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Effects of Ethephon, Ethylene, and 2,4-Dichlorophenoxyacetic Acid on Asexual Embryogenesis *in Vitro*[1, 2]

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ABSTRACT

Asexual embryogenesis in *Daucus carota* L. 'Queen Anne's Lace' callus was suppressed by Ethephon, ethylene, and 2,4-dichlorophenoxyacetic acid (2,4-D). The Ethephon effect could be attributed to volatile and nonvolatile substances. The volatile component was probably entirely ethylene. Ethylene was liberated in the cultures in direct proportion to Ethephon added to the medium. Autoclaving of Ethephon caused a substantial decrease of measurable ethylene. Continuous exposure of callus to 5 μl/l ethylene depressed somatic cell embryogenesis, but not markedly. Depression of embryogenesis by 2,4-D was unrelated to ethylene evolution.

Ethephon, a source of ethylene when in aqueous solution (3, 5), has been reported to reduce asexual embryogenesis in cell cultures of *Daucus carota* L. 'Queen Anne's Lace' (4). Our initial experiments with the wild carrot callus confirmed the repressive effect, but suggested at least two active components in Ethephon, one volatile and another nonvolatile. Wochok and Wetherell's (4) inference that ethylene affected somatic cell embryogenesis adversely was based on their use of Ethephon, rather than on direct experimentation with ethylene alone. This investigation was intended to determine the extent to which the Ethephon effect was attributable to ethylene. In addition, this research explored the possible relationship between the antiembryogenetic and the ethylene-generating effects of the auxin, 2,4-D.

MATERIALS AND METHODS

Tissue Cultures. Somatic cell embryogenesis in *D. carota* L. 'Queen Anne's Lace' callus cultures was employed as the test system. The callus was obtained from petiole sections of greenhouse-grown seedlings and maintained in a medium containing Murashige and Skoog salts and the following, in mg/1: 2,4-D, 0.1; thiamine·HCl, 0.4; *myo*-inositol, 100; sucrose, 30,000; and Difco Bacto Agar, 7,000. The callus was maintained as stock by subculturing 100-mg portions in fresh medium at monthly intervals. Embryogenesis occurred when the callus was recultured in the above medium without 2,4-D. The pH of all media was set at 5.7 with 1 N NaOH prior to addition of agar. The nutrient media were distributed in 25-ml aliquots in culture tubes (25 × 150 mm) unless noted otherwise. The tubes were capped with Bellco Kaputs, except in experiments where gas evolution was monitored. Bittner rubber serum stoppers were used as culture tube closures when gas monitoring was desired. For detection of the nonvolatile component of Ethephon, the media were dispensed into 100-ml Pyrex beakers at a rate of 25 ml/beaker. At the center of some of the beakers was placed a glass vial, about 25 mm in diameter and 40 mm tall, and to the vial was added 17 ml of nutrient agar. The beakers were covered with aluminum foil for autoclaving and the foil was replaced with Parafilm following planting of tissue. In studying effects of exogenous ethylene the nutrient medium was contained in 500-ml Erlenmeyer flasks, in 100-ml aliquots. The flasks were closed with autoclavable two-holed rubber stoppers. All media were sterilized by autoclaving 15 min at 121 C. Media in culture tubes were allowed to solidify as slants.

Ethephon (2-chloroethylphosphonic acid, Amchem 69-37, 98% active ingredient) was added to the embryogenetic medium following Millipore filter sterilization, except when studying autoclaving effects. The auxin, 2,4-D, was autoclaved together with the basal medium constituents. Ethylene from a gas cylinder was filtered via nonabsorbent cotton and percolated through autoclaved water before introduction into culture flasks. The gas contained 5 μl/l C_2H_4 was introduced at a rate of 10 ml/min; it entered the 500-ml flasks through one opening of its stopper, was circulated internally, and exited through the second. Compressed air was used for the control.

Stock callus was maintained at constant 27 C and in darkness. Embryogenesis was allowed to occur at 27 C and under 16-hr daily exposure to 1,000 lux Gro-Lux light. After 4 weeks the number of embryos and fresh weight/culture were recorded. Deviations in general quality of embryos and tissues were also noted. In the test with exogenous ethylene, treatment and control employed five flasks of cultures each. In determining effects of nonvolatile substances from Ethephon, 10 cultures were used/treatment. To correlate monitored gases with embryogenesis, embryo and tissue measurements and gas analyses were performed on three cultures/week for 4 weeks.

In counting embryos the contents of each culture were weighed and transferred to 125-ml DeLong flasks containing 25 ml 2% HCl. The suspension was agitated 1 hr on a gyrotor shaker at 150 rpm. Fifty ml of tap water were added and the mixture was filtered through a series of stainless steel screens of 400-, 275-, and 150-μm pore sizes. The screens were washed with 500 ml of water and embryos of heart-shaped and more advanced stages were counted.

Monitoring Evolved Gases. The cultures were capped continuously with Bittner rubber serum stoppers. The gas analyses followed the procedure of Negm *et al.* (2). Using a sterile Plaspak disposable glass syringe, fitted with a Yale 22 stainless steel needle, 0.5-ml samples were withdrawn from the cultures. The samples were analyzed by gas chromatography, using a Beckman GC-4 dual hydrogen flame instrument. They were injected through a 285-cm stainless steel column, 0.35 cm outer diameter, packed with 50 to 80 mesh Poropak Q at 70 C. The gases were passed through a thermal conductivity detector to

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determine CO_2 and through a dual hydrogen flame detector to measure ethylene. Identification was confirmed by comparing with elution patterns of standard gas mixtures.

RESULTS AND DISCUSSION

Suppression of Embryogenesis by Ethephon. The Ethephon was tested in 0, 3, 10, and 30 mg/l concentrations. Prolific formation of embryos and development of advanced embryonic stages occurred only in the medium devoid of Ethephon (Fig. 1). The number of embryos produced/culture was reduced drastically by the lowest concentration, 3 mg/l. The higher concentrations virtually eliminated any embryogenesis. Associated with the reduction of embryos was a corresponding decrease in fresh weights. Low embryo numbers were not compensated by increased callus formation. Furthermore, embryos that developed in Ethephon media were smaller and chlorotic. The results agree with findings of Wochok and Wetherell (4).

Volatile and Nonvolatile Components of Ethephon. To test the effects of volatile substances released by Ethephon some of the callus was cultured in vials of Ethephon-free medium, surrounded by media containing the chemical. The Ethephon was incorporated in the surrounding medium in a 30 mg/l concentration. Embryo number in the tissue allowed to develop in the Ethephon-free central well was reduced to about 60% (Table I). In contrast the callus cultured directly in Ethephon-containing medium showed a reduction in embryo yield to nearly 6%. Thus, while the embryogenetic repression by Ethephon could be attributed to a volatile component, most probably ethylene, an equally significant suppression was associated with nonvolatile substances. Perhaps the phosphonate reportedly produced by the decomposition of Ethephon (3) might have been the repressive agent in the nonvolatile portion. At the end of the 4-week period both types of Ethephon treatments revealed equal concentrations of ethylene in the culture vessels (Table I).

In another experiment some of the Ethephon was incorporated and autoclaved with the constituents of the medium. After 4 weeks, cultures in unautoclaved Ethephon had accumulated 400 μl/l ethylene, whereas those in autoclaved Ethephon had 5 μl/l, or only slightly more than the control Ethepon-free medium. Nevertheless, the autoclaved Ethephon caused a 50% reduction in embryo yield, as compared to a complete suppression by the cold sterilized sample. A 30 mg/l concentration of autoclaved Ethephon gave a repression comparable to 3 mg/l of the unautoclaved sample. The ethylene released by the lower concentration of the unautoclaved Ethephon was, however, 10 times higher.

Effects of Exogenous Ethylene. A continuous exposure to 5 μl/l ethylene over a 4-week culture period failed to reduce embryogenesis substantially. The untreated controls produced approximately 550 embryos/culture, whereas the ethylene treatment gave roughly 400, or 30% fewer embryos. The fresh weight of the cultures was reduced by ethylene and severe epinasty resulted among the seedlings. Perhaps the dosage of ethylene was inadequate; nevertheless, a direct effect on asexual embryogenesis has been demonstrated.

Ethylene Emanating from Ethephon. The cumulative release of ethylene was proportional to the Ethephon concentration of the nutrient medium (Fig. 1). The 4-week totals of ethylene were 60 μl/l for 3 mg/l Ethephon, 225 μl/l for 10 mg/l, and 450 μl/l for 30 mg/l. The bulk of the ethylene from any of the treatments was released within the first 2 weeks of culture. The high concentrations of ethylene found in each of the Ethephon treatments would indicate that the 5 μl/l ethylene employed in the exogenous ethylene experiment were inadequate.

For supplemental information the CO_2 evolution was also monitored in cultures treated with Ethephon. A progressive decrease of CO_2 was associated with increasing concentrations of Ethephon. Similar measurements in other situations of embryogenetic repression, *e.g.* in the presence of 2,4-D, also showed less CO_2 evolution. The physiological relationship is unclear, although it suggests a repressed respiration. Indeed, over-all growth of cultures is inhibited when embryogenesis is depressed. The antagonism between CO_2 and ethylene is an interesting possibility, but perhaps not applicable here; as described below

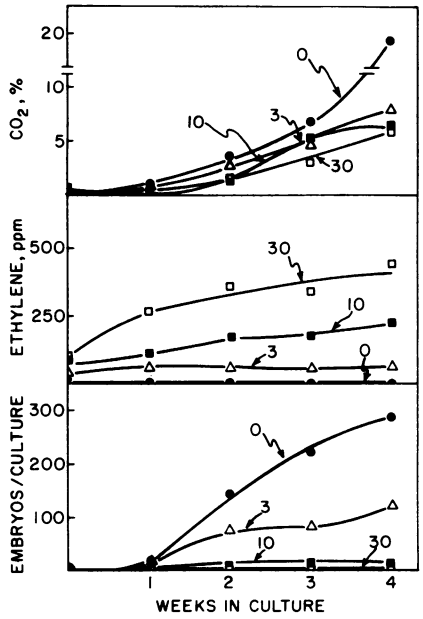

FIG. 1. Relationship between Ethephon effect on asexual embryogenesis in carrot callus and gases evolved. Numbers with arrows indicate mg/l Ethephon in medium.

Table I. Repression of Asexual Embryogenesis in Carrot Callus by Volatile and Non-volatile Components of Ethephon. Ethephon was added at a rate of 30 mg/l. Ethylene was determined at the end of the 4-wk culture period. Cultures were contained in beakers sealed with parafilm.

Treatment	Embryos/ Culture	ppm Ethylene
No ethephon in culture	757.0 ± 19.4	0.34 ± 0.9
Tissue cultured directly in ethephon medium	54.3 ± 27.3	4.31 ± 0.98
Tissue in ethephon-free central well, surrounded by ethephon medium	431.0 ± 71.0	4.19 ± 1.04

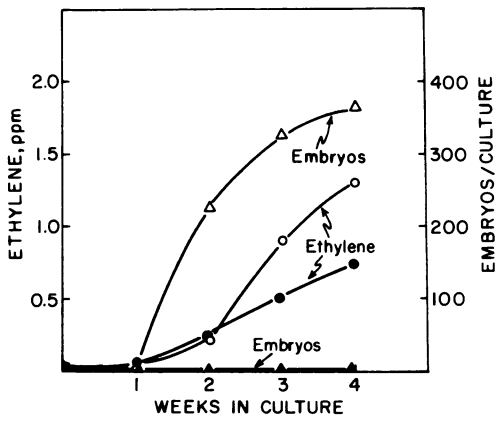

FIG. 2. Relationship between 2,4-D effects on ethylene evolution and asexual embryogenesis in carrot callus. 2,4-D was included in a 0.1 mg/l concentration. Control cultures: ○△; cultures with 2,4-D: ●▲.

the repression by 2,4-D was associated with also a slight depression of ethylene release.

2,4-D Effects on Ethylene Evolution and Embryogenesis. A 0.1 mg/l concentration of 2,4-D repressed embryogenesis in carrot callus completely. However, the release of ethylene by the cultures showed only a slight depression. At the end of 4 weeks the untreated cultures had accumulated slightly over 1 μl/l ethylene, whereas the 2,4-D-treated cultures contained slightly less than 1 μl/l (Fig. 2). In addition to the small difference in ethylene production, the total yield of gas was too low to account for the severe embryogenetic repression by 2,4-D. The auxin effect must be by a mechanism other than an effect on ethylene production, as has been suggested in some other morphogenetic studies (1).

Conclusions. The conclusion that Ethephon inhibits asexual embryogenesis in carrot (4) is justified. The inhibitory effect could be attributed in large part to the ethylene it generates. However, some of the repression can be associated with a non-volatile component. A relatively high dose is required for embryogenesis to be suppressed by ethylene. The negative action of 2,4-D on the process cannot be related to an effect on ethylene evolution.

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