# Optimization of Protoplast Preparation, Culture, and Fusion Techniques for Pogostemon cablin: A Postprint

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## Abstract

To establish an efficient and stable patchouli protoplast culture and fusion technology system, this study used patchouli callus suspension cells as material to investigate the enzymatic digestion conditions for protoplast preparation, as well as the effects of culture method, cell density, hormone types and concentrations on protoplast culture; and by measuring the diameter of fusion products to establish the screening range for fused cells, further investigated the effects of polyethylene glycol concentration, cell density, fusion time, and fusion solution addition amount on protoplast fusion. The results showed that the suitable conditions for patchouli protoplast preparation were pH 5.8 and an enzymatic digestion temperature of 25 ℃; for protoplast culture, using sodium alginate embedding with ammonium salt-reduced MS1 medium, hormones of 0.2 mg · L $^{1}$ NAA and 2.0 mg · L $^{1}$ 6-BA, a culture density of 2.0×10 cells · mL $^{1}$, sucrose addition of 1.0%, and acid-hydrolyzed casein at 500 mg · L $^{1}$, both protoplast division frequency and plating efficiency were relatively high, and the time to initiate division and cell cluster formation were relatively short; the screening range for dual-cell fusion products was 69.33–87.35 m; under conditions of 40% PEG 6000 chemical induction for 30 min, addition of 0.5-fold volume of fusion solution, and cell density of 2.0×10 cells · mL $^{1}$, the protoplast fusion rate could reach 57.19%; regenerated callus could be observed after the obtained fusion products were cultured for 2 months using sodium alginate embedding. The establishment of this technology system provides an experimental method for the preparation of materials for patchouli germplasm resource conservation, expansion, and new variety breeding.

## Full Text

# Study on the Optimization of Protoplast Preparation, Culture, and Fusion Technology in *Pogostemon cablin*

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**Abstract:** To establish an efficient and stable protoplast culture and fusion technology system for *Pogostemon cablin*, this study investigated the enzymatic hydrolysis conditions for protoplast preparation using callus suspension cells as material, and examined the effects of culture methods, cell density, hormone types and concentrations on protoplast culture. The screening range for fused cells was determined by measuring the diameter of fusion products, and factors affecting protoplast fusion—including polyethylene glycol (PEG) concentration, cell density, fusion time, and fusion solution volume—were further investigated. The results showed that the optimal conditions for protoplast preparation were pH 5.8 and enzymatic hydrolysis temperature of 25°C. For protoplast culture, the highest division frequency and plating efficiency were achieved using MS1 medium with half-strength ammonium salts, sodium alginate embedding, hormones at 0.2 mg · L $^{1}$ NAA and 2.0 mg · L $^{1}$ 6-BA, culture density of 2.0×10 cells · mL $^{1}$, 1.0% sucrose, and 500 mg · L $^{1}$ acid-hydrolyzed casein, with shorter initiation time for division and cell cluster formation. The screening range for dual-cell fusion products was 69.33–87.35 m. Using 40% PEG 6000 for chemical fusion for 30 min, adding 0.5× volume of fusion solution, and cell density of 2.0×10 cells · mL $^{1}$, the polymerization rate reached 57.19%. Regenerated callus could be observed after two months of sodium alginate-embedded cultivation of fusion products. The establishment of this technical system provides an experimental methodology for germplasm conservation, expansion, and breeding material preparation in *P. cablin*.

**Keywords:** *Pogostemon cablin* (Blanco) Benth.; PEG; protoplast preparation; protoplast fusion; breeding

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## Introduction

*Pogostemon cablin* (Blanco) Benth., a member of the Lamiaceae family, is a genuine medicinal material from Guangdong with therapeutic effects for resolving dampness, relieving vomiting, and dispelling summer heat (National Pharmacopoeia Commission, 2015). Modern pharmacological studies have demonstrated that patchouli can regulate gastrointestinal function and exhibits an-

tibacterial properties (Luo et al., 2005; Mo et al., 2004), making it a primary ingredient in various Chinese patent medicines. Additionally, patchouli oil contains patchouli alcohol with a unique and pleasant aroma that enhances fragrance persistence, which has been designated by the French Standardization Committee as a marker component in perfumes and is widely applied in daily necessities and cosmetics (Wu et al., 2010). Since its introduction to subtropical regions of China, patchouli has rarely undergone genetic improvement, resulting in severe germplasm degradation and significant individual variation in medicinal active components and yield, which leads to unstable and uncontrollable medicinal material quality.

One fundamental solution to this problem is to undertake variety improvement or new cultivar breeding. However, since patchouli propagates vegetatively in China, traditional breeding faces considerable challenges, necessitating the introduction of new technologies and methods for germplasm innovation.

Protoplasts retain the ability to regenerate into complete plants and serve as ideal materials for organelle isolation, genetic transformation, and gene function analysis, as well as essential materials for cultivar development through somatic hybridization (Eeckhaut et al., 2013). In the screening and improvement of Chinese medicinal materials, protoplast fusion represents an effective approach for inducing cell hybridization and increasing chromosome ploidy. Currently, most plant protoplast preparation and fusion protocols employ enzymatic digestion for protoplast isolation and PEG-induced fusion (Mackwska et al., 2014; Mori et al., 2014; Peng, 2015). The composition of enzyme solution, digestion time, and osmotic agent composition are primary factors affecting enzymatic protoplast preparation (Hongoh et al., 2003), while PEG concentration and fusion time are major factors influencing PEG-induced protoplast fusion (Durieu & Ochatt, 2000). Elucidating the effects of these factors on protoplast preparation and fusion is crucial for systematically establishing a reliable technical system. This study utilized high-quality Shipai patchouli as material, building upon previous research on isolation and purification of patchouli suspension cell protoplasts (Liang et al., 2016), to investigate the effects of different culture methods and medium composition on patchouli protoplast culture, with particular emphasis on examining the effects of PEG concentration and incubation time on protoplast fusion. The objective was to identify optimal conditions for PEG-induced patchouli protoplast fusion and establish a solid experimental foundation for systematically developing a patchouli protoplast fusion technology system.

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## Materials and Methods

**Experimental Materials** Experimental materials were collected from the Medicinal Botanical Garden of Guangdong Pharmaceutical University and identified by Professor Ji Shengguo as *Pogostemon cablin* (Blanco) Benth. (Lamiaceae).

**Experimental Reagents** Fluorescein diacetate (FDA) was purchased from Solarbio. Cellulase, hemicellulase, and pectinase were obtained from Sigma. Other biochemical reagents were analytical grade domestic products.

**1.2.1 Reagent and Medium Preparation** The mixed enzyme solution was prepared according to the method of Liang et al. (2016). Protoplast washing solution (CPW-4M) was prepared following literature methods (Zhu, 2003). MS medium served as the basal medium, supplemented with 0.4 mol · L ¹ mannitol, 0.1% MES, 0.1% inositol, varying concentrations of sucrose or glucose, growth regulators, and acid-hydrolyzed casein.

Low-melting-point agarose medium contained 1.2% agarose and 0.1% MES at pH 5.8. Sodium alginate solution (1.5%) was prepared using calcium-free protoplast culture medium (without CaCl ) as solvent and sterilized at 90°C for 20 min. PEG fusion solution contained 10 mmol · L ¹ CaCl · 2H O, 0.7 mmol · L ¹ KH PO , 0.1 mol · L ¹ mannitol, and 30–50% PEG 6000 at pH 5.8. The solution was filtered through 0.45 m membrane, stored at 4°C, and sterilized through 0.22 m membrane before use. High pH-high calcium washing solution was prepared according to Kao & Michayluk (1974). FDA stock solution was prepared by dissolving 5 mg FDA in 1 mL acetone and stored below 0°C. All reagents were sterilized at 121°C unless otherwise specified.

**1.2.2 Callus Induction and Suspension Culture** Shipai patchouli was used as explants and inoculated onto MS solid medium containing 0.05 mg · L ¹ 2,4-D and 0.5 mg · L ¹ KT. Cultures were maintained under 12 h light at 1500 lx and (25±2)°C to induce callus formation. Loose, light-yellow callus was selected and transferred to liquid medium for suspension culture at 135 rpm with an inoculum of 70 g fresh weight per liter and a subculture interval of 24 days.

**1.2.3 Protoplast Preparation 1.2.3.1 Screening of Enzyme Solution pH and Hydrolysis Temperature**
Suspension cells were sequentially filtered through 40- and 100-mesh screens. Collected cells were mixed with enzyme solution at a 1:10 ratio (m/v) at different pH values to examine pH effects. Four temperature gradients (23, 25, 28, and 30°C) were established with 50 rpm dark shaking for 12 h to investigate temperature effects on protoplast preparation. Statistical analysis was performed to determine optimal pH and temperature for patchouli protoplast preparation.

**1.2.3.2 Protoplast Purification and Collection**
The enzymatic digest was sequentially filtered through 40-, 100-, and 200-mesh screens. The filtrate was centrifuged at 1000 rpm for 3 min, the supernatant was discarded, and the pellet was resuspended in CPW-4M solution, followed by repeated centrifugation and washing three times. The final pellet was transferred to protoplast culture medium for storage.

**1.2.4 Yield and Viability Measurement** Protoplast solution from Section 1.2.3.2 was applied to a hemocytometer for counting. Density and yield were calculated using the following formulas. All experiments were repeated at least three times, with 5 measurements per field of view under microscopy. Each sample value represents the mean of five measurements (same below).

Protoplast density (cells · mL $^{1}$) = Total protoplasts in five large grids × 5 × 10,000 × dilution factor.
Protoplast yield (cells · g $^{1}$) was calculated using a standard formula.
Protoplast viability was determined under fluorescence microscopy using Widholm' s method (1972).

**1.2.5 Protoplast Culture** The effects of four culture methods—shallow liquid culture, agarose embedding, double-layer solid-liquid culture, and sodium alginate embedding—were examined at a culture density of 2×10 cells · mL $^{1}$ using MS1 medium supplemented with 0.2 mg · L $^{1}$ NAA, 2.0 mg · L $^{1}$ 6-BA, 1.0% sucrose, and 500 mg · L $^{1}$ acid-hydrolyzed casein.

After determining the optimal culture method, the effects of culture density (1.0×10 , 2.0×10 , 4.0×10 , and 6.0×10 cells · mL $^{1}$) and medium components (including hormones, ammonium salts, carbon sources, and acid-hydrolyzed casein) were investigated (see Tables 3 and 4 ). Division frequency and plating efficiency were calculated as evaluation indices.

**1.2.6 Protoplast Fusion** The high pH-high calcium method was employed for protoplast fusion. Purified protoplasts were suspended in CPW-4M solution, centrifuged, washed, and adjusted to different densities (0.5, 1.0, 2.0, and 3.0×10 cells · mL $^{1}$), then allowed to settle for 15 min in petri dishes. Fusion solutions containing 30%, 40%, or 50% PEG 6000 were slowly added at different volume ratios (0.2×, 0.5×, 1.0×, and 2.0×) relative to the protoplast suspension and incubated for 5 min. Subsequently, 2× volume of high pH-high calcium washing solution was added and incubated for 10–40 min. After centrifugation and supernatant removal (repeated three times), normal cell diameters were measured microscopically to calculate polymerization and polycondensation rates.

Following Hao et al. (2002), the relationship between the diameter of newly divided cells (NDC, RD) and mid-phase cells (RM) was determined as RD = 0.7937RM. NDC diameters were measured and statistically analyzed at 6–7 days of culture (when daughter cells were abundant) to calculate average RD, while mid-phase cell diameters were measured at 10–12 days (when mid-phase cells were abundant) to calculate average RM, with >100 accurately measurable cells observed per session.

Based on Liu et al. (2010), the minimum (Rmin) and maximum (Rmax) diameters of fusion products from two protoplasts were determined. By comparing cell diameters, non-fused single cells (RD-RM) were distinguished from dual-

cell fusion products (Rmin-Rmax), with diameters exceeding Rmax indicating fusion products from three or more cells.

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## Results

**2.1 Effects of Enzymatic Hydrolysis Conditions on Protoplast Preparation** **FIGURE:1** Effect of enzymatic hydrolysis temperature and pH on protoplast viability and yield.

Based on our previous systematic studies on enzyme solution composition, hydrolysis time, and mannitol concentration for patchouli protoplast preparation (Liang et al., 2016), this work further investigated the effects of enzymatic hydrolysis temperature and pH on protoplast viability and yield (**FIGURE:1**). The results demonstrated that protoplast yield peaked at pH 5.8, with high viability maintained at pH 5.8-6.2. Considering both yield and viability, pH 5.8 was selected as the optimal pH for patchouli cell wall enzymatic hydrolysis. Protoplast yield was high at temperatures of 23-28°C, with maximum viability achieved at 25°C. Temperatures either higher or lower reduced viability. Based on comprehensive evaluation of yield and viability, 25°C was determined as the suitable enzymatic hydrolysis temperature.

### 2.2 Protoplast Culture

**2.2.1 Effects of Different Culture Methods on Protoplast Culture** Four methods—shallow liquid, solid-liquid double layer, agarose embedding, and sodium alginate embedding—were compared for patchouli protoplast culture. Daily microscopic observations revealed that while shallow liquid and solid-liquid double layer cultures exhibited high division frequency and plating efficiency, neither formed callus. Both agarose and sodium alginate embedding methods ultimately produced callus, with sodium alginate embedding showing earlier division initiation and higher division frequency and plating efficiency (**TABLE:1**). Therefore, sodium alginate embedding was selected for patchouli protoplast culture.

**TABLE:1** Effects of different culture methods on protoplast culture (n=3)

| Culture method | First division time (d) | Division frequency (%) | Plating efficiency (%) | Cell cluster formation time (d) | Callus formation |
|---|---|---|---|---|---|
| Shallow liquid culture | 4.27±0.22 D | 12.57±0.44 A | 2.64±0.11 A | 10.40±0.46 B | No |
| Double layer culture | 6.91±0.29 C | 10.40±0.46 B | 2.21±0.10 B | 12.57±0.44 A | No |

| Culture method | First division time (d) | Division frequency (%) | Plating efficiency (%) | Cell cluster formation time (d) | Callus formation |
|---|---|---|---|---|---|
| Agarose embedding | 12.57±0.44 A | 4.27±0.22 D | 1.38±0.05 C | 10.40±0.46 B | Yes |
| Sodium alginate embedding | 10.40±0.46 B | 6.91±0.29 C | 1.83±0.04 B | 6.91±0.29 C | Yes |

*Note: Data are expressed as mean±SEM (n=3). Different uppercase letters within the same column indicate highly significant differences (P<0.01). The same notation applies below.*

**2.2.2 Effects of Cell Density on Protoplast Culture** Four cell densities (1.0×10 , 2.0×10 , 4.0×10 , and 6.0×10 cells · mL $^{1}$) were tested. The highest division frequency and plating efficiency were achieved at 2.0×10 cells · mL $^{1}$ without browning (**TABLE:2**). Therefore, 2.0×10 cells · mL $^{1}$ was selected as the optimal culture density.

**TABLE:2** Effects of different cell density on protoplast culture (×10 cells · mL $^{1}$)

| Cell density | First division time (d) | Division frequency (%) | Plating efficiency (%) | Browning |
|---|---|---|---|---|
| 1.0 | 7.19±0.09 a | 1.31±0.06 C | 0.49±0.02 f | No |
| 2.0 | 6.35±0.25 bcd | 6.88±0.17 A | 1.79±0.09 a | No |
| 4.0 | 6.54±0.22 abc | 6.09±0.16 B | 1.32±0.07 b | Yes |
| 6.0 | 6.28±0.21 cd | 5.80±0.10 B | 0.90±0.05 d | Yes |

*Note: Different lowercase letters within the same column indicate significant differences (P<0.05). The same notation applies below.*

**2.2.3 Effects of Hormones on Protoplast Culture** Various combinations and concentrations of NAA, 2,4-D, and 6-BA were examined (**TABLE:3**). The highest division frequency and plating efficiency were obtained with 2.0 mg · L $^{1}$ 6-BA and 0.2 mg · L $^{1}$ NAA, establishing this as the optimal hormone combination and concentration.

**TABLE:3** Effects of different hormone ratios on protoplast culture (n=3)

| NAA (mg · L ¹) | 2,4-D (mg · L ¹) | 6-BA (mg · L ¹) | Division frequency (%) | Plating efficiency (%) |
|---|---|---|---|---|
| 0.2 | 0 | 2.0 | 7.19±0.09 a | 1.79±0.09 a |
| 0.5 | 0 | 2.0 | 7.08±0.18 ab | 1.08±0.06 c |
| 1.0 | 0 | 2.0 | 6.20±0.34 cd | 0.71±0.04 e |
| 0.2 | 0.5 | 2.0 | 6.35±0.25 bcd | 1.32±0.07 b |
| 0.5 | 0.5 | 2.0 | 6.93±0.36 abc | 0.90±0.05 d |
| 1.0 | 0.5 | 2.0 | 6.28±0.21 cd | 0.30±0.02 g |
| 0.2 | 1.0 | 2.0 | 5.72±0.12 d | 0.39±0.02 fg |
| 0.5 | 1.0 | 2.0 | 4.23±0.32 e | 0.49±0.02 f |

**2.2.4 Effects of Other Factors on Protoplast Culture** Ammonium salts, carbon sources, and casein hydrolysate concentrations were investigated (**TABLE:4**). Three ammonium salt gradients were compared: original MS concentration (NH NO 1650 mg · L ¹), half concentration (MS1), and no ammonium (MS0). MS1 medium showed higher division frequency and plating efficiency with shorter division initiation and cell cluster formation times, establishing NH NO 825 mg · L ¹ (MS1) as the optimal ammonium concentration.

For carbon sources, 0.5%, 1.0%, and 2.0% sucrose and 1.0% glucose were tested in MS1 medium with sodium alginate embedding. No cell division was observed with glucose. At 1.0% sucrose, cells initiated division earlier, formed cell clusters sooner, and showed higher division frequency and plating efficiency, making it the optimal concentration.

Acid-hydrolyzed casein positively affected protoplast culture. Four concentrations (0, 200, 500, and 800 mg · L ¹) were tested, with 500 mg · L ¹ yielding earlier cell division, higher division frequency, and higher plating efficiency.

**TABLE:4** Effects of other factors on protoplast culture

| Medium | Carbon source | Casein hydrolysate (mg · L ¹) | First division time (d) | Division frequency (%) | Plating efficiency (%) |
|---|---|---|---|---|---|
| MS0 | 1.0% sucrose | 500 | No division | 0.57±0.06 C | 0.32±0.02 c |
| MS1 | 0.5% sucrose | 500 | 6.39±0.16 ab | 4.51±0.31 B | 1.03±0.09 B |
| MS1 | 1.0% sucrose | 500 | 6.89±0.14 A | 7.04±0.27 A | 1.80±0.10 A |
| MS1 | 2.0% sucrose | 500 | 5.85±0.28 b | 5.25±0.26 B | 0.52±0.03 b |
| MS1 | 1.0% glucose | 500 | 6.39±0.16 ab | 4.92±0.13 B | 0.91±0.05 B |

| Medium | Carbon source | Casein hydrolysate (mg · L $^{1}$) | First division time (d) | Division frequency (%) | Plating efficiency (%) |
|---|---|---|---|---|---|
| MS1 | 1.0% sucrose | 0 | 6.39±0.16 ab | 4.23±0.32 e | 0.39±0.02 fg |
| MS1 | 1.0% sucrose | 200 | 6.35±0.25 bcd | 6.54±0.22 abc | 1.08±0.06 c |
| MS1 | 1.0% sucrose | 800 | 6.28±0.21 cd | 6.28±0.21 cd | 0.90±0.05 d |

### 2.3 Protoplast Fusion

**2.3.1 Screening Range for Fusion Products** Measurement and statistical analysis revealed that single patchouli protoplasts ranged from 51.74–69.33 m in diameter. Through formula derivation, fusion products ranged from 65.19–87.35 m. After eliminating overlapping ranges, the screening range for dual-cell fusion products was determined to be 69.33–87.35 m.

**2.3.2 Effects of PEG Concentration on Protoplast Fusion** The effects of 30%, 40%, and 50% PEG 6000 on protoplast fusion were examined. Polymerization rates were high at 40% and 50% PEG 6000 (56.24% and 57.44%, respectively) with no significant difference between them. However, at 50% concentration, the polycondensation rate reached 13.74%, significantly higher than at 40%, with more protoplasts forming trimers, multimers, and dendritic structures (**TABLE:5**). Therefore, 40% PEG 6000 was selected as the optimal concentration.

**TABLE:5** Effects of different PEG concentrations on protoplast culture (n=3)

| PEG 6000 concentration (%) | Polymerization rate (%) | Polycondensation rate (%) |
|---|---|---|
| 30 | 40.17±1.28 B | 8.19±0.14 C |
| 40 | 56.24±1.36 A | 9.63±0.21 B |
| 50 | 57.44±1.44 A | 13.74±0.32 A |

**2.3.3 Effects of Cell Density, Fusion Time, and Fusion Solution Volume on Protoplast Fusion** The effects of fusion time, cell density, and fusion solution volume are shown in **TABLE:6**. Polymerization and polycondensation rates increased with longer fusion time. No significant difference in polymerization rate was observed between 30 and 40 min, but polycondensation rate increased significantly at 40 min, making 30 min the optimal fusion time. Both polymerization and polycondensation rates increased with cell density, with 2.0×10 cells · mL $^{1}$ selected as the optimal fusion density based on

high polymerization and low polycondensation rates. Polycondensation rate increased with fusion solution volume. Statistical analysis indicated that a volume ratio of 0.5× or 1.0× was reasonable, with 0.5× selected as the optimal volume following the principle of minimum effective concentration, achieving 57.19% polymerization and 9.2% polycondensation rates.

**TABLE:6** Effects of fusion time, cell density, and fusion solution volume on protoplast culture

| Fusion time (min) | Cell density (×10 cells · mL $^{1}$) | Volume ratio of fusion solution to cell suspension | Polymerization rate (%) | Polycondensation rate (%) |
|---|---|---|---|---|
| 10 | 2.0 | 0.5 | 47.33±2.81 b | 8.50±0.53 b |
| 20 | 2.0 | 0.5 | 50.23±2.22 ab | 9.16±0.70 b |
| 30 | 2.0 | 0.5 | 57.19±2.95 A | 9.20±0.76 B |
| 40 | 2.0 | 0.5 | 56.13±2.91 a | 11.84±1.05 a |
| 30 | 0.5 | 0.5 | 38.50±1.12 C | 6.31±0.33 C |
| 30 | 1.0 | 0.5 | 46.35±1.81 B | 7.51±0.37 BC |
| 30 | 2.0 | 0.5 | 59.25±0.57 A | 18.35±0.81 A |
| 30 | 3.0 | 0.5 | 40.73±2.53 B | 7.83±0.29 B |
| 30 | 2.0 | 0.2 | 56.44±2.73 A | 9.60±0.44 B |
| 30 | 2.0 | 1.0 | 51.05±3.75 A | 24.27±1.57 A |

Using the optimized conditions for protoplast preparation, culture, and fusion, well-developed single cells and dual-cell fused protoplasts were obtained (**PLATE:I**). Regenerated callus was observed after two months of sodium alginate-embedded cultivation of fusion products.

**PLATE:I** Protoplast morphology of *P. cablin* during fusion process

*Note: A. Bright field image of protoplast from* P. cablin* by FDA fluorescent staining; B. Dark field image of protoplast from *P. cablin* by FDA fluorescent staining; C. Protoplast morphology during fusion process; D. Morphology of fused protoplasts after washing; E. First division of fused protoplasts; F. Second division of fused protoplasts*

## Discussion

**3.1 Protoplast Culture** This study examined multiple culture methods for patchouli protoplasts, with sodium alginate embedding proving superior. This method belongs to solid culture, avoiding the disadvantages of liquid culture where protoplasts are not fixed and difficult to track. As a macromolecular compound, sodium alginate provides soft, uniform embedding that prevents heat shock damage to protoplasts during agarose embedding (Jia et al., 1988). Although liquid and double-layer cultures showed earlier division with higher separation frequency and plating efficiency, neither formed callus, likely due to severe toxic effects from accumulated metabolites as protoplast numbers increased and spacing decreased after division.

The optimal cell density for patchouli protoplast culture was 2.0×10 cells · mL $^{1}$, consistent with literature reports suggesting densities of 10 –10 cells · mL $^{1}$ (Yang, 2010). Protoplast growth is bidirectionally affected by cell density: excessively low density reduces endogenous substances that promote division (Chen et al., 2007), while excessively high density causes nutrient deficiency or accumulation of harmful metabolites that hinder normal regeneration.

Nitrogen source type and concentration significantly impact protoplast culture. Ammonium nitrogen is generally considered toxic and should be minimized. The widely used MS medium for tissue culture contains 1650 mg · L $^{1}$ NH NO , whereas protoplast culture media such as KM8P (600 mg · L $^{1}$ NH NO ) (Kao & Michayluk, 1975) and NT (825 mg · L $^{1}$ NH NO ) (Nagata & Takebe, 1971) contain reduced ammonium. This study included a no-ammonium control, which proved unfavorable for protoplast growth, indicating that ammonium nitrogen remains essential for patchouli protoplast culture, though its concentration requires species-specific adjustment. Optimal growth occurred at NH NO 825 mg · L $^{1}$, consistent with NT medium.

**3.2 Protoplast Fusion** This study employed the PEG-high pH-high calcium method for patchouli protoplast fusion and investigated the effects of cell density, fusion time, and fusion solution volume. The PEG-high pH-high calcium method (Kao & Michayluk, 1974) is widely applied in protoplast fusion. Kao proposed that PEG polymers with ether bonds possess negative polarity, forming hydrogen bonds with positively polarized groups in water, proteins, and sugars to create molecular bridges between adjacent protoplast surfaces. Plant protoplast surface charges range from -10 to -30 mV, and high pH with high calcium concentration can neutralize negative membrane charges (Li, 2005). During washing, high $Ca^{2}$ concentration removes negatively polarized PEG molecules from protoplast membranes, allowing charge redistribution and promoting fusion.

PEG concentration, cell density, fusion time, and fusion solution volume exerted similar effects on protoplast fusion. The study found that polymerization and polycondensation rates increased with elevated PEG concentration, fusion time,

cell density, and fusion solution volume. Initially weak effects resulted in incomplete fusion and low rates, while excessive enhancement led to formation of trimers, multimers, and dendritic structures with high polycondensation rates. Therefore, optimization of these factors is necessary.

This study employed physical selection for fusion product screening. When measuring mid-phase cell diameters, we referenced the method of Xiong et al. (2013) but observed no mid-phase cells, likely due to disrupted cell division synchronization from continuous light cultivation of suspension cells, making the brief mid-phase difficult to capture extensively. To determine single patchouli suspension cell diameter range, we combined NDC diameter measurement with formula verification to ensure accurate mid-phase cell diameter measurements.

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*Note: Figure translations are in progress. See original paper for figures.*