are subsequently formed. In addition, the results of endogenous hormone analysis under MG indicated that the high content of AUX and low ABA and the high ratio of AUX / GA and CKs / GA could be the cause of shorter SE induction time than the control. From the results, it is likely that MG accelerated the initiation of SE via altering the homeostasis of endogenous hormone and carbohydrate content in p-tTCL explants.

In this study, regardless of MG conditions, SE still takes place with complete and sequential through 4 main stages: globular; heart; torpedo and mature. Besides, somatic embryos regenerated under MG without any defects were observed. Similar results have been reported in orchardgrass [9] and carrot [18] which were both regenerated from single cells under real MG conditions. It can be seen that somatic cells can fully express their totipotency to regenerate somatic embryos under MG. On the other hand, embryogenesis via sexual reproduction proved to be a failure under MG; such as on wheat [6], arabidopsis [11] and *Brassica rapa* [12,13]. These results reveal that SE is subject to a limited

Table 1
Effect of MG on the ratio of endogenous hormones after 30, 60, 90 and 120 days of culture.

Exposure time	30 d		60 d		90 d		120 d	
Hormone ratios	Control	Clinostat	Control	Clinostat	Control	Clinostat	Control	Clinostat
AUX / ABA	–	–	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
AUX / CKs	0.93 ± 0.04a	0.83 ± 0.02b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
AUX / GA	0.02 ± 0.00b	0.07 ± 0.01a	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CKs / ABA	–	–	5.25 ± 0.44a	4.42 ± 0.43b	2.33 ± 0.38c	2.07 ± 0.59c	1.29 ± 0.18d	0.77 ± 0.12e
CKs / GA	0.02 ± 0.00b	0.08 ± 0.01a	0.01 ± 0.00c	0.01 ± 0.00c	0.01 ± 0.00c	0.01 ± 0.00c	0.01 ± 0.00c	0.01 ± 0.00c
ABA / GA	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.00 ± 0.00b	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a

*Different letters (a, b, …) in the same row indicate a significant difference ($P \leq 0.05$) between treatments according to Tukey's test. Data represent the mean ± standard error of six replicates.

Table 2
Effect of MG on starch and reducing sugar content after 30, 60, 90 and 120 days of culture.

Exposure time	30 d		60 d		90 d		120 d	
Hormone ratios	Control	Clinostat	Control	Clinostat	Control	Clinostat	Control	Clinostat
Starch (% w/w DW)	29.37 ± 0.23 g	34.06 ± 0.75e	38.28 ± 0.15b	39.91 ± 0.45a	38.13 ± 0.96b	35.06 ± 0.66d	30.02 ± 0.55f	35.91 ± 0.16c
Sugar (mg/g DW)	103.01 ± 0.15d	88.74 ± 0.58e	84.67 ± 0.37f	68.19 ± 0.36 g	124.43 ± 1.12c	153.61 ± 0.52a	105.67 ± 0.54d	150.00 ± 0.38b

*Different letters (a, b, …) in the same row indicate a significant difference ($P \leq 0.05$) between treatments according to Tukey's test. Data represent the mean ± standard error of six replicates.

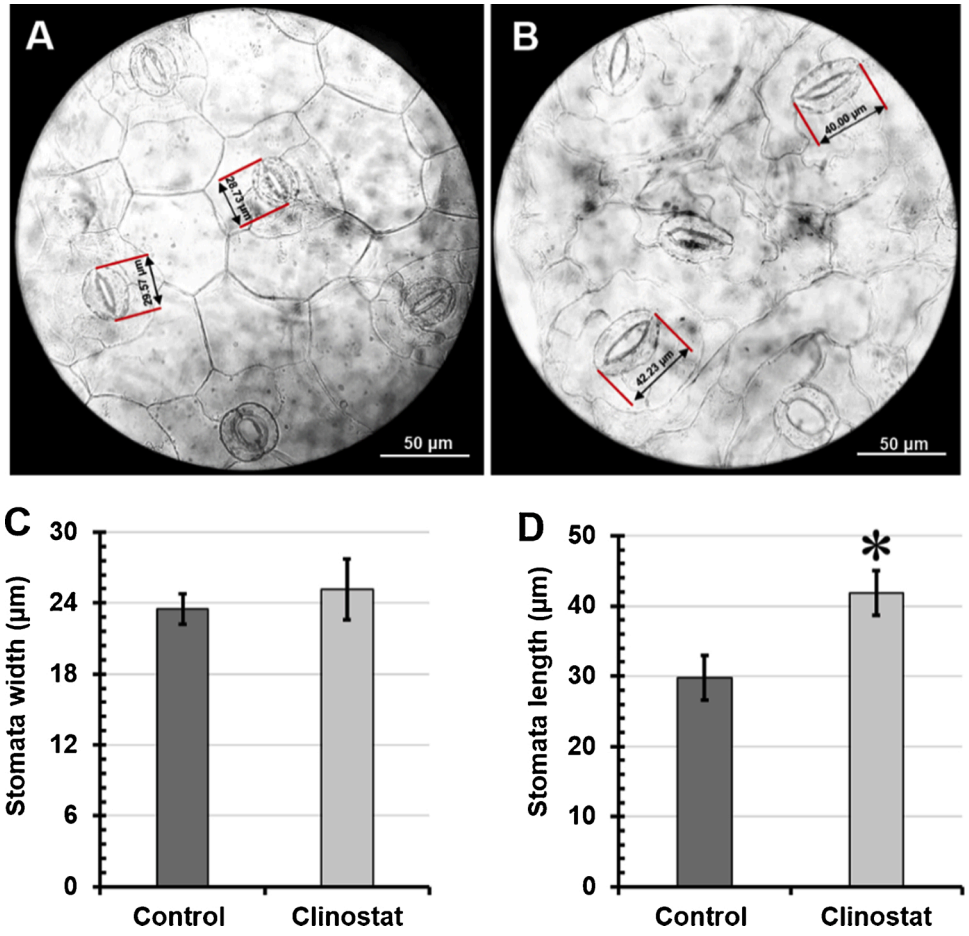

Fig. 6. The changes in stomatal morphology of *B. tuberous* under different gravity conditions. **A** Control. **B** Clinorotation. **C** Length and width of the stomata. Images were recorded at X40 magnification. Data represent the mean ± standard error of six replicates. *Indicates that the values are significantly different according to Tukey's test ($p < 0.05$).

effect of MG conditions, while zygote embryogenesis should be carefully considered for the purpose of seed generation in space environments.

Results on the effect of MG conditions on SE have been reported in several previous studies [9,18,30]; however, there were differences between these reports. In the case of carrot [18] and arabidopsis [30] the number of somatic embryos regenerated under MG remained unchanged. In contrast, a negative effect of real MG conditions on SE was reported in orchardgrass [9], accordingly, SE in orchardgrass was reduced by 70 % after 21 h of space flight. However, centrifugation treatment of 1*g* (*in situ* control) was not available in this flight for related experiments; therefore, the authors suggested more rigorous studies before reaching a conclusion whether MG conditions or other factors of spaceflight had an effect on SE [9]. In the present case of *B. tuberous*, the number of somatic embryos regenerated from p-tTCL explants was significantly elevated under clinorotating. This may be the result of changes in the content of endogenous hormones and energy substances under MG. Experimental results showed that high levels of GA, CKs and low levels of ABA are highly correlated with a high number of somatic embryos after 90 days of culture under MG. It is well documented that high starch accumulation in explants plays a role in promoting plant regeneration in *in vitro* culture [27,33,34] because starch is an energy source that supports growth and development in metabolically active tissues [34], as well as carbohydrates, are a major component of the carbon skeleton that makes up cells [29,35]. In the present study, the conversion of a large amount of starch to sugar after 90 days of culture may have promoted SE under clinostating. An increase in starch accumulation and metabolism under MG was detected in *Brassica rapa* L. [13], sweet potato [36], *Matricaria chamomilla* [31], mung bean [37]. In a recent study, clinorotation was reported to induce water uptake in mung bean seedlings, thereby enhancing amylase activity as well as seedling growth [38]. In addition, plants have been shown to produce more ethylene under MG stress [39,40]; while SE is stimulated by the presence of ethylene at appropriate levels and inhibited at higher concentrations [41]. Hence further studies will be required to verify

whether SE in plants is related to ethylene hormone under MG stress. All of this demonstrates that long-term exposure to MG significantly affects SE potential through a pathway of biochemical alterations in the cell. However, the SE potential of plants is not the same under MG. Therefore, to support life in space, consideration and selection of suitable plant species are of paramount importance.

Based on previous studies, plants respond to gravitational (microgravity) stress through an increase in biomass [31,42–46]. This increase may be due to the effect of MG on the mechanical properties, cellulose and lignin content of the cell wall - the framework that shapes and strictly regulates cell proliferation [47]. Recently, the maintenance of plant cell wall loosening under MG has been confirmed [46,48]. Besides, under MG, plants tend to save energy to serve growth [46] or improve the activity of antioxidant enzymes that also promote the increase of biomass [31]. Furthermore, MG has been shown to accelerate cell division even though they have not reached their critical size [49]. In the present case, the increase in biomass of *B. tuberous* somatic embryos under clinostating were highly correlated with high levels of GA, CKs and sugars. The starch accumulation before the peak biomass period (90 days) was determined to be higher in the MG treatment than in the control; therefore it was possible that this starch was converted to sugar and released the energy needed for proliferation after 90 days of culture. In addition, a high CKs / ABA ratio that may also benefit the proliferative phase has been confirmed. Although further studies are needed to elucidate this increase at the molecular level, it is clear that the increase in biomass under MG has the potential to generate sufficient food to serve the crew in long term flight. Furthermore, the physiology of biomass increase by MG stimulation can be applied to plant biomass production.

Analysis of growth indexes, rate of mature embryos and endogenous hormone content showed that somatic embryos cultured under MG after 120 days had begun to enter the aging stage. Total chlorophyll content in leaves of mature embryos was clearly increased under MG conditions. However, the content decreased as the number of days of clinorotation increased; similar results have been reported in carnation [50] and rice [43]. The decline of chlorophyll content in leaves is a characteristic manifestation of senescence in plants [27]. Besides, the fresh and dry weight of somatic embryos under MG did not increase significantly during this time period; therefore, it can be seen that the embryos have stopped growing to move to the aging stage. This result is highly correlated with a strong increase in ABA content as well as a low CKs / ABA ratio in somatic embryos after 120 days of culture under MG. On the other hand, a significantly higher percentage of mature embryos under clinostating compared with 1 g control was observed. Similar results have been reported in different plant species with different interpretations. According to [40], MG promotes catabolic processes in cells that lead to accelerated cell differentiation and consequent senescence of plants under this stress. Furthermore, MG enhances ethylene production causing changes in membrane permeability and results in the release of abnormally high levels of hydrolytic enzymes [39]. Another possible explanation is that MG stimulates respiration and succinate dehydrogenase activity and consequent overload on mitochondria [51]. Our experiments show that long-term exposure to MG increases the biosynthetic ABA content, thereby causing physiological aging in plants.

Stomata are the portals that control the gas exchange of plants, while ABA is a phytohormone that directly affects the growth, opening/closing of stomata and the expansion of pavement cells (the origin of stomata). In this case, long-term exposure to MG stimulated ABA synthesis and this may be related to the abnormal elongation of *B. tuberous* stomata guard cells. It is well documented that the high organization of microtubules and actin filaments is involved in stomatal morphogenesis [52–54]; ie., they controlled the state and morphology of the stomata [55]. As described by [54], cortical microtubules play an important role in coordinating the orientation of depositing cellulose microfibrils and this directly determines the thickness and mechanical properties of the stomatal cell walls, which in turn control their shape. However, disturbances in the organization of the cytoskeleton under reduced gravity have been reported [56,57]; and as a result, the cells showed an abnormal increase in size [56]. Consistently, our preliminary results also indicated that the size of the *B. tuberous* stomatal cells was abnormally increased under clinorotation. In addition, it is well known that calcium ions (Ca^{2+}) alter stomatal opening [58] by regulating the activity of many actin-binding proteins [59]. However, the available evidence has shown that Ca^{2+} concentration is significantly altered under MG conditions [60] and it may also affect stomatal morphogenesis. As reported by [61], MG caused an abnormal accumulation of IAA in leaves and this caused the *Chlorophytum comosum* stomata to remain open; thereby, improving the efficiency of benzene removal in the closed chamber. Considering all the above information, it can be seen that the morphogenesis of the stomata under MG was altered possibly related to ABA and Ca^{2+} signaling or reorientation of the cytoskeleton. Furthermore, the results raised significant concerns about the effects of MG on the gas exchange ports of plants. Therefore, more extensive research is needed on the change of stomata under the MG because it is important in cleaning the air in space [61], and stomata determine plant growth by controlling the amount of air entering and leaving the plant.

5. Conclusions

Microgravity conditions did not affect the morphogenesis program but it did favor SE induction and embryo regeneration potential. The increase in the number of embryos demonstrated that MG simulation was not a limiting factor for SE. An increase in biomass and chlorophyll content was observed under clinorotating as a normal plant response to gravitational stress. The higher accumulated starch content with a greater increase in sugar conversion in somatic embryos treated with clinostat suggests that energy metabolism is profoundly influenced by gravity. The change in AUX, CKs, GA and ABA content under clinorotation changed the homeostasis in the cell and as a result changed the growth and development of somatic embryos. ABA biosynthesis clearly increased with long-term exposure to MG-induced senescence of somatic embryos. MG caused abnormalities in the stomata morphology of *B. tuberous* and may also affect their function which warrants further investigation. The results led to the conclusion that the induction, differentiation and senescence of *B. tuberous* somatic embryos were promoted by MG in which endogenous hormone balance and energy metabolism played a central role.

Author contributions

DTN and HDK designed research. HDK, LTB, HTT, NTNM, VQL, DMC performed MG experiments. NQV, NDN, DMD performed biochemical experiments. HDK, NQV, LTB, HTT analyzed the data. DTN and HDK wrote the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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