COMMUNICATIONS

An Analytical Procedure for Determining Residues of the Quaternary Ammonium Plant Regulator AMCHEM Z-2088

To elucidate the relatively short-term (14 day) growth retardant effects of the plant regulator dimethylheptyl-(1-hydroxy-*p*-menth-2-yl)ammonium bromide (AMCHEM Z-2088) on grapefruit trees (*Citrus paradisi* MacF.), an analytical procedure has been developed to determine its biological half-life in the leaves. Isolation and purification were accomplished by column and paper chromatography. Regulator levels were measured colorimetrically as the dipicrylamine complex at 415 nm. At a treatment level of 30 g per tree, the biological half-life for this plant regulator in grapefruit leaves was 10.8 days.

The synthesis and characterization of 11 *n*-alkyl quaternary ammonium plant regulators from the citrus terpene, (+)-limonene, have been reported previously (Pieringer and Newhall, 1968). The ability of these compounds to retard the growth of young bean plants varied with the length of the alkyl chain, dimethylheptyl-(1-hydroxy-*p*-menth-2-yl)ammonium bromide (Newhall, 1971) being most active in all tests. This plant regulator, when applied in an aqueous foliar spray (3000 ppm) to young greenhouse-grown grapefruit seedlings, effectively retarded vegetative growth by approximately 70% during the first 14 days (Pieringer and Newhall, 1970). On 3-year-old commercially grown lemon trees, two spray applications at the same rate retarded growth for about 4 weeks. Relatively long-term (136 day) growth retardation of grapefruit seedlings was achieved in the greenhouse (Newhall and Pieringer, 1972). However, this required the application of four successive sprays (3000 ppm) which would not be commercially feasible.

The present study was undertaken to determine why vegetative growth retardation by this plant regulator is of such relatively short duration. This necessitated the development of a method for determining residue levels in citrus leaves in order to determine the biological half-life of this compound. Mature 15-year-old grapefruit trees were used because the preponderance of testing done with this compound was on grapefruit seedlings in the greenhouse. Attempts to adapt the method of Mooney and Pasarela (1967) developed for the determination of the quaternary ammonium plant regulator chlormequat (2-chloroethyltrimethylammonium chloride) in wheat were not successful.

MATERIALS AND METHODS

Reagents used included: ethanol, 95% and absolute (U.S. Industrial Chemicals Co., New York, N.Y.) distilled in glass; methylene chloride (Fisher Scientific Co. D-37), distilled in glass through a 38-cm Widmer column; 2-propanol (Eastman Organic Chemicals, 13031, Catalog No. 47), distilled in glass; ethyl ether (anhydrous) (Fisher Scientific Co. E-138); methanol, distilled in glass by Burdick and Jackson Laboratories, Inc., Muskegon, Mich.; dipicrylamine reagent (2,2′,4,4′,6,6′-hexanitrodiphenylamine) (Eastman Chemical Co.) (a solution of 13.0 mg in 100 ml of purified methylene chloride was prepared); aluminum oxide, Fisher Scientific Co. A-540.

Analytical Procedure. *Leaf Extracts.* Fifty grams of mature grapefruit leaves was collected at random from the last growth flush of an unsprayed tree. The leaves were sliced with scissors and mixed thoroughly in a 600-ml beaker. Ten grams of this tissue was placed in a 400-ml stainless steel cup of a Sorvall omni-mixer. The extraction solvent was 200 ml of 95% ethanol acidified with 1 ml of 6 N HCl. Fifty milliliters of this acidified ethanol was added to the leaf tissue and the mixture homogenized for 1 min at speed setting 8. The mixture was filtered, using aspirator vacuum, through 12.4-cm diameter qualitative filter paper in a Buchner funnel and the filtrate collected in a 500-ml filter flask. The filter cake was transferred back to the 400-ml omni-mixer cup and rehomogenized with another 50-ml portion of acidified ethanol. This procedure was repeated two more times and the total filtrate was concentrated on a film evaporator (water bath temperature 50 °C) to a volume of about 50 ml. This solution was transferred to a 100-ml volumetric flask and diluted to the mark with 95% ethanol. This comprised the extract tissue blank.

Similar leaf extracts were prepared using this same procedure and adding known microgram amounts of growth regulator, dimethylheptyl-(1-hydroxy-*p*-menth-2-yl)ammonium bromide, to the leaf homogenate at the first extraction in the omni-mixer cup. In this manner, all extracts contained the same amount of leaf tissue (0.1 g/ml) and from 0 to 100 μg of plant regulator per ml. Control extracts and extracts containing known added amounts of plant regulator were processed individually and simultaneously as follows.

Ether Extraction. Five milliliters (0.5 g) of tissue extract was pipetted into a 25-ml round-bottomed flask and reduced to dryness on a film evaporator (water bath 50 °C). Two 10-ml portions of absolute ethanol were added separately and evaporated to remove the last traces of water and cause the precipitation of chlorophyl compounds. Fifteen milliliters of water and 3 Hengar (alundum) granules were added, the flask contents swirled to wash solid material from the sides, and the mixture filtered (gravity) through qualitative paper into a 125-ml separatory funnel. The flask was washed with three 10-ml portions of water, each filtered, and a final 10 ml of water was used to wash the filter. The filtrate was washed three times with 15–20-ml portions of ethyl ether and the ether washes discarded. About 15 ml of 95% ethanol was added to the aqueous phase to prevent foaming before it was concentrated to dryness on a film evaporator (water bath 50 °C).

Column Chromatography. A 10 × 100 mm alumina column was prepared in methylene chloride. The flask residue above was treated with two successive 0.5-ml

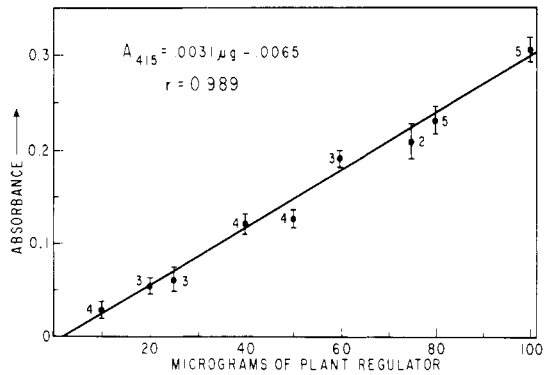

Figure 1. Plot of absorbance vs. micrograms of plant regulator per 5 ml of leaf extract.

portions of methanol–methylene chloride (50:50, v/v) for transfer to the column. One milliliter of methanol–methylene chloride (20:80) was used to complete the transfer. The column was developed with methanol–methylene chloride (20:80) and the first 50 ml of eluate collected was concentrated to dryness on a film evaporator.

Paper Chromatography. The residue from column chromatography was dissolved using three 0.5-ml portions of absolute ethanol and streaked on 19 × 18.5 cm Whatman No. 3MM paper, prewashed with ethanol–NH_4OH (28%) (95:5). The chromatogram was run ascending in ethanol–NH_4OH (28%) (95:5), and the upper 5 cm of the paper, where the plant regulator was located, cut off and eluted with 40 ml of 95% ethanol. This eluate was transferred to a round-bottomed flask and evaporated to dryness with a film evaporator. The residue was dissolved using three 0.5-ml portions of absolute ethanol and streaked on 9.5 × 43.5 cm Whatman No. 1 paper, prewashed with 2-propanol–NH_4OH (2.8%) (30:70). The chromatogram was run descending in 2-propanol–NH_4OH (2.8%) (30:70). The lower 8 cm of the chromatogram, containing the plant regulator, was eluted into a 100-ml flask with 40 ml of absolute ethanol and concentrated to dryness on a film evaporator. Drying was completed at high vacuum (<1 mmHg) in a vacuum oven at 40 °C.

Colorimetric Analysis. Five milliliters of standard dipicrylamine solution was added to each flask. The flasks were stoppered and left at room temperature for 1 h. A Bausch and Lomb Spectronic-20 was used to measure absorbance of the solutions at 415 nm using the reagent blank as a reference. Absorbance vs. micrograms of plant regulator per 5 ml of leaf extract was plotted on linear graph paper (Figure 1).

Field Experiment. One mature grapefruit tree was selected from a grove in which the subject plant regulator was to be applied as a foliar spray at a concentration of 500 ppm (30 g per tree). A leaf sample was picked from this tree prior to spraying to serve as a control. Mature leaves from the last growth flush were selected. After the spray had thoroughly dried on the leaves, another leaf sample was picked to establish the initial plant regulator residue level. Similar samples were picked from this same tree at 2 and 4 weeks after spray application. Leaf samples from an unsprayed grapefruit tree three rows away from the sprayed tree were picked at 2 and 4 weeks after spraying to serve as controls. All samples were immediately frozen in the grove with dry ice, transported to the laboratory, and kept frozen until analyzed. No rainfall occurred in the grove for 1 week after spray application.

RESULTS AND DISCUSSION

Quantitative recovery of quaternary ammonium compounds from plant tissue is difficult due to the tendency of the ammonium cation to become strongly adsorbed on negatively charged cell fragments. This is why it was found to be essential to use hydrochloric acid to flood the system with protons during the initial alcohol extraction. Even then repeated extractions of the filter cake were necessary for complete removal of growth regulator.

Table I. Residues of Plant Regulator (Dimethylheptyl-(1-hydroxy-*p*-menth-2-yl)ammonium Bromide) in Grapefruit Leaves

Days after spraying	Spectronic-20 readings[a]		Residue	
	Control	Treatment	μg	ppm
0	0.136 (±0.021)	0.285 (±0.029)	51	102
14	0.158 (±0.017)	0.213 (±0.030)	21	42
28	0.111 (±0.001)	0.121 (±0.001)	6	12

[a] Control and treatment samples were run in quadruplicate at 0 and 14 days and in duplicate at 28 days.

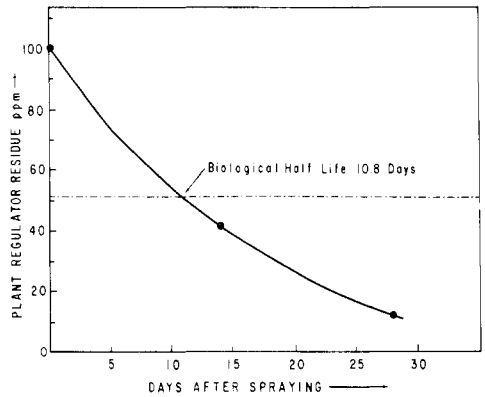

Figure 2. Plot of growth regulator residues vs. number of days after spray application.

The colorimetric reaction between quaternary ammonium compounds and 2,2′,4,4′,6,6′-hexanitrodiphenylamine (dipicrylamine) as a means of quantitative analysis has been reported (Schill, 1959; Schill and Danielsson, 1959). However, the analytical procedure reported herein is a much simplified adaptation of the original procedure employed by these authors.

In Figure 1, the vertical bars on the plotted points show the standard deviation for the number of replicates indicated by the numbers at each point. The equation for this standard curve is A_{415} = 0.0031 μg − 0.0065. The sample correlation coefficient determined by linear regression analysis is 0.989.

Residues of plant regulator in grapefruit leaves at 0, 14, and 28 days after spray application, determined using the standard curve (Figure 1), are presented in Table I. Standard deviations for Spectronic-20 readings on replicates are indicated. These residue data, expressed as parts per million of growth regulator, are plotted against number of days after spray application in Figure 2. The disappearance of growth regulator from the leaves with time is represented by a smooth curve which indicates a biological half-life in leaves of 10.8 days. Since other parts of the tree were not sampled for growth regulator residues, it is possible that this disappearance from the leaves could be due to translocation downward to the root system. In fact, two other closely related quaternary ammonium plant regulators synthesized from (+)-limonene have been shown to translocate downward from the stems of small grapefruit seedlings to the root system (Newhall and Pieringer, 1967). However, this study also indicated that rapid metabolism of these similar regulators occurred within all parts of the

plant. This was evident from changes in infrared absorption of residues isolated from both leaves and roots. The greatest change was the disappearance of the 1-hydroxyl group which suggests glycoside formation at this position. This is similar to the reported metabolism of abscisic acid to a water-soluble complex with glucose in apple (Powell and Seeley, 1974).

For these reasons, it is considered more likely that the disappearance of the plant regulator dimethylheptyl-(1-hydroxy-*p*-menth-2-yl)ammonium bromide from grapefruit leaves is also due to its metabolism and deactivation by the tree. The biological half-life determined (10.8 days) explains the short term (14 day) growth retardant effect invariably observed when this regulator is applied to grapefruit trees.

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Nutritive Value of Rye and Wheat Breads Assessed with *Aspergillus flavus*

The relative nutritive value of crust and crumb portions of rye and wheat breads has been assessed using the fungus *Aspergillus flavus*. Rye bread crumb was rated superior to wheat bread crumb. Crusts of both the breads showed a reduced nutritive value. The fungus graded these samples in the same order as has been done by the protozoan *Tetrahymena pyriformis* and by rat bioassays using the same material.

While looking for simple, rapid, and inexpensive biological tests for evaluating relative nutritive value (RNV) in plant breeder's material, we found that *Aspergillus flavus* produced a biomass response which was negatively correlated with protein content and positively correlated with lysine in protein. The material was tested at equal nitrogen level and included barley (Mohyuddin et al., 1976a) and rye, wheat, and triticale cultivars (Mohyuddin et al., 1976b). Encouraged by these results, we tested this fungus on a set of wheat and rye bread samples comprised of crust and crumb portions. Data on protein efficiency ratio (PER) using rat and RNV determined by *Tetrahymena pyriformis* were available with the same material (Kaestner et al., 1976).

MATERIALS AND METHODS

A non-toxin-producing strain of *Aspergillus flavus* Link ex. Fr. was initially isolated from spoiling bread crumbs and maintained on 2% malt extract agar. Spores from a 10-day old culture were harvested according to Mohyuddin and Skoropad (1972). The rye and wheat breads were prepared in the Bundesforschungsanstalt für Getreideverarbeitung, Detmold, West Germany. The preparation and further fractionation into crust and crumb portions have been reported by Brümmer and Seibel (1975). The data on the PER values using rat and RNV determined with *T. pyriformis* have been taken from Menden et al. (1975) and Kaestner et al. (1976), respectively.

The samples were weighed at 3 mg nitrogen equivalent in duplicate in 100-ml Erlenmeyer flasks. Soluble potato starch was added in a quantity that brought the total weight to 500 mg. Thus, the samples were tested at isonitrogenous and isoweight levels. Twenty milliliters of distilled water was added to each flask, allowed to stand for 1 h, and then autoclaved at 1 kg/cm^2 pressure (121 °C) for 10 min. A vitamin solution (Stott and Smith, 1966) was similarly autoclaved. One milliliter of spore suspension ($\sim 10^6$) and 1 ml of vitamin solution were added to each flask.

The contents were shaken on a flat flask shaker for 72 h at 26–28 °C. The mycelium produced was filtered through a nylon sieve, washed with water, transferred to a preweighed filter paper, and dried at 100 °C for 1 h, and the dry weight was calculated. This dry weight in milligrams was used as an index of biomass. Available lysine data were kindly supplied by Dr. Kaestner and were determined by the method of Booth (1971).

Table I. Biomass Development of *Aspergillus flavus* on Crust and Crumb Portions of Rye and Wheat Breads, in Comparison with RNV Determined by *Tetrahymena pyriformis* and PER Values Obtained with Rat

Sample	Nitrogen, %	Avail Lys, g/16 g of N	*A. flavus* biomass,[a] mg	*T. pyriformis*[b] RNV	Rat[c] PER
Rye bread crumb	1.57	2.6	101.5	33.3	1.7
Wheat bread crumb	2.00	2.1	86.9	22.1	1.2
Rye bread crust	1.50	1.2	77.2	10.3	−0.3
Wheat bread crust	1.96	1.0	72.2	10.3	−0.6

[a] Each value is the mean of two replicates. [b] RNV = relative nutritive value, expressed as direct microscopic counts of cells/milliliter in relation to casein; casein counts taken as 100. [c] PER = protein efficiency ratio.

RESULTS AND DISCUSSION

Table I presents the data on the biomass produced by *Aspergillus flavus* on the crust and crumb components of rye and wheat breads when administered at an equal