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PROBABLE IDENTITY OF SUBSTANCES IN CITRUS THAT REPRESS ASEXUAL EMBRYOGENESIS

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SUMMARY

The embryogenetic response of cultured *Daucus carota* L. 'Queen Anne's Lace' callus was employed to attempt fractionation and identification of a repressive factor produced by *Citrus medica* L. ovules. The factor was evidently synthesized and released into the medium continuously, inasmuch as citron ovules that had been autoclaved with the medium were completely ineffective. The inhibition could be attributed to volatile and nonvolatile components. A substantial part of the inhibition was prevented by continuously refreshing the atmosphere within the cultures with filtered air. Monitoring of the gases produced by citron ovule sections under conditions simulating bioassays disclosed significant evolution of carbon dioxide, ethylene and ethanol. Repression of embryogenesis was not averted by trapping the liberated ethylene. On the other hand, ethanol in concentrations equivalent to those released by citron ovules suppressed asexual embryogenesis dramatically. The adverse effect of ethanol was reversed immediately upon transfer to ethanol-free medium. Another investigation had disclosed anti-embryogenetic effects of auxin, abscisic acid and gibberellin. Analysis of *Citrus* ovules excised from young fruits disclosed those of monoembryonic citron to contain concentrations of IAA, ABA and GA_3 several times higher than those of polyembryonic Ponkan mandarin. The nonvolatile portion might be identified with these hormonal substances.

Key words: asexual embryogenesis; nucellus; *Citrus reticulata; Daucus carota; Citrus medica;* repression.

INTRODUCTION

In attempting to elucidate the basis for monoembryonic and polyembryonic cultivars of *Citrus*, Esan (1) discovered that the nucellus of the former contained a graft-transmissible and diffusable embryogenetic repressant. The concentration of this factor was lowest in the most polyembryonic cultivar. The chalazal end of the nucellus or the ovule contained more repressor than the micropylar region. The *Citrus* factor also was effective in suppressing asexual embryogenesis in carrot callus, implicating a possible general significance.

An immediate concern has been the identification of the repressive factor in order that the mechanism of its action could be explored subsequently. The relative irreversibility of the anti-embryogenetic action of *Citrus* cultures suggested a mechanism at the gene transcription level. In another investigation we examined the effects of inclusion in the culture medium of known plant growth regulators, in an attempt to achieve indirect identification. The results are being prepared for publication elsewhere. This research attempted to identify more directly the inhibitory substances produced by citron ovules.

MATERIALS AND METHODS

Tissue culture. Initial experiments employed *Citrus reticulata* Blanco 'Ponkan' mandarin nucellus as well as *Daucus carota* L. 'Queen Anne's Lace' callus cultures as embryogenetic materials. However, the bulk of the experiments was based on carrot cultures. Fruits approximately 100 days after pollination served as source of Ponkan mandarin nucellus. They were washed, severed of their stem and stylar ends, and sterilized by immersion in dilute bleach (Purex diluted 10-fold with water and containing 2 drops Tween 20 emulsifier per 100 ml) for 15 min. They were bisected to expose the ovules. The ovules were

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transferred to sterile Petri dishes. By holding the chalazal region with a pair of microforceps and with the use of a surgeon's scalpel fitted with a No. 11 blade, a shallow incision was made longitudinally through the integuments, from chalazal region to micropylar tip. The ovule was halved transversely. The endosperm and integuments, as well as any embryos, then were removed from the micropylar half of the nucellus. The nucellus section was transferred to culture vessels, with its cut base placed in contact with the agar surface. One nucellus section was planted per vessel.

The carrot callus was obtained from a stock that originated in seedling-petiole sections. The callus was initiated and maintained in a medium that contained Murashige and Skoog salts plus the following (in mg per l): thiamine·HCl, 0.4; 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1; *myo*-inositol, 100; sucrose, 30,000; and Difco Bacto agar, 7000. The stock was maintained by monthly subcultures of 100-mg quantities.

Citrus medica L. ovules served as source of embryogenetic repressor. The ovules were obtained from fruits also about 100 days after pollination. Ovules were prepared by the procedure described above for Ponkan mandarin. They were halved transverely and planted in the nutrient medium with their cut surfaces exposed, together with the carrot and Ponkan mandarin tissues. The citron ovule sections were positioned in a ring at a distance 1 cm from the centrally located carrot or Ponkan mandarin tissue.

Embryogenesis in Ponkan mandarin nucellus was attained in a medium composed of Murashige and Skoog salts and the following (in mg per l): *myo*-inositol, 100; thiamine·HCl, 0.2; pyridoxine·HCl, 1.0; nicotinic acid, 1.0; glycine, 4.0; Difco Bacto malt extract, 500; sucrose, 50,000; and Difco Bacto agar, 10,000. Embryo formation in carrot was achieved by excluding the 2,4-D from the callus medium. The pH of all media was adjusted to 5.7 with 1 *N* NaOH before addition of agar. In most experiments the nutrient media were dispensed into 50-ml DeLong flasks at a rate of 25 ml per vessel. The flasks were capped with Morton stainless-steel closures through the autoclaving step.

To test the effects of removing ethylene produced by the cultures, 125-ml DeLong flasks were used as culture vessels; each held 50 ml nutrient medium. Polypropylene test tubes, 16- by 41-mm, were placed in the nutrient agar and to one side of the flasks. The tubes contained 2 ml of 0.25 M mercuric perchlorate in 2.0 M perchloric acid solution, which was added to the tubes after the culture medium had been autoclaved. A 2- by 55-mm polypropylene rod was inserted into the tube to prevent tipping. A 25- by 35-mm strip of Whatman No. 1 filter paper was folded lengthwise and inserted into the tubes to maximize gas absorption. The larger DeLong flasks also were capped with Morton stainless-steel closures through the autoclaving step. All stainless-steel closures were replaced with Bittner rubber serum stoppers following planting of tissues to enable gas monitoring.

For detection of the nonvolatile components of citron ovules, aliquots of 20 ml of nutrient medium were placed in 50-ml Pyrex beakers. A glass vial, 25- by 40-mm, filled with 17 ml nutrient medium was placed in the center of some of the beakers. The beakers were covered with aluminum foil to allow autoclaving; the foil was replaced with Parafilm after planting of tissues. Effects of continuously refreshing the atmosphere within culture vessels was explored by using 500-ml Erlenmeyer flasks, each containing 100 ml nutrient agar and capped with two-hole autoclavable rubber stoppers.

All media were sterilized by autoclaving 15 min at 121°C. Tests of ethanol effects employed 95% alcohol in doses of 0, 10, 30 and 100 μl per culture. The alcohol was added to the nutrient medium after autoclaving of the medium; also, the stainless-steel closures of the DeLong flasks were replaced with Parafilm. Continually refreshing of the air within culture vessels was accomplished by using compressed air, provided at a rate of 10 ml per min. The air entered through one opening of the rubber stopper that closed the large Erlenmeyer flask, circulated through the culture and exited through the second. The air was filtered through nonabsorbent cotton and percolated through autoclaved water before use.

Stock carrot callus was initiated and maintained in constant darkness at 27°C. Embryogenesis of carrot and *Citrus* occurred at 27°C and under 16-hr daily exposure to 1000 lux of Gro Lux light. The number of embryos and fresh weight were determined after 4 weeks for carrot cultures and after 5 weeks for Ponkan mandarin. Ten cultures were employed per treatment, except in the gas monitoring experiment with citron ovules in which three cultures were sampled each week. The carrot tissue was weighed and transferred to 125-ml DeLong flasks containing 25 ml of 2% HCl solution. The suspension was agitated for 1 hr on a gyratory shaker at 150 rpm, followed by

addition of 50 ml tap water. The suspension was filtered through a series of stainless-steel screens of 400-, 275- and 150-μm pore sizes. Water (500 ml) was passed through the screens to remove fine debris, and embryos of heart-shaped and more advanced stages that collected on the screens were counted.

Analysis of liberated gases. Gases found in the culture vessels were analyzed according to the procedure of Negm, Smith and Kumamoto (2). Aliquots of 0.5 ml were withdrawn from the culture vessels, using a Plaspak glass syringe fitted with a Yale 22 stainless-steel needle. The samples were analyzed via a Beckman GC-4 dual hydrogen flame gas chromatograph. They were passed through a 0.32- by 285-cm stainless-steel column, packed with 50-80 mesh Porapak Q (Water's Associated, Inc., Framingham, Mass.). A thermal conductivity detector monitored CO_2 and a dual hydrogen flame detector measured ethylene and other hydrocarbons. Identification was achieved by comparing with elution patterns of standard gas mixtures.

Analysis of growth regulator contents of citrus ovules. Ovules were obtained from Ponkan mandarin and citron fruits harvested 100 days after pollination. The ovules were frozen immediately in liquid nitrogen and stored at −20°C until extraction. Ethyl acetate extracts and trimethylsilyl derivatives (TMS) were prepared according to methods of Shindy and Smith (3). The derivatives were analyzed with the Beckman GC-4 gas chromatograph. The instrument was equipped with a temperature programmer and a 0.64- by 183-cm glass column packed with 3% QF-1. The instrument was operated with these specifications (in ml per min): air flow, 104; helium flow, 28; and hydrogen flow, 24. Temperature programming was started at 95°C and increased linearly to 230°C at a rate of 6°C per min. The retention time and temperature for each peak were recorded and compared with those of TMS derivatives of standards.

RESULTS AND DISCUSSION

Effects of coculturing with monoembryonic citrus ovule sections. Progessive decreases in embryo yield were encountered when the nucellus of the polyembryonic Ponkan mandarin was cocultured with increasing numbers of chalazal halves of ovules from the monoembryonic citron (Table 1), thus confirming previous observations. The same repression could be observed with carrot callus (Table 2). In view of numerous advantages, including greater range and faster response, year-round availability of material and easier quantitation, the carrot callus was chosen over Ponkan mandarin nucellus as the standard bioassay tissue. Perhaps of interest, coculturing of carrot callus with Ponkan mandarin sections in numbers equal to citron showed little if any repression of asexual embryogenesis in carrot. Evidently, the repressive factor is synthesized and released continuously by the citron ovules. The effect of coculturing was lost when the citron ovules had been autoclaved.

TABLE 1

INHIBITION OF ASEXUAL EMBRYOGENESIS IN PONKAN MANDARIN NUCELLUS BY CITRON OVULE SECTIONS

No. Citron Ovules/Culture	No. Mandarin Embryos/Culture
0	39.0±4.5
1	14.0±5.5
3	6.6±2.6
10	4.0±1.7
20	0.0±0.0

Volatile substances from citron ovules. When the carrot callus was cultured in wells containing no citron tissue, but with the wells surrounded by cultured citron ovules and the entirety enclosed in a sealed beaker, there occurred some inhibition, suggesting a volatile component emanating from the citron. However, the extent of embryogenetic repression by volatile substances was short of the total attained when the carrot callus was placed directly in an agar medium in which citron ovules were being cultured. A supplemental experiment showed that part of the depressed embryogenesis could be recovered by continually replacing the atmosphere within the cultures with fresh filtered air, but the recovery was only 50 to 60%. Thus at least two components, a volatile and a nonvolatile, could be distinguished.

Analysis of the gas in the culture vessels disclosed conspicuous levels of CO_2, ethylene and

TABLE 2

INHIBITION OF ASEXUAL EMBRYOGENESIS IN CARROT CALLUS BY CITRON OVULE SECTIONS

No. Citron Ovules/Culture	No. Carrot Embryos/Culture
0	783.0±154.0
5	384.6±160.3
10	103.3± 48.9
20	24.3± 9.7

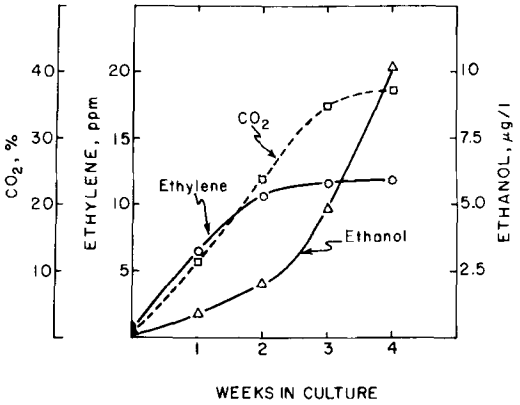

FIG. 1. Gases liberated by citron ovule section in vitro. Twenty ovules were employed per culture in the absence of carrot or Ponkan mandarin tissue.

ethanol. The pattern of evolution of these substances can be seen in Fig. 1. The data in Fig. 1 were obtained from cultures that contained only citron ovules, 20 per culture. The CO_2 showed a rapid accumulation during the first 3 weeks and a leveling thereafter. Ethylene evolved with the same rapidity, but leveled off after 2 weeks. Ethanol increased slowly during the first 2 weeks and exponentially thereafter. The effects of ethylene and ethanol on somatic embryogenesis in carrots were examined. Other experiments suggested no repression of embryo yield by CO_2.

Inclusion in the culture vessels of vials containing perchlorate resulted in no measurable ethylene in the cultures (Table 3). However the removal of ethylene was not accompanied by the recovery of depressed embryogenesis. Nevertheless, this observation does not preclude an antiembryogenetic effect of ethylene; other experiments have shown that ethylene suppresses embryogenesis in carrot and *Citrus* when present in substantially higher concentrations.

TABLE 3

CORRELATION BETWEEN ETHYLENE RELEASED BY OVULE SECTIONS AND ASEXUAL EMBRYOGENESIS IN CARROT CALLUS[a]

Treatment	No. Embryos/Culture	Ethylene Evolved (ppm)
No ovules, no trap	346.3±36.8	0.84±0.05
Ovules, no trap	161.3±25.1	9.13±1.03
Trap, no ovule	323.0±37.7	0.00±0.00
Trap and ovule	153.7±51.9	0.00±0.00

[a] Vials containing 0.25 M mercuric perchlorate in 2.0 M perchloric acid solution were placed in cultures to trap the evolved ethylene. Ten citron ovule sections were planted per culture.

TABLE 4

INHIBITION OF ASEXUAL EMBRYOGENESIS IN CARROT CALLUS BY ETHANOL[a]

Ethanol in Culture Atmosphere (µg/l)	No. Embryos/Culture
0.38	469.0±18.7
1.70	203.3±49.2
2.94	44.3± 7.2
10.20	0.0± 0.0

[a] These concentrations were obtained by adding 0, 10, 30 and 100 µl of 95% ethanol per culture.

Addition of ethanol to carrot cultures in doses comparable to those released by citron ovules disclosed marked reductions in embryo yields (Table 4). A concentration of 1.7 µg ethanol per l of sampled gas reduced the number of embryos to less than half of the controls. A 10 µg per l concentration eliminated embryogenesis entirely. The effect of ethanol, however, was not irreversible. One reculture in ethanol-free medium was sufficient to enable normal levels of embryogenesis to reoccur.

Nonvolatile constituents in citrus ovules. The ethyl acetate extracts showed that monoembryonic citron ovules contained significantly more IAA, ABA and GA_3 than the polyembryonic Ponkan mandarin ovules (Table 5). Bioassays of the substances, singly and in combination and using the concentrations found in citron ovules, showed no effect on embryogenesis. This does not exclude the substances from the antiembryonic process, since the concentrations tested were very low. Their continued synthesis in the citron ovules during the course of culture could result in substantially higher levels than those observed in the freshly excised tissue. Investigations by others, e.g. Fujimura and Komamine (4), as well as by us have demonstrated repressive actions of auxin, gibberellin and abscisic acid.

Probable identity of the citrus factor. This research has revealed that the repression of asexual

TABLE 5

HORMONAL CONTENTS OF ETHYL ACETATE EXTRACTS OF FRESHLY EXCISED MONOEMBRYONIC CITRON AND POLYEMBRYONIC PONKAN MANDARIN OVULES[a]

Constituent	µg/120 ovules	
	Ponkan Mandarin	Citron
Indole-3-acetic acid	0.43	3.76
Abscisic acid	14.32	38.76
Gibberellin A_3	0.36	59.04

[a] Gas chromatographic analyses were performed on trimethylsilyl derivatives.

embryogenesis by ovules of monoembryonic *Citrus* can be identified with several substances, some of which are ubiquitous in plants and associated with basic developmental processes. The significance of ethanol is unclear. The possibility of its production in vitro being an artifact cannot be ruled out. It is noteworthy that severe repression of embryogenesis can be obtained by very low ethanol concentrations. Ethylene by itself has little anti-embryogenetic effect, mainly because it is produced in only small amounts. The other hormones, including auxin, abscisic acid and gibberellin, by virtue of their higher concentrations in monoembryonic than in polyembryonic *Citrus* tissues and their observed inhibitory effects in other investigations, also are implicated in the suppressive role. It is most probable that the repression is attributable to a combined action of the substances identified in this research as well as others not yet identified.

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