Residue Analysis of Gibberellic Acid in Grapes by Bioassay and Isotope Methods

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Two varieties of seedless grapes were sprayed with nonlabeled and tritium-labeled gibberellic acid. The initial deposit and final residues were determined by bioassay, using dwarf maize as a test plant, and total counting by liquid scintillation spectrometry. The two methods are compared, but the bioassay is preferred.

THE ACTION OF GIBBERELLIC ACID on grapes has been reported recently by Weaver (*7*). Thompson seedless grapes, treated shortly after bloom with gibberellic acid, increased in size considerably. This observation has led to the possible application of gibberellic acid in agricultural practices. In order to obtain U. S. Department of Agriculture registration for gibberellic acid, it was necessary to furnish residue data for treated grapes. For this purpose two methods of analysis were chosen: the bioassay of Neely and Phinney (*4*, *5*), and the radioisotope assay using tritium-labeled gibberellic acid. The bioassay is based on the specific growth response to gibberellic acid of certain dwarf mutants of maize (*6*).

Analytical Methods

Bioassay. Seeds of dwarf mutant of maize (d-1) were soaked overnight in tap water. Flats were prepared by mixing 1 to 1 (w./w.) of vermiculite and sterile soil. The flats were watered for uniform wetting and allowed to stand overnight. All experiments were run in a greenhouse with temperatures ranging from 54° to 92° F. No attempts were made to control light, temperature, or humidity.

The corn was planted in eight rows about 0.75 inch deep, 40 seeds per row. After the corn plants had emerged in 4 to 5 days, the seedlings segregated into three tall to one dwarf mutant. (The dwarf plant had a short broad first leaf.) The tall plants were removed, with the exception of one or two which were kept for comparison.

Ten milligrams of gibberellic acid (Abbott product) were dissolved in 1.0 ml. of ethyl alcohol and diluted to 100 ml. with 0.05% of aqueous Tween 20. Appropriate dilutions were made with Tween 20 solutions. One-tenth milliliter of standard solutions, containing 0.001 to 1.0 γ of gibberellic acid were applied with a 100-μl. pipet to the cups formed by the leaves of the seedlings. The experiment was arranged as a randomized complete-block, and five replicates were run for each treatment, including five controls with Tween 20 solution.

After 7 days, the plants were harvested and measured by the following method: the distance (mm.) from the prop root to the ligule of the first leaf (1), and from the prop root to the ligule of the second leaf (2). "Log concentration gibberellic acid" was plotted *vs.* log of (1 + 2) and a straight line resulted, using three concentration points.

Radioisotope Assay. Uniformly tritium-labeled gibberellic acid (specific activity, 108 μc. per mg.) was dissolved in a minimum amount of absolute ethyl alcohol (not to exceed 1.0 ml.) and diluted with 15.0 ml. of toluene containing 0.3% 2,5-diphenyloxazole (DPO). For very dilute samples, 0.01% 1,4 - bis - 2 - (5 - phenyloxazole) benzene (POPOP) (Arapahoe Chemicals Co.) was added to increase the counting rate. The samples were kept in low potassium-content glass vials (Wheaton Co.), and were counted usually for 10 minutes in a Tri-Carb manual liquid scintillation spectrometer at Tap 7 at a window width of 10 to 50 volts. Identical results were obtained at this setting on two different Tri-Carb instruments.

For unknown sample counting, the internal standard method was chosen. This consisted of counting the unknown solution by itself and counting it again after the addition of a known amount of tritium-labeled gibberellic acid. The amount of gibberellic acid in the unknown was determined by the simple proportionality equation:

$$\gamma \text{ gibberellic acid} = \frac{\text{net c.p.m. unknown}}{\text{net c.p.m. known}} \times \gamma \text{ known gibberellic acid}$$

Residue Analysis in Grapes

Treatment and Sampling. On April 30, 1958, ten vines each of Thompson-seedless grapes at the Harry Caviar vineyards in Indio, Calif., were sprayed to run-off by hand atomizer with 100 p.p.m. of gibberellic acid and 100 p.p.m. of tritium-labeled gibberellic acid dissolved in 0.1% of aqueous Tween-20. This date was approximately 1 week after bloom. About 20 clusters of each series of treated grapes and controls were harvested immediately after spray treatment in order to obtain the "initial spray deposit." These samples were extracted 1 day later in the Davis laboratories, and another batch was shipped to Abbott, North Chicago, Ill., via air express. Samples were taken on June 3, 1958 (about 20 days before normal harvest) and on June 20, 1958. Samples were again independently analyzed by the laboratories of the University of California at Davis, and Abbott, North Chicago, Ill.

On May 27, 1958, eight vines of Black Corinth grapes in the University of California, Davis, vineyard were sprayed as above. Initial residues were run. They were harvested on August 13, 1958, for bioassay and radioassay.

On June 11, 1958, another group of Thompson-seedless grape vines at Davis was treated with gibberellic acid and tritium-labeled gibberellic acid. No initial residue was run. The grapes were harvested on August 12, 1958, and assayed at the Davis and Abbott Laboratories.

Extraction of Grapes. Between 250 and 500 grams of grapes, including stems, were homogenized in a Waring Blendor with one volume of acetone at a low speed for 2 minutes. The slurry was filtered by suction with Celite filter aid, and the solids were re-extracted with the same volume of acetone. The acetone was evaporated in vacuo in a Rinco evaporator, and the remaining water phase was filtered. This step removed the chlorophyll, which was insoluble. The water phase was extracted with three 100-ml. volumes of ethyl acetate, and

Table I. Gibberellic Acid Residue Analysis of Bioassay

Sample	Aliquot Analyzed, G.	Distance (1) + (2), Mm.	Log [(1) + (2)]	Gibberellic Acid γ	Gibberellic Acid P.p.m.
Standard Curve					
Control	..	50.4	1.7024	...	...
Gibberellic acid, γ					
0.001	..	50.4	1.7024	...	...
0.010	..	50.8	1.7059	...	...
0.100	..	74.8	1.8739	...	...
1.000	..	106.4	2.0270	...	...
Thompson seedless, Indio, 34 days after application					
Control grapes	1.03	48.2	1.6830	0	0
Gibberellic acid	1.99	64.6	1.8102	0.042	0.021
H^3-gibberellic acid	1.84	65.8	1.8182	0.046	0.025

Table II. Analysis of Variance (Data from Table I)

Source	Degrees of Freedom	Sums of Squares	Mean Squares	$F_{exp.}$	$F_{0.95}$
Total	39	14,951	383.35		
Replicates	4	224	56.00	1.34	2.69
Treatments	7	13,559	1,937.00	46.43	2.33
Error	28	1,168	41.71		

$\Sigma X^2 = 6,538,249$

$n = 40$

$$s_{\bar{x}} = \sqrt{\frac{41.71}{5}} = 2.89$$

Table III. Duncan's Multiple Range Test (3)

(Data from Tables I and II)

Treatment	Treatment Ranks of Means, Mm.	Least Significant Range 5% (LSR) x $s_{\bar{x}}$	Means − LSR	Results[a]
Grape control	48.2	...	...	
GA control	50.4	8.38	42.02	
0.001 γ GA	50.4	8.78	41.62	
0.01 γ GA	50.8	9.04	41.76	
GA	64.6	9.24	55.36	
H^3–GA	65.8	9.42	56.38	
0.1 γ GA	74.8	9.53	65.27	
1.0 γ GA	106.4	9.62	96.78	

[a] Values within same bracket are not significantly different.

the combined extracts were dried over anhydrous sodium sulfate. This was filtered again and the sodium sulfate was washed with small volumes of ethyl acetate. The combined ethyl acetate fractions were taken to dryness in vacuo at about 25° C. The residue was taken up in 1.0 ml. of ethyl alcohol and an appropriate volume of 0.05% of aqueous Tween 20 for bioassay. For direct isotope counting the sample was dissolved in 1.0 ml. of ethyl alcohol and in 15.0 ml. of 0.3% of diphenyloxazole in toluene.

Residue Analyses by Bioassay. Table I gives a detailed account of one residue sample of Thompson-seedless grapes harvested 34 days after they were sprayed with 100 p.p.m. of gibberellic acid and tritium-labeled gibberellic acid. As the environmental conditions of the bioassay were not closely controlled, it was necessary to run a standard curve for each analysis.

Each bioassay was analyzed statistically by analysis of variance (Table II) and Duncan's multiple range test (*3*). As shown by Duncan's multiple range test (Table III), the response due to 0.01 γ of gibberellic acid in this bioassay was not significantly different from the control. In all subsequent bioassays, the values for 0.001 γ of gibberellic acid and control were discarded because of nonsignificance, and a three-point standard curve (0.01, 0.1, 1.0 γ of gibberellic acid) was used throughout. Because in these tests only five replicates per treatment were chosen, it might be expected that Neely (*4*) was able to attain a sensitivity of 0.001 γ of gibberellic acid by using 10 replicates. As seen in Table II, the variance of replicates was not significant, while that of the treatments was highly significant.

Table IV is a summary of residue analyses of Thompson-seedless and Black Corinth grapes. The residue, as measured by this technique, decreased appreciably 34 days after the application of 100 p.p.m. of gibberellic acid. The gibberellic acid residue in Black Corinth grapes decreased in a similar manner.

Some of the extracts of Thompson-seedless grapes at one week after bloom seemed to have a stimulating effect on dwarf maize as was recorded after visual inspection. Duncan's test, however, proved that this apparent stimulation was not statistically significant. Coombe (*2*) has actually found gibberellin-like response in extracts of seedless grapes shortly after bloom. This observation has now been confirmed in the authors' laboratories using extracts of pollen of Thompson seedless grapes.

The extraction efficiency of the method was tested by adding 0.1 p.p.m. of gibberellic acid and 0.1 p.p.m. of tritium-labeled gibberellic acid to a weighed amount of control grapes at harvest time. The recovery was 120% (Table IV) at this concentration level.

Residue Analysis by Radioisotope Counting. The results by the direct "total counting technique" are found in Tables IV and V. Table V is a detailed account of four replicate samples of initial residue which were assayed independently by two laboratories. The good agreement of the results indicates the reproducibility of the extraction and counting procedures.

The lower counting efficiency of samples II *A*, *B*, as compared with I *A*, *B*, is presumably due to the quenching effect by plant pigments, as a greater aliquot size was chosen.

Values by the bioassay technique were consistently lower than those by the isotope technique (Table IV). This discrepancy may be due to a partial breakdown of the gibberellic acid to a compound which is inactive when tested by the response on d-1 dwarf corn. The extraction efficiency, as measured by per cent recovery (Table III) was only 56.5% which would make the "isotope residue values" even higher if corrected for complete recovery. The chemical nature of these radioactive compounds, other than gibberellic acid, is under study at this time. Baumgartner *et al.* (*1*) have confirmed that some of the radioactive material is due to gibberellic acid.

On the basis of the results of residue analysis of gibberellic acid, the biological assay gives a more reliable level than the total radioactivity and should, therefore, be used for the establishment of tolerance. These residues were obtained from an initial spray of 100 p.p.m. which

is at a higher concentration than recommended for agricultural practices (*7*).

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Table IV. Summary of Gibberellic Acid Residues in Grapes

Sample	Days after Application	Bioassay, P.P.M.	Isotope Analysis, P.P.M.	Recovery, %
Thompson seedless, Indio				
Control	0	0.024[a]	..	....
Gibberellic acid	0	2.60	..	....
H^3-gibberellic acid	0	1.79	3.320	....
Control	34	0.00[b]	..	....
Gibberellic acid	34	0.021	..	....
H^3-gibberellic acid	34	0.025	0.086	....
Thompson seedless, Davis				
Gibberellic acid	62	0.024	..	....
H^3-gibberellic acid	62	0.008	0.032	....
Control	..	0.00[b]	..	....
Control + 0.1 p.p.m. GA	0	0.24	0.056	120% (bioassay)
Control + 0.1 p.p.m. H^3-GA				56% (isotope analysis)
Black Corinth, Davis				
Control	0	0.32[a]	..	....
Gibberellic acid	0	0.75	..	....
H^3-gibberellic acid	0	4.33	12.630	....
Control	78	0.00[b]	..	....
Gibberellic acid	78	0.019	..	....
H^3-gibberellic acid	78	0.008	0.028	....

[a] Not significant—Duncan's test (5% level). [b] Less than 0.002 p.p.m.

Table V. Gibberellic Acid Residue Analysis by Isotope Counting

Initial deposit, Thompson Seedless, April 30, 1958

Sample	Aliquot Counted, G.	Counts per Minute: Sample, net	Counts per Minute: Standard net	H^3-Gibberellic Acid Added, γ	Gibberellic Acid Found, P.P.M.
I A	0.029	2014	25,689	1.23	3.36
I B	0.029	2060	28,148	1.14	3.12
II A	1.00	569	203	1.14	3.23
II B	1.00	566	195	1.23	3.58

LEACHED ZONE PHOSPHATES

Initial and Residual Effectiveness of Two Leached Zone Phosphate Fertilizers

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Two NPK fertilizers prepared from Florida leached zone phosphate were compared with concentrated superphosphate for successive crops of Sundangrass, wheat, and Sudangrass in greenhouse and field experiments. The two leached zone fertilizers differed in the water solubility of their available phosphorus content, 4 and 32%. The available phosphorus was citrate-soluble. The initial effectiveness, as measured by the first-crop yields of Sudangrass, was related to water solubility; the 4% fertilizer was less effective than and the 32% fertilizer was comparable to concentrated superphosphate. No differences were found in residual effectiveness for the second or the third crop.

THE LEACHED ZONE ORES overlying the phosphate rock in the Florida deposits have been used by the Tennessee Valley Authority in the production of nitric phosphate fertilizers. The methods of fertilizer manufacture have been described in detail by Hignett *et al.* (*2*). The phosphate in the ores is present primarily as the minerals wavellite and pseudowavellite, although some ores contain varying amounts of apatite.

Starostka, Norland, and MacBride (*4*) reported that a leached zone fertilizer in which 6.7% of the available phosphorus was water-soluble was less effective than concentrated superphosphate, but one with 21% water-soluble phosphorus was comparable to concentrated superphosphate. DeMent and Seatz (*1*) concluded from the results of a number of field and greenhouse experiments that leached zone fertilizers were satisfactory sources of phosphorus for cotton, small grain, and corn, even though they were somewhat less effective than concentrated superphosphate.

The experiments reported were conducted to measure both the initial and residual effectiveness of the leached zone fertilizers as compared with concentrated superphosphate in greenhouse and field experiments.

Experimental Procedure

Similar field experiments were con-