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VOLATILE EMISSIONS OF PLANT TISSUE CULTURES

I. Identification of the Major Components

DONOVAN DES S. THOMAS[1] AND TOSHIO MURASHIGE[2]

Department of Botany and Plant Sciences, University of California, Riverside, California 92521

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SUMMARY

The low-molecular-weight volatiles released by a variety of plant tissue cultures were examined by gas chromatography. Callus cultures invariably produced carbon dioxide, ethylene, acetaldehyde and ethanol. In cultures with developed shoots, ethanol was absent and acetaldehyde was detected only rarely.

Key words: carbon dioxide; ethylene; acetaldehyde; ethanol; plant tissue culture.

INTRODUCTION

During the course of development in vitro, plant tissues not only deplete the nutrients that are furnished in the medium, but also release substances that can accumulate in the cultures. These substances, some of which may have profound physiological effects on the cultured tissues, include volatile and nonvolatile compounds. This research was focused on the volatile products that accumulated in a variety of plant tissue cultures. Identifications were made of the low-molecular-weight emanations that were ubiquitous or encountered in substantial quantities. Their physiological significance and factors that might influence their synthesis and release remain under investigation.

Autogenous volatile growth factors have been reported in cell suspension cultures. In *Acer pseudoplatanus* L. cultures, growth was enhanced by a substance that was absorbed by KOH but could not be replaced by carbon dioxide (1). The growth inhibition by an unidentified factor produced by cultures of *Atropa belladonna* was not attributable to oxygen depletion or toxic levels of carbon dioxide or ethylene (2). Talbot and Street (3) reported enhanced growth of cultured wheat roots in the presence of an unidentified volatile root metabolite. The substance was not absorbed by mercuric perchlorate, indicating that it was not ethylene. Ethylene production is common to a number of plant cell cultures (4,5). Recently, ethanol was shown to be a product of excised citrus ovules (6). Volatiles of higher molecular weight, such as terpenoids and volatile oils, also have been reported in some tissue cultures, e.g. in *Andrographis paniculata* (7) and *Ruta graveolens* (8).

MATERIALS AND METHODS

Tissue culture. Cultures were grown in 25- by 150-mm glass culture tubes, each containing 25 ml nutrient agar and capped with a polypropylene closure (Kaput, Bellco). Four types of cultures were examined: (a) callus only; (b) cultures with fully developed shoots, identified in the data as "proliferating shoot cultures"; (c) "woody nodal explants," excised to include the nodal region of a woody stem with an unemerged bud; and (d) "herbaceous nodal explants" from herbaceous species that have proved difficult to culture in vitro. The *Daucus* and *Nicotiana* callus cultures were grown in the dark. All other cultures were maintained at 27° C under a daily regime of 16 hr illumination with 1000 lx from Gro Lux lamps. The nutrient media varied with the species and type of tissue being cultured. All cultures were allowed to develop for at least 2 weeks within the passage before sampling for gas analysis. Triplicate cultures were sampled. No ethanol was used in medium preparation or in disinfestation of tissues or instruments.

Gas sampling. Cultures were prepared for gas sampling by replacing the polypropylene Kaputs

[1]On sabbatical leave from Department of Biology, University of Windsor, Windsor, Ontario N9B 3P4, Canada.

[2]To whom requests for reprints should be addressed.

(Bellco) with rubber vaccine caps or Kaputs that had been modified for gas sampling. The modification of the Kaput involved a 2-mm perforation at its top center, sealed with two layers of builders' duct tape (Frost King; Thermwell Products Co., Inc., Los Angeles, California). Flaming of the culture tube rim was omitted since this caused evolution of methane and other volatiles from the vaccine caps. Gas samples were obtained by inserting a syringe needle through the vaccine cap or the duct tape seal. Glass tuberculin syringes (B-D Glaspak, 1-cc), with Yale 22 stainless-steel needles were used to remove the gas. Samples were obtained when 9 to 12 hr of the light cycle had elapsed; times between preparation for sampling and sample extraction are given in the Results.

Gas analysis. The procedure of Negm, Smith and Kumamoto (9), which is restrictive (under the conditions used) to low-molecular-weight volatiles, was employed. Gas samples (0.5 ml) were analyzed on a Beckman GC-4 dual hydrogen flame gas chromatograph with a 285- by 0.32-cm stainless-steel column packed with 50- to 80-mesh Porapak Q (Waters Associates Inc., Milford, Massachusetts). Column eluates were passed, via a thermal conductivity detector to monitor CO_2, to the hydrogen flame ionization detector to determine the other gases. The concentrations of carbon dioxide, ethylene and ethane were determined by calibration with standard gas mixtures. Ethanol and acetaldehyde were identified by cochromatography with standards at 78° and 100° C, and the quantities in the gas phase within the cultures (volume taken as 30 ml) were estimated from calibration curves established from aqueous solutions of standards. After completion of gas analysis, plant tissues were weighed and gas values were adjusted to a 1-g fresh-weight basis. Ethylene values were corrected to compensate for loss of approximately 6% per day from vaccine-capped tubes.

RESULTS AND DISCUSSION

Gases detected and identified from tissue cultures were carbon dioxide, ethylene, ethane, acetaldehyde and ethanol, with gas chromatographic retention times at 78° C of 1.3, 1.9, 2.3, 18.5 and 58 to 60 min, and at 100° C of 1.0, 1.3, 1.6, 8.3 and 19.7 min. Nutrient media alone showed no emission of volatiles. *Daucus* and *Lactuca* callus sometimes also showed a small unidentified peak (possibly formaldehyde or methanol) immediately preceding the acetaldehyde. Volatiles determined in cultures 1 day after closing tubes with vaccine caps are shown in Table 1.

Carbon dioxide levels tended to be lowest in cultures with well developed shoots (Table 1*B*). Some of these cultures, such as *Clerodendrum* and *Dicksonia*, maintained a virtually carbon-dioxide-free atmosphere in the light. Since these cultures accumulated carbon dioxide in the dark, the low levels in light are presumably a result of photosynthetic utilization.

Ethylene production showed a variable pattern, with the highest levels in two of the callus cultures, *Nicotiana* and *Phoenix* (Table 1*A*). The rate of ethylene production was low for *Nicotiana* callus (19.8 nmol per g fresh weight per day) compared with values for *Glycine* (123.1 nmol per g fresh weight per day) and *Rosa* (438.01 nmol per g fresh weight per day) calculated from maximum rates obtained in suspension cultures by other investigators (5). The average ethylene production in cultures with shoots was 0.74 nmol per g fresh weight per day, which may be compared with a rate of 0.43 nmol per g fresh weight per day from etiolated pea hypocotyl tissue calculated from data of Burg and Burg (10). Ethylene production was not detected in *Adiantum cuneatum* and *Ficus benjamina* cultures; the amounts observed did not exceed the background level of 0.04 ± 0.02 ppm.

The ethane levels, observed frequently, approximated those in room air (0.05 ± 0.06 ppm). Acetaldehyde and ethanol, absent from most cultures with shoots, were characteristic of the callus cultures and the woody nodal explants (Table 1*C*).

The acetaldehyde and ethanol cannot be attributed to increased levels of carbon dioxide. The highest levels of accumulated carbon dioxide (as evident in *Apium* and *Daucus* callus cultures) were associated with a relatively low content of acetaldehyde and ethanol. In the cultures with shoots, none of which yielded ethanol, the levels of carbon dioxide sometimes exceeded those observed in callus cultures.

The herbaceous nodal explants (Table 1*D*) represented cultivars which, under the culture conditions used, failed to manifest bud emergence or callus formation and died within a relatively short period (about four weeks). These explants showed high levels of CO_2 and ethylene production compared to most cultures with profuse shoot development. Ethanol was present in *Pelargonium* (but not in *Begonia*) cultures.

TABLE 1

VOLATILE EMISSIONS OF PLANT TISSUE CULTURES SAMPLED 24 HR AFTER SEEDING WITH VACCINE CAPS

Plant Material	Volatiles/g Fresh Weight/Day					
	Fresh Wt.	CO_2	C_2H_4	C_2H_6	CH_3CHO	CH_3CH_2OH
	g	%	*ppm*	*ppm*	*nmol*	*nmol*
A. CALLUS						
Arecaceae						
Phoenix dactylifera L.	1.04±0.13 [a]	3.58±2.01	12.27±0.35	0.06±0.03	60.39±24.24	811.58±295.21
Geraniaceae						
Pelargonium graveolens 'Old-Fashioned Rose'	0.43±0.4	2.28±0.12	1.50±0.06	0.30±0.05	44.17±31.25	244.25±207.45
Solanaceae						
Nicotiana tabacum L. 'Wisconsin 38'	0.69±0.10	2.27±0.14	16.93±2.63	0.08±0.07	1.20± 0.17	19.00± 3.175
Umbelliferae						
Apium graveolens L. var. *dulce*	1.05±0.11	19.56±3.29	0.17±0.04	0.04±0.03	7.09± 1.02	28.78± 13.95
Daucus carota L. var. *sativus* Hoffm.	0.82±0.09	6.87±1.82	0.04±0.03	0.12±0.01	14.01± 0.46	3.79± 1.24
B. PROLIFERATING SHOOTS						
Araliaceae						
Tupidanthus calyptratus Hook. f. and T. Thoms.	6.25±2.44	0.05±0.01	0.20±0.07	0.02±0.01	0.15± 0.07	0
Berberidaceae						
Nandina domestica Thunb.	0.31±0.03	0.39±0.24	1.39±0.44	0.32±0.14	0	0
Compositae						
Gerbera jamesonii H. Bolus ex Hook f. cv.	2.85±1.06	2.53±0.91	0.78±0.07	0.08±0.06	0	0
Dicksoniaceae						
Dicksonia antarctica Labill.	0.30±0.00	0	0.90±0.42	0.43±0.23	6.61± 5.01	0
Lauraceae						
Persea americana var. drymifolia (Schlechtend and Cham.) S.F. Blake cv. Topa	0.30±0.17	0.01±0.02	0.68±0.65	0.06±0.11	0	0
Liliaceae						
Ophiopogon japonicus Thunb. cv. nana	2.30±0.73	1.49±0.85	0.40±0.12	0.06±0.03	0	0
Moraceae						
Ficus elastica Roxb. ex Hornem.	2.08±0.68	4.81±0.69	1.24±0.80	0.26±0.26	0	0
Ficus benjamina L.	4.64±0.13	0.12±0.15	0.01±0.20	0.03±0.01	0	0
Ficus lyrata Warb.	6.39±0.73	1.33±0.05	0.24±0.07	0.02±0.00	0	0
Polypodiaceae						
Adiantum caudatum L.	0.85±0.15	0.13±0.22	0.31±0.08	0.13±0.09	0	0
Adiantum cuneatum Langsd. and Fisch.	5.44±0.01	0.21±0.03	0.04±0.00	0.02±0.00	0	0
Davallia trichomanoides Blume.	3.75±0.11	0.29±0.06	0.15±0.00	0.03±0.00	0	0
Nephrolepis exaltata (L.) Schott.	1.68±0.44	0.61±1.05	0.41±0.33	0.13±0.07	0	0
Polystichum pungens	1.60±0.35	0.55±0.74	0.60±0.25	0.22±0.08	0	0
Saxifragaceae						
Saxifraga stolonifera Meerb. 'Tricolor'	0.39±0.17	3.58±0.60	2.06±1.08	0.46±0.24	0	0
Verbenaceae						
Clerodendrum thompsoniae Balf.	0.43±0.05	0	0.67±0.14	0.35±0.06	0	0

TABLE 1 (continued)

Plant Material	Fresh Wt.	Volatiles/g Fresh Weight per Day				
		CO_2	C_2H_4	C_2H_6	CH_3CHO	CH_3CH_2OH
	g	%	*ppm*	*ppm*	*nmol*	*nmol*
C. WOODY NODAL EXPLANTS						
Lauraceae						
Persea americana var. drymifolia (Schlechtend and Cham.) S.F. Blake						
'Topa'	0.52±0.28	4.24±1.73	1.01±0.26	0.17±0.05	21.75±14.71	142.37± 92.81
'Duke 7'	0.40±0.11	2.16±0.44	0.89±0.32	0.17±0.07	18.94± 3.70	145.66± 58.05
D. HERBACEOUS NODAL EXPLANTS						
Begoniaceae						
Begonia X hiemalis Fotsch.						
'Fantasy'	0.02±0.00	8.93±6.18	0.91±0.99	3.20±5.54	0	0
'Pink Fantasy'	0.02±0.00	10.41±4.92	1.44±1.26	3.67±4.45	0	0
Geraniaceae						
Pelargonium X hortorum						
'Penny'	0.70±0.29	5.22±0.06	1.53±0.22	0.45±0.02	0	1.88± 2.66
'Snowball'	0.40±0.02	6.45±0.19	1.83±0.63	0.42±0.11	5.26±7.43	22.47± 31.77
Pelargonium peltatum						
'Galilee'	0.43±0.24	3.64±3.20	1.03±0.60	0.25±0.10	0	39.48± 55.83

[a] Mean ± standard deviation; $n = 3$.

Cultures, sealed with vaccine caps prior to the gas determinations reported in Table 1, might be expected to generate metabolites, such as acetaldehyde and ethanol, which are associated with anaerobic respiration. Fig. 1*A,B* shows the results of experiments in which restricted aeration was imposed for 7 days on cultures of *Phoenix* and *Nicotiana* callus. Volatiles that accumulated during the 1-day period before sealing with vaccine caps were measured in cultures with polypropylene Kaputs modified for gas sampling. Hence the gas levels characteristic of Kaput-closed cultures were determined. These levels are represented in Fig. 1*A,B* as day 0 values.

All gases in this study, namely carbon dioxide, ethylene, acetaldehyde and ethanol, were present. The acetaldehyde and ethanol in cultures sealed for 1 day with vaccine caps (Table 1) were not simply a response to respiratory stress imposed by the sealing procedure. The levels of ethanol in the *Phoenix* cultures were approximately two orders of magnitude higher than in those in *Nicotiana* (necessitating different scales in Fig. 1*A,B*). The effects of sealing, initiated on day 0, varied with the gases, but were strikingly similar in callus of both *Phoenix* and *Nicotiana*. Linear increases in carbon dioxide indicated a fairly constant respiratory rate over the 7-day period.

Ethylene increased rapidly in response to sealing, especially in *Nicotiana*. However, the experimental method did not distinguish between increased rate of synthesis and decreased rate of loss. The eventual decline in ethylene levels in *Nicotiana* callus cultures, despite the correction made for loss through the vaccine caps, may be attributable to the solution of ethylene in the aqueous culture medium or to catabolism (11). Abeles (12) reported that carbon dioxide could, depending on the tissue, inhibit or promote ethylene synthesis, whereas low levels of oxygen typically inhibited ethylene production. Acetaldehyde showed little increase between days 3 and 7 after sealing. Ethanol levels showed a 3-day lag before the onset of stress-induced increases.

Volatiles during the 1st day after sealing showed increases only in carbon dioxide and ethylene. Control cultures, enclosed with Kaputs instead of vaccine caps during the experiment, showed relatively small or no increase in volatiles over the 7-day period of the experiment.

Conspicuous in the survey was the presence of ethanol and acetaldehyde in all callus cultures examined. This contrasted with undetectable levels of these metabolites in cultures with well developed shoots. Whereas the status of carbon dioxide and ethylene as regulators of plant development is established, other volatiles such as ethanol have received less study. Growth of certain plants or their organs has been reported to be stimulated by ethanol (3,13–16). An alco-

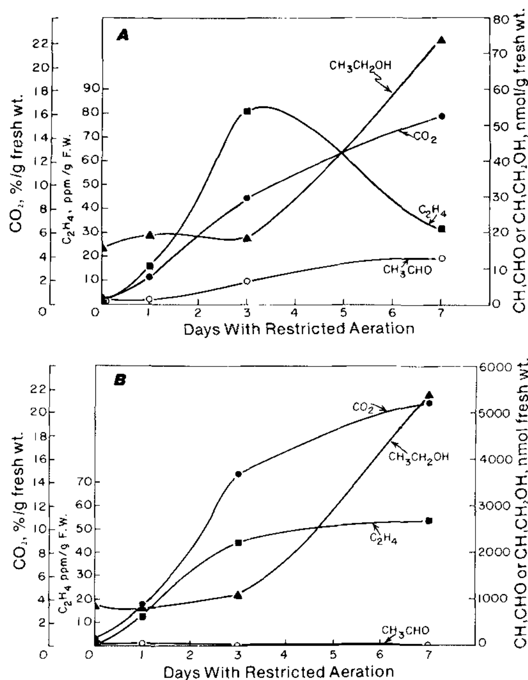

FIG. 1. *A*, Accumulation of volatiles in *Nicotiana tabacum* callus cultures sealed (day 0) with vaccine caps. In controls with Kaputs, increases over the 7-day period were obtained in ethylene (28%), acetaldehyde (64%) and ethanol (41%). *B*, Accumulation of volatiles in *Phoenix dactylifera* callus cultures sealed (day 0) with vaccine caps. In controls with Kaputs, only acetaldehyde and ethanol increased (by 32% and 50%, respectively) during the experiment.

hol:NAD^+ oxidoreductase that produces ethanol is considered likely to be ubiquitous in plants (17). Recently, exogenous ethanol, in concentrations approximately 10-fold lower than those we now report for *Phoenix dactylifera* callus cultures, was shown to repress, completely and reversibly, somatic embryogenesis in *Daucus* (6).

The volatiles observed in this study include potentially important components of the plant tissue culture environment, suggesting a need for improved regulation of endogenous volatile production and of the levels of gases permitted to accumulate in cultures.

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