



## OPEN In vitro strategy to enhance the production of bioactive polyphenols and caffeoylputrescine in the hairy roots of *Physalis peruviana* L.

Yi-jia Zhong<sup>1</sup>, Shao-fu Wu<sup>1</sup>, Lu Zhang<sup>2</sup>, Zhong-ping Yin<sup>1</sup>, Yi-hai Xie<sup>1</sup> & Ji-guang Chen<sup>1</sup>✉

The *Rhizobium rhizogene*-transformed root culture from *Physalis peruviana* L. (*P. peruviana*) may be a promising and novel source of valuable phenolics, including caffeoylputrescine (CP), which is known for antioxidant, antidiabetic, insect-resistant, disease-resistant, and neuroprotective properties. In this study, to improve the production efficiency of phytochemical components in *P. peruviana* hairy root cultures, we optimized various culture conditions, including the inoculum size, liquid volume, culture media type, carbon source, sucrose concentration, initial pH, and application of elicitors, to enhance the total phenolic content and CP yield in these hairy root cultures. The findings indicate that the use of sucrose as carbon source resulted in the highest biomass (13.28 g DW/L), total phenolic content (6.26 mg/g), and CP yield (2.40 mg/L). The White medium excelled in enhancing the total phenolic content (9.35 mg/g), whereas the B5 medium was most effective for the biomass (13.38 g DW/L) and CP yield (6.30 mg/L). A sucrose concentration of 5% was best for the biomass (18.40 g DW/L), whereas a sucrose concentration of 4% was ideal for the CP yield. Optimal culture conditions were as follows: an inoculum size of 0.5 g/100 mL, a liquid volume of 100 mL in a 250-mL flask, B5 medium, 4% sucrose, and a pH of 5.5. Among the tested elicitors, methyl jasmonate (MeJA) at 100  $\mu$ M significantly increased the biomass (21.3 g/L), total phenolic content (23.34 mg/g), and CP yield (141.10 mg/L), which represent 0.96-, 2.12-, and 13.04-fold increases, respectively, over the control after 8 days. The optimized HR culture of *P. peruviana* provides a promising system to enhance the production of CP for pharmaceutical applications.

**Keywords** *Physalis peruviana* L., Hairy root, Caffeoylputrescine, Polyphenols, Culture conditions

Plants are valuable sources of secondary metabolites, and they play crucial roles in nutrition and pharmaceuticals<sup>1</sup>. However, plants can be negatively impacted by various biotic and abiotic stresses in their natural environment, which reduces the productivity and affects the production of secondary metabolites<sup>2</sup>. To address this issue, in vitro culture technology can be effectively utilized for the mass propagation, conservation, and extraction of secondary metabolites from these plants<sup>3</sup>. Hairy root (HR) cultures of plants are established by infecting explants with a T-DNA, which has been integrated into the genomic DNA of the target plant through the natural genetic engineering process of *Agrobacterium rhizogenes* at the site of plant injury<sup>4</sup>. The T-DNA region contains genes that are responsible for auxin and cytokinin synthesis in the roots and genes that produce opines (unusual amino acids)<sup>5</sup>. The resulting HR tissues are genetically stable and have a high growth rate in a hormone-free medium<sup>6</sup>. Compared to their parent plants, HR cultures can often better produce and increase secondary metabolites<sup>7</sup>. In vitro culture enables the application of diverse strategies to enhance the accumulation of secondary metabolites.

Previous studies have shown that factors such as the type and concentration of the carbon source, inoculum size, liquid volume in the flask, mediums, and initial pH of the medium can impact the growth and secondary

<sup>1</sup>Jiangxi Key Laboratory of Natural Products and Functional Food, College of Food Science and Engineering, Jiangxi Agricultural University, Nanchang 330045, China. <sup>2</sup>Collaborative Innovation Center of Jiangxi Typical Trees Cultivation and Utilization, College of Forestry, Jiangxi Agricultural University, Nanchang 330045, China. ✉email: chenjiuguang@jxau.edu.cn

metabolism of HR cultures<sup>8,9</sup>. Therefore, optimizing these parameters can improve the growth and secondary metabolite biosynthesis in this culture system.

In addition, elicitation is a valuable biotechnological tool to alter the metabolic pathways of medicinal plants by stimulating the plant defense and stress-induced responses<sup>10</sup>. Elicitors, which are classified as biotic or abiotic based on their origin, can enhance the secondary metabolism in plants<sup>11</sup>. Methyl jasmonate (MeJA), salicylic acid (SA), and sodium nitroprusside (SNP) are powerful elicitors for the secondary metabolite production in medicinal plants. Jasmonic acid and its methyl esters are crucial in the signal transduction process that regulates the defense mechanisms in plants<sup>12,13</sup>. MeJA has been successfully used to increase the production of 3,5-dicaffeoylquinic acid in *Cichorium intybus* L<sup>14</sup>. SA, which is a phenolic phytohormone, triggers various plant defense responses and promotes the synthesis of secondary metabolites<sup>15</sup>. SA treatment can enhance the yield of phenolic acids in *Salvia miltiorrhiza*<sup>16</sup>. SNP, which acts as a donor of nitric oxide (NO), plays a role in plant signaling, defense mechanisms, and processes such as plant growth and development<sup>17</sup>. SNP has been effective as an elicitor in increasing flavonoids, including rutin, in *Haplophyllum virgatum*<sup>18</sup>.

Phenolic compounds represent a group of secondary metabolites that plants produce<sup>19</sup>. These compounds are subdivided into different groups, including phenolic acids, flavonoids, stilbenes, lignans, and polymeric lignans<sup>20</sup>. There are two main groups of phenolic acids: hydroxybenzoic and hydroxycinnamic<sup>21</sup>. Phenolic compounds have been linked to various beneficial properties, including antioxidant, antidiabetic, anticancer, and anti-inflammatory effects<sup>20,22</sup>. Caffeoylputrescine (CP) is a hydroxycinnamic acid amide compound that results from the conjugation of caffeic acid and putrescine<sup>23</sup>. The molecular architecture of CP features a conjugated double bond, which bestows upon it the potential to exist as two geometric isomers: (E)-N-caffeoylputrescine and (Z)-N-caffeoylputrescine. It was initially discovered in the callus tissue culture of *Nicotiana tabacum*<sup>24</sup> and subsequently found in various plants of the Solanaceae family such as *Scopolia tangutica*<sup>25</sup>, *Solanum lycopersicum*<sup>13</sup>, and *Capsicum annuum*<sup>26</sup>. Our preliminary research also detected CP in *Physalis peruviana* L. (*P. peruviana*). In *Nicotiana attenuata*, CP is believed to have defensive and developmental functions. It functions acts as a direct defense mechanism by inhibiting the growth of *Manduca sexta* caterpillars<sup>27</sup>. In *Capsicum annuum* L., CP compounds act as the phytoalexin to defend against the *Colletotrichum gloeosporioides* pathogen<sup>28</sup>. CP demonstrates moderate antimicrobial activity against various bacterial strains and exhibits anti-inflammatory properties in tomato leaves<sup>29</sup>. Additionally, the CP compound in pepper leaves may enhance antidiabetic effects and provide a natural approach to improve the human glucose metabolism<sup>30</sup>. Kim et al.<sup>31</sup> have reported that CP and anthocyanin from *Capsicum annuum* exhibit the greatest antioxidant activity, with potent radical-scavenging potential. Furthermore, CP enhances the analgesic and sedative properties of *Anisodus tanguticus* (Maxim) Pascher, bolstering its role as an auxiliary in managing neurodegenerative diseases<sup>32</sup>.

Although numerous studies investigated the optimal culture conditions and elicitors of HR and their impact on secondary metabolites in various plants, no research has specifically evaluated the production of phytochemical components (e.g., polyphenols and CP) in *P. peruviana* HRs. The production of these phytochemical components must be optimized to enhance the industrial potential of *P. peruviana* HRs. This study aimed to establish the optimal culture conditions to improve the growth, total phenolic content, and CP content of *P. peruviana* HRs. Additionally, *P. peruviana* HRs were treated with different elicitors (MeJA, SA, and SNP), and the most effective elicitor was selected for further treatment. The findings of this study are expected to provide a reference for the cultivation of *P. peruviana* HRs and synthesis and regulation of secondary metabolites.

## Materials and methods

### Plant material and hairy root culture establishment

All plant experiments complied with relevant institutional standards. *P. peruviana* seeds were purchased from China National Seed Group Co., Ltd., Beijing, China, and stored at the Jiangxi Key Laboratory of Natural Products and Functional Food, in Nanchang, Jiangxi, China. The seeds were identified by Professor Lu Zhang of the College of Forestry at Jiangxi Agricultural University. HRs were induced using the method described by Fu et al.<sup>33</sup>. These pre-cultured explants were immersed in a culture medium of *Agrobacterium rhizogenes* for 10 min; then, the surface bacteria and moisture were removed using sterile filter paper. The infected explants were placed on the surface of a 1/2 MS solid medium and cultured at 28 °C in the dark. After 2 d of co-culture, the explants were transferred to a 1/2 MS solid medium supplemented with cefotaxime sodium (Cef) to eliminate any remaining bacteria. To confirm the plant transformation, a DNA polymerase chain reaction (PCR) analysis was performed. To obtain the optimal harvest time for the biomass of *P. peruviana* HRs in liquid culture, liquid cultivation was first conducted following the method of Hajati et al.<sup>34</sup>, and a growth curve was plotted. Then, 0.5 g fresh weight (FW) of HR was inoculated in 250-mL flasks containing 100 mL of 1/2 MS liquid medium supplemented with 30 g/L of sources. Samples were collected every 4 days until day 36. The FW and dry weight (DW) were recorded.

### Optimization of culture conditions

To establish the optimal growth conditions to produce phytochemical components, the effects of different carbon sources, sucrose concentrations, inoculum sizes, liquid volumes in the flask, and media were evaluated on in vitro HR cultures of *P. peruviana*. In total, 500 mg of HRs was inoculated in 250-mL Erlenmeyer's flasks that contained 100 mL of 1/2 MS medium supplemented with 30 g/L of different carbon sources (maltose, glucose, sucrose, or fructose). Different inoculum sizes (0.1, 0.3, 0.5, 0.7, or 0.9 g/100 mL), liquid volumes in the flask (60, 80, 100, 120, or 140 mL), media (MS, 1/2 MS, B5, WPM, or White), sucrose concentrations (1%, 2%, 3%, 4%, 5%, or 6%), and initial pH values of the medium (4.5, 5.0, 5.5, 6.0, or 6.5) were applied depending on the objective of the experiment. The increases in total biomass, total phenolic content, and secondary metabolite content were evaluated after 24 days of subculture.

### Elicitor treatments of the hairy root culture

To improve the HR biomass and phytochemical component production, different elicitor treatments were studied to assess their influence on the amount of phytochemical components. The study used elicitors such as SA, SNP, and MeJA at concentrations of 50, 100, 200, and 400  $\mu\text{M}$ . These elicitors were added to the B5 medium and individually applied to 20-day-old liquid HR cultures. The control group was treated without using any elicitors. The HRs were harvested at 2, 4, 6, and 8 days after being treated with the elicitors.

### Determination of root biomass

To calculate the FW of the collected HRs, they were washed twice with distilled water, delicately pressed onto filter papers to eliminate excess water, and weighed. The DW was determined by dehydrating the fresh HRs in an oven set at 60 °C until a stable weight was reached.

### Extraction of phenolic

The ultrasound-assisted extraction was performed as reported by Alcántara et al.<sup>35</sup> Then, 200 mg of the dried and grinded HR sample was extracted using 2 mL of 70% methanol through sonication at 80 °C for 1 h, followed by centrifugation at 3000 rpm for 15 min. The supernatant was collected, and the solution was filtered through a 0.22- $\mu\text{m}$ -pore-size filter to determine the total phenolic content (TPC) and for the high-performance liquid chromatography (HPLC) analysis. The ultrasonic plant material extraction procedure was repeated three times.

### Total phenolic content determination and quantitative analysis of caffeoylputrescine

The TPC of transgenic HR and non-transformed roots was determined using the Folin-Ciocalteu method, as described by Singleton and Rossi<sup>36</sup> and Cheng et al.<sup>37</sup> with modifications. A mixture was prepared with 0.1 mL of sample solution, 0.5 mL of Folin-Ciocalteu reagent, 7.9 mL of water, and 1.5 mL of 10% sodium carbonate (w/v). After 1 h, absorbance was measured at 760 nm using a MicroplateReader (SpectraMax M2; Molecular Devices, Shanghai, China). The TPC was determined in milligrams of gallic acid equivalents using an equation from the gallic acid calibration curve.

CP in the HR was identified via HPLC-TOF-MS (6430 HPLC-MS system; Agilent Technologies, Santa Clara, CA, USA) and quantified via HPLC (1260 HPLC system; Agilent Technologies, Santa Clara, CA, USA). The HPLC conditions were as follows: the chromatographic column was a Waters Symmetry C18 column (4.6 mm  $\times$  250 mm, 5  $\mu\text{m}$ ; MA, USA). The mobile phase consisted of 0.3% formic acid (A) and acetonitrile (C) using the following gradient program: 0–10 min, 97% (A); 10–18 min, 97–85% (A); 18–28 min, 87–75% (A); 28–30 min, 75–5% (A); 30–40 min, 5% (A); 40–41 min, 5–97% (A); 41–45 min, 97% (A). The column temperature was maintained at 40 °C with a flow rate of 1 mL/min and an injection volume of 3  $\mu\text{L}$ . The target compound was quantified using specific calibration curves and reported as mg/g with respect to the DW of the roots.

### Data statistics and analysis

Each experiment was repeated thrice, and the results are presented as mean values  $\pm$  standard deviation. Data were analyzed using the GraphPad Prism 8.0.2 (<https://www.graphpad-prism.cn/>) and SPSS Statistics 17 software (<https://www.ibm.com/cn-zh/spss>). Statistical analysis was performed using an analysis of variance followed by Duncan's test (at  $P \leq 0.05$ ).

## Results

### Establishment of a suspension culture system for *P. peruviana* hairy roots.

Figure 1A–D show the time course of *P. peruviana* HR growth in the 1/2 MS liquid medium. The entire growth cycle can be divided into four stages: adaptive phase (0–4th day), exponential phase (4th – 24th day), stationary phase (24th – 28th day), and decline phase (after the 28th day). The maximal biomass concentration of 18.6 g DW/L was achieved on the 24th day of cultivation, and it represented a significant increase compared to the initial inoculation. Subsequently, the biomass concentration of HRs decreased, and the liquid culture medium gradually became yellow after the 28th day.

*P. peruviana* HRs were analyzed using HPLC-TOF-MS and HPLC to identify the extracts. The results showed that a compound identified as CP had pseudo-molecular ions at  $m/z$  251  $[\text{M} + \text{H}]^+$ , which matched those reported by Baumert et al.<sup>38</sup> We confirmed this compound by comparing the retention times of authentic standards and extracts from the treated HR cultures (Fig. 1E,F). The standard curve was generated using a series of CP solutions of known concentrations of 1–1000  $\mu\text{g}/\text{mL}$ , which were prepared with methanol. The solutions were analyzed using an HPLC method, and the peak area was plotted against the concentration to obtain the standard curve equation:  $y = 9.0559x - 8.7285$  with a high correlation coefficient ( $R^2 = 0.9999$ ), which indicates excellent linearity.

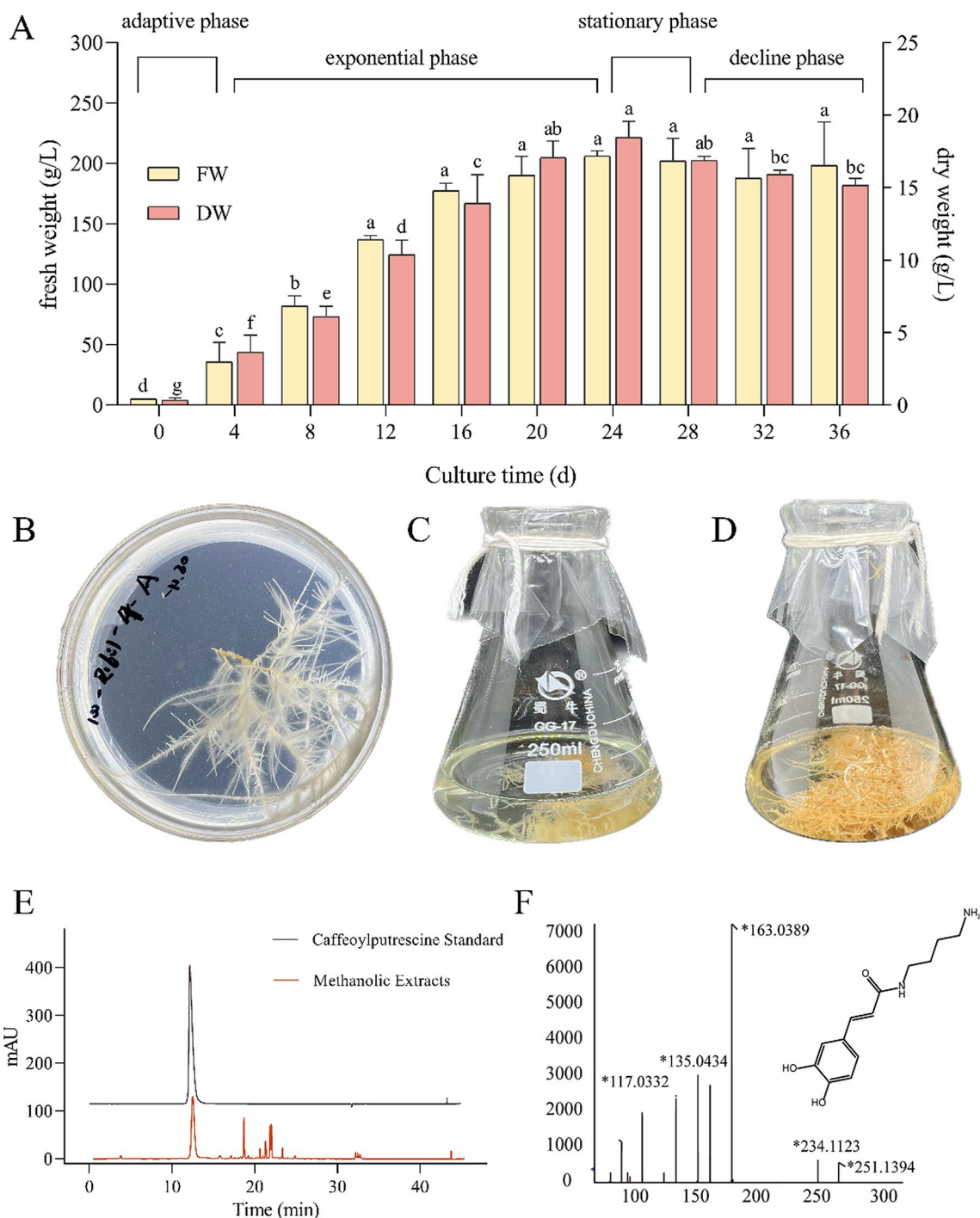
### Optimizing the culture condition effects

#### *Effect of the inoculum size on the TPC and CP synthesis of P. Peruviana HRs*

This study identified that an optimal HR inoculum size of 0.5 g FW/100 mL resulted in the highest accumulation of biomass (16.57 g DW/L), TPC (10.39 mg/g DW), CP (0.53 mg/g DW), and CP yield (8.03 mg/L) after 24 days of culture (Fig. 2 [1(a), 1(b)]). A higher or lower inoculum size was not advantageous because they decreased the biomass and phytochemical component accumulation.

#### *Effect of the liquid volume on the TPC and CP synthesis of P. Peruviana HRs*

In this experiment, 250-mL triangular bottles were used with varying liquid volumes (60, 80, 100, 120, and 140 mL). The results in Fig. 2(2(a) and 2(b)) illustrate the effects of these different liquid volumes on the biomass and production of secondary metabolites. Among the tested volumes, HRs at 140 mL exhibited the highest biomass

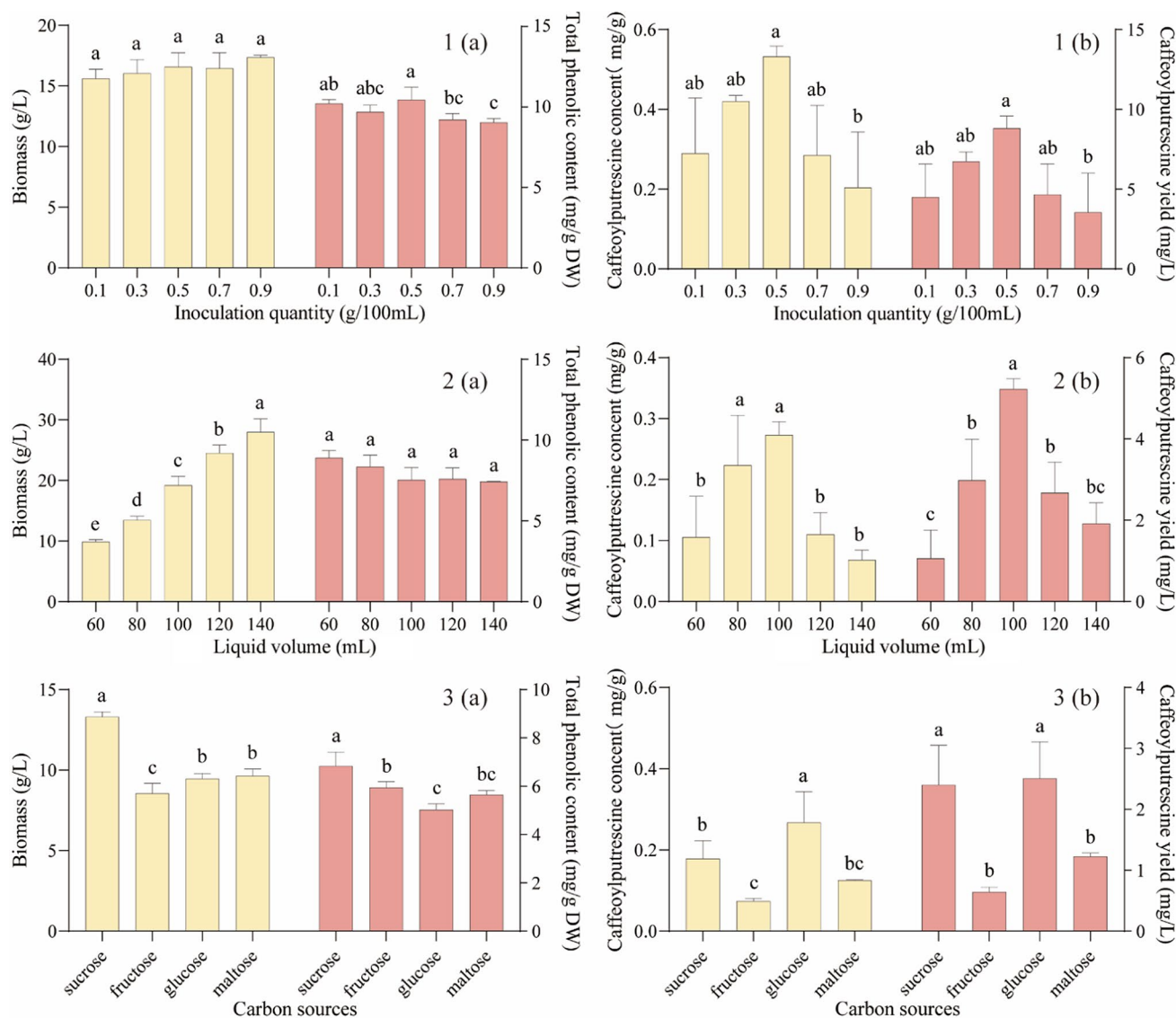


**Fig. 1.** (A) Fresh weight and dry weight of hairy root of *Physalis peruviana* L. (*P. peruviana*) on different days after a sucrose treatment. (B) Identified hairy roots. Growth of hairy roots in a liquid 1/2MS medium: (C) 4 days - adaptive phase; (D) 24 days - stationary phase. Mass spectra of the identified caffeoylputrescine (CP) in the positive ion mode. (E) HPLC chromatograms of standard CP and methanolic extracts of *P. peruviana* HRs. (F) Secondary mass spectrometry of CP.

accumulation (27.97 g DW/L) after 24 days of incubation. Additionally, the highest total phenolics accumulation (8.90 mg/g DW) was observed at 60 mL, whereas the highest accumulation of CP content (0.27 mg/g DW) and CP yield (5.23 mg/L) occurred at 100 mL.

#### Effect of the carbon sources on the TPC and CP synthesis of *P. Peruviana* HRs

This study assessed the effects of four carbon sources on the HR biomass and production of secondary metabolites (Fig. 2[3(a) and 3(b)]). Among these sources, sucrose was the most optimal for the biomass (13.28 g DW/L)



**Fig. 2.** Optimization of culture conditions to enhance the biomass, total phenolic content (TPC), and caffeoylputrescine production from hairy roots. The effects of the inoculum size (1(a) and 1(b)), liquid volume (2(a) and 2(b)), and carbon source sucrose (3(a) and 3(b)) were investigated. The presented values are the mean  $\pm$  standard error of three replicates; the data recorded were after 24 days of culture.

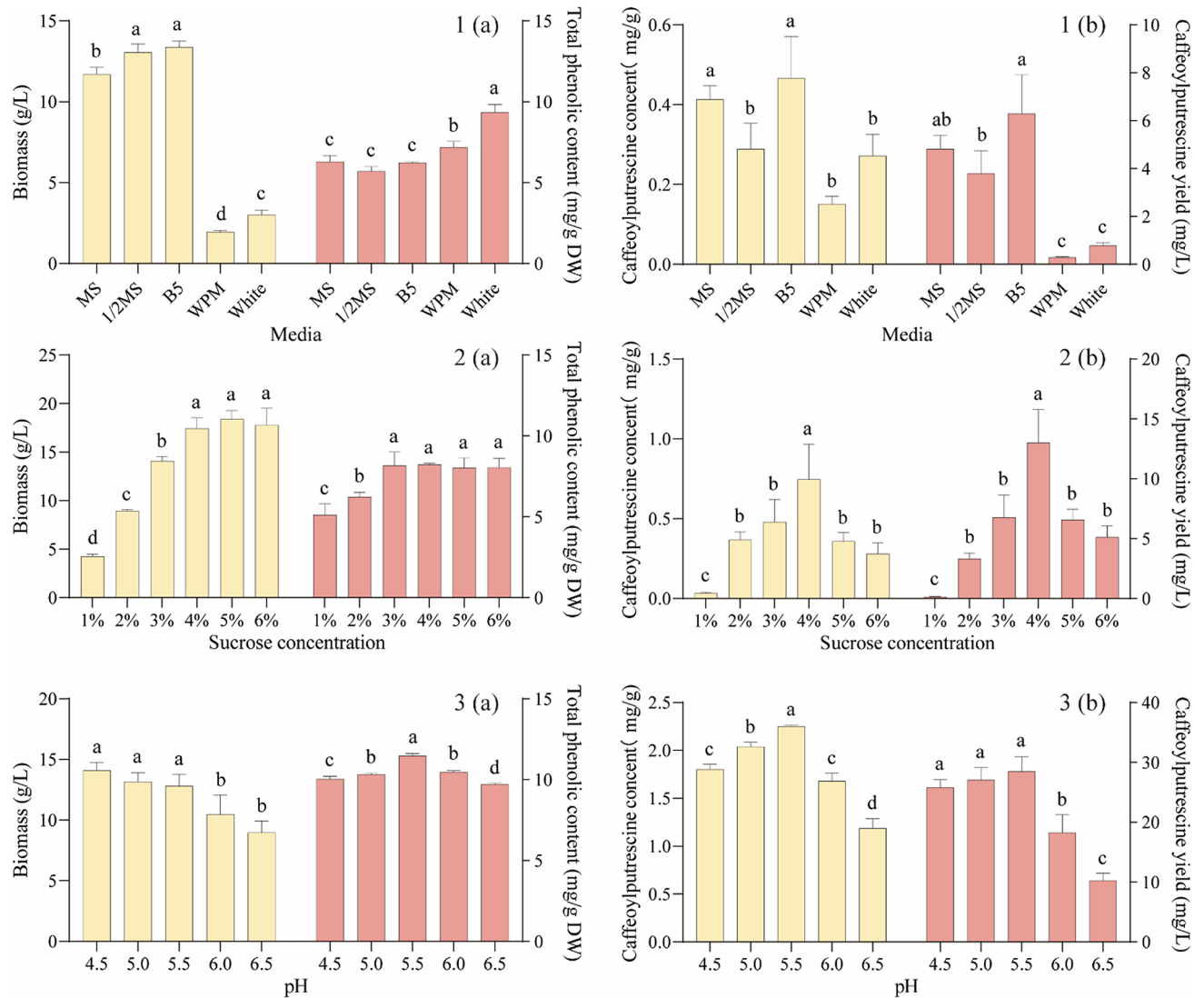
and TPC (6.83 mg/g DW) accumulation in the HRs, and glucose was more favorable for CP (0.27 mg/g DW) accumulation. Interestingly, there was no significant difference in CP production between sucrose (2.40 mg/L) and glucose (2.50 mg/L). As a result, sucrose was selected for this study due to its commonality and cost-effectiveness.

#### *Effect of the media on the TPC and CP synthesis of P. Peruviana HRs*

To determine the most suitable medium for HR growth, five different media were evaluated: MS, 1/2 MS, WPM, White, and B5. The results in Fig. 3(1(a) and 1(b)) show significant variations in growth capacity and CP production among different media, and B5 was superior. The B5 medium exhibited the highest biomass accumulation (13.38 g DW/L), the maximum CP content (0.47 mg/g DW), and the highest CP yield (6.30 mg/L). Although the White medium showed the highest total phenol accumulation, its CP yield (0.78 mg/L) was only 0.12 times higher than that of the B5 medium. Therefore, B5 was identified as the most effective medium for CP production.

#### *Effect of the concentration of sucrose on TPC and CP synthesis of P. Peruviana HRs*

The increasing trend was notably apparent with sucrose levels of 1–4% but inversely proportional at concentrations exceeding 4% which affected the CP content and yield in HRs. Optimal results were observed with 4% sucrose: the CP content and yield increased by 1.56 and 1.92 times, respectively, compared to the 3%



**Fig. 3.** Optimization of culture conditions to enhance the biomass, total phenolic content (TPC), and caffeoylputrescine production of HRs. The effects of the media (1(a) and 1(b)), sucrose concentration (2(a) and 2(b)), and initial pH (3(a) and 3(b)) were investigated. The presented values are the mean  $\pm$  standard error of three replicates; the data were recorded after 24 days of culture.

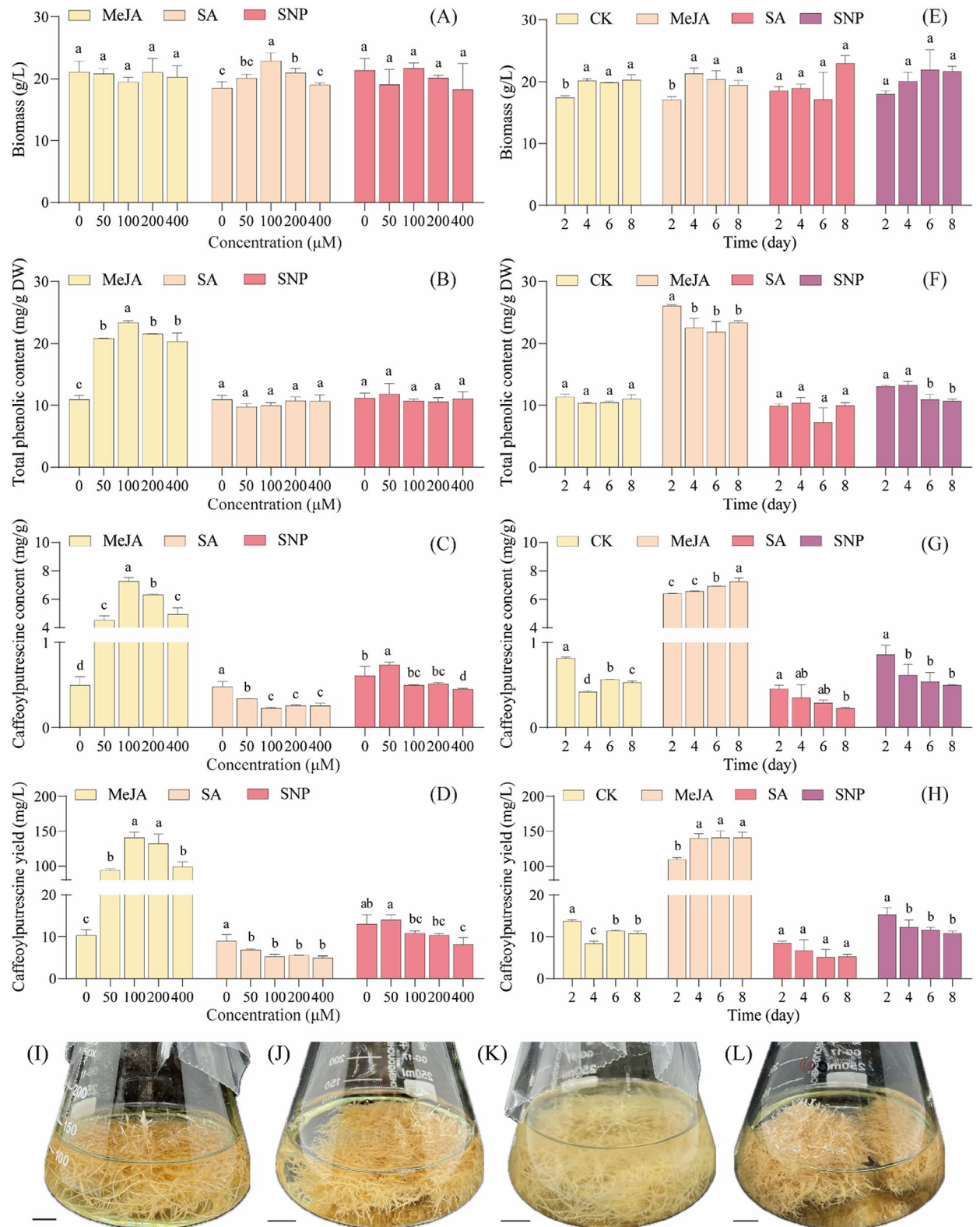
sucrose levels (Fig. 3[2(a) and 2(b)]). However, there was no significant difference in the production levels of the growth capacity and TPC at higher sucrose concentrations.

#### *Effect of the initial pH on the TPC and CP synthesis of P. Peruviana HRs*

The study evaluated the effect of the initial pH (4.5–6.5) on the HR growth and secondary metabolite production on the 24th day of culture (Fig. 3[3(a) and 3(b)]). The pH levels of 4.5–5.5 showed no significant effect on the biomass and CP productivities, whereas pH levels above 5.5 had an inverse relationship with these factors and affected the biomass, TPC, CP content, and yield in HRs. Notably, HRs grown at pH 5.5 exhibited the highest TPC (10.31 mg/g DW), CP content (2.25 mg/g DW), and CP yield (28.49 mg/L).

#### **Effect of different elicitors on the TPC and CP synthesis of *P. peruviana* HRs**

Figure 4I–L show the production of HRs after treatment with elicitors. The MeJA-induced HRs (Fig. 4J) exhibited a yellow-brown color, which was darker than that of the control group (Fig. 4I). In contrast, the HRs induced with SA (Fig. 4K) and SNP (Fig. 4L) showed an orange-yellow color, which was lighter than that of the control group. The SA-induced HRs exhibited a finer root system than MeJA-induced HRs. Furthermore, the results in Fig. 4A–H clearly demonstrate that different types of elicitors have varying effects on the accumulation of phytochemicals in *P. peruviana* HRs. Compared to the non-treated control, significant changes in CP content (14.52-, 0.47-, and 0.82-fold increases) and yield (13.54-, 0.58-, and 0.83-fold increases) were obtained when MeJA, SA, and SNP (100  $\mu$ M) were induced in *P. peruviana* HRs for 8 days respectively (Fig. 4C,D). The TPC significantly increased by 2.13-, 0.91-, and 0.96-fold when *P. peruviana* HRs were subjected to MeJA, SA, and



**Fig. 4.** Elicitor effects on the HR parameters of *P. peruviana* HR. (A–D) Impact of MeJA, SA, and SNP at concentrations of 50, 100, 200, and 400  $\mu\text{M}$  on the (A) biomass, (B) total phenolic content (TPC), (C) caffeoylputrescine (CP) content, and (D) CP yield in 20-day-old *P. peruviana* HRs after an 8-day exposure. (E–H) Impact of the same elicitors at a fixed concentration of 100  $\mu\text{M}$  on the biomass (E), TPC (F), CP (G), and CP yield (H) under different exposure durations (2, 4, 6, and 8 days). (I–L) Morphological effects of various elicitors on *P. peruviana* HRs: (I) non-treated control roots, (J) MeJA (100  $\mu\text{M}$ ) treated, (K) SA (100  $\mu\text{M}$ ) treated, and (L) SNP (100  $\mu\text{M}$ ) treated. CK denotes the untreated control group. The presented values are the mean  $\pm$  standard error of three replicates. Bars = 1 cm.

SNP (100  $\mu\text{M}$ ) for 8 days, respectively (Fig. 4B). Thus, the best MeJA concentration to further enhance CP in *P. peruviana* HRs was 100  $\mu\text{M}$ , which yielded the highest level of CP (7.26 mg/g).

The *P. peruviana* HRs exhibited varying levels of TPC and CP when treated with SA, MeJA, and SNP at different time points (2, 4, 6, and 8 days). The CP content was 0.42–0.82 mg/g, whereas TPC was 10.42–11.31 mg/g in the non-treated control group. The CP content significantly increased to 7.26 mg/g after 8 days of MeJA treatment (Fig. 4G). Notably, similar yields of CP were observed at 2, 6, and 8 days of treatment with values of 140.13, 141.11, and 141.10 mg/L, respectively (Fig. 4H). In contrast, both TPC and biomass were lower after 8 days of MeJA treatment than after 2 days of treatment, with TPC levels at 23.34 mg/g and biomass at 19.43 g/L after 8 days of treatment. With SA treatment, the TPC level also maximized (10.45 mg/g) at 4 days. Concurrently, the maximum content and yield of CP were observed at 2 days, with values of 0.46 mg/g and 8.58 mg/L, respectively. In comparison, SNP significantly increased the TPC (13.05 mg/g), CP (0.86 mg/g), and CP yield (15.43 mg/L) after 2 days of treatment. No significant differences were noted between treated samples and controls at 2, 4, 6, and 8 days.

Overall, MeJA was more effective at enhancing the accumulation of TPC and CP in *P. peruviana* HRs than SA and SNP. MeJA showed no significant impact on the biomass, whereas SA slightly increased the biomass production (Fig. 4A,E).

## Discussion

This study aimed to optimize the culture conditions for HRs of *P. peruviana* and to screen for the most effective elicitors to increase the production of phenolic compounds, especially CP. Our results comprehensively optimized the culture parameters, specifically the inoculum size, liquid volume, carbon source, medium, sucrose concentration, and initial pH, to effectively cultivate *P. peruviana* HRs.

The inoculum size and liquid volume play a crucial role in the efficiency of tissue culture to produce secondary metabolites<sup>39,40</sup>, which directly impacts the nutrient availability, oxygen exchange, and overall growth kinetics<sup>41</sup>. Our results indicate that inoculum sizes of 0.1–0.9 g/100 mL had a minimal effect on the biomass of HRs. The optimal inoculum size of 0.5 g/100 mL provided a balanced starting point for growth without overcrowding the culture vessel, which can lead to localized nutrient depletion or uneven growth distribution. Meanwhile, the liquid volume significantly affected the biomass and metabolite accumulation. The ideal liquid volume of 100 mL in a 250-mL flask well balanced the nutrient and oxygen availability and promoted efficient growth and metabolic activity. Interestingly, we noted a decrease in CP accumulation at high inoculum sizes (0.7–0.9 g/100 mL) and liquid volumes (120–140 mL), possibly due to nutrient limitations, oxygen limitations, and flask volume constraints<sup>42</sup>.

A suitable culture medium plays a crucial role in regulating the plant cell growth and secondary metabolite accumulation<sup>43</sup>. This study revealed that the highest biomass production in *P. peruviana* HRs occurred when they were cultured on B5 and 1/2 MS media, whereas WPM and White media were considered unsuitable. The greatest accumulation of CP was observed in HRs grown on the B5 medium, followed by those grown in MS, 1/2 MS, White, and WPM media. The high salt levels and nitrogen content in the MS medium may impact the growth and secondary metabolite accumulation of HRs<sup>44</sup>. The 1/2 MS medium, which has lower nutrient concentrations than MS, was more effective for the biomass accumulation in *P. peruviana* HRs, although it resulted in a lower CP content than the B5 medium. Thus, a low inorganic nitrogen source (1/2 MS) and a high organic nitrogen source (B5 vitamin) were more favorable for the HR growth and secondary metabolite accumulation<sup>45</sup>. The WPM, which is commonly used for woody plant tissue culture, was not conducive to the growth and CP production of *P. peruviana* HRs, possibly due to its low total ion concentration<sup>46</sup>. Similarly, the White medium significantly decreased the biomass for *P. peruviana* HRs, which was also observed in *Anisodus acutangulus* HRs due to the suboptimal total nitrogen content in the White medium<sup>47</sup>. Notably, the distinctive feature of the B5 medium was its high thiamine concentration, a crucial element for cell biosynthesis and metabolism. Thiamine is essential for the continuous growth of isolated root cultures in vitro<sup>48</sup>. Among the five evaluated media compositions (MS, 1/2 MS, B5, WPM, and White), the B5 medium emerged as the most suitable option. Similar results were observed in *Artemisia vulgaris*<sup>49</sup> and *Picrorhiza kurroa*<sup>2</sup>.

This research also enhanced the biomass and secondary metabolite yields by optimizing the medium pH before autoclaving. Specifically, an initial pH of 5.5 was found to be conducive for the biomass accumulation and CP production. Similar results were observed in the HR cultures of *Plumbago indica*, where the initial pH of 5.5 was favorable for the production of plumbagin production<sup>50</sup>. In *Isatis tinctoria* HRs, an initial pH of 5.8 favored the biomass accumulation and bioactive alkaloid production<sup>51</sup>.

Carbon sources were recently recognized as compounds that act as energy sources and can serve as signaling molecules that affect the growth, development, and metabolism of cultured cells<sup>52</sup>. Sucrose was identified as the optimal carbon source for biomass accumulation and CP yield, followed by glucose and maltose. Conversely, the lowest accumulation of biomass and CP content was observed in the fructose-supplemented medium. Similar to our results, Fu et al.<sup>33</sup> demonstrated that sucrose significantly enhanced the biomass accumulation in *Stevia rebaudiana* and resulted in higher levels of TP-CADs than other carbon sources. Praveen and Murthy<sup>53</sup> found that sucrose was the optimal carbon source for the biomass accumulation and withanolide A production in *Withania somnifera* HR. Sucrose is commonly preferred as a carbohydrate source in cell and tissue culture media due to its abundance in the phloem sap of many plants, effective absorption through the cell membrane, economic feasibility, and easy accessibility<sup>54</sup>. Previous studies showed that *P. peruviana* HRs and seedlings exhibited poor growth when glucose and fructose were used as carbon sources, but certain plant species can thrive on alternative carbohydrates aside from sucrose<sup>55,56</sup>. The level of sucrose can affect the productivity of secondary metabolites in plant tissue cultures, particularly the growth of transformed roots<sup>57</sup>. In the present study, lower sucrose concentrations (1–3%) negatively impacted the biomass production and CP accumulation in *P. peruviana* HRs. In contrast, higher sucrose concentrations of 4.0–6.0% had a significant effect on the CP



accumulation but minimal impact on the biomass yield. Optimal CP accumulation was observed in cultures supplemented with approximately 4% sucrose, and this concentration was also suitable for biomass production. Similar findings were noted in the HR cultures of *Picrorhiza kurroa*, where a 4% sucrose concentration favored biomass accumulation and picroliv production<sup>58</sup>. Generally, sucrose serves a dual role as a carbon source and an osmotic agent in plant cell and organ culture media<sup>59</sup>. Increased sucrose consumption was associated with faster root growth, but higher sucrose concentrations led to elevated osmolality in the culture media, as reported by Jiao et al.<sup>39</sup>. This behavior may reduce the cell viability due to the dehydration and enhanced diffusion of metabolites from tissues into the media, which ultimately decreases the CP accumulation in *P. peruviana* HRs.

The optimal culture conditions for HRs were determined to be an inoculum size of 0.5 g/100 mL, a liquid volume of 100 mL in a 250-mL flask, sucrose as a carbon source, B5 medium, a sucrose concentration of 4%, and a pH of 5.5. Compared to *P. peruviana* HRs before optimization, the TPC, CP content, and CP yield significantly increased (by 1.26-, 8.24-, and 5.45-fold, respectively) after the optimization of the cultivation conditions.

Furthermore, the production levels can be enhanced by the application of elicitors in HR culture systems. The secondary metabolism is activated by intricate interactions between the plant tissue and the elicitor, and the response is contingent on various factors, including the elicitor specificity and concentration, duration of elicitation, and specific developmental stage of the plant tissue<sup>60</sup>. Various elicitors have distinct impacts on the accumulation of secondary metabolites in plants. For example, MeJA has been applied to enhance the production of scopolamine in *Duboisia genus*<sup>61</sup> and glucoraphanin and sulforaphane in *Brassica oleracea var. italica*<sup>62</sup>. The SA treatment increased the yield of friedelin and epifriedelanol in HRs of *Cannabis sativa* L.<sup>63</sup>. SNP acted as a nitric oxide donor and enhanced the growth of *Artemisia annua* HRs<sup>64</sup> and the accumulation of four tanshinone compounds in *Salvia miltiorrhiza* HRs<sup>65</sup>. Our study indicates that compared to the elicitors SA and SNP, MeJA is more effective at enhancing CP accumulation in *P. peruviana* HRs. Based on the results obtained from this experiment, adding MeJA to the culture media increased the CP to be 14.52-fold higher than that in the non-treated control. Recently, several studies have been reported to enhance secondary metabolites of respective abiotic elicitors in various Solanaceous. Piñeros-Castro et al.<sup>66</sup> reported that 10 mM SA significantly increased the production of 4 $\beta$ -Hydroxywithanolide E in *Physalis peruviana* L. HR. Halder et al.<sup>67</sup> also demonstrated that both SA and MeJA substantially stimulated the accumulation of withaferin A in *Physalis minima* L. HRs, with SA and MeJA inducing 21.40-fold and 20.39-fold increases, respectively, compared to non-treated control. Similarly, Halder et al.<sup>68</sup> showed that SA and MeJA significantly boosted withanolides production in *Withania somnifera* (L.) Dunal HRs, with SA (4.14 mg/L) and MeJA (6.75 mg/L) inducing 11.49-fold and 11.00-fold increases, respectively, compared to the non-treated control. These findings underscore the potential of SA and MeJA as potent elicitors for enhancing the production of bioactive compounds within the *Solanaceae* family.

MeJA is a well-known elicitor, and it has been reported as a signal transduction elicitor for plant defense response and production of plant secondary metabolites. It plays an important role in plants from morphological to molecular functions. Therefore, the jasmonate (JA) signaling pathway is generally considered an integral signal to biosynthesize many secondary plant products<sup>69</sup>. Chen et al.<sup>13</sup> demonstrated that the production of CP in tomato plants was tightly regulated by the JA signaling pathway, and exogenous MeJA elicited a massive accumulation of CP in leaves. The general timing and high level of induction of this response appear to be similar to those in *N. attenuata*<sup>70</sup>. Pieterse et al.<sup>71</sup> demonstrated that SA suppressed JA accumulation. S-nitrosoglutathione reductase (GSNOR) is associated with NO levels. Furthermore, GSNOR is required for the MeJA-induced accumulation of defense-related CP<sup>72</sup>. Sodium nitroprusside has been used as an NO donor in plant physiological studies to increase the NO level and CP accumulation. Notably, the biochemical mechanisms by which MeJA alterations activate CP biosynthesis in *P. peruviana* HRs remain unknown. A more comprehensive understanding of this issue will provide invaluable insight into the mechanisms of jasmonate homeostasis, which may inform the development of novel approaches to manipulate the plant secondary metabolism. Furthermore, the combined application of different elicitors in bioreactors may be considered a useful strategy to increase the yield of effective compounds.

## Conclusions

In this study, a culture system of *P. peruviana* HRs was successfully established. The optimal culture conditions for HRs were as follows: an inoculum size of 0.5 g/100 mL, a liquid volume of 100 mL in a 250-mL flask, sucrose as a carbon source, B5 medium, a sucrose concentration of 4%, and a pH of 5.5. The effects of MeJA, SA, and SNP on enhancing the biomass accumulation and CP production in *P. peruviana* HR cultures were investigated. Compared to the non-treated control, the CP yield significantly increased by 13.54-, 0.58-, and 0.83-fold when SA, MeJA, and SNP (100  $\mu$ M) were induced in *P. peruviana* HRs for 8 days, respectively. This established protocol will serve as a valuable guide for the large-scale production of *P. peruviana* biomass accumulation and CP production.

## Data availability

All data generated or analysed during this study are included in this published article.

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## Author contributions

Y.J.Z.: Methodology, Validation, Investigation, Data curation, Writing—original draft, Writing—review, and editing. S.F.W.: Conceptualization, Methodology. L.Z.: Investigation, Validation, Formal analysis. Z.P.Y.: Concep-

tualization, Methodology, Supervision, Funding Acquisition. Y.H.X: Data curation. J.G.C: Conceptualization, Writing - review and editing, Visualization, Project administration, Funding acquisition.

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### Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

**Correspondence** and requests for materials should be addressed to J.-g.C.

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