An In Vitro–Ex Vitro Micropropagation System for Hemp

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Additional index words. *Cannabis sativa*, retipping, tissue culture

Summary. **Hyperhydricity of shoots initiated in vitro, poor shoot extension, inability of shoot cultures to maintain good growth over an extended time, and unsuccessful ex vitro rooting have limited the development of a commercial scale micropropagation system for hemp (*Cannabis sativa*). We present a culture initiation method that prevents shoot hyperhydricity using vented-lid vessels with 0.2-µm pores and medium containing agar at 1% (w/v). To optimize shoot multiplication in vitro, a control medium (medium A) and four treatment media (medium B, C, D, and E), with varying inorganic nutrients and vitamins were tested. Control medium A consisted of 1× Murashige and Skoog (MS) with vitamins plus 3% (w/v) sucrose, 0.5 mg·L^{-1} metatopolin, 0.1 mg·L^{-1} gibberellic acid, and 0.8% agar (w/v) at pH 5.7. The four treatment media differed from the control medium as follows: medium B, 2.5× MS with vitamins; medium C, 1× MS with vitamins plus added mesos [calcium chloride (anhydrous), magnesium sulfate (anhydrous), and potassium phosphate (monobasic) nutrients]; medium D, 1× MS with vitamins plus added vitamins; and medium E, 1× MS with vitamins plus added mesos and vitamins. Medium C and medium E produced more microcuttings than the control at 6 weeks after the initial subculture with shoot multiplication media and all other treatments at 9 and 12 weeks. Shoots grown on these two media displayed optimal extension and leaf lamina development; however, they exhibited slight chlorosis by 12 weeks after subculture with shoot multiplication media. In a separate experiment, medium E was supplemented with ammonium nitrate at 0, 500, 1000, or 1500 mg·L^{-1}, and cultures grown with 500 mg·L^{-1} produced the most microcuttings and exhibited the best combination of shoot extension and leaf lamina development. We provide a method of prerooting microshoots in vitro that has resulted in 75% to 100% rooting ex vitro in rockwool. Using 10 recently micropropagated plants, ≈300 retip cuttings (cuttings taken from new shoots from recently micropropagated plants) were harvested over 10 weeks. The average weekly rooting was more than 90%. Retipping can produce ninetimes as many plants in a similar amount of floor space as stem cuttings derived from traditional stock mother plants. The micropropagation/retipping method proposed can be a more efficient way to generate clonal liner plants for commercial-scale production.**

There is increased interest in the production of hemp (*Cannabis sativa*) because of its medicinal properties (Small, 2015). For commercial production purposes, hemp is propagated by seed or stem cuttings to take advantage of superior genotypes (Cervantes, 2015). Many indoor hemp production facilities propagate cultivars by taking stem cuttings from stock mother plants, which they must maintain (Bechtel, 2019). Mother plants are large (10-gal container size) and require a significant amount of grow space to provide enough cuttings to meet production quotas. Growers must maintain mother plants in triplicate, with each replicate grown in a separate area of the facility, to reduce the risk of losing valuable cultivars to sudden disease outbreaks. Mother plants lose vigor because of the serial removal of shoots for cuttings, and they must be replaced every 6 months. Additionally, over time, mother plants accumulate insects and diseases, thus limiting their useful life as donors of cuttings. Overall, this propagation process is labor-intensive and inefficient. Hemp growers are interested in micropropagation as an alternative method of generating clones for commercial production (Rosslee, 2020).

Micropropagation provides unique benefits to growers and has several advantages over traditional plant cloning systems. These include the production of a large number of genetically clonal plants, uniform plants with enhanced vigor, disease-free plants, and preservation of maternal germ lines (Hartmann et al., 2002). Micropropagation also requires substantially fewer mother plants to be maintained compared with traditional stem cutting propagation, and in vitro cultures can be stored for longer in a smaller area than mother plants.

There are few published reports of hemp micropropagation. Wang et al. (2009) evaluated the effects of growth regulator additions to Murashige and Skoog (MS) medium on in vitro shoot multiplication and rooting of hemp cultures started from seed. Using nodal stem segments and MS medium, Lata et al. (2009) similarly tested rates of three growth regulators alone and in combination with gibberellic acid (GA_3) on shoot multiplication. Lata et al. (2016) published a protocol refinement of their previous work (Lata et al., 2009) and introduced the growth regulator meta-topolin (MT), which was found to be superior to thidiazuron (TDZ) for in vitro shoot multiplication. Unfortunately, these published protocols have not translated well to large-scale micropropagation of clones necessary for commercial production. Noted shortcomings of published micropropagation methods

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
29.5735	fl oz	mL	0.0338
0.0929	ft^2	m^2	10.7639
3.7854	gal	L	0.2642
2.54	inch(es)	cm	0.3937
25.4	inch(es)	mm	0.0394
1	micron(s)	µm	1
28.3495	oz	g	0.0353
1	ppm	mg·L^{-1}	1
(°F – 32) ÷ 1.8	°F	°C	(°C × 1.8) + 32

Table 1. Various Murashige and Skoog (MS)-based media formulations for initiation and shoot multiplication of hemp.

Formula ($mg \cdot L^{-1}$)[z]	Initiation medium: 1× MS with vitamins	Medium A: 1× MS with vitamins	Medium B: 2.5× MS with vitamins	Medium C: 1× MS with vitamins + added mesos[y]	Medium D: 1× MS with vitamins + added vitamins	Medium E: 1× MS with vitamins + added mesos and vitamins
Ammonium nitrate	1,650	1,650	4,125	1,650	1,650	1,650
Boric acid	6.2	6.2	15.5	6.2	6.2	6.2
Calcium chloride, anhydrous	332.2	332.2	830.5	830.5	332.2	830.5
Cobalt chloride·$6H_2O$	0.025	0.025	0.063	0.025	0.025	0.025
Cupric sulfate·$5H_2O$	0.025	0.025	0.063	0.025	0.025	0.025
$Na_2EDTA \cdot 2H_2O$	37.26	37.26	93.15	37.26	37.26	37.26
Ferrous sulfate·$7H_2O$	27.8	27.8	69.5	27.8	27.8	27.8
Magnesium sulfate, anhydrous	180.7	180.7	451.8	451.8	180.7	451.8
Manganese sulfate·H_2O	16.9	16.9	42.3	16.9	16.9	16.9
Molybdic acid (sodium salt)·$2H_2O$	0.25	0.25	0.625	0.25	0.25	0.25
Potassium iodide	0.83	0.83	2.075	0.83	0.83	0.83
Potassium nitrate	1,900	1,900	4,750	1,900	1,900	1,900
Potassium phosphate, monobasic	170	170	425	425	170	425
Zinc sulfate·$7H_2O$	8.6	8.6	21.5	8.6	8.6	8.6
Glycine (free base)	2	2	5	2	5	5
Myo-inositol	100	100	250	100	250	250
Nicotinic acid (free acid)	0.5	0.5	1.25	0.5	1.25	1.25
Pyridoxine HCl	0.5	0.5	1.25	0.5	1.25	1.25
Thiamine HCl	0.1	0.1	0.25	0.1	0.25	0.25
Sucrose	30,000	30,000	30,000	30,000	30,000	30,000
Metatopolin	0.5	0.5	0.5	0.5	0.5	0.5
Gibberellic acid	0	0.1	0.1	0.1	0.1	0.1
Agar	10,000	8,000	8,000	8,000	8,000	8,000
pH	5.7	5.7	5.7	5.7	5.7	5.7

[z]1 $mg \cdot L^{-1}$ = 1 ppm.
[y]Mesos components include the calcium chloride (anhydrous), magnesium sulfate (anhydrous), and potassium phosphate (monobasic) nutrients.

include development of hyperhydricity during establishment of shoots in vitro, lack of consistent shoot elongation in culture, and inability of shoot cultures to maintain quality growth for an extended period of time (Monthony et al., 2021).

Microshoots from in vitro cultures are miniaturized, have altered physiology, and root easily (Hartmann et al., 2002). Nursery producers use a process called retipping to take advantage of and extend the period of time that micropropagated plants retain this miniaturized physiology so that more cuttings can be rooted (Keith and Brand, 1995). Retipping is the repeated harvesting of new shoots from recently micropropagated plants. The retipping process stimulates shoot growth from latent buds originating from the region of the stem that was miniaturized in culture. Retipping is routinely used to substantially increase the yield of propagules for crops such as rhododendron (*Rhododendron* sp.), mountain laurel (*Kalmia latifolia*), and lilac (*Syringa vulgaris*).

The objective of this work was to enhance hemp micropropagation by reducing hyperhydricity, improving in vitro shoot extension and performance through adjustment of the media nutrient content, and developing a method of ex vitro rooting. An additional objective was to evaluate retipping of recently micropropagated plants as a method of obtaining large quantities of clones for commercial-scale hemp production.

Materials and methods

Plant material. The hemp cultivars Wife (US Hempcare, Niantic, CT) and Dinamed CBD (Dinafem seeds, San Sebastian, Spain) were used. 'Wife' was used for all studies and 'Dinamed CBD' was used for the retipping study only. Cultivars were maintained as 2-gal stock plants in a greenhouse with set points of 21/17 °C day/night temperatures under long-day (18-h) conditions provided by 600-W high-pressure sodium (HPS) lamps (Phantom HPS 600W;

Hydrofarm, Petaluma, CA) to maintain vegetative growth.

In vitro shoot initiation. To initiate shoots in vitro, 4-cm-long stem tips were collected from stock plants. After leaves were removed, stems were disinfected by immersion in a solution of 0.54% (w/v) sodium hypochlorite and 0.1% surfactant (Tween 20; Croda Intl., Snaith, UK) for 15 min with intermittent agitation by hand, followed by rinsing in sterile distilled water. Then, stems were aseptically trimmed to remove damaged tissue and cultured in boxes (GA-7; Magenta, Lockport, IL) with vented lids featuring a vent with a diameter of 10 mm and pore size of 0.2 µm (Caisson Laboratories, Smithfield, UT) and containing 45 mL of medium. Explants were trimmed to ≈2-cm-long segments and four explants were placed per box. The initiation medium (Table 1) was MS with vitamins (Murashige and Skoog, 1962) plus 3% (w/v) sucrose, 0.5 $mg·L^{-1}$ metatopolin (MT), and 1.0% (w/v) agar (Millipore Sigma, St. Louis, MO) at pH 5.7. After 3 weeks, sterile shoots were subcultured with the same medium and maintained for another 3 weeks, at which time they were subcultured with shoot multiplication medium. Cultures were maintained in a growth chamber (Percival, Perry, IA) at 25 °C with an 18-h photoperiod provided by cool white fluorescent lamps at an intensity of 40 $µmol·m^{-2}·s^{-1}$.

In vitro shoot multiplication. Two studies were conducted to optimize the nutrient composition of MS medium for in vitro shoot growth and performance. For these studies, in vitro shoots were initiated as described. The first shoot multiplication study evaluated the mesos components of MS, which included the calcium chloride (anhydrous), magnesium sulfate (anhydrous), and potassium phosphate (monobasic) nutrients. The control medium for this study was medium A, which consisted of 1× MS with vitamins plus 3% (w/v) sucrose, 0.5 $mg·L^{-1}$ MT, 0.1 $mg·L^{-1}$ GA_3, and 0.8% agar (w/v) at pH 5.7 (Table 1). In addition to control medium A, there were four other treatment media (formulations provided in Table 1): medium B, 2.5× MS with vitamins; medium C, 1× MS with vitamins plus added mesos; medium D, 1× MS with vitamins plus added vitamins; and medium E, 1× MS with vitamins plus added mesos and vitamins. The second shoot multiplication study evaluated the addition of 0, 500, 1000, or 1500 $mg·L^{-1}$ ammonium nitrate (NH_4NO_3) to medium E.

For both studies, the experimental unit was a box. At each subculture, four microcuttings were placed per box. There were 10 and 8 boxes per treatment for the mesos and NH_4NO_3 studies, respectively. For each study, experimental units were arranged in a completely random design. Cultures were subcultured every 3 weeks and maintained as described for in vitro shoot initiation. At each subculture, shoot extension and leaf lamina development were visually assessed by the lead author. Data were collected at 6, 9, and 12 weeks after shoots were initially subcultured with shoot multiplication media. For the mesos study, the numbers of ≈2-cm

Fig. 1. Microshoots of hemp cultivar Dinamed CBD (A) at the end of the 6-week shoot initiation step and (B) after 3 additional weeks on initiation medium containing 0.1 $mg·L^{-1}$ gibberellic acid and 0.8% agar. 1 $mg·L^{-1}$ = 1 ppm.

Table 2. Number of 'Wife' hemp apical and two-node nonapical microcuttings per box subcultured on various modified Murashige and Skoog (MS) shoot multiplication media every 3 weeks during the first 12 weeks.

	Apical and two-node nonapical microcuttings (no./box)		
Treatment medium[z]	6 weeks	9 weeks	12 weeks
Medium A: 1× MS with vitamins (control)	4.9 c[y]	4.1 b	4.2 b
Medium B: 2.5× MS with vitamins	5.1 c	2.9 c	3.1 b
Medium C: 1× MS with vitamins + added mesos[x]	6.4 a	7.1 a	6.6 a
Medium D: 1× MS with vitamins + added vitamins	6.0 a	4.7 b	3.0 b
Medium E: 1× MS with vitamins + added mesos and vitamins	5.9 a	6.5 a	6.9 a

[z]All media contained 3% (w/v) sucrose, 0.5 $mg·L^{-1}$ metatopolin, 0.1 $mg·L^{-1}$ gibberellic acid, and 0.8% agar (w/v) at pH 5.7. 1 $mg·L^{-1}$ =1 ppm.
[y]Mean separation within columns indicated by different letters according to Fisher's least significant difference test at $P \leq 0.05$ (n = 10).
[x]Mesos components include the calcium chloride (anhydrous), magnesium sulfate (anhydrous), and potassium phosphate (monobasic) nutrients.

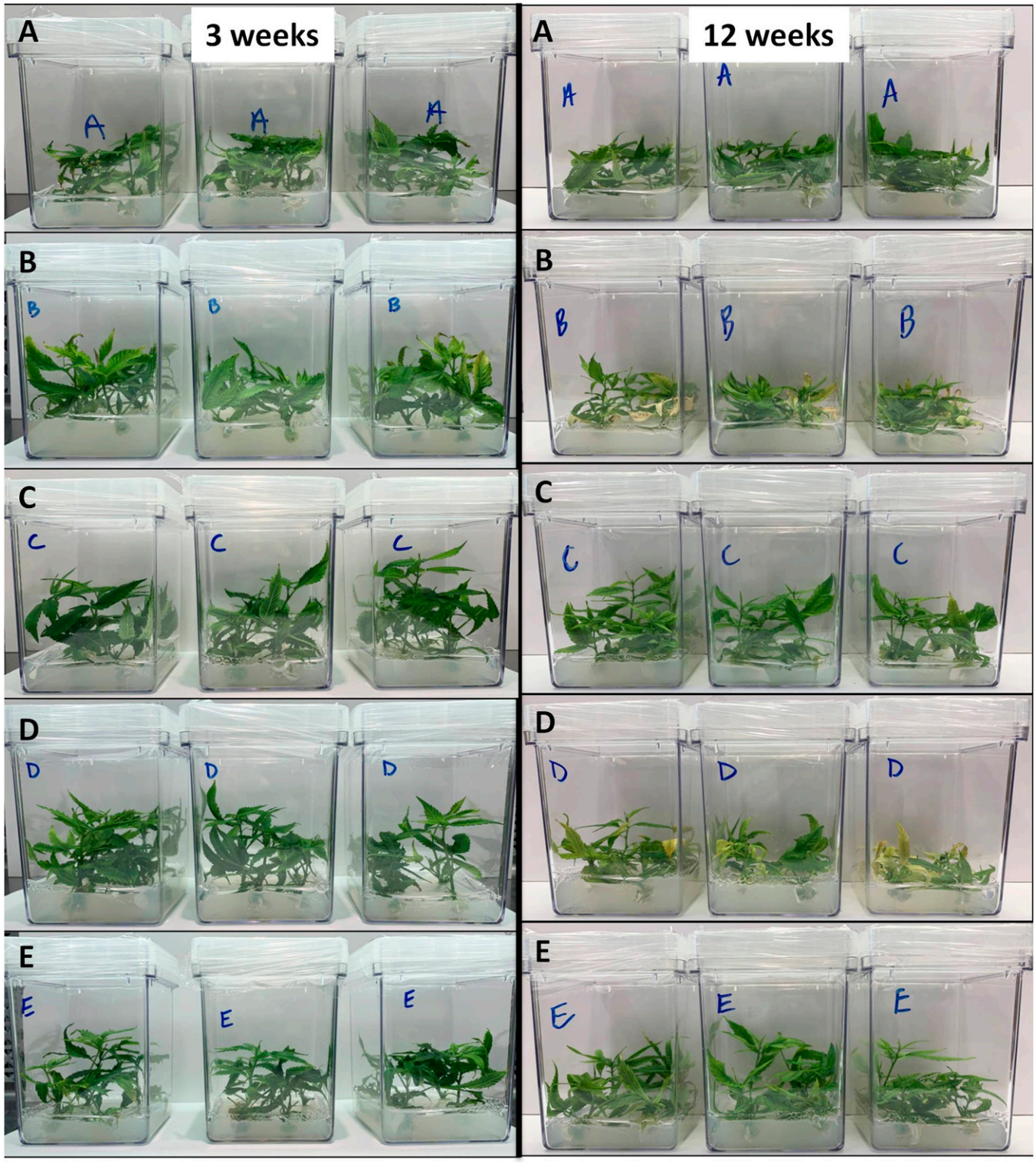

Fig. 2. In vitro cultures of hemp cultivar Wife after 3 weeks and 12 weeks of growth on experimental shoot multiplication media: (A) control medium A, 1× Murashige and Skoog (MS) medium with vitamins; (B) medium B, 2.5× MS with vitamins; (C) medium C, 1× MS with vitamins plus added mesos; (D) medium D, 1× MS with vitamins plus added vitamins; and (E) medium E, 1× MS with vitamins plus added mesos and vitamins.

apical and two-node nonapical microcuttings per experimental unit were recorded. For the NH_4NO_3 study, the number of ≈2-cm apical microcuttings per experimental unit was recorded. Data were subjected to an analysis of variance (PROC GLM) and mean separation with Fisher's least significant difference test ($P \leq 0.05$) using SAS (version 9.4; SAS Institute, Cary, NC).

Microcutting rooting and acclimation. Microshoots were

prerooted in vitro using MS with vitamins plus 3% (w/v) sucrose, 1 mg·L^{-1} indole-3-butyric acid (IBA), and 0.8% agar (w/v) at pH 5.7; boxes with vented lids were used. For prerooting, 8 to 10 microshoots were placed per box and cultures were maintained as described for in vitro shoot initiation. After 14 d, microshoots had formed visible white root initials (≈1 mm) and were transferred ex vitro to 1-inch rockwool cubes with preformed holes, set in 96-plug trays, and covered with clear plastic propagation domes to maintain humidity. Domed trays were maintained with 18-h photoperiods provided by cool white fluorescent lamps at an intensity of 30 μmol·m^{-2}·s^{-1}. Microcuttings rooted at 21 d and initiated new shoot growth. Rooted microcuttings were acclimated to greenhouse conditions by gradually decreasing atmospheric relative humidity around the microcuttings and gradually increasing light levels. This was accomplished by melting 30 holes (diameter, 0.5 cm) in the domes and, 4 d later, melting 30 more holes in the domes. After 4 d with 60 holes in the domes, rooted microcuttings were potted in 307-mL containers filled with a peatmoss-based potting mix (Promix BK25; Premier Tech Horticulture, Quakertown, PA). Plants were top-dressed with 15N–3.9P–10K controlled-release fertilizer (Osmocote Plus 5- to 6-month formulation; Everris NA, Dublin, OH) at 2.5 g per container. Potted plants were placed in the greenhouse with an 18-h photoperiod under 50% shade for 5 d before they were exposed to full light greenhouse conditions. Plants received a 20N–8.7P–16.6K soluble fertilizer (Peters; Scotts, Marysville, OH) providing 100 ppm nitrogen (N) at every irrigation, which occurred as needed.

Retipping. For each of the three experimental time replications of retipping, 10 recently potted micropropagated plants were used. Plants were potted 10 d before taking the first retip cuttings, and plants were placed pot-to-pot on a greenhouse bench. The first two experimental time replications with 'Dinamed CBD' were initiated in July and Aug. 2019, and the third experimental time replication with 'Wife' was initiated in Apr. 2020. Retip cuttings with lengths of 5 to 8 cm were taken every week for 10 consecutive weeks. Cuttings were treated with talc-based IBA at 1000 ppm (Hormodin #1; OHP, Mainland, PA) and stuck in 1-inch rockwool cubes. Rockwool cubes were set in 96-plug trays in a staggered plug cell arrangement and covered with clear plastic propagation domes to maintain humidity. Before sticking the cuttings, a hole with a diameter of ≈3 mm was bored into rockwool cubes using a forceps, which prevented damaging the stem upon sticking. Retip cuttings were rooted and acclimated as described for microcuttings. The percent rooting of retip cuttings for each sticking date was recorded at 28 d after sticking.

Results and discussion

Lata et al. (2016) proposed a one-step in vitro propagation protocol using MS medium containing 3% (w/v) sucrose, 0.5 mg·L^{-1} MT, 0.8% (w/v) agar, and 500 mg·L^{-1} activated charcoal at pH 5.7 in glass culture vessels with nonvented caps. We improved this protocol by adjusting the culture vessel to boxes with vented lids and changing the medium components for the shoot initiation and shoot multiplication steps. For shoot initiation, the amount of agar was increased; for shoot multiplication, the amounts of mesos components and NH_4NO_3 were adjusted and GA_3 was added. Activated charcoal, used by Lata et al. (2016), was not included in our medium at any step. If care is used to remove all unfurled leaves down to the smallest leaves spread from the shoot apical meristem, then contamination rates can be reduced to close to 0%. Low contamination rates with hemp shoot initiation may be attributable to the rapid shoot growth of greenhouse hemp plants that do not allow shoot tips to accumulate high microbial loads.

Using the method of Lata et al. (2016), shoots were hyperhydric and developed leaves that were light green in color, thick, translucent, and brittle. Possible causes of hyperhydricity are excessive humidity and/or high ethylene levels in vitro (Ivanova and Van Staden, 2009; Kevers et al., 1984). The physiological abnormalities caused by hyperhydricity are a serious problem for micropropagation and can result in up to 90% multiplication losses (Nairn et al., 1995). We were able to eliminate the development of hyperhydric shoots in vitro by using vessels with vented lids and a 6-week initiation step with medium containing increased agar (1% w/v). The use of vented lids and/or higher agar content effectively reduced hyperhydricity and improved the growth of cultures for aloe (*Aloe vera*), carnation (*Dianthus caryphyllus*), jones' cycladenia (*Cycladenia humilis* var. *jonesii*), passion fruit (*Passiflora edulis* f. *favicarpa*), serviceberry (*Amelanchier arborea*), and other plants (Brand 1993; Ivanova and Van Staden, 2009; Majada et al., 1998; Pence et al., 2020; Trevisan and Mendes, 2005). By potentially reducing the relative humidity within the vessel using vented lids and the ψ_S of the medium using more agar, shoots may have accumulated less water. Additionally, vented lids may have allowed for ethylene to leave the vessel

Table 3. Number of 'Wife' hemp apical microcuttings per box subcultured every 3 weeks during the first 12 weeks on shoot multiplication media E [1 × Murashige and Skoog medium with vitamins + added mesos[z] and vitamins with 3% (w/v) sucrose, 0.5 mg·L^{-1} metatopolin, 0.1 mg·L^{-1} gibberellic acid, and 0.8% agar (w/v) at pH 5.7] with additional ammonium nitrate (NH_4NO_3) at 0, 500, 1000, or 1500 mg·L^{-1}.

	Apical microcuttings (no./box)		
NH_4NO_3 (mg·L^{-1})[y]	6 weeks	9 weeks	12 weeks
0	6.8 b[x]	7.3 a	5.2 b
500	8.8 a	8.4 a	6.7 a
1000	6.7 b	5.1 b	5.0 b
1500	6.6 b	6.2 b	5.0 b

[z]Mesos components include the calcium chloride (anhydrous), magnesium sulfate (anhydrous), and potassium phosphate (monobasic) nutrients.
[y]1 mg·L^{-1} = 1 ppm.
[x]Mean separation within columns indicated by different letters according to Fisher's least significant difference test at $P \leq 0.05$ (n = 8).

rather than accumulate in vitro. At the end of the 6-week initiation step, shoots were not hyperhydric and had produced healthy, dark green leaves with expanded lamina, but the stems were not elongated (Fig. 1A). Preliminary studies performed to enhance shoot elongation tested concentrations of filter-sterilized GA_3 from 0.05 to 0.9 mg·L^{-1} (data not shown). It was observed that 0.1 mg·L^{-1} GA_3 enhanced stem elongation (Fig. 1B) and that rates of GA_3 greater than 0.1 mg·L^{-1} resulted in shoot tip collapse and necrosis.

Shoots grown on a multiplication medium modified from our initiation medium to contain 0.1 mg·L^{-1} GA_3 and 0.8% agar improved the performance we observed using the medium reported by Lata et al. (2016), but it still demonstrated a reduction in performance over two subculture cycles. Cultures developed chlorosis and had less vigor, and multiplication rates declined. Commercial hemp producers conducting tissue culture tests have also experienced declines in shoot quality as cultures age during the short term. The observed decline of cultures was an indication that the shoot multiplication medium nutrients were not optimal for growth. Several plants, including gerbera (*Gerbera hybrida*), hazelnut (*Corylus avellana*), pear (*Pyrus* sp.), red raspberry (*Rubus idaeus*), and stevia (*Stevia rebaudiana*), have benefited from adjustments to the mesos nutrients and/or NH_4NO_3 of MS medium (Hand et al., 2014; Niedz et al., 2014; Poothong and Reed, 2015; Poothong et al., 2017, 2018; Wada et al., 2013).

Medium C and medium E produced the greatest number of usable microcuttings (Table 2). Cultures grown on these media (Fig. 2C and E) were observed to be greener than those grown on medium B and medium D (Fig. 2B and D). Shoots on medium C and medium E were observed to have better extension and leaf lamina development compared with the control medium A at 12 weeks after the first subculture with shoot multiplication media (Fig. 2A, C, and E). The numbers of microcuttings were 1.5-times greater for medium C and medium E than the control at 12 weeks after the first subculture (Table 2). For medium C and medium E, the numbers of microcuttings produced at 6, 9, and 12 weeks after the first subculture were the same or increased slightly. For medium B and medium D, the numbers of microcuttings produced decreased from 6 to 12 weeks after first subculture. Medium E supplemented with 500 mg·L^{-1} NH_4NO_3 produced more usable microcuttings than medium E and medium E supplemented with higher rates of NH_4NO_3 (Table 3). Shoots produced on medium E with 500 mg·L^{-1} NH_4NO_3 exhibited the best combination of shoot extension and leaf lamina development (Fig. 3B), which is necessary for good rooting and acclimation to ex vitro conditions. We conclude that using this modified shoot multiplication medium, which contains added mesos and vitamins plus 500 mg·L^{-1} NH_4NO_3, a 2× shoot multiplication rate can be achieved for hemp and maintained over a minimum of 12 weeks after the initiation step. Cultivars of pear and red raspberry similarly benefitted from additions of ≥2.5× mesos

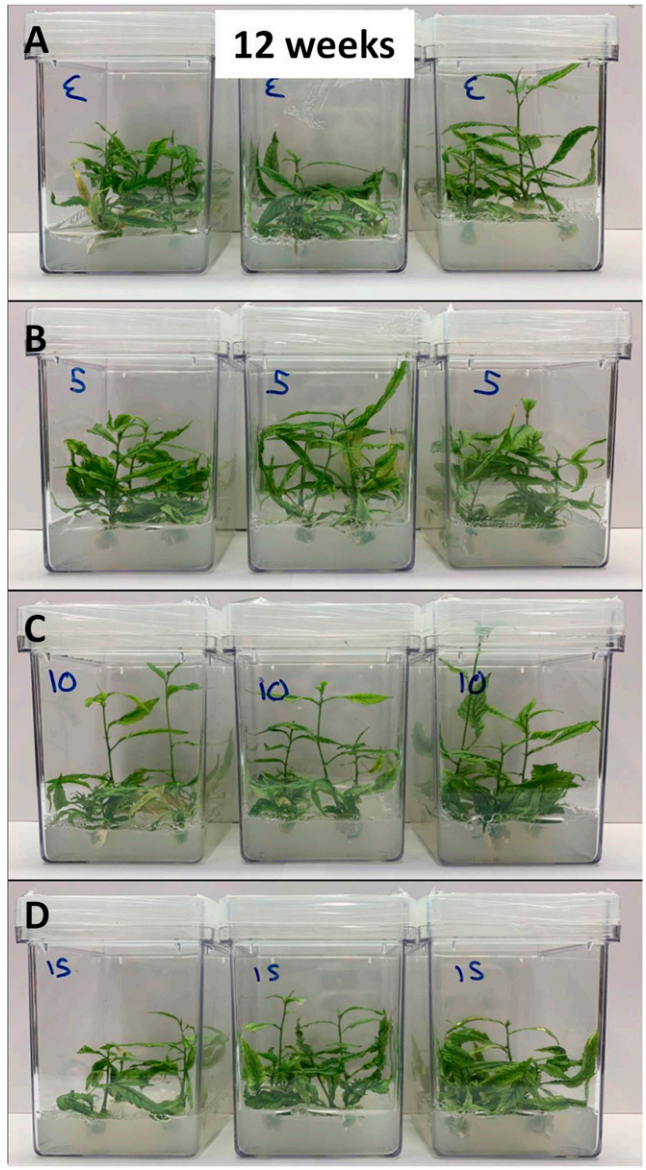

Fig. 3. In vitro cultures of hemp cultivar Wife after 12 weeks of growth on medium E comprising 1× Murashige and Skoog (MS) medium with vitamins plus added mesos and vitamins plus 3% (w/v) sucrose, 0.5 mg·L^{-1} metatopolin, 0.1 mg·L^{-1} gibberellic acid, and 0.8% agar (w/v) at pH 5.7 and supplemented with ammonium nitrate at: (A) 0 mg·L^{-1}; (B) 500 mg·L^{-1}; (C) 1000 mg·L^{-1}; and (D) 1500 mg·L^{-1}. 1 mg·L^{-1} = 1 ppm.

(Poothong and Reed, 2015; Wada et al., 2013). Other plants have benefited from higher amounts of N salts, such as stevia, which preferred 3× N (Poothong et al., 2018).

In vitro rooting of hemp microcuttings can be accomplished, but it is not practical for commercial production purposes (Lata et al., 2016). Growers have reported that rooting microcuttings ex vitro in humidity vessels with peat-based medium is largely unsuccessful. Our method of prerooting microcuttings in vitro followed by rooting ex vitro in rockwool has proven to be highly successful (Fig. 4). For five replications of 'Dinamed CBD' microcuttings ranging from 18 to 56, we achieved rooting of 100% (n = 18), 81% (n = 17), 75% (n = 52), 84% (n = 21), and 75% (n = 56). Two replications of 'Wife' with microcuttings of 12 and 56 rooted at 100% and 95%, respectively. According to our observations, the larger the microshoot, the better the rooting success and in vitro shoot multiplication potential.

We demonstrated that retipping using recently micropropagated hemp plants produces a sufficient number of liner plants for large-scale commercial production. Micropropagated plants that were recently acclimated to greenhouse conditions yielded ≈10 retip cuttings during weeks 1 and 2 of the 10-week propagation time course (Figs. 4 and 5). After the first 1 or 2 weeks, cutting productivity increased to a maximum of 50 cuttings per week (Fig. 5). For all three replications of retipping, cutting productivity remained high over the course of 10 weeks. Generally, ≥90% rooting success was achieved weekly, and rooting never decreased to less than 78%. Over the course of 10 weeks, averages of 90.8%

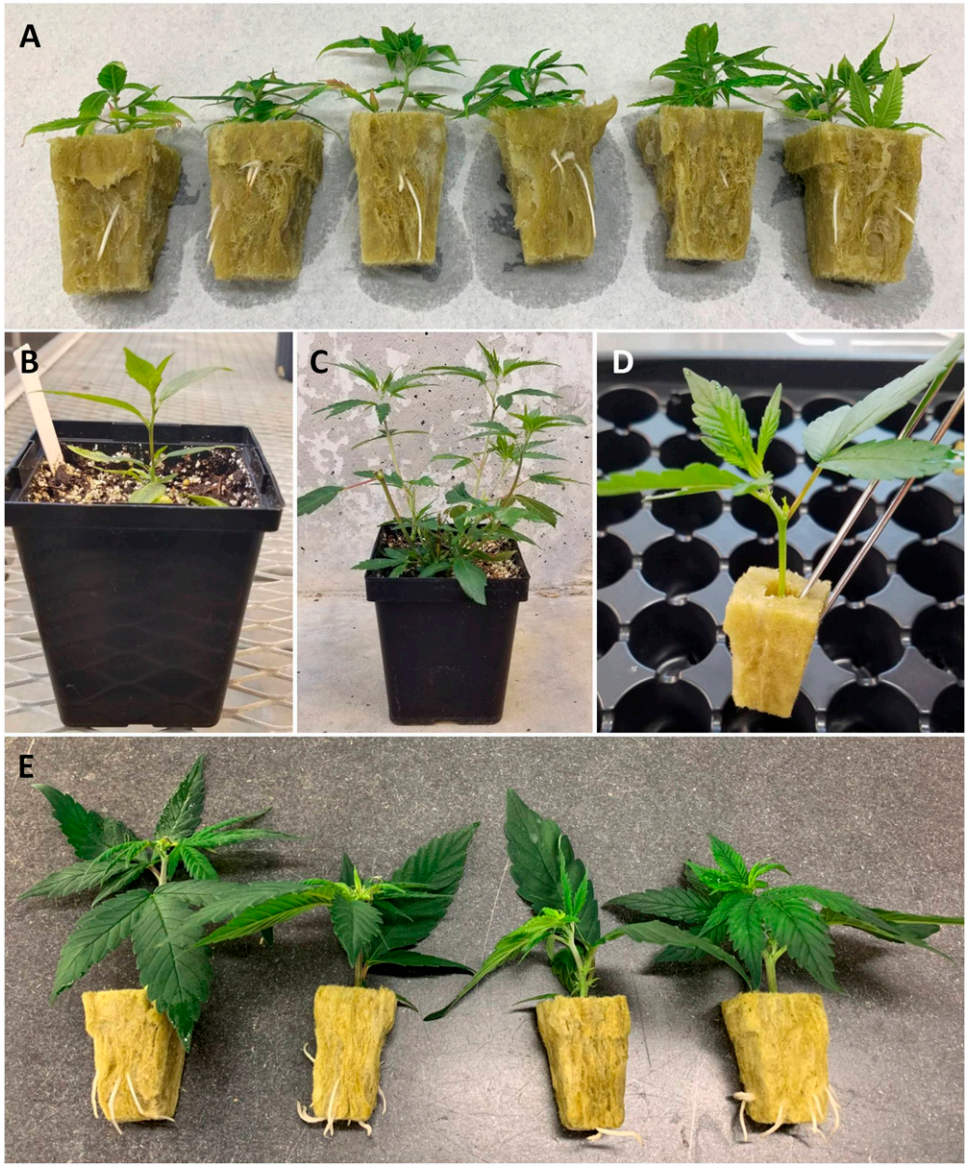

Fig. 4. Micropropagated and retip (cuttings of new shoots from recently micropropagated plants) hemp plantlets of 'Dinamed CBD' (A) 21 d after transfer to rockwool cubes ex vitro; (B) greenhouse-acclimated micropropagated plant 10 d after potting; (C) new shoot breaks on micropropagated stock plants after retipping; (D) retip cutting stuck in rockwool cube; and (E) rooted retip cuttings 21 d after sticking.

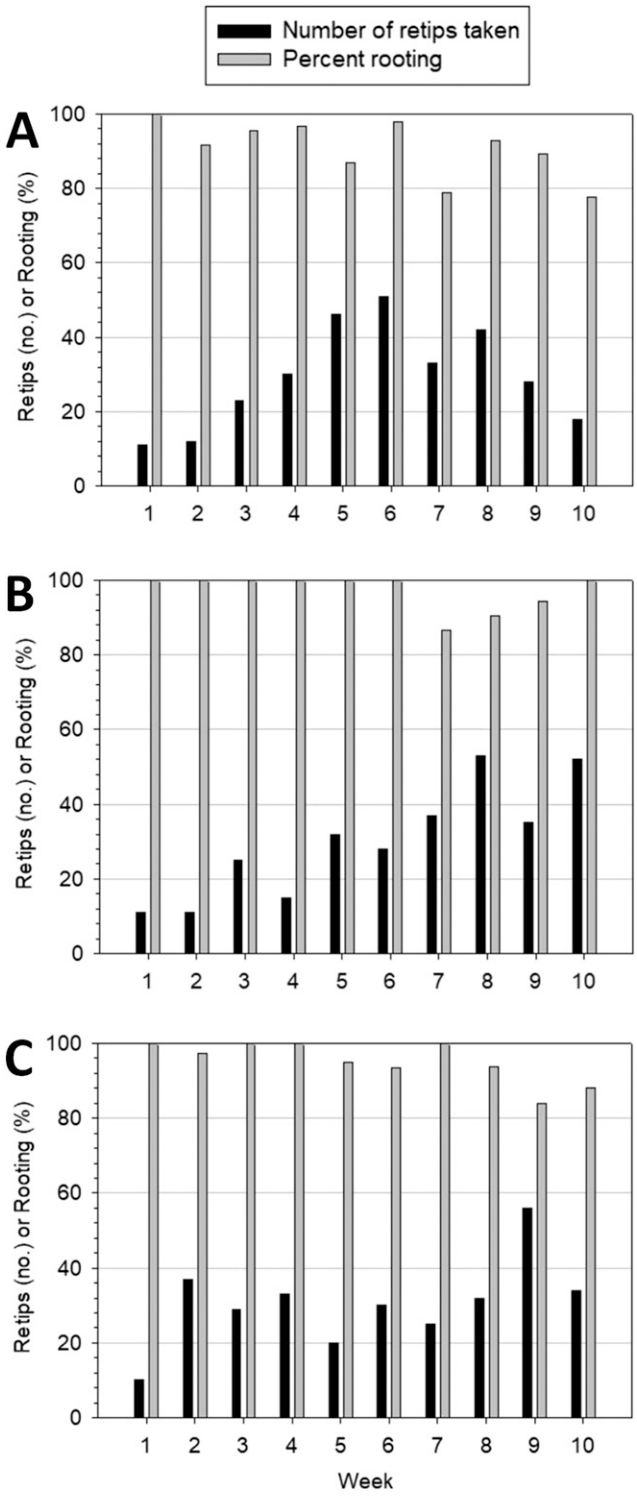

Fig. 5. Number of retips (cuttings of new shoots from recently micropropagated plants) taken per week from 10 micropropagated hemp stock plants and cutting percent rooting for (A) 'Dinamed CBD' initiated in July 2019; (B) 'Dinamed CBD' initiated in Aug. 2019; and (C) 'Wife' initiated in Apr. 2020.

and 96% rooting were achieved for 'Dinamed CBD' replication 1 and replication 2, respectively, and an average of 93.8% rooting was achieved for 'Wife'. Using traditional stem cuttings from mother plants, Caplan et al. (2017) reported maximum rooting success of 71%. Rooting success varied from 35% to 100% for three distinct hemp cultivars propagated from stem cuttings (Campbell et al., 2019). The rooting ability of retip cuttings was steady over 10 weeks, and it is likely that strong rooting would have continued for several more weeks. Using 10 micropropagated plants that use less than 0.25 m^2 of grow space, ≈300 retip cuttings were generated. Using a conservative 90% rooting rate, this translated to 270 plants within 10 weeks.

Our propagation method using retipping of micropropagated hemp yields more liners than the traditional method using mother plants and stem cuttings. Mother plants grown in 10-gal pots to provide cuttings for propagation occupy floor space of ≥1 m^2. A large mother plant may provide 50 to 60 cuttings every 2 weeks and, under ideal circumstances, cuttings root at 80%. This translates to 200 plants within 10 weeks from floor space of 1 m^2. The same 1 m^2 of floor space could hold 67 micropropagated plants that would produce nearly 1800 plants, which is nine-times the number of liners from traditional cutting mother plants. Furthermore, because of the small size of stock plants used for retipping, it is possible to have three sets of 67 stock plants stacked in a three-tier, shelved bench arrangement in 1 m^2 of floor space. Such a three-tier growing system containing 201 plants would produce 5400 retip liners. Plants from retip cuttings have been observed to grow vigorously at a similar or faster rate than plants from traditional vegetative cuttings.

The retip micropropagation system described will generate nine-times more hemp liners for commercial production in the same grow space as traditional mother plants and stem cuttings. Furthermore, liner plants produced through retipping are clonal, uniform, disease-free, and vigorous. Our in vitro shoot initiation and multiplication methods demonstrate improved hemp micropropagation results. We have eliminated shoot hyperhydricity, enhanced culture growth, and extended the time during which in vitro cultures remain productive. Although our method uses some micropropagated plants, much of the process only requires conventional propagation facilities and labor. Retipping takes advantage of all the benefits of micropropagation

while limiting the amount of tissue culture infrastructure that is needed by producers.

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