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OPTIMIZATION OF THE ADVENTITIOUS ROOT CULTURE SYSTEM AND PETROLEUM ETHER SOXHLET EXTRACTION PROCESS FOR *VALERIANA OFFICINALIS* L.

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Abstract

Valeriana officinalis L. is an important medicinal plant widely used for sedative and neuroactive applications. However, limited studies have integrated optimized adventitious root culture with downstream extraction process optimization for efficient utilization of V. officinalis biomass. To establish an efficient *In vitro* production and utilization route for *V. officinalis*, Single-factor experiments and Box–Behnken response surface methodology were applied to optimize adventitious root induction, suspension culture, and Soxhlet extraction conditions. Our results indicated that adventitious roots in *V. officinalis* were efficiently induced using 0.2 mg·L^{-1} NAA combined with 0.05 mg·L^{-1} 6-BA. The culture conditions of 42 g·L^{-1} sucrose, 39.854 mmol·L^{-1} total N, and 1.590 mmol·L^{-1} total P were suitable for adventitious root growth. Using adventitious roots produced under these conditions as the raw material, optimization of the Soxhlet extraction process gave a petroleum ether extract yield of (3.13 ± 0.12) %. The developed response surface models were statistically significant ($p<0.0001$) with non-significant lack-of-fit. In summary, this study established an integrated experimental framework from adventitious root induction and suspension culture to the preparation of lipophilic extracts. Although chemical profiling of valerenic acid-related compounds was not performed in this study, the optimized system provides a basis for future phytochemical investigations. Bioactivity evaluation of the obtained extracts remains necessary for confirming functional relevance. The optimized culture and extraction conditions provide a reproducible platform for future phytochemical characterization and large-scale utilization of V. *officinalis* adventitious roots.

Key words: *Valeriana officinalis* L.; Adventitious root induction; Suspension culture; Valerenic acid; Petroleum ether; Soxhlet extraction

Introduction

Despite extensive studies on valerian pharmacology and extraction technologies, limited research has integrated adventitious root culture optimization with downstream extraction process optimization using tissue-cultured biomass. Furthermore, quantitative optimization of suspension culture nutrients and petroleum ether Soxhlet extraction parameters for *V. officinalis* adventitious roots remains insufficiently explored.

The genus *Valeriana* in the family Valerianaceae consists of perennial herbs. There are more than 300 *Valeriana* species worldwide, and most are distributed in regions with mild and humid climates across Europe, northern Asia, South America, and the United States (Müller *et al*., 2012).*V. officinalis* L. has long been used as a medicinal plant, particularly for sedative and neuroactive purposes. Previous studies have identified important constituents such as valerenic acid, valerenal, and valeranone, which are associated with several biological activities including sedative, antidepressant, anti-inflammatory, antioxidant, and neuroprotective effects (Hendriks *et al*., 1981; Occhiuto *et al*., 2009; Orhan, 2021). At present, valerian has been reported to show sedative, antidepressant, anti-inflammatory, antioxidant, and neuroprotective activities (Hendriks *et al*., 1981; Occhiuto *et al*., 2009; Xu *et al*., 2011; Wang *et al*., 2016; Wang *et al*., 2019; Mulyawan *et al*., 2020; Xue *et al*., 2020; Orhan, 2021; Sánchez *et al*., 2021; Wang *et al*., 2022; Afzal *et al*., 2023; Erdoğan *et al*., 2023; Celik & Kirmizibekmez, 2025).

In recent years, many researchers have used several spectroscopic methods to isolate, purify, and identify chemical constituents from the roots of *Valeriana* species, and many compounds have been reported, including petroleum ether-soluble components, lignans, phenylpropanoids, flavonoids, and alkaloids (Zhou *et al*., 2023). However, the yield and composition of valerian extracts can vary greatly with species, geographic origin, harvest time, and extraction method (Letchamo *et al*., 2004; Sermukhamedova *et al*., 2017). These variations indicate that both biomass production conditions and extraction parameters critically influence the quality and yield of valerian-derived products, highlighting the need for integrated optimization strategies. Previous studies have reported substantial variability in valerian extract yield and composition depending on extraction technique, harvest timing, and plant source, emphasizing the importance of systematic process optimization. Therefore, this study designed and screened an efficient adventitious root culture system for *V. officinalis* and focused on optimizing a Soxhlet extraction process using petroleum ether as the solvent. Adventitious roots obtained by tissue culture were also compared with cultivated root materials collected at different harvest times to evaluate the feasibility of replacing part of the traditional cultivated material with tissue-cultured material and of building a continuous, controllable, and

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potentially useful technical route for *In vitro* production of *V. officinalis* adventitious roots and extraction of lipophilic volatile-related components. Common extraction methods for valerian include steam distillation, Soxhlet extraction, supercritical CO_2 extraction, subcritical butane extraction, and microwave-assisted extraction (Yang, 2009; Li *et al*., 2018; Tan *et al*., 2024a; Tan *et al*., 2024b). Among these, Soxhlet extraction offers advantages in operational simplicity, reproducibility, and scalability for laboratory optimization studies (Ma *et al*., 2022). Therefore, petroleum ether Soxhlet extraction was selected in the present study. . Therefore, establishing a reproducible system that combines efficient biomass production with optimized extraction conditions is important for improving the utilization potential of *V. officinalis*.

Therefore, this study used Soxhlet extraction with petroleum ether as the solvent. Single-factor tests and a Box–Behnken design were used to optimize the extraction conditions, improve the recovery of lipophilic components from *In vitro* materials of *V. officinalis*, and provide an experimental basis for later component analysis and resource utilization. Despite extensive studies on valerian pharmacology and extraction technologies, limited research has integrated adventitious root culture optimization with downstream extraction process optimization using tissue-cultured biomass. Furthermore, quantitative optimization of suspension culture nutrients and petroleum ether Soxhlet extraction parameters for *V. officinalis* adventitious roots remain insufficiently explored. The novelty of this study lies in combining optimized adventitious root induction, suspension culture, and petroleum ether Soxhlet extraction into a unified experimental workflow for controlled *In vitro* biomass utilization. Therefore, this study aimed to: (i) optimize adventitious root induction using different combinations of NAA and 6-BA, (ii) optimize suspension culture conditions through response surface methodology, and (iii) optimize petroleum ether Soxhlet extraction conditions for adventitious roots of *V. officinalis*. The study further sought to establish an experimentally reproducible framework linking *In vitro* biomass production with downstream extraction optimization.

Materials and Methods

Plant materials: Seed material of *Valeriana officinalis* L. was provided by the College of Agriculture, Utah State University, USA, and subsequently cultivated in the greenhouse of the College of Agriculture, Yanbian University, Yanji, Yanbian Prefecture, Jilin Province, China. Flowering plants were identified by Professor Quan Xueli of the College of Agriculture, Yanbian University, as *Valeriana officinalis* L. Seeds collected in autumn were air-dried and inoculated onto standard MS medium under sterile conditions. Stems from aseptic seedlings obtained after several subcultures were used as explants for adventitious root induction.

Adventitious roots cultured for 28 days under the optimized suspension culture conditions described in *Optimization of adventitious root suspension culture*, as well as underground roots and rhizomes of 2-year-old cultivated *V. officinalis* plants grown on the north side of the greenhouse of the College of Agriculture, Yanbian University and harvested on the 30th day of each month from April to September, were washed several times with tap water and then with distilled water. The materials were air dried and then ground. After sieving through 50 mesh, the samples were sealed and stored in a refrigerator at 4°C until use. Soxhlet extraction efficiency is strongly influenced by solvent diffusion, solute solubility, and concentration gradients between plant matrices and extraction solvent.

Optimization of the medium for adventitious root induction: MS solid medium was supplemented with 30 $g \cdot L^{-1}$ sucrose, and the pH was adjusted to 5.7. Different concentrations of NAA and 6-BA were added, giving a total of 16 combinations of plant growth regulators (Table 1). Stems of uniform thickness were cut into 1 cm segments and used as explants, which were inoculated onto the prepared media. Eight explants were inoculated into each medium, and each treatment was repeated 7 times. Cultures were maintained in the dark at (25±1)°C. Rooting was recorded after 28 days. The rooting rate was calculated as follows:

$$\text{Rooting rate} = \frac{\text{Number of rooted explants}}{\text{Number of inoculated explants}} \times 100$$

Table 1. Treatment combinations of different concentrations of NAA and 6-BA.

Treatment number	NAA concentration ($mg \cdot L^{-1}$)	6-BA concentration ($mg \cdot L^{-1}$)
1	1.0	0.10
2	0.2	0.05
3	0.5	0
4	1.0	0
5	0	0.05
6	0.5	0.20
7	1.0	0.20
8	0	0.10
9	0	0.20
10	0.5	0.05
11	0.2	0
12	0.2	0.20
13	0.2	0.10
14	0	0
15	1.0	0.05
16	0.5	0.10

Table 2. Factors and levels of the Box–Behnken design.

Level	A: Sucrose concentration ($g \cdot L^{-1}$)	B: Total N concentration ($mmol \cdot L^{-1}$)	C: Total P concentration ($mmol \cdot L^{-1}$)
-1	30	32.31	1.0
0	40	48.46	1.5
1	50	64.61	2.0

Optimization of adventitious root suspension culture: Adventitious roots of *V. officinalis* induced under the optimal conditions described in Section 2.1 were used as the experimental material. Based on the single-factor study of Li *et al*., (2020), sucrose concentration (A), total N concentration (B), and total P concentration (C) were selected as independent variables, and harvested fresh weight was used as the response value. Each factor had three levels coded as −1, 0, and 1. Based on the single factor

results, the variation ranges and the zero levels of the three factors were determined, and a three factor, three level Box–Behnken design was created using Design Expert 13. The coded levels of the factors are listed in Table 2.

Root tips cut to 2 cm were inoculated into 17 treatment groups of RCM liquid medium containing double-strength macronutrients and different combinations of sucrose concentration, total N concentration, and total P concentration according to the Box–Behnken design. Forty root tips were inoculated into each medium, and each treatment was repeated three times. After inoculation, the cultures were placed on a shaker at 100 r·min^{-1} and maintained in the dark at (25 ± 1) °C. The proliferation of adventitious roots was recorded after 28 days.

Single-factor test of petroleum ether extract yield: Using petroleum ether as the solvent, Soxhlet extraction was performed on adventitious root powder of *V. officinalis*. Although supercritical extraction methods may provide higher selectivity and lower thermal stress, Soxhlet extraction remains advantageous because of lower equipment cost and greater operational simplicity. The experiment was divided into 6 groups. In each group, 1 g of *V. officinalis* adventitious root powder was accurately weighed and placed in a Soxhlet extractor, and extraction was carried out under different liquid-to-solid ratios, extraction temperatures, and extraction times. After petroleum ether was removed by evaporation, the yield was calculated according to Equation (3).

$$\text{Petroleum ether extract yield (\%)} = \frac{\text{Net weight of petroleum ether extract}}{\text{Net weight of dried valerian powder}} \times 100$$

The single factor testing scheme is described below. First, with the extraction temperature fixed at 60°C and the extraction time fixed at 4 h, the yield of petroleum ether extract from *V. officinalis* was determined at liquid to solid ratios of 12:1, 16:1, 20:1, 24:1, 28:1, and 32:1 mL·g^{-1}. Second, with the liquid to solid ratio set to 24:1 mL·g^{-1} and the extraction time maintained at 4 h, the extract yield was determined at extraction temperatures of 30, 40, 50, 60, 70, and 80°C. At a liquid-to-solid ratio of 24:1 mL·g^{-1} and an extraction temperature of 60°C, the yield was measured at different extraction times (2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 h). Then, using the extraction process optimized by the response surface design, the yield of petroleum ether extract from *V. officinalis* was measured in different months (April, May, June, July, August, and September).

Response: surface design for optimization of the petroleum ether Soxhlet extraction process of *V. officinalis* adventitious roots: A response surface design was developed in Design Expert 13. The response surface analysis included three factors: liquid to solid ratio, extraction temperature, and extraction time. Based on the results of the single-factor tests, the extraction process of petroleum ether extract from *V. officinalis* was optimized. The factors and levels are shown in Table 3.

Table 3. Factor levels of the Box–Behnken design.

Level	A: Liquid-to-solid ratio (mL·g^{-1})	B: Extraction temperature (°C)	C: Extraction time (h)
-1	20:1	50	3.5
0	24:1	60	4
1	28:1	70	4.5

Results

Optimization of the medium for adventitious root induction: SPSS 26.0 was used to analyze the total number of roots and the rooting rate of *V. officinalis* stem segments treated with different concentrations of NAA and 6-BA. In Levene's test for homogeneity of variance, all P values were greater than 0.05, indicating small experimental error and reliable results. This trend may be associated with physiological saturation effects and altered metabolic balance under excessive treatment levels. The results of the significance analysis are shown in Figs. 1 and 2.

As shown in Figs. 1 and 2, no roots were produced when NAA was not added to the medium. Figure 1a showed that, at different NAA concentrations, the number of roots were first increased and then decreased with increasing 6-BA concentration. When the NAA concentration was 0.2 mg·L^{-1}, the number of roots was higher than that of the other combinations. Among them, 0.1 mg·L^{-1} 6-BA gave a significantly higher root number compared to other combinations. Fig. 1b showed that, under different 6-BA concentrations, high NAA concentrations inhibited root formation, and the number of roots was decreased with the increase of NAA concentration. Excess auxin concentrations may disturb endogenous hormone balance and suppress normal root differentiation. Based on Fig. 1a and 1b, the combination with the highest number of roots was 0.1 mg·L^{-1} 6-BA plus 0.2 mg·L^{-1} NAA (Treatment 13).

Figure 2a showed that, when the NAA concentration was fixed at 0.5 mg·L^{-1}, the rooting rate was first increased and then decreased as the 6-BA concentration changed. However, when the NAA concentration was 0.2 or 1.0 mg·L^{-1}, the rooting rate showed a three-stage trend of increase, decrease, and increase again with changes in 6-BA concentration. Figure 2b showed that, except for the combination with 0.2 mg 6-BA, which also showed a three-stage pattern, the rooting rates of the other combinations were first increased and then decreased as the NAA concentration increased. However, Treatment 2 (0.2 mg·L^{-1} NAA combined with 0.05 mg·L^{-1} 6-BA) produced a rooting rate that was significantly higher than those obtained with the other combinations.

Under Treatment 13, the total number of roots per explant reached (36.6 ± 2.51), which was the highest among all treatments. Although Treatment 13 produced the highest root number, Treatment 2 was selected because it combined a very high rooting rate with relatively vigorous and morphologically healthy roots, making it more suitable for stable biomass production." In addition, the adventitious roots under Treatment 2 were relatively thick (Fig. 3). Therefore, based on these results, 0.2 mg·L^{-1} NAA combined with 0.05 mg·L^{-1} 6-BA was adopted for inducing adventitious roots in *V. officinalis*. Similar auxin–cytokinin interactions affecting adventitious root induction have been reported in other medicinal plant tissue culture systems (Cao & Huang, 2016; Ren *et al*., 2019).

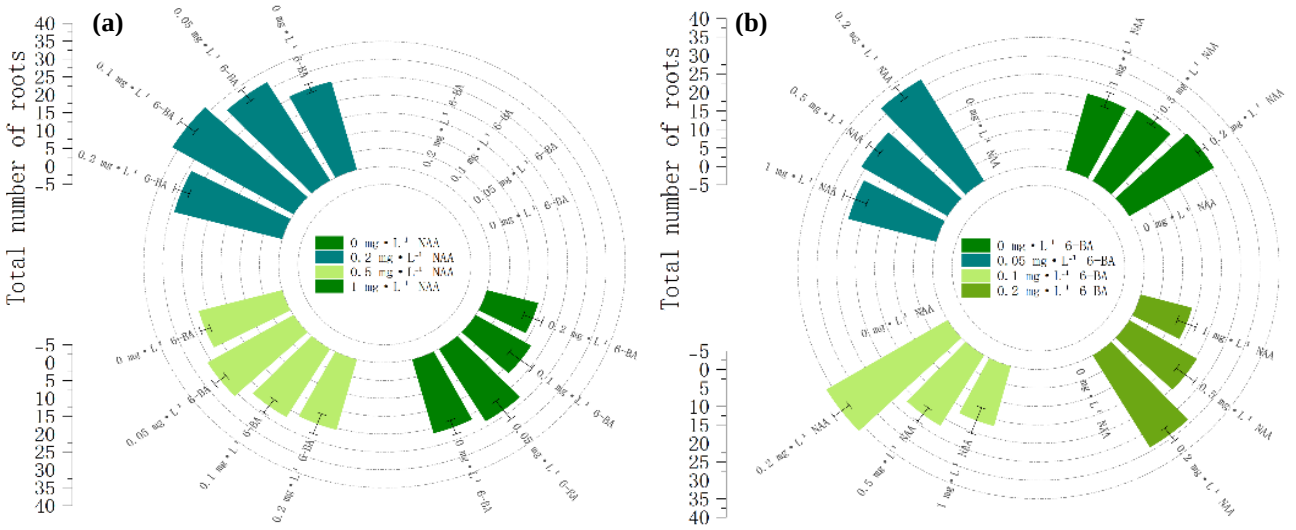

Fig. 1. Total number of roots of *V. officinalis* stem segments under combined treatments with different concentrations of NAA and 6-BA

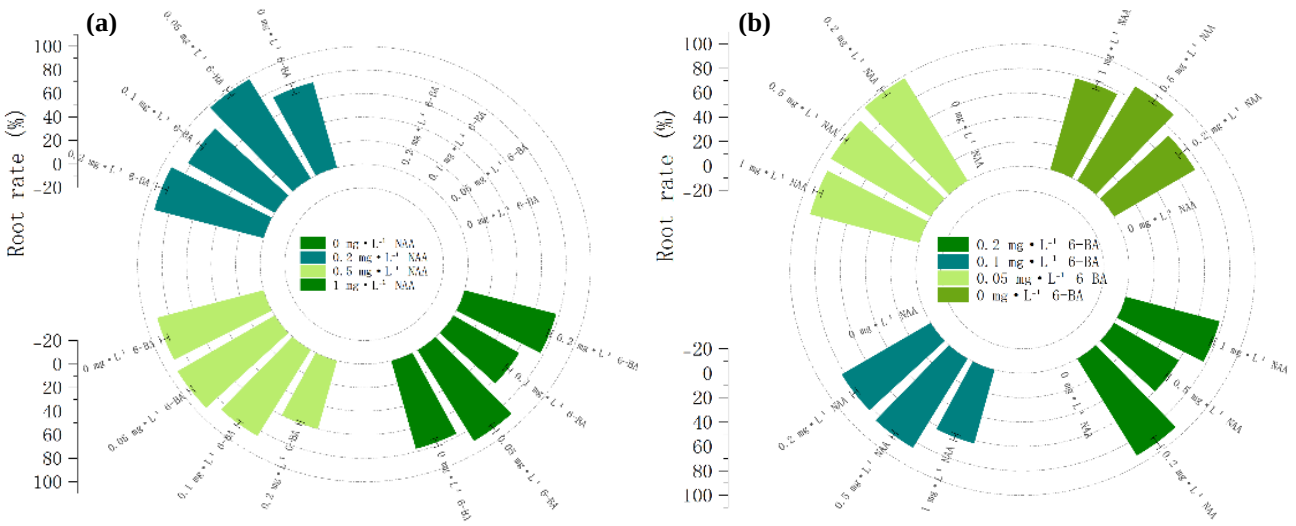

Fig. 2. Rooting rate of *V. officinalis* stem segments under combined treatments with different concentrations of NAA and 6-BA.

Optimization of adventitious root suspension culture: Response surface data were analyzed by Design-Expert 13 to examine interactions among variables and predict the optimal conditions for adventitious root culture of *V. officinalis*. A Box–Behnken design with 17 treatments was used for this analysis.

The analysis of variance and the significance of regression coefficients are shown in Table 4. The model was highly significant ($p<0.0001$), indicating that it was suitable for optimizing suspension culture conditions for *V. officinalis* adventitious roots. A non-significant lack-of-fit was observed ($F = 4.56$, $p = 0.0884 > 0.05$), which implies a small experimental error. Sucrose concentration had a highly significant effect on harvested fresh weight ($p<0.01$), total P concentration had a significant effect ($0.01< p<0.05$), and total N concentration had no significant effect ($p>0.05$) suggesting that the tested range was close to the optimal range for the growth of *V. officinalis*. The p values reflect the relative contribution of each factor to the response; smaller p values correspond to stronger effects. Accordingly, the influences of the three factors on harvested fresh weight ranked as follows: sucrose concentration >total P concentration >total N concentration. Among the interaction terms, sucrose concentration × total N concentration was highly significant, total N concentration × total P concentration was significant, and sucrose concentration × total P concentration showed no significant effect. After removing the non-significant interaction term, regression Equation (1) was established, and the response surface and contour plots were obtained. The use of response surface methodology enabled simultaneous evaluation of individual and interaction effects among culture variables.

The steepness of the 3D response surfaces and the contour patterns suggest that the interaction between sucrose concentration and total N concentration was more pronounced than that between total N concentration and total P concentration. Figure 4a showed that, when the total N concentration was 48.46 mmol·L^{-1}, the harvested fresh weight first increased and then gradually decreased as the sucrose concentration was increased. Elliptical contour patterns indicated stronger interaction effects among variables, whereas circular contours would suggest weaker interactions. Figure 4b showed dense contour changes

along the sucrose concentration axis, indicating that sucrose concentration had a greater effect on harvested fresh weight than total N concentration. Figure 4c showed that, when the total N concentration was 48.46 mmol·L^{-1}, the harvested fresh weight first increased to a peak and then gradually decreased as the total P concentration increased. Total P concentration had a greater effect on harvested fresh weight than total N concentration (Fig. 4d).

Solving Equation (1) gave the following optimal combination of the three factors: sucrose concentration 41.905 g·L^{-1}, total N concentration 39.382 mmol·L^{-1}, and total P concentration 1.590 mmol·L^{-1}, with a predicted response value of 13.796. To verify the reliability of this result and to simplify practical operation, seven repeated experiments were carried out under the following conditions: sucrose concentration 42 g·L^{-1}, total N concentration 39.854 mmol·L^{-1}, and total P concentration 1.590 mmol·L^{-1}. The measured value was (14.53 ± 3.52) g, which was close to the theoretical value and confirmed the validity of the model. The regression model showed satisfactory predictive performance with acceptable agreement between predicted and experimental values.

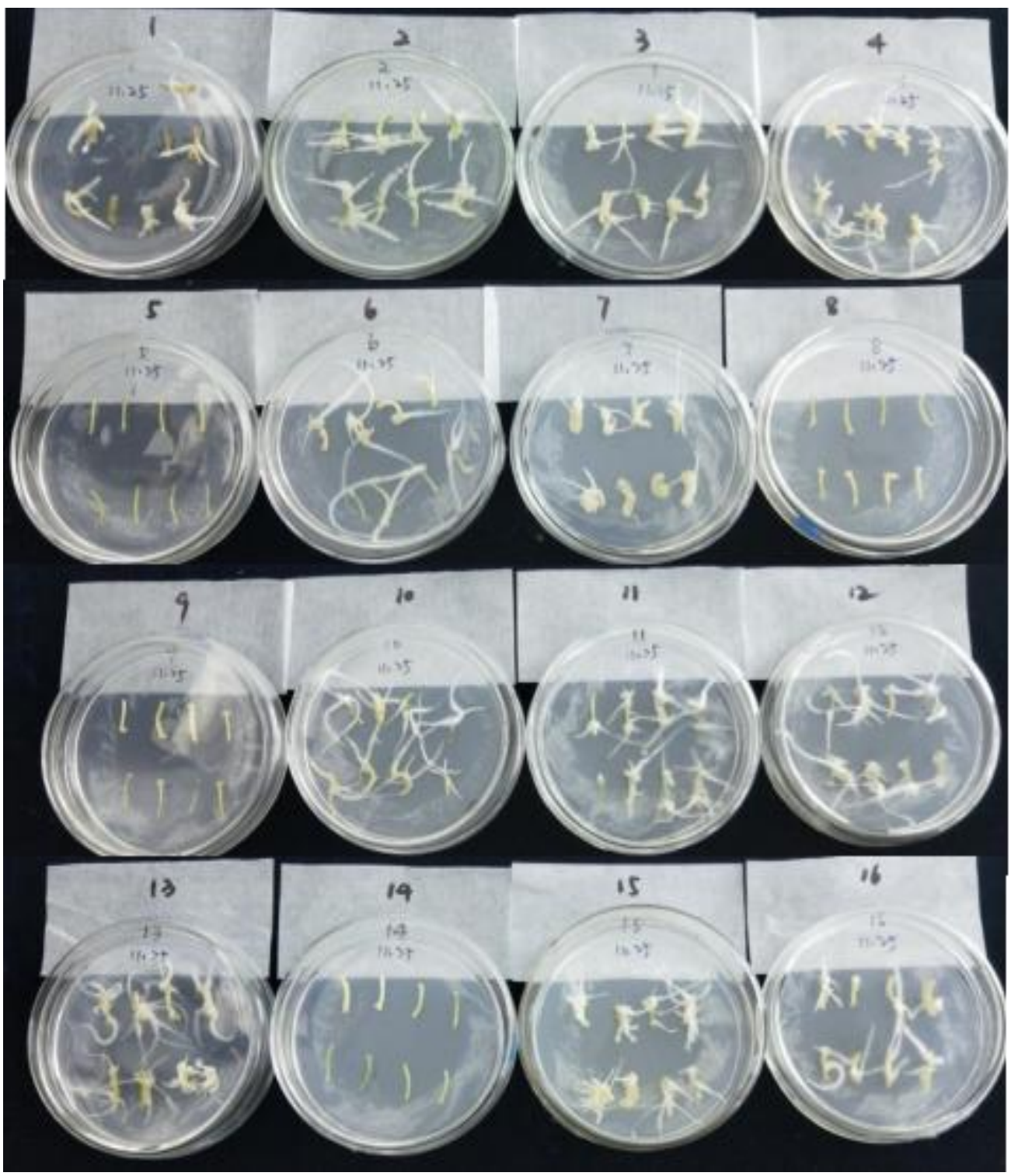

Fig. 3. Adventitious roots induced by combinations of different concentrations of plant growth regulators.

Table 4. ANOVA of the response surface regression equation describing adventitious root suspension culture.

Source	Sum of squares	df	Mean square	F value	p value	Significance
Model	51.55	9	5.730	31.54	<0.0001	**
A	4.90	1	4.900	26.97	0.0013	**
B	0.068	1	0.068	0.38	0.5587	
C	2.21	1	2.210	12.14	0.0102	*
AB	2.99	1	2.990	16.48	0.0048	**
AC	0.36	1	0.360	1.98	0.2020	
BC	1.21	1	1.210	6.66	0.0364	*
A^2	23.01	1	23.01	126.67	<0.0001	**
B^2	1.99	1	1.990	10.96	0.0129	*
C^2	11.50	1	11.50	63.31	<0.0001	**
Residual	1.27	7	0.180			
Lack of Fit	0.98	3	0.330	4.56	0.0884	---
Pure Error	0.29	4	0.072			
Cor Total	52.82	16				

Note: "**" Indicates a highly significant difference ($p<0.01$), "*" Indicates a significant difference ($p<0.05$), and "---" Indicates no significant difference

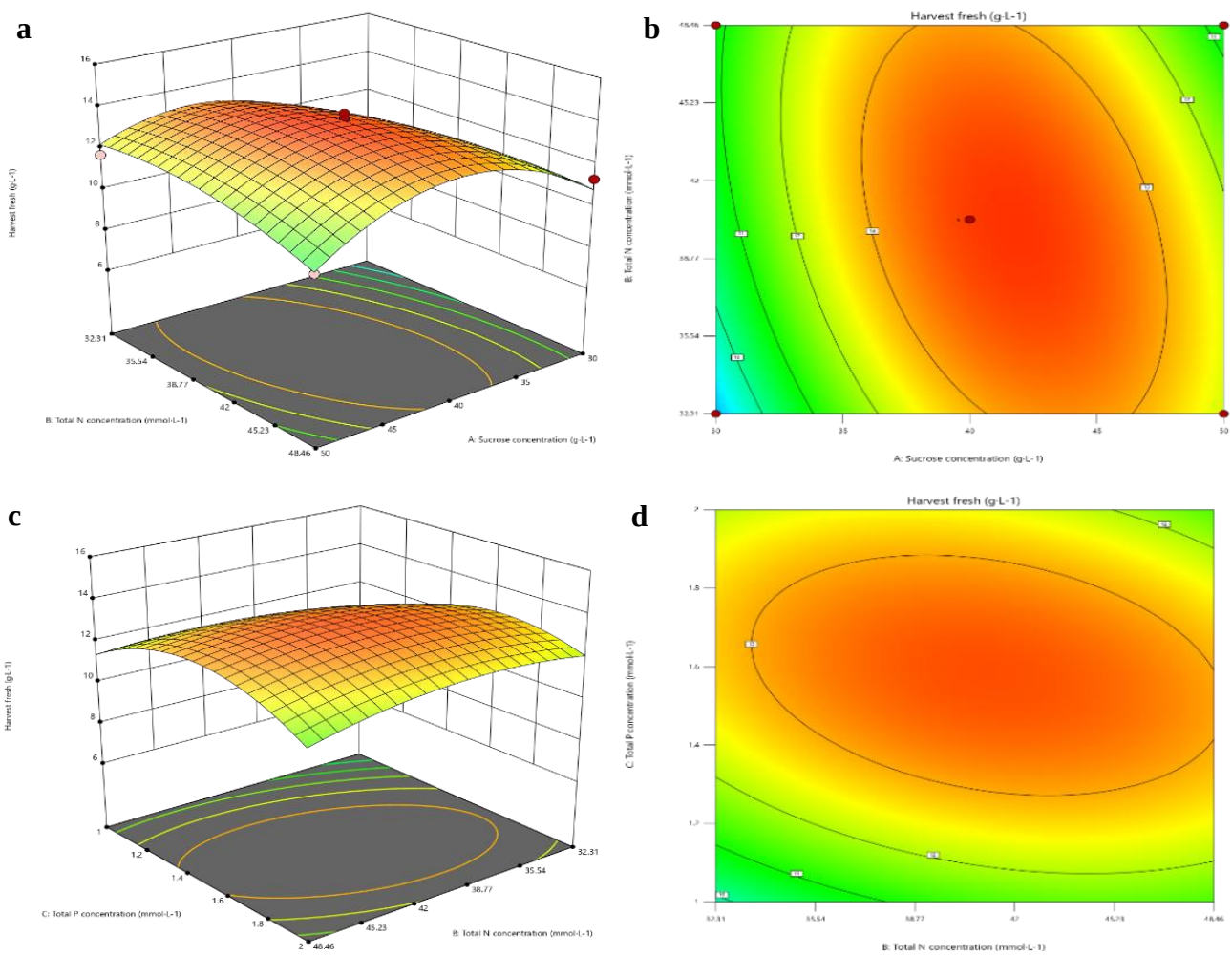

Fig. 4. Response surface and contour plots illustrating how sucrose concentration and total N concentration influence harvested fresh weight: (a) response surface plot; (b) contour plot; and plots depicting the relationship between total N concentration, total P concentration, and harvested fresh weight: (c) response surface plot; (d) contour plot.

Harvest fresh = -86.512457710703 + 2.380857120743 *Sucrose concentration + 1.495876506053 *Total N concentration + 26.381362229102 *Total P concentration -0.010712074303405 *Sucrose concentration *Total N concentration -0.13622291021672 *Total N concentration *Total P concentration -0.023375 *Sucrose concentration^2-0.010543568902223 *Total N concentration^2-6.61 *Total P concentration^2 ---------- (1)

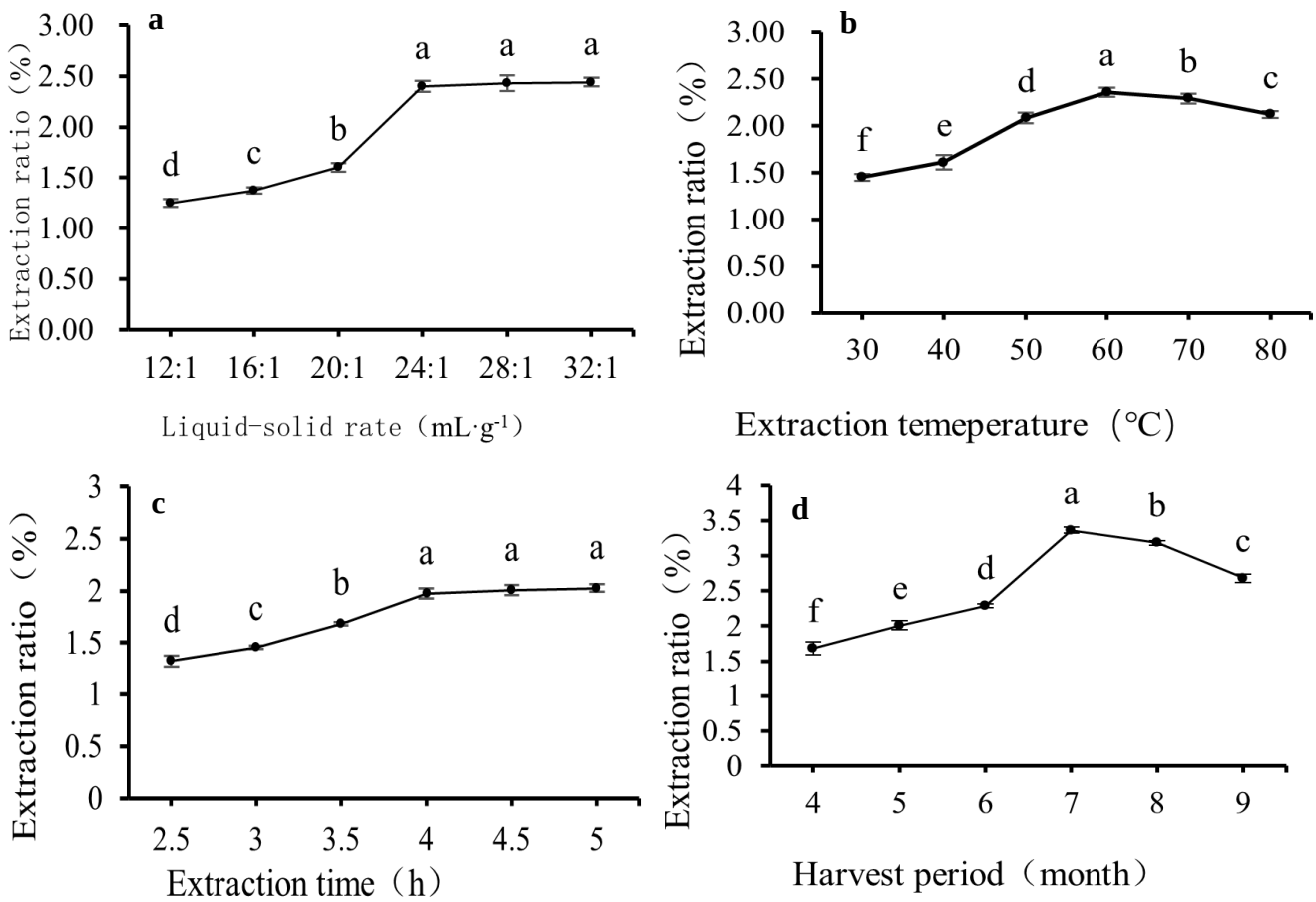

Fig. 5. Yield of the petroleum ether extract as a function of (a) liquid-to-solid ratio, (b) extraction temperature, (c) extraction time, and (d) harvest time. Different letters indicate significant differences ($p<0.05$).

Table 5. ANOVA results for the response surface regression model describing the petroleum ether extract extraction process.

Source	Sum of squares	df	Mean square	F value	p value	Significance
Model	1.38	9	0.1537	582.99	< 0.0001	**
A	0.0036	1	0.0036	13.71	0.0076	
B	0.4232	1	0.4232	1605.64	< 0.0001	**
C	0.316	1	0.316	1198.96	< 0.0001	**
AB	0.0004	1	0.0004	1.52	0.2578	
AC	0.1406	1	0.1406	533.54	< 0.0001	**
BC	0.0324	1	0.0324	122.93	< 0.0001	**
A^2	0.0275	1	0.0275	104.17	< 0.0001	**
B^2	0.0014	1	0.0014	5.32	0.0545	
C^2	0.4198	1	0.4198	1592.67	< 0.0001	**
Residual	0.0018	7	0.0003			
Lack of Fit	0.0013	3	0.0004	3.4	0.1341	
Pure Error	0.0005	4	0.0001			
Cor Total	1.38	16				

Note: ** Highly significant difference ($p<0.01$); *Significant difference ($p<0.05$)

Extraction rate = -19.2455-0.1274375 *Liquid-to-feed ratio + 0.1169 *Extraction temperature + 9.3314999999999 *Extraction time + 0.09375 *Liquid-to-feed ratio * Extraction time-0.018 *Extraction temperature *Extraction time-0.005046875 Liquid-to-feed ratio²-0.0001825 *Extraction temperature²-1.263 *Extraction time² ---------- (2)

The ANOVA results, together with the significance of the regression coefficients, are summarized in Table 5. The model was highly significant ($p<0.0001$), indicating that the model was suitable for optimizing the extraction process of petroleum ether extract from adventitious roots of *V. officinalis*. The lack-of-fit term was not significant ($F = 3.4$, $p = 0.1341 > 0.05$), suggesting small experimental error. The p value of the AB term was >0.05, indicating that this interaction was not significant. After removing the non-significant interaction term, regression Equation (2) was established, and the response surface and contour plots were obtained.

Optimization of the Soxhlet extraction process

Effects of single-factor treatments on the yield of petroleum ether extract from *V. officinalis*: The observed extraction trends likely reflect changes in solvent penetration efficiency and diffusion kinetics during Soxhlet extraction. Figure 5a shows that increasing the extraction solvent allowed fuller contact between valerian powder and the solvent, which favored the dissolution of petroleum ether-soluble components. The yield of the petroleum ether extract increased initially with a higher liquid to solid ratio, and then stabilized. When the liquid-to-solid ratio reached 24:1 (mL:g), the yield remained at about (2.40 ± 0.06)%, indicating that extraction was close to saturation. Figure 5b shows that, within the range of 30–80°C, the yield first increased and then decreased as the extraction temperature increased. Higher temperature helped solute molecules diffuse from the cells into the extraction solution, but excessively high temperature may have caused loss of some petroleum ether extract with solvent volatilization. The highest yield, (2.36 ± 0.04) %, was obtained at 60°C. Figure 5c shows that the yield increased with longer extraction time, but the increase became slow after 4 h. Therefore, 4 h was selected as the optimal extraction time, giving a yield of (1.98 ± 0.05) %. Figure 5d showed that the yield of petroleum ether extract from cultivated roots of *V. officinalis* harvested from April to September first increased and then decreased, and the highest yield, (3.36 ± 0.04) %, was obtained in July, with significant differences from the other months. Collectively, these results indicate the presence of optimal extraction conditions beyond which extraction efficiency no longer improves substantially. Comparable extraction behavior has also been reported for Soxhlet extraction of Valeriana species and other medicinal plants using non-polar solvents.

Results of response surface optimization of the extraction process for petroleum ether extract: Response surface data were analyzed by Design-Expert 13 to examine interactions among variables and predict the optimal conditions for extracting petroleum ether extract from adventitious roots of *V. officinalis*. A Box–Behnken design with 17 treatments was used for this analysis.

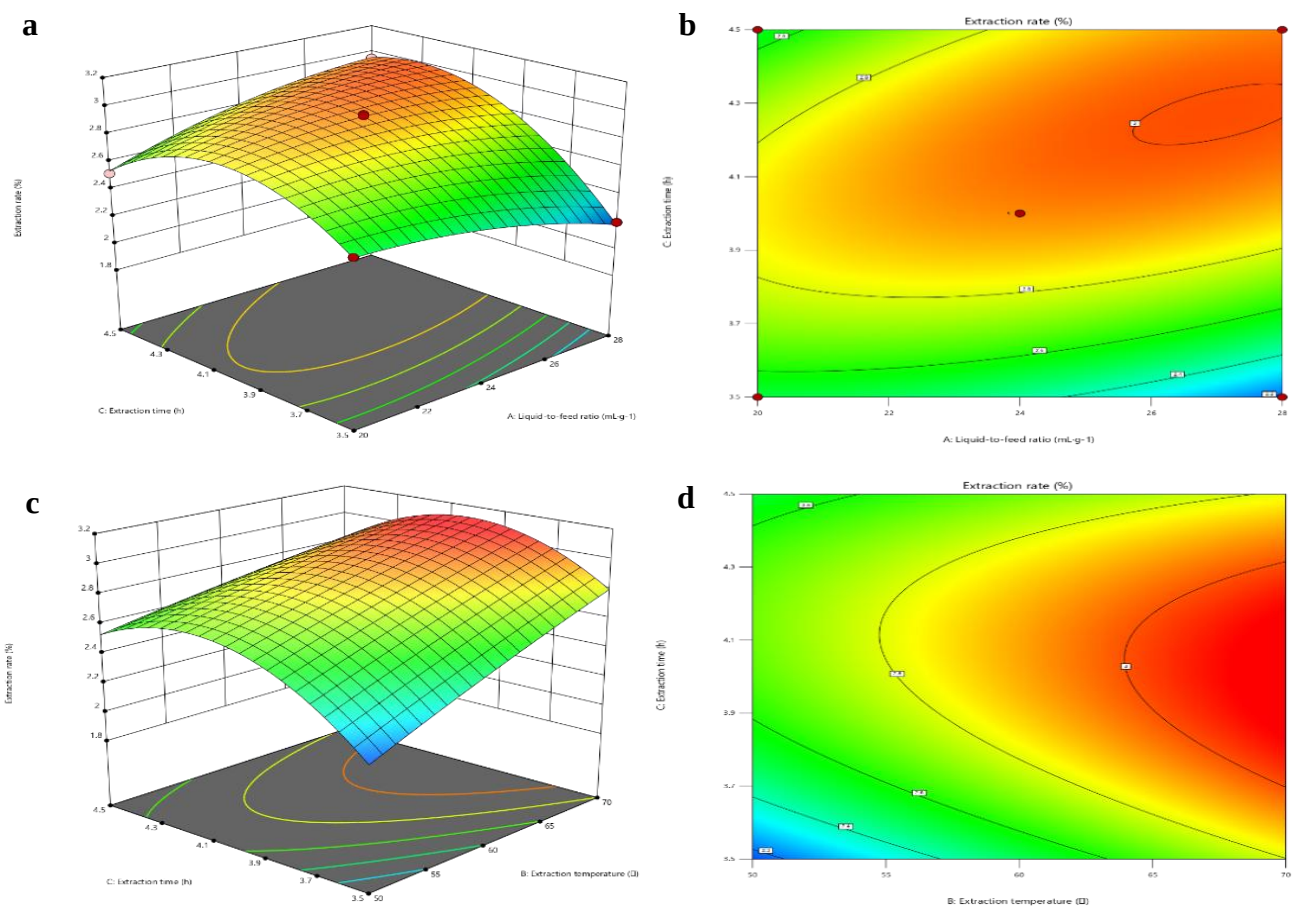

Fig. 6. Response surface (a, c) and contour (b, d) plots of petroleum ether extract yield from V. officinalis as a function of liquid to solid ratio and extraction time (a, b), and as a function of extraction temperature and extraction time (c, d).

The interaction between liquid-to-solid ratio and extraction time on the yield of petroleum ether extract from *V. officinalis* is shown in Figs. 6a and 6b. The response surface was steep and the contour plot was clearly elliptical, indicating that this interaction had a highly significant effect on the yield. The highest yield was obtained when the liquid-to-solid ratio was 24:1–28:1 $mL \cdot g^{-1}$ and the extraction time was 4.0–4.4 h. The interaction between extraction temperature and extraction time is shown in Figs 6c and 6d. The response surface was also steep and the contour plot was elliptical, indicating a highly significant interaction. The yield reached its maximum when the extraction temperature was between 65 and 70°C, closer to 70°C, and the extraction time was between 3.8 and 4.3 h.

To verify the reliability of the above results, Equation (2) was solved and the maximum predicted response value, 3.15%, was obtained under the following conditions: liquid-to-solid ratio 26.649: 1, extraction temperature 68.541°C, and extraction time 4.248 h. For operational convenience, we adjusted the optimized conditions to a liquid to solid ratio of 26.7:1 mL·g^{-1}, 68.5°C for extraction temperature, and 4.3 h for extraction time. Three repeated experiments under these conditions gave a measured value of (3.13 ± 0.12) %, only 0.02 different from the theoretical value, which confirmed the validity of the model. The close agreement between predicted and experimental values further confirmed the reliability and adequacy of the regression model.

Discussion

6-BA and NAA are two commonly used plant growth regulators, and both can promote root growth. However, the use of a single regulator often has some limitations (Liang *et al*., 2024). Previous studies have shown that 6-BA alone can inhibit root formation, and that induction with NAA alone is comaratively less effective than induction where the two regulators are used together (Cao & Huang, 2016; Ren *et al*., 2019; Huang *et al*., 2024). This is consistent with the results of the present study, in which the combinations containing only 6-BA gave a rooting rate of 0, and the combinations containing only NAA or excessively high concentrations of 6-BA showed low root number and low rooting rate. In the study by He & Shi (2014) on the combined effects of 6-BA and NAA on hairy root growth, biomass also showed a three-stage trend of increase, decrease, and increase again as the 6-BA concentration changed, which was similar to the pattern observed here. Previous studies using Soxhlet and related extraction techniques for valerian have similarly reported strong influences of solvent ratio and extraction temperature on extraction efficiency (Tan *et al*., 2024a). This trend may be related to the simultaneous synergistic and interactive effects of NAA and 6-BA. In addition, the combination of relatively high NAA and low 6-BA used in this study allowed direct redifferentiation of *V. officinalis* stem segments into adventitious roots, without the intermediate step of callus redifferentiation. This greatly shortened the time required for adventitious root growth and improved culture efficiency. Auxin promotes root primordia initiation and cell elongation, whereas low cytokinin concentrations may support controlled cell division during root differentiation.

At present, most studies on the extraction of constituents from *Valeriana* species are still based on steam distillation (Samaneh *et al*., 2010; Ren *et al*., 2022). The product obtained here by petroleum ether Soxhlet extraction was not exactly the same as the valerian volatile oil obtained by steam distillation, so the extract yields cannot be compared directly. Even so, Soxhlet extraction still has certain advantages in sample powder compatibility, equipment accessibility, operational stability, and repeatability during process optimization. Based on the culture conditions and extraction parameters identified in this study, later work may combine HPLC, GC–MS, or LC–MS to analyze the petroleum ether fraction of *V. officinalis* adventitious roots, while also considering adventitious root biomass and the contents of characteristic components. Higher extraction temperature may enhance solvent diffusivity and mass transfer, thereby facilitating release of lipophilic compounds from plant tissues. However, excessively high temperatures may also increase volatilization or degradation of thermolabile constituents. Experimental verification under optimized conditions demonstrated good reproducibility of the model predictions.

Importantly, the optimized suspension culture system provided sufficient biomass quality and consistency for downstream Soxhlet extraction optimization, demonstrating the feasibility of integrating *In vitro* production with extraction processing.

Conclusions

In this study, the induction method, suspension culture conditions, and petroleum ether extraction process of *V. officinalis* adventitious roots were optimized. A growth regulator combination of 0.2 mg·L^{-1} NAA and 0.05 mg·L^{-1} 6-BA gave an induction rate of (98.00 ± 3.80) % for adventitious roots. Response surface optimization showed that the best suspension culture conditions for *V. officinalis* adventitious roots were 42 g·L^{-1} sucrose, 39.854 mmol·L^{-1} total N, and 1.590 mmol·L^{-1} total P. Response surface optimization of the petroleum ether extraction process showed that, at a liquid-to-solid ratio of 26.7:1 mL·g^{-1}, an extraction temperature of 68.5°C, and an extraction time of 4.3 h, the yield of petroleum ether extract from *V. officinalis* adventitious roots reached (3.13 ± 0.12) %. Several limitations should be acknowledged. First, chemical characterization of petroleum ether extracts was not performed. Second, bioactivity validation of the obtained extracts was beyond the scope of this study. Third, scale-up feasibility under bioreactor conditions remains unexplored. Future studies should integrate phytochemical profiling, bioactivity validation, and pilot-scale culture systems to further evaluate the industrial applicability of the optimized framework.

Overall, this study demonstrates the feasibility of integrating optimized tissue-culture-based biomass production with Soxhlet extraction process optimization for *V. officinalis*. The proposed framework may support future phytochemical production, resource standardization, and controlled utilization of valerian-derived materials.

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