The Effect of Simulated Microgravity On The Antioxidant Capacity And Alkaloid Formation In The *Hyoscyamus Niger*

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Abstract

Microgravity is one of the most important abiotic stresses in space. In the case of plant exposure to short term microgravity, plants establish strategies to response to these stresses and promote growth and survival. We hypothesized that the simulated microgravity can promote the antioxidant capacity and the formation of secondary metabolites such as tropane alkaloids in the *Hyoscyamus niger*. Callus induction was conducted by putting hypocotyl segments of *H. niger* seedlings in solid MS medium supplemented with 1 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP growth regulators. Then, the sub-cultured calli were placed on a clinostat for 3, 7 and 10 days. We performed Atropine and Scopolamine determination through HPLC. PAL (Phenyle alanine amonalyase) and antioxidant activity were also determined. Gene expression analysis of jasmonic acid (JA), Hyoscyamine 6-beta Hydroxylase (H6H), Putrescine N-methyltransferase (PMT), mitogen-activated protein kinase (MAPK) and ethylene responsive element binding (EREB) was performed using quantitative real time PCR. Findings showed that microgravity had a positive effect on the antioxidant capacity, Atropine and Scopolamine production in the *H. niger* calli. However, microgravity had a negative effect on the PAL activity. Furthermore, gene expression analysis indicated that microgravity significantly induced gene expression of the H6H, PMT and JA. It was also revealed that callus growth, carbohydrate and protein content increased in response to microgravity treatment. We conclude that microgravity can be considered as a potent factor to induce plant antioxidant activity and tropane alkaloids formation to be applicable in the pharmaceutical and medicinal industries.

Introduction

The genus *Hyoscyamus niger* belongs to the Solanaceae family and has various pharmaceutical properties. *H. niger*, which is a highly used medicinal plant for its anti-inflammatory, painless, and antipyretic effects (Begum et al. 2010; Sengupta et al. 2011) contains tropane alkaloids, phenols, flavonoids and some other non-alkaloid compounds (Bahmanzadegan et al. 2009). It is generally known for the synthesis of anticholinergic tropane alkaloids such as Atropine, Hyoscyamine and Scopolamine (hyoscyne) (Duke 1990; Lee 2006). These compounds are medicinally used as analgesic, sedative and antispasmodic agents (Alizadeh et al. 2014).

Generally, plants are considered as major living chemical factories, producing a broad range of secondary metabolites (SMs), which serve as a basis for commercial pharmaceutical drugs. SMs are low-molecular weight compounds and derivatives of primary metabolites produced in plants as the result of several physiologic variations (Kliebenstein 2013; Zandalinas et al. 2018). Under different environmental stresses, the SMs exert important long-term effects on the plant growth and survival rate (Agostini-Costa et al. 2012; Kurepin et al. 2017). SMs comprises nitrogen-containing compounds (cyanogenic glycosides, alkaloids and glucosinolates), phenolic compounds (flavonoids and phenylpropanoids) and terpenes (isoprenoids), which are being increasingly considered for drug development (Fang et al. 2011). One of the most widely used methods for the induction of SM production in plants is the application of biotic and abiotic stresses (Akula and Ravishankar 2011; Jan et al. 2021).

Microgravity is one of the abiotic environmental stresses that has been proved to be capable of changing plant growth and development (Kordyum 2014). The science of plant microgravity is one of the most interesting scientific fields that has been introduced since the first satellite was launched in 1957. Clinostats are the analog devices which induce partial or reduce gravity in a similar way as the Moon and Mars conditions (Kiss 2014). This device rotates the specimens around one or more axes, and the created microgravity may be altered with changes in rotational speed (rpm) and sample placement radius (Hassanpour and Ghanbarzadeh 2021; Kiss et al. 2019).

Plant cell gravisensitivity is based on very sensitive mechanisms that rearrange both metabolism and structure of plant cells (Kordyum 2014). These mechanisms include hypersensitive reactions, lipid oxidation and production of reactive oxygen species (ROS), which are involved in secondary metabolism and detoxification (Dumanović et al. 2020; Edwards and Dixon 1991; Garcia-Brugger et al. 2006). However, to our knowledge, there is no clear information about the effect of simulated microgravity (clinorotation) on the antioxidant capacity and production of secondary metabolites, especially alkaloids, in the medicinal plants. Therefore, this study aimed to understand the effect of clinorotation on the alkaloid formation and antioxidant capacity of *Hyoscyamus niger*.

Materials And Methods

Callus culture and microgravity application

H. niger seeds were collected from Maragheh, *Azerbaijan Province* of Iran. Seeds were sterilized in a 10 % (v/v) NaOCl solution for 10 min and then, washed three times with sterile distilled water. Then, sterilized seeds were placed in 70% alcohol for 1 min and 10% sulfuric acid for 7 min, followed by three times of washing with distilled water. Next, the disinfected seeds were placed in half strength ($\frac{1}{2}$) Murashige and Skoog (MS) basal medium (Murashige 1962), supplemented with 7% agar and 3% sucrose under dark condition, at 25 ± 2 and pH 5.7. For callus induction, hypocotyl segments (0.4-0.5 cm) of 10 days old seedlings were transferred into solid MS medium, supplemented with 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg L^{-1} benzyl amino purine (BAP) hormones and incubated in the darkness for three weeks. After that, the calli were sub-cultured using the same MS solid medium. For simulated microgravity exposure, calli were placed at the center of Petri dish with a 2 cm rotational radius and located on a developed two-dimensional clinostat turning at the rate of 2 rpm, for 0 (control), 3, 7 and 10 days. The centrifugal force was evaluated from zero on the center to $2.24 \times 10^{-5} \text{ g}$ on the edge of the callus ring. Finally, 3-5 calli per treatment were used for the evaluation of *H. niger* cell growth and biochemical analysis.

Hydrogen peroxidase (H_2O_2) measurement

To evaluate H_2O_2 content of the *H. niger*, we homogenized microgravity-treated samples in 1 ml solution containing 0.25 Trichloroacetic acid (TCA) (0.1% (w:v)), 0.5 ml KI (1M) and 0.25 ml potassium phosphate buffer (10 mM), at 4 for 10 min. Simultaneously, control samples were prepared with H_2O instead of KI

for tissue coloration background. Homogenized samples were centrifuged at 12000 ×g, 4 for 15 min and then, 200 µl supernatant of each sample was placed in a UV-microplate well, followed by a 20 min incubation at room temperature. Finally, the absorbance of all samples was read at 350 nm, using Shimadzu UV-1800 spectrophotometer. The standard curve was also obtained with H₂O₂ standard solutions, prepared in 0.1% TCA (Velikova et al. 2000)

Lipid peroxidation

For the estimation of lipid peroxidation, we measured the malondialdehyde (MDA) content of *H. niger* calli, based on the Heath and Packer modified protocol (Heath and Packer 1968). To do this, 0.3 g fresh callus samples were homogenized in 25% Thiobarbituric acid (TBA) reagent and 10 % TCA. The reaction mixture was boiled in a water bath, at 95°C for 20 min. After incubation, the mixture was quickly cooled on ice and centrifuged at 10000 ×g, 4 (Sigma Centrifuge 1-16K, German). Measurement of the supernatant absorbance was performed at 532 and 600 nm, using a UV–visible spectrophotometer (UV-160, Shimadzu, Tokyo, Japan). MDA content was determined by the extinction coefficient of 155 mM⁻¹ cm⁻¹.

Total protein content and enzymatic activity

Following the extraction of 0.2 g fresh *H. niger* calli with the use of 0.1 M sodium phosphate extraction buffer, at 4 and pH 6.8 (EMSURE®, MerckKGaA), samples were centrifuged at 10,000 rpm for 15 min, at 4 . The obtained supernatant was stored at -70 and used for the measurement of the protein and enzyme activity. Soluble protein content was evaluated through Bradford method (Bradford 1976). The enzymatic extract (100 µL) was added to the reaction mixture containing 25 mg Coomassie Brilliant Blue, 12.5 mL ethanol 96%, and 25 mL phosphoric acid 85%. After 10 min of incubation, the absorbance was measured at 595 nm (Shimadzu UV-1800 spectrophotometer).

Catalase activity

Catalase activity was evaluated according to the proposed method of Dhindsa, et al (Dhindsa et al. 1981). The enzymatic extract (100 µL) was added to the reaction mixture containing 0.2 mL H₂O₂ 3% and 2.8 mL phosphate buffer. Subsequently, the absorbance was measured at 240 nm through Shimadzu UV-1800 spectrophotometer.

Peroxidase activity

The evaluation of peroxidase activity was performed through the method of Abeles and Biles (Abeles and Biles 1991). The enzymatic extract (100 µL) was added to the reaction mixture containing 0.2 mL H₂O₂ 3%, 2 mL acetate buffer 0.2 M at the pH of 4.8 and 0.1 mL benzidine at the concentration of 20 mM. The absorbance was recorded at the wavelength of 530 nm (Shimadzu UV-1800 spectrophotometer).

Determination of Proline and sugar content

The analysis of proline content was conducted according to the acid ninhydrine procedure of Bates et al (Bates et al. 1973). The callus samples (0.2 g) were pulverized with 4 ml sulfosalicylic acid 3% and clarified through centrifugation. Then, 2 ml of the obtained supernatant was mixed with the same volume (2 ml) of acid ninhydrin and acetic acid, followed by incubation in the oven, at 100 for 1 h. The reaction was stopped by putting the mixture on the ice. In order to separate the reaction mixture, 4 ml toluene was added and vortexed for 15-20 s. The absorbance of the samples was measured at 520 nm, using Shimadzu UV-1800 spectrophotometer.

To evaluate the content of soluble sugars, calli samples (0.2 g) were dissolved in 10 mL (80% v/v) ethanol and centrifuged at 6000 ×g, for 10 min. Total soluble sugars were determined through preparing the mixture of 1 mL supernatant and 3 mL anthrone reagent [200 mg anthrone in 100 ml H₂SO₄ (70% v/v)] (EMSURE®, MerckKGaA), and the following incubation in the boiling water bath for 10 min. Then, the absorbance was recorded at the wavelength of 620 nm, using Shimadzu UV-1800 spectrophotometer. The total soluble sugar content was also determined through a calibration curve of Glucose, being expressed as gram of tissue weight equivalent.

Determination of Phenyl-alanine amonialyase activity

To measure the activity of PAL enzyme, we prepared a stock solution of 0.006 mM (5 ml) phenyl alanine and 0.5 M (pH 8; 120 ml) Tris-HCL buffer as the reaction mixture. Then, 0.4 ml enzymatic extraction was added to 6 ml reaction mixture. Control samples consisted of 6 ml reaction mixture and 0.4 ml water, instead of enzymatic extraction. After stirring and incubation at 37 for 1 h, 0.1 ml HCL was added to stop the reaction. The absorbance was recorded at the wavelength of 290 nm (Shimadzu UV-1800 spectrophotometer)(Beaudoin-Eagan and Thorpe 1985).

Determination of Atropine and Scopolamine by HPLC

Atropine and Scopolamine were extracted from the callus samples (0.2 g of which were dried samples) and then underwent an overnight incubation in the mixture of 28% ethanol and NH₄OH at the ratio of 9:1, respectively. Then, the extracted samples were incubated in the ultrasonic bath for 30 min and centrifuged at 1,500 rpm, for 3 min. The sediment was dissolved in 1.5 mL HCl 0.1 N and the prepared acidic aqueous solution was filtered to produce alkaline with diluted KOH (final pH 8-9). Next, 6 ml chloroform was added and after a vigorous shake, the tube was centrifuged at 1,500 rpm, for 2 min. The lower layer which contained alkaloids underwent a double precise pipetting with 6 mL chloroform and subsequently was evaporated at 40 until dryness. The dry sediment was dissolved in 99% methanol to determine the Atropine and Scopolamine content through High Performance Liquid Chromatography (HPLC, Cecil Company, England), using an ODS (C18) column (25 cm × 4.6 mm, partial size 5 µm). The mobile phase contained 450 mM Acetonic-K₂HPO₄ pH 3 by phosphoric acid; flow rate: 1.5 mL/min; UV K2501 detector at 280 nm, and was analyzed through an external standard (Kamada et al. 1986).

Gene expression analysis

H. niger samples (50 mg) were homogenized in liquid nitrogen and RNA was isolated using DENAzist Column RNA Isolation Kit (DENAzist Asia Co., Mashhad, Iran). In order to avoid any DNA contamination, 3 µg of total RNA was treated with DNase I kit (EN0521, fermentase) and then, the purity of the extracted RNA was checked through NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis. After reverse transcription of RNA into cDNA (SMOBIO cDNA synthesis kit, Taiwan), the relative expression of target genes (Table 1) was analyzed by Applied Biosystems Step One real-time PCR systems in triplicate reactions per samples, using SYBR Green Master Mix (Amplicon RealQ Plus 2x, High ROX™, Denmark) with an initial denaturation step (5 min at 95) and the subsequent 40 cycles of 15 s at 94°C, 25 s at 57 and 20 s at 72 . The EF1α was used as the internal reference gene. Relative expression of treatments were calibrated with control samples and calculated by $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak 2008).

Statistical analysis

All data were expressed as mean $\pm$ SD of at least three replications. Data analysis was done by one-way analysis of variance (ANOVA), with the Brown-Forsythe test and the use of GraphPad Prism 9.0 (San Diego, USA). P values lower than 0.05 were considered as statistically significant.

Results

The effect of simulated microgravity on the MDA content

To investigate the effect of microgravity on the MDA content of *H. niger*, we treated the callus with microgravity for 3, 7 and 10 days and compared with the control. As it is appeared from figure 1, compared to control samples, MDA content increased significantly after 3 days of treatment ($p < 0.05$). However, it was revealed that the MDA content decreased at day 7 and finally reached down to the control MDA level at day 10.

The effect of simulated microgravity on the H₂O₂ content

The hydrogen peroxidase content of *H. niger* plant was evaluated after microgravity treatment and through H₂O₂ colorimetric assay. Figure 1 shows the effect of microgravity on the H₂O₂ content of *H. niger* callus. According to this figure, microgravity significantly increased the concentration of H₂O₂ after 3 and 7 days. However, it was found that 10 days of microgravity treatment led to decreased H₂O₂ concentration.

Simulated microgravity impacts on the activity of antioxidant enzymes

After treatment with microgravity for 3, 7 and 10 days, we determined the catalase activity through Dhindsa method (Dhindsa et al. 1981). Our result which is displayed in figure 1, showed that microgravity treatment increased catalase activity during 3 and 7 and 10 days after treatment, in a time dependent manner ($p < 0.05$).

Figure 1 shows the effect of microgravity treatment on the peroxidase activity of *H. niger* plant. From this figure, it can be seen that the activity of peroxidase significantly increased following all time spans of microgravity treatment.

The effect of simulated microgravity on the phenyl alanine amonialyase activity and proline content

As figure 2 shows, *H. niger* callus treatment with microgravity indicated a significant decrease in the activity of PAL enzyme after 3, 7 and 10 days ($p < 0.05$). This result also showed that 10 days of microgravity treatment led to a remarkable reduction in the PAL activity, which was also statistically significant, compared to the control and other treatment periods ($p < 0.05$).

The analysis of proline content showed no statistically significant difference between proline content of the control sample and other microgravity-treated samples (figure 2).

The effect of simulated microgravity on the Atropine and Scopolamine content

The effect of microgravity on the production of Atropine and Scopolamine in the *H. niger* callus was investigated through HPLC. From figure 2, it is clear that Atropine production increased after three days of microgravity treatment ($p < 0.05$), while decreased after 7 and 10 days. Figure 2 shows that Scopolamine content was increased after all microgravity treatment time spans, with the most remarkable surge after three days of treatment ($p < 0.05$).

Simulated microgravity impacts on the *H. niger* protein content

In order to determine total protein content of the *H. niger*, we conducted Bradford assay after microgravity treatment. Table 2 highlights the effect of microgravity on the protein content of callus samples after 3, 7 and 10 days. This table indicates that microgravity treatment significantly increased protein content of samples after all time spans.

Simulated microgravity impact on the *H. niger* sugar content

The investigation of *H. niger* sugar content under the effect of microgravity, which has been illustrated in the Table 2, showed that microgravity treatment was capable of increasing sugar content of callus samples after 3, 7 and 10 days, which was statistically significant compared to the control sample ($p = 0.001$).

The impact of simulated microgravity on the *H. niger* callus growth

The effect of clinorotation on fresh and dry weights of callus is summarized in Table 2. The table shows that clinorotation remarkably increased the weight of fresh and dry callus in comparison with control samples. Clinorotation resulted in a 23%, 29% and 63% increase in the callus fresh weight during 3, 7 and 10 days as compared to their controls, respectively. Furthermore, it was observed that clinorotation caused 26%, 36% and 89% increase in the dry weight of callus after 3, 7 and 10 days, respectively.

The effect of simulated microgravity on the gene expression of H6H, PMT, jasmonic acid, EREB and MAPK.

To examine the effect of microgravity on the H6H, PMT, jasmonic acid, MAPK and EREB gene expression, we treated callus with microgravity for 3, 7 and 10 days and then, analyzed gene expression through quantitative real-time PCR. Gene expression analysis of the H6H, which catalyzes the synthesis of Scopolamine from Hyosciamine, revealed that H6H gene expression remarkably increased after microgravity treatment (Figure 3, $p < 0.05$). Figure 3 shows PMT gene expression under the effect of microgravity. From this figure, it can be seen that microgravity significantly induced PMT gene expression after 7 and 10 days of treatment. According to figure 3, it was revealed that microgravity treatment induced jasmonic acid expression after 10 days of treatment ($p = 0.0001$). The impact of microgravity on the EREB gene expression is shown in figure 3. It is apparent from the figure that microgravity could significantly induce EREB gene expression after 3 and 7 days ($p < 0.001$), while had no significant effect after 10 days of treatment. MAPK gene expression under the effect of microgravity is represented in figure 3. According to this figure, MAPK gene expression was reduced after all microgravity treatment groups. There was a slight increase in the MAPK gene expression after 10 days of microgravity treatment, however, it was insignificant as compared with the control sample.

Discussion

Plants sense the gravity as a uniform, directional and environmental cue to control their growth reorientation and body structure for the purpose of promoting their survival through efficient mechanisms (Kiss 2000; Morita 2010). The current study was set out with the aim of assessing the impact of microgravity on the molecular and biochemical behavior of *Hyoscyamus niger*. One of the effects of abiotic stress is cell membrane lipid peroxidation, which leads to the impaired membrane integrity and is proportionate to the cellular MDA content (Candan and Tarhan 2012). Investigation on the MDA content of *H. niger* in this study showed that after three days of microgravity treatment, the MDA content increased significantly. However, we found that MDA content decreased after 7 and 10 days of microgravity treatment, which may be due to the increased antioxidant activity in response to membrane destruction. These findings are in agreement with an earlier study which reported a notably increased MDA content in the clinorotated plant chloroplasts (Baranenko 2001). Our findings also accord with previous observations which showed that simulated microgravity like other types of stresses such as drought or water logging can induce a remarkable accumulation of MDA in plants, serving as a hallmark of oxidative stress responses to altered gravity (Xu et al. 2014).

Peroxidase and catalase, key antioxidant enzymes associated with plant defense system, protect plants and lessen the ROS free radical levels for its survival (Nikraves et al. 2012). Findings of this study indicated that microgravity treatment was capable of increasing catalase and peroxidase activity after 3, 7 and 10 days of treatment. Along with this finding, enhanced antioxidant activity under the effect of clinorotation (2 rpm) has been reported previously (Al-Awaida et al. 2020; Nakajima et al. 2019). Our

finding corroborates earlier studies, which reported that microgravity leads to increased level of antioxidant enzymes activity (Chen et al. 2015; Lu et al. 2009).

It is interesting to note that compared to control sample, we observed reduced levels of H_2O_2 content in the microgravity-treated samples. This can be due to increased peroxidase and catalase activity, which uses H_2O_2 as its substrate and converts it to water and oxygen.

In this study, microgravity was found to increase callus growth of *H. niger* during all treatment time spans. This finding is in consistence with findings of Hassanpour and Ghanbarzadeh, which showed a significant increase in callus growth of *Matricaria chamomilla* L., after 7 days of clinorotation (Hassanpour and Ghanbarzadeh 2021).

In this study, microgravity was found to increase protein content of *H. niger* during all treatment time spans. The proteomic profiles of *A. thaliana* calli under the effect of microgravity and the on-board centrifuge (1 g of the control) in an orbital flight were compared in a previous study and the findings showed that significant differences were identified among proteins, of which 24 proteins increased and 21 ones decreased; these proteins have a major role in several cellular processes (Zhang et al. 2015).

The most important finding of this study was decreased level of PAL enzyme under the effect of microgravity treatment. PAL enzyme catalyzes the biotransformation of L-phenylalanine to trans-cinnamic acid and ammonia (MacDonald and D'Cunha 2007). Hence, this enzyme leads to decreased L-phenylalanine concentration in plant, which is one of the most important factors for alkaloid production (Bedewitz et al. 2014). From these information, it can be interpreted that microgravity-mediated PAL reduction in this study is in favor of producing more alkaloid components in the *H. niger*. This finding corroborates the idea of Raghad et al, who suggested that treatment of the pea, rice, corn and wheat seeds with simulated microgravity has positive effects on the amino acid profile (tryptophan, phenylalanine, etc) (Mouhamad et al. 2016). Therefore, microgravity is probably capable of inducing increased phenylalanine content and the subsequent alkaloid formation.

Another important finding of this study was that microgravity had no significant effect on the proline content of *H. niger*. This can be due to the fact that proline is produced from ornithine and glutamate (Sekhar et al. 2007), which are of the most essential factors in the pathway of tropane alkaloid production (Kohnen-Johannsen and Kayser 2019). From these findings, we can infer that in the microgravity-treated samples, glutamate is rather involved in alkaloid formation than proline production. It is also interesting to note that all microgravity treatments in this study, significantly increased carbohydrate content in the *H. niger* callus samples. It has been previously reported that carbohydrate not only acts as the carbon and energy source, but also as an osmotic agent (Verma and Dougall 1977). This osmotic effect induces putrescine production and the ensuing tropane alkaloid formation (Flores and Galston 1982).

The results of this study indicated that after microgravity treatment, the expression of H6H and PMT increases significantly. In agreement with our results, previous studies have showed that increased

expression of H6H and PMT leads to increased production of tropane alkaloids such as Hyoscyamine and Scopolamine (Wang et al. 2011; Zhang et al. 2007). One unanticipated finding was that microgravity treatment was capable of inducing jasmonic acid biosynthesis in *H. niger* samples. It is encouraging to compare this finding with that found by Kang et al. who found that methyl jasmonate treatment in *Scopolia Parviflora* induces H6H and PMT gene expression and consequently leads to the increased tropane alkaloid formation (Kang et al. 2004). More precisely, jasmonic acid and methyl jasmonate has been considered as signal transducers that lead to the expression of particular enzymes that catalyze biochemical reactions to produce low molecular weight compounds such as polypeptides, polyphenols, quinones, alkaloids and terpenoids (Court et al. 1996; Mizukami et al. 1993). During this study, we observed that microgravity reduced MAPK gene expression, while induced mRNA expression of EREB. We have no evidence from previous studies to discuss about this part of our research. Therefore, there is a crucial need for further investigations in the future, regarding the exact signaling pathway which leads to alkaloid formation under the effect of altered gravity.

Conclusion

The main goal of the current study was to determine the effect of microgravity on the antioxidant enzymes and alkaloid production in the *H. niger*. This study has shown that microgravity is capable of inducing antioxidant enzymes and increasing tropane alkaloid formation in the *H. niger* through various mechanisms and pathways. Taken together, the results of the present study suggest a promising role for the microgravity in the way of manipulating plants for pharmaceutical and medicinal purposes. Thus, this study can pave the way for the future investigations in order to achieve the most useful plant-driven tropane alkaloids and other influential secondary metabolites.

Abbreviations

PAL-Phenyle alanine amonalyase, JA- Jasmonic acid, H6H- Hyoscyamine 6-beta Hydroxylase, PMT- Putrescine N-methyltransferase, MAPK- Mitogen-Activated Protein Kinase, EREB- ethylene responsive element binding,

Declarations

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Conflict of interest

All authors declare that they have no financial or personal competing interests.

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Tables

Table 1. Specific primer sequences for H6H, PMT, JA, EREB, MAPK and EF-1α

GENE name	Forward Primer	Reverse Primer
H6H	ATCTGTGAAGGACTTGGGCTT	GTGTCCTCCTGATCCCAATGT
PMT	CTCTTAGGCCAGGAGGAGTTG	ACCCCTGTTGGATAGGTTGGA
JA	GCCATTGACAGCCCATTATCC	GATGAGGTACCACAACCCCAT
EREB	AATGAGGGCAGGGATGGTTG	GACCCCACTGGCTCCTAAAC
MAPK	CTGTTCTCTCCCTCTTCCAC	GCCACTACCGATGCGATTAAC
EF-1α	TTCACATCAGCATCGTGGTCA	TCAGCAGCTTCCTTCTCGAAC

Table 2. Effect of simulated microgravity on callus weight, catbohydrate and total protein content of *H. niger* callus samples.

Treatment	Fresh weight callus (g)	Dry weight Callus (g)	Catbohydrate (g/DW)	Total protein (g/ml FW)
Control	1.28	0.083	3042	928
Mic- 3 days	1.59 ^{ns}	0.103 ^{**}	11813 ^{**}	1005.5 [*]
Mic- 7 days	1.66 [*]	0.112 ^{****}	12426 ^{**}	1116.5 ^{***}
Mic- 10 days	2.089 ^{****}	0.155 ^{****}	1137 ^{**}	1365 ^{****}

Figures

Figure 1

The MDA, H2O2 Peroxidase and Catalase content under the effect of simulated microgravity in *H. niger* callus samples. Data are represented as mean ± SD of at least three replicates (p value < 0.05).

Figure 2

The effect of simulated microgravity on the PAL, Proline, Atropine and Scopolamine content of *H. niger* callus samples. Data are represented as mean $\pm$ SD of at least three replicates (p value < 0.05).

Figure 3

The effect of simulated microgravity on the H6H, PMT, jasmonic acid, MAPK and EREB (p value = 0.0001), gene expression in the *H. niger* callus samples. Data are represented as mean $\pm$ SD of at least three replicates (p value = 0.0001).