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ORIGINAL ARTICLE

Simulated microgravity improved secondary metabolism in *Physalis alkekengi* via influencing gene expression and DNA methylation in a light quality-dependent manner

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Abstract

This study aimed to investigate the effect of microgravity and light spectral composition on DNA methylation levels and the expression of genes responsible for secondary metabolism in *Physalis alkekengi*. Seedlings were exposed to the microgravity treatments under two different light conditions, including white and red + blue (R + B). The microgravity treatments were more capable of rapidly influencing growth performance than the light spectrum quality. The microgravity treatment up-regulated the expression of the 7-sterol Δ7 reductase (DWF5) gene. The highest expression of 4–1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) gene was recorded in the microgravity-treated seedlings grown under the white conditions. Microgravity treatment and R + B irradiation synergistically stimulated the 3–1-deoxy-D-xylulose-5-phosphate synthase 2 (DXS2) gene. The expression pattern of the 1-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) and 2-sterol C-7,8 isomerase (HYD1) genes was similar to that of the DXS2 gene. The transcription of the mevalonate kinase (MK) gene was slightly changed in response to the microgravity and light composition. In the microgravity-treated seedlings, the 5-squalene synthase (SQS) gene displayed a similar upward trend, whereas the R + B radiation conditions contributed to the slight down-regulation of this gene. The microgravity factor was more effective in generating epigenetic responses in terms of DNA methylation than the light spectrum quality. However, light conditions affected the molecular responses of the plant to the microgravity factor. The microgravity treatment under both radiation conditions also increased the concentration of soluble phenols and proline in leaves. These findings refer to the high efficacy of simulated microgravity conditions to up-regulate the secondary metabolism in medicinal plants.

Keywords Clinorotation · DNA methylation · Light quality · Physalis · Secondary metabolites

Abbreviations

R + B	Red + blue light
R + B + Microgravity	Microgravity treatment and R + B irradiation
DWF5	7-Sterol Δ7 reductase
DXR	4–1-Deoxy-D- xylulose-5-phosphate reductoisomerase
DXS2	3–1-Deoxy- D-xylulose-5-phosphate synthase 2
HMGR	1-Hydroxy-3-methylglutaryl coenzyme A reductase
HYD1	2-Sterol C-7,8 isomerase
MK	Mevalonate kinase
SQS	5-Squalene synthase
IPP	Isoepentenyl pyrophosphate
DMAPP	Dimethylallyl pyrophosphate
MVA	Mevalonate
MEP	Methylerythritol phosphate
MSAP	Methylation-sensitive amplification polymorphism
NML	Non-methylated loci
MSL	Methylation-susceptible loci

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Introduction

The man-made technology of simulated microgravity has been employed to modify growth performance, morphogenesis, and metabolism in crops. The simulated microgravity was associated with changes in both primary and secondary metabolism in diverse plant species such as wheat (Al-Awaida et al. 2020), *Brassica napus* (Frolov et al. 2017), and *Arabidopsis* (Hausmann et al. 2014). Moreover, current knowledge supports the hypothesis that the microgravity conditions may be potentially associated with significant variations in the transcription of a plethora class of genes involved in critical processes such as photosynthesis, ribosome biosynthesis, respiration (Vandenbrink et al. 2019), water uptake (Nakajima et al. 2021), and phytochemical properties (Nakajima et al. 2019).

The fundamental roles of light quality and intensity in regulating various aspects of plant growth, metabolism, organogenesis, morphogenesis, and immunity have been well established (Esmaelpour et al. 2022). On the other hand, the photo-inhibition phenomenon results from excess light intensity or inappropriate short wavelengths, thereby impairing metabolism and reducing crop productivity. In addition to the well-proven role of light as an energy source in carbon assimilation (photosynthesis), light perception and signaling are associated with fundamental changes in morphology/organogenesis (photomorphogenesis). It has been affirmed that light acts as a morphogenic signaling factor that contributes to regulating the function of stem cells in the meristem zone (Yoshida et al. 2011; Esmaelpour et al. 2022). For instance, light signaling participates in leaf initiation and development by influencing hormonal pools of auxin and cytokinin in the stem apical meristem (SAM) (Yoshida et al. 2011). Light-emitting diodes (LEDs) can be utilized to precisely manipulate the light spectral composition, thereby optimizing plant growth performance and production of primary/secondary metabolites.

Plant species of the *Physalis* genus, including *P. alkekengi* contain valuable resources for medicinally important bioactive secondary metabolites such as steroids including physalins (Mirzaee et al. 2019). It has been validated that physalins exhibit significant antiproliferative and apoptotic activities against human cancer cells by arresting the G2/M cell cycle and altering mitochondrial function (Cao et al. 2019). That is why optimizing the cultivating conditions for stimulating secondary metabolism in this medicinal plant is of great importance. For instance, Fukushima et al (2016). provided RNA-Seq and metabolite profiling in *P. alkekengi* and *P. peruviana* (Fukushima et al. 2016) Moreover, the utilization of methyl jasmonate as an elicitor agent increased the accumulation of secondary metabolites, including physalins D and H in *Physalis angulata* hairy roots (Fukushima et al. 2016).

According to the earlier literature, light quality (Esmaelpour et al. 2022) and microgravity (Nakajima et al. 2019; Kamal et al. 2019) can act as efficient factors affecting secondary metabolism in plants. Therefore, this study hypothesizes that light quality and microgravity have good efficiency in triggering molecular and biochemical changes in *P. alkekengi*. To test and validate this hypothesis, several marker genes that participate in the secondary metabolism of *P. alkekengi* were selected as candidate targets. These genes include HMGR, HYD1, DXS2, DXR, SQS, MK and DWF5. Active isoprene IPP or DMAPP is the building block of terpenoid compounds in plants. The biosynthesis of this five-carbon subunit is mediated through two routes called (i) MVA pathway in plastid organelle and (ii) MEP in the cytoplasm. The MK gene is considered as one of the candidate index genes in the MEP pathway to explore the effect of environmental cues on the production of secondary metabolites (Tabatabaee et al. 2021).

Exploring changes in DNA methylation profile offers a remarkable way to illustrate the plant epigenetic responses to different environmental cues (Rajaee Behbahani et al. 2020). The role of DNA methylation during the plant responses to the light and gravity signals is poorly understood. This study intends to assay the hypothesis that microgravity and light spectral composition can substantially affect the expression of genes, and DNA methylation levels.

Materials and methods

Seeds of *Physalis alkekengi* were purchased from Pakan Bazr company (Isfahan, Iran). The seeds were sterilized in 10% sodium hypochlorite for 20 min and then thoroughly washed with distilled water. Sterilized seeds were placed on Murashige and Skoog medium in the center of Petri dish in 2 cm radius, and transferred in culture roon at 25 ± 2 °C temperature. After 5 days, Petri dishes were placed on a two-dimensional clinostat (Paya Kesht Company, Iran) with rotational speed (ω) of 3 rpm and clockwise at 90° for 1 week according the method of Hassanpour and Ghanbarzadeh (2021). The acceleration force (g) was calculated by the equation of $g' = (2\pi/60)^2 r\omega^2$ from 0 at the center to 3.02×10^{-4} at the edge of the ring. The clinostat was placed under two LED lights, including white (7000 K, 100%) (as a control light) and red-blue (red, 660 nm, 75%, and blue, 440 nm, 25%) with a light intensity of 40–45 µMol m^{-2} s^{-1}, a 16/8 h (light/dark) photoperiod, 25 ± 2 °C temperature, and 55% relative humidity. Selection of LED light was conducted according to the method described by Hassanpour (2022). After clinoration application, seedlings were harvested for biochemical and

molecular analyses. At each experiment, three Petri dishes were placed on the clinostat, and three of them were put on the ground with $g = 1$ as control.

Real-time quantitative PCR (qRT-PCR)

An RNA isolation kit (Denazist, Iran) was employed to isolate total RNA. This step was followed by producing complementary DNA (cDNA). The list of forward and reverse primers of target genes, including HMGR (KX574810.1), HYD1 (KX574835.1), DXS2 (KX574818.1), DXR (GQ921844.1), SQS (KX574827.1), MK (KX574812.1) and DWF5 (AB839751.1) and reference gene (elongation factor) are described in Table 1. Real-time PCR was performed using the specific kit and specialized primers to monitor CT values for calculating gene expression in terms of fold change.

Table 1 The list of forward and reverse primers of target genes

Primer name	Sequence (5′–3′)	Tm	Amplicon (bp)
DWF5a-F	ACACCATGGATATTGCGC ATG	61	101
DWF5a-R	GACAAGGTACATGCCAGG AGA	61	
HMGR-F	CTTCCCTCTCAAGACGAC GAA	60	109
HMGR-R	GAATCGAAGCAGCTCGGT AAC	60	
HYD-F	AACAGCTATCTTGGAAGG CGA	60	147
HYD-R	TGGACCTTGATAGCAGCA CAT	60	
DXS-F	TGCTGAAGAATGTCTTTG CGG	60	112
DXS-R	ATTATGCAGAACGGCTTC AGC	60	
DXR-F	AGGAAAGGACATTGCTTT GGC	60	140
DXR-R	AGCCTTGTACGCACTGGA ATA	60	
SQS-F	GCGTGATCCTTCCATCTT TCG	60	129
SQS-R	TTAGCAGTCAGACCACGT CTC	60	
MK-F	ATTCATGGGAAGCCATCT GGT	61	100
MK-R	GCATGTTTGTTTTGAGGC GTG	61	
EFa-F	TTCACATCAGCATCGTGG TCA	61	127
EFa-R	TCAGCAGCTTCCTTCTCG AAC	60	

Investigating DNA methylation level: the MSAP method

The MSAP protocol was utilized to explore DNA methylation levels in the microgravity-treated seedlings grown under two irradiation conditions. In this procedure, the samples were subjected to the isoschizomers (*Msp*I and *Hpa*II). The genomic DNA was extracted according to the instruction of the kit (GeneAll, South Korea). The MSAP method includes several steps, including digestion with isoshimerases, adapter ligations, pre-amplification, selective amplification, fragment visualization, scoring, and data mining using the MSAP package. Table 2 present the sequences (5′–3′) of the primers/adapters applied in the current study.

The concentration of proline, H_2O_2, and soluble phenols

The proline levels in leaves and roots were quantified according to the method described by Bates et al. (1973). The H_2O_2 level was determined based on a procedure described by Velikova et al. (2000). The reaction solution was 0.5 ml plant extract, 1 ml KI (1 M) and 0.5 ml potassium phosphate buffer (10 mM, pH 7.0). The absorbance was recorded at 390 nm. The total phenolic content was determined using the Folin–Ciocalteu method (Singleton et al. 1999).

Statistical analysis

All data were subjected to analysis of variance (ANOVA) using GraphPad software. The mean values of three independent replications were statistically compared using Tukey's test at a level of 5% of probability.

Table 2 The sequences (5′–3′) of the primers/adapters

Adapter/primer	Sequence (5′–3′)
MsHp-A1	GACGATGAGTCTAGAA
MsHp-A2	CGTTCTAGACTCATC
*Eco*RI-A1	CTCGTAGACTGCGTACC
*Eco*RI-A2	AATTGGTACGCAGTCTAC
MsHp-pre	GATGAGTCTAGAACGGT
*Eco*RI-pre	GACTGCGTACCAATTCA
*Eco*RI-ACT	GACTGCGTACCAATTCACT
*Eco*RI-AAG	GACTGCGTACCAATTCAAG
MsHp-TAC	GATGAGTCTAGAACGGTAC
MsHp-TC	GATGAGTCTAGAACGGTC

Results

Recording the rapid responses of seedlings revealed that the microgravity efficiency in inducing rapid changes in growth and morphology was greater than the light spectrum quality. The microgravity treatment significantly increased root length, whereas the light quality did not make a significant change (Fig. 1a). Microgravity treatment under R + B irradiation synergistically enhanced root biomass compared to the white control (Fig. 1b). The observed difference in shoot fresh weight among the treatment groups was not statistically significant and may be attributed to the evaluation time, immediately after the microgravity treatment (Fig. 1c).

The microgravity variable up-regulated the expression of the DWF5 gene, while light quality did not trigger a statistically significant change (Fig. 2a). The highest expression of DXR gene was recorded in the microgravity-treated seedlings grown under white conditions (Fig. 2b). The R + B irradiation slightly up-regulated the DXR gene (Fig. 2b). Microgravity treatment and R + B irradiation synergistically stimulated the DXS2 gene when compared to the white control (Fig. 2c). The expression pattern of the HMGR2 (Fig. 2d) and HYD1 (Fig. 2e) genes was similar to that of the DXS2 gene. The transcription of the MK gene was slightly changed in response to the microgravity and light composition (Fig. 2f). In the microgravity-treated seedlings, the SQS gene displayed a similar upward trend when compared to the white control (Fig. 2g). However, the R + B radiation conditions contributed to the slight down-regulation of the SQS gene (Fig. 2g).

The number of NML and MSL were 28 and 81, respectively. The number of polymorphic MSL and NML was, respectively, 15 and 23. As depicted in Fig. 3a and b, the microgravity factor was more effective in generating epigenetic responses than the light spectrum quality. However, light conditions affected the molecular responses of the plant to microgravity.

Depending on the type of treatment, a slight difference was observed in terms of the amount of hydrogen peroxide (Fig. 4a). The microgravity treatment under both radiation conditions increased the concentration of soluble phenols (Fig. 4b) and proline (Fig. 4c) in leaves compared to the white control group. However, the applied treatments made no significant effect on the root proline concentration (Fig. 4d).

According to the heatmap of the correlation matrix, moderate to strong statistical correlations were found among the genes explored (Fig. 5). Besides, the soluble phenols, a secondary metabolite index, displayed a moderate positive correlation with the investigated genes.

Discussion

According to the physiological and molecular assessments, the microgravity treatment was more capable of rapidly influencing the transcription of genes and growth performance than the light spectrum quality. Considering growth characteristics, the most noticeable effect of microgravity was recorded in the root length and biomass. The microgravity-mediated alteration in the root may be attributed to significant mechanisms such as (i) cytoskeleton (Soga et al. 2018), (ii) cell cycle progression (Kamal et al. 2019), and (iii) metabolism of hormones (Xu et al. 2018). The microgravity conditions led to epigenetic changes of DNA methylation profile in a light quality-dependent manner. DNA

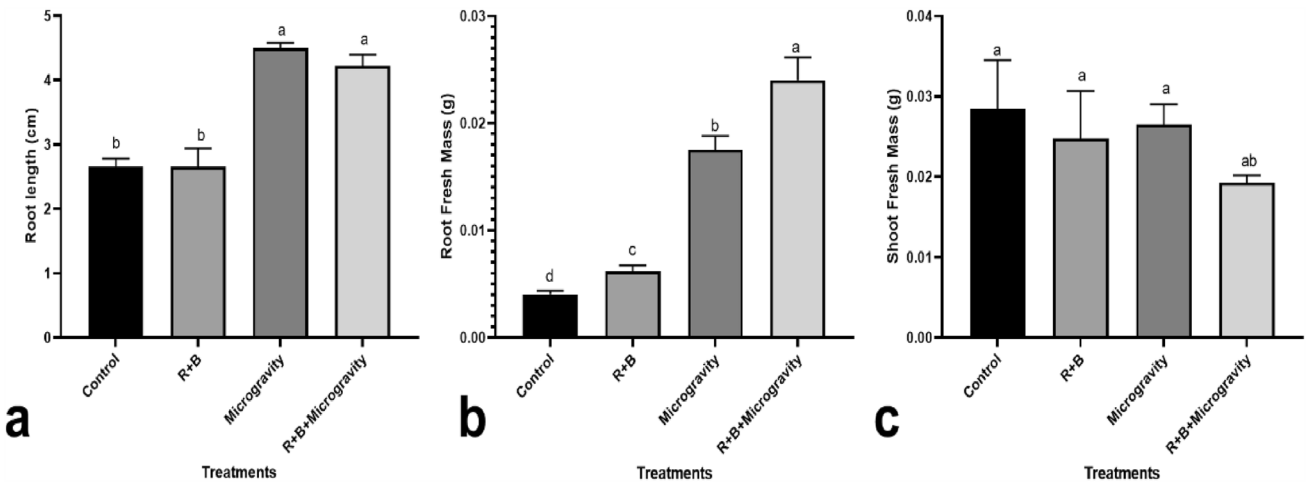

Fig. 1 The effect of microgravity treatment on the several traits, including root length (**a**), root fresh mass (**b**), and shoot fresh mass (**c**) in plants grown under two different light spectral qualities

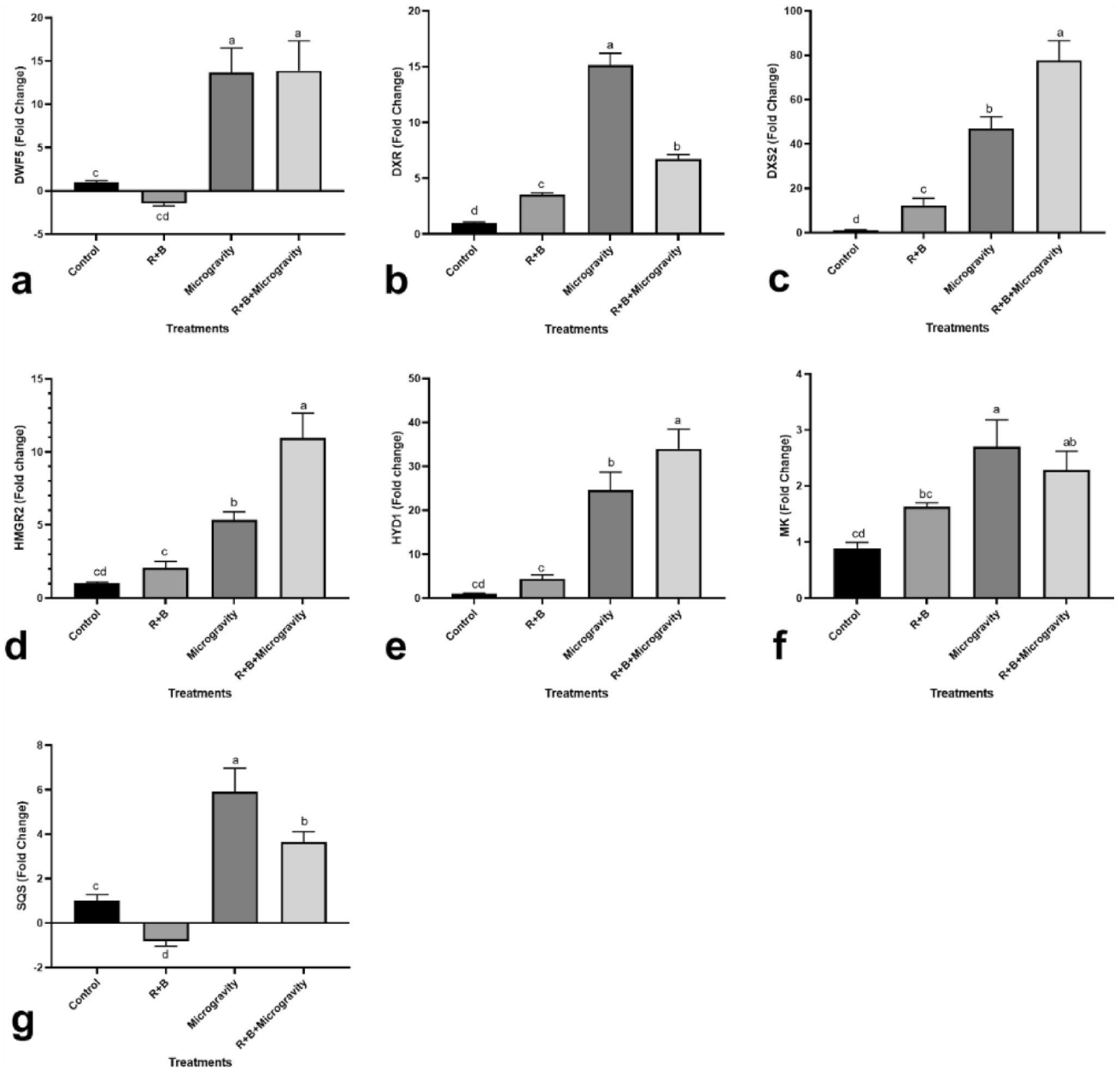

Fig. 2 The effect of microgravity treatment on the expression of several genes, including DWF5 (**a**), DXR (**b**), DXS2 (**c**), HMGR2 (**d**), HYD1 (**e**), MK (**f**), and SQS (**g**) in plants grown under two different light spectral qualities

methylation, which can occur at the locations of CG, CHG, and CHH (H = A, C, or T), is one of the most significant changes that can alter the ultrastructure of chromatin and genome stability. The dynamic nature of DNA methylation permits plants to epigenetically regulate cellular events in response to both internal and external stimuli (Iranbakhsh et al. 2021, 2022). It has been affirmed that the microgravity reception and signaling may affect the expression of genes (Xu et al. 2018; Kamal et al. 2019; Vandenbrink et al. 2019). However, the mechanisms participating in microgravity-triggered changes in the expression pattern of genes remain controversial. Singh et al. (2010a, b) provided molecular evidence demonstrating the epigenetic responses in human T-lymphocyte cells (Singh et al. 2010a, b). Their findings suggested epigenetic events as a significant mechanism for changing gene expression following the microgravity conditions. Exposure to microgravity was associated with alteration in DNA methylation in *Arabidopsis* (Xu et al. 2018). It has been recently reported that the expression of histone deacetylase (an epigenetic marker) varied depending on the light spectral composition (Esmaelpour et al. 2022). However, focus on the earlier studies indicates that little attention

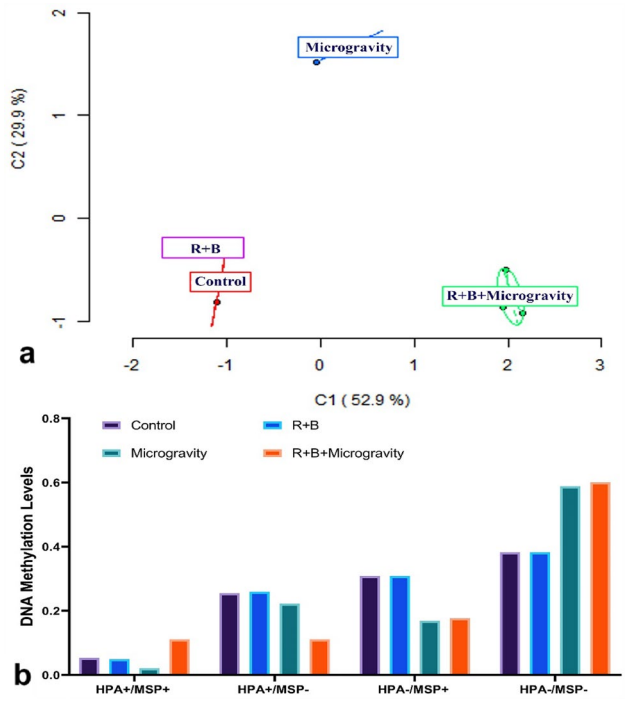

Fig. 3 The principal coordinates analysis (PCoA) plot for methylation-susceptible loci (MSL) (**a**) and methylation levels (**b**) in terms of unmethylation (HPA + /MSP +), internal cytosine methylation (HPA − /MSP +), hemi-methylation (HPA + /MSP −), full methylation, or absence of target (HPA − /MSP −)

has been paid to recognizing the epigenetic responses of plants to microgravity and light quality.

Both light quality and microgravity, especially the latter factor, altered the expression of genes involved in secondary metabolism. Moreover, statistical assessment confirmed the correlation among the genes explored. These findings refer to the high efficacy of simulated microgravity conditions to up-regulate secondary metabolism. One of the main significances of exploring the plant responses to microgravity conditions includes the production of secondary metabolites that can be exploited in medicinal plants (Nakajima et al. 2019). Earlier studies support this opinion that the manipulation of light incubation conditions in a greenhouse or in vitro conditions may considerably influence early growth events, primary metabolism (Lafuente et al. 2021; Esmaelpour et al. 2022), and the production of secondary metabolites (Zhang et al. 2020; Esmaelpour et al. 2022). Moreover, a light signaling network can affect chromatin ultrastructure, transcriptome, and proteome (Parrine et al. 2018; Iranbakhsh et al. 2022). The cellular responses to the microgravity factor are mediated through the involvement of Ca^{2+} and redox status (Xiao et al. 2012; Hausmann et al. 2014), thereby converting signals into responses such as transcription of genes (Cai et al. 2019), DNA methylation (Xu et al. 2018), and membrane function (Kamal et al. 2019). The simulated reduced gravity altered chromatin structure, ribosome biogenesis, and the distribution of cell cycle phases, as well as cell proliferation (Kamal et al. 2019). In rice calluses, transcriptomic experiment revealed that transcription factors, hormone metabolism, respiration, cell wall, protein modification/degradation, and calcium regulation are influenced by the microgravity factor (Jin et al. 2018). It has been validated that the microgravity conditions may be potentially associated with variations in the transcription of a multitude of genes involved in critical processes such as photosynthesis, ribosome biosynthesis, respiration (Vandenbrink et al. 2019), water uptake (Nakajima et al. 2021), and phytochemical properties (Nakajima et al. 2019). According to the RNA-Seq assessment, the microgravity conditions induced changes in the expression of genes in *Arabidopsis thaliana* (Vandenbrink et al. 2019). The findings of Vandenbrink et al (2019). indicated that several photosynthesis-related genes were among the microgravity-responsive genes. The expressions of genes involved in the metabolism of photosynthesis pigments displayed a down-regulation trend in response to the microgravity conditions, implying potential cross-links between the signaling networks of these two factors (gravity and light signals) (Vandenbrink et al. 2019). The microgravity conditions led to changes in the metabolism of photosystem components, chloroplast ultrastructure, and starch metabolism (Vandenbrink et al. 2019). The genes involved in ribosome biosynthesis and respiration were among the differentially expressed genes in response to microgravity (Vandenbrink et al. 2019).

Conclusion

This study evaluated the microgravity efficiency and light quality toward stimulating the secondary metabolism in *P. alkekengi*. The results confirmed that microgravity has a high potential for inducing the expression of genes involved in secondary metabolism. Epigenetic response in terms of DNA methylation was also validated following microgravity treatment. R + B radiation synergistically enhanced the beneficial roles of microgravity. These findings refer to the high efficacy of simulated microgravity conditions to up-regulate secondary metabolism in medicinal plants.

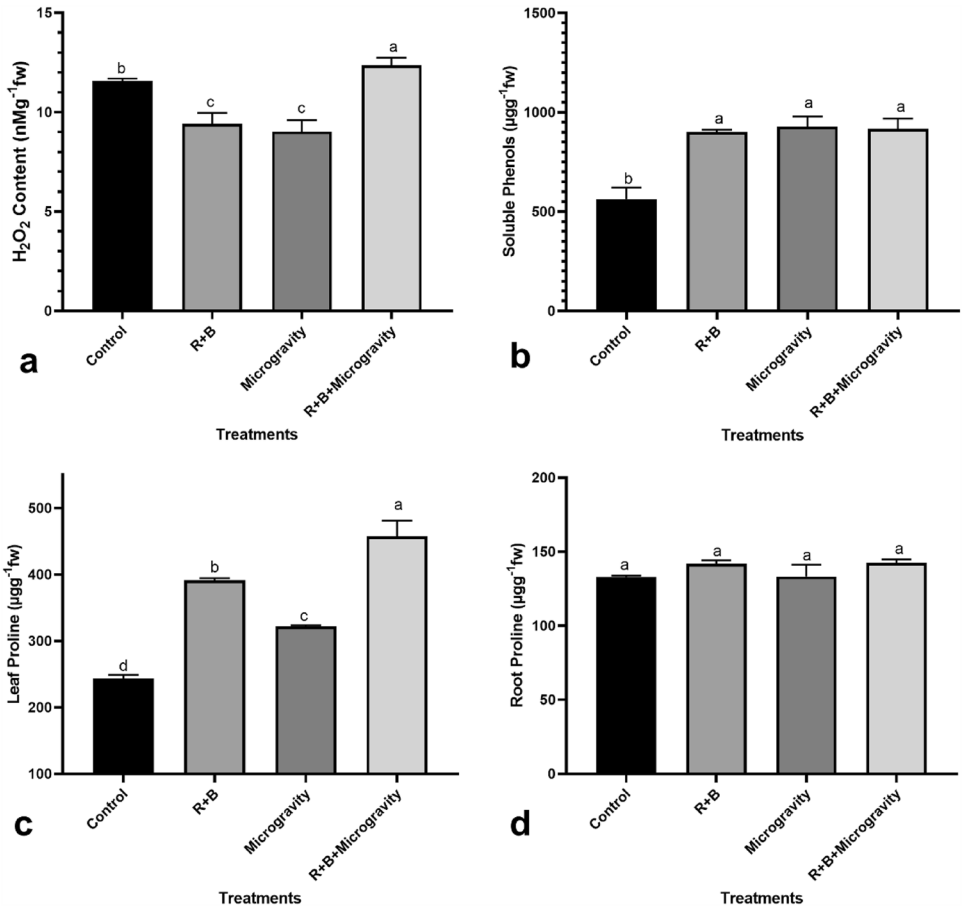

Fig. 4 The effect of microgravity treatment on H_2O_2 content (**a**), soluble phenols (**b**), leaf proline (**c**), and root proline (**d**) in plants grown under two different light spectral qualities

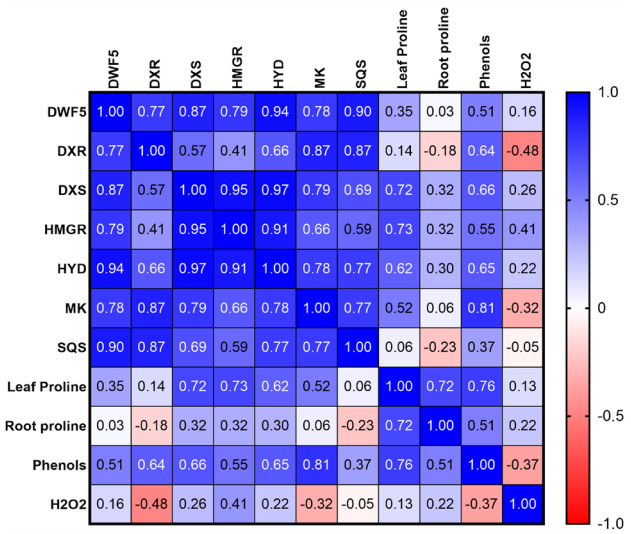

Fig. 5 A heatmap of the correlation matrix regarding traits investigated

Author contribution statement The present study was accomplished with the collaboration of all authors. AI and ME designed the experiments. AI was the supervisor of this project. FA performed physiological analyses. AH was a writer of the manuscript. HH did the statistical analysis.

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Data availability The data of this study are available on request from the corresponding author.

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