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ORIGINAL ARTICLE

The apical bud as a novel explant for high-frequency in vitro plantlet regeneration of *Perilla frutescens* L. Britton

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Abstract In this study, we established an in vitro regeneration system to maximize the recovery of leafy perilla (*Perilla frutescens* L. Britton) plantlets as part of developing a molecular biotechnology-based metabolic engineering program for this crop plant. Hypocotyl segments including the apical buds were used as explants for the direct production of shoots without an interim callus phase. The number of shoots produced from the apical buds peaked within 3–4 weeks, and the shoots were subsequently cultured on Murashige and Skoog (MS) media supplemented with 2 mg l^{-1} benzylaminopurine (BA). Spontaneous rhizogenesis was observed after 7–10 days of culture on MS media without hormonal additives. The rooted shoots developed into normal plants in soil after hardening on distilled water for 3–4 days. The average plantlet regeneration frequency was higher for the apical buds (64.33%) than for the top (15.66%), middle (4%), and basal (1.33%) segments of the hypocotyls. This regeneration system demonstrates a capacity for high-frequency plantlet recovery and thus should be considered for use in the genetic manipulation of leafy perilla.

Keywords Apical bud · *Perilla frutescens* L. Britton · Regeneration · Somatic organogenesis

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Abbreviations

BA	6-Benzylaminopurine
IAA	Indole acetic acid
MS	Murashige and Skoog

Introduction

Perilla (*Perilla frutescens* L. Britton), an oil-yielding and leafy vegetable crop that has been cultivated in Southeast and East Asian countries since time immemorial, is a self-fertilizing short-day annual crop plant in the family Lamiaceae. The crop is characterized by two major species of the genus *Perilla* that can be distinguished based on their morphology and uses: *P. frutescens* L. var. *frutescens* is used as an oil crop and *P. frutescens* L. var. *crispa* is used as either a medicine or a vegetable (Lee et al. 2002). Both are also considered essential parts of Southeast and East Asian cuisine (Li 1969; Choi et al. 1980; Kurita and Koike 1981; Koezuka et al. 1985). Nevertheless, to date, only *P. frutescens* L. var. *frutescens* has been extensively cultivated (Nitta 2001) for use as a medicine, oil-yielding crop, and leafy vegetable in Korea (Honda et al. 1990; Nitta 2001).

Beginning in the 1990s, researchers began trying to produce a genetically improved version of perilla, and consequently several reports on plant regeneration using organs such as anthers (Lee et al. 1994), leaves and cotyledons (Kim et al. 1993), hypocotyls (Kim et al. 2004), and indirect organogenesis via a callus phase from cotyledons, leaves, and hypocotyls as explants (Lee et al. 2003) are available. Notably, in all cases, scientists have relied on the perilla cultivars Yeopsil, Daesil, and Dasil, which are

considered to be good sources of oil in Korea, but yet another important commercial cultivar (cv. Manbaeg) is used as a leafy vegetable and as a wrap in the preparation of almost every Korean meal. Few reports, however, have described the regeneration protocols for genetically manipulating this leafy species of *Perilla* via plant biotechnical approaches.

Recently, a shoot induction frequency of 27.3% was achieved using cotyledons as explants (Kim and Lee 2007). A similar in vitro system with an improved regeneration frequency is essential in leafy perilla to further augment its qualities through a metabolic engineering program. Therefore, here we report the development of a reproducible, reliable, and simple in vitro regeneration system that will pave the way for further genetic manipulation of leafy perilla. The regeneration protocol described in this study can be used to facilitate the *Agrobacterium*-mediated genetic transformation of leafy perilla.

Materials and methods

Seed disinfection

Leafy perilla seeds (cv. Manbaeg) were obtained from the Rural Development Administration's National Crop Experimental Station (Suwon, Korea); the seeds were free of concomitant contaminants with special reference to the varietal admixture. The seeds were disinfected using a two-step procedure. First, about 2 g of seed were surface-sterilized with 70% ethanol for 1 min in a 50 ml Falcon tube and then washed several times with distilled water. Second, the seeds were treated with different concentrations of mercuric chloride ($HgCl_2$) or commercial bleach (Yuhan Clorox, 4% sodium hypochlorite), as shown in Table 1. Immediately following the second treatment, the seeds were washed three to four times using sterile water in a laminar airflow cabinet. The seeds were then rinsed three to four times every hour and immersed in sterile water overnight at room temperature.

In vitro germination of the seeds

The sterilized seeds were blot-dried on filter paper then germinated aseptically on basal 0.5× MS (Murashige and Skoog 1962) media containing 1.5% (w/v) sucrose without plant hormones. The pH of the medium was adjusted by adding 0.1 N NaOH or 1.0 N HCl to 5.7 ± 0.1 prior to the addition of plant agar (Duchefa Biochemie, Haarlem, The Netherlands) at 0.4% (w/v). The medium was autoclaved in an HB-506-6 vertical autoclave (Hanback Scientific Co., Daejeon, Korea) for 20 min at 1.5 kg cm^{-2} and 121°C. To obtain seedlings with sufficiently enlarged hypocotyls, 22–25 seeds were plated on plant culture dishes containing the above medium. The plates were incubated at 25 ± 2°C under a 16-/8-h (day/night) photoperiod provided by 40 W cool-white fluorescent lamps.

Explant source

We developed a direct somatic organogenic method to facilitate embryonic induction directly from hypocotyls without going through an intervening callus phase. Most attempts at regenerating perilla have utilized leaf segments and cotyledons (Kim et al. 1993) or anthers (Lee et al. 1994). However, the relative efficacy of using hypocotyl segments in the regeneration of perilla plantlets has not yet been reported. Thus, we selected hypocotyls, which have been used as explants for other *Perilla* species, as our explant source. We divided individual 8- to 10-day-old hypocotyls into pieces (excluding the apical bud), each of which was 5–6 mm long. The pieces, which were designated top, middle, or bottom (Fig. 1), were then analyzed for their relative regeneration potential under various hormone concentrations. The segments were prepared using a sharp surgical blade and forceps over a sterile paper tray. Special care was taken to avoid damaging the cells at either end during the dissection process. The regeneration efficiency was also checked using explants produced from seedlings of different ages (10, 12, and 14 days old).

Table 1 Comparative efficacies of seed sterilization methods

	Sterilization method			Germination percentage (%)	Seedling development (%)	Number of contaminated seeds
	EtOH	$HgCl_2$	Bleach			
A	70%, 1 min	0.3%, 15 min	100%, 20 min	25 ± 1.45	4 ± 1.57	0
B	70%, 1 min	–	100%, 20 min	95 ± 1.66	20 ± 1.75	0
C	70%, 1 min	–	100%, 15 min	95 ± 1.53	23 ± 1.89	5–6
D	70%, 1 min	–	50%, 15 min	96 ± 1.76	23 ± 2.58	8–10
E	70%, 1 min	0.15%, 15 min	–	51 ± 1.86	8 ± 1.88	1–2

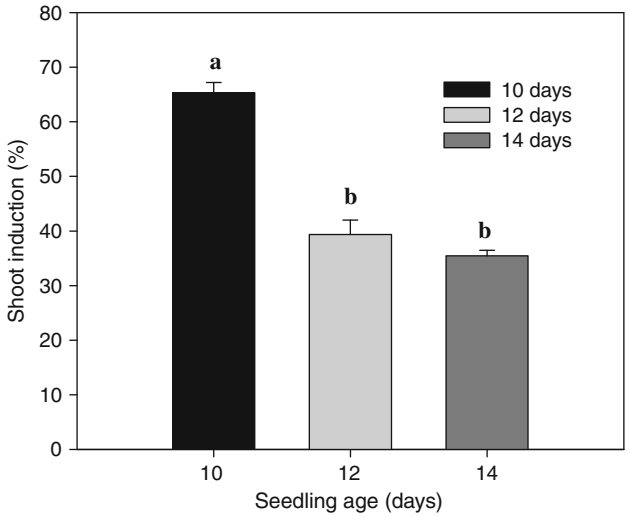

Fig. 1 Effect of age on shoot induction efficiency (%) in hypocotyl explants of leafy perilla. The data represented in the graph are the averages of three separate experiments

Shoot production

The organogenic capacity of the hypocotyl sections was explored by incubating them on solidified MS basal medium supplemented with various concentrations of 6-benzyl-aminopurine (BA) at pH 5.7 ± 0.1. The temperature and light conditions were the same as those described for the in vitro germination of the seeds. Initially, we tested 15 different treatment combinations derived from five concentrations of BA (0, 2.0, 4.0, 6.0, and 8.0 mg l^{-1}) and the three explant sources (bottom, middle, or top of the hypocotyl). Each treatment regimen was applied to 30 randomly selected hypocotyl sections with three replications to produce a completely randomized design. On the basis of this trial, we added the apical buds and compared their relative performances in the regeneration of perilla. The explants were subcultured every two weeks for two months on the refreshed media.

Shoot elongation and root induction

The growing shoots were placed on fresh hormone-free MS basal medium solidified with 0.8% plant agar to allow shoot elongation and root development. Those plantlets with well-developed roots were uprooted from the medium and washed with water. The rooted plants were then acclimatized both in water for three days and in small plastic pots filled with autoclaved soil (1:1 compost and vermiculite) for two weeks. The plants were then transplanted to larger pots filled with soil and transferred to a glasshouse.

Statistical analysis

The average value and standard deviation for each treatment as well as the mean separation by Duncan's multiple range test at the 5% probability level were determined using SPSS (release 12.0.1; SPSS Inc., Chicago, IL, USA).

Results

Seed sterilization and seedling germination

To maximize the number of developing explants in vitro, we treated perilla seeds with various sterilizing agents under several different conditions. The disinfectants (0.3% $HgCl_2$ and 100% commercial liquid bleach) removed all contaminants when applied for 15 and 20 min in a sequential manner, respectively. Using this method, of the seeds that germinated (25%), approximately 4% developed into seedlings (Table 1). In contrast, those seeds treated with commercial bleach alone for the times indicated above showed no evidence of contamination; moreover, the percent germination increased to 95% with a concomitant increase in the number of seedlings (20%).

The duration of treatment also had an impact on the removal efficiency of the microbial contaminants from the seeds. Those seeds that were immersed in 100% (v/v) commercial bleach and incubated for 15 and 20 min showed no evidence of contamination during incubation, whereas those seeds treated for only 15 min showed contamination, even though a greater number of seedlings were produced (3%) (Table 1). The germination of the seedlings was unaffected in both of the situations described. Although a similar frequency of contamination was detected, no significant variation was observed in germination (%) or in the number of seedlings raised in the seeds treated with 100 and 50% (v/v) commercial bleach for 15 min. Similarly, the use of 0.15% (v/v) $HgCl_2$ alone resulted in almost no contamination; however, germination as well as the number of seedlings were twice those produced using 0.3% $HgCl_2$ and 100% commercial bleach (v/v), respectively (Table 1). Due to the reduced efficiency of seedling production compared to the other methods, treatment with $HgCl_2$ was disregarded as a means of disinfection in this study. Overall, treatment with 100% commercial bleach (v/v) for 20 min was considered to be the best in terms of the number of seedlings produced by sterile culture in vitro.

Seedling age and its effect on shoot induction

On the basis of the differential growth rates (data not shown) of the seedlings that were generated through the

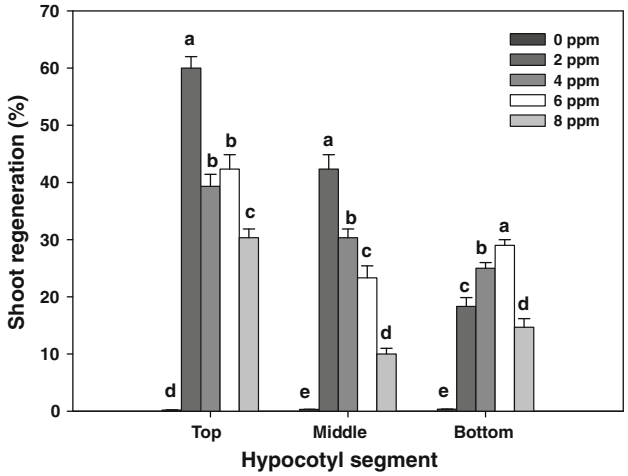

Fig. 2 Effects of hypocotyl segment (*top*, *middle*, and *bottom*) and the level of BA on shoot regeneration in leafy perilla. The *vertical bars* indicate the standard deviations; 40 explants were used in this experiment

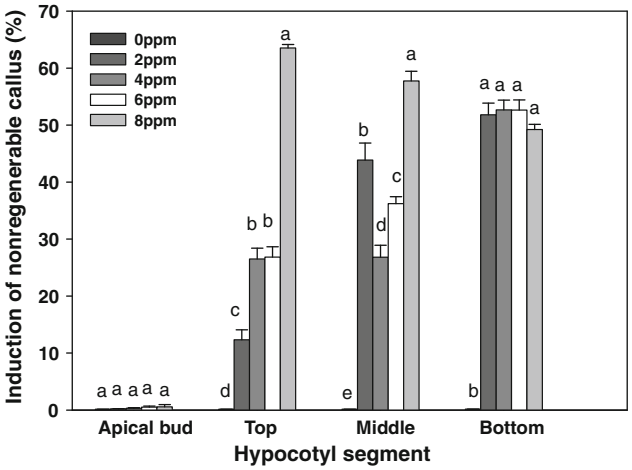

Fig. 3 Effect of BA on the induction of nonregenerable calli from segmented hypocotyl explants in leafy perilla. The *vertical bars* indicate the standard deviations; 40 explants were used in this experiment

various disinfection methods, we sought to optimize the age of the seedlings used as explants for shoot induction. From the time of planting of the sterile seeds on the germination medium, 9–10 days were required to produce explants for shoot induction. In some cases, the hypocotyls of 15- to 16-day-old seedlings were found to be suitable for use as explants. A comparison of 10-, 12-, and 14-day-old seedlings revealed that the 10-day-old seedlings had a higher frequency of shoot induction (65.24%) compared to the 12- (39.15%) and 14-day-old (34.67%) seedlings (Fig. 1); thus, 10-day-old seedlings were selected as the best source of hypocotyls as explants for shoot induction in perilla (Fig. 1). Our results also revealed a gradual decrease in shoot induction frequency (%) with increasing seedling age.

Shoot production

In our preliminary experiment, the regenerative capacities of the various hypocotyl segments (top, middle, and bottom) and their responses to BA varied widely (Fig. 2). The top segment appeared to be the best source of explants for accelerated and spontaneous shoot regeneration compared to the middle and basal segments when placed on MS medium supplemented with 2 mg l^{-1} BA. Although 2 mg l^{-1} BA was found to be the best among the hormone levels tested for the top and middle segments, shoot regeneration from the bottom portion of the hypocotyls was highest at 6 mg l^{-1} BA (Fig. 2). In addition, the induction of nonregenerable calli was observed. The level of BA had a dramatic effect on the induction of this type of callus, which was found to increase in accordance with the BA concentration in all of the hypocotyl segments studied (Fig. 3).

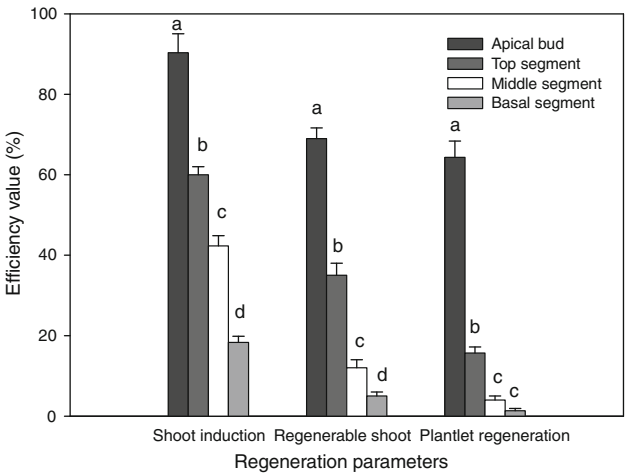

Fig. 4 The gradient response from the base to the apical bud of the hypocotyl explants as measured by various regeneration parameters in leafy perilla. The *vertical bars* indicate the standard deviations; 40 explants were used in this experiment

On the basis of these findings, we investigated the organogenic response of the apical buds to explore the feasibility of using them as an alternative source of explants for the in vitro regeneration of perilla. Thus, in addition to the hypocotyl segments described, apical buds were plated onto regeneration medium supplemented with 2 mg l^{-1} BA (the optimal level in our previous experiment). Among the explants tested, the frequency of shoot regeneration, the total number of shoots induced, and the number of regenerated plantlets were highest for the apical buds (Fig. 4), followed by the

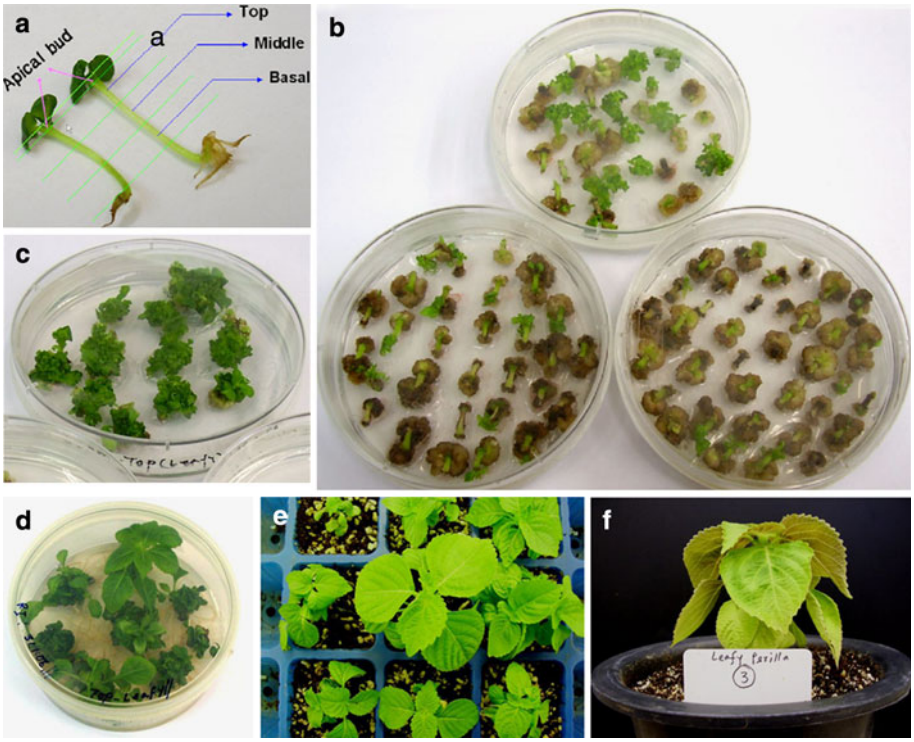

Fig. 5 Regeneration of leafy perilla plants using hypocotyls as explants. **a** Hypocotyl segments from 9- and 10-day-old seedlings. **b** Explants on growth media; the *top*, *bottom-left*, and *bottom-right* plates contain the top, middle, and basal hypocotyl segments, respectively. **c** The explants from the top hypocotyl segments in **b** after removal of the calli. **d** Induced shoots on rooting media. **e** Regenerated plants in small pots. **f** Perilla plant growing in a glasshouse

top, middle, and basal hypocotyl segments. The average frequency of plantlet regeneration from the apical buds was higher (64.33%) than those from the top (15.66%), middle (4%), and basal (1.33%) hypocotyl segments, respectively. Thus, apical buds appear to be the best explants for the regeneration of leafy perilla in vitro (Fig. 4).

Root induction and the establishment of acclimatized plants

The induced shootlets exhibited spontaneous rhizogenesis on full-strength MS media without hormonal additives (Fig. 5d). Usually, roots appeared within 7–10 days of culture; however, some rapidly growing shoots that failed to produce roots within that time period were successfully rooted by 1–2 weeks of culture in distilled water in a 100 ml flask. In all cases, only those regenerated shootlets with well-developed roots survived the transfer to the glasshouse (Fig. 5e). The survival rate of the plantlets in soil (data not shown) was improved by hardening in distilled water for 3–4 days followed by transplantation into small plastic pots filled with sterile soil (1:1 vermiculite and compost). All of the acclimatized plants resembled leafy perilla in terms of their morphology (Fig. 5f); moreover, all of the plants grew to maturity with normal flowering and produced well-developed seeds.

Discussion

Given the potential for producing quality oils and leafy vegetables (Park et al. 2000; Nitta 2001) as well as important pharmacological agents (Makino et al. 2002; Takeda et al. 2002; Sawabe et al. 2006), a great deal of interest is focused on developing a genetic engineering program for perilla; however, in vitro regeneration is an inevitable part of this process. In this regard, proper disinfection is considered to be an essential part of producing a sterile culture. Note that even in the field, perilla seed germination is a major problem due to limited seed viability and vigor following storage (Choi et al. 1980). As such, the low percent germination of seedlings in vitro may have occurred because the initial germination of the seeds (data not shown) was inhibited by the concentration of the sterilants used (A and E in Table 1); moreover, in some cases, the germinated seeds failed to grow into usable seedlings with long hypocotyls due to poor seed vigor (B–D in Table 1). A few of the treated seeds (five to six) were not completely free of contaminants; thus, maintaining a sterile culture environment was difficult. During the plating of the seeds after sterilization, the seeds bearing contaminants were distributed randomly in terms of their number and location on the plates. Overall, five to six spots of contamination were detected on one to two culture dishes, which were subsequently discarded. Nevertheless, each of the methods used in this study should be considered useful

for the production of perilla seedlings in vitro except for the methods involving $HgCl_2$ as a sterilant.

A prerequisite for the successful application of plant biotechnology to agriculture is to have a suitable in vitro protocol for regenerating whole plants via organogenesis or somatic embryogenesis (Rey and Mroginski 2006). Somatic embryogenesis, whereby somatic embryos are induced through callus cultures, has been shown to result in a lower probability of somaclonal variation, which gives it an advantage over other propagation methods (Vasil 1987; Ahloowalia 1991). However, a recent report revealed that a significantly higher percentage of abnormal shoots was regenerated through somatic embryogenesis compared to organogenic development in perilla (Kim and Lee 2007). Moreover, a risk exists of morphological abnormalities and sterility in plants regenerated from calli in vitro (Hansena and Wright 1999). Thus, our regenerated plants, which were developed through direct organogenesis from hypocotyls, were identical in terms of morphology and fertility because no callus phase occurred in our experiment. Therefore, a protocol for the regeneration of whole leafy perilla plants from hypocotyl explants without callogenesis was developed. In all cases in our study, shoot organogenesis occurred in the presence of the cytokinin BA (Torres 1989). In fact, supplementation of the media with 2 mg l^{-1} BA significantly increased the number of shoots produced.

That the physiological activity induced by BA during shoot organogenesis was reliant on such a low concentration of the cytokinin is somewhat consistent with the observations of Lee et al. (2003) and Kim et al. (2004). The responses of the explants derived from various hypocotyl segments to media containing 2 mg l^{-1} BA were variable. For example, the highest number of shoots was produced from explants derived from apical buds. The use of hypocotyl segments as explants is preferable for the regular transformation of perilla as an oil-yielding crop (Kim et al. 2004); however, the apical buds of hypocotyls have not been considered as a starting material for in vitro regeneration in either oil-type or leaf-type perilla. The use of the apical part of the hypocotyl as an explant has been reported in various micropropagation systems in other genera, including elm (*Ulmus glabra*) (Mala et al. 2005), sugarcane (Vazquez Molina et al. 2005), wheat (Haliloglu 2006), and *Alstroemeria* (Lin et al. 1998). In each of these cases, efficient in vitro propagation using explants isolated from the apical segment of the source organ was reported except for wheat, in which the basal segment offered the highest level of regeneration efficiency.

The auxin:cytokinin ratio influences the organogenic response during micropropagation in vitro. Specifically, when the auxin dominates or the levels of auxin and cytokinin are the same, shoot organogenesis results because auxin inhibits shoot induction in plants via apical dominance (Dimech et al. 2007). Moreover, Mala et al. (2005) determined the amount of endogenous free auxin (IAA), and found that it was approximately three times higher in the basal parts than in the apical parts of elm (*U. glabra*) shoots. In addition, significantly higher amounts of free bases, ribosides, and ribotides of isopentenyl adenine, zeatin, and dihydrozeatin were found in the apical segments of that plant. Thus, the response may be an indication of the dominance of cytokinin, as detected by Mala et al. (2005), which would favor shoot organogenesis in the apical bud rather than in the other segments of the hypocotyl in perilla. Additional experiments are needed to clarify the propensity for shoot organogenesis among the lower hypocotyl segments (top, middle, and basal) and determine whether it can be improved using a combination of plant growth regulators in sterile culture media. In addition to the gradient response of the hypocotyl segments, the age of the hypocotyls influenced the regenerative ability of perilla. The age of the cultured explants is believed to be a crucial regeneration factor in *Geranium* and *Brassica* species (Chang et al. 1996; Choi et al. 1996). However, this could be a kind of physiological age-related response. The frequency and number of shoots were found to decrease with age in explants of mungbean (*Vigna radiata* L. Wilczek) (Sonia et al. 2007).

Root induction from regenerated shoots appears to be a crucial factor in micropropagation in vitro. Endogenous auxin is widely accepted to play a role in initiating adventitious roots in plants (Gaspar and Coumans 1987). Similarly, cytokinins at high concentrations are known to inhibit rooting; however, a particular level of them is probably essential for the induction of cell division during the initial step of root formation (Deklerk et al. 2001). In our experimental system, the absence of auxin/cytokinin was not a limiting factor for rhizogenesis, as the shoots were rooted without the presence of additional hormones in the media. Evidence indicates that the inclusion of auxin and cytokinin in the rooting medium for perilla during somatic embryogenesis is unnecessary (Lee et al. 2003; Hou and Jia 2005). Similarly, studies have reported on perilla rooting in the absence of external hormones (Kim et al. 2004; Zhang et al. 2005). In the latter case, perilla was regenerated from cultured tissues via organogenesis, which is consistent with our results in leafy perilla. Although root induction in perilla is not dependent on the presence of growth regulatory additives in the media, the timing of root formation may be delayed in some explants. In such cases, a low level of auxin and lignin precursors may be the cause of delayed rooting, as described by Mala et al. (2005). Therefore, we hypothesize that successful rooting in propagating shoots is favored by the availability of IAA and lignin precursors, which appeared to be

rate-limiting for rooting in some of our explants on the MS + 0 medium. The in vivo rooting of elongated shoots on water, which was suspended in vitro, suggests that perilla shoots can be rooted hydroponically.

In conclusion, we successfully developed a reliable method of regeneration for leafy perilla in vitro. Our protocol for shoot organogenesis, which produces plantlets at a high frequency, opens up the possibility of developing transgenic leafy perilla, which may lead to new cultivars with novel traits.

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