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In Vitro Adventitious Shoot Regeneration of Native Spearmint Using Internodal Explants

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Abstract. **An in vitro shoot regeneration procedure was developed for native spearmint (*Mentha spicata* L.) using internodal explants. Shoot regeneration from internodes was evaluated on Murashige and Skoog (MS) media supplemented with individual cytokinins thidiazuron (TDZ), benzylaminopurine (BA), kinetin (KT), or zeatin (ZT) or various pair wise combinations of these. The highest regeneration was achieved by the second internode on a medium containing MS basal salts, B5 vitamins, 10% coconut water, 1.0 mg·L^{-1}TDZ, 2.5 mg·L^{-1} ZT, and solidified with 0.2% phytagel. Unlike previous protocols this medium does not need sub culturing and produces elongated shoots in 4 weeks, rather than 6 weeks. Maximum number of shoots (36 per explant after 4 weeks) was observed when internodes from 2-week-old stock plants were used as explant source. The shoots were removed and roots were initiated on medium containing MS basal salts, 0.4 mg·L^{-1} thiamine-HCL, 100 mg·L^{-1} myo-inositol, 7.5 g·L^{-1} agar and 0.01 mg·L^{-1} ∝-napthaleneacetic acid (NAA) and then plants were transferred to the greenhouse 2 weeks after root initiation, where 100% of the plantlets developed into healthy plants.**

The genus *Mentha* (Lamiaceae) is grown throughout the world and contains numerous economically important essential-oil producing plants, with *Mentha* ×*piperita* L. (peppermint) and *Mentha spicata* L. (native spearmint) being the most important species. The essential oils are synthesized and produced in the glandular trichomes on leaves and stems. Oil from spearmint contains mostly carvone (Croteau et al., 1994a), which is responsible for the characteristic spearmint flavor and is widely used in dental care and food products, including candies and gums. By comparison, the oil from peppermint is high in menthol, which is responsible for the characteristic peppermint flavor. Peppermint production is limited by a soil fungus that causes verticillium wilt (*Verticillium dahliae* Kleb.), which can persist in the soil for up to fifteen years. In contrast, native spearmint is highly resistant to verticillium wilt and can be grown in fields infected with the verticillium wilt fungus. Spearmints do not express the limonene-3-hydroxylase enzyme, which is required for menthol synthesis from geranylpyrophosphate (Croteau, 1991; Croteau et al., 1994b). However scientists have speculated that the activity of limonene-3-hydroxylase might be increased in spearmint using genetic approaches to make it produce oil with high menthol content (Goddijin and Pren, 1995; Lange and Croteau, 1999). Potentially then, a high menthol oil could be produced by native spearmint, even in soils infected with verticillium. Genetic improvement of spearmint to modify oil quality through conventional breeding methods however is constrained by near complete sterility of the species (Constabel, 1990; Croteau et al., 1991; Larkin, 1981). To overcome these constraints, biotechnological approaches may provide a means to insert the genes required for converting limonene to menthol. However, the lack of an efficient regeneration system has limited our ability to genetically modify native spearmint using gene transfer or mutagenesis and there is a need for a highly efficient in vitro regeneration protocol for native spearmint if genetic improvements using these new technologies are to be applied. Regeneration of spearmint using cotyledons, hypocotyls (Van Eck and Kitto, 1990), and protoplast (Sato et al., 1993) have had limited success. The best spearmint regeneration system published to date employed leaf explants, however, these produced low shoot regeneration (60%), and in the process, required excessive culture periods (Li et al., 1999). Internodes had high regeneration capacity in other species of *Mentha* (Bhat et al., 2002; Shasany et al., 1998), leading us to speculate that native spearmint might also respond well to internode culture. In this paper, we report significant improvement in the regeneration efficiency of native spearmint from internodal explants using specific pair wise combinations of cytokinins as media supplements, and the optimization of explant size, position, and stock plant age.

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Material and Methods

Stock plant culture. Stock cultures of a single genotype (accession PI557787) of native spearmint were obtained from shoot tips derived clonally from stolons of greenhouse grown plants originally obtained from USDA National Clonal Germplasm Repository, Corvallis, Ore. Stolon pieces with nodes were surface sterilized by immersing them in 70% v/v ethanol for 1 min, and then in a 20% solution of Clorox (1.05% w/v sodium hypochlorite) with 3 drops/L of Tween 20 for 20 min. The explants were then rinsed 7 times with sterile deionized water. The sterilized stolon was placed in Magenta (GA7) boxes (Bio-world, Dublin, Ohio) on stock culture medium containing MS salts (Murashige and Skoog, 1962), 100 mg·L^{-1} myo-inositol, 0.4 mg·L^{-1} thiamine-HCl, 2.0% sucrose, and 7.5 g·L^{-1} of agar (Sigma-Aldrich Chemical Co., St Louis, Mo.) and supplemented with 0.01 mg·L^{-1} ∝-napthaleneacetic acid (NAA). The pH of all the media described in this report was adjusted to 5.8 before adding agar and all media (with growth regulators) was autoclaved for 20 min at 121 °C. Plants were recultured on stock culture medium every 3 weeks from shoot tip explants and maintained under 16-h photoperiod provided by cool-white fluorescent light at 27 °C.

Explants. Two types of explants were tested in regeneration experiments. The first were internodal explants taken from the first, second, third and fourth internode counting downward from the top of the unbranched stock plants. Secondly, leaf explants were excised from in vitro grown shoots, and represented fully expanded leaves with petioles but without axillary buds. The edges of the leaves were trimmed on three sides leaving a piece of the petiole with the leaf explant as described by Li et al., (1999). The leaf and internode explants were compared on regeneration medium developed by Li et al., (1999). For subsequent regeneration experiments internodal explants were used. Internodal explants were placed on the surface of a regeneration medium consisting of MS basal salts, B5 vitamins, 2.0% sucrose, 10% coconut water, and 2.0 g·L^{-1} phytagel (referred to as basal medium) and thidiazuron (TDZ), zeatin (ZT), kinetin (KT), or 6-benzyladenine (BA) at 0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, or 32.0 mg·L^{-1}. For growth regulator combination experiments, basal medium was supplemented with a combination of 1.0 mg·L^{-1} TDZ with one other cytokinin at 0**,** 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, or 32.0 mg·L^{-1}. Coconut water was shown to promote greater adventitious shoot regeneration from native spearmint (Li et al., 1999). Coconut water was taken from ripe brown coconuts purchased from the market and filtered through four layers of cheesecloth, and then through two layers of Whatman No.1 filter paper. The filtered coconut water was stored at –20 °C until use. The length of the internodal explants for testing growth regulators was 3 to 6 mm. To test the effect of explant size on shoot regeneration, explants of 2 to 4, 5 to 7, and 8 to 10 mm length were compared on medium optimized for growth regulator supplements. In all shoot regeneration experiments, explants were first placed in the dark at 25 °C for a period of 3 weeks before being transferred to light (16-h photoperiod provided by cool-white fluorescent light at 27 ± 1 °C).

Rooting of regenerated shoots. After reaching about 1.5 cm in length, the in vitro regenerated shoots were excised from the callus and

placed in GA7 vessels on the same medium as used for stock plant culture described above. Conditions for growth were the same as described for stock plant cultures.

Planting in soil and establishing in greenhouse. Plantlets with well-developed root systems (about 5 cm long) and shoots (7 to 10 cm long) were removed from the GA7 boxes and planted in prewatered cell packs in flats filled with Metromix 360 medium (SUNGRO Horticulture Inc. Bellevue, Wash.). The flats were covered with transparent plastic domes to maintain 100% humidity for 2 d. Humidity was then decreased gradually by removing the plastic dome for varying periods of time every day with complete removal after 1 week. Plants were transplanted into individual 10 cm plastic pots containing Metromix 360 medium after 3 weeks growth in flats.

Experimental design. For all experiments, a completely randomized experimental design, with four replications per treatment for each explant type, was used. In the in vitro studies, each treatment consisted of four replicate petri dishes with 16 explants per dish. In the final analysis, all data was expressed as the mean of replicate petri dishes ± standard error.

Results

Formation of meristemoidal callus. In all studies in which callus was produced, callus initiated primarily from cut ends of freshly excised explants after 2 to 4 d in the dark. Callus first initiated on the proximal and then 2 d later on the distal side of the internodal explants. On leaf explants, callus initiated first on the cut end of the petiole and 4 d later on the cut blade surface. Dark grown callus was loosely packed and transparent, yellowish and friable. The callus on internodal explants differentiated into meristemoid regions in about 3 weeks in the dark, whereas obvious meristemoidal regions did not form on leaf explant callus. Meristemoidal regions were a more compact and roundish mass of cells that emerge above the initially formed callus and are referred to as meristemoidal because they represented regions from which shoots arose about 1 week after transfer to light.

A comparison of the leaf and stem internodal explants. The regeneration efficiency (percent of explants regenerating shoots) of the leaf and stem internodal explants was compared using the optimum leaf medium previously developed by Li et al. (1999). Adventitious shoots were first visible on internodal explants in 5 weeks, while adventitious shoots were first visible on leaf explants in 6 weeks. On this medium, internodal explants had a regeneration efficiency of 85% with 18.3 shoots per explant while the leaf explants had a regeneration efficiency of 60% with 9.7 shoots per explant. Subsequent experiments were conducted with internodal explants only.

Effect of individual cytokinins on shoot regeneration from stem internodes. Callus formation was the greatest with 1.0 mg·L^{-1} TDZ supplementation and occurred within 2 weeks (data not shown). Callus that formed on explants on media with >4.0 mg·L^{-1} TDZ appeared waterlogged and necrotic after 2 weeks, whereas callus development on media supplemented with TDZ below 4.0 mg·L^{-1} formed distinct meristemoidal tissue after 3 weeks. Callus formed on all ZT concentrations, but this was lower than observed using TDZ. Callus was formed on media supplemented with BA below 16.0 mg·L^{-1} or KT below 32.0 mg·L^{-1}. The greatest adventitious shoot regeneration was observed on medium supplemented with 32.0 mg·L^{-1} ZT, followed by 1.0 mg·L^{-1} BA, and then 4.0 mg·L^{-1} KT, quantified after 4 weeks of culture (Table 1). ZT and KT supplementations below the concentration of 4.0 mg·L^{-1}, and BA below 0.5 mg·L^{-1}, resulted in no shoots. Explants on medium supplemented with TDZ alone did not regenerate shoots (Table 1), even though they produced the most callus.

Effect of pair-wise combinations of cytokinins on in vitro shoot regeneration. The effect of combinations of TDZ at 1.0 mg·L^{-1} with various cytokinins (ZT, BA, and KT), was examined. Callus and meristemoidal regions were evident on all cut surfaces on media supplemented with two cytokinins. Interestingly, when combinations of cytokinins were used, meristemoidal regions were also observed on the noncut surfaces of explants, between the explant ends, and those on surfaces closest to the medium. The highest regeneration efficiency (92%) was observed on medium supplemented with 2.0 mg·L^{-1} ZT and 1.0 mg·L^{-1} TDZ (Table 2). Adventitious shoot regeneration was most profuse from the callus that originated from epidermal and sub epidermal regions of the fresh-cut explant. For BA and KT combinations with TDZ, regeneration efficiency was highest on basal medium supplemented with 1.0 mg·L^{-1} BA or 1.0 mg·L^{-1} KT in combination with 1.0 mg·L^{-1} TDZ at 43.6% and 39%, respectively (Table 2). Additional experiments were conducted to further optimize regeneration by examining the effect of ZT and TDZ pair-wise combinations around a finer range of growth regulator concentrations (Table 3). Better regeneration efficiency (97% of explants regenerating shoots) and higher shoot numbers (31 per explant) were obtained when ZT concentration was 2.5 mg·L^{-1} with TDZ at 1.0 mg·L^{-1} (Table 3).

Table 1. Effect of individual cytokinins on shoot regeneration (mean shoots per explant) by internodes of *Mentha spicata* cultured on basal medium supplemented with individual cytokinins over a range of concentrations from 0 to 32 mg·L^{-1}. Percentage of the explants producing shoots in parentheses.

Concn	Mean shoots/explant ± SE			
(mg·L^{-1})	Thidiazuron	Benzyladenine	Zeatin	Kinetin
0.0	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)
0.5	0.0 ± 0.0 (0)	11.5 ± 1.5 (23)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)
1.0	0.0 ± 0.0 (0)	15.9 ± 1.7 (46)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)
2.0	0.0 ± 0.0 (0)	12.4 ± 1.7 (38)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)
4.0	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	9.6 ± 1.6 (21)	9.6 ± 1.7 (30)
8.0	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	11.9 ± 1.7 (33)	0.0 ± 0.0 (0)
16.0	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	15.3 ± 1.9 (33)	0.0 ± 0.0 (0)
32.0	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	19.4 ± 2.6 (27)	0.0 ± 0.0 (0)

Table 2. Effect of pair-wise combinations of cytokinins on shoot regeneration (mean shoots per explant) by internodes of *Mentha spicata* cultured on basal medium supplemented with 1.0 mg·L^{-1} TDZ and a second cytokinin over a range of concentrations from 0 mg·L^{-1} to 32 mg·L^{-1}. Percentage of the explants producing shoots in parentheses.

Concn	Mean shoot per explant ± SE		
(mg·L^{-1})	Zeatin	Benzyladenine	Kinetin
0.0	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)
0.5	9.2 ± 2.1 (25.0)	2.5 ± 0.6 (28.1)	2.1 ± 0.5 (29.7)
1.0	15.8 ± 2.3 (45.3)	11.1 ± 1.6 (43.6)	9.9 ± 1.6 (39.1)
2.0	29.2 ± 1.8 (92.1)	1.5 ± 0.5 (15.6)	3.8 ± 0.8 (25.0)
4.0	13.4 ± 1.2 (75.0)	0.8 ± 0.3 (7.8)	1.3 ± 0.4 (18.8)
8.0	10.8 ± 1.5 (48.4)	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)
16.0	7.6 ± 1.3 (35.9)	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)
32.0	5.1 ± 1.2 (25.0)	0.0 ± 0.0 (0.0)	0.0 ± 0.0 (0.0)

Table 3. Effect of thidiazuron and zeatin combinations on shoot regeneration (mean shoots per explant) by internodes of *Mentha spicata* cultured on basal medium with thidiazuron and zeatin supplements. Percentage of the explants producing shoots in parentheses.

Zeatin	Mean shoots per explant ± SE					
	Thidiazuron (mg·L^{-1})					
(mg·L^{-1})	0.0	0.5	0.75	1.0	1.5	2.0
0.0	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)	0.0 ± 0.0 (0)
1.5	0.0 ± 0.0 (0)	14.2 ± 2.2 (41)	16.8 ± 1.7 (61)	19.4 ± 1.5 (73)	15.5 ± 1.2 (77)	9.7 ± 1.8 (34)
2.0	0.0 ± 0.0 (0)	15.5 ± 2.1 (48)	19.2 ± 1.8 (67)	28.5 ± 1.6 (88)	17.2 ± 1.5 (72)	13.4 ± 2.0 (44)
2.5	7.3 ± 1.5 (31)	20.3 ± 1.9 (66)	26.5 ± 1.7 (81)	31.1 ± 1.7 (97)	26.3 ± 2.3 (69)	15.1 ± 2.3 (42)
3.0	9.7 ± 1.8 (34)	15.2 ± 1.7 (59)	17.0 ± 1.3 (75)	25.8 ± 1.3 (91)	19.2 ± 1.6 (70)	11.8 ± 1.9 (39)
3.5	11.4 ± 1.9 (39)	12.4 ± 1.6 (48)	14.1 ± 1.5 (63)	17.4 ± 1.1 (84)	8.8 ± 0.9 (66)	5.4 ± 1.8 (13)

Effect of explant size on shoot regeneration efficiency. The size of the internodal explants (excised from the second internode of the stock plant) affected regeneration efficiency of native spearmint. Explants 8 to 10 mm long formed less callus than 5 to 7 mm or 2 to 4 mm long explants on the optimal regeneration medium (supplemented with 1.0 mg·L^{-1} TDZ and 2.5 mg·L^{-1} ZT), and the callus on larger explants turned brown earlier. Explants 2 to 4 mm long had the highest regeneration efficiency of 97%, while 66% of the 8 to 10 mm long explants produced shoots. The 2 to 4 mm long explants produced the greatest number of shoots per explant (33) on the optimized regeneration medium (Table 4).

Effect of stock plant age and internode position on shoot regeneration efficiency. Native spearmint shoot tips placed in stock culture medium supplemented with 0.01 mg·L^{-1} NAA produced about one internode per week. All internodes from 1-, 2-, 3-, and 4-week-old stock plants were evaluated for their regeneration efficiency. On optimized shoot regeneration medium, there was no visible difference in callus formation on explants from different age stock plants. The internode position was counted backwards from the tip of the stock plant (apical most fully expanded internode designated internode one). The first internode of 1-week-old stock plants had high regeneration efficiency of 100%. The second internode of 2-week-old stock plants likewise had high regeneration efficiency of 100%, whereas the first internode of 2-week-old plants had slightly lower regeneration percentage of 97%. In 3-week-old stock plants, the third internode had the highest regeneration efficiency (100%) followed by the second internode (97%) and then the first internode (95%) (Table 5). The highest number of adventitious shoots of 36 per explant occurred in 4 weeks on the second internode from 2 week old plants. The first internode of the 1-week-old plant and the second internode of the 2-week-old plant had similar response.

To address the issue regarding the clonal origin of shoots, we quantified the number of distinct meristemoidal regions produced on the callus of explants varying in position and age, and growing on optimized shoot regeneration medium (Table 6). These meristemoid formations became visible increasingly as shoots began to emerge. After 30 d in culture, the maximum number of meristemoidal regions was 10.2 per explant on the second internode of 2-week-old stock plants (Table 6). On average, the maximum number of shoots per meristemoidal region was 3.9 on explants derived from the first internode of 2-week-old plants, followed by the first internode of 1-week-old plants, and the second internode of 2-week-old plants, at 3.6 and 3.5 shoots per meristemoidal region, respectively (Tables 5 and 6).

Rooting of regenerated shoots in vitro and acclimatization of the in vitro produced plantlets to greenhouse environment. Shoots about 2.0 cm long were excised and transferred to rooting medium in GA7 boxes where explants initiated roots in 4 to 6 d and developed a well branched root system within 2 weeks (Fig. 1E). The rooted plants were transplanted to greenhouse and acclimatized where 100% of the transplants grew vigorously (Fig. 1F).

Table 4. Effect of internode explant size (excised from the second internode counting back from the terminal shoot bud) on shoot regeneration efficiency (mean shoots per explant) of *Mentha spicata* internodes on basal medium supplemented with 1.0 (mg·L^{-1}) TDZ and 2.5 (mg·L^{-1}) ZT. Percentage of the explants producing shoots in parentheses.

Explant size (mm)	Mean shoots/ explant ± SE
2–4	33.1 ± 1.6 (97)
5–7	27.3 ± 1.9 (82)
8–10	14.2 ± 1.5 (66)

Discussion

To develop an efficient system for in vitro adventitious shoot regeneration of native spearmint, we tested different cytokinin supplements, and the size, age and position of internodal explants for their effect on regeneration. In contrast to a previous report, our preliminary studies using the same genotype of *Mentha spicata* (PI 557787) showed that internodal explants regenerated better than leaf explants on medium optimized for leaf explant regeneration (Li et al., 1999) with internode explants producing an average of 18.3 shoots per explant while leaf explants produced only 9.7 shoots per explant. This provided evidence that internodes have higher regeneration capacity than leaf explants. It is unclear why the internodes performed better than leaf explants in our studies, however, it is possible (as discussed later) that our internodal explants were younger and more morphogenic than those tested by Li et al. (1999). Internodal explants but not leaf explants produced adventitious roots and little callus on medium without cytokinin, potentially due to the presence of high levels of endogenous auxin. Internodal explants on medium with TDZ alone produced only callus, with no shoot regeneration. When TDZ induced callus was moved to a cytokinin free media, a few shoots subsequently arose, but shoots were formed only on explants grown on medium supplemented with <4 mg·L^{-1} TDZ (data not shown). TDZ was thus necessary for initiating shoot meristemoids, but appeared to have an inhibitory effect on shoot elongation. Similar results were reported for native spearmint (Li et al., 1999) as well as a few woody plant species (Ellis et al., 1991; Huetteman and Preece, 1993). In *Picea glauca* (Moech) Voss, however, the addition of a second cytokinin to a TDZ containing medium led to efficient shoot elongation from meristemoidal callus produced on embryo and epicotyl explants (Ellis et al., 1991). Likewise, our study with native spearmint shows that 1.0 mg·L^{-1} TDZ containing medium plus 2.5 mg·L^{-1} ZT promoted both maximum shoot initiation and the elongation of adventitious shoots from meristemoidal regions, and this occurred without the need for subculture onto a cytokinin free medium. Therefore, ZT thus appears to counteract the inhibitory effect of TDZ on shoot elongation but the mechanism for this effect is unclear.

On optimized shoot regeneration medium, smaller explants regenerated more shoots than larger explants. Why larger explants produced less callus and fewer shoots than smaller explants in unknown.

Explants from younger plants produced more shoots than explants from older plants, in contrast to previous reports that leaf explants from older stock plants were more regenerative (Li et al., 1999). Higher explant response from younger plants may be due to the fact that they were physiologically more active and easily influenced by the presence of exogenous hormones (Dong and Jia, 1991). Furthermore, there was a decrease in the regeneration efficiency of the internodal explants from the same position as the age of the stock plants increased. The position of the internode also affected the regeneration efficiency of native spearmint, with regeneration efficiency increasing on explants taken closer to the proximal end (apical or teminal bud end) of the stock plant. Higher regenerative capacity in leaf explants of native spearmint taken from the proximal portions of the stock plants was likewise reported (Li et al., 1999), a response possibly influenced by the distance of the explant from the apical meristem and the basipetal transport of auxin.

Table 5. Effect of stock plant age (in weeks) and internode position (counting backward from the terminal bud of the stock plant) on adventitious shoot regeneration (mean shoots per explant) by internodes of *Mentha spicata.* Percentage of the explants producing shoots in parentheses.

Internode position	Mean shoots/explant ± SE			
	Week 1	Week 2	Week 3	Week 4
1	33.6 ± 1.5 (100)	30.6 ± 2.0 (97)	20.7 ± 1.7 (95)	16.5 ± 1.3 (82)
2		36.4 ± 2.2 (100)	20.0 ± 1.6 (97)	21.8 ± 1.1 (93)
3			27.2 ± 1.4 (100)	18.4 ± 1.4 (87)
4				15.7 ± 1.3 (75)

Table 6. Effect of stock plant age and internode position on the mean visible meristemoidal regions per explant (± SE) after 25 d.

Internode position	Stock plant age (weeks)			
	1	2	3	4
1	9.1 ± 0.2	8.0 ± 0.3	7.9 ± 0.3	5.2 ± 0.4
2		10.2 ± 0.2	7.2 ± 0.2	7.0 ± 0.3
3			9.1 ± 0.2	6.9 ± 0.3
4				5.1 ± 0.4
Week avg	9.1	9.1	8.1	6.1

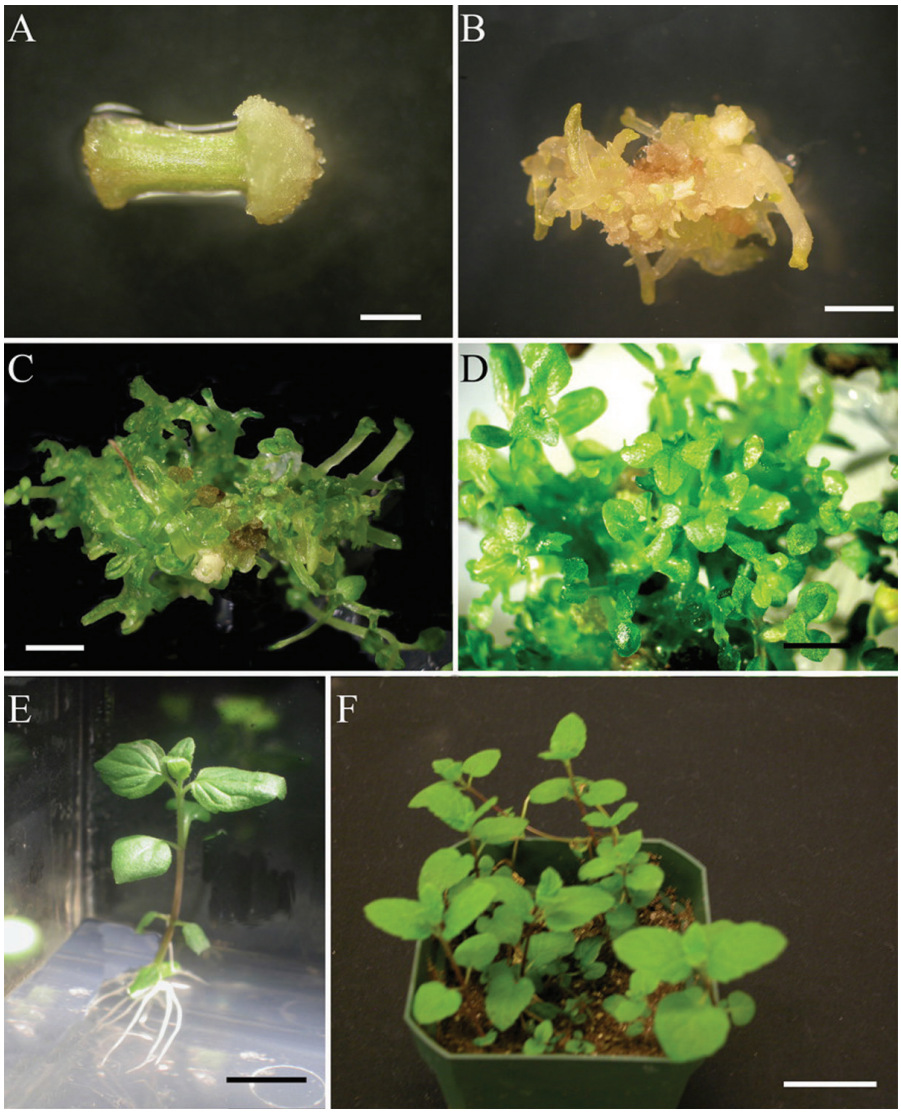

Fig. 1. Regeneration of native spearmint internodes on basal medium, TDZ 1.0 mg·L^{-1} and ZT 2.5 mg·L^{-1}. (**A**) Callus formation in dark (7 d after culture initiation, bar = 1.0 mm) (**B**) Shoot initiation in dark (21 d after culture initiation, bar = 1.0 mm) (**C**) Shoot elongation 4 d after transfer to light (25 d after culture initiation, bar = 1.5 mm). (**D**) Shoot growth 9 d after transfer to light (36 d after culture initiation, bar = 1.6 mm). (**E**) Plantlets on rooting medium showing vigorous root growth (40 d after culture initiation, bar = 3.0 cm). (**F**) Rooted plantlets in greenhouse (56 d after culture initiation, bar = 9cm).

Where callus formed, it was produced over the entire cut surface of both leaf and internodal explants and from epidermal, subepidermal and vascular regions. Most callus was capable of forming meristemoidal regions, except that initiating from vascular tissues, which was distinctively less compact. Meristemoidal regions also formed from the explant surface between the cut ends, apparently due to the direct differentiation of epidermal cells on the uncut surface. Shoots were observed to arise simultaneously from multiple meristemoidal regions of the same explant, regions first visible in about 3 weeks. A single shoot arose from a single meristemoidal area, followed within 3 to 5 d by additional shoots near its base. It is unclear what induces the secondary shoots to form. Potentially growth of the first shoot alters the local hormonal balance, inducing meristemoidal centers to organize into shoot initials and then elongate. For transformation efficiency, it is desired that many transgenic plants arise from each explant, and each from unique transformation events of single cells. It is unclear whether the multiple shoots that arise from individual meristemoidal regions originate from the same or different cells of the original explant. Studies of cell lineage in callus and meristemoid formation by native spearmint are needed.

The highest adventitious shoot regeneration by native spearmint of 36 shoots per explant was obtained with basal medium supplemented with 1.0 mg·L^{-1} TDZ and 2.5 mg·L^{-1} ZT when the explant was 2 to 4 mm long and taken from the second internode of 2-week-old stock plants. The use of internodal explants rather than leaf explants, and a medium that contained two cytokinins rather than one, as in previous studies (Li et al., 1999), resulted in a greatly improved adventitious shoot regeneration protocol. The research presented here describes a new, highly efficient regeneration system that provides a critical step toward the development of a genetic transformation protocol for native spearmint.

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