Peppermint and Spearmint Tissue Culture II

Dual-Carboy Culture of Spearmint Tissues

By CHING-JU WANG† and E. JOHN STABA

A 5-day old, 15 per cent (v/v) spearmint cell inoculum in a dual-carboy system containing modified T-medium with 2,4-dichlorophenoxyacetic acid (0.5 p.p.m.), Dow Corning-B antifoam (500 p.p.m.), and bacitracin (5 p.p.m.) is suggested for good cell suspension growth.

APPLICATIONS of plant tissue cultures may be forthcoming for evaluating herbicides and plant growth regulators, for the production of food, for testing drug efficacy and toxicity, and for the production of economically useful compounds. Basic microbial techniques have been used to grow large quantities of plant tissue in suspension cultures (1–3).

Air has been supplied to plant suspension cultures containing less than 0.5 L. of medium by agitating glass flasks on various shakers (4–7), or to larger volumes of medium by forcing sterile air through open-end tubing or spargers in a carboy or pilot-plant fermentor (1, 3, 8, 9). Agitation may be produced by the aeration process itself (1, 2) or by magnetic bar stirring devices (9).

This study reports the growth characteristics of spearmint (*Mentha spicata* L.) cell inocula of known cell number in dual-carboys receiving constant air flow and agitation. The effect of certain antifoam and antibiotic compounds on spearmint tissue growth is also reported.

EXPERIMENTAL

The spearmint stem-callus tissues studied were approximately 1-year-old and had been established by the method previously published (7). Suspension cultures in this study were maintained and subcultured on our modification of Murashige's and Skoog's tobacco medium (T-medium) with 0.5 p.p.m. of 2,4-dichlorophenoxyacetic acid (2,4-D). Murashige's and Skoog's tobacco medium (10) was referred to as modified Murashige's medium in our previous publications (7, 11).

Erlenmeyer Flask Cultures.—Preliminary experiments were performed in 250-ml. Erlenmeyer flasks containing 50 ml. of medium to determine the effects of cell inoculum concentrations, antifoams, and antibiotics on the growth of spearmint suspension cultures.

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The 15-day-old cell inocula for these experiments were prepared by a method previously published (12). After inoculation, the flasks were incubated on a reciprocal shaker (stroke: 9 cm.; rate/minute: 88), at room temperature (about 27°) and light. Relative growth was calculated as:

$$\text{Relative Growth (\%)} = \frac{\text{Growth average (four flasks) of each experiment}}{\text{Growth average (four flasks) of control}} \times 100$$

Dual-carboy Cultures.—Each carboy of the dual-carboy system (Fig. 1) was simultaneously aerated by a forced flow of sterile, moistened air through a T tube attached to a stainless steel sparger (porosity:

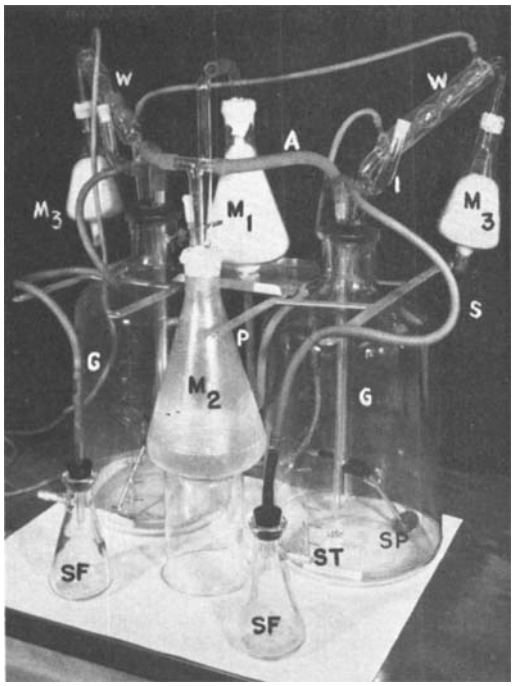

Fig. 1.—Dual-carboy apparatus. *A*, air inlet tubing connected to stainless steel tube and sparger; *I*, innoculation tube; *G*, 12-L. carboy; *M*, modified 1-L. flask with cotton for air sterilization; *M*2, 2-L. flask with distilled water for water saturation of air; *M*3, modified 500-ml. flask with cotton as air vent; *P*, air inlet tube connected to a gas flowmeter; *S*, sampling tube; *SF*, sampling flask; *SP*, stainless steel sparger; *ST*, magnetic bar; *W*, water condenser.

TABLE I.—OXYGEN ABSORPTION RATES (OAR) OF ERLENMEYER FLASKS AND CARBOYS I AND II

Aeration Condition	Volume Sulfite Soln., L.	DC-B Antifoam, p.p.m.	OAR (m*M* O_2/L./min.) Erlenmeyer[a]	Carboy I[b]	Carboy II[b]
Reciprocating shaker[c]	0.05	...	0.235	...	...
I[d]	3.0	500	...	0.068	0.066
	6.0	500	...	0.026	0.024
II[e]	3.0	500	...	0.117	0.117
	3.0	...	...	0.181	0.158
	6.0	500	...	0.058	0.055

[a] Erlenmeyer flask (250 ml.) with cotton plug. [b] Twelve-liter capacity. [c] Stroke: 9 cm.; rate/min.: 88, at room temperature. [d] Air flow: 4.5 L./min. at 28°. [e] Air flow: 27 L./min. at 28°.

TABLE II.—EFFECT OF INOCULUM SIZE ON THE GROWTH OF SPEARMINT TISSUE IN ERLENMEYER FLASKS[a]

	Inoculum Size[b] 0.25	1.0	2.0	3.0
Days	Growth[c]	Growth[c]	Growth[c]	Growth[c]
15	22.6	211.6	361.6	474.4
30	480.8	495.6	477.3	474.0

[a] Flasks (250 ml.) with 50 ml. of medium. [b] Milligrams cellular dry weight per flask. [c] Milligrams cellular dry weight after 15 or 30 days culture (average of four replicates).

TABLE III.—EFFECTS OF ANTIFOAM AGENTS ON SPEARMINT SUSPENSION GROWTH

Antifoam	Source	Compn.	Concn. Studied[a] Low	High	Relative Growth, %[b] Low Concn.	High Concn.
DC-A	Dow Corning (Mich.)	Silicone 100%	10	100	94.3	99.4
DC-B	Dow Corning (Mich.)	Silicone 10%	100	1000	99.3	96.5
FD-62	Hodag (Ill.)	Silicone 10%	100	1000	99.5	95.1
GE-60	General Electric (N. Y.)	Silicone 30%	30	300	97.7	98.2
Troy-333	Troy (N. J.)	Non-silicone 100%	10	100	104.5	99.1
Safflower oil	.....	Fatty acids	100	..	109.7	..

[a] Expressed in p.p.m. [b] Relative growth was determined after 21 days of culture. Each flask received 1 ml. of inoculum containing 1.9×10^6 cells (3.5 mg. cellular dry weight).

10 μ).[1] Agitation was produced by a 7.5-cm. Teflon-coated magnetic bar rotating at 90 r.p.m. on the base of each carboy. Two rates of air flow were studied; 4.5 L./minute (aeration condition I) and 27 L./minute (aeration condition II). The air flow was measured through a gas-flow meter[2] at room temperature. Pressures over the sulfite medium were 1.16 Atm. for the slower flow rate and 1.33 Atm. for the faster one. The dual-carboy system was mounted in a water bath maintained at 28°. Measured temperature variance of the medium within the carboys was negligible.

Oxygen absorption rates (OAR) of aqueous sulfite solutions in the dual-carboy system and Erlenmeyer flasks under the conditions specified in Table I were determined by standard procedures (13, 14).

The inocula for the dual-carboy system were prepared as follows. Approximately 100 ml. of 15-day-old cell suspension was transferred aseptically into a 2-L. Erlenmeyer flask containing 300 ml. of medium and grown for 5 days on the reciprocal shaker. This new cell suspension was then aseptically passed through a 602μ-mesh nylon sieve[3] and used as inocula for the carboys. Each carboy was inoculated with 15% by volume of new cell suspension and cultured in the water bath at 28° for 8 days. A sample was taken daily from each carboy for 8 days and examined for cell concentration, cellular dry weight, and the pH change in the medium.

Semiquantitative determinations were made on each medium sample for dextrose, fructose, and sucrose by a chromatographic procedure previously published (12). Aqueous standard solutions of sucrose (1.5%), fructose (1.5%), and dextrose (1.0%) were used. Whatman No. 1 chromatographic papers were spotted with the standard solutions (2, 4 and 10 μl.), control nutrient medium (10 μl.), and the medium filtrates (10 μl.). The presence of dextrose was confirmed by Clinistix[4] test paper.

RESULTS

Erlenmeyer Flask Cultures.—The heaviest inoculum (3 mg. cellular dry weight/flask) produced a maximum yield within 15 days (Table II). With smaller inocula (0.25, 1 and 2-mg. cellular dry weight/flask), proportionately less growth resulted within 15 days, but the cultures achieved a maximum growth within 30 days.

None of the six antifoam compounds studied at the low or high concentration levels appreciably affected spearmint tissue growth (Table III).

Amphotericin, griseofulvin, and/or oxytetracycline (5 p.p.m.) inhibited spearmint tissue suspension growth. The effects of penicillin G, bacitracin, tylosin, tylosin with nystatin, and nystatin are shown in Table IV. Tylosin and griseofulvin were autoclaved with the medium, but the other antibiotics were added aseptically to the medium after dilution with sterilized double-distilled water.

[1] Scientific Glass Apparatus Co., Inc., Bloomfield, N. J.
[2] Brooks Rotameter Co., Lansdale, Pa.
[3] Trobler, Ernst and Traber, Inc., New York, N.Y.
[4] Ames Co., Inc., Elkhart, Ind.

TABLE IV.—EFFECT OF ANTIBIOTICS ON SPEARMINT SUSPENSION GROWTH

Antibiotic	Concn.[a]	Relative Growth, %[b] Generation 1	Generation 2	Generation 3
Penicillin G[c]	60	101.7	...	...
Bacitracin[c]	5	92.8	106.3	...
Tylosin[c]	100	46.9	112.3	100.7
Nystatin and tylosin[c]	25 20	23	...	...
Nystatin[d]	25	52.2	...	...
	50	11.8	...	...
	125	0.0	...	...

[a] Expressed in p.p.m., except nystatin which is expressed as u./ml. [b] Determined after 15 days of culture. [c] Each flask received 4 ml. of inoculum containing 872 × 10^3 cells (2.3 mg. cellular dry weight). [d] Each flask received 4 ml. of inoculum containing 338 × 10^3 cells (1.6 mg. cellular dry weight).

Bacitracin (5 p.p.m.) was routinely used as a constituent in the medium for dual-carboy cultures.

The OAR values obtained under certain specified conditions for 250-ml. Erlenmeyer flasks and the dual-carboy system are shown in Table I. At no time did the OAR values for the sulfite medium in the dual-carboy system exceed the value for the flasks.

Dual-carboy Cultures.—Preliminary growth studies in the dual-carboy system indicated that a 15% (v/v) inoculum resulted in greater yields than a 5% (v/v) inoculum, and that a 5-day-old inoculum resulted in greater yields than did a 30-day-old inoculum.

The results obtained from carboy I under aeration condition I are shown in Fig. 2. The growth yield of spearmint tissue in carboy I under aeration condition I was 112.13 Gm. fresh weight, and that in carboy I under aeration condition II 95.70 Gm. fresh weight after an 8-day growth cycle. The growth yield in carboy II was 10% of that of carboy I under aeration condition I and 40% of carboy I under aeration condition II.

The utilization of sucrose, fructose, and dextrose by tissues in carboy I grown under aeration condition I is shown in Fig. 3.

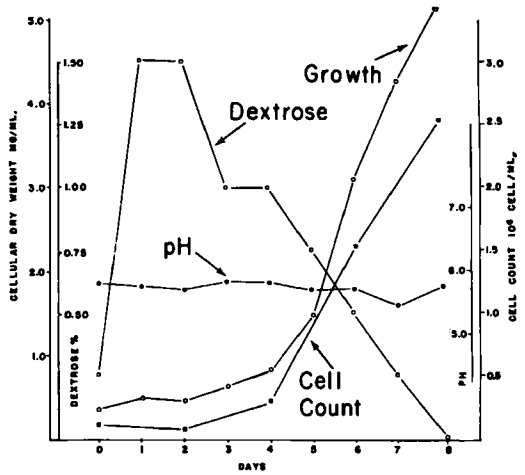

Fig. 2.—Spearmint suspension growth in carboy I containing 3 L. of modified T-medium with DC-B antifoam (500 p.p.m.) and bacitracin (5 p.p.m.). Inoculum: age, 5 days; quantity, 15% v/v; dry weight, 2 mg./ml.; cell number, 824 × 10^3 cells/ml. Final growth yield under aeration condition I: 112.13 Gm. fresh weight.

DISCUSSION

Torrey (15) has suggested that the smaller the inoculum the more complex the medium must be to reduce the time required for cells to "condition" the medium. Blakely and Steward (16) have reported that filtered and rigorously washed low-cell-content inocula of angiosperm cells on agar medium exhibited a very low frequency of colony formation. Our results indicate that the smaller the inoculum concentration of rigorously washed spearmint cells, the longer the lag phase of suspension growth. After 30 days of culture, however, the four inoculum concentrations tested resulted in a similar growth yield (Table II).

Tulecke and Nickell (2) used 300 p.p.m. of Dow Corning-A (DC-A) in their carboy cultures of various plant tissues with no apparent toxicity. We selected DC-B (500 p.p.m.) for routine use because of its lack of toxicity to spearmint suspension culture and the ease with which it may be dispersed in an aqueous medium.

Certain antibiotics have been reported to control microbial contamination in plant tissue cultures (2, 3, 17–19). However, none appear to have been accepted as a routine constituent of plant-growth media. Although nystatin (25 u./ml.) with tylosin (20 p.p.m.) is reported not to inhibit carrot, grape, and rose suspension cultures, we found that nystatin (25 u./ml.) or nystatin (25 u./ml.) with tylosin (20 p.p.m.) will inhibit spearmint suspension growth. Penicillin (10 u./ml.) and bacitracin (5 p.p.m.) is reported to stimulate the growth of sorrel tumor tissue (17), and penicillin (20 p.p.m.) not to inhibit Ginkgo-pollen, holly-stem, or rose-stem-callus suspension growth (2). Penicillin (60 p.p.m.) and bacitracin (5 p.p.m.) were not inhibitory to spearmint suspension cultures. Bacitracin (5 p.p.m.) was routinely used in media for dual-carboy cultures.

Convolvulus cell suspensions are reported to divide most frequently 1 week after inoculation and *Haplopappus* cell suspensions 3 days after inoculation (20). Similarly, our preliminary studies indicated that a 5-day-old, 15% v/v inoculum passed through a 602 μ-mesh nylon sieve gave more growth in the dual-carboy system than either 15 or 30-day-old tissue inocula.

Plant tissue culture growth may be reported as Growth Index (GI) values

$$GI = \frac{\text{Final fresh weight growth}}{\text{Initial fresh weight inoculum}} \quad (2).$$

The Growth Efficiency Index (GEI) value suggested

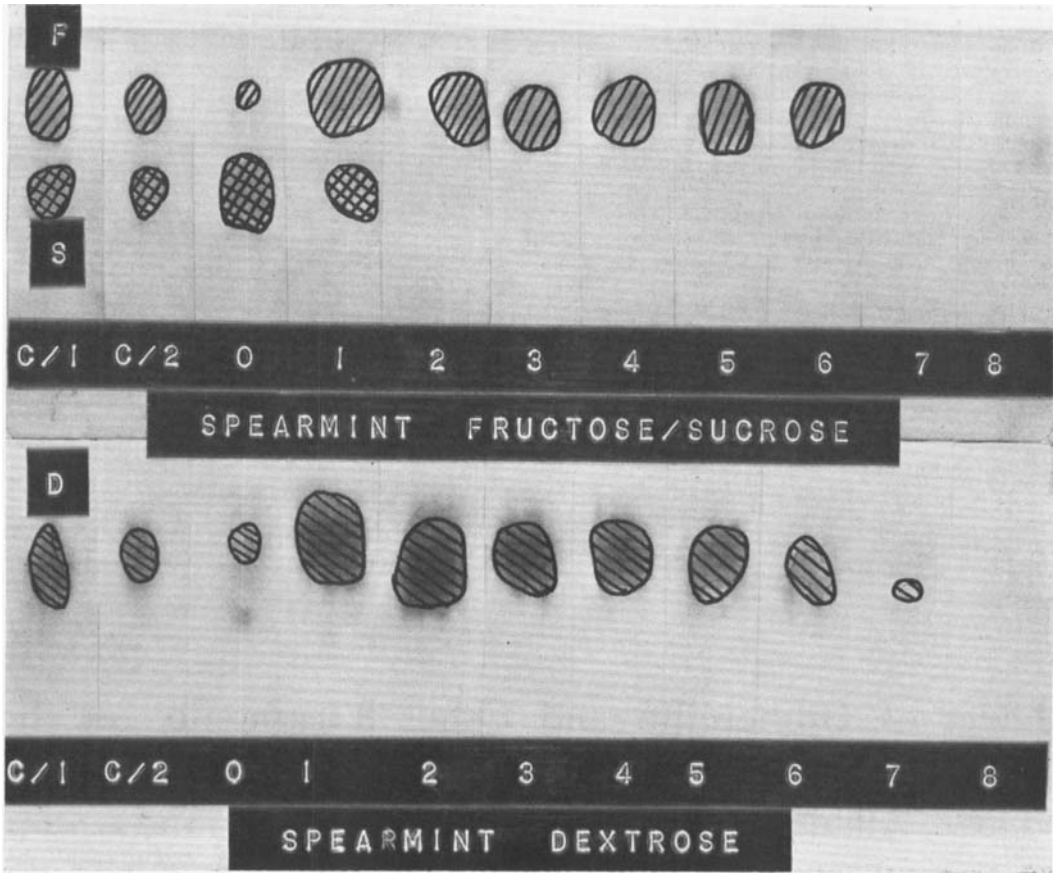

Fig. 3.—Chromatograms of fructose (*F*), sucrose (*S*) and dextrose (*D*) samples (10 μl.) taken at daily intervals (0–8) from the spearmint suspension culture described in Fig. 2. C/1: control *F*, *S*, *D*, (0.15 mcg.); C/2: control *F*, *S*, *D*, (0.075 mcg.).

below would consider the GI at maximal growth, as well as the volume of medium used and the time required for maximal tissue growth. The GEI is expressed as

$$\text{GEI} = \frac{\text{GI at maximal growth}}{\text{Medium (L.)} \times \text{days required for maximum growth}}$$

The GI and GEI values calculated for Ginkgo-carboy cultures were 8.7 and 0.03, respectively (2), and 14.9 and 0.11 for rose carboy cultures, respectively (1). The GI and GEI values for the spearmint carboy culture shown in Fig. 2 are 20.8 (112.13/5.4) and 0.87 (20.8/3 x 8), respectively. The GI and GEI values shown in Table II for the 3.0-mg. dry weight inocula grown in 250-ml. Erlenmeyer flasks are 157.3 (3.46/0.22) and 209.7 (157.3/0.05 × 15), respectively. Dry weight of spearmint cell tissues may be converted to approximate tissue fresh weight by the factor 7.3.

The poor growth repeatedly obtained in carboy II of the dual-carboy system suggested that the stainless steel sparger used in carboy II might be toxic to spearmint suspension cultures. Dyer and Richardson (21) reported that types 316 and 304 stainless steels occasionally slightly inhibited algal cultures. The effects of the presence of six different stainless steel spargers on spearmint suspension growth were tested by growing each sparger separately with cells in Erlenmeyer flasks. Various degrees of growth inhibition were observed, varying from complete inhibition or good growth with blackening of the suspension medium, to normal growth.

Spearmint suspensions grown in carboy I gave similar growth yields with either 4.5 L. of air/minute or 27 L./minute. Therefore, it appears that the amount of air supplied by the lower flow rate is adequate for good growth of spearmint tissue in the dual-carboy system used.

Sequoia semipervirens callus cultures and *Vinca rosea* crown-gall callus cultures hydrolyze certain oligosaccharides (22). Tulecke, *et al.* (23), reported that fructose and dextrose predominated in Ginkgo pollen itself. Bürstrom (24) demonstrated that excised roots hydrolyze sucrose in liquid media. In carboy I of the dual-carboy system (Figs. 2 and 3) sucrose was completely hydrolyzed to dextrose and fructose by the third day of culture. Both dextrose and fructose were absent in the culture medium after 8 days.

CONCLUSIONS

A dual-carboy system enabled 112.13 Gm. of spearmint tissue (GI, 20.8; GEI, 0.87) to grow in one carboy receiving an inoculum of known cell volume (15% v/v), age (5 days), cell number

(824×10^3 cells/ml.), and air flow (4.5 L. air/minute) within 8 days. An air flow rate of 27 L./minute did not increase growth materially.

Spearmint suspensions in the dual-carboy system completely hydrolyzed sucrose to dextrose and fructose by the third day of culture. Both dextrose and fructose were absent in the culture medium after 8 days.

None of six antifoam compounds studied appreciably affected spearmint suspensions. Dow Corning-B (500 p.p.m.) is suggested for foam control.

Five parts per million of either amphotericin, griseofulvin, and/or oxytetracycline inhibited spearmint suspension growth. Bacitracin (5 p.p.m.) is suggested for bacterial prophylaxis.

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Effect of Gibberellin and Other Treatments on the Germination and Subsequent Biogenesis of Alkaloids in *Datura stramonium* Linné

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Stramonium **seeds were subjected to two "cold treatments," to treatment with gibberellic acid (GA) and with concentrated sulfuric acid (SA). The rate and percentage of germination were markedly reduced by the cold treatments, whereas they were significantly increased by the GA and SA treatments. A threefold increase in germination percentage was noted in the former group and a twofold increase in the latter. Plants from the GA, SA, and control groups were allowed to grow to maturity. Characteristic gibberellin effects were not noted in plants receiving a gibberellin-seed treatment. However, the early growth of this group was markedly reduced. At the final harvest total plant weight was slightly less than controls. Fluctuations in the alkaloid concentration of the plant organs were noted. At the final harvest the total content of alkaloids was about 87 per cent of controls. The growth rate of the SA group was considerably lower than controls initially, but, the total dry weight at the final harvest approximated the controls. Both decreases and increases in the concentration of alkaloids in the plant organs were observed. The total plant alkaloids of this group at the final harvest were about the same as controls.**

Treatment of seeds with gibberellic acid (GA) has hastened germination and promoted an earlier and more uniform emergence of the seedlings of many crop plants (1–8) and several medicinal plants (9–11). Treatment of belladonna seeds with concentrated sulfuric acid resulted in an increase in the germination rate (12). Acid scarification and various types of "cold treatments" have been recommended as pregermination treatments of seeds to stimulate germination (13, 14). A review of the literature, however, has indicated that a study of such treatments on the subsequent growth and alkaloid biogenesis of stramonium has not been conducted.

The purpose of this study was (*a*) to determine and compare the effects on the germination of stramonium seeds of a freeze-thaw method (FT), a constant freeze method (CF), treatment with GA, and treatment with concentrated sulfuric acid

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