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Ethylene Production by Plant Cell Cultures

VARIATIONS IN PRODUCTION DURING GROWING CYCLE AND IN DIFFERENT PLANT SPECIES[1]

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ABSTRACT

Suspension cultures of *Rosa* sp., soybean (*Glycine max* L.), wheat (*Triticum monococcum* L.), sweet clover (*Melilotus alba* Desc.), *Haplopappus gracilis* Nutt., and rue (*Ruta graveolens*) produced ethylene. The amount varied with the species. The rate of formation in rose and *Haplopappus* cells paralleled growth but accelerated when the stationary phase was reached, after which the rate declined sharply. Light was not required for ethylene production. Exogenous ethylene could not replace 2,4-dichlorophenoxyacetic acid or naphthaleneacetic acid in the cell cultures, and there was no stimulation of growth in the normal medium. Ethylene at 20 mM reduced growth of *Ruta* and rose cells by 30 and 20%, respectively. The amounts of ethylene produced by the cultures do not affect growth.

Ethylene is produced by a wide variety of microorganisms and plants and acts as a gaseous plant hormone (1, 13, 18, 19). Its production is generally higher in tissues containing auxins or when these are applied to tissues (2, 3, 13, 17); thus it is difficult to ascribe a physiological effect to the auxin or the ethylene (1).

The study of ethylene synthesis and action in whole plants or plant tissues is complicated by the presence of differentiated cells which may react differently than meristematic cells. In a plant cell culture the cells are fairly uniform and undifferentiated. Moreover, the level and kind of growth hormone in the nutrient medium may be manipulated at will. Therefore, plant cell cultures should provide a useful tool for the study of ethylene metabolism. We reported briefly that cell suspension cultures produce ethylene (8). This paper presents methods in more detail and reports on ethylene production in relation to growth cycle and the influence of nutritional and environmental factors. The effect of plant hormones on ethylene production is presented in a subsequent paper (9).

MATERIAL AND METHODS

Cultures. Earlier papers (5–7, 20) describe the establishment and growth of cell cultures of *Rosa* sp. var. Scepter (rose), *Glycine max* L. (soybean), *Triticum monococcum* L. (wheat), *Melilotus alba* Desc. (sweet clover), *Haplopappus gracilis* Nutt., and *Ruta graveolens*. The cells grew on B5 medium (10) containing mineral salts, sucrose, nicotinic acid, pyridoxine, thiamine, myoinositol, 2,4-D, or NAA[2] (1 mg/liter). Rose, *Ruta*, and sweet clover cultures required 0.2% casein hydrolysate (N-Z Amine; Sheffield Chemical, Norwich, N.Y.). The rose cultures could grow in a medium lacking exogenous hormones.

Ethylene Determination. Ethylene was determined by gas chromatography using a Varian model 204C with a flame ionization detector. The column was 1.5 m × 3.2 mm, packed with Porapak R (Waters Association Inc., Framingham, Mass.), maintained at 75 C. The identity of ethylene was confirmed on a similar column packed with activated alumina, at 45 C.

Dry Weight. Cells were harvested, washed on tared filters, and dried for 18 hr *in vacuo* at 60 C.

Cell Growth and Ethylene Production. Cells were grown in modified 125- or 250-ml Erlenmeyer flasks (Fig. 1A). A small port fitted with a serum cap permitted sampling of the gas phase. The side arm contained a foam plastic plug (Gaymar Industries Inc., Buffalo, N.Y.) and could be sealed with a serum cap. The flasks were kept at 27 C in continuous light on gyrotory shakers operated at 150 rpm. Prior to ethylene determinations, the flasks were flushed with sterile air, the serum cap was fitted in the side arm, and gas samples were obtained immediately and after 1 to 2 hr. The rate of ethylene production was constant for 4 hr and is calculated on a per hour basis. Total ethylene refers to the amount of ethylene produced by a culture from inoculation to harvest.

For light-dark experiments, the flasks were covered with black tape and aluminum foil.

Chemicals. Ethylene, CP grade 99.5%, was obtained from Matheson of Canada, Whitby, Ont. *cis*-3-Chloroacrylic acid was a gift from Union Carbide Corp., Clayton, N.C. The compound was recrystallized before use [MP 59–60, reported 60–62° (11)]. 2,4-D, NAA, and *p*CPA were obtained from commercial suppliers and recrystallized.

Respiration. Respirometers which could be fitted to standard Warburg manometers were made of 50-ml Warburg flasks (Fig. 1B). Large side arms permitted rapid flushing of the flask with a desired atmosphere. The center well contained KOH to absorb CO_2. The vessels were flushed with air twice daily and closed, and oxygen consumption was observed in the conventional way for 2 or 4 hr. Then a sample of the gas phase was taken through the serum cap for ethylene determination, after which the vessel was opened until the next determination.

Results. The cell cultures of rose produced ethylene throughout the growing cycle (Figs. 2 and 3). Ethylene measurements

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[2] Abbreviations: NAA: naphthaleneacetic acid; CPA: *p*-chlorophenoxyacetic acid; CAA: *cis*-3-chloroacrylic acid.

were taken regularly over the growing period, which lasted 4 to 6 days. The rate was nearly parallel with growth in the earlier phases of the growing cycle of rose cells (Fig. 2). When the stationary phase was reached, there was a sharp increase in ethylene evolution, after which the rate rapidly decreased.

The *Haplopappus* cells also produced ethylene throughout the growing period and appeared to follow the same pattern as the rose cells. The sweet clover and particularly the soybean cells produced measurable amounts only when a later phase in growth was reached (Fig. 3).

The total amount of ethylene produced varied between the species (Table I). The wheat cells consistently produced very low amounts. Soybean and sweet clover cells were intermediate producers while *Haplopappus*, rose, and *Ruta* cells produced high amounts throughout the growing period. The wheat, soybean, and *Haplopappus* cells were grown on defined media without amino acids, and the sweet clover, rose, and *Ruta* cells were cultured in the same medium but with casein hydrolysate added. The addition of casein hydrolysate, therefore, did not seem to be a factor in determining the degree of ethylene formation. This was verified by growing soybean cells on media with glutamine, casein hydrolysate, or ammonium citrate. The cells grew well in all of the media, but no difference in the rate of ethylene production could be observed. Addition of methionine at 0.5 or 2.0 mM to sweet clover cells inhibited ethylene production, and β-alanine or ethanol added at 1 mM had no effect. The absence of light did not affect the growth of *Ruta*, sweet clover, and rose cells, but lower rates of ethylene production by rose cells were observed when light was excluded (Table II).

Several experiments were conducted to determine if ethylene had any effect on cell growth. Ethylene was applied to soybean cells growing in hormone-free medium. These cells require 2,4-D for growth (10). The gas was passed through the cultures at 14.8 ml/hr delivered by syringes on a Harvard pump using concentrations of 10^{-5}, 10^{-7} and 10^{-9} mole/hr. There was no cell growth in any of the flasks, indicating that ethylene did not replace 2,4-D at the concentrations tested. In a comparable experiment with sweet clover cells the ethylene also failed to replace the hormone. The sweet clover cells usually grew equally well with 2,4-D or NAA as the growth hormone.

The effect of ethylene on yields of cells grown in normal hormone-containing media was determined. Cells of *Ruta*, rose, and wheat were grown in modified Warburg flasks (Fig. 1b), and the cells were gassed twice daily for 30 min with air containing ethylene at 4 and 20 mM, respectively. The data in Table III show that ethylene at these concentrations had relatively little effect on cell yield. There was a 20 to 30% reduction in yield at the higher concentration. This difference may have been caused by a change in oxygen concentration due to the addition of ethylene.

The rose cells divided and produced ethylene in the absence of exogenous hormones, although there was a slight increase in cell growth when 2,4-D or NAA was added to the medium. Results of studies on the effect of auxins on ethylene production and respiration are shown in Table IV. There was no consistent increase in respiration rate due to the hormones, but ethylene production was enhanced when NAA or *p*CPA was added to the medium. The results suggest that respiration and ethylene production are independent processes, and that auxin-like compounds may stimulate ethylene production without affecting respiration.

Ethylene production may be inhibited selectively in *Penicillium digitatum* by CAA (15). Respiratory CO_2 production was not affected.

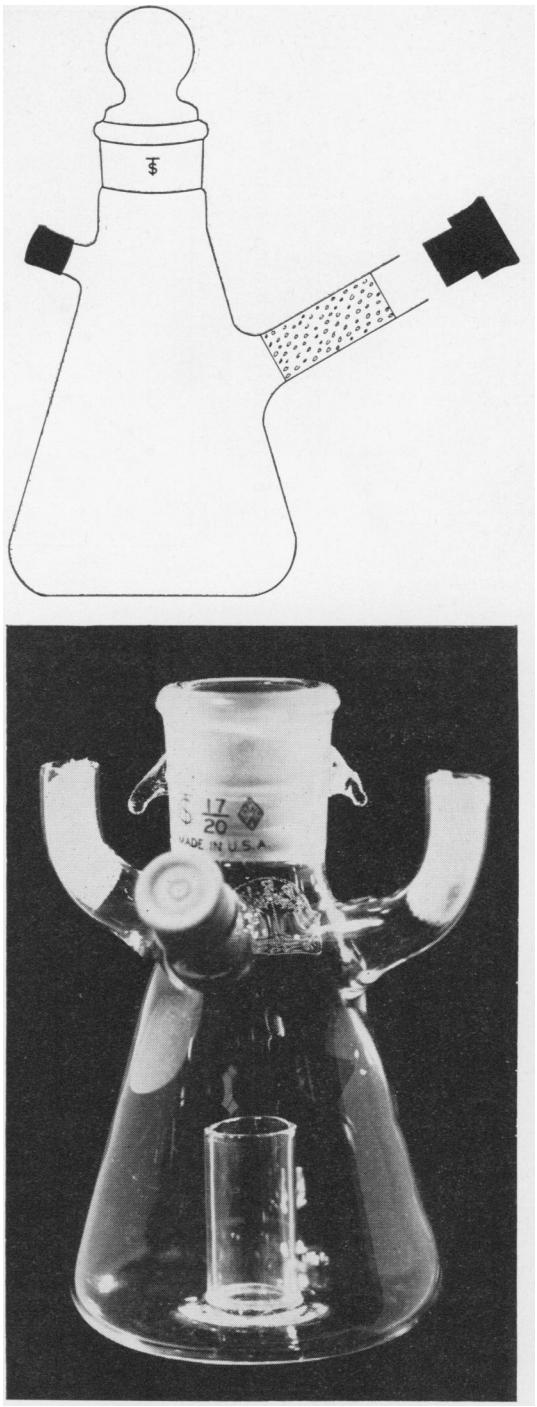

FIG. 1. A: Vessel for culturing cells used in measuring ethylene production; B: modified Warburg flask for simultaneously measuring ethylene production and respiration.

The effect of CAA on respiration and ethylene formation in rose cells was tested. The data in Table V show that the compound inhibited cell growth. The rate of respiration and ethylene formation also declined at the higher concentrations. There was no indication that CAA inhibited specifically the production of ethylene. On the contrary, the results of several experiments demonstrated that CAA caused an initial, rapid stimulation in the formation of ethylene (Fig. 4). There was no corresponding increase in respiration rate.

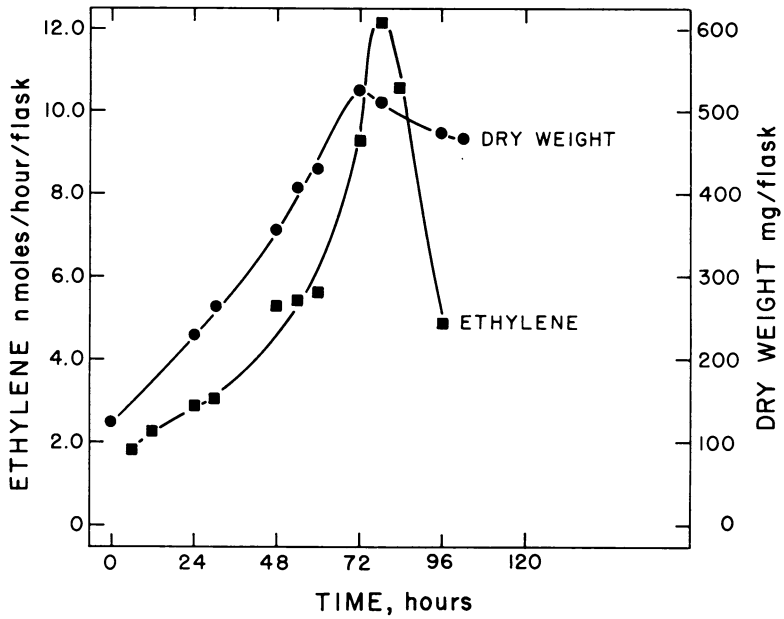

FIG. 2. The relationship between growth rate and ethylene production in rose cell cultures. The results are based on triplicate samples. The cells were cultured in 40 ml of B5 medium with 0.2% casein hydrolysate and 1 mg/liter of NAA.

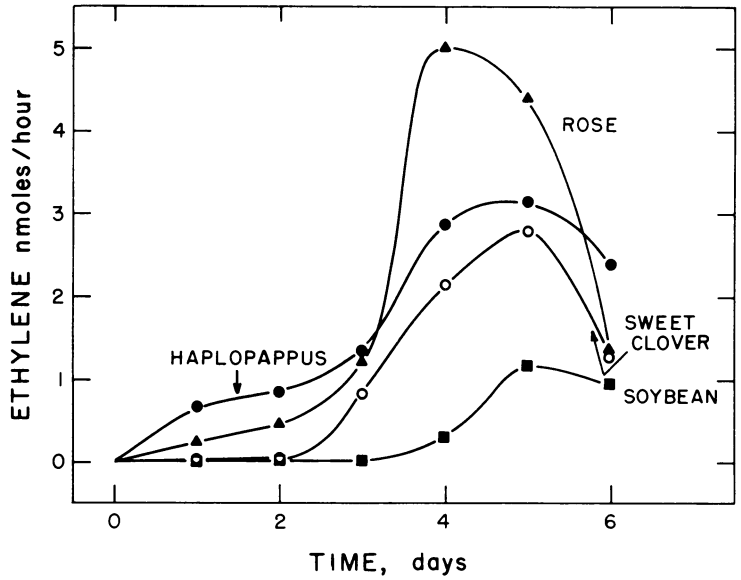

FIG. 3. Ethylene formation by soybean, *Haplopappus*, sweet clover, and rose cell cultures during growth. The cells were grown in flasks (Fig. 1A) on a shaker. The initial and final dry weights of the samples were rose, 19 and 274 mg; soybean, 9 and 234 mg; sweet clover, 14 and 281 mg; and *Haplopappus gracilis*, 16 and 206 mg.

DISCUSSION

Ethylene appears to be a regular metabolite of plant cells in culture. The production of the compound is not associated with a particular growth phase, although the rates of production appeared to increase toward the early part of the stationary growth phase. A similar pattern was observed with sycamore cells (16). When the culture approached the stationary phase, the nutrients became limiting and most of the cells were senescing. Ethylene production may thus be enhanced as a result of senescence and may be associated with deterioration of functional structures such as mitochondria within the cells (19). In whole plants ethylene accelerates certain aging processes (1), and the production of ethylene increases when a particular stage of senescence is reached prior to leaf abscission (14).

The total amounts of ethylene depend on the species from which the cell culture were derived. Cells of wheat and other cereals produce low amounts of ethylene (8), a property which may be characteristic for cereal plants (4, 17).

Addition of ethylene did not significantly affect cell growth. The concentrations used in the present experiments were more than 1000 times higher than those accumulating in cell cultures, indicating that ethylene produced by the cells does not limit the growth rate of these cells. On the other hand, exogenous ethylene does not replace the growth substances required for cell division. Neither does ethylene stimulate growth in the normal medium. MacKenzie and Street (16) added Ethrel (2-chloroethylphosphonic acid), which is a potential ethylene source (13), to suspension cultures of sycamore cells and similarly failed to observe any stimulation of cell division and growth.

Ethylene production and respiration appear to be two separate processes in plant cell cultures. The stimulation of ethylene formation by CAA was not reported for *Penicillium digitatum* (15). In this fungus ethylene production was blocked but CO_2 evolution continued. The concentrations of CAA used in the fungal experiments were 100 times higher than those used in the present experiments. There is no apparent

Table I. *Ethylene Formation by Cell Cultures of Different Plant Species*

The cultures were grown in flasks (Fig. 1a) on the shaker for 6 days. Each experiment was done in triplicate.

Culture	Experiment	Cell Mass		Total Ethylene
		Initial	Final	
		mg		*nmoles*
Triticum monococcum	1	24	196	Trace
	2	14	145	Trace
Soybean	1	8.7	234	60
	2	8.6	278	22
Sweet clover	1	22	248	139
	2	14	258	140
Haplopappus gracilis	1	16	206	268
Rose	1	18	274	307
Ruta graveolens	1	34	218	237

Table II. *Influence of Light on Growth and Ethylene Formation by Cell Cultures of Ruta, Sweet Clover, and Rose*

Culture	Conditions	Cell Mass		Ethylene
		Initial	Final	
		mg		*nmoles/mg dry wt*
Ruta	Dark[1]	34	206	1.13
	Light	34	218	1.09
	Dark	53	225	1.00
	Light	53	183	1.17
Sweet clover	Dark	14	281	0.61
	Light	14	258	0.54
Rose	Dark	13	131	0.1
	Light	13	130	0.49
	Dark	39	274	6.7
	Light	39	290	18.8

[1] Light was excluded by covering the flasks with black tape and aluminum foil.

Table III. *Influence of Ethylene on Cell Growth*

The cells were cultured in the flasks (Fig. 1b) adapted for the Warburg and were gassed twice daily for 30 min. The initial dry weights of the cultures were: *Ruta*, 29 mg; rose, 6 mg; wheat, 4.8 mg. The gas mixture flow rate was 50 cc/min.

Gas Mixture, C_2H_4:Air	Ethylene Concn	*Ruta*		Rose		Wheat	
		Final wt	Percentage	Final wt	Percentage	Final wt	Percentage
	mM	*mg*		*mg*		*mg*	
Air	0	83.2	100	50.0	100	7.2	100
1:10	4	78.6	95	42.5	85	7.2	100
1:1	20	58.6	70	40.2	80	6.8	95

Table IV. *Effect of Auxins on Respiration and Ethylene Production by Rose Cell Cultures*

The auxins were added at 1 mg/liter. The inoculum was 8.4 mg, and the cells were grown in flasks (Fig. 1b) adapted for the Warburg.

Time of Analysis	No Auxin		2,4-D		NAA		*p*CPA	
	O_2[1]	C_2H_4[2]	O_2	C_2H_4	O_2	C_2H_4	O_2	C_2H_4
3 PM	7.2	112	7.3	163	9.5	343	9.4	366
4 AM	10.1	246	11.0	334	10.9	733	11.1	864
4 PM	9.1	230	11.0	322	10.6	676	10.2	847
5 AM	8.4	373	9.5	491	11.6	1263	11.2	1309
Final dry wt (mg)	42		46		61		58	

[1] In μmoles/hr. [2] In pmoles/hr.

Table V. *Effect of 3-Chloroacrylic Acid on Respiration and Ethylene Production of Rose Cell Cultures*

The inoculum was 12.8 mg, and the cells were grown in flasks (Fig. 1b) adapted for the Warburg.

Day of Analysis	Concn of 3-Chloroacrylic Acid (M)							
	0		2×10^{-6}		10^{-5}		2×10^{-5}	
	O_2[1]	C_2H_4[2]	O_2	C_2H_4	O_2	C_2H_4	O_2	C_2H_4
1	11.2	376	12.5	390	7.8	559	7.6	677
2	12.5	482	10.6	520	7.7	180	4.5	121
3	8.9	830	7.2	830	8.3	420	5.4	205
4	7.9	610	7.1	931	6.9	488	7.4	326
5	13.4	539	2.6	591	4.8	684	3.6	389
7	0.86	501	1.6	422	1.8	607	1.9	297
C_2H_4	80,112		88,416		68,592		48,360	
Dry wt	76.7		76.6		75.7		40.0	

[1] In μmoles/hr. [2] In picomoles/hr.

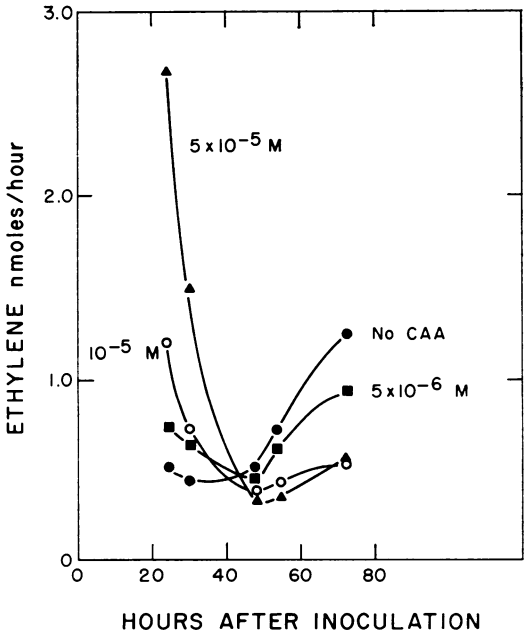

FIG. 4. The effect of 3-chloroacrylic acid on ethylene production by rose cell cultures.

explanation as to why CAA should cause an increase in ethylene production.

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