Plant Cell, Tissue and Organ Culture (PCTOC) (2026) 167:61
https://doi.org/10.1007/s11240-026-03663-0

BRIEF REPORT

PGR and LED light-dependent optimisation of isoflavonoid productivity in long-term cultivated calli of *Pueraria lobata* (Wild.) Ohwi

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Received: 16 August 2026 / Accepted: 17 November 2026
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Abstract

Various species of the genus *Pueraria* are common native plants of Southeast Asia and produce species-specific isoflavonoids and the classical isoflavonoids of leguminous plants. Isoflavonoids of *Pueraria lobata* (Wild.) Ohwi exhibits potent antioxidant and phytoestrogenic effects. The Russian Far East represents the northernmost area of the native range of *P. lobata*. However, *P. lobata* occurs in isolated, scattered locations and is highly conserved, which complicates research efforts. This study aims to develop a methodology for the initiation and long-term maintenance of calli derived from leaf explants of cultivated *P. lobata*. We tested 24 and 40 variants of media for the induction and subculturing of *P. lobata* calli, respectively. The optimal variants were selected: 2 mg L^{-1} tidiazuron for inducing callus formation and a combination of 5 mg L^{-1} benzylaminopurine and 0.1 mg L^{-1} 1-naphthaleneacetic acid for long-term subculturing in both solid and liquid mediums. The isoflavonoid profiles of the initial and subcultured *P. lobata* calli were studied. The isoflavonoids are represented primarily by glycosylated forms of daidzein and genistein. Prenylated forms include prenyl-genistein 5 and its glycosylated forms. The highest content and productivity of isoflavonoids were observed in calli grown on the optimal medium, 12 mg g^{-1} DW and 75 mg L^{-1} DW, respectively. Monochromatic light-emitting diode (LED) red lighting increased the content of prenylated forms of genistein more than twofold. Green LED light was the most effective treatment for increasing the levels of the prenylated isoflavonoid form—up to 4 times.

Key message

Green LED light and optimal medium increased prenyl-genistein production up to 4 times in *P. lobata* calli. These results provide a foundation for research on the relict northernmost *P. lobata* population.

Keywords Artificial light · Callus culture · Kudzu · LED · Long-term cultured calli · *Pueraria lobata* · Prenylated isoflavonoids

Introduction

Members of the genus Pueraria belong to the Leguminosae family and are generally referred to as “kudzu”. Because kudzu roots contain high concentrations of healthy isoflavones, such as the unique puerarin (8-C-glycoside of daidzein), they have been utilised in traditional Chinese medicine. The Chinese Pharmacopoeia formally lists the dried root of *Pueraria lobata* (Wild.) Ohwi (kudzu) is one of the 50 essential herbs in traditional Chinese medicine. In Southeast Asia, kudzu is frequently utilised as a herbal remedy, functional food, and dietary supplement (Mocan et

Communicated by Ali Ramazan Alan

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Published online: 20 November 2026

al. 2018). Kudzu is considered a hazardous weed that harms the environment and the economy in the United States. However, not all kudzu species are equally invasive or produce puerarin. Because the morphology of different *Pueraria* species differs and the terminology is conflicting, the recognition of kudzu is still challenging (Adolfo et al. 2022; Haynsen et al. 2018; Zhou et al. 2025). Moreover, in the southern part of the Russian Far East, this plant is found in only a few isolated areas and is known as *Pueraria lobata* (Wild.) Ohwi. In 1989, there were just three known locations. Consequently, *P. lobata* is listed in the Federal Red Book as an extremely rare plant (edbookrf.ru/puerariya-dolchataya-pueraria-lobata/). However, it remains unstudied and undescribed in terms of its genetics and chemistry—data that would be of great scientific interest. First, such information would allow for a comparison of the phytochemical profile of the plant from this northernmost range with profiles already known. Second, genetic and chemical analyses make accurate classification of this northern *P. lobata* possible.

Flavonoid profiles of kudzu and Pueraria plants indicate high biological activity (Aboushanab et al. 2023). Of the species in the genus Pueraria, two have been the most extensively studied, namely, *Pueraria candollei* var. *mirifica* or *P. mirifica* and *P. lobata*. A total of 106 different types of secondary metabolites, such as benzopyrans, isoflavones and their glycosides, coumarins, steroids, fatty acids and their derivatives, and other metabolites, were found in *P. mirifica* rhizomes according to phytochemical investigations (Monthakantirat et al. 2014). Kudzu is widely used in alternative Chinese medicine for treating chronic conditions such as liver diseases, cardiovascular diseases, respiratory diseases, gastric diseases, diabetes, Alzheimer's disease, and cancer (Liga and Paul 2024). Clinical research has indicated that high concentrations of potent phytoestrogens successfully reduce vasomotor symptoms and urogenital syndromes in menopausal women and positively affect lipid metabolism and bone mineral density (Tiyasatkulkovit et al. 2014; Zeng et al. 2026).

Leguminous plants are the main source of isoflavonoid phytoalexins, which are produced by the central phenylpropanoid pathway and legume-specific isoflavonoid branch pathways (Chu et al. 2014). The unique isoflavonoids of Pueraria provide it with a more potent phytoestrogenic effect than other legumes do. *P. mirifica* is characterised by standards for the legumes daidzein, daidzin, genistein, and genistin and unique puerarin, kwakhurin, and mirificin, as well as minor but potent estrogenic chromenes, including derivatives of miroestrol (Jearapong et al. 2014; Chatuphonprasert et al. 2013). Up to 10 mg/g of total isoflavonoids (more than half of puerarin) accumulated in the tubers during the summer (Cherdshewasart et al., 2007a). The main bioactive component extracted from the roots of *P. lobata* and *P. mirifica* is puerarin. Its pharmacological characteristics have since been thoroughly studied. Puerarin's wide range of pharmacological qualities, including vasodilation, cardioprotection, neuroprotective, antioxidant, anticancer, anti-inflammatory, pain relief, bone formation promotion, alcohol intake inhibition, and attenuation of insulin resistance, may be responsible for its beneficial effects on various medical purposes (Chen et al. 2026). Nevertheless, the direct molecular mechanisms and targets are still unknown (Gray et al. 2015; Zhou et al. 2014).

As noted above, we have a unique opportunity to study the natural distribution of *P. lobata* plants from the northernmost range. The climatic conditions include a long winter period (at least five months) characterised by subzero temperatures and heavy snow cover. In this climate, *P. lobata* plants propagate exclusively vegetatively, as the warm season is too short for seeds to mature. The phytochemical composition is of particular interest because the isoflavonoid content in wild *P. lobata* and *P. mirifica* plants varies depending on the location (Cherdshewasart et al. 2007b). The inaccessibility of northernmost *P. lobata* plants and their status as a protected species pose major obstacles to research. In this regard, we conducted a pilot experiment on the initiation and stabilisation of a callus culture from a commercially available *P. lobata* cultivar. This successful preliminary work will enable us to proceed with rare specimens and, in the long term, gain a comprehensive understanding of this unique population. Tissue and callus cultures are vital tools for working with rare plants for both industrial and medical purposes (Ozyigit et al. 2023). It is known that obtaining and maintaining cell cultures of legumes is difficult. The success of long-term legume culture depends largely on the medium formulation, explant type, and callus morphology (Susunaga-Gómez et al. 2025).

In the present work, we investigated the possibility of initiating and stabilising a callus culture of the cultivated form of *P. lobata* as a springboard and the rationale for working with a rare population. Moreover, we investigated the effect of monochromatic light-emitting diode (LED) lighting on isoflavonoid production in culture. Monochromatic-modulated LED lighting is currently a promising, reproducible, and eco-friendly approach for modulating secondary metabolism in plant cells. Both the amount and quality of light, including photoperiod, spectra, and light intensity, affect the growth and metabolism of plant cells (Taulavuori et al. 2017). Compared with traditional fluorescent lights, LED lights are widely utilised in the growth of various plant species, especially horticulture plants, because of their higher luminous efficiency and reduced power consumption. LED light affects gene expression, enzyme activity, cell wall formation, plant defense, and postharvest quality (Sena et al.

2024). Modulation of the basic parameters of LED light treatments can affect a range of bioactive phytochemicals, including alkaloids, phenolics, and terpenoids, without compromising growth characteristics (Veremeichik et al. 2024, 2025a; b).

Materials and methods

Induction and stabilisation of *P. lobata* calli

Obtaining cotyledons. Commercially available (https://blog-travushka.ru/shop) seeds of cultivated *P. lobata* were used to obtain a callus culture. *P. lobata* seeds were surface-sterilised by incubation in 0.02% diocidum for 5 min, followed by five rinses with sterile distilled water and sacrifice. For germination and cultivation, in-culture vessels with MS/2 medium were used (Murashige and Skoog 1962). The conditions of cultivation in a climate chamber (KS-200, Russia) were as follows: photoperiod, 16/8 h with illumination during the day of 140 µmol m^{-2} s^{-1}; temperature, 24/22°C; and humidity, 70%. The resulting 20-day-old plantles were used to establish cell cultures.

Induction of calli. Cotyledons *P. lobata* were used to induce the formation of calli. Basal media with variations in plant growth regulators (PGRs) was used for the induction of calli and subsequent cultivation. In addition to standard macro- and microelements, modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with (mg L^{-1}) ammonium nitrate (400), nicotinic acid (0.5), thiamine-HCl (0.2), meso-inositol (100), peptone (100), pyridoxine-HCl (0.5), sucrose (25000), and agar (6000) was used. Cytokinins (thidiazuron, TDZ and benzylaminopurine, BAP) and auxins (1-naphthaleneacetic acid, NAA; 4-chlorophenoxy acetic acid, CPA; 2,4-dichlorophenoxyacetic acid, 2,4-D) were used, both in combination and individually, at concentrations ranging from 0 to 5 mg L^{-1} to induce callus formation (Supplemental Table S1). Thus, a medium supplemented with TDZ (2 mg L^{-1}) was optimal for callus induction and was designated the initial medium (i). The obtained callus culture of *P. lobata* was designated Pl.

Long-term cultivation. For successful subculturing, the initial *P. lobata* callus culture was grown for two subculture cycles (40 days) on media with varying PGR contents. PGR combinations and concentrations are listed in Supplemental Table S1. The conditions of cultivation were as follows: constant darkness or photoperiod, 16/8 h with illumination during the day of 140 µmol m^{-2} s^{-1}; temperature, 24/22°C; and humidity, 70%. To determine the effects of medium composition and culture conditions on growth and isoflavonoid content, 2 g of initial callus tissue was inoculated into 50 ml of experimental media. After 40 days of cultivation, the callus tissue was weighed, dried, and prepared for chemical analysis. The medium variant that yielded the greatest increase in biomass and isoflavonoid content after the second subculture was selected for long-term subculturing, adaptation to liquid culture, and light experiments. To calculate the biomass accumulation (g) per 1 l of medium, the growth obtained from 2 g of inoculum in 50 mL of medium over 40 days was used.

The study employed specimens (strains) deposited in the Bioresource Collection of the Federal Scientific Centre of East Asia Terrestrial Biodiversity of the Far East Branch of the Russian Academy of Sciences (reg. number 2797657).

Light treatments and growth chamber construction

Experimental design. Thus, for subsequent experiments, a medium supplemented with BAP (5 mg L^{-1}) and IAA (0.1 mg L^{-1}) was used and designated as the optimal solid (S) and liquid (L) subculturing media. For the experiments, after 2 successive subcultures, *P. lobata* calli were cultured for 8 successive subcultures on the same optimal solid media. Three sets of experiments were conducted to optimise isoflavonoid production in *P. lobata* callus culture: (1) dynamic analysis of isoflavonoid production on solid medium during eight subcultures; (2) dynamic analysis of isoflavonoid production on liquid medium during eight subcultures; and (3) the effect of monochromatic lighting.

After inoculation (2 g of inoculant per 50 mL of the same solid medium in 250-mL *Erlenmeyer* flasks; Simax, Czech Republic), the *P. lobata* calli were immediately transferred under different light conditions for one passage (40 days). Calli growing in the dark were used as a control. In this study, the following varieties of monochromatic light were used: red (660 nm, designated "R") and green (520 nm, designated "G"). Each section of the chamber was equipped with LED lamps with a photosynthetic photon flux density (PPFD) of 100 µmol $m^{-2}s^{-1}$. The calli were cultivated in four-section chambers (100 × 50 × 50 cm, Supplementary Fig. S2) with light sources designed and manufactured at the IACP FEB RAS. The light source matrices were made up of 24 three-watt (CHANZON, China) LEDs of various colours, creating one integrated light source as described previously (Veremeichik et al. 2025b). The chambers were covered with reflective aluminum foil to diffuse light, and an FFB1212SH 12,025 exhaust fan (power: 14.8 W; speed: 3700 rpm; air volume: 140.16 cfm; China) was used to maintain the temperature (24 °C). 70% air humidity was maintained, and the photoperiod (light/dark) lasted 16–8 h. Chambers of different wavelengths were distant from one another to reduce the mutual influence of light of different spectra on each other. The spectra and actual PPFD were measured with a PG200N spectrophotometer (UPRtek,

Taiwan). Currents in the driver supply system were managed by a UT61A digital multimeter (Uni-T, China). The normalised spectra of the light sources and the main characteristics of the light are listed in Supplementary Table S2.

After 40 days of cultivation under different light conditions, the calli were harvested, weighed, and dried (45 °C for 12–16 h) for further chemical analysis. Three separate experiments were conducted as biological replicates. As a technical replication, ten jars were exposed to each type of light for each experiment.

Phenolic acid extraction and HPLC–DAD–ESI–MS/MS) conditions

Chemicals

Analytical standards of daidzein and genistein were obtained from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water (Millipore, Bedford, MA, USA) was used for the preparation of all the extraction solutions and eluents. All the solvents were of analytical grade.

Sample preparation for analytical chromatography

Polyphenol extraction was performed according to a previously published protocol (Veremeichik et al. 2021). Briefly, ultrasonic extraction of dried and powdered callus material in 80% aqueous methanol was performed. The extracts were subsequently filtered through a 0.45-µm membrane (Millipore, Bedford, MA, USA) before HPLC analysis.

Analytical chromatography and mass spectrometry

This study utilised an analytical method previously developed in our laboratory for the determination of soy isoflavones (Veremeichik et al. 2019, 2021). The analytical chromatography and mass spectrometry procedure was performed according to our previously reported protocol (Veremeichik et al. 2021). For sample preparation, dried and powdered callus tissue was sonicated in 80% v/v methanol, and the supernatant was filtered (0.45-µm membrane, Millipore, Bedford, MA, USA) and used for analysis. Isoflavonoid determination was performed on an Infinity 1260 analytical HPLC instrument (Agilent Technologies, Santa Clara, California, USA) interfaced with an ion-trap electrospray ionisation mass spectrometer (IT-ESI-MS) Bruker HCT ultra PTM Discovery System (Bruker Daltonik GmbH, Bremen, Germany) using an analytical Zorbax C18 column (150 mm, 2.1 mm i.d., 3.5 µm part size; Agilent Technologies, USA) for separation. The linear gradient profile from A (0.1% aqueous formic acid) to B (acetonitrile) was as follows: 0–5 min 10% B, 20% B, 45 min 40% B, and 50–65 min 95% B. The elution was carried out at a flow rate of 0.2 ml/min and a column temperature of 40 °C. UV spectra were recorded with a diode array detector (DAD) in the range of 200–400 nm, and chromatograms were obtained at a wavelength of 254 nm. Negative/positive ion mode ESI–MS experiments were conducted according to the following parameters: the range of *m/z* detection was 100–1,000, the drying gas (N_2) flow rate was 8.0 l/min, the nebuliser gas (N_2) pressure was 25 psi, and the drying gas temperature was 325 °C in auto-MS^2 mode. High-resolution MS studies for isoflavone identification were performed using a Shimadzu LCMS-IT-TOF instrument (Shimadzu, Japan) including a tandem ion-trap/time-of-flight mass spectrometer. The mass spectra were collected under ESI conditions with simultaneous negative and positive ion detection at a resolution of 12,000. The following settings were used: the drying gas (N_2) pressure was 100 kPa, the nebuliser gas flow rate was 1.5 l/min, the ion source potential changed from −3.8 to 4.5 kV, and the interface temperature was 200 °C. The quantitative determination of isoflavonoids was carried out using the calibration curves of available standards.

The productivity of isoflavonoids was calculated as follows:

Productivity (mg/L) = Content × DW,

where Content denotes the content of individual compounds (mg/g DW) and DW denotes the dry weight (g) of the callus biomass per liter of medium (g/L).

Statistical analysis

The STATISTICA software package (StatSoft, Inc., USA) was used for the statistical analysis. All values are presented as the mean ± standard error (SE). Student's t test was employed for the statistical assessment to compare two independent groups. Analysis of variance (ANOVA) was used, together with a multiple comparison approach, to compare several datasets. The cut-off point for statistical significance was fixed at $p < 0.05$, Fisher's LSD.

Results

Establishment and stabilisation of the *P. lobata* callus line

As stated above, the primary objective of the study was to establish a working method for the induction and long-term cultivation of a high-producing *P. lobata* cell line. Two-week-old in vitro germinated cotyledons from commercially available seeds of cultivated *P. lobata* were used to obtain a callus culture. Using different variations of PGRs in basal media (Supplementary Table S1), we succeeded in inducing

callus formation only on medium supplemented with 2 mg L^{-1} TDZ. Callus formation was notably delayed. Obtaining cell material suitable for propagation took more than six months. Subculturing the primary callus also took more than 60 days. After four successive subcultures on these initial media, *P. lobata* callus tissue growth was significantly impaired, and the culture became morphologically compact and lost its loose texture (Fig. 1, a, Pl-i). Thus, TDZ (2 mg L^{-1}) without auxins is the optimal supplement in initial media (i) for inducing calluses in *P. lobata* cotyledons. The obtained callus culture of *P. lobata* was designated Pl. However, successful subsequent subculturing requires another PGR component in the medium.

For successful subculturing, the initial *P. lobata* callus culture was grown for two subculture cycles (40 days) on media with varying PGR contents. Cytokinins (thidiazuron, TDZ and benzylaminopurine, BAP) and auxins (1-naphthaleneacetic acid, NAA; 4-chlorophenoxy acetic acid, CPA; 2,4-dichlorophenoxyacetic acid, 2,4-D) were used, both in combination and individually, at concentrations ranging from 0 to 5 mg L^{-1} to stabilise callus growth. PGR combinations and concentrations are listed in Supplemental Table S1. For all the tested media, *P. lobata* callus tissue was grown under both dark and light conditions. After 40 days of cultivation, the callus tissue was weighed, dried, and prepared for chemical analysis. Among the many combinations of cytokinins and auxins, only the combination of BAP and NAA at concentrations of 5 and 0.1 mg L^{-1} had a consistently positive effect on callus tissue growth under dark conditions (Fig. 1, b). Most notably, the callus tissue became friable. Loosening of the compact initial culture was already observed during the second subcultivation (Fig. 1, a Pl-SII).

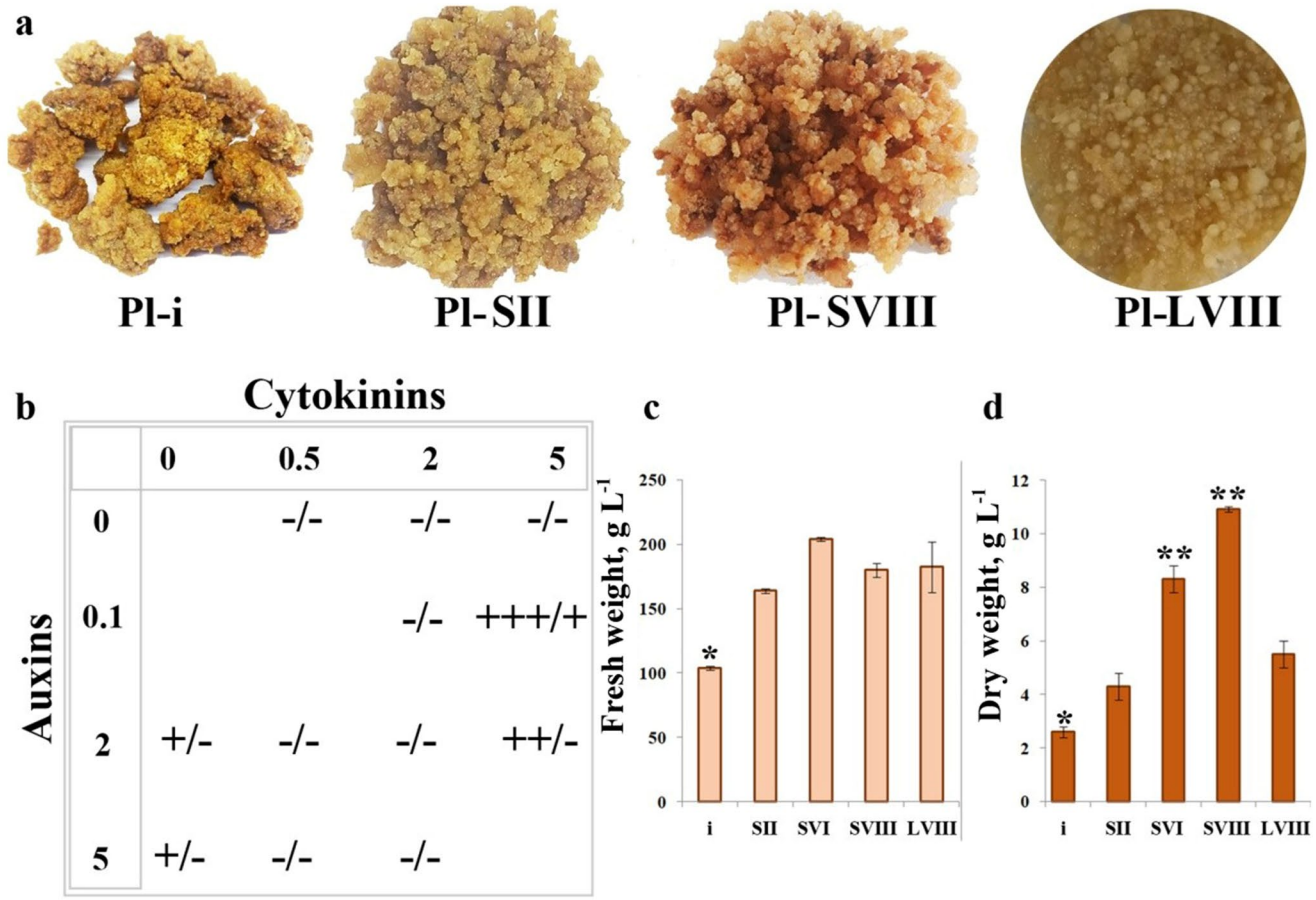


Fig. 1 Morphology and weight dynamics of *P. lobata* calli growing on media supplemented with different combinations of PGRs. Morphology (**a**) of *P. lobata* calli growing for two subcultures on induction media (Pl-i; TDZ, 2 mg L^{-1}) and eight subcultures on optimal solid (Pl-S) and liquid (Pl-L) subculturing media (BAP, 5 mg L^{-1} and NAA, 0.1 mg L^{-1}). The effects of different combinations of auxins and cytokinins on the total isoflavonoid content (plusses or minuses before the slash) and the growth parameters (plusses or minuses after the slash) of *P. lobata* calli (**b**) are visualised schematically, where "-", no effect; "+", "++", and "+++" indicate low, moderate, and high positive effects, respectively. Dynamics of biomass accumulation (**c**, fresh weight; **d**, dry weight) during continuous maintenance (eight 40-day-long subcultures, I–VIII) for c *P. lobata* calli growing on the optimal solid (S) and liquid (L) and subculturing media (BAP, 5 mg L^{-1} and NAA, 0.1 mg L^{-1}) compared with calli growing for two subcultures on the induction media (i; TDZ, 2 mg L^{-1}). The data are presented as the means ± standard errors. The asterisks above the bars indicate significantly different means ($P < 0.05$; Fisher's LSD)

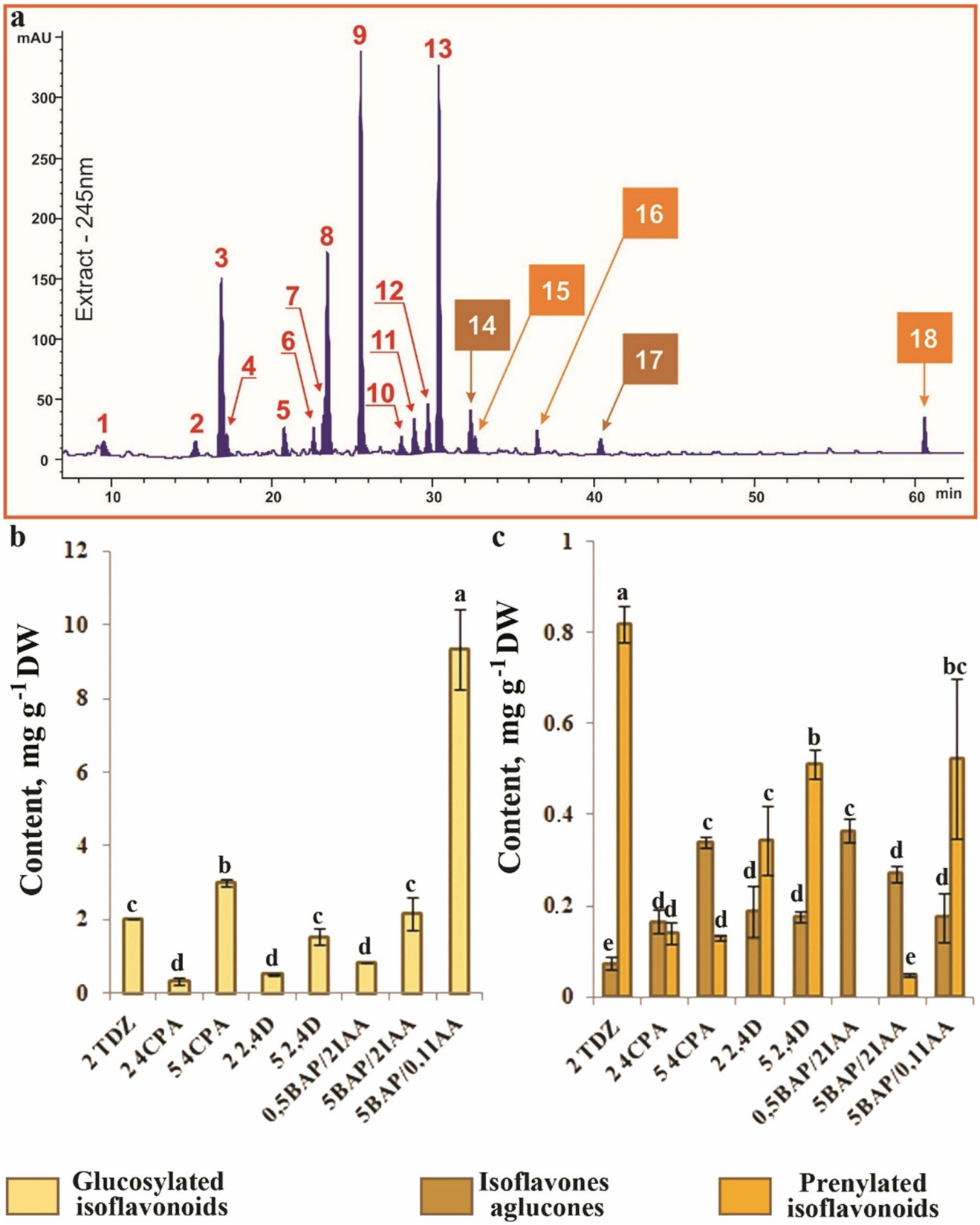
The medium variant (5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) that yielded the highest biomass growth content after the second subculture was selected for long-term subculturing, adaptation to liquid culture, and light experiments. The *P. lobata* callus line was adapted for cultivation in liquid media supplemented with the same combination of plant growth regulators. Biomass growth dynamics were analysed over eight consecutive subcultures for solid and liquid media variants. As shown in Fig. 1, c–d, the accumulation of fresh biomass of the *P. lobata* callus line on these solid media was stable across the 8 subcultures and was twofold greater than the initial accumulation of the initial calli. The dry biomass accumulation of the stable *P. lobata* calli line reached 10 g L^{-1} after eight subcultures as well as for the solid and liquid media, which was 5-fold greater than the dry biomass accumulation of the initial calli. Thus, from 40 subculturing media, only the medium variant (5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) was suitable for long-term cultivation of *P. lobata* calli: we observed good, stable biomass growth and the loosening of callus tissue. On most of the tested media, the calli died. However, in addition to the optimal medium, the callus culture survived, but the growth was poor, and the callus tissue was highly compact. The following media variants were used: 2 and 5 mg L^{-1} CPA; 2 and 5 mg L^{-1} 2,4-D; 0.5 mg L^{-1} BAP; 2 mg L^{-1} NAA; and 5 mg L^{-1} BAP and 2 mg L^{-1} NAA. After two successive subcultures on these media, qualitative and quantitative chemical analysis of the isoflavonoid content was performed.

Isoflavonoid determination

To determine the optimal PGR composition for high *P. lobata* callus productivity, a chemical analysis of *P. lobata* callus tissue cultured during 2 consecutive subcultures on 8 media variants was carried out. The initial calli were grown on initial media supplemented with 2 mg L^{-1} TDZ; stabilised calli were grown on optimal media supplemented with 5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA; and the following six media variants were grown: 2 and 5 mg L^{-1} CPA; 2 and 5 mg L^{-1} 2,4-D; 0.5 mg L^{-1} BAP and 2 mg L^{-1} NAA; and 5 mg L^{-1} BAP and 2 mg L^{-1} NAA. The isoflavonoid profile of *P. lobata* calli extracts was studied by high-performance liquid chromatography with a diode array detector and an ion-trap electrospray ionisation detector (HPLC-DAD-IT-ESI-MS/MS) in both positive and negative modes and supplemented by high-resolution mass spectrometry data (a mass error of less than 4 mDa). The identification results are summarised in Supplemental Table S3.

In this study, 18 compounds, specifically isoflavones and their derivatives, were defined. Seventeen of these genes were detected in *P. lobata* callus tissue grown on an optimal medium. Only genistin-O-hexoside was identified in callus tissue grown on medium supplemented with 5 mg L^{-1} CPA. A chromatographic profile of isoflavonoids detected in *P. lobata* callus tissue grown on an optimal medium is presented in Fig. 2, a. Six compounds turned out to be daidzein and its derivatives, and twelve were genistein and its derivatives, as confirmed by the UV spectral patterns. All the compounds generated a molecular ion $[M+H]^+$ of good intensity, and the remaining well-visible positive and negative adduct/fragment ions confirmed the identification (Supplemental Table S3). Two isoflavone aglycones, daidzein (**14**) and genistein (**17**), were identified because of their complete agreement with the analytical standards. Additionally, the mono-glycoside *O*-conjugates of these compounds were found to be daidzin (**3**) and genistin (**8**). The characteristic fragment ions $[(M+H)\text{-}162]^+$ and $[(M\text{-}H)\text{-}162]^-$ indicated that the hexoside moieties were attached to the oxygen atom in the form of an *O*-glycosidic group. The fragmentation pattern of the protonated molecules of component **1** ($[M+H]^+$ at *m/z* 595 and $C_{27}H_{30}O_{15}$) showed signals at *m/z* 433 and 271 because of the loss of one and two sugar moieties (162 Da), respectively. The same pattern was observed in the negative mode. It was then possible to determine that **1** was a di-*O*-glycoside derivative of genistein. Compound **18** ($[M+H]^+$ at *m/z* 339, $C_{20}H_{18}O_5$) produced fragmentation products with *m/z* 283 $[(M+H)–56]^+$ and *m/z* 281 $[(M\text{-}H)–56]^-$, indicating the characteristic neutral loss of the prenyl moiety (C_4H_8). Thus, prenylated genistein was recognised. With respect to compound **15** ($[M+H]^+$ at *m/z* 663, $C_{32}H_{38}O_{15}$), the MS^2 spectrum revealed the presence of product ions at *m/z* 501 $[(M+H)–162]^+$, *m/z* 445 $[(M+H)–162-56]^+$, *m/z* 339 $[(M+H)–162–162]^+$ and *m/z* 283 $[(M+H)–162-56]^+$ because of the elimination of one/two sugars and one prenyl moieties. Therefore, compound **15** was determined to be a di-*O*-glycoside derivative of prenylgenistein (**18**). The formation of product ions due to elimination of CO_2 (44 Da), the malonic acid moiety ($C_3H_2O_3$, 86 Da), and the malonylated sugar moiety ($C_9H_{12}O_8$, 248 Da) is characteristic of malonate ester fragmentation.

Fig. 2 Qualitative and quantitative analysis of isoflavonoids identified in crude extracts of *P. lobata* calli. (**a**) HPLC–UV profiling of isoflavonoids identified in crude extracts of *P. lobata* calli recorded at 325 nm. Compounds 14 and 17 (light beige squares) are identified as isoflavone aglycones, and compounds 15, 16 and 18 (brown squares) are identified as prenylated isoflavonoids. Other compounds (red numbering) are identified as glycosylated derivatives of isoflavones. The identification results are summarised in Supplemental Table S3. Contents (mg g^{-1} DW) of the sum of glycosylated isoflavone derivatives (**b**) and isoflavone aglycones and prenylated isoflavonoids (**c**) determined for *P. lobata* calli growing for eight subcultures on induction media (TDZ, 2 mg L^{-1}) and for two subcultures on media supplemented with different combinations of auxins and cytokinins. The contents of the individual compounds are presented in Supplemental Table S4. The data are presented as the means ± standard errors. The different letters above the bars indicate significantly different means ($P < 0.05$; Fisher's LSD)


a
mAU
Extract - 245nm
min
b
Content, mg g-1DW
c
Content, mg g-1DW
2 TDZ
2 4CPA
5 4CPA
2 2,4D
5 2,4D
0,5BAP/2IAA
5BAP/2IAA
5BAP/0,1IAA
Glucosylated isoflavonoids
Isoflavones aglucones
Prenylated isoflavonoids

Malonylated daidzin had two isomeric forms (compounds **7** and **9**), as did malonylated genistin (compounds **11** and **13**). In addition, several malonyl di-*O*-glycoside derivatives were found among the minor components. Compound **5** was determined to be the malonate ester of genistin *O*-hexoside (compound **1**). Malonylylated di-glycosylated daidzein was detected as two isomers: **2** and **4**. Compound **12** ($[M+H]^+$ at *m/z* 651, $C_{29}H_{30}O_{17}$) clearly produced visible fragment ions at *m/z* 519 and 271 with compositions of $[(M+H)\text{-}132]^+$ and $[(M+H)\text{-}132\text{-}248]^+$, respectively (due to loss of the pentoside and malonylhexoside moieties, respectively). Therefore, it was identified as a malonate ester of genistin *O*-pentoside. In addition, in a similar manner, malonylated diglycosylated prenylgenistein (**16**) was determined. Notably, two minor compounds from our extracts were not precisely identified. The presence of fragment ions with *m/z* 271 in both cases indicated the presence of the aglycone genistein, which was consistent with their UV spectral data. Notably, two minor compounds (**6** and **10**) from our extracts were not precisely identified. The presence of fragment ions with *m/z* 271 in both cases indicated the presence of the aglycone genistein, which was consistent with their UV spectral data. The neutral loss of two sugar moieties, one (product ions at *m/z* 685, $[(M+H)\text{-}162]^+$) and two (product ions at *m/z* 523, $[(M+H)\text{-}162\text{-}162]^+$), by compound 6 allowed us to identify it as a di-*O*-glycoside derivative. Thus, compounds 6 ($[M+H]^+$ at *m/z* 847, $C_{39}H_{42}O_{21}$) and 10 ($[M+H]^+$ at *m/z* 933, $C_{42}H_{44}O_{24}$) were assigned as genistein derivative di-*O*-hexoside X and its malonyl conjugate, respectively.

Effects of PGR combination on isoflavonoid composition in *P. lobata* calli

Qualitative and quantitative chemical analysis of the cell culture revealed that, regardless of the PGR composition, isoflavonoids are represented primarily by glycosylated forms of daidzein and genistein. High concentrations of auxins (2 mg L^{-1} or more), as well as their complete absence, have adverse effects on isoflavonoid accumulation. The total content of isoflavonoids and their glycosylated derivatives increases at extremely low auxin concentrations and sufficiently high cytokinin concentrations. In the first case, we observe only 6 of the 13 glucosylated forms, whereas in the second case, all identified forms are present. Moreover, their total content is 5–10 times greater (Fig. 2, b; Supplemental Table S3). Prenylated forms in calli grown over 2 subcultures on the optimal medium (5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) are represented by three compounds (prenylgenistein 5 and its glycosylated forms), whereas in the other variants, only one, prenyl-genistein 5. The initial *P. lobata* callus content was approximately 3 mg g^{-1} DW of total isoflavonoids (Fig. 2, b, c; Supplemental Table S3). Among them, the major compound was prenyl-genistein (0.8 mg g^{-1} DW); the minor compound was daidzein (less than 0.1 mg g^{-1} DW); and the other compounds were daidzin, genistin and their malonylated derivatives (approximately 1 mg g^{-1} DW in sum). Thus, after two subcultures, the optimal medium (5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) ensures stable growth and the highest isoflavonoid content compared with those of the other varieties—up to 12 mg g^{-1} DW of total isoflavonoids, including approximately 1 mg g^{-1} DW of bioactive aglucones and their prenylated forms (Fig. 2, b, c; Supplemental Table S3).

Dynamics of isoflavonoid accumulation during long-term cultivation of *P. lobata* calli

The results showed that solid medium supplemented with 5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA is optimal for the cultivation of *P. lobata* calli. Next, we investigated the stability of the culture during prolonged serial cultivation on this medium over eight subcultures, each lasting 40 days. Thus, by the end of the experiment, the age of the culture exceeded two years. To optimise culture productivity, the culture was adapted to a liquid medium, and growth and isoflavonoid biosynthesis were analysed every two subcultures on both liquid and solid optimal media.

As shown in Fig. 3, the total isoflavonoid content was slightly lower in the eight subcultures than in the second subcultures as well as in the calli grown on solid and liquid media. Moreover, the content of prenylated isoflavonoids decreased, whereas the content of isoflavonoid aglucones significantly increased over time (Fig. 3, b, c). On the basis of the growth characteristics of the culture (Fig. 1, b) at the eighth subculture, productivity was determined (Fig. 3, d, e). The production of total isoflavonoids was almost two times greater in the calli grown on the solid media than on the liquid media and reached 75 mg L^{-1} DW. Moreover, the productivity of the more bioactive aglucones and prenylated forms was the same in both variants.

Effects of monochromatic LED light on isoflavonoid content and productivity in *P. lobata* calli

Previously, it was shown that single-stage cultivation of plant callus culture under red and green LED light at an intensity of 100 µmol $m^{-2}s^{-1}$ can increase the accumulation of phytochemicals without affecting growth (Veremeichik et al. 2024, 2025a). In the present study, we investigated the effects of monochromatic red and green LED light on the content and productivity of different groups of isoflavonoids in *P. lobata*-stabilised calli.

Compared with the dark-grown control, different light treatments had different effects on the biosynthesis of

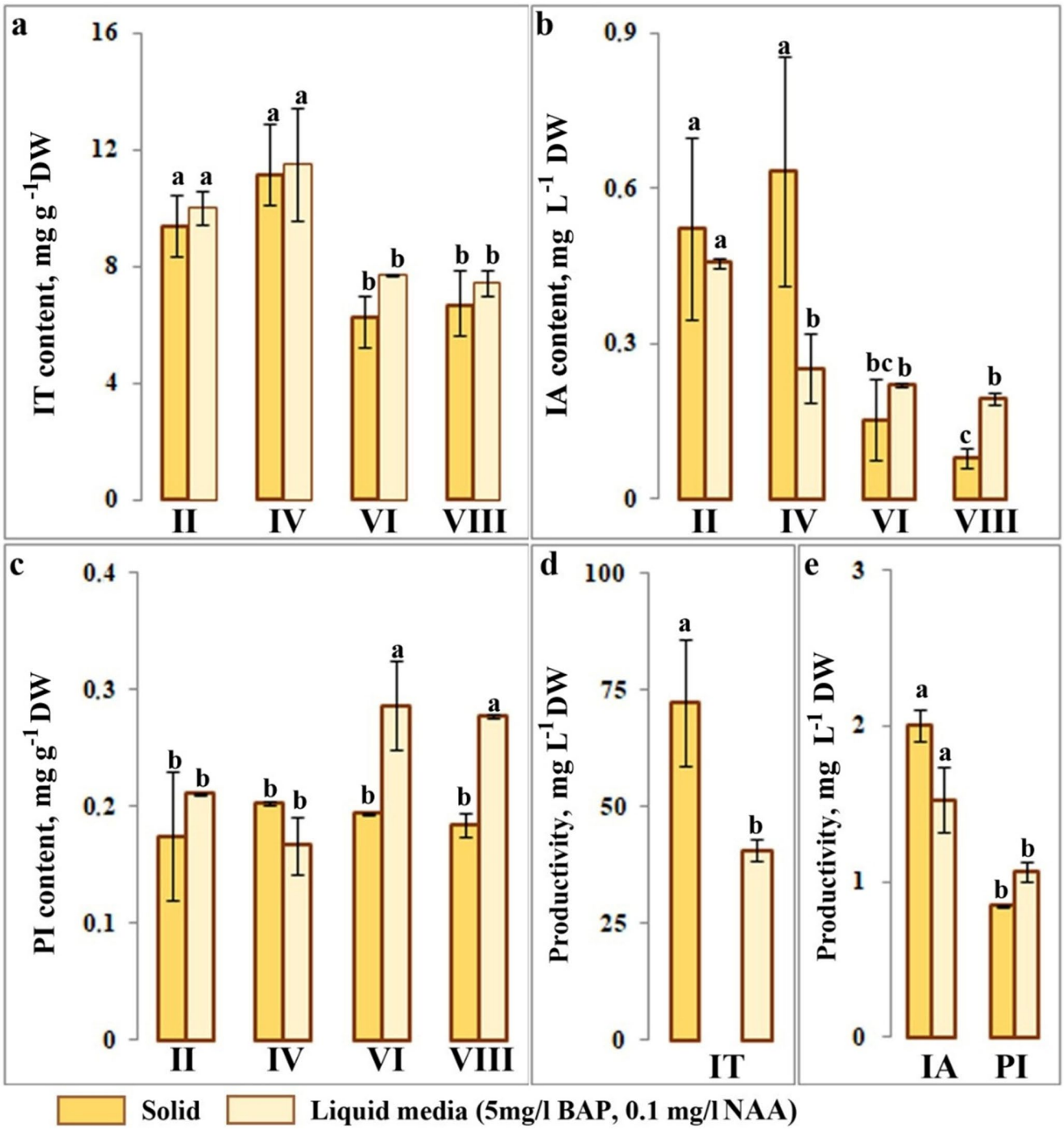


Fig. 3 Content and productivity of isoflavonoids in crude extracts of stabilised long-term-cultured *P. lobata* calli. Contents (mg g^{-1} DW) of isoflavones total (**a**, **IT**), isoflavones aglycones (**b**, **IA**), and prenylated isoflavonoids (**c**, **PI**) determined for *P. lobata* calli growing on optimal solid (dark beige bars) and liquid (light beige bars) subculturing media (BAP, 5 mg L^{-1} and IAA, 0.1 mg L^{-1}) for eight subcultures. For analysis, II, IV, VI, and VIII subcultures were used. The contents of the individual compounds are presented in Supplemental Table S5. The productivities (mg L^{-1}) of isoflavonoids total (IT, **d**), isoflavone aglycones (IA, **e**) and prenylated isoflavonoids (PI, **e**) were calculated for the VIII subcultures. The data are presented as the means ± standard errors. The different letters above the bars indicate significantly different means ($P < 0.05$; Fisher's LSD)

individual phytochemical compounds in *P. lobata* calli. To evaluate which light treatments were most effective, we analysed the content of the main group of isoflavonoids (Fig. 4, Supplementary Table S6). Interestingly, light exposure had different effects on the derivatives of two independent biosynthetic branches: genistein from naringenin (Fig. 4, purple blocks and purple frames) and daidzein from liquiritigenin. Neither red nor green LED light affects the content of genistein aglycone. Moreover, the balance of genistein derivatives in light-treated calli noticeably shifted toward the prenylated forms, and the content of glycosylated forms decreased. Thus, compared with the dark-grown control, red light increased the content of prenylated forms of genistein by more than twofold, whereas green light increased it by nearly fourfold. Similarly, exposure to light affected the daidzein derivatives (Fig. 4, blue blocks and blue framed diagrams). The glycosylated forms of daidzein remained unaffected, whereas the content of the more active aglycone daidzein increased 1.5- and 2-fold in red and green light-treated *P. lobata* calli, respectively, compared with the dark-grown control. Neither red nor green light affected the growth of the *P. lobata* calli (Fig. 5, a, b). Red and green light treatments had similar effects on aglycone productivity—which increased by 1.5-fold in both cases (Fig. 5, c). In terms of growth and content, green light was the most effective treatment for increasing the abundance of the biologically active prenylated isoflavonoid form—up to 4 times (Fig. 5, d).

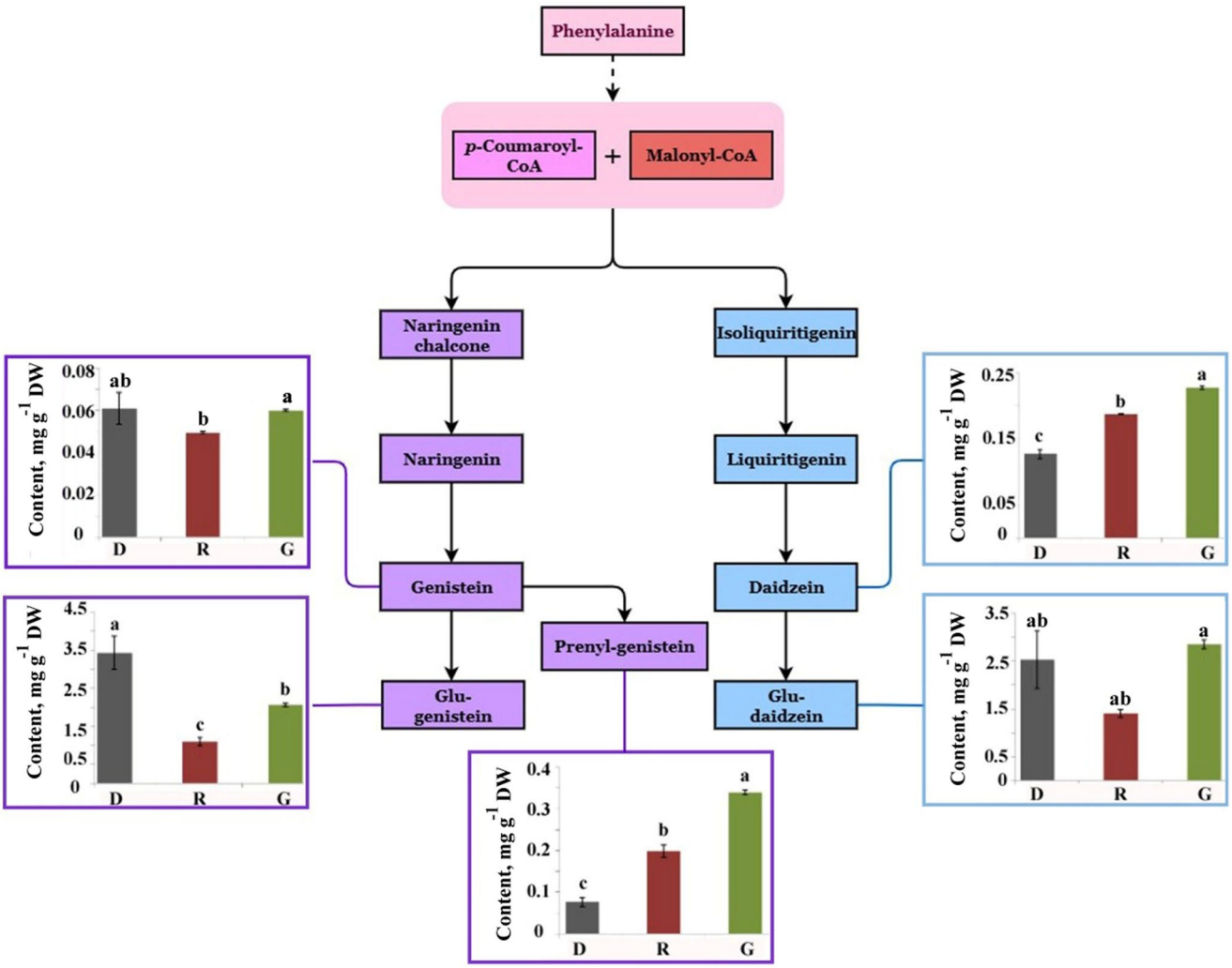


Fig. 4 Effects of monochromatic LED light on the contents of genistein and daidzein derivatives. The central part of the figures schematically illustrates the biosynthesis pathway of genistein (purple blocks) and daidzein (blue blocks) derivatives. Schematic diagrams illustrate the effect of monochromatic red (R) and green (G) light with an intensity of 100 µmol m^{-2} s^{-1} on the compound content (mg g^{-1} DW) after one month of treatment (one subculture) compared with the control dark-grown (D) *P. lobata* calli on optimal solid subculturing media (BAP, 5 mg L^{-1} and NAA, 0.1 mg L^{-1}). The contents of the individual compounds are presented in Supplemental Table S6. The data are presented as the means ± standard errors. The different letters above the bars indicate significantly different means ($P < 0.05$; Fisher's LSD)

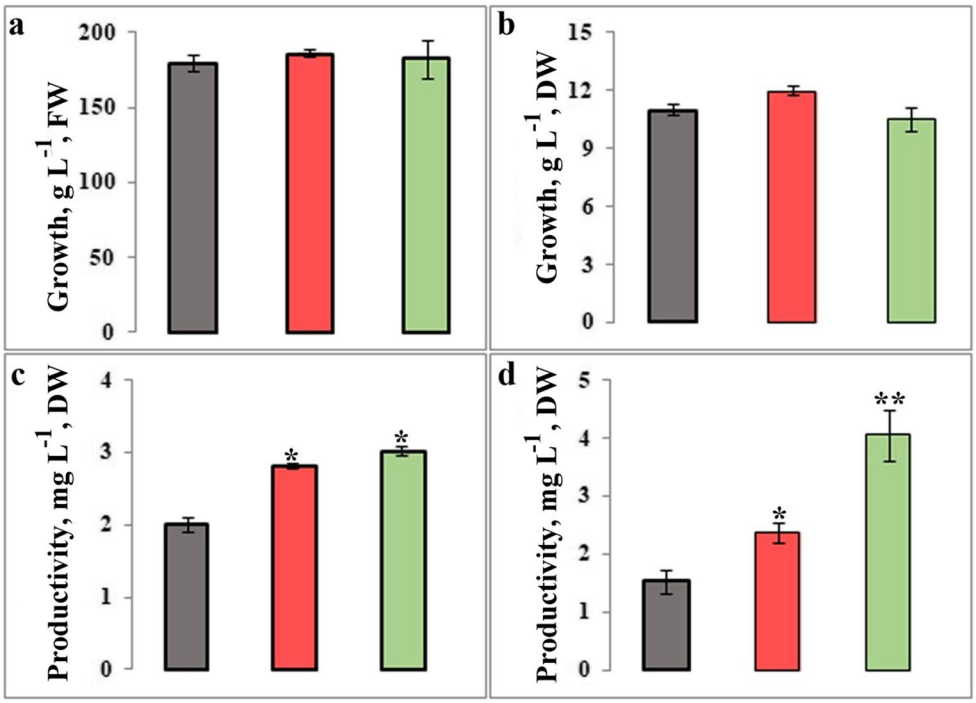


Fig. 5 Effect of monochromatic LED light on the growth and isoflavonoid productivity of *P. lobata* calli. Fresh (**a**) and dry (**b**) biomass accumulation of *P. lobata* calli growing on optimal solid subculturing media (BAP, 5 mg L^{-1} and NAA, 0.1 mg L^{-1}) after one month of treatment with monochromatic red (red bars) and green (green bars) light with an intensity of 100 µmol m^{-2} s^{-1} compared with the control dark-grown calli (dark gray bars). Productivity (mg L^{-1}) was calculated for isoflavone aglycones (**c**) and prenylated isoflavonoids (**d**) in light-treated and dark-grown *P. lobata* calli. The data are presented as the means ± standard errors. The asterisks above the bars indicate significantly different means ($P < 0.05$; Fisher's LSD)

Discussion

Widely known by the name "kudzu", members of the genus Pueraria contain high concentrations of healthy isoflavones, such as the unique puerarin (Mocan et al. 2018). In the southern part of the Russian Far East, this plant is found in only three isolated locations and is listed in the Federal Red Book as an extremely rare plant. The inaccessibility of northernmost *P. lobata* plants and their status as a protected species pose major obstacles to research. In this regard, we conducted a pilot experiment on the initiation and stabilisation of a callus culture from a commercially available *P. lobata* cultivar. This successful preliminary work will enable us to proceed with rare specimens and, in the long term, gain a comprehensive understanding of this unique population.

Obtaining and stabilising P. lobata calli. As stated above, the primary objective of the study was to establish a working method for the induction and long-term cultivation of a high-producing *P. lobata* cell line. Using different variations of PGRs in basal media, we succeeded in inducing callus formation only on medium supplemented with 2 mg L^{-1} TDZ. Notably, callus induction proceeded very slowly. After four successive subcultures on these initial media, *P. lobata* callus tissue growth was significantly impaired, and the culture became morphologically compact and lost its loose texture. Thus, TDZ without auxins is the optimal supplement in initial media for inducing calluses in *P. lobata* cotyledons. Our data match those published previously. It has been shown that whitish or light yellow friable calli of different legume species tend to disaggregate more efficiently, whereas compact or brown calli exhibit reduced proliferation and metabolic activity (Susunaga-Gómez et al. 2025). Among the many combinations of cytokinins and auxins, only the combination of BAP and NAA at concentrations of 5 and 0.1 mg L^{-1} had a consistently positive effect on callus tissue growth under dark conditions. Most notably, the callus tissue became friable. Loosening of the compact initial culture was observed during the second round of subcultivation. Thus, from 40 variants of subculturing media, only the medium variant (5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) was suitable for long-term cultivation of *P. lobata* calli. As shown previously, friable, whitish to yellow calli are generally associated with the successful establishment of suspensions, whereas compact or darker callus types tend to lose viability over time (Susunaga-Gómez et al. 2025). Organogenesis was not recorded in the calluses under any of these treatments. Similarly, the absence of organogenesis or morphogenesis in *P. lobata* callus cultures is consistent with previously obtained data (Vaishnav et al. 2006).

Isoflavonoid composition and content. To determine the optimal PGR composition for high *P. lobata* callus productivity, a chemical analysis of *P. lobata* callus tissue cultured during 2 consecutive subcultures on 8 media variants was carried out. The isoflavonoid profile of *P. lobata* callus extracts was studied by HPLC-DAD-IT-ESI-MS/MS. In this study, 18 compounds, specifically isoflavones and their derivatives, were defined. Two isoflavone aglycones that are well known for *P. lobata* (Lin et al. 2005; Yang et al. 2022), namely, daidzein (**14**) and genistein (**17**), were identified

because of their complete agreement with analytical standards. Additionally, the mono-glycoside *O*-conjugates of these compounds were found to be daidzin (**3**) and genistin (**8**). The characteristic fragment ions $[(M+H)\text{-}162]^+$ and $[(M\text{-}H)\text{-}162]^-$ indicated that the hexoside moieties were attached to the oxygen atom in the form of an *O*-glycosidic group (Lin et al. 2005; Yang et al. 2022). Notably, a significant portion of the extract components were various malonyl conjugates of glycosylated isoflavones, as we reported earlier for the callus culture of *G. max* (Veremeichik et al. 2019). The formation of product ions due to elimination of CO_2 (44 Da), the malonic acid moiety ($C_3H_2O_3$, 86 Da), and the malonylated sugar moiety ($C_9H_{12}O_8$, 248 Da) is characteristic of malonate ester fragmentation. Moreover, these compounds are often present in several isomeric forms, and the UV and MS patterns of the isomers are similar (Veremeichik et al. 2019). Six compounds turned out to be daidzein and its derivatives, and twelve were genistein and its derivatives. Qualitative and quantitative chemical analysis of the cell culture revealed that, regardless of the PGR composition, isoflavonoids are represented primarily by glycosylated forms of daidzein and genistein. Prenylated forms in calli grown over 2 subcultures on the optimal medium are represented by three compounds (prenyl-genistein 5 and their glycosylated forms), whereas in the other variants, only one, prenyl-genistein 5.

The total isoflavonoid content in the initial *P. lobata* callus was approximately 3 mg g^{-1} DW. The major compound was prenyl-genistein (0.8 mg g^{-1} DW), and the minor compound was daidzein (less than 0.1 mg g^{-1} DW). Thus, after two subcultures, the optimal medium (5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA) ensures stable growth and the highest isoflavonoid content (12 mg g^{-1} DW), including approximately 1 mg g^{-1} DW of bioactive aglucones and their prenylated forms. However, we did not detect puerarin. This does not contradict previously published data and is likely related to the specific characteristics of biosynthesis during long-term cultivation. High amounts of 0.65% puerarin accumulate in the tubers and stems of *P. tuberosa* (Vaishnav et al. 2006). Notably, puerarin was absent from the obtained long-term cultivated callus culture (Matkowski 2004). This is attributed to prolonged *P. lobata* callus cultivation—specifically, more than three rounds of subculturing (Boonsnongcheep et al. 2010). The presence and high content of puerarin have been demonstrated in cell cultures up to the fourth round of subculturing. Thus, the primary callus of *P. lobata* (up to 60 days before subculturing) contains up to 1 mg g^{-1} DW puerarin (Li et al. 2022). After three rounds of subculture at 21-day intervals, the friable *P. lobata* callus content was 0.33 mg g^{-1} DW of puerarin and 3 mg g^{-1} DW of total isoflavonoids vs. 14 mg g^{-1} DW and 30 mg g^{-1} DW in roots, respectively (Prasain et al. 2007). After 2 subcultures, 16-week-old calli were grown on media supplemented with different concentrations of PGR and accumulated up to 11–50 mg g^{-1} DW of total isoflavonoids and 0.3–2 mg g^{-1} DW of puerarin (Udomsuk et al. 2009). In the other case, after 3 subcultures, root-derived *P. lobata* produced only 0.57 mg g^{-1} DW of total isoflavonoids, with 0.18 mg g^{-1} DW of puerarin as a major aglucone (Matkowski 2004). Notably, in the present study, the total isoflavonoid content was approximately 3-fold (12 vs. 4.8 mg g^{-1} DW) greater than that in *G. max* long-term cultivated call (Veremeichik et al. 2019).

Productivity of the long-term cultured P. lobata calli. Next, we investigated the stability of the culture during prolonged serial cultivation on this medium over eight subcultures, each lasting 40 days. Thus, by the end of the experiment, the age of the culture exceeded two years. To optimise culture productivity, the culture was adapted to a liquid medium. The total isoflavonoid content was slightly lower in the eight subcultures than in the second subculture, as well as in the calli grown on solid and liquid media. The production of total isoflavonoids was almost two times greater in the calli grown on the solid media than on the liquid media and reached 75 mg L^{-1} DW. Moreover, the productivity of the more bioactive aglucones and prenylated forms was the same in both variants. We hypothesise that a different combination of plant growth regulators (PGRs) might be required for cultivation in a liquid medium. A comparison with previously published data revealed that cultivation in a liquid medium allows for an almost two-fold increase in isoflavonoid content compared with that in a solid medium (Li and Zhang 2006). However, this has been demonstrated only for short-term cultures—specifically, those involving just two rounds of subculturing. Moreover, we demonstrated for the first time that *P. lobata* callus culture is capable of producing high levels of isoflavonoids during long-term cultivation (approximately two years) while remaining fully viable and exhibiting good growth characteristics. Notably, the total isoflavonoid productivity of *P. lobata* calli was approximately 2-fold greater than that of *G. max* long-term cultivated call (72 vs. 39 mg L^{-1} DW). Therefore, compared with *G. max* calli, *P. lobata* calli are a more potent alternative source of isoflavonoids (Veremeichik et al. 2019).

LED light effect. Modern LED lighting control capabilities allow the qualitative and quantitative parameters of light exposure to be modulated. This approach enables the selection of a noninvasive, reproducible, and eco-friendly method for activating secondary metabolism as a whole or specific branch without compromising growth parameters (Veremeichik et al., 2023; Veremeichik et al.,2024b; Taulavuori et al. 2017). Previously, it was shown that single-stage cultivation of plant callus culture under red and green LED

light at an intensity of 100 µmol $m^{-2}s^{-1}$ can increase the accumulation of phytochemicals without affecting growth (Veremeichik et al. 2024, 2025a). In the present study, we investigated the effects of monochromatic red and green LED light with an intensity of 100 µmol $m^{-2}s^{-1}$ on the content and productivity of different groups of isoflavonoids in 2-year-old *P. lobata*-stabilised calli. Compared with the dark-grown control, different light treatments had different effects on the biosynthesis of individual phytochemical compounds in *P. lobata* calli. Neither red nor green LED light affects the content of genistein aglycone. Moreover, the balance of genistein derivatives in light-treated calli noticeably shifted toward the prenylated forms, and the content of glycosylated forms decreased. Thus, compared with the dark-grown control, red light increased the content of prenylated forms of genistein by more than twofold, whereas green light increased it by nearly fourfold. Similarly, exposure to light affected daidzein derivatives. Thus, green light was the most effective treatment for increasing the abundance of the biologically active prenylated isoflavonoid form—up to 4 times. We managed to find only one publication investigating the effect of monochromatic lighting on isoflavonoid biosynthesis in cell culture. The total isoflavonoid content in the callus culture of *P. candollei* increased by 3- and 2-fold under red and white LED lights, respectively (Sae-Foo et al. 2025).

Prospects for further research

It is well known that establishing and maintaining cell cultures of legumes is challenging. This process takes much more effort for the woody plants in the family. The success of initiating and long-term maintenance of legume callus cultures depends largely on the composition of the nutrient medium, the type of explant, and the morphology of the callus. (Susunaga-Gómez et al. 2025). The aim of this study was to establish an efficient method for callus induction and proliferation, ultimately enabling the successful in vitro conservation of a protected relict plant species. There is a misconception that *P. lobata* grows throughout the Russian Far East (Kou et al. 2025). That region covers a vast area characterised primarily by very harsh climatic conditions. In the 1960s, the plant was discovered at three or four locations in the southernmost part of Primorsky Krai and was described as a native species (Kozhevnikov et al. 2017). Since then, the presence of *P. lobata* at these locations has not been confirmed. Mori, Hokkaido (Japan), is generally considered the northernmost natural range of *P. lobata*. Owing to a lack of international publications, the global scientific community is virtually unaware of the species' limited natural range within the Russian Federation. There is only one publication that mentions it; however, that work lacks information regarding the species' specific location and a detailed description. Notably, the temperature conditions in southern Primorsky Krai are harsher than those at the Japanese site (according to data from http://russia.pogoda360.ru/926582/avg/and climate data.org). Consequently, both phylogenetic and phytochemical studies of this species at its northernmost location are of great scientific interest. As previously noted, *P. lobata* is highly protected; however, while this aids in preserving its natural range, it complicates research on plants in the wild. We believe that the present study will serve as a foundation for comprehensive research into the northernmost representative of the genus *P. lobata*—encompassing everything from precise location mapping to taxonomic clarification and successful in vitro cultivation. Moreover, we suggest that the results obtained (optimal media variants) may prove useful for establishing callus cultures of other rare woody legume species.

Conclusion

Widely known kudzu contains high concentrations of healthy isoflavones, such as the unique form of puerarin. In the southern part of the Russian Far East, this plant is found in only three isolated locations. The inaccessibility of northernmost *P. lobata* plants and their status as a protected species pose major obstacles to research. In this regard, we conducted a pilot experiment on the initiation and stabilisation of a callus culture from a commercially available *P. lobata* cultivar. More than 50 mediums for the induction and subculturing of *P. lobata* calli were tested; the optimal variants for inducing callus (2 mg L^{-1} TDZ) and for long-term subculturing (a combination of BAP and NAA at concentrations of 5 and 0.1 mg L^{-1}) were selected. The isoflavonoid profile of *P. lobata* calli was studied. The isoflavonoids are represented primarily by glycosylated forms of daidzein and genistein. Prenylated forms include prenylgenistein 5 and its glycosylated forms. The total isoflavonoid productivity in the calli reached 75 mg L^{-1} DW. Thus, compared with *G. max* calli, *P. lobata* calli are more potent alternative sources of isoflavonoids. Monochromatic LED red lighting increased the content of the prenylated forms of genistein more than twofold. Green LED light was the most effective treatment for increasing the levels of the prenylated isoflavonoid form—up to 4 times. We believe that the present study will serve as a foundation for comprehensive research into the northernmost representative of the genus *P. lobata*—encompassing everything from precise location mapping to taxonomic clarification and successful in vitro cultivation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-0

26-03663-0.

Acknowledgements The analyses described in this work were performed *via* equipment from the Instrumental Centre for Biotechnology and Gene Engineering at the Federal Scientific Centre of East Asia Terrestrial Biodiversity of the Far East Branch of the Russian Academy of Sciences using lighting equipment from IACP FEB RAS in state assignments of the Ministry of Science and Higher Education of the Russian Federation (FWFW-2024-0004).

Author contributions G.N. : Conceptualization, Data curation, Project administration, Supervision.G.N. , E.V. , V.P. , S.A. , O.A. , S.O. , E.P.: Investigation, Methodology, Formal Analysis Validation, Visualization, Writing – original draft. All authors reviewed the manuscript.

Funding The research was carried out within the state assignment of the Ministry of Science and Higher Education of the Russian Federation (theme No. 124012200181-4).

Data availability No datasets were generated or analysed during the current study.

Code availability Not applicable.

Declarations

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication All the authors whose names appeared on the submission approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that the questions related to the accuracy of integrity of any part of the work were appropriately investigated and resolved.

Competing interests The authors declare no competing interests.

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