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BRIEF COMMUNICATION

Plant regeneration *in vitro* directly from cotyledon and hypocotyl explants of *Perilla frutescens* and their morphological aspects

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Abstract

A rapid plantlet regeneration system for *Perilla frutescens* was established from cotyledon and hypocotyl explants. A maximum of 91.06 % cotyledon and 76.4 % hypocotyl explants could directly produce shoots (3.09 ± 0.18 shoots per explants) on Murashige and Skoog (MS) medium. The optimum hormone combinations were 4.44 μM 6-benzylaminopurine (BA) for cotyledon and 2.22 μM BA + 2.85 μM indole-3-acetic acid (IAA) for hypocotyls. Rooting was induced on half-strength hormone-free MS medium. After transplantation to soil, approximate 80 % of the regenerated plantlets could survive, flower and fruit. Moreover, some morphological abnormalities were found among the regenerated plants.

Additional key words: growth regulators, direct organogenesis, morphological abnormalities.

Perilla frutescens L. is an annual herb of *Lamiaceae* used in traditional medicine of China as well as modern medicine (Makino *et al*. 2002). Moreover, *Perilla frutescens* usually have two forms, pigmented red form, whose leaves and stems are deep red-purple, and non-pigmented green form, whose leaves and stems are green. Therefore, it was considered as a model plant to study molecular mechanism of anthocyanin biosynthesis (Saito and Yamazaki 2002). In present study, we focused on establishing a high frequency *in vitro* regeneration system of *Perilla frutescens*, aiming to get useful somatic mutants and to pave a way for further genetic manipulation of this plant. To our knowledge, such exploitation on *Perilla frutescens* has not been done so far.

The seeds, kindly provided by Gansu Academy of Agricultural Science, were surface-sterilized in 70 % ethanol for 30 s, then in 0.1 % mercuric chloride for 10 min, and rinsed 4 times (5 min each) in sterile distilled water. The sterilized seeds were placed on Murashige and Skoog (1962; MS) basal medium without any growth regulator in a 200 cm^3 conical flask to germinate at 25 ± 2 °C with 16-h photoperiod (irradiance of 65 μmol m^{-2} s^{-1}, cool white fluorescent light). About 3 - 5 mm long hypocotyls and 0.2 cm^2 cotyledon segments of 5-d-old seedlings were inoculated onto solidified MS medium supplemented with 3 % sucrose, 0.7 % agar and various combinations of BA, IAA, NAA and 2,4-D. Each combination contained 5 replicates and was repeated three times. The frequencies of shoot regeneration via cotyledon and hypocotyl explants and the number of shoots formed on each explants were counted after 40 d. Meanwhile, the regenerated shoots of 2 - 3 cm in length were excised and cultured in half-strength hormone-free MS medium for further elongating and rooting. All the media were adjusted to pH 5.8 using NaOH or HCl and autoclaved at 121 °C for 15 min and all the cultures were maintained at 25 ± 2 °C, 16-h photoperiod (65 μmol m^{-2} s^{-1}) in a chamber.

After 5 to 10 d of culture on MS media supplemented with different concentrations of BA and IAA, the cotyledon explants began to swell and produce granular structures on their margins and cut surfaces. Two weeks

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Abbreviations: BA - 6-benzylaminopurine; IAA - indole-3-acetic acid; NAA - α-naphthaleneacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; MS medium - Murashige and Skoog medium.
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later, the granular structures developed into dark green shoots (Fig. 1*A*). For the hypocotyl explants, the condition was similar to cotyledon, but the initiation of shoot differentiation was not observed until the 15^{th} - 20^{th} day of culture.

For cotyledon and hypocotyl explants, direct shoot regeneration could take place on MS media with BA and IAA, but they remarkably differed from each other in response to hormone and concentrations (Table 1). On media with 0.88 - 17.76 µM BA, 35.73 % to 91.06 % of cotyledon explants were able to regenerate shoots. The optimum concentration was 4.44 µM BA. But on media with 2.22 µM BA along with 2.85, 5.71, 11.42 and 22.84 µM IAA, the frequencies of shoot regeneration of cotyledon explants were reduced by 15.80, 25.76, 42.38, and 53.3 %, respectively, in comparison to 2.22 µM BA alone. For the explants from hypocotyls, the frequency of shoot regeneration was zero on medium with BA alone, but it increased up to 76.4 % on MS media with 2.22 µM BA plus 2.85 µM IAA. The results indicated that BA alone was effective for cotyledon explants but combinations between BA and IAA inhibited shoot regeneration contrarily. Whereas, for hypocotyl explants, shoot regeneration must depend on the synergism of BA and IAA, and BA or IAA alone was incapable for it. This difference may be contributed to the different endogenous BA/IAA content between cotyledon and hypocotyls. Both of BA and IAA possibly are necessary for the direct shoot regeneration from cotyledon and hypocotyls, but the cotyledon may contain higher content of IAA, so it do not need additional IAA during shoot regeneration. Moreover, the average number of regenerated shoots per explants varied between these two different types of explants and between different hormone concentrations (Table 1). For the explants from hypocotyls, it was up to 3.09 shoots per explants (at 2.22 µM BA plus 2.85 µM IAA), and for that from cotyledon 2.76 shoots per explants (at 4.44 µM BA) at maximum. Under the higher concentrations of BA (> 4.44 µM for cotyledon) or IAA (> 2.85 µM for hypocotyls), the frequency of shoot regeneration and the number of shoots per explants significantly decreased, simultaneously accompanied by formation of little gray callus. It was consistent with the results of Syamkumar *et al*. (2003/4) who found that high concentrations of BA did not enhance shoot induction. Furthermore, the shoot differentiation from cotyledon explants mostly initiated at its proximal ends, being consistent with the reports of Singh *et al*. (2002) that shoot differentiation *via* cotyledon depends upon the removal of embryonic axis and the presence of the proximal region of cotyledon.

2,4-D and NAA alone or in combination with BA had no efficiency for the shoot regeneration from cotyledon or hypocotyls both, but could stimulate callus formation (data not shown). The callus usually appeared pale yellow and become brown soon with prolonged culture, suggesting that 2, 4-D and NAA prompted the explants callusing and inhibited shoot organogenesis in this plant species.

After 40 d, the regenerated shoots (2 - 5 cm) excised and transferred onto half-strength MS media without growth regulators rooted easily. 10 d later, the resultant plantlets with well-developed roots (Fig. 1*B*) were pulled up from media, washed with tap water and then transplanted to pots filled with autoclaved *Vermi-compost*. The pots were covered with polyethylene film and kept in the growth chamber. Two days later, the film was removed and the plantlets were irrigated with half-strength MS solution. After two weeks, the alive were transplanted to larger pots containing garden soil to allow a further growth for 10 d, and then moved to outdoors, where approximate 80 % of them survived and eventually flowered and seeded (Fig. 1*F*).

A primary morphological evaluation was conducted between the regenerated plantlets and the seedlings from seeds (Table 2). It was found that most of the regenerated plantlets flowered when they had only 3 - 6 pairs of leaves,

Table 1. Effects of BA and IAA on shoot induction from cotyledon and hypocotyls explants of *Perilla frutescens* on MS medium. Means ± SE of three repeated experiments with about 60 explants used in each treatment. Means followed by the same letter are not significantly different at 5 % level according to Duncan's Multiple Range Test. Data were scored after 40 d culture.

BA [µM]	IAA [µM]	Explants forming shoots [%]		Number of shoots [explants^{-1}]	
		cotyledon	hypocotyls	cotyledon	hypocotyls
0	0	14.26 ± 2.54f	0	0.17 ± 0.02e	0
0.88	0	64.33 ± 5.47c	0	0.67 ± 0.06d	0
2.22	0	81.00 ± 3.40b	0	2.21 ± 0.14b	0
4.44	0	91.06 ± 2.56a	0	2.76 ± 0.09a	0
8.88	0	83.93 ± 2.36ab	0	2.31 ± 0.21b	0
17.76	0	35.73 ± 5.81e	0	0.46 ± 0.06de	0
2.22	2.85	68.20 ± 2.90c	76.40 ± 3.64a	1.36 ± 0.12c	3.09 ± 0.18a
2.22	5.71	60.13 ± 2.41c	69.80 ± 5.13a	1.21 ± 0.12c	2.46 ± 0.14b
2.22	11.42	46.66 ± 1.87de	53.61 ± 6.15b	0.52 ± 0.02d	1.24 ± 0.12c
2.22	22.84	37.86 ± 1.24e	39.60 ± 3.10c	0.42 ± 0.05de	0.60 ± 0.04d

Table 2. Primary characteristics of plants originated from explants of cotyledon and hypocotyl and from seeds of *Perilla frutescens.* Means ± SE of 30 plantlets. Means followed by the same letter are not significantly different at 5 % level according to Duncan's Multiple Range Test.

Plant origin	Plant height [cm]	Number of flowers [plant^{-1}]	Number of pods [plant^{-1}]	Number of seeds [plant^{-1}]
From explants	5.83 ± 1.37a	17.47 ± 1.83a	7.06 ± 1.61a	1.61 ± 0.67a
From seeds	10.87 ± 1.48b	29.86 ± 2.82b	14.53 ± 2.02b	1.58 ± 0.48a

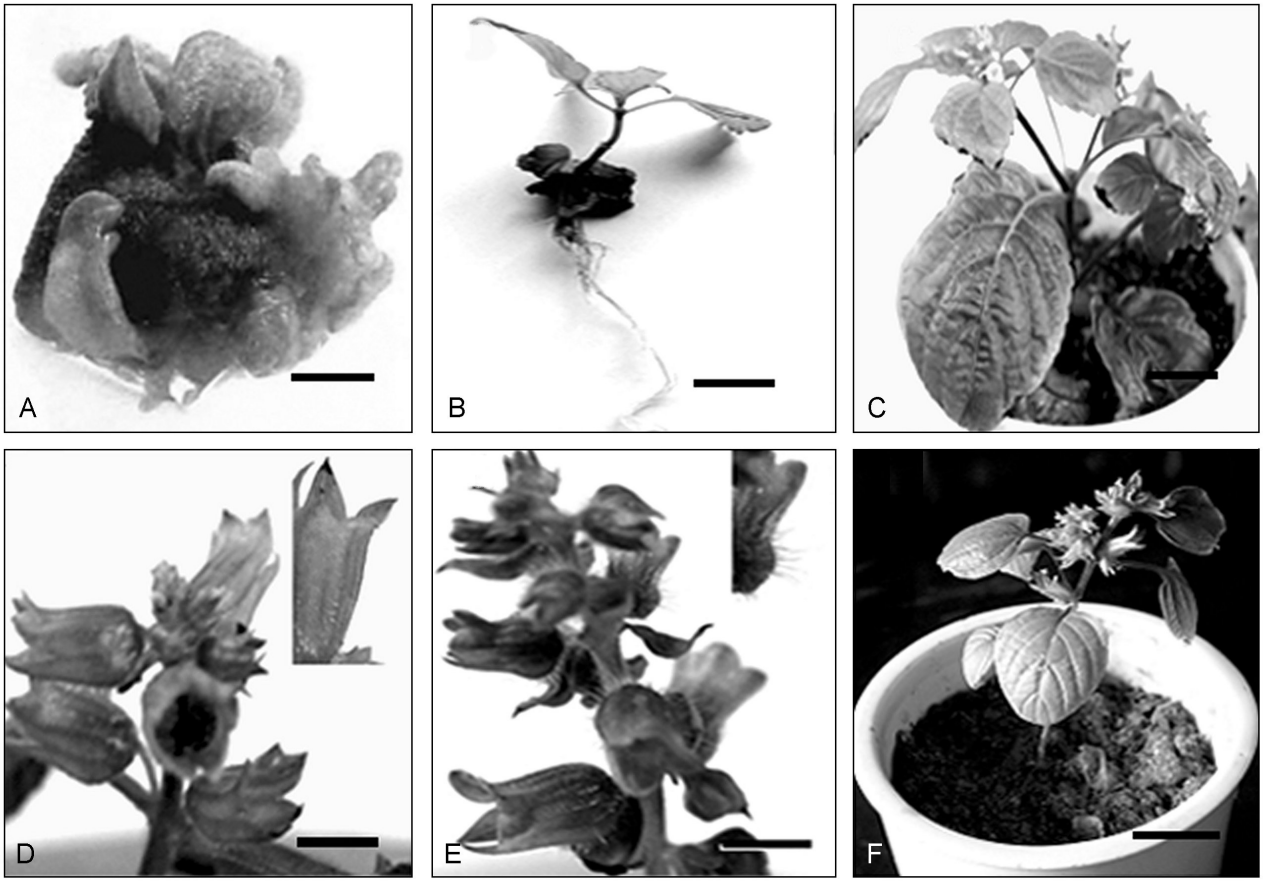

Fig. 1. Direct organogenesis of *Perilla frutescens: A* - the cotyledon explant cultured for 14 d on MS medium containing 4.44 μM BA, which still are dark green, but has produced many adventurous shoots along its edge (*bar* = 0.2 cm); *B* - a regenerated plantlet from cotyledon with well developed roots after 45 d culture (*bar* = 1.0 cm); *C* - a plantlet regenerated from hypocotyls with initial stem showing inhibited apical growth and new stems growing up from its axillary bud (*bar* = 1.5 cm); *D* - a plantlet regenerated from cotyledon that has fruited, whose calyxes have little surface hairs (*bar* = 0.4 cm); *E* - a regenerated plantlet with fruits, whose calyxes surface are covered by very thick and long hairs (*bar* = 0.4 cm); *F* - an adult plantlet from cotyledon whose flowers have withered and seeded (*bar* = 2.5 cm).

suggesting a shortened vegetative growth period and advanced flowering time. Furthermore, they were apparently dwarf (mean height being only 5.83 cm) and some of them showed very dark green leaves with many deep wrinkles. The flower numbers varied from 4 to 28 between different individuals and only 40.4 % flowers were able to fertilize and bear fruits, the rest gradually degenerated. Moreover, we found two morphological abnormalities, one with initial stem showing inhibited apical growth and new branches growing up from axillary bud (Fig. 1*C*), and another having calyxes without epidermal hairs (Fig. 1*D*). The regenerated plantlets and wild types of *Perilla frutescens* generally having very dense and long hairs on intra- and extra-epidermis of their calyxes (Fig. 1*E*). However, the possibility that the abnormalities were induced by environmental factors,

such as nutrient, hormones and illumination period cannot be excluded. Our further efforts will be concentrated on the investigation of their offspring. The plantlets regenerated from cotyledon and hypocotyls appeared no significant difference in morphology.

In conclusion, a system for direct plantlet regeneration of *Perilla frutescens* was established in the present research. It takes less than 45 d from initial explants inoculation to the formation of well-developed plantlets and most of the regenerated plantlets could survive, flower and bear seeds when transferred to soil. All the parameters imply that this protocol would be a way for further genetic transformation and *in vitro* molecular biology study of *Perilla frutescens*. Also this is the first report on plant regeneration directly from cotyledon and hypocotyls of *Perilla frutescens* via organogenesis.

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