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Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study

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Abstract

Background: Lettuce (*Lactuca sativa* L.) is an economically important leafy vegetable that is cultivated worldwide. Advances in plant biotechnology have enabled the development of transgenic and transplastomic lettuce lines with specific agronomic traits that produce pharmaceutical proteins and biological compounds. Plant regeneration efficiency is a critical and highly cultivar-dependent step in plant genetic transformation. No morphological markers have been identified that predict the regeneration ability or cytokinin requirement of lettuce cultivars, hindering the establishment of efficient regeneration systems.

Objective: This study aimed to optimize the direct shoot regeneration efficiency of leaf lettuce cultivars and identify a morphological trait that predicts the optimal cytokinin concentration for each cultivar.

Methods: The direct shoot regeneration of two cultivars (Chima-sanchi and Chirimen-chisya) was tested on media containing various concentrations of the cytokinin 6-benzylaminopurine (BAP). Four additional cultivars with different seed coat colors were analyzed to determine the relationship between seed coat color and the optimal BAP concentration. Statistical significance was evaluated using the Student *t* test, with significance set at $P<.01$.

Results: The highest regeneration efficiencies in Chima-sanchi (80.5%, SE 3.0%; 103 of 128 explants) and Chirimen-chisya (50%, SE 4.4%; 64 of 128 explants) were obtained with 0.05 and 0.5 mg/L BAP, respectively. Therefore, the optimal BAP concentration differed significantly between the cultivars ($P<.01$). The seed coat color and the optimal BAP concentration required for efficient direct shoot regeneration were strongly correlated among the six cultivars.

Conclusions: Seed coat color is a useful morphological marker for predicting the optimal BAP concentration required for efficient direct shoot regeneration in leaf lettuce cultivars. These findings contribute to optimizing lettuce shoot regeneration systems for specific cultivars.

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KEYWORDS

leaf lettuce; shoot regeneration efficiency; 6-benzylaminopurine; seed coat color; CIELAB color scale; flavonoid; BAP

Introduction

Lettuce (*Lactuca sativa* L.) is a major vegetable crop cultivated worldwide that belongs to the *Asteraceae* family. The total world production of lettuce and chicory has increased 1.3-fold in the 20 years since 2005 according to the Food and Agriculture Organization of the United Nations [1]. Asia produced 18.1 million tons of lettuce in 2023, which was 64.4% of global production; Japan produced 0.6 million tons of lettuce, ranking

seventh highest in the world [1]. Lettuce is a dietary source of vitamins and minerals [2]. Thus, lettuce cultivars with increased yield and resistance to biotic and abiotic stresses have been developed using conventional breeding methods [3].

Transgenic and transplastomic lettuce lines with specific agronomic traits that accumulate pharmaceutical proteins and biocompounds have been developed using transformation procedures mediated by particle bombardment and *Agrobacterium* [3]. The major lettuce varieties worldwide include leaf, crisphead, butterhead, and romaine lettuce [3]. Leaf lettuce varieties have wrinkled leaves with frilly edges and no head; their fresh shoots are heavier than those of butterhead varieties under light-emitting diode lighting [4]. Therefore, leaf lettuce varieties are more suitable for indoor growth. Moreover, leaf lettuce contains more β-carotene, a precursor to vitamin A, and more lutein per dry weight than either crisphead or butterhead lettuce [5].

Plant tissue cultures have been widely used in plant breeding and industrial applications, such as for propagating virus-free plants, producing valuable compounds, and producing somaclonal variations [6]. The shoot regeneration efficiency of most plant species is highly dependent on the explant sources, the basal salt mixtures, sugars, and plant growth regulators [7]. Combining the cytokinin 6-benzylaminopurine (BAP) and the auxin 1-naphthaleneacetic acid (NAA) effectively regenerates lettuce shoots, but the optimal combination differs among cultivars [8-10]. Optimizing plant tissue culture parameters is labor-intensive and time-consuming because culturing plant tissues is a slow process. The molecular mechanisms regulating shoot regeneration in lettuce have been examined [11]. The effects of auxins and cytokinins on lettuce regeneration have been studied: the response is mostly cultivar-specific [8-10], and no reliable morphological marker has been identified to predict regeneration ability. For example, Bull and Michelmore [11] molecularly characterized the genetic and regulatory mechanisms underlying regenerative competence in lettuce, but how visible traits relate to hormonal responsiveness was not examined. Certain visible traits may reflect the regenerative capacity, as demonstrated in *Cymbidium* [12]; however, these morphological cues have not been explored in lettuce. We found that seed coat color strongly correlates with the cytokinin requirements for efficient shoot regeneration in leaf lettuce cultivars. Seed coat color is a simple, nondestructive morphological marker that can be used to accelerate the optimization of regeneration systems for genetic transformation. Therefore, this study aimed to evaluate whether seed coat color can reliably predict the cytokinin concentration required for efficient direct shoot regeneration across multiple leaf lettuce cultivars.

Methods

Plant Materials and Growth Conditions

Six leaf lettuce cultivars were used in this study: Chima-sanchi and Chirimen-chisya (purchased from Tohoku Seed), Red fire and Green wave (purchased from Takii Seed), and Fringe green and Shiki-beni (purchased from Sakata Seed). Chima-sanchi, Red fire, and Shiki-beni are white seed cultivars; Chirimen-chisya, Green wave, and Fringe green are brown seed cultivars . The seeds were stored in a constant humidity chamber (SD-302 - 01, Toyo Living) at 25 °C and a relative humidity of 0% - 1% until sowing. Seeds were surface-sterilized via immersion in 70% ethanol for 1 minute. The seeds were treated with 20% commercial bleach (Kao) containing 6% sodium hypochlorite, resulting in a final NaOCl concentration of 1.2%, for 15 minutes. The seeds were then rinsed 3 times with sterile distilled water. The sterilized seeds were placed on a germination medium containing half-strength Murashige and Skoog medium (2.3 g/L, Wako Pure Chemical Industries) [13] supplemented with 10 g/L sucrose and 2.5 g/L Phytagel (Sigma-Aldrich) in Petri dishes with a diameter of 9 cm. The pH of the medium was adjusted to 5.8 with 1N KOH and 1N HCl. The medium was then autoclaved at 120 °C and 0.1 MPa for 20 minutes. Seeds were germinated in an environmentally controlled growth chamber (LPH-411S, NK systems) fitted with fluorescent light (FLR40SW/M/36, NEC) at a photosynthetic photon flux density of 300 µmol photons/m^2/s under continuous white light conditions at 20 °C. All experiments were conducted at Takasaki University of Health and Welfare, Takasaki City, Gunma Prefecture, Japan (36.33°N, 139.00°E) between September 2021 and September 2022, in a humid subtropical climate (Köppen climate classification: Cfa).

Media Composition for Shoot Regeneration

Shoot regeneration efficiency was examined using a medium supplemented with different basal salt mixtures, sugars, and concentrations of BAP and NAA (Nacalai Tesque) following a previously described method [14] (Table 1). NAA was dissolved in a 10 mM NaOH solution and BAP was dissolved in a 10 mM HCl solution before either was added to the culture media. All media contained 0.5 g/L polyvinylpyrrolidone (Nacalai Tesque) and 2.5 g/L Phytagel (Sigma-Aldrich) at pH 5.8. The medium was sterilized via autoclaving at 121 °C for 20 minutes. Cotyledons from 7-day-old seedlings were used as explants and placed on the medium in Petri dishes with a diameter of 9 cm. Each treatment included 16 explants that were cultured per dish, with 8 dishes per treatment, for a total of 128 explants. The explants were maintained for 4 weeks under continuous white light conditions (photosynthetic photon flux density=300 µmol photons/m^2/s) at 25 °C and transferred to fresh medium every 2 weeks.

Table . Media composition and growth regulators for shoot regeneration of lettuce.

Medium	Basal salt mix	Sugar	BAP[a] (mg/L)	NAA[b] (mg/L)
M1	1 × MS[c]	3% sucrose	0.5	0.1
M2	1 × MS	3% sucrose	0.05	0.01
M3	1 × MS	3% sucrose	0.05	0.1
M4	1 × MS	3% sucrose	0.05	1
M5	1 × MS	3% sucrose	0.5	0.01
M6	1 × MS	3% sucrose	0.5	1
M7	1 × MS	3% sucrose	5	0.01
M8	1 × MS	3% sucrose	5	0.1
M9	1 × MS	3% sucrose	5	1
M10	1/2 × MS	3% sucrose	0.5	0.1
M11	1 × B5	3% sucrose	0.5	0.1
M12	1 × MS	6% sucrose	0.5	0.1
M13	1 × MS	1.5% glucose	0.5	0.1

[a]BAP: 6-benzylaminopurine.

[b]NAA: 1-naphthaleneacetic acid.

[c]MS: Murashige and Skoog.

Seed Coat Color Measurement

The color parameters of the seeds from each cultivar were measured with an SD 7000 spectrophotometer (Nippon Denshoku Industries) using the CIELAB *L**, *a**, and *b** color scale. The *L** axis represents the degree of brightness ranging from black (*L**=0) to white (*L**=100). The *a** and *b** axes represent redness (positive number) to greenness (negative number) and yellowness (positive number) to blueness (negative number), respectively [15].

Total Flavonoid Content Analysis

The total flavonoid content was analyzed according to a previously described method, with some modifications [16]. A 50 µg aliquot of seeds was homogenized in 0.5 mL of 80% methanol with 5.0 mm stainless beads (Biomedical Science) at 1100 rotations per minute for 45 seconds using a Shake Master (Biomedical Science). The homogenized solutions were incubated for 15 minutes at 70 °C, then centrifuged at 10,000 × g for 10 minutes at 4 °C. The resulting supernatants were incubated at 60 °C, and the dried pellets were dissolved in 20 µL of 80% methanol. The extracts were spotted on a 5 × 5 cm TLC Silica gel 60 $F_{2\ 4}$ plate (Merck). For staining, the blots were sprayed with a methanolic solution containing 1% diphenylboric acid 2-aminoethylester (DPBA, Tokyo Chemical Industry), then sprayed with a methanolic solution containing 5% PEG 4000 (Nacalai Tesque). The fluorescence was visualized using an iBright CL1000 imaging system (Thermo Fisher Scientific).

Statistical Analyses

All statistical analyses were performed using EZR software [17], a free graphical interface for R that is widely used for standard biostatistical analyses. Significance was determined using a Student *t* test for two-group comparisons or a one-way ANOVA followed by a Tukey test for multiple group comparisons. The statistical significance was set at $P<.01$ for Student *t* tests and $P<.05$ for one-way ANOVA. All values are expressed as means and SE.

Results

In this study, we found that seed coat color is strongly associated with the cytokinin requirement for efficient direct shoot regeneration across 6 leaf lettuce cultivars. White seed cultivars exhibited their highest regeneration efficiency at low BAP concentrations (0.05 mg/L), whereas brown seed cultivars required higher BAP levels (0.5 mg/L) to achieve comparable regeneration. Seed brightness (L*) and yellowness (b*) positively correlated with the M3/M1 ratio, supporting seed coat color as a predictive morphological marker.

Effects of Medium Composition on Shoot Regeneration

BAP and NAA are commonly used as plant growth regulators for regenerating lettuce shoots [8,10,14,18]. We examined the effects of different concentrations of BAP and NAA on the efficiency of shoot regeneration in Chima-sanchi and Chirimen-chisya cultivars (Figure 1). We used different concentrations of BAP with 0.1 mg/L NAA (M1, M3, and M8). The shoot regeneration efficiency was highest in Chima-sanchi (Figure 1C) and Chirimen-chisya (Figure 1D), with 0.05 mg/L BAP (M3) and 0.5 mg/L BAP (M1), respectively. We then tested different concentrations of NAA with 0.5 mg/L BAP (M1, M5, and M6). The efficiency was highest in Chima-sanchi using 0.1 mg/L NAA (M1) (Figure 1C). The Chirimen-chisya shoot regeneration efficiency did not differ between the 0.1 mg/L (M1) and 1 mg/L (M6) NAA treatments (Figure 1D). The shoots of both cultivars weakly regenerated when treated with 5 mg/L BAP (M7-9; Figure 1). The BAP concentration strongly and cultivar-dependently influenced the shoot regeneration

efficiency, whereas the basal salt mixture and sugar composition did not (Multimedia Appendices 1 and 2).

Figure 1. Shoot regeneration from cotyledon segments of (A) Chima-sanchi and (B) Chirimen-chisya on medium M1 after 4 weeks of culture. Bar=1 cm. Effects of different concentrations of BAP and NAA on shoot regeneration from cotyledon segments of (C) Chima-sanchi and (D) Chirimen-chisya after 4 weeks of culture (n = 16 explants per dish × 8 dishes). All media were supplemented with 1 × MS, 30 g/L sucrose, and 0.5 g/L polyvinylpyrrolidone. Different letters indicate statistically significant differences (one-way ANOVA followed by Tukey test, $P<.05$). BAP: 6-benzylaminopurine; MS: Murashige and Skoog; NAA: 1-naphthaleneacetic acid.

Seed Coat Color Phenotype and Relationship With Cytokinin Requirement

The seed coat color is a key phenotypic trait in many crops such as lettuce [19-21]. The Chima-sanchi seeds were lighter, redder, and yellower than those of Chirimen-chisya (Figure 2). The ratio of shoot regeneration efficiency under 0.05 mg/L BAP to that under 0.5 mg/L BAP (M3/M1) positively correlated with seed brightness (L*) and yellowness (b*) in all 6 cultivars (r=0.834 and 0.722, respectively; Figure 3B and D; Multimedia Appendix 3). The brown-seeded cultivars contained more flavonoids than the white-seeded cultivars (Multimedia Appendix 4). The regeneration efficiency of the white-seeded types was higher than that of the brown-seeded types, indicating that flavonoid accumulation negatively modulates cytokinin responsiveness.

Figure 2. (A) Seed samples of Chima-sanchi (left) and Chirimen-chisya (right). Average values of CIE (B) L*, (C) a*, (D) b* color coordinates of seed coat color in the cultivars (n=5). Horizontal bars within the box indicate the median value of the data, and the outer vertical bars represent the maximum and minimum values of the data. **P<.01 (Student t test).

Figure 3. (A) Samples of 6 lettuce cultivars. Genotype names are as follows, clockwise from the upper left: Chima-sanchi, Red fire, Shiki-beni, Green wave, Fringe green, and Chirimen-chisya. Bar=1.0 cm. Correlations between values of CIE (B) L*, (C) a*, (D) b* color coordinates of seed coat color (n=5) and ratio of shoot regeneration efficiency using M3 to the efficiency using M1 (M3/M1) in the 6 cultivars (n = 16 explants per dish × 8 dishes) are shown.

Relationship Between Flavonoid Biosynthesis and Shoot Regeneration Capacity

Flavonoids are secondary metabolites that play multiple roles in auxin transport, oxidative stress tolerance, and cell division [22]. In *Arabidopsis*, *transparent testa* (*tt*) mutants are defective in enzymes or regulatory factors involved in the flavonoid biosynthetic pathway. These mutants have a pale seed coat and show altered responses to phytohormones [23]. The *Arabidopsis tt4* mutant is deficient in chalcone synthase, a key enzyme in the flavonoid biosynthetic pathway. The *Arabidopsis tt4* mutant has a pale yellow seed coat and markedly reduced shoot regeneration efficiency compared with the wild type [24]. This finding suggests that certain downstream flavonoid metabolites produced after chalcone synthase catalysis are required for efficiently regenerating shoots in *Arabidopsis*. The brown seed cultivars contained higher levels of flavonoids than the white seed cultivars in this study (Multimedia Appendix 4); however, the shoot regeneration efficiencies of the brown seed cultivars were considerably lower (Figure 3). These patterns imply that although flavonoid metabolism may influence regenerative competence, the mechanisms underlying this metabolism differ between lettuce and *Arabidopsis*. However, the strong correlation we observed between seed coat color and the optimal BAP concentration provides a practical morphological marker for predicting the cytokinin requirements of leaf lettuce.

Discussion

Principal Findings

Seed coat color is a simple morphological marker for predicting the most appropriate cytokinin concentration required for efficiently regenerating shoots regardless of lettuce cultivar. This approach can be quickly and easily used for optimizing regeneration conditions, thereby increasing the efficiency of transgenic and genome-edited lettuce production. However, this study was limited to 6 leaf lettuce cultivars, which were tested under controlled laboratory conditions. Further validation with additional genotypes and under different environmental conditions is necessary. This marker-based method is more cost- and labor-efficient for laboratories; however, scaling up tissue culture systems for industrial use remains challenging because maintaining aseptic conditions, controlling the environment, and performing manual subculture steps substantially increases labor and energy costs. Further technical improvements and validation studies are required before genotype-specific protocols can be applied to large-scale propagation or transformation systems. However, integrating visible traits, such as seed coat color, into regeneration and transformation workflows could help balance the overall costs and benefits of cultivar-specific plant biotechnologies and support the broader use of lettuce as a bioproduction platform.

Conclusion

Seed coat color is strongly correlated with the cytokinin required for efficiently regenerating shoots in leaf lettuce. The maximum shoot regeneration occurred with 0.05 and 0.5 mg/L BAP for white and brown seed cultivars, respectively. Pigmentation-related metabolites, possibly flavonoids, may modulate cytokinin responsiveness during the regeneration process. This simple visual marker can accelerate the optimization of regeneration systems, reduce experimental costs, and facilitate cultivar-specific transformation. Our findings directly support the development of lettuce cultivars with specific traits, such as enhanced yield, stress tolerance, and nutritional value, by enabling the rapid establishment of efficient regeneration and transformation protocols. Additionally, understanding the interplay between flavonoid metabolism and cytokinin signaling may provide new options for engineering regenerative competence in other horticultural crops. Our findings not only clarify the physiological basis of genotype-dependent regeneration but also provide a practical framework that connects the fundamental knowledge of plant regeneration mechanisms to their application in lettuce breeding and commercial production.

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Data Availability

All data are available from the corresponding author upon reasonable request.

Authors' Contributions

MK and TY conceived, designed, and supervised the project. MK performed all experiments. TY provided some important suggestions. MK wrote the paper. TY critically reviewed and approved of the final version.

Conflicts of Interest

None declared.

Multimedia Appendix 1
Effects of different basal media on shoot regeneration from cotyledon segments of (A) Chima-sanchi and (B) Chirimen-chisya after 4 weeks of culture (n = 16 explants per dish × 8 dishes). All media were supplemented with 30 g/L sucrose, 0.5 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, and 500 mg/L polyvinylpyrrolidone. Different letters indicate statistically significant differences (one-way ANOVA followed by a Tukey test, $P<.05$).
[PNG File, 21 KB - xbio_v4i1e70496_app1.png]

Multimedia Appendix 2
Effects of different sugar concentrations and types on shoot regeneration from cotyledon segments of (A) Chima-sanchi and (B) Chirimen-chisya after 4 weeks of culture (n = 16 explants per dish × 8 dishes). All media were supplemented with 1 × Murashige and Skoog, 0.5 mg/L 6-benzylaminopurine, 0.1 mg/L 1-naphthaleneacetic acid, and 500 mg/L polyvinylpyrrolidone. Different letters indicate statistically significant differences (one-way ANOVA followed by a Tukey test, $P<.05$).
[PNG File, 21 KB - xbio_v4i1e70496_app2.png]

Multimedia Appendix 3
Shoot regeneration efficiency using M1 and M3 and CIELAB values of seed coat color in 6 lettuce cultivars. 1: w=white seed cultivar, b=brown seed cultivar. 2: L* corresponding to the brightness, a* to the red/green coordinates, and b* to the yellow/blue coordinates.
[PNG File, 32 KB - xbio_v4i1e70496_app3.png]

Multimedia Appendix 4
The flavonoid content of methanolic extracts of seeds in 6 lettuce cultivars. Bar=0.5 cm.
[PNG File, 118 KB - xbio_v4i1e70496_app4.png]

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Abbreviations

BAP: 6-benzylaminopurine
NAA: 1-naphthaleneacetic acid
***tt*:** *transparent testa*

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Machine Learning Ensemble Investigates Age in the Transcriptomic Response to Spaceflight in Murine Mammary Tissue: Observational Study

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Abstract

Background: Spaceflight presents unique environmental stressors, such as microgravity and radiation, that significantly affect biological systems at the molecular, cellular, and organismal levels. Astronauts face an increased risk of developing cancer due to exposure to ionizing radiation and other spaceflight-related factors. Age plays a crucial role in the body's response to the cellular stresses that lead to cancer, with younger organisms generally exhibiting more efficient response mechanisms than older ones. The vast majority of research investigating breast cancer risk from spaceflight uses cell lines exposed to simulated radiation and microgravity, but cell lines cannot capture the combinatorial response expressed across tissues, organs, and systems to real radiation and microgravity in space.

Objective: The primary objective of this in silico observational study is to characterize the molecular response to spaceflight of in vivo murine mammary tissue. We use an ensemble of linear binary classifiers to identify the molecular biomarkers enriched in this response using mice flown on the International Space Station. The secondary objective is to determine if age plays a role in this response.

Methods: The National Aeronautics and Space Administration (NASA) Open Science Data Repository has curated transcriptomic data obtained from 10 BALB/cAnNTac female mice flown on the International Space Station and 33 control mice kept on earth (OSD-511). In this observational study focused on two age groups (old/young), we used an ensemble of 4 machine learning binary classifiers with linear decision boundaries (logistic regression, support vector machine, stochastic gradient descent, and single-layer perceptron) to analyze gene expression profiles to predict age (old vs young) and condition (spaceflight vs ground control). Using the genes our ensemble identified as most predictive, we performed pathway enrichment analysis to investigate the molecular pathways involved in spaceflight-related health risks, particularly in the context of breast cancer.

Results: The pathway enrichment analyses revealed age-differentiated responses to spaceflight (false discovery rate–adjusted q values<.05). Among the 10 mice flown in space, younger mice exhibited significantly enriched pathways related to lipid metabolism and inflammatory stress signaling. All space-flown mice demonstrated evidence of adaptation in retinoid metabolism and peroxisome proliferator-activated receptor signaling in response to microgravity and radiation relative to their 33 ground control counterparts.

Conclusions: Spaceflight-induced breast cancer risk manifests through distinct age-specific mechanisms: younger individuals face risk through maladaptive metabolic hyperactivity and oxidative cycling, while older individuals are vulnerable due to impaired

stress responses and accumulated metabolic dysfunction. Both age groups ultimately face elevated carcinogenic potential through different but converging pathways. These findings highlight the critical role of age in modulating the response to spaceflight-induced stress and suggest that these molecular pathways may contribute to differential outcomes in tissue homeostasis, metabolic disorders, and breast cancer susceptibility.

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KEYWORDS

machine learning; spaceflight; mammary tissue; gene expression; mice; breast cancer; feature importance

Introduction

Spaceflight exposes living organisms to a unique set of environmental challenges, including microgravity [1], radiation [2], and altered gas composition [3], which can significantly impact biological systems at the molecular, cellular, and organismal levels. Several systems have been shown to be impacted in both male and female organisms, including the cardiovascular [4], musculoskeletal [5], immune [6], neurologic [7], hepatic [8], and ophthalmologic [9] systems, to name a few. Although there is currently no evidence of increased gynecological cancer incidence among female astronauts [10], earth-based mouse studies using ionizing radiation, including simulated galactic cosmic radiation, suggest that they may face an increased risk of breast cancer when exposed to space radiation [11]. Exposure to ionizing radiation is well established as a risk factor for breast cancer [12], and both microgravity and simulated microgravity have been shown to enhance the tumorigenic potential of breast cancer cells grown in vitro [13-15]. Furthermore, spaceflight disrupts circadian rhythms, and consequent lower levels of melatonin reduce its efficacy in inhibiting cancer cells [16,17]. Mammary cellular response to spaceflight has been shown to differ with age, as younger organisms typically exhibit more efficient cellular repair and adaptive mechanisms than their older counterparts [18]. Adolescent murine mammary glands exposed to ionizing radiation show increased activation of mammary stem cell and Notch signaling pathways, heightened mammary repopulating activity, and an increased propensity to develop estrogen receptor–negative tumors [19]. A history of ionizing radiation to the chest is a risk factor for breast cancer. The Childhood Cancer Survivor Study indicates that breast cancer risk is highest in young women treated for Hodgkin lymphoma, but it is also increased in those who received moderate-dose chest radiation for other pediatric or young adult cancers [20]. In summary, current research suggests that female astronauts are at a higher risk of developing breast cancer than their terrestrial counterparts, with age being a contributing factor to this increased vulnerability.

The vast majority of research into the risk of breast cancer due to spaceflight has been conducted using simulated radiation and microgravity on either female mice or human breast cell lines. Monti et al [21] found that normal and cancerous breast cell response to microgravity varies drastically, depending on whether the cells are adhered or attached in the organoid model. Kannan et al [22] exposed breast cancer cells to simulated microgravity and compared cells exposed to 10 *g* and 1 *g* forces and the respective response in proliferation, cell-cell interaction, and formation of 3D structures, migration, and invasiveness. Although in vitro studies are valuable for mechanistic insights, high-throughput screening, and controlled manipulations, they cannot fully replicate the physiological context of an intact organism. Although simulated microgravity and radiation experimentation on cell lines are much less expensive and resource-intensive approaches than controlled spaceflight experiments, they fail to reproduce the full combinatorial spectrum of the spaceflight environment. Sarkar and Pampaloni [23], in their study of bone marrow remodeling and immune dysfunction in space, note that it remains uncertain how well various microgravity simulation methods replicate the conditions of actual microgravity. They also emphasize that differences in equipment may influence experimental reproducibility, as past studies have frequently produced conflicting results [23].

Bioinformatic approaches have been used to study the effect of spaceflight on health. Many methods in bioinformatics, such as genome-wide association studies and differential gene expression analysis, leverage statistical hypothesis testing as a mechanism to discover new insights. Integrating machine learning (ML) into established bioinformatics and computational biology frameworks has significantly advanced the development of predictive models and analytical tools across molecular evolution, proteomics, systems biology, and disease genomics [24]. ML and artificial intelligence (AI) models are becoming more complex, trained on larger datasets, and run on faster hardware. These trends are accelerating adoption across domains, including bioinformatics. Casaletto et al [25] leveraged an ensemble of ML algorithms to identify genes most predictive of lipid density in murine liver tissue. Building accurate models, particularly with high-dimensional predictors such as gene expression, typically benefits from large sample sizes [24]. To mitigate this, researchers use some form of feature selection—a broad collection of techniques that reduces the dimensionality of the feature space [26,27]. Filtering methods such as coefficient of variation and feature correlation to a target are examples of feature selection techniques. Traditional ML algorithms such as single-layer perceptrons and logistic regression may be considered weak learners in the context of high-dimensional datasets—but, leveraged together in an ensemble, such weak learners can achieve excellent performance [28].

The use of ML to study spaceflight-induced changes in mammary gene expression can offer valuable insights into the mechanisms of breast cancer development. In this study, we examine the gene expression profiles from a controlled in vivo experiment in which young and old mice were exposed to spaceflight. The mammary glands were dissected and the tissue

used for transcriptomic analysis. We are repurposing the data from this study to explore the use of traditional ML methods including random forest, logistic regression, support vector machine, and the single-layer perceptron to determine how murine mammary tissue responds to spaceflight and whether age is a factor. Using the coefficients of simple models such as these to determine feature importance makes this approach very transparent and easy to understand, and combining models into an ensemble makes it a powerful and robust approach.

Methods

In this section, we discuss the data on which this research is based and how we preprocessed it for our ML ensemble. We describe the ensemble of ML algorithms we leveraged, how we derived feature importance from the trained models, and how we combined and filtered the results of the models to form a final set of gene results from our experiments.

Ethical Considerations

We used OSD-511 as the source of data for our observational study. All National Aeronautics and Space Administration (NASA) rodent research missions, including Rodent Research Reference Mission 1 (RRRM-1) from whence our data are derived, are required by US federal law to follow strict humane care and use of laboratory animals under the provisions of the Health Research Extension Act of 1985 [29]. As an observational study, our research was conducted on data from an already-published experiment. The authors believe the repurposing of existing datasets not only maximizes the cost-effectiveness of those studies, it also eliminates the need to further expose animals to the conditions of spaceflight and ultimately sacrifice animals for novel research.

Data

In the RRRM-1, a total of 43 female BALB/cAnNTac mice were included in the study, consisting of 21 younger mice (aged 9‐12 weeks, YNG) and 22 older mice (aged 32 weeks, OLD). Among the younger mice, 5 were flown in space, 8 were kept in the Animal Enclosure Module (AEM), and 8 were housed in regular vivarium cages (VIV). Vivarium controls are included in spaceflight studies to distinguish the effect of the cage used in spaceflight (ie, AEM) from the ambient effects of spaceflight (eg, radiation, microgravity). In this research, we do not explore that distinction, so we combined the VIV and AEM control groups into a single ground control group called “GC.” For the older mice (OLD), 5 were flown in space, 7 were housed in flight hardware, and 10 in vivarium cages. Note that there was no basal group included in the design of their experiment. After 40 days in space, the mice were safely returned to Earth, given 2 days to recover (Live Animal Return), and then euthanized. Mice flown in space and kept in standard cages are denoted FLT. Table 1 summarizes the distribution of mice in the experiment.

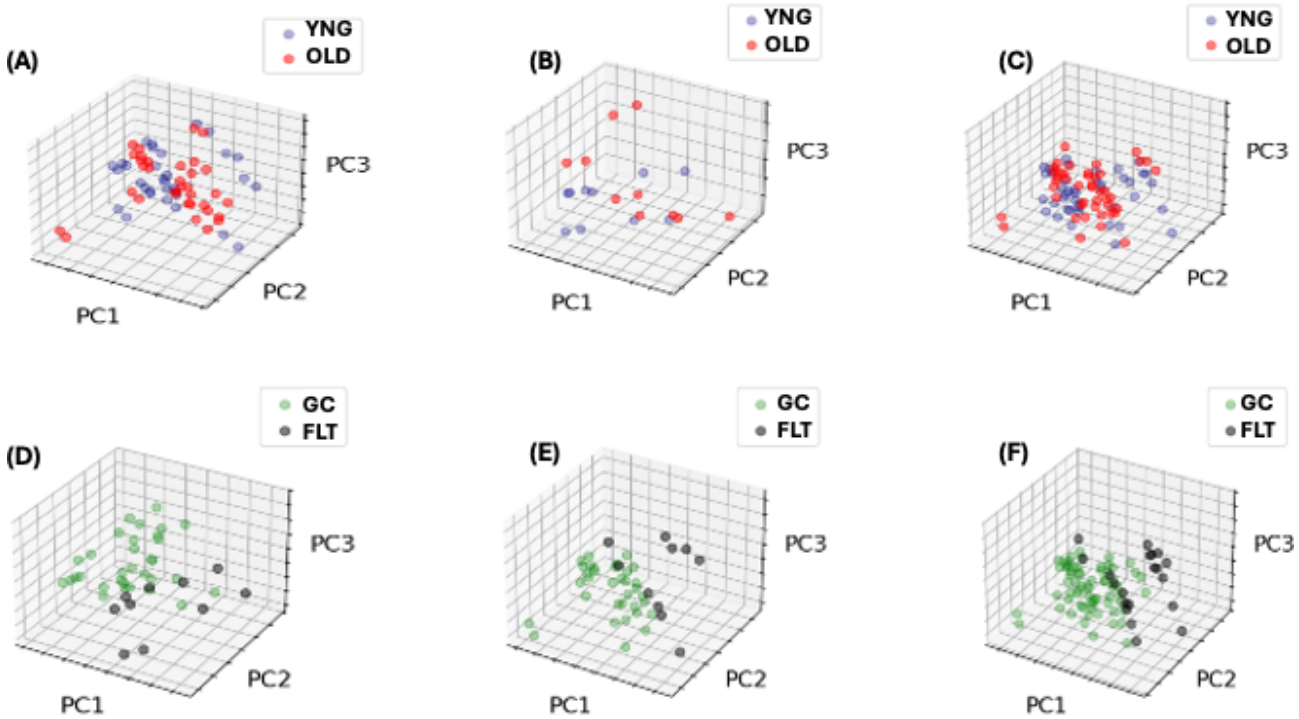
Table . Distribution of mice in different experimental groups, including flight habitat (AEM) and vivarium (VIV), which together constitute the overall ground control (GC=AEM+VIV), and spaceflight (FLT) groups for both the old (OLD) and young (YNG) cohorts. Marginal totals are provided in the last column of the table.

	OLD (32 weeks)	YNG (9-12 weeks)	Total
AEM (Animal Enclosure Module)	7	8	15
VIV (vivarium)	10	8	18
GC (ground control)	17	16	33
FLT (spaceflight)	5	5	10

The dataset contains ribo-depleted total RNA sequencing (RNA-seq) data from mammary glands. The sequences for each mouse were aligned once using *Mus musculus* Spliced Transcripts Alignment to a Reference (STAR; version 2.7.10a) and once with RNA-Seq by Expectation-Maximization (RSEM version 1.3.1) to the Ensembl release 107, genome version GRCm39. These data are available in the Open Science Data Repository [30] as dataset OSD-511 [31]. Both datasets (RSEM, STAR) are published with OSD-511.

The principal component analysis (PCA) plots of the data are shown in Figure 1. PCA projections that display approximately linearly separable classes suggest that binary classifiers with linear decision boundaries, such as those in our ensemble, may achieve strong classification performance.

Figure 1. PCA plots for each of the experiments (augmented datasets with RSEM, STAR). Figures 1A-C are PCA plots of the ground control mice, spaceflight mice, and all mice, respectively, and are colorized by age. Figures 1D-F are PCA plots of the young mice, old mice, and all mice, respectively, and are colorized by condition. PC: principal component; PCA: principal component analysis; RSEM: RNA-Seq by Expectation-Maximization; STAR: Spliced Transcripts Alignment to a Reference.



Based on the 3D PCA plot in Figure 1A, age among ground control mice did not seem to be predictable from gene expression with a linear decision boundary. This supportd the use of the control group and provided a neutral baseline for later comparisons. In Figure 1B, gene expression differed between young and old mice in response to spaceflight. Figures 1D and E showed a clear distinction between ground control and spaceflight among young and old mice, respectively. This pattern suggested an age-related response to spaceflight and motivated us to investigate further. Figure 1C did not clearly distinguish young from old mice, but Figure 1F showed a clear separation between the unmarginalized age groups. This suggests that the impact of age on gene expression is not as significant as the impact of spaceflight.

ML model performance generally improves with more data points. Additionally, training and testing must be performed on a sufficient number of data points to accurately quantify model performance. Data augmentation is a collection of methods used to increase the size of a dataset for training and testing. In our research, we combined the RSEM and STAR datasets by creating 2 data points per biological sample: one for the RSEM quantification and one for the STAR quantification. This increased the size of our dataset by a factor of 2, with the caveat that the augmented samples are not independent (see points in Figure 1). Because ML model performance improves with fewer dimensions, we performed the filtering methods described in Table 2 to reduce the dimensionality of the dataset. We removed genes that have nonnumeric values or not-a-number values, genes that do not code for proteins, genes with counts below 30 in 80% of the samples, genes with a coefficient of variation lower than 0.4, and nondifferentially expressed genes at an α level of 0.1.

Table . Data-filtering methods applied to this dataset include removing genes with not-a-number values, noncoding genes, and genes that are not correlated to the binary targets (old vs young or ground control vs spaceflight). Columns include the total count of genes before the filter was applied, the total number of genes removed by the filter, and the count of genes remaining after the filter was applied. These filters were executed in order from top to bottom, leaving a total of 750 genes for training our models.

Filtering method	Count before filter	Number removed by filter	Count after filter
Remove genes with not-a-number values	56,840	0	56,840
Remove non–protein-coding genes	56,840	35,159	21,681
Remove noncorrelated genes	21,681	20,931	750

After reducing the dimensionality of the data, we applied three transformations. First, we transformed the data into transcripts per million to account for sequencing depth and gene length, thus making the gene expression values comparable within a sample. Second, we applied a log transformation to stabilize the variance inherent in transcriptomic count data. Third, since coefficient-based ML algorithms require all the feature values to be on the same scale, we used the StandardScaler method from *scikit-learn* to convert all feature values to z-scores.

Figure 2 shows the graphical summary of the methods we used in our in silico experiments to create sets of genes that are predictive of their respective targets. We introduce the notation "GROUP:target" to denote the experiment where GROUP represents the subsets ground control (GC) and spaceflight (FLT) or the subsets young mice (YNG) and old mice (OLD), and the target represents the binary class age or condition (cnd) that the ML model is trained to predict.

Figure 2. Graphical summary of the methods used in this research. (A) The OSD-511 dataset contains RNA-seq data for mouse mammary tissue. (B) The data were filtered to reduce dimensionality, normalized, log-transformed, and standardized. (C) Data were divided into GC and FLT groups to predict age and divided into YNG and OLD groups to predict condition. (D) Each subset of data was used to build 4 models in the ensemble. (E) Each model generated two sets of genes most predictive of the target. (F) The two sets from each model were unioned into a single set per model. (G) The four sets from each model were majority-intersected to yield the intermediate set of genes per experiment. cnd: condition; FLT: spaceflight; GC: ground control; OLD: old; YNG: young.

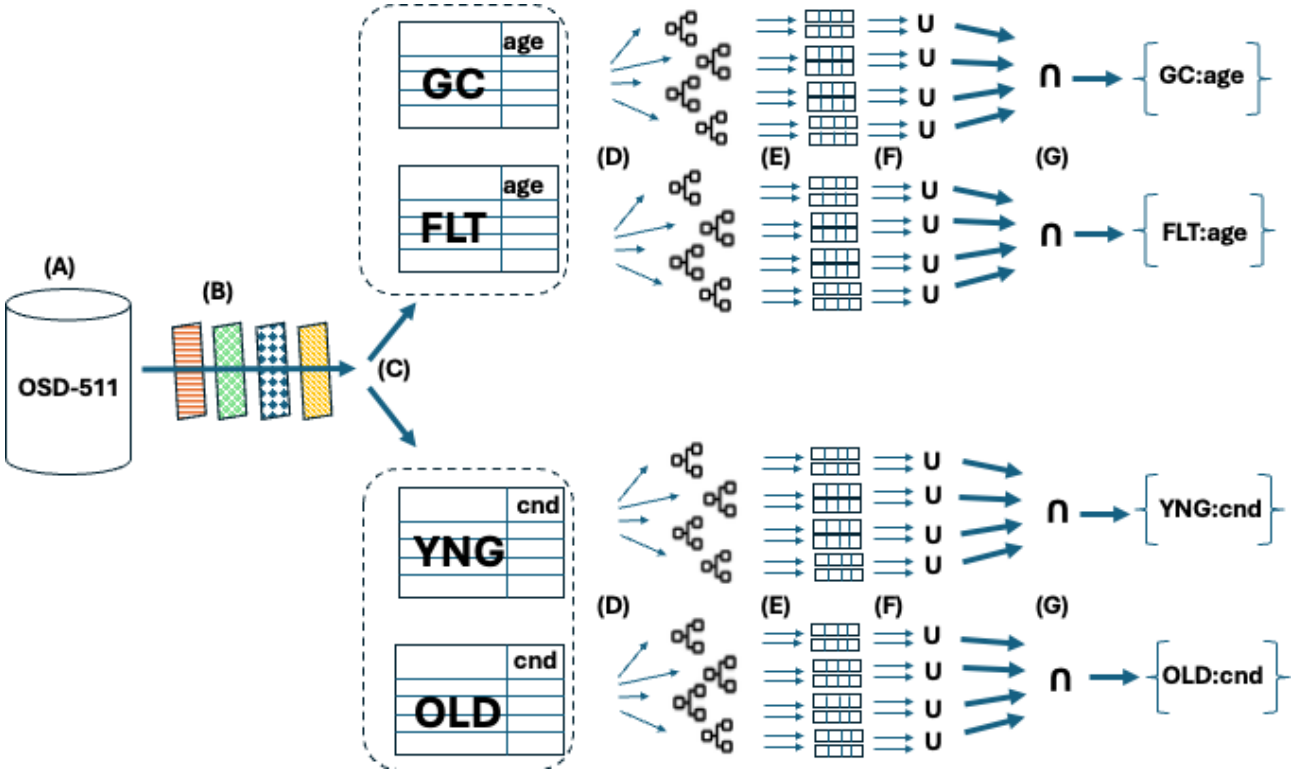
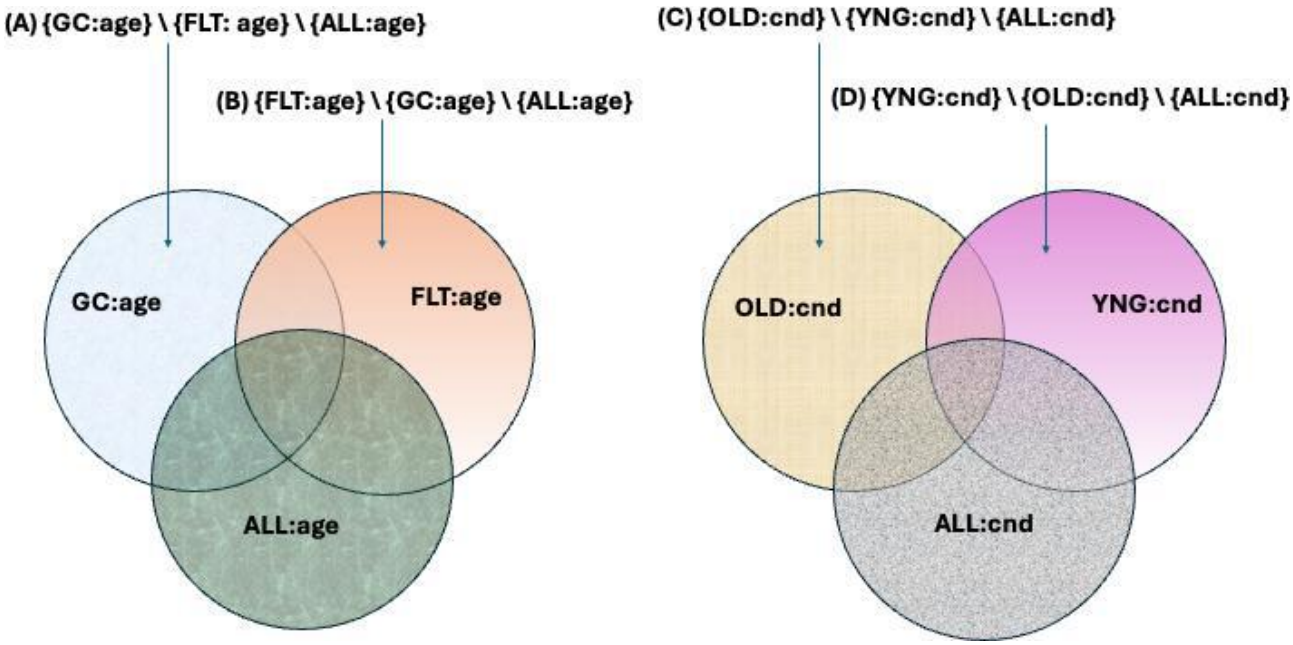


Figure 2 shows all the steps in the pipeline to produce the intermediate result set of genes, which were further processed as described in Figure 3.

Figure 3. Venn diagrams depicting set difference operations to identify genes uniquely predictive of age (A and B) and condition (C and D) for a given subset of mice. In Figure 3A, we remove ALL:age genes and FLT:age genes that intersect with GC:age to obtain those genes that uniquely predict age for ground control mice. These genes are represented by the light blue part of the Venn diagram. Similarly, we remove ALL:age genes and GC:age genes that intersect with FLT:age genes to obtain those genes that uniquely predict age for space-flown mice. These genes are represented by the light orange part of that Venn diagram. In Figures 3C and 3D, we use the same logic to obtain those genes that uniquely predict the condition of old mice in the yellow, textured part of the Venn diagram and those genes that uniquely predict the condition for young mice in the pink part of the Venn diagram. These set operations yielded the final gene results we discuss in the next section. cnd: condition; FLT: spaceflight; GC: ground control; OLD: old; YNG: young.



Algorithms

We leveraged 4 supervised ML algorithms on the gene expression data to predict labels associated with each sample. These models were trained and tested to classify binary labels (spaceflight vs ground control and old vs young) and include stochastic gradient descent (SGD), logistic regression (LR), single-layer perceptron (SLP), and support vector machine (SVM). These models were specifically selected to capture linear decision boundary classification patterns.

The SGD classifier from *scikit-learn* trains a linear classifier using stochastic gradient descent to update the coefficients of the input features. SGD iteratively updates the coefficients based on the gradient of the loss function, using one training sample at a time to compute each gradient step, rather than the whole dataset. We used the *scikit-learn* implementation of SGDClassifier with all default hyperparameters. LR, despite its name, is a binary classification algorithm that provides a probability for the binary target prediction based on a set of discrete or continuous features [32]. Because it does use regression, there are model coefficients associated with the features that may be used for feature importance. We used the *scikit-learn* implementation of LR as a binary classifier with all default values for the hyperparameters. The SLP was developed in the 1950s by Frank Rosenblatt and is the most basic form of neural network [33]. The input features are weighted in a linear combination that can either be sent through a sigmoidal activation function for binary classification or through a linear activation function for regression. Feature importance is conveniently derived directly from the feature weights, which makes the SLP an easy-to-interpret ML algorithm. We used the *scikit-learn* implementation of SLP as a binary classifier with all default hyperparameter values. The SVM was created by Hava Siegelmann and Vladimir Vapnik as a margin-based classifier using so-called support vectors to separate classes in the feature space [34]. Feature importance is derived directly from the coefficients of the support vectors of linear kernels. We used the *scikit-learn* implementation of the linear SVM with all default hyperparameter values.

All four models were trained using a train/test split of 80/20 with GroupShuffleSplit() from *sklearn.model_selection*. This method allows users to specify which samples must be grouped together after the split, permitting us to keep the RSEM and STAR replicates in the same train and test groups and thereby prevent target leakage. The models were validated using the *scikit-learn* implementation of k-fold cross validation, and we used k=5 as the number of folds because we had such few samples. Because of the small number of samples, we repeated the experiments several times using different seeds for the random number generators used throughout the pipeline. We deployed the four classification algorithms as binary classifiers in two experiments: predicting age (OLD vs YNG) and predicting condition (FLT vs GC) using gene expression data as predictors. After training each model, we identified the features most predictive of the classes using the two methods described in the next section.

Per-Model Feature Importance

In our method, we combined multiple ML algorithms into an ensemble classifier to predict either experimental condition (ground control vs spaceflight) or age (young vs old). We quantified feature importance by coefficient magnitude in two

parts of the pipeline: cross-validation and a standard train-test split. In the cross-validation setting, the data were partitioned into 5 folds, and models were then trained on a single fold and evaluated on the other 4 folds. This procedure yielded 5 fitted estimators. For each estimator, *scikit-learn* provided coefficients from which we derived feature importances. We then averaged the importances for each feature across the folds, ranked the features according to this mean value, and kept the top 50 highest-coefficient features. In the train-test approach, we fitted the model to the training set, ranked the coefficients by magnitude and selected the top 50 as the most predictive features. We combined these two gene sets together into a single set of genes using the union set operation and then removed genes overlapping with other experiments as described in the next section.

Per-Experiment Ensemble Voting

Ensemble predictions are commonly aggregated by majority voting [35]. For each experiment, we first formed, for each algorithm, the union of the two feature importance lists. We then applied majority voting across the 4 algorithm-specific unions, retaining genes that were present in at least 3 of them. We obtained the final label predictive set with a difference operation, as described in the next section.

Final Gene Set Formulation

To determine the genes that are most predictive of a target (age or condition) for a given subset of mice (eg, YNG vs OLD or FLT vs GC), we removed those genes that are generally predictive of the target, regardless of their subset. In this way, we identified the marginal set of genes that are uniquely predictive of the target within that subset. For example, in the experiment in which we predicted age, we ran 3 experiments: one in which we used only ground control samples to predict age (GC:age), one in which we used only spaceflight samples to predict age (FLT:age), and one in which we used all the samples combined to predict age (ALL:age). Each of these 3 experiments produced a set of gene results as previously described. In Figure 3, we showed how we formulated our final set of gene results for analysis. We adopted the notation {X} \ {Y} to represent the difference in set membership between sets X and set Y.

Results

In this section, we discuss the final results of our 4 experiments: predicting age for ground control samples, predicting age for spaceflight samples, predicting condition for old samples, and predicting condition for young samples.

Model Performance

Since our models do not classify outcomes as “positive” and “negative” with different associated costs, metrics such as the false positive rate and false negative rate offer limited insight. Given the imbalanced class distribution between ground control and spaceflight groups, accuracy is an inadequate performance measure. To evaluate model performance using a single comprehensive metric, we selected the F_1-score, which represents the harmonic mean of precision and recall, as our primary performance indicator. Table 3 displays the F_1-score (averaged over 5 different random number generator seeds) of each of the 4 classification models in the ensemble for the experiments predicting age in FLT, GC, and ALL groups. The train and test scores were obtained using the 80/20 train/test split data sets, and the cross-validate score is the mean score across the 5 folds.

Table . Average F_1-score for training, testing, and cross-validation of each of the classification models (stochastic gradient descent, support vector machine, logistic regression, and single-layer perceptron) for the experiments predicting age (FLT[a]:age, GC[b]:age) for those mice in the FLT and GC groups.

Model and experiment		Train	Test	Cross-validate
Stochastic gradient descent				
	FLT:age	1.0	0.96	0.89
	GC:age	1.0	0.99	0.98
Support vector machine				
	FLT:age	1.0	1.0	0.91
	GC:age	1.0	1.0	0.99
Logistic regression				
	FLT:age	1.0	1.0	1.0
	GC:age	1.0	1.0	1.0
Single-layer perceptron				
	FLT:age	1.0	1.0	1.0
	GC:age	1.0	0.99	1.0

[a]FLT: spaceflight.

[b]GC: ground control.

Table 4 displays the performance of each of the 4 classification models in the ensemble for the experiments predicting the condition for YNG and OLD groups.

Table . Average F_1-score for training, testing, and cross-validation of each of the classification models for the experiments predicting condition (OLD[a]:cnd[b], YNG[c]:cnd) for those mice in the OLD and YNG groups.

Model and experiment		Train	Test	Cross-validate
Stochastic gradient descent				
	OLD:cnd	1.0	0.97	0.84
	YNG:cnd	1.0	0.99	0.91
Support vector machine				
	OLD:cnd	1.0	1.0	0.90
	YNG:cnd	1.0	1.0	0.92
Logistic regression				
	OLD:cnd	1.0	1.0	1.0
	YNG:cnd	1.0	1.0	1.0
Single-layer perceptron				
	OLD:cnd	1.0	0.96	0.94
	YNG:cnd	1.0	1.0	1.0

[a]OLD: old.

[b]cnd: condition.

[c]YNG: young.

As shown in Tables 3 and 4, all the train scores had a perfect F_1-score, and all but 3 of the test scores in each table were also perfect. The cross-validate score is useful in determining to what extent there is bias in the model due to how the train and test data were split, or how much the model is otherwise overfit. The experiments predicting condition for young mice outperformed the same experiments for old mice. The SLP and LR models outperformed SGD and SVM in all experiments. In Table 2, SGD scored the lowest F_1-scores in both experiments (OLD:cnd, YNG:cnd) predicting the condition. Because we used the majority consensus for our feature voting algorithm, we acknowledge SGD as the weakest learner for those experiments and accept the results from the rest (majority) of the ensemble. After training each model, we identified those genes most predictive of their respective target. We present these results in the next section.

Most Predictive Genes

In this section, we discuss the genes most predictive of the targets for each experiment. Textbox 1 lists the genes most predictive of the label for each of the experiments.

Textbox 1. List of genes most predictive of the target (age, condition [cnd]) for the given subset (GC [ground control], FLT [spaceflight], YNG [young], OLD [old]).

GC:age

- *Aip, Aldh2, Ceacam10, Ciao2b, Clec4d, Csn1s2a, Ctsz, Dmbt1, Gng2, Gstm1, Klk4, Lrrc30, Mrgprb1, Myh8, Nudt9, Or13a27, Pam, Park7, Prom1, Psmc4, Psmd2, Slc5a5, Smyd2, Syngr2, Tle5, Vmn1r38, Wap, Wdr18, Yif1b, Znhit2*

FLT:age

- *Acsm1, Acss2, Adamdec1, Adcy10, Ahsg, Aldoa, Aldob, Ap2b1, Apoa1, Apoa2, Apoa4, Atp1a3, Atp6ap1, Bmp2k, Ces1g, Chrna5, Cps1, Cyp2c29, Cyp2c50, Elovl3, Epyc, Fabp1, Fga, Fgb, Fmo3, Gbp11, Gc, Gnl1, Hadha, Hspa5, Immt, Lmod2, Lrrc59, Maob, Mat1a, Mogat2, Mrpl30, Mtch1, Ncan, Psmb7, Ptk7, Rad23b, Ramp2, Rdh11, Scgb1c1, Serpinf2, Slc10a1, Slc25a3, Slc25a39, Slc27a5, Slc38a3, Ssx2ip, Stfa3, Sult3a1, Tat, Tdrd9, Tmem259, Ugt2b34, Uox, Urod, Zfp747*

YNG:cnd

- *Aar2, Abcc6, Acot11, Apcdd1, Aspg, Cdcp3, Elovl3, Ergic1, Gale, H1f0, Hspb8, Kcng4, Ltc4s, Maff, Map3k4, Mogat2, Mrpl47, Mrps18a, Ncan, Odad4, Pnpla5, Postn, Ppcs, Prune2, Rdh11, Scd2, Sfxn5, Smtnl2, Tekt1, Tmprss11a, Vstm2b*

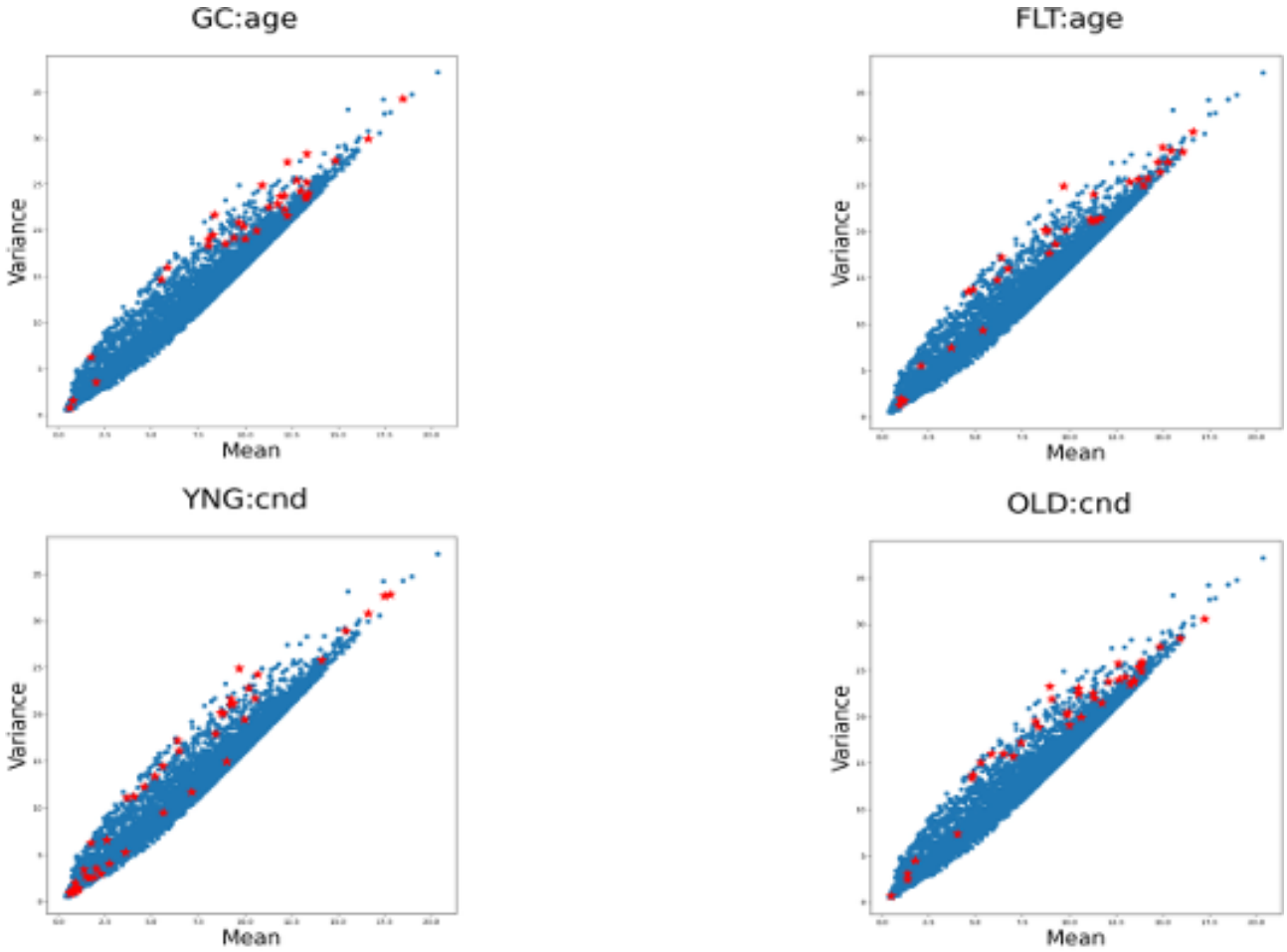
OLD:cnd

- *6430571L13Rik, Acad10, Actl6b, Agtr1a, Ambp, B3gnt7, Begain, Calca, Ceacam20, Cuta, Dgat2, Fgf21, Glud1, Igfbp4, Igsf21, Jmjd8, Krt12, Krtap6-7, Map6d1, Mrpl42, Or2y1e, Or51r1, Or56b35, Rgs16, S100a9, Tcap, Trim9, Ttr, Vmn1r32*

The genes listed in constitute the final results of our ML ensemble that resulted from the set operations portrayed in Figure 3.

In Figure 4, we show the distribution of gene expression for the most predictive genes of each experiment across the distribution of all the genes that were used to train the models.

Figure 4. Scatter plots of variance versus mean for the experiments predicting age (top row) and predicting condition (bottom row). The blue points are the background genes (ie, all 750 genes that were used to train the model), and the red points are most predictive of their respective target. cnd: condition; FLT: spaceflight; GC: ground control; OLD: old; YNG: young.



As shown in Figure 4, the distribution of the genes identified by our ML ensemble across the spectrum of expression is approximately uniform. From that, we can infer that the ML algorithms do not portray any bias based on the magnitude (mean or variance) of the distributions of gene counts. This indicates that the models and their ensemble are not vulnerable to the heteroskedastic nature of gene expression count data. Note that the distribution of genes predicting age is different than the distribution of genes predicting condition because we used the 750 genes most correlated to the respective target. We next show which biological pathways are enriched by the GC:age, FLT: age, YNG:cnd, and OLD:cnd gene sets.

Pathway Enrichment Analysis

We submitted our lists of most predictive genes to ShinyGO (version 0.81)—an online pathway enrichment analysis tool [36]—using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [37], a false discovery rate cutoff of .05, and minimum gene set intersection size of 2, and displayed the top 5 most enriched pathways. The results of these analyses are captured in Table 5.

Table . Pathway enrichment analyses for the machine learning experiments. All corresponding false discovery rate *q* values were statistically significant to an α level of less than .05.

Experiment and pathways	Genes	False discovery rate *q* value
GC[a]:age		
No enrichment	—[b]	—
FLT[c]:age		
Metabolic pathways	• *Ugt2b34, Cyp2c50, Maob* • *Aldoa, Acsm1, Mat1a* • *Elovl3, Cyp2c29, Fmo3* • *Rdh11, Uox, Urod, Cps1* • *Aldob, Mogat2, Tat* • *Slc27a5, Adcy10, Atp6ap1* • *Acss2, Hadha*	1.456e-07
Fat digestion and absorption	• *Apoa1, Apoa4, Fabp1, Mogat2*	0.00037
Biosynthesis of amino acids	• *Aldoa, Mat1a, Cps1, Aldob*	0.00264
Peroxisome proliferator-activated receptor signaling pathway	• *Apoa1, Apoa2, Fabp1, Slc27a5*	0.00331
Retinol metabolism	• *Ugt2b34, Cyp2c50* • *Cyp2c29, Rdh11*	0.00347
YNG[d]:cnd[e]		
Biosynthesis of unsaturated fatty acids	• *Elovl3, Scd2*	0.02312
Fatty acid metabolism	• *Elovl3, Scd2*	0.03802
Metabolic pathways	• *Ppcs, Elovl3, Ltc4s, Rdh11, Scd2, Mogat2, Gale* • *Rdh11, Scd2, Mogat2* • *Gale*	0.00779
OLD[f]:cnd		
No enrichment	—	—

[a]GC: ground control.

[b]Not applicable.

[c]FLT: spaceflight.

[d]YNG: young.

[e]cnd: condition.

[f]OLD: old.

The most important genes predicting the age in the ground control group (GC:age) and those predicting the condition in the old group (OLD:cnd) did not significantly enrich any of the KEGG pathways. The genes most predictive of age in the spaceflight group (FLT:age) enriched several KEGG pathways, the top 5 of which are shown in . The metabolic pathways enrichment represents a very broad class of biological functions including lipid metabolism, energy metabolism, and xenobiotic metabolism. The peroxisome proliferator-activated receptor (PPAR) signaling pathway represents fatty acid oxidation, lipoprotein metabolism, and an anti-inflammatory response. Because retinoids are antioxidants, the retinol metabolism pathway is likely responding to oxidative stress. The genes most predictive of condition for the young mice (YNG:cnd) also primarily enriched membrane lipid metabolism, inflammatory stress signaling, and overall metabolic capacity. All these pathways being enriched suggests that spaceflight amplifies age-related differences in metabolic flexibility, especially in pathways that manage lipid metabolism in response to inflammation and oxidative stress. In the Discussion section, we will explore this theme further in the context of breast cancer.

Discussion

Principal Findings

In this study, we used a novel approach combining results from an ensemble of 4 linear classifier ML models to predict condition (spaceflight or ground control) and age (young or old) using features derived from gene expression data. The results reveal distinct gene expression signatures that differentiate both

age and exposure to spaceflight in mice, revealing some of the molecular mechanisms that may underpin the effects of spaceflight and aging and their potential impact on breast cancer. In this section, we discuss the principal findings of our research in the context of breast cancer, compare our approach to other ML approaches on transcriptomic data, describe strengths and limitations to our methods, and conclude with considerations toward future directions of this research.

Our research finds that the younger mouse cohort mounted a differential response to spaceflight with respect to their older counterparts. One reason for this may be that younger cells have higher plasticity, and therefore their tissue has greater capacity to respond to the environment [38]. Older cells may have blunted responses because they have exhausted their capacity to respond due to accumulated stress [39]. Another reason may be signal saturation: older tissue has chronic low-grade inflammation and is already expressing a stress response to oxidative damage at a baseline [40]. In the context of breast cancer risk due to spaceflight, our research paradoxically suggests that the younger cohort may have an increased risk due to the simultaneous modulation of PPAR signaling and fatty acid biosynthesis. The younger cohort gene expression enriched unsaturated fatty acid metabolism pathways in the *Elovl3* and *Scd2* genes. Galactic cosmic radiation generates reactive oxygen species, which attack unsaturated fatty acids in membranes, leading to lipid peroxidation [41]. Damaged lipids, if left unchecked, can cause mitochondrial and nuclear membrane damage, leaving cells struggling to maintain basic homeostasis [42]. The genes enriching the PPAR signaling pathway (*Apoa1*, *Apoa2*, *Fabp1*, and *Slc27a5*) are all PPAR-α genes, which promote the breakdown of damaged fatty acids so they may be used as an energy source [43]. This can lead to a vicious cycle whereby fatty acids are synthesized and then oxidized, inducing reactive oxygen species production, which causes more lipid peroxidation [44]. The subsequent proliferation of peroxisomes would put these younger mammary cells under chronic oxidative stress and increase carcinogenic potential [45].

Our research suggests that older mice may be at increased risk of breast cancer for different reasons. In the experiment predicting condition (spaceflight vs ground control) for all mice, their most predictive genes enriched pathways in retinol metabolism and PPAR signaling. The genes enriching the retinol metabolic pathway include *Ugt2b34*, *Cyp2c50*, *Cyp2c29*, and *Rdh11*. The *Rdh11* enzyme, or retinol dehydrogenase 11, synthesizes retinoids, which regulate cell proliferation, promote cell differentiation, and induce apoptosis—all of which help prevent and suppress mammary gland tumor formation [46]. However, the *Cyp2c50* and *Cyp2c29* genes are degradation enzymes in this pathway and lead to retinoid depletion. Moreover, the *Ugt2b34* gene is an excretion enzyme that eliminates active retinoids. The overall metabolic impact on this pathway may lead to the degradation of retinoids, which would greatly increase the risk of developing breast cancer [47]. The simultaneous disruption of PPAR signaling and retinoid metabolism in mammary tissue following spaceflight represents a synergistic increase in breast cancer risk [48-50]. This two-hit disruption is normally more severe in older animals due to depleted antioxidant reserves and reduced metabolic flexibility [51], suggesting that older individuals may face substantially elevated breast cancer risk from spaceflight exposure.

Comparison to Prior Work

Zhang et al [52] built an ML model that leverages a transformer architecture, incorporating phenotype prediction, biomarker discovery, and identification of implicated biological processes into a single model using transcriptomic data as features. Our research provides similar types of analyses, but we use binary classification models for phenotype prediction and two forms of feature importance to identify biomarkers; we also leverage an existing, well-used framework (ie, KEGG pathways) for identifying biological processes. Smith et al [53] use a similar set of data processing steps in their pipeline (converting gene counts to transcripts per million, applying log transformations) in an ML ensemble, but they use regression rather than classification to predict phenotypes. Arnold et al [18] examined the same dataset (OSD-511) as the one explored in this research but used differential gene expression analysis to identify the biomarker genes that distinguish young from old and spaceflight from ground control mice. Differential gene expression analysis is a commonly used technique for high-dimensional data but suffers from multiple test burden and an inability to distinguish between true and spurious correlations.

Strengths and Limitations

The first strength and motivating factor for studying this data is to maximize the utility of underpublished in vivo research in controlled spaceflight experiments. Murine experimentation in space is very costly, time-consuming, and requires sacrificing animals. As an observational study, we obtained real-world insights without further cost and sacrifice. The second strength of our approach is model interpretability. Particularly in the context of predicting biomedical outcomes, using whitebox, linear decision-boundary models such as SGD classifier, SVM, LR, and SLP enables transparency, engenders trust, and provides more straightforward biological insight into a high-dimensional feature space such as gene expression data. The third strength of our approach is the use of simple set operations (union, intersection, and difference) to improve interpretability. The fourth strength of our approach is the use of the KEGG database as a trusted, well-known pathway enrichment analysis database to further promote simplicity and trust.

The first limitation of our approach is that we excluded many ML methods, such as multilayer perceptrons and other deep learning architectures, that may outperform the ones we used at the expense of simplicity and interpretability. The second limitation of our study is the sensitivity of the results to our preprocessing. For example, removing genes that have low counts and are not correlated to the target reduces the signal-to-noise ratio in a high-dimensional feature space. However, because some biological processes are sensitive to slight variations in gene expression, we may have removed some of the genes that contribute to the phenotypes that our models predicted. Filtering out genes that do not code for proteins allows our pathway enrichment analysis to focus on well-understood genes, though again, we understand that noncoding genes may also have contributed to the phenotypes. The third limitation of our study is the paucity of data. We would

feel more confident in our results if we could explore a larger and more varied collection of samples. The fourth limitation of our study is the lack of an in vivo or in vitro validation of our findings. Although the gold standard in biomarker identification is the randomized controlled trial, our observational research serves to inform such a study and can restrict the search space of an otherwise very resource-intensive endeavor. The last limitation of our research is that it relies on a single point-in-time snapshot of the mammary transcriptome via bulk RNA-seq. A better approach would be a longitudinal investigation that elucidates time as a contributing factor to spaceflight response.

Future Directions

Our research has identified putative genes and pathways implicated in age-differentiated pathological responses to spaceflight in mammary tissue. Future work may include single-cell RNA sequencing and proteomic sequencing to give higher resolution and downstream validation, respectively. Combining multiple datasets from similarly controlled experiments to increase the number of biological replicates would, in turn, increase confidence in our ML results. These findings offer valuable information for further studies into the impact of spaceflight on female astronaut health, reiterates well-established roles between spaceflight and breast cancer risk, and provides a straightforward ML approach to leverage a vast array of unexplored data.

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Data Availability

The notebook for this research is available at [54]. The OSD-511 dataset is available at [55].

Authors' Contributions

JC designed the experiments and wrote most of the manuscript. TZ and JY organized the efforts of the student researchers (AA, AR, AM, AF, KS, SL, WG, AL) who explored alternative approaches to processing the data and validated the references. The ensemble approach was conceived with SC; using linear decision boundary classifiers for ease of interpretation was conceived by MSC. All authors proofread the manuscript and provided their feedback.

Conflicts of Interest

None declared.

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Abbreviations

AI: artificial intelligence
cnd: condition
FLT: flight
GC: ground control
KEGG: Kyoto Encyclopedia of Genes and Genomes
LR: logistic regression
ML: machine learning
NASA: National Aeronautics and Space Administration
OLD: old
PCA: principal component analysis
PPAR: peroxisome proliferator-activated receptor
RNA-seq: RNA-sequencing

RRRM-1: Rodent Research Reference Mission 1
RSEM: RNA-Seq by Expectation Maximization
SGD: stochastic gradient descent
SLP: single-layer perceptron
STAR: Spliced Transcripts Alignment to a Reference
SVM: support vector machine
VIV: vivarium
YNG: young

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Peer Review of “Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study”

Ahmed Madi Waheed Al-Mayahi

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KEYWORDS

leaf lettuce; shoot regeneration efficiency; 6-benzylaminopurine; seed coat color; CIELAB color scale; flavonoid; BAP

This is the peer-review report for “Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study.”

Round 1 Review

General Comments

Major Comments

- The presented work [1] brings new information.
- At the beginning of your abstract, you should write a paragraph about the problem you want to solve.
- The abstract mentions statistical significance but does not provide any details about how these were assessed or the significance level (eg, *P* value). Details on the statistical analysis methods used (eg, “significant at *P*<.05”) should be added.
- The Introduction contains well-documented data that are widely known. Hormonal information has been extensively reported and reviewed. Against this background, authors have to point out how this work is different from the earlier reported work; what are the innovative findings reported here? A strong and convincing justification is required.
- Introduction: While references are important, the paragraph reads as somewhat overloaded with citations. Many sentences contain a high number of citations, which can disrupt the readability of the text. Try to reduce the frequency of citations by grouping them more effectively and summarizing the findings rather than listing individual sources for every claim. This will help make the text more fluid.
- The Methods section in its current form is not acceptable because it requires more details, such as the latitude and longitude of the culture area. Write a simple paragraph describing the climate of the area and date of study.
- It is necessary to mention the active ingredient of commercial chlorine bleach.
- Tween-20 is used with disinfectants to reduce surface tension, thus increasing the disinfectant’s effectiveness.
- State the manufacturer of the MS medium and the quantity used to prepare it half-strength.
- How were the hormone solutions prepared and dissolved?
- KOH and HCl are used in the pH adjustment process.
- It is necessary to mention the lighting intensity during the incubation period of the cultures.
- The statistical analysis mentions EZR software, but there is no explanation of why this particular software was chosen.
- In the Discussion, authors have explained various biochemical interactions and mechanisms that are widely known and reported. Authors should give their own reflections of the work. It is essential to include the advantages and shortcomings of the work; what are the limitations of this technology and its shortfalls? Authors’ own scrutiny of the data clarifications is decisive for the impending research on this subject. This work is field-oriented, the cost-benefit ratio is very significant, and micropropagation will increase the cost, but this has not been commented on in the text. Scale-up of the tissue culture plant is not an easy task and would be challenging work.
- Conclusion: What does this infer for lettuce production? Need a little more work to show the significance of your work.
- References: It is advised to refer only to recent work and not old citations.

Round 2 Review

General Comments

After reviewing the manuscript, I found substantial improvements, which positively impacted its scientific value. Therefore, the manuscript meets the requirements for publication.

Conflicts of Interest

None declared.

Reference

1. Kimura M, Yoshizumi T. Relationship between seed coat color and cytokinin concentration in efficiently regenerating leaf lettuce shoots: in vitro experimental study. JMIRx Bio 2026;4:e70496. [doi: 10.2196/70496]

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Peer Review of “Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study”

Hamidreza Soufi

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leaf lettuce; shoot regeneration efficiency; 6-benzylaminopurine; seed coat color; CIELAB color scale; flavonoid; BAP

This is the peer-review report for “Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study.”

Round 1 Review

Reviewer’s Comments on the Manuscript

The manuscript [1] presents a well-structured and novel study exploring the correlation between seed coat color and the optimal concentration of 6-benzylaminopurine (BAP) for shoot regeneration in leaf lettuce cultivars. The research is timely and addresses a significant challenge in plant tissue culture—genotypic variability in regeneration efficiency.

Strengths

The experimental design is solid, involving 6 cultivars with distinct seed coat colors.

The use of the CIELAB color scale adds objectivity to phenotypic assessments.

The identification of seed coat color as a potential morphological marker for shoot regeneration efficiency is innovative and potentially valuable for breeding and transformation programs.

Suggestions for Improvement

Language and clarity: While the scientific content is strong, the manuscript would benefit from careful language editing for grammar and fluency.

Statistical reporting: The statistical significance (eg, *P* values) is noted, but a more detailed description of the statistical models and effect sizes would enhance reproducibility.

Figures and tables: Ensure that all figures and tables referenced (eg, Figure 1, Table S1) are clearly labeled and formatted for clarity. Including a visual summary (graphical abstract) could further enhance impact.

Discussion depth: The discussion of mechanisms linking seed coat pigmentation to shoot regeneration could be expanded, possibly integrating flavonoid biosynthesis and tissue culture responsiveness more.

Conclusion: Consider sharpening the Conclusion to emphasize the practical applications of the findings, especially in the context of lettuce transformation systems.

Overall, this is a meaningful contribution to plant biotechnology literature and warrants publication after minor revisions.

Conflicts of Interest

None declared.

Reference

1. Kimura M, Yoshizumi T. Relationship between seed coat color and cytokinin concentration in efficiently regenerating leaf lettuce shoots: in vitro experimental study. JMIRx Bio 2026;4:e70496. [doi: 10.2196/70496]

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Authors' Response to Peer Reviews of "Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study"

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KEYWORDS

leaf lettuce; shoot regeneration efficiency; 6-benzylaminopurine; seed coat color; CIELAB color scale; flavonoid; BAP

This is the authors' response to peer-review reports for "Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study."

Round 1 Review

Reviewer DQ [1]

The reviewer acknowledged the novelty and robustness of our study [2] but suggested improving the language, statistical reporting, and discussion depth.

Language and clarity: While the scientific content is strong, the manuscript would benefit from careful language editing for grammar and fluency.

- **Response:** The manuscript was professionally proofread for grammar and clarity.
- *Statistical reporting: The statistical significance (eg, P values) is noted, but a more detailed description of the statistical models and effect sizes would enhance reproducibility.*
- **Response:** Statistical methods are now detailed in the Methods section (one-way ANOVA with Tukey test, $P<.05$).
- *Figures and tables: Ensure that all figures and tables referenced (eg, Figure 1, Table S1) are clearly labeled and formatted for clarity.*
- **Response:** All figures and tables have been relabeled and referenced in the correct order.
- *Discussion depth: The discussion of mechanisms linking seed coat pigmentation to shoot regeneration could be expanded, possibly integrating flavonoid biosynthesis and tissue culture responsiveness more.*
- **Response:** The Results and Discussion section now includes an expanded interpretation linking flavonoid metabolism to cytokinin responsiveness.
- *Conclusion: Consider sharpening the Conclusion to emphasize the practical applications of the findings, especially in the context of lettuce transformation systems.*
- **Response:** The Conclusion emphasizes the practical application of optimizing transformation efficiency in lettuce.
- *Including a visual summary (graphical abstract) could further enhance impact.*
- **Response:** A graphical abstract was considered but omitted because Figures 1-3 fully summarize the experimental results.

Reviewer FA [3]

- *At the beginning of your abstract, you should write a paragraph about the problem you want to solve.*
- **Response:** The Abstract begins with a clear problem statement: cultivar-dependent shoot regeneration efficiency.
- *The abstract mentions statistical significance but does not provide any details about how these were assessed or the*

significance level (eg, P value). Details on the statistical analysis methods used (eg, "significant at P<.05") should be added.

- **Response:** Quantitative and statistical details ($P<.05$) have been added to the Abstract.
- *The Introduction contains well-documented data that are widely known. Hormonal information has been extensively reported and reviewed. Against this background, authors have to point out how this work is different from the earlier reported work; what are the innovative findings reported here? A strong and convincing justification is required.*
- **Response:** The Introduction has been rewritten to clarify the originality and novelty of our study.
- *The Methods section in its current form is not acceptable because it requires more details, such as the latitude and longitude of the culture area. Write a simple paragraph describing the climate of the area and date of study.*
- *It is necessary to mention the active ingredient of commercial chlorine bleach.*
- *KOH and HCl are used in the pH adjustment process.*
- **Response:** These Methods have been expanded to include climate information (humid subtropical, Cfa), bleach composition (6% NaOCl, final 1.2%), and pH adjustment (KOH/HCl).
- *Tween-20 is used with disinfectants to reduce surface tension, thus increasing the disinfectant's effectiveness.*
- **Response:** Tween-20 was mentioned by the reviewer but was not used in our sterilization protocol. Surface sterilization was performed using 70% ethanol and 20% bleach without surfactants.
- *It is necessary to mention the lighting intensity during the incubation period of the cultures.*
- **Response:** The light intensity during incubation was approximately 300 µmol m^{-2} s^{-1} under cool white fluorescent lamps.
- *The statistical analysis mentions EZR software, but there is no explanation of why this particular software was chosen.*
- **Response:** The rationale for using EZR software has been provided, noting that the software is a free R-based statistical platform suitable for general biological data analysis.
- *In the Discussion, authors have explained various biochemical interactions and mechanisms that are widely known and reported. Authors should give their own reflections of the work. It is essential to include the advantages and shortcomings of the work; what are the limitations of this technology and its shortfalls? Authors' own scrutiny of the data clarifications is decisive for the impending research on this subject. This work is field-oriented, the cost-benefit ratio is very significant, and micropropagation will increase the cost, but this has not been commented on in the text. Scale-up of the tissue culture plant is not an easy task and would be challenging work.*
- **Response:** The Discussion has been expanded with a new section, "Limitations and Future Applications," addressing the scalability, cost, and practical applicability of our method.
- *Conclusion: What does this infer for lettuce production? Need a little more work to show the significance of your work.*
- **Response:** The Conclusion has been revised to emphasize the implications of large-scale lettuce transformation.
- *References: It is advised to refer only to recent work and not old citations.*
- **Response:** The references have been updated to include recent literature (2022 - 2025).

References

1. Soufi H. Peer review of "Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study". JMIRx Bio 2026;3:e89399. [doi: 10.2196/89399]
2. Kimura M, Yoshizumi T. Relationship between seed coat color and cytokinin concentration in efficiently regenerating leaf lettuce shoots: in vitro experimental study. JMIRx Bio 2026;4:e70496. [doi: 10.2196/70496]
3. Al-Mayahi AMW. Peer review of "Relationship Between Seed Coat Color and Cytokinin Concentration in Efficiently Regenerating Leaf Lettuce Shoots: In Vitro Experimental Study". JMIRx Bio 2026;4:e89401. [doi: 10.2196/89401]

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Authors' Response to Peer Reviews of "Machine Learning Ensemble Investigates Age in the Transcriptomic Response to Spaceflight in Murine Mammary Tissue: Observational Study"

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KEYWORDS

machine learning; spaceflight; mammary tissue; gene expression; mice; breast cancer; feature importance

This is the authors' response to peer review reports related to "Machine Learning Ensemble Investigates Age in the Transcriptomic Response to Spaceflight in Murine Mammary Tissue: Observational Study."

Live Review Round [1]

List of Major Comments

- *The title of this paper [2] should be more specific with respect to the source of mammary tissue: identify "mouse mammary gland tissue" in the title or, perhaps, simply "murine mammary tissue."*
- **Response:** We changed the title as suggested to "Machine learning ensemble investigates age in the transcriptomic response to spaceflight in murine mammary tissue: observational study."
- *While the methodology is interesting and the findings certainly warrant further study, this should be clearly identified as formative research: there was no preregistration of hypotheses and methods, and the findings (list of key genes and of pathways differing according to age) are just suggestive and not at all robust or convincing. Accordingly, some detail about the experiences of the mice and physiological values is beside the point, so we suggest it is moved to a "Supplements" section along with more specifics about machine learning parameters, etc, that could help researchers attempting similar approaches.*
- **Response:** We describe in the newly-added Strengths and Limitations section of the manuscript that our in silico findings need to be validated in vitro. We also make the software (as a Jupyter notebook) available so that our approach may be repurposed or reproduced.
- *With respect to the OSD-511 dataset, the details of Rodent Research Reference Mission 1 need revision, as it was mentioned that there are 40 female BALB/cAnNTac mice, while the total number of animals used was 43: 21 younger mice and 22 older mice. Moreover, the 8 younger mice that were kept in standard cages were exposed to different conditions from the 7 older mice that were housed in flight hardware.*
- **Response:** We rectified the counts and created Table 1 for clarity.
- *In addition, it was mentioned that each group of space-flown mice had corresponding control groups (ground control), but it is not clear which basal controls (10 mice euthanized 1 day post launch) are used to compare which group. This is important to explain the single group called "non-flight" that is mentioned later in the paragraph, and indicate if these latter details from the original experiment are not available to the authors.*

- **Response:** We added explanations to specify which mice were used in which grouping.
- *In the Discussion section, or as a separate Limitations sections, consider explicitly pointing out that data of experimental mice that were collected just once after 40 days in space and 2 days post return recovery provides only cross-sectional data and does not capture changes in the mice that could be evident while in space or longer after return from space. Also, the description for Figure 1 mentions Figure 1E and F, which are not available in the figure.*
- **Response:** We added this and several others to a dedicated section called "Strengths and Limitations" in the Discussion.
- *The small sample size should be acknowledged, which means the outcome models may not be able to generalize well on unseen data in downstream tasks.*
- **Response:** We describe how we augmented the data in the Methods section. We also call out the paucity of data in the Strengths and Limitations part of the Discussion.

Minor Comments

- *The title could be enhanced to make it clear that this was an experiment based on a model organism (mouse) and not human.*
- **Response:** We changed the title as suggested to "Machine learning ensemble investigates age in the transcriptomic response to spaceflight in murine mammary tissue: observational study."
- *The reviewers acknowledge the availability of details that enable the reproducibility of the study, such as publicly accessible data sources and detailed description of data handling and analysis procedures. However, the reviewers wondered whether the source code used could be availed for enhancing the reproducibility.*
- **Response:** Per this suggestion, we made the code available to the reader.
- *The total number of mice stated that were used in the study does not correspond with the total number used, based on the breakdown of individual group numbers. Authors need to cross-check the numbers to ensure that they tally with the numbers used.***Response:** We rectified the counts and created Table 1 for clarity.
- *Clarify the composition of the control cohort, refer to those mice in a consistent way, and discuss differences that were found to exist between the subsets of controls.*
- **Response:** We rectified the counts and created Table 1 for clarity.
- *In page 4, under the Data Transformation section, it is stated that "four filtering methods were performed," but Figure 2B only represents three filters. Kindly clarify if the fourth filtering method was used but not included in the figure or whether there was a mistake in either the figure or the text for the sake of consistency.*
- **Response:** We updated Figure 2B to include four filter icons.
- *On page 6, the last paragraph, a linear regression model was used to predict the weight of mice at euthanasia, but the significance of this prediction was not discussed. The significance should be discussed for a better understanding of its applicability. Add a brief discussion of the significance of the model, which may include a statistical test validation such as P values and/or CIs.*
- **Response**: We removed the linear regression model from the ensemble.
- *On page 15, under the Conclusion section, it is also mentioned that "The dysregulation of ECM [extracellular matrix] remodeling, cytoskeletal function, and stress response pathways was observed in radiation-exposed mice," but radiation exposure was not the intervention applied. Revise this statement to accurately reflect the intervention applied in this study (spaceflight) and ensure the conclusion is per the experimental conditions.*
- **Response:** We updated the model and the subsequent pathway results do not include extracellular matrix remodeling.
- *In the Discussion section, some results are repeated instead of being analyzed in depth. Focus more on interpreting the results, compare them with similar studies, and discuss their significance.*
- **Response:** We added a lot of content interpreting the results in the Discussion section, along with comparing to similar studies and discussing their relevance.
- *Only accuracy is reported for model performance metrics. Add other metrics, including area under the receiver operating characteristic curve, sensitivity, specificity, and F_1-score, to enhance the assessment of the model's predictive ability.*
- **Response:** We changed our model performance metric to use the F_1-score.
- *Under the algorithms discussion, remove possessive apostrophe from the "1950's."*
- **Response:** We removed the possessive apostrophe.
- *It may help to add a statement to make it explicit whether ethics approval was necessary for the study. In addition, it would add value in discussing ethical implications of collecting the dataset used in the manuscript with reference to any discussion in previous publications or from the authors who collected the original data.*
- **Response:** We added an entire section dedicated to ethics approval.

Concerns with Figures and Tables

- *Most figures have poor resolution, which makes them difficult to understand or interpret. It would be helpful to regenerate the figures with better resolution.*
- **Response:** We increased the resolution of all our images.
- *It would be helpful to add details to the captions to include what's represented in each panel and any elements of statistics.*
- **Response**: We added additional explanations to the captions of all figures and tables.

- *Creating a table to present the various groups and their characteristics, including ground control, would help improve readability.*
- **Response:** We created Table 1 for this purpose.
- *Figure 1 lacks an adequate explanation of each panel, which will clarify what they represent.*
- **Response:** We added additional explanations to the caption of Figure 1.
- *Table 1 is not clear, making it difficult to read.*
- **Response:** We made Table 1 more clear and legible.
- *The top and left parts of Figure 7 are cropped, and it is possible important information is omitted.***Response:** We omitted Figure 7.
- *The legend refers to plots by layout (left/right), duplicating the role of (a)-(d) labels. Also, plot titles are not the most prominent text and are not referenced in the text.*
- **Response:** We removed the "left/right" language from the caption and removed the plot titles from the figure.
- *In Figure 4, the term "accuracy" is used without clarification.*
- **Response:** We replaced Figure 4 with Table 2. Also, we replaced "accuracy" with "F_1-score" as the performance metric.
- *Abbreviations used in Figures 2 and 3 are not explained.*
- **Response:** We added explanations for all abbreviations and created an abbreviation table at the end of the manuscript.
- *The Figure 3 legend does not clearly describe the difference between the left and right diagrams.*
- **Response:** We removed "left/right" language from the figure caption and replaced it with letters and colors to be more clear.
- *The manuscript refers to Table 1 subsections "e" and "f," which are not present. Some figures are also unclear and not explanatory enough.*
- **Response:** We added Figure 1E and F to Figure 1. We also added more explanations to all of the figure and table captions.
- *Figure 5: Fonts are too small to read, and part of the legend is cropped.*
- **Response:** Figure 5 is now Figure 4 and has been updated with larger fonts, and we removed the legend.
- *In Figure 1, the caption states that the left plots represent ground mice and the right plots represent space mice, which is not reflected in the figure.*
- **Response:** We removed "left" and "right" language from the figure caption.
- *On page 4, the principal components analysis statement interpreting Figure 1A and D is misleading. The statement suggests that both Figure 1A and D show principal components analysis for spaceflight, whereas Figure 1A only represents ground mice.*
- **Response:** We updated the figure caption and interpretation to properly reflect the principal components analysis plots.
- *The text for Figure 1 describes Figure 1E and F, but these panels are not present.*
- **Response:** We added Figure 1E and F to Figure 1.

Additional Comments

- *Consider revising the title and abstract to identify that the study was conducted with data collected in a model organism or murine model.*
- **Response:** We changed the title as suggested to "Machine learning ensemble investigates age in the transcriptomic response to spaceflight in murine mammary tissue: observational study."
- *The second page, second sentence of the first paragraph: "Female astronauts in particular have an increased risk of breast cancer due to exposure to galactic cosmic radiation (7)." Please revise the reference, as Kumar et al [3] did not investigate or conclude the mentioned data.*
- **Response:** We modified the text further to be more inclusive in terms of breast cancer risk from ionizing radiation, including cosmic radiation.
- *On the second page, in the last sentence of the first paragraph, "Female astronauts...this increased vulnerability." Please provide a reference for the mentioned data.*
- **Response:** It is a summary statement of the previous statements encompassing 20 references.
- *The second page, second paragraph: "Machine learning (ML) has been leveraged but to a much lesser extent (15)." Please revise the reference as Larrañaga et al [4], as ML's role in bioinformatics has been widely expanded since 2006.*
- **Response:** We updated the sentence and changed the reference to a more recent one.
- *Page 6, second paragraph: It was mentioned that "The support vector machine was created by Hava Siegelmann and Vladimir Vapnik," and there is a reference to Cortes and Vapnik [5], while this work [6] was published in 2001.*
- **Response:** We are not using support vector clustering in our method.
- *Page 11, pathway enrichment analysis: Please identify the abbreviation "KEGG" as "Kyoto Encyclopedia of Genes and Genomes."*
- **Response:** We expanded the acronym at its first use. We also created a table of acronyms at the end of the manuscript.
- *Page 11, pathway enrichment analysis: Please identify the abbreviation "FDR" as "False Discovery Rate."*
- **Response:** We expanded the acronym on first use. We also created a table of acronyms at the end of the manuscript.

Concluding Remarks

- *In the Data Transformation section, groups were introduced for the first time in the manuscript (FLT vs GC and YNG vs OLD); these categories are defined later, but it would be good to spell out the names the first time they are mentioned. That's true for any other acronym used.*

- **Response:** We added an explanation for those and all other acronyms on first mention. We also created a table at the end of the manuscript that defines each acronym.

- *The article did not introduce a Limitation section. It is helpful to the reader to emphasize the limitations of the methods.*
- **Response:** We added a Strengths and Limitations section to the Discussion.

References

1. Sakilay S, Collier M, Rahgozar A, et al. Peer review of "Machine Learning Ensemble Identifies Distinct Age-Related Response to Spaceflight in Mammary Tissue". JMIRx Bio 2025;3:e75688. [doi: 10.2196/75688]
2. Casaletto JA, Zhao T, Yeung J, et al. Machine learning ensemble identifies distinct age-related response to spaceflight in mammary tissue. BioRxiv. Preprint posted online on Oct 6, 2025. [doi: 10.1101/2025.02.17.638732]
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