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BRIEF COMMUNICATION

# *In vitro* regeneration of *Perilla frutescens* from hypocotyl and cotyledon explants

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## Abstract

Organogenetic buds were induced from hypocotyl and cotyledon explants of oil crop *Perilla frutescens* in Murashige and Skoog (MS) medium supplemented with 5.7 μM indole-3-acetic acid (IAA) and 8.9 - 13.3 μM 6-benzylaminopurine (BA). Shoots were rooted on MS medium with 2.9 μM IAA and 1.4 μM gibberellic acid ($GA_3$) and the regenerated plants flowered and set seeds normally.

*Additional key words*: growth regulators, organogenesis.

*Perilla frutescens* (L) Britt, an important oilseed crop and traditional Chinese medicinal herb, is widely cultivated in China especially in arid areas for over two thousands years. This plant grows under various conditions because of its tolerance to drought, cold, water-logging, disease, pest and barren land. It was gradually recognized worldwide as a new economic plant with multiple uses (Liu *et al.* 1996). The perilla seeds contain about 45 % oil and most of which (up to 92 %) is composed of unsaturated fatty acids, especially α-linolenic acid (about 68 %) and linoleic acid (about 14 %). Perilla oil also contains 17 amino acids and does not include unhealthy components such as sinapic acid that is rich in rape oil. It is therefore a high quality edible oil and also has wide applications in industry (Yao and Yu 1999). In addition, perilla essential oil extracted from its leaves and stems has been used in medicine, food and industry (Liu *et al.* 1996). Meanwhile, as a traditional Chinese medicine, the whole plant of *P. frutescens* is used to treat cold, pain, fever and cough (Guo *et al.* 1987), and has the effects against bacteria, fungi and tumor (Liu *et al.* 2000). Although there are extensive investigations of plant regeneration in oil crops such as *Brassica* species and flax (Eapen and George 1997, Chen *et al.* 1998), to date there have been no reports on the *in vitro* regeneration of *Perilla frutescens*. In this communication, we described its regeneration through tissue culture from hypocotyl and cotyledon explants.

The basal medium consisted of MS (Murashige and Skoog 1962) medium supplemented with 500 mg $dm^{-3}$ casein hydrolysate, 3 % (m/v) sucrose and 0.8 % (m/v) agar. All cultures were carried out in 100-$cm^3$ glass Erlenmeyer flasks containing 35 $cm^3$ of medium. The pH of all media was adjusted to 5.6 before sterilization by autoclaving at 121 ºC for 20 min. The cultures were incubated at 25 ± 2 ºC with a 16-h photoperiod of 30 μmol $m^{-2}$ $s^{-1}$ under cool-white fluorescent light. Seeds of *Perilla frutescens* were obtained from Gansu Seed Company, (Lanzhou, China). All seeds were sterilized in 70 % (v/v) ethanol for 30 s, followed by 0.1 % (m/v) mercuric chloride solution for 10 min, and then rinsed six times with sterile distilled water. The seeds germinated aseptically on MS medium without growth regulators. Hypocotyl segments (5 mm long) dissected from 2-week-old seedlings (1.0-1.5 cm in length) were placed on medium for initiation of calli or adventitious buds. The entire cotyledons from the same seedlings were cut into three strips and then were placed on medium with abaxial surface in contact with the culture medium. The callus or adventitious bud induction medium was MS containing

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*Abbreviations*: BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; $GA_3$ - gibberellic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid.
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different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), α-naphthalene-acetic acid (NAA) or indole-3-butyric acid (IBA) in combination with various concentrations of 6-benzyl-aminopurine (BA). Twenty explants were used for each treatment with three replicates. The percentage of callus or adventitious bud induction was evaluated after 4 weeks of culture. Growth index of callus was calculated as (final callus mass - initial callus mass)/initial callus mass after the second 4 weeks of subculturing on the same medium as primary culture.

Calli were transferred onto MS medium containing 5.7 μM IAA and BA (4.4, 8.9, or 13.3 μM) in order to induce morphogenesis. Each treatment consisted of 20 pieces of calli with three replicates. After 4 weeks in culture, data on the frequency of produced adventitious buds were recorded. All of the shoot-buds were transferred on MS medium with 5.7 μM IAA, 2.2 μM BA and 1.4 μM gibberellic acid ($GA_3$) for enhancing growth for 3 weeks. The strong shoots of 1.5 - 3 cm were detached and cultured on MS with 2.9 μM IAA and 1.4 μM $GA_3$ for rooting. The percentage of rooting and root number per shoot were calculated after 10 d of culture. Rooted plantlets with four to seven leaves were washed with tap water to remove all traces of medium and transferred to plastic boxes (25 × 20 × 12 cm) containing autoclaved *Perlite* for acclimatization. Hardened plantlets were transplanted to pots with soil in a greenhouse after 2 weeks.

After 1 week in culture, calli were formed from the cut ends of hypocotyls or the excised edges of cotyledons and they subsequently spread to the whole explants on MS medium supplemented with 2,4-D and BA. However no callus was induced on the medium in absence of growth regulators. The freshly induced calli were yellow-white in colour, and then gradually turned dark-brown after 1 week on all the media tested due to release of apparent phenolic compounds and essential oils. Most of the developed calli were loose and watery, some of them were friable and had small granular structure on the surface. The highest frequency of explants inducing calli was 100 % after 4 weeks in culture, and there were no differences between hypocotyl-derived calli and cotyledon-derived ones except their growing rates. The growth index of cotyledon-derived calli was lower than that of hypocotyl-derived calli on the same culture medium composition. Although calli from different explants were induced on all the tested media, 4.5 μM 2,4-D and 4.4 μM BA gave the best response with 100 % of explants forming callus and 2.4 - 3.7 of growth index (Table 1).

When hypocotyls and cotyledons were cultured on the medium containing various concentrations of IAA, NAA or IBA with BA, direct adventitious buds and calli were induced simultaneously. Green spots appeared at the cut ends of hypocotyls or at the excised edges of cotyledons within 2 weeks of culture; they developed further into adventitious shoots after another 2 - 4 weeks of culture (Fig. 1*A*). Of the three auxins tested, IAA with suitable concentrations of BA was the most effective in the direct shoot-bud induction. The medium with 5.7 μM IAA and 8.9 μM BA supported the highest frequency (43.7 and 68.1 %) of hypocotyl- and cotyledon-producing directly adventitious buds. About 1 - 3 shoot-buds were developed in each explant. The percentage of hypocotyl-forming calli was significantly higher than that of cotyledon (Table 1). Texture and colour of the calli were similar to that induced on all the concentrations of 2,4-D and BA.

All of the calli induced through different ways

Table 1. Effect of different concentrations of auxins and BA on induction of calli and direct adventitious buds from hypocotyls and cotyledons of *Perilla frutescens*. Percentage of explants inducing calli and adventitious buds was evaluated after the first 4 weeks of culture. Callus growth index was calculated after the second 4 weeks of subculturing on the same medium as primary culture. Means ± SE of three independent experiments.

| Auxin | | BA | Hypocotyl callus induction | growth index | explants forming buds | Cotyledon callus induction | growth index | explants forming buds |
|---|---|---|---|---|---|---|---|---|
| | [μM] | [μM] | [%] | | [%] | [%] | | [%] |
| 2,4-D | 4.5 | 4.4 | 100.0 | 3.7 ± 0.4 | 0.0 | 100.0 | 2.4 ± 0.2 | 0.0 |
| | 4.5 | 8.9 | 100.0 | 2.9 ± 0.2 | 0.0 | 95.8 ± 2.6 | 1.8 ± 0.1 | 0.0 |
| | 9.0 | 4.4 | 100.0 | 2.6 ± 0.1 | 0.0 | 97.3 ± 3.4 | 1.9 ± 0.0 | 0.0 |
| IAA | 5.7 | 4.4 | 95.5 ± 3.7 | 1.2 ± 0.1 | 28.6 ± 3.0 | 62.3 ± 4.8 | 0.9 ± 0.0 | 51.7 ± 5.1 |
| | 5.7 | 8.9 | 93.2 ± 2.6 | 1.6 ± 0.0 | 43.7 ± 0.9 | 57.6 ± 4.3 | 1.0 ± 0.1 | 68.1 ± 7.6 |
| | 5.7 | 13.3 | 79.8 ± 1.9 | 0.8 ± 0.0 | 32.3 ± 1.4 | 45.4 ± 1.7 | 0.5 ± 0.0 | 50.3 ± 1.3 |
| | 11.4 | 8.9 | 88.5 ± 2.1 | 1.3 ± 0.2 | 35.4 ± 3.1 | 50.6 ± 2.4 | 1.1 ± 0.0 | 43.2 ± 2.7 |
| NAA | 5.4 | 8.9 | 85.2 ± 5.4 | 1.4 ± 0.0 | 21.6 ± 1.5 | 58.9 ± 1.6 | 0.9 ± 0.0 | 39.1 ± 0.4 |
| IBA | 4.9 | 8.9 | 90.5 ± 6.1 | 1.3 ± 0.1 | 15.4 ± 0.8 | 65.1 ± 3.5 | 1.0 ± 0.2 | 35.7 ± 1.1 |

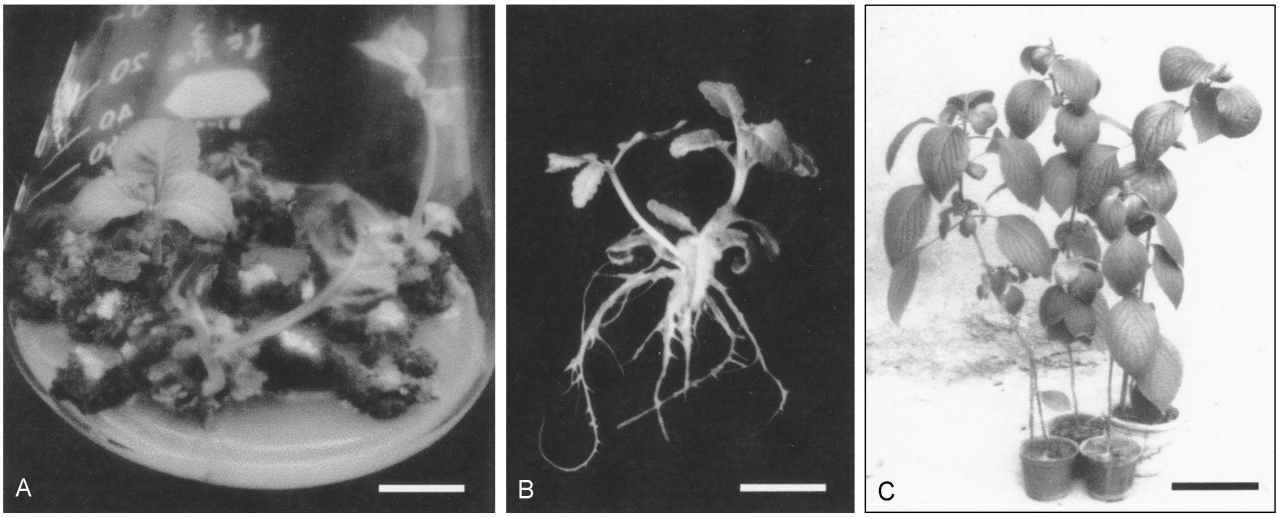


Fig. 1. Plantlet regeneration and development of *Perilla frutescens*. *A* - adventitious shoots regenerated from hypocotyls and calli induction on MS medium supplemented with 5.7 μM IAA and 8.9 μM BA after 6 weeks of culture (*bar* = 1.2 cm); *B* - rooted plant on MS medium with 2.9 μM IAA and 1.4 μM $GA_3$ after 10 d of culture (*bar* = 1.5 cm); *C* - established plantlets flowering in pots 3 months after transfer (*bar* = 20 cm).

multiplied rapidly on the optimal callus induction medium (4.5 μM 2,4-D and 4.4 μM BA) at 4 weeks intervals. After the proliferated calli were transferred on MS medium with 5.7 μM IAA and BA (4.4, 8.9, or 13.3 μM), they expressed organogenetic potential. Within 2 weeks in culture, numerous green spots emerged from the surface of friable yellow-white calli, and they continued to develop into shoot-buds after another 2 weeks. The highest shoot regeneration frequency (37.6 ± 3.8%) was obtained from the hypocotyl-derived calli on medium with 5.7 μM IAA and 8.9 μM BA. However, when 5.7 μM IAA and 4.4, or 13.3 μM BA were used, the frequency was only 10.4 ± 1.0 and 29.7 ± 1.4 %. Meanwhile, in comparison with the calli cultured in medium with 5.7 μM IAA and 4.4 or 8.9 μM BA (with frequencies of 21.7 ± 2.4 and 35.1 ± 1.6 %, respectively), the cotyledon-derived calli presented the highest percentage (45.8 ± 0.9 %) of shoot regeneration on medium with 5.7 μM IAA and 13.3 μM BA. No significant difference in plant regeneration was observed among the calli that were induced in different initial culture media.

The elongation of the shoot-buds was promoted when transferred on MS medium with 5.7 μM IAA, 2.2 μM BA and 1.4 μM $GA_3$. The shoots grew up to 1.5 - 3 cm height and had 5 - 7 leaves after 3 weeks of culture. On average, each explant produced 2 - 3 shoots. About 60 shoots were detached and cultured on MS medium supplemented with 2.9 μM IAA and 1.4 μM $GA_3$ for rooting. The roots were induced within 3 d at the cut ends of shoots. 100 % of the excised shoots developed roots after 10 d of culture (Fig. 1*B*). An average of 6.8 roots was formed per shoot and the mean root length was about 2.1 cm. Sixty plantlets with fully expanded leaflets and well-developed roots were acclimatized and transplanted to the pots with soil, and about 94 % of them survived. The regenerated plants flowered and set seeds three months after transfer to pots in greenhouse, and they were normal in morphology, growth features and floral characters (Fig. 1*C*).

To sum up, the present study reported for the first time a reproducible procedure for plant regeneration from hypocotyl and cotyledon explants of oil crop *Perilla frutescens* through organogenesis within 9 - 13 weeks. This protocol is useful for the exploitation and improvement of *P. frutescens* by means of biotechnological approaches.

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