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RESEARCH

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Tailoring enhanced production and identification of isoflavones in the callus cultures of *Pueraria thomsonii* Benth and its model verification using response surface methodology (RSM): a combined in vitro and statistical optimization

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Abstract

Background: Scientifically, isoflavones from *Pueraria thomsonii* Benth possess diverse pharmacological activities and have been used to treat various diseases. In vitro propagation of callus has contributed to the reliability for large-scale production of target compounds. However, the factors affecting the biosynthesis of major isoflavones daidzin, puerarin and daidzein in the callus culture of *P. thomsonii* are still not known. Therefore, we aimed to enhance the in vitro production of daidzin, puerarin and daidzein by optimizing three independent factors such as temperature, NAA and 6-BA concentrations.

Results: Our findings showed that the optimal concentrations for in vitro biomass production and efficient synthesis of puerarin, daidzin and daidzein were found to be 0.158%, 0.463% and 0.057%, respectively. In addition, the HPLC fingerprint with chemo-metrics analysis was constructed by linear regression of the puerarin, daidzin and daidzein which was found to be in the range of 1.0–36.0, 5.0–72.0 and 1.0–15.0 mg/mL and the LODs and LOQs were found to be 0.15, 0.52, 0.35 and 0.28, 1.50, 0.50 mg/mL for puerarin, daidzin and daidzein, respectively. Surprisingly, our results were also in agreement with the concentration obtained from the model verification for optimal and efficient production of puerarin, daidzin and daidzein which was found to be 0.162%, 0.458% and 0.049%, respectively.

Conclusions: In summary, our present investigation provides new insights that could facilitate the enhanced production of valuable isoflavones in *P. thomsonii* using plant cell cultures treated with appropriate elicitor combinations and temperature. As far as the authors are concerned, this is the first report on production of daidzin, puerarin and daidzein at higher yield at laboratory level for a wide range of applications in future food, medicinal and pharmaceutical companies.

Keywords: *Pueraria thomsonii*, Callus induction, Isoflavones, Elicitors, HPLC analysis, RSM

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1 Background

Pueraria thomsonii is a creeping, climbing and trailing perennial vine belonging to the plant family Leguminosae. It is a variety of *Pueraria lobata* mainly distributed

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in East Asian countries, such as China and Japan [1]. The dried root of *P. thomsonii* is an important edible Chinese medicinal material named Fen-Ge which is used to treat influenza, cervical spondylosis, alcoholism, chest stuffiness, hemiplegia, vertigo, headache and other illnesses [2, 3]. The main bioactive components, especially isoflavones occurring in *P. thomsonii*, are puerarin, daidzin and daidzein which are proved to possess diverse pharmacological properties in the treatment of cardiovascular [4, 5], hypolipidemic [6], hyperglycemic disorders [7, 8], cancer [9, 10], epileptic [11], bladder dysfunction [12] and multiple myeloma [13], knee osteoarthritis [14], breast cancer [15, 16], hepatic fibrosis [17] and Parkinson disease [18]. These bioactive molecules are synthesized during environmental stress and are very important in the interactions between producer plant and other organisms for key protective role against oxidative stress-induced cell damage [19]. Such reports put these therapeutic biomolecules in high demand in order to meet the needs of medicinal and pharmaceutical industries. Therefore, the production of these valuable bioactive isoflavones needs to be enhanced using in vitro elicitation technique. In this line, the callus culture could be a realistic source of isoflavonoids production with no time and space limitations and the technique has been widely used as an alternative for the production of plant secondary metabolites [20, 21]. Similarly, some authors have reported flavones accumulation in callus culture of *Phaseolus coccineus* and *Glycine max* [22], antioxidants in Thai Basil callus [23], plumbagin production in root callus of *Plumbago zeylanica* L. [24] and biosynthesis of phenylpropanoid in purple basil callus [25]. However, the bioactive isoflavonoids production in callus is influenced by many complex factors, including genetic and environmental factors [26]. In our previous studies, we have found the potential biosynthetic pathways and transcriptional regulation of isoflavones in *P. thomsonii* by comprehensive transcriptome analysis [27]. However, the effects of environmental factors and plant growth regulators (PGRs) such as 6-benzylaminopurine (6-BA) and 1-naphthaleneacetic acid (NAA) on accumulation mechanism of total isoflavonoids such as puerarin, daidzin and daidzein in *P. thomsonii* are still unknown in the in vitro callus cultures [20, 28]. Similarly, Downey et al. (2012) reported that the ratio of NAA and 6-BA to have profound effect on both callus growth and isoflavonoids accumulation. RSM is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the relative significance of multiple independent variables and determining the optimal conditions for the desired response and is widely used to optimize the production conditions of bioactive compounds [29–31]. Therefore, the induction of callus culture from *P. thomsonii* is thought to be important for large-scale production of isoflavones. In this context, the present study was aimed to enhance the production of isoflavones such as puerarin, daidzin and daidzein in the callus cultures of *P. thomsonii* by optimizing the individual and interacting effects of different temperature and elicitors NAA and 6-BA at different concentrations and to validate by Central Composite Rotatable Design (CCRD). Further, the accumulation of isoflavones in the in vitro propagated callus was identified and quantified by simple and rapid HPLC metabolic profiling.

2 Methods

2.1 Plant material and chemicals

The young shoots of *P. thomsonii* were collected from Central China Medicinal Botanical Garden (Enshi, China, E109°45′24″; N30°10′51″). The nomenclature of the collected plant material was authenticated by Dr Meijun He, Hubei Academy of Agricultural Sciences. 1-naphthaleneacetic acid (NAA, ≥ 98% purity), 6-benzylamino purine (6-BA, ≥ 98% purity), sucrose and agar were purchased from Sangon Biotech (Shanghai, China). Murashige and Skoog Basal Salt medium (MS) was purchased from Sigma-Aldrich (China). The organic solvents used for extraction were procured from Beijing chemical factory (Beijing, China). HPLC-grade methanol for quantitative HPLC was obtained from Merck KGaA, Darmstadt, Germany. Distilled water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA).

2.2 Callus Induction and cultivation of *P. thomsonii*

Sterile solid MS culture medium containing 3% sucrose, PGRs-free and 0.8% agar was adjusted to pH-5.8 before sterilization (121 °C, 15 psi, for 15 min). The young shoots of *P. thomsonii* were surface-sterilized following the method of Su et al. [32]. Briefly, the young shoots were washed 5 times with sterile water and then soaked in 75% ethanol for 45 s followed by intermittent soaking in 0.1% $HgCl_2$ for 20 min. The shoots were washed with sterile water 3 times and then were cut into pieces of about 2 cm long. Initially, four explants were transferred into jars containing 40 mL of MS medium with NAA (0.25 mg/L) and 6-BA (1.5 mg/L). Explants were incubated at 25 ± 2 °C under photoperiod of 16 h with white fluorescent light (50 μmol m^{-2} s^{-1}) for 14 days. After incubation, the uninfected and well-grown primary callus of *P. thomsonii* were harvested for extraction.

2.3 Delineating the influence and establishing variability ranges of single factors on production of puerarin, daidzin and daidzein

To optimize the individual and interacting effects of temperature, elicitors such as NAA and 6-BA at different concentrations on accumulation of isoflavones in the in vitro callus culture. Initially, the callus was sub-cultured

under six different temperature, viz. 16, 20, 24, 28, 32 and 36 °C containing 40 mL of MS medium with NAA (0.25 mg/L) and 6-BA (1.5 mg/L), under photoperiod of 16 h and white fluorescent light (50 μmol m^{-2} s^{-1}), the callus formation rate (CRF) was calculated after 5 weeks. The elicitation studies were carried out with 40 mL of MS medium, supplemented with various concentrations of NAA (0, 0.50, 1.00, 1.50, 2.00 and 2.50 mg/L) and the other culture conditions were 6-BA (1.5 mg/L), under photoperiod of 16 h and white fluorescent light (50 μmol m^{-2} s^{-1}) for 30 days. In order to evaluate the effect of 6-BA concentration on production of puerarin, daidzin and daidzein, 40 mL MS medium was supplemented with various concentrations of 6-BA (0.00, 0.50, 1.00, 1.50, 2.00 and 2.50 mg/L) and other culture conditions were NAA (0.25 mg/L) under photoperiod of 16 h and white fluorescent light (50 μmol m^{-2} s^{-1}) for 30 days. After incubation, the isoflavones accumulations in callus were quantified to evaluate the effect of temperature, NAA and 6-BA on production of puerarin, daidzin and daidzein using HPLC chemical fingerprinting. The data was collected from three independent assays conducted separately. The obtained data correlating with the most promising range for production of puerarin, daidzin and daidzein were further chosen to be included within the second-phase multifactorial runs.

2.4 HPLC quantification of isoflavones content in the cultured callus

Five grams of well grown callus was extracted with 50 mL of 100% ethanol by sonication (500 w) for 1 h. Callus extracts were then filtered through nylon filter papers (0.22 μm, Kewei, China) and evaporated to dryness under reduced pressure and analyzed for its secondary metabolites using HPLC. Briefly, the solvent system consisted of solvent A (0.1% HOAc/10% MeOH in H_2O) and solvent B (0.1% HOAc/100% MeOH). The HPLC conditions were at the flow rate of 1.0 mL/min with a stepwise gradient from 0 to 80% of solvent B (0–20 min), 80% to 100% of solvent B (20–21.5 min), 100% of solvent B (21.5–27.0 min), and 0% of solvent B (27.1–30.0 min). The DAD detector (Agilent) was programmed to scan the entire UV range from 190 to 400 nm whereas the 270 nm and 360 nm wavelengths were used for isoflavones detection.

Chromatographic profiles of the standards and callus extract were identical with regard to retention time mentioned above. In addition, no interference was observed at the retention times of any analytes in the chromatogram of the extract. The calibration curves for puerarin, daidzin and daidzein were made by plotting the peak area versus the concentration for each analyte using least-square regression analysis. Three calibration curves were obtained using six levels of concentrations in three ranges of 5.0–72.0 mg/mL, 1.0–36.0 mg/mL, and 1.0–15.0 mg/mL in triplicate. The ranges of all the calibration curves were adequate for simultaneous analysis of the three flavonoids in the callus samples. The linear correlation coefficient (R^2) for all calibration curves was higher than 0.999 for all analytes, indicating good linearity over the investigated range. The limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation (σ) of the response and the slope (S): LOD $= 3.3 \times \sigma/S$, LQD $= 10 \times \sigma/S$. The LOD and LOQ for puerarin, daidzin and daidzein were in the ranges of 0.15–0.28, 0.52–1.50 and 0.35–0.5 mg/mL, respectively.

2.5 Optimization of callus induction using RSM

In order to obtain maximum yield of isoflavones from callus, a CCRD was created with the aid of the Design Expert software 8.0.6. Based on the literature and our previous data, three factors were selected as independent variables: temperature, NAA and 6-BA concentration. The design comprised a total of 20 experiments, 3 experimental variations, 5 levels (−alpha, low, middle, high and + alpha coded as −1.68179, −1, 0, +1 and +1.68179, respectively) and 6 center points (Additional file 1: Table S1). All runs were incubated with white fluorescent light (50 μmol m^{-2} s^{-1}) for 30 days. Each run was repeated three times. The effect of the variables and their interactions were considered statistically significant when $p < 0.05$. Nonsignificant factors were excluded from the model.

2.6 Statistical analysis

Design Expert 8.0.6 software was used to analyze the results of CCRD. The quality of the regression of the model equation was evaluated by the coefficients R^2, adjusted R^2 and predicted R^2. The significance of the factors such as temperature, NAA and 6-BA concentrations and the interactions were determined by the tabulated and calculated F value at $p = 0.05$. The lack-of-fit test was used to evaluate the differences of experimental and pure error. An insignificant lack-of-fit test (p value > 0.05) stated that the model correctly represented the correlation between response and predictors. The normal probability plot was used to observe the distribution of the residuals [33]. In general, the adjusted coefficient of determination (R^2adj) and predicted coefficient of determination (R^2pre) should be within approximately 20% of each other to be in reasonable agreement [34].

3 Results

3.1 Callogenesis and isoflavones production

The surface-sterilized explants of *P. thomsonii* were inoculated on sterile MS medium amended with varying

concentration of elicitor's 6-BA and NAA at different temperatures for callus induction and efficient biosynthesis of isoflavones. After five weeks of incubation, the explants gave rise to white to brownish compact to crisp callus (Fig. 1a, g, k). In order to evaluate the proportion isoflavones, the dry weight of the callus was identified by HPLC (Additional file 1: Table S2–S4). Figure 1 h, m shows the biosynthesis of daidzin and daidzein was significantly enhanced when culture temperature gradually increased up to 24 °C with decline at higher temperature more than 24 °C. In contrast, the content of puerarin increased when culture temperature increased gradually up to 28 °C and then its content did not increase with the rise in temperature over 28 °C (Fig. 1c). Our results clearly showed that the culture temperature had a significant effect on biosynthesis of puerarin, daidzin and daidzein**.** These results suggested that low temperature (≤ 24 °C) enhanced the production of isoflavones such as daidzin, daidzein and puerarin content in the callus culture. In order to determine the optimum 6-BA concentration for maximum production of puerarin, daidzin and daidzein, the concentrations ranging from 0.00 to 2.50 mg/L were used. Interestingly, the maximum production of daidzin, daidzein and puerarin was observed at 2.00 and 1.50 mg/L, respectively (Fig. 1d, i, n). Further increase in the concentration of 6-BA greatly affected the biosynthesis of puerarin, daidzein and daidzin and it started to decline. To determine the influence of NAA concentration on production of puerarin, daidzin and daidzein, six different concentrations such as 0.00, 0.50, 1.00, 1.50, 2.00 and 2.50 mg/L were investigated. The content of puerarin, daidzin and daidzein gradually increased as the concentration of NAA increased up to 2.50 mg/L (Fig. 1e, j, o). Based on the above observation, culture temperature from 20 to 28 °C and 6-BA concentration ranging from 1.00 and 2.50 mg/L, were chosen for the Central Composite design (CCD) to determine the optimal culture temperature and 6-BA concentration. Further, NAA concentrations were selected representing low and high values of this particular factor as the starting point for the second round of optimization.

3.2 HPLC analysis of *P. thomsonii* callus extract and standard compounds

In order to determine the content of three major isoflavones-related compounds in *P. thomsonii* callus extract; retention time and UV absorption are used for peak identification and simultaneous qualitative analysis of the main isoflavones were monitored at 270 and 370 nm. Figure 2 shows the superposition of the representative chromatograms of callus extracts under elicitor treatments at different temperatures. The retention times of puerarin, daidzin and daidzein in callus extract were 3.48 min, 4.72 min and 9.78 min, respectively, and these isoflavones are proved to be the most important and abundant components of this species.

3.3 Linearity and range

The in-house HPLC–DAD method is presented in detail in Sect. 2.6. HPLC procedure was applied to identify and quantify isoflavones compounds in *P. thomsonii*. The calibration curves of the isoflavones were constructed by linear regression of the analytes concentration against peak area at 270 and 370 nm resulting in linear ranges from 1.0–36.0, 5.0–72.0 and 1.0–15.0 mg/mL and the LODs and LOQs were 0.15, 0.52; 0.35 and 0.28; 1.50, 0.50 mg/mL for puerarin, daidzin and daidzein, respectively. Interestingly, the HPLC was found to have linear calibration over the entire working ranges for all the analytes shown in Fig. 3a–c (Table 1). Therefore, the HPLC was specific and sensitive enough for simultaneously quantitating three isoflavones in the callus culture extracts.

3.4 Optimization of callus culture parameters for isoflavones synthesis and the effect of temperature, 6-BA and NAA using CCD

After establishing the preliminary effect of each parameter of the culture conditions (culture temperature, 6-BA and NAA concentration) individually on production of puerarin, daidzin, and daidzein, the influence of these factors collectively on productions of puerarin, daidzin and daidzein was delineated through a RSM approach using the CCD. We utilized three variables-five levels (− alpha, low, middle, high and + alpha coded as − 1.68179, − 1, 0, + 1 and + 1.68179, respectively) and 20 different runs recording the hypothetical and experimental outcomes

(See figure on next page.)

Fig. 1 **a** Macroscopic image of the callus with high production of puerarin. **b** Chemical structure of puerarin. **c** Effect of different culture temperature on production of puerarin. **d** Effect of different 6-BA concentration on production of puerarin, **e** Effect of different NAA concentration on production of puerarin. **f** Chemical structure of daidzein. **g** Macroscopic image of the callus with high production of daidzein. **h** Effect of different culture temperature on production of daidzein. **i** Effect of different culture temperature on production of daidzein. **j** Effect of different NAA concentration on production of daidzein. **k** Macroscopic image of the callus with high production of daidzin. **l** Chemical structure of daidzin. **m** Effect of different culture temperature on production of daidzin. **n** Effect of different culture temperature on production of daidzin. **o** Effect of different NAA concentration on production of daidzin

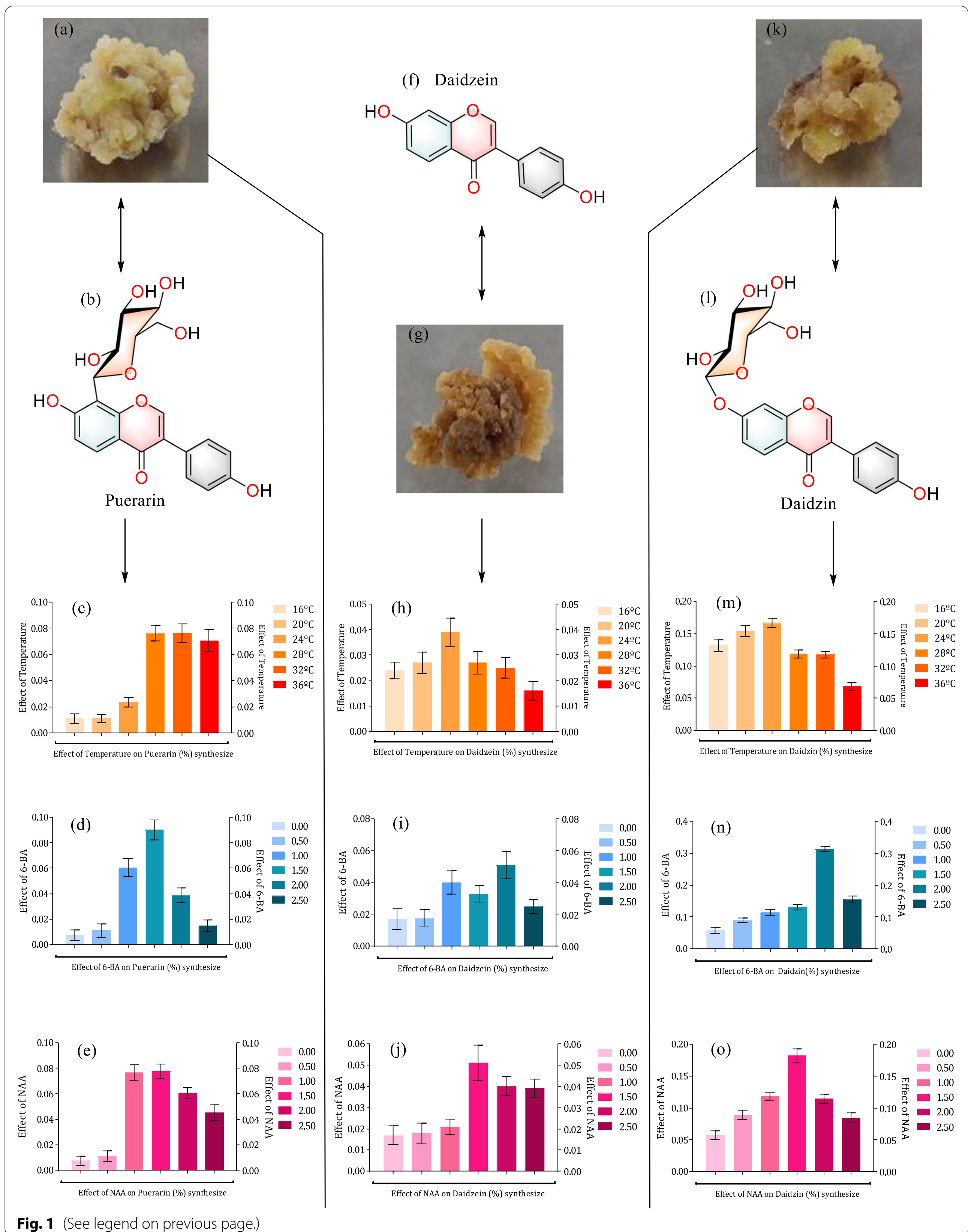

Fig. 1 (See legend on previous page.)

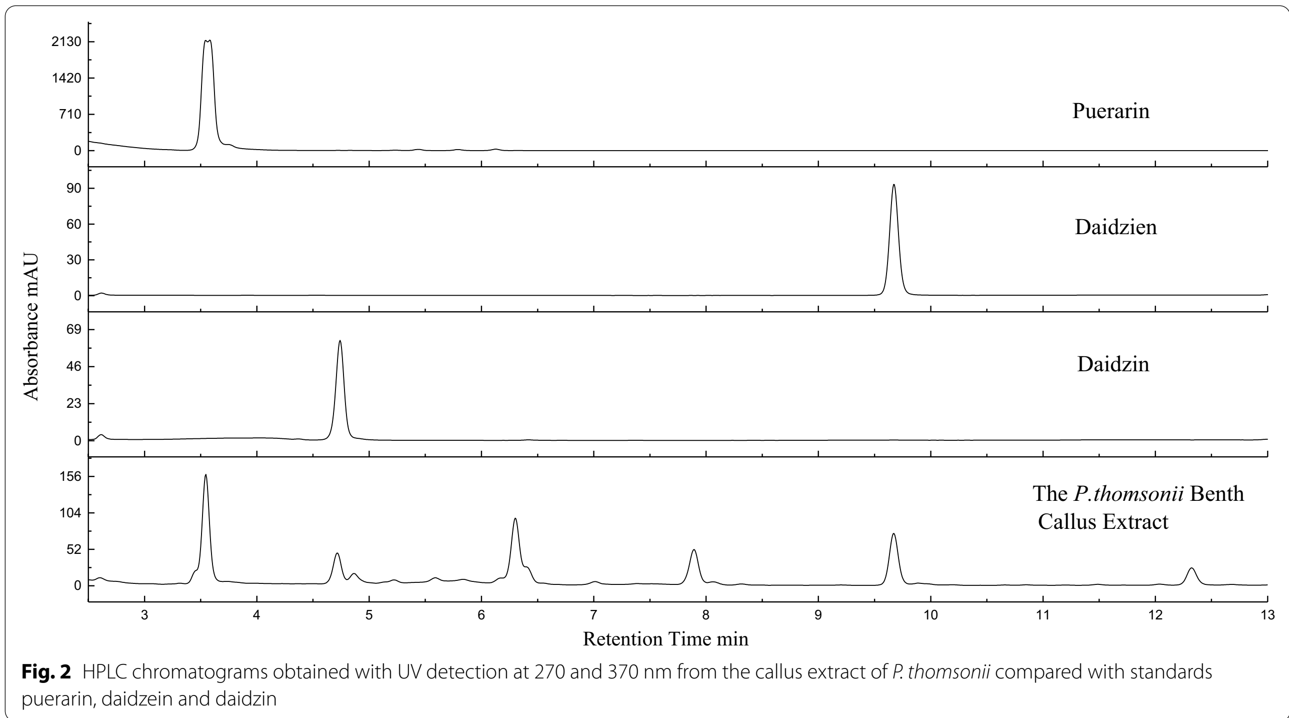

Fig. 2 HPLC chromatograms obtained with UV detection at 270 and 370 nm from the callus extract of *P. thomsonii* compared with standards puerarin, daidzein and daidzin

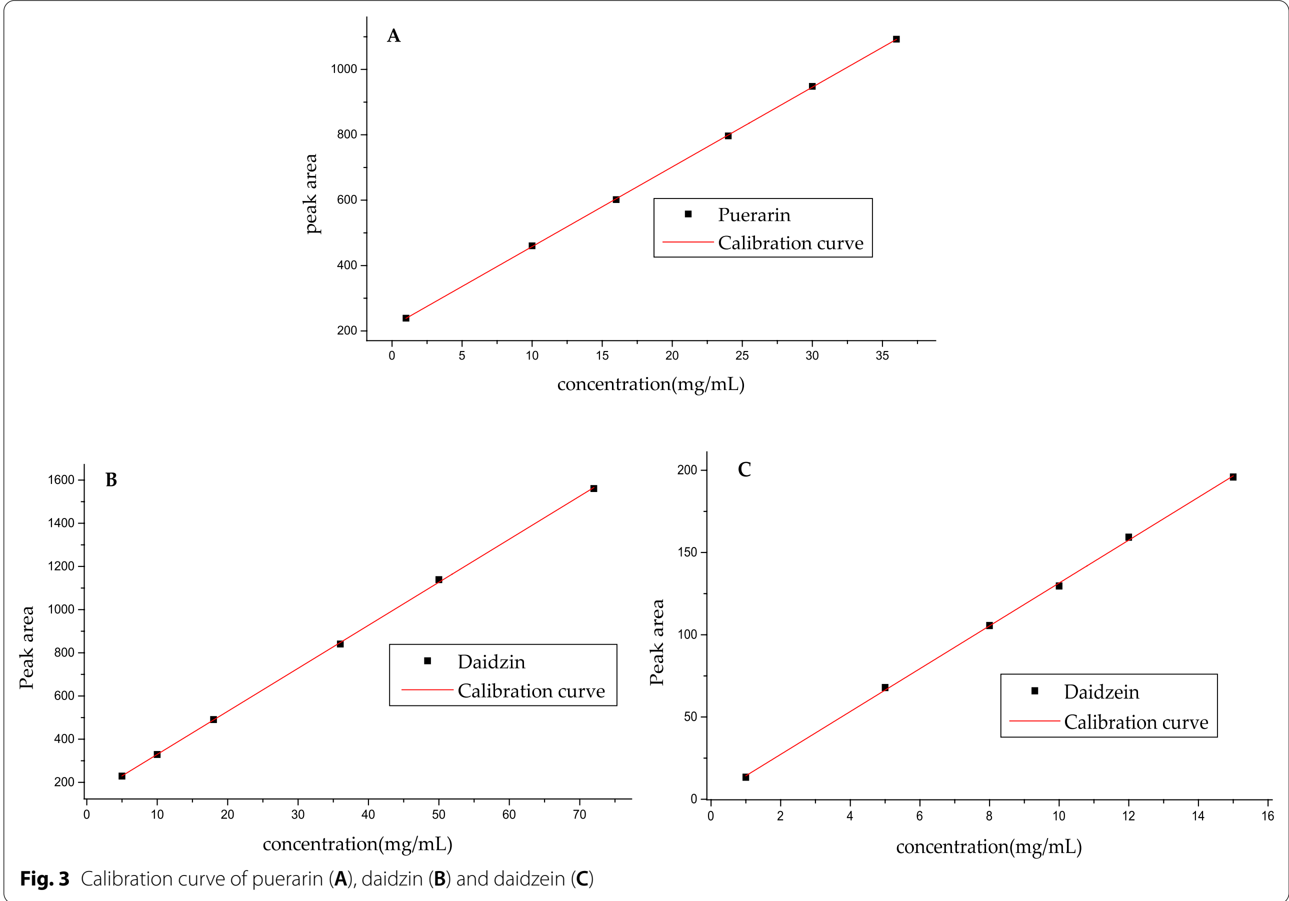

Fig. 3 Calibration curve of puerarin (**A**), daidzin (**B**) and daidzein (**C**)

Table 1 Calibration curve analysis for puerarin, daidzin and daidzein

Analyte	Calibration curve	R^2	Linear range (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)
Puerarin	$y = 24.38686x + 214.09593$	0.99994	1.0–36.0	0.15	0.28
Daidzin	$y = 29.93579x + 130.22225$	0.99980	5.0–72.0	0.52	1.50
Daidzein	$y = 12.3412x + 13.02450$	0.99941	1.0–15.0	0.35	0.50

(content of puerarin, daidzin and daidzein) for each of these combination treatments (Table 2).

Furthermore, to find the competency of the CCD-developed model and the significance of the associated factors, an analysis of variance (ANOVA) was performed on the obtained results. The outcomes are summarized in Table 2. Puerarin varied between 0.017 and 0.165%, daidzin from 0.115 to 0.521% and daidzein from 0.008 to 0.069%.

The effect of temperature, 6-BA and NAA concentration on content of puerarin, daidzin and daidzein is depicted in Table 3. Both the 6-BA concentration ($p = 0.0011$) and the interaction ($p = 0.0398$) between temperature and 6-BA concentration showed a positive significant effect on puerarin. The cytokinin 6-BA promoted callus cell growth and increased callus isoflavonoid suggesting that the puerarin accumulation was keeping pace with the cell growth in the elevated concentration of 6-BA. The interaction between NAA and 6-BA concentration and all quadratic factors was significant and had negative effect on this response. The quadratic effect of the temperature presented the major influence on puerarin (the lowest coefficient, +1.86), which made sense as values below or above the optimum temperature range would diminish cellular metabolism. Figure 4 A shows optimum temperature and concentrations of 6-BA for production of puerarin near the middle of the applied concentrations of 6-BA and temperature (6-BA/1.75 mg/L and temperature/24.0 °C). The response surface plot for NAA (B)

Table 2 Central composite design matrix of three variables (temperature, 6-BA and NAA) in coded and actual concentration units along with the study of responses with respect to puerarin, daidzin and daidzein synthesis in *P. thomsonii* callus culture

Std	Run	Independent variables			Response values		
		Tem (°C)	6-BA (mg/L)	NAA (mg/L)	Puerarin (%)	Daidzin (%)	Daidzein (%)
8	1	28.0 (+1)	2.5 (+1)	2.50 (+1)	0.0685 ± 0.0088	0.3063 ± 0.0254	0.0430 ± 0.0045
9	2	17.3 (−1.68)	1.75 (0)	1.75 (0)	0.0750 ± 0.0094	0.2875 ± 0.0342	0.0250 ± 0.0036
16	3	24.0 (0)	1.75 (0)	1.75 (0)	0.1560 ± 0.0143	0.3909 ± 0.0458	0.0620 ± 0.0065
1	4	20.0 (−1)	1.00 (−1)	1.00 (−1)	0.0315 ± 0.0063	0.1711 ± 0.0123	0.0180 ± 0.0078
17	5	24.0 (0)	1.75 (0)	1.75 (0)	0.1354 ± 0.0153	0.4511 ± 0.0458	0.0690 ± 0.0094
15	6	24.0 (0)	1.75 (0)	1.75 (0)	0.1654 ± 0.0241	0.5206 ± 0.0463	0.0510 ± 0.0057
3	7	20.0 (−1)	2.50 (+1)	1.00 (−1)	0.0612 ± 0.0073	0.1889 ± 0.0243	0.0090 ± 0.0087
4	8	28.0 (+1)	2.50 (+1)	1.00 (−1)	0.0356 ± 0.0026	0.2545 ± 0.0378	0.0080 ± 0.0049
7	9	20.0 (−1)	2.50 (+1)	2.50 (+1)	0.0557 ± 0.0037	0.1145 ± 0.0145	0.0420 ± 0.0037
11	10	24.0 (0)	0.49 (−1.68)	1.75 (0)	0.0645 ± 0.0095	0.1856 ± 0.0183	0.0450 ± 0.0098
10	11	30.7 (+1.68)	1.75 (0)	1.75 (0)	0.0167 ± 0.0042	0.3689 ± 0.0253	0.0310 ± 0.0082
6	12	28.0 (+1)	1.00 (−1)	2.50 (+1)	0.1508 ± 0.0115	0.3974 ± 0.0328	0.0330 ± 0.0048
20	13	24.0 (0)	1.75 (0)	1.75 (0)	0.1600 ± 0.0187	0.4207 ± 0.0661	0.0530 ± 0.0018
5	14	20.0 (−1)	1.00 (−1)	2.50 (+1)	0.0655 ± 0.0068	0.3113 ± 0.0502	0.0110 ± 0.0034
14	15	24.0 (0)	1.75 (0)	3.01 (+1.68)	0.1045 ± 0.0121	0.2613 ± 0.0467	0.0310 ± 0.0048
12	16	24.0 (0)	3.01 (+1.68)	1.75 (0)	0.0511 ± 0.0069	0.2635 ± 0.0183	0.0410 ± 0.0079
19	17	24.0 (0)	1.75 (0)	1.75 (0)	0.1490 ± 0.0211	0.4807 ± 0.0834	0.0480 ± 0.0041
13	18	24.0 (0)	1.75 (0)	0.49 (−1.68)	0.0327 ± 0.0071	0.1667 ± 0.0282	0.0230 ± 0.0028
2	19	28.0 (+1)	1.00 (−1)	1.00 (−1)	0.0272 ± 0.0054	0.2167 ± 0.0516	0.0340 ± 0.0019
18	20	24.0 (0)	1.75 (0)	1.75 (0)	0.1601 ± 0.0214	0.4414 ± 0.0918	0.0540 ± 0.0083

Data are expressed as means ± standard deviations (SD) of three independent assays

Table 3 Estimated effects for temperature, 6-BA and NAA concentration on production of puerarin, daidzin, and daidzein

Factors	Puerarin (%)			Daidzin (%)			Daidzein (%)		
	Effect	SE	*p* value	Effect	SE	*p* value	Effect	SE	*p* value
Intercept	0.150	4.801×10^{-3}	< 0.0001*	0.450	0.019	< 0.0001*	0.056	3.324×10^{-3}	< 0.0001*
A:Temp	–	–	–	0.039	0.013	0.0097*	–	–	–
B:NAA	–	–	–	–	–	–	–	–	–
C:6-BA	0.021	3.185×10^{-3}	< 0.0001*	0.033	0.013	0.0207*	5.379×10^{-3}	2.205×10^{-3}	0.0286*
AB	–	–	–	–	–	–	–	–	–
AC	0.014	4.162×10^{-3}	0.0064*	–	–	–	–	–	–
BC	− 0.014	4.162×10^{-3}	0.0056*	− 0.043	0.017	0.0228*	9.500×10^{-3}	2.881×10^{-3}	0.0053*
A2	− 0.029	3.101×10^{-3}	< 0.0001*	− 0.043	0.012	0.0041*	− 0.011	2.147×10^{-3}	0.0001*
B2	− 0.034	3.101×10^{-3}	< 0.0001*	− 0.080	0.012	< 0.0001*	-6.025×10^{-3}	2.147×10^{-3}	0.0140*
C2	− 0.030	3.101×10^{-3}	< 0.0001*	− 0.083	0.012	< 0.0001*	− 0.012	2.147×10^{-3}	< 0.0001*

*: the effect of factors and their interactions considered statistically significant

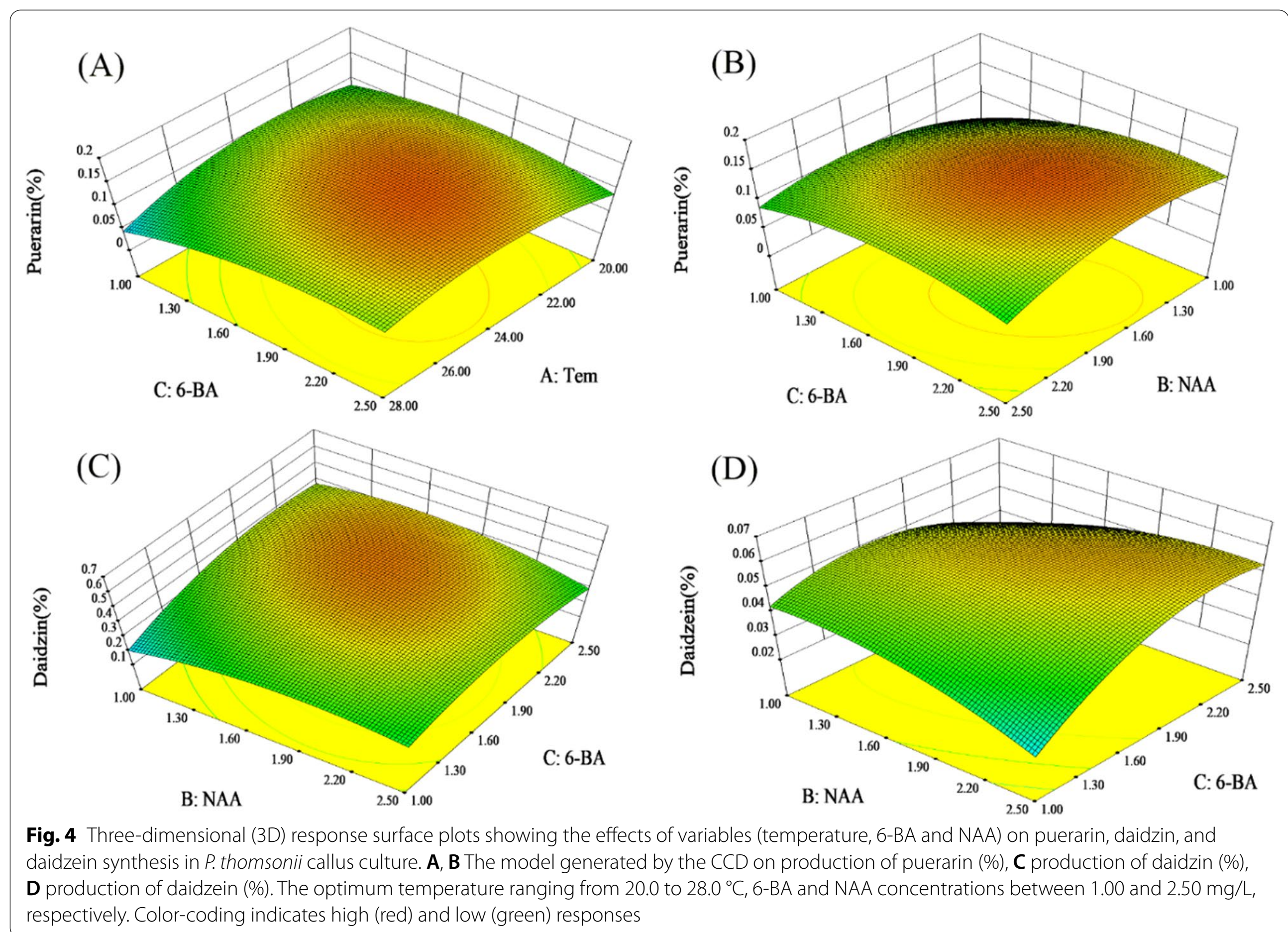

Fig. 4 Three-dimensional (3D) response surface plots showing the effects of variables (temperature, 6-BA and NAA) on puerarin, daidzin, and daidzein synthesis in *P. thomsonii* callus culture. **A**, **B** The model generated by the CCD on production of puerarin (%), **C** production of daidzin (%), **D** production of daidzein (%). The optimum temperature ranging from 20.0 to 28.0 °C, 6-BA and NAA concentrations between 1.00 and 2.50 mg/L, respectively. Color-coding indicates high (red) and low (green) responses

and 6-BA (C) showed a rising peak point toward the middle of concentrations of 6-BA and NAA (Fig. 4B). The ANOVA table showed that the empirical model and the lack of fit were statistically insignificant (Table 4).

The temperature ($p=0.0097$) and 6-BA concentration ($p=0.0207$) showed a positive significant effect on the production of daidzin, while the interaction between temperature and 6-BA concentration, and all quadratic factors had negative significant effect on this response

Table 4 Analysis of variance (ANOVA) of the influence of temperature, 6-BA and NAA concentration on puerarin, daidzin and daidzein synthesis

Source of variation	Puerarin (%)					Daidzin (%)					Daidzein (%)				
	Sum of squares	***df***	**Mean square**	***F* value**	***p* value**	**Sum of squares**	***df***	**Mean square**	***F* value**	***p* value**	**Sum of squares**	***df***	**Mean square**	***F* value**	***p* value**
Model	0.044	6	7.291×10^{-3}	52.620	< 0.0001*	0.240	6	0.039	17.840	< 0.0001*	4.817×10^{-3}	5	9.634×10^{-4}	14.510	< 0.0001*
Residuals	1.801×10^{-3}	13	1.386×10^{-4}	–	–	0.029	13	2.212×10^{-3}	–	–	9.298×10^{-4}	14	6.642×10^{-5}	–	–
Lack of fit	1.224×10^{-3}	8	1.530×10^{-4}	1.320	0.3944ns	0.018	8	2.301×10^{-3}	1.110	0.4750ns	6.230×10^{-4}	9	6.922×10^{-5}	1.130	0.4722ns
Pure Error	5.775×10^{-4}	5	1.155×10^{-4}	–	–	–	5	2.070×10^{-3}	–	–	3.068×10^{-4}	5	6.137×10^{-5}	–	–

*: The correlation between response and predictors in the model is significant; *NS*: The lack of fit is statistically insignificant

(Table 3). Figure 4C shows that optimum concentrations of 6-BA and NAA for production of daidzin were observed around the middle of the applied concentrations of 6-BA and NAA (6-BA, 1.75 mg/L and NAA, 1.75 mg/L), which showed a rising peak point toward the middle of concentrations of 6-BA and NAA. The ANOVA table showed that the empirical model was statistically significant ($p < 0.0001$), and the lack of fit was statistically insignificant (Table 3).

The 6-BA concentration and the interaction between NAA and 6-BA showed a positive significant effect on production of daidzein, but all quadratic factors (A^2, B^2, C^2) had negative significant effect on this response (Table 3). 6-BA increased the content of daidzein in callus by enhancing callus proliferation and rapid induction of the isoflavonoid daidzein. The effect of quadratic temperature showed that low temperature increased the daidzein content in *P. thomsonii* callus. For daidzein, the response surface plot for NAA (B) and 6-BA (C) showed a rising ridge shape toward higher concentrations (Fig. 4D). The ANOVA table showed that the empirical model was statistically significant, and the lack of fit was statistically insignificant (Table 4).

3.5 Testing model compliance with the quadratic fit using the CCD

To further adjust and fine-tune a quadratic model that better explained the relationship between three independent variables such as temperature/X1, NAA/X2 and 6-BA/X3 and three dependent variables on content of puerarin, daidzin and daidzein, the CCD-RSM fitted models were carefully analyzed by the analysis of variance (ANOVA). The final model for production of puerarin, daidzin and daidzein was developed (Eqs. 1–3). The experimental data for content of puerarin, daidzin and daidzein showed good correlation between actual and predicted values ($R^2 = 0.961$/puerarin, 0.892/daidzin and 0.838/daidzein) predicted by the model (Eqs. 1 –3).

By using the models (Eqs. 1–3), the predicted highest production of puerarin was found to be 0.158% at the predicted optimal parameters: 24.4 °C, 2.04 mg/L 6-BA and 1.69 mg/L NAA; the predicted highest production of daidzin was found to be 0.463% at the predicted optimal parameters: 25.8 °C with 1.91 mg/L of 6-BA and 1.71 mg/L of NAA; the predicted highest production of daidzein was found to be 0.057% at the predicted optimal parameters: 24.0 °C with 1.95 mg/L and 2.00 mg/L, respectively. To validate the model generated by the RSM design, we performed three independent runs at predicted optimal condition for both dependent variables. The results obtained were very close to those predicted by the model. On an average, $0.162 \pm 0.011\%$ puerarin, $0.458 \pm 0.031\%$ daidzin and $0.049 \pm 0.008\%$ daidzein were produced (Additional file 1: Table S5).

4 Discussion

Medicinal plants are able to synthesis several unique secondary metabolites in response to abiotic and biotic stress for their biological functions [35]. Since human origin plants have been employed in traditional medicine to treat various ailments because of their curative effects with little or no adverse side effects [36], numerous bioactive compounds extracted from plants such as alkaloids, flavonoids, phenolic, steroids, tannins and terpenes are the main ingredients responsible for various biological activities. Currently, these bioactive compounds are acquiring solid interest for production due to their clinical and pharmaceutical importance [37]. However, the biosynthesis of these important secondary metabolites are specific to particular plant parts depends on developmental and growth stages, stress and availability of nutrients they occur in very low quantity [38]. Through use of recent advancement in plant tissue culture technology such as adventitious root culture, callus culture, hairy root culture, shoot culture and suspension culture have proved to be potential tool to uncover active principles which can be used in the

$$\begin{aligned}\text{Puerarin}\,(\%) = {} & 0.107 - 0.015 \times \text{6-BA} + 7.145 \times 10^{-3} \times \text{Tem} \\ & \times \text{6-BA} + 0.016 \times \text{NAA} \times \text{6-BA} - 2.502 \times 10^{-4} \\ & \times \text{Tem}\,2 - 0.011 \times \text{NAA2} - 0.045 \times \text{6-BA2}\end{aligned} \tag{1}$$

$$\begin{aligned}\text{Daidzin}\,(\%) = {} & -1.573 + 0.119 \times \text{Tem} + 0.470 \times \text{6-BA} + 0.030 \\ & \times \text{NAA} \times \text{6-BA} - 2.281 \times 10^{-3} \times \text{Tem}\,2 \\ & - 0.023 \times \text{NAA2} - 0.137 \times \text{6-BA2}\end{aligned} \tag{2}$$

$$\begin{aligned}\text{Daidzin}\,(\%) = {} & -4.384 \times 10^{-3} + 0.043 \times \text{6-BA} + 0.017 \times \text{NAA} \\ & \times \text{6-BA} - 1.306 \times 10^{-5} \times \text{Tem}\,2 - 8.535 \times 10^{-3} \\ & \times \text{NAA2} - 0.019 \times \text{6-BA2}\end{aligned} \tag{3}$$

various treatments [39]. Moreover, this technique has several advantages compared to natural resources including continuous and high-quality compounds production, production of compounds free of the chemical contaminants and without environmental constraints [40].

However, artificially controlled culture conditions such as light, temperature, pH, nutritional availability, plant growth regulators (PGRs) strongly affect accumulation of secondary metabolites under in vitro conditions [41, 42]. Optimization of culture conditions for enhanced production of secondary metabolites in in vitro condition is the most effective way [43]. In the current study, the accumulation of isoflavones in different in vitro regenerated callus cultures of *P. thomsonii* was evaluated following temperature, NAA and 6-BA elicitations. Initially, our study started with callus initiation from healthy shoot tips that were cultured at different temperature and changing concentrations and combinations of NAA and 6-BA. Interestingly, a good amount of daidzin, daidzein and puerarin was significantly high at 24 °C and 28 °C with decline at higher temperature more than 24 °C and 28 °C, respectively. Although temperature is not often considered for callus cultures, the influence of temperature in enhanced in vitro isoflavones production to be species specific, which temperature strongly affects bioflavonoid accumulation in *C. subternata* callus [44].

An undesirable pH of the medium may strongly affect accumulation of secondary metabolites and medium pH adjustment strongly regulates the nutrients uptake and metabolic activities of in vitro cultures [45]. Furthermore, the cellular pH adjustment, cellular growth, gene expression and transcription of secondary metabolite biosynthesis are highly regulated by the medium pH [46]. pH acts as an elicitor of non-biological origin, triggering the cultured cells to produce secondary metabolites beyond the normal levels [47]. In future, our focus will be to evaluate the effect of pH of culture medium on biosynthesis of isoflavones.

In addition to physical factor, the other parameter has been studied in order to increase the production of isoflavones in the callus culture of *P. thomsonii*. Fascinatingly, in the present work the supplementation with NAA and 6-BA proved to be essential to support increased biomass as well as high isoflavones synthesis. Similarly, Lee et al. [48] also reported the enhanced production of puerarin in *Pueraria lobata* callus cultured in the medium containing 1.0 mg/L NAA. 6-BA further activates some of the enzymes that take part in the biosynthetic pathway of secondary metabolite, deriving some primary metabolites such as L-phenylalanine, L-tyrosine, L-arginine, L-asparagine, and D-proline into secondary metabolites such as umbelliferone, chlorogenic acid, glutathione and isoflavones [49]. However, the exact mechanisms underlying regulation of primary and secondary metabolism by NAA are not yet fully known. The PGRs affect metabolism differentially [50], our focus will be to evaluate the effect of more variety of plant regulators on biosynthesis of isoflavones in *P. thomsonii* callus in future.

These findings of optimal conditions to get the highest production of isoflavones using statistical model such as RSM are an important method for optimization [51, 52]. Further, the statistical-RSM models of cultivation conditions for estimating the content of daidzin, puerarin and daidzein in *P. thomsonii* were successfully validated using RSM were also in agreement with the concentration obtained from in vitro callus culture. Therefore, the success obtained with increased production of isoflavones in callus culture of *P. thomsonii* makes this protocol a suitable and potential biotechnological reliable strategy for the synthesis of high valuable secondary metabolites.

5 Conclusions

In the present study, the optimal conditions for callus induction and enhanced production of puerarin, daidzin and daidzein were found to be 0.162 ± 0.011%, 0.458 ± 0.031% and 0.049 ± 0.008%, respectively, and were positively identified and quantified by HPLC. Further, models of cultivation conditions for the accumulation of puerarin, daidzin and daidzein in *P. thomsonii* callus cultures were successfully validated using RSM. The optimal conditions for obtaining maximum accumulation of puerarin (0.158%), daidzin (0.463%) and daidzein (0.057%) were at temperature 24.4 °C, 6-BA (2.04 mg/L) and NAA (1.69 mg/L), 25.8 °C, 6-BA (1.91 mg/L) and NAA (1.71 mg/L) and 24.0 °C, 6-BA (2.00 mg/L) and NAA (1.95 mg/L), respectively. Further, the described RSM models for estimating the content of daidzin, puerarin and daidzein in *P. thomsonii* callus induction by different levels of NAA and 6-BA concentrations and temperature were in agreement with the in vitro callus culture conditions. Therefore, RSM studies on the effect of cultivation variables on the content of daidzin, puerarin and daidzein would further facilitate the economic importance of *P. thomsonii* when scaling up production at a factory.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43088-022-00220-4.

Additional file 1: Table S1. Independent variables and levels of the experimental design. **Table S2.** Effect of different temperature on puerarin, daidzin and daidzein synthesis in *P. thomsonii* callus culture. **Table S3.** Effect of different 6-BA concentration on puerarin, daidzin and daidzein synthesis in *P. thomsonii* callus culture. **Table S4.** Effect of different NAA concentration on puerarin, daidzin and daidzein synthesis in *P. thomsonii* callus culture. **Table S5.** Validation experiments on production of the Puerarin, daidzin and daidzein.

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Authors' contributions
We declare that this work was done by the authors named in this article. YL and MJH were involved in the conceptualization; YXF, RJZ, JZ and XHT contributed to the methodology; MJH and JQ contributed to the software and validation; YL, MYY, YLR and PSK were involved in the formal analysis, investigation and resources; MJH was involved in the data curation; YL, YLR and PSK contributed to writing—original draft preparation and writing—review and editing; YLR and PSK contributed to the visualization; QJ was involved in the supervision; MJH and QJ contributed to the project administration and funding acquisition. All the authors read and approved the final manuscript.

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Availability of data and material
The supplementary data of the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest.

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