Determination of Gibberellins in Ovaries and Young Fruits of Navel Oranges and their Correlation with Fruit Growth

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Abstract. After refinement, a fluorometric technique was found adequate for quantitatively determining certain gibberellins in the flowers and young fruits of sweet oranges. Using this method, significant changes in gibberellins were shown to occur, both in tissue concentrations and total amounts per fruit, in samples collected during the bloom and early period of fruit growth.

Two relationships, a correlation between gibberellin concentration and rate of fruit growth, and an effect of total gibberellins per fruit on cumulative fruit growth, were found. These data indicated a cause and effect relationship between endogenous gibberellins and the early stages of fruit growth of the navel orange.

Introduction

The navel oranges constitute a group of very similar cultivars of sweet oranges, Citrus sinensis Osbeck, long known as low or erratic bearers (30). Because of pollen sterility and defective embryo sacs, very few seeds are produced, which results in unfruitfulness. Parthenocarpic production of fruit is commonplace but not sufficient for consistent, satisfactory crops (7). Attempts to improve yields through cross pollination (29), cultural practices (3), budwood selection (25), girdling (17), and application of growth regulators (12, 18) have met with limited success.

Gibberellic acid has shown promise in overcoming this problem. In both California and Florida fruit-set response of navel oranges to gibberellic acid (GA) (hereafter referred to as GA) has been shown to occur. GA applied to individual flowers and flowering shoots has increased fruit-set over a wide range of concentrations but has not been effective when applied to entire trees (12, 18).

The reason for this inconsistent response is not known. Moreover, basic information on which to predicate further research, such as the amounts of endogenous gibberellins in the navel orange is unavailable (16, 26). This has been mostly due to the lack of an accurate and rapid analytical method for determining the amounts of gibberellin-like compounds in partially purified extracts from the plants. This is a report on the suitability of a fluorometric method for measuring certain naturally-occurring gibberellin-like compounds (hereafter referred to as gibberellins) in young navel oranges, the amounts of these gibberellins in the young navel oranges during early growth, and the relationship between gibberelin content and fruit growth during this period.

Materials and Methods

Analytical method. GA, has been identified from vegetative citrus tissue and GA has increased fruit-set when applied to navel orange flowers (12, 18, 26). GA and GAA have shown high growth promoting activity in many plant tissues (10, 11, 21, 23, 28) and can be isolated in the acidic, ethyl acetate soluble portion of plant tissue extracts (6, 11, 13, 28). GA and GA, have similar fluorescence characteristics (6, 27), and relative fluorescence intensity (RFI) has been used for quantitative determination of gibberellins (8, 15, 27). Therefore, RFI was investigated as a suitable quantitative method for determining amounts of GA and GA, in young navel orange fruits.

A fluorometric method has been used to determine GA in several fruits (8). A similar method was used in the present research. A weighed sample of young, whole navel orange fruits was ground for 2 to 4 min with acetone and water in an Omni-mixer, the amounts of acetone and water depending upon the weight of the sample. After filtration of the homogenate on Whatman 1 paper, the pulp and paper were ground again with acetone in the original Omni-mixer container. The second homogenate was filtered on a Whatman 1 paper on the original filter funnel and the mixer jar and filter cake were washed with additional acetone. The combined filtrates were collected in a Florence flask and the acetone removed under vacuum. The resulting aqueous solution was then quantitatively filtered through a glasswool pledget overlaid on a filter paper.

The filtrate was measured and a portion taken which represented the sample size desired for further study. This aliquot was acidified to pH 2.5 with 25% sulfuric acid and extracted 3 times with 10 ml portions of ethyl acetate. The combined extracts were then extracted 3 times with 10 ml portions of water buffered at pH 7.0. The combined aqueous extracts were acidified to pH 2.5 and extracted again 3 times with 10 ml portions of ethyl acetate. The combined extracts were transferred to a 50 ml Florence flask and dried under vacuum.

The sample residue was redissolved in 0.5 ml of ethyl acetate and streaked 2 cm from the base of an Eastman K301R2 silica gel thin-layer chromatographic plate. Markers of GA were applied at both edges of the chromatogram and separated from the sample by scraping 0.2 cm-wide vertical bands of silica gel from the polyester backing of the chromatogram.

The chromatogram was developed with chloroform, acetic acid, methanol (75:24:3) until the solvent front had traversed 15 to 16 cm. The solvent front was marked, the chromatograms dried for 5 minutes and the marker strips cut from the chromatogram. After spraying with 90% ethanolic sulfuric acid, the marker strips were heated at 100°C for 2–3 min and viewed under UV light. The fluorescing GA spots were marked and these marker strips used to locate the GA and GA, zone of the chromatogram. A strip 4 cm wide, centered on the marker spots, was cut from the chromatogram. The silica gel from this strip was scraped into a fritted glass funnel, the polyester backing rinsed with chloroform-methanol (20:10) and the silica gel on the funnel eluted with additional chloroform-methanol. The resulting solution was dried under vacuum in a large test tube. For fluorescence development, 2 ml of water were introduced into the test tube, the tube cooled for 5 min in an ice bath and 10 ml of chilled 85% sulfuric acid (10°C) was added. After an additional 10 min in the
ice bath, the tubes were removed and allowed to remain overnight at room temperature prior to determination of RFI.

Fluorescence spectra and RFI determinations were made using an Amino-Bowman spectrophotofluorometer. Solution temperature was maintained at 22°C. RFI's were determined at 420 mµ excitation and 460 mµ emission wavelengths, the maximums for GA, GA1, and processed samples. Fluorescence of samples was compared to fluorescence of known amounts of GA and amounts of gibberellins in the samples was determined as GA equivalents per gram fresh weight tissue.

Inconsistent results in the early stages of this research indicated the possibility of glassware contamination by fluorescent substances. All subsequent processing was accomplished in glassware treated to remove such substances. After routine washing, glassware was rinsed with hexane, then methylene chloride and finally with methanol.

The analytical technique was evaluated to determine its sensitivity, both in the presence and absence of interfering materials from the plant tissue. Additional tests were made to determine the most appropriate sample size and demonstrate the ability of the technique to detect both GA-like and GA1-like substances in the tissues. Further, several tests were made to verify the gibberellin-like character of the processed samples.

To establish the sensitivity of the spectrophotofluorometer to GA in the absence of interfering substances, aqueous solutions of GA ranging in concentration from 4.88 mµg per ml to 20 mµg per ml were prepared. These were treated with 85% sulfuric acid at 0°C, and their RFI's determined using the technique and instrument parameters previously described.

The sensitivity of the technique in the presence of interfering substances in plant tissue extracts was determined by adding GA to young fruit samples. The samples plus GA were processed and their RFI's determined. For this test, a large sample of young fruits was ground with acetone-water, filtered and the acetone removed from the filtrate. Eighteen identical portions of the aqueous extract of the sample were taken. Each portion represented 1 g of the original fruit sample. One of each of 5 different GA amounts was added to 3 portions of the sample, the sample portions were processed and RFI's determined using methods previously described.

The effect of size of sample portion on RFI's of processed samples was investigated. Previous workers used large tissue samples (8, 13, 16) or concentrated broths (15, 27). Due to the small size of ovaries and young fruits of navel orange, collection of large samples could seriously limit the scope of any contemplated research. Sample size was reduced to 1 g during development of the quantitative procedure. Later improvements in sample collection methods permitted an increase in sample size. Therefore RFI's of 1-, 3- and 10-g portions of aqueous sample extracts were compared to establish the effect of increased sample size on fluorescence of processed samples. All sample portions were processed and RFI's determined using previously described techniques and instrument parameters.

Similarity of movement of GA and GA1 on Eastman K301R2 TLC plates was verified. GA and GA1 were spotted at the base of an Eastman plate and the plate developed with chloroform, acetic acid, methanol (74:24:3). Movement was detected by spraying with 90% ethanolic sulfuric acid, heating and viewing under UV light.
Fluorescence excitation and emission characteristics of GA and GA$_1$ are identical but comparative RFI of the 2 gibberellins had not been firmly established (6, 27). Since temperature, sulfuric acid concentration and mixing conditions affect RFI of gibberellins, equal concentrations of both gibberellins were reacted with 85% sulfuric acid at 0°C, and RFI's of both gibberellin solutions determined following routine procedures as previously stated.

Since several modifications were made of the basic procedure (8), additional verification of the gibberellin-like character of the samples was made. Gas chromatographic comparison of propylated samples to propylated solutions of GA and GA$_1$, activity in the barley endosperm bioassay (22) and fluorescence spectra were used.

For the gas chromatographic comparisons, a sample of fruit tissue was extracted and processed, up to the point of fluorescence development with sulfuric acid, using the routine analytical procedure previously described. This processed plant sample, pure GA and GA$_1$, and a solvent blank were propylated with an ethereal solution of diazopropane. The propylated solutions were injected into an F and M Model 400 gas chromatograph equipped with a 15% SE 30 on Chromosorb W column and flame ionization detector. Injector, column and detector temperatures were 275°, 250° and 280°C, respectively.

For the verification of gibberellin-like activity in the processed samples, using the barley endosperm bioassay, 2 samples of fruit tissue were extracted and processed up to the stage of fluorescent development with sulfuric acid. The 2 samples were assayed using the barley endosperm bioassay as outlined by Paleg et al. (22).

**Endogenous gibberellins in navel orange fruits.** Tests were designed to determine GA-like and GA$_1$-like gibberellins in fruit samples during the period of fruit-set and early fruit-drop. Therefore, the length of the period of early fruit-drop was determined by counting the abscised fruits from full bloom until early drop ceased. Fruit-drop counts were obtained both from fruit collected from screens placed below the tree and by periodically counting fruits on tagged branches. The counts included fruits from both the bouquet bloom and the leafy bloom.

Samples for analysis were from singly-occurring, leafy-bloom fruits collected weekly from full bloom until 9 weeks after petal fall (March 21 to May 30, 1967) from each of 4, 20-year-old 'Washington' navel orange trees in a commercial orange orchard. The samples, varying in size from 6 to 150 fruits, depending on the size of the fruits at the time of collection, were frozen immediately and stored frozen until analyzed.

For processing, each sample was removed from the freezer, poured from the collection container into an insulated cup on a torsion balance, weighed and the diameter of the individual fruits measured. The sample was extracted and a sample portion representing 3 g of fresh tissue was taken from the first aqueous solution and quantitatively analyzed for gibberellins using the analytical technique previously described. Each sample, after processing up to final fluorescence development, was frozen and stored until processing of all samples was completed. To reduce variability, fluorescence development with sulfuric acid was performed at the same time for all samples. RFI's of the processed samples, GA standards and blanks were determined with the Aminco-Bowman spectrophotofluorometer and the RFI's were converted to GA equivalents.

Finally, the gibberellin concentration (GA equivalents per gram of fresh tissue), total amount of gibberellin per fruit (GA equivalents per individual
fruit at the time of sampling), mean weight of fruit, mean volume of fruit, and % growth rate of the fruits were calculated for the 11 weekly sampling periods. Data were then analyzed statistically and comparisons made between GA-like and GAl-like gibberellins in the fruit and fruit growth.

**RESULTS AND DISCUSSION**

**Analytical method.** RFI of GA was determined and found to be approximately linearly related to concentration in the concentration range from 4.88 mg to 20 µg per ml. RFI's were determined with an Aminco-Bowman spectrophotofluorometer using parameters previously described. Although not the most sensitive assay (22) for gibberellins, the fluorometric method was more sensitive than most available bioassay methods and was less complex.

The sensitivity and precision of the fluorometric method in the presence of interfering substances was established by adding each of 5 different amounts of GA to 3 identical 1-g sample portions and determining the RFI's of the processed samples (Fig. 1). The lowest concentration tested (2.65 mg per ml) did not give RFI's which were significantly higher than RFI's of samples to which no GA had been added. Differences in GA concentration from 5.2 mg to 41.7 mg per ml were detected by the spectrophotofluorometer.

When RFI's of 1-, 3- and 10-g aliquots of samples were compared (Fig. 2), RFI increased in proportion to increase in sample size. The increase in RFI as sample portion was increased from 1 to 3 g was approximately the same as the calculated increase. The increase in RFI as sample portion was increased to 10 g was less than the calculated response. This appeared due to dampening of fluorescence by increasing amounts of impurities in the processed samples. Therefore, sample size was increased to 3 g for determination of gibberellin-like substances in subsequent fruit samples.

GA and GAl movement on Eastman K301R2 TLC plates using a chloroform, acetic acid, methanol solvent system was identical and the Rf of both substances was 0.57.

When equal concentrations of GA and GAl were treated with sulfuric acid under the experimental conditions used in this research (85% sulfuric acid at 0°C), RFI of GAl was 47% of the RFI of GA. Both substances, however, fluoresced strongly under these conditions and changes in both GA and GAl would be detected using the prescribed procedure.

The results of the gas chromatographic comparisons gave positive evidence of gibberellin-like substances in the processed samples. When the gas chromatographs were compared, all peaks appearing in the prophylated sample, except one, were found in either the propylated GA or GAl solutions, or in both.

Further, the 2 processed samples which were assayed using the barley endosperm bioassay gave a positive response. Comparison of fluorescence excitation and emission spectra of processed samples agreed with published data for GA and GAl (6, 27) and with spectra of standards.

The basic analytical method (8) was developed for determining GA alone and it has been shown in this research that GAl, the only gibberellin definitely isolated from citrus tissue (26), was detectable by this method. Both GA and GAl, separated from plant tissue extracts by partitioning from an acidic aqueous solution into ethyl acetate (6, 11, 13, 28), moved in a similar

![Fig. 5. Seasonal changes in average weight and volume of ovaries and young fruits of navel orange.](image)

![Fig. 6. Mean weekly % growth and gibberellin concentration of ovaries and young fruits of navel orange.](image)
manner on silica gel thin-layer chromatograms, as shown in this and other research (6, 13, 14), fluoresced when treated with sulfuric acid and had identical fluorescence spectra when treated with sulfuric acid (6, 27). Thus, any GA applied to the plant for promotion of fruit-set, or GA or GA₁, naturally present in the tissue, could be detected by this fluorometric method. This method, however, does not discriminate between GA-like and GA₁-like substances.

Endogenous gibberellins in navel orange fruits. The early fruit-drop period occurred from full bloom until 4 weeks after petal fall (Table 1). Since the fruit-drop data included fruits from both leafy and bouquet bloom, and samples were taken of leafy bloom fruits only, no direct comparisons were possible between fruit-drop and gibberellins in the fruit. These data showed, however, that the sampling period included the early fruit-drop period.

Amounts of GA-like and GA₁-like gibberellins in the samples for the 11 sampling dates were determined and expressed on bases on concentration and total amounts per fruit (Fig. 3). Significant differences were found using Duncan’s Multiple Range Test (5, 9). Concentration of these gibberellins was high at full bloom (FB), decreased at petal fall (PF), increased 1 week after petal fall (PF + 1), and remained higher than the amount at petal fall for the remainder of the sampling period. Concentration of gibberellins changed significantly during the period from 1 week after petal fall (PF + 1) to 6 weeks after petal fall (PF + 6). Total amounts of gibberellins per fruit did not change significantly during the first 4 weeks of sampling but increased significantly 4 weeks after petal fall (PF + 4) and each week thereafter.

Indirect evidence has been reported (4) that gibberellins are a controlling factor in fruit growth through regulation of both cell division and cell enlargement. Therefore, changes in concentration and amounts of gibberellins were examined to determine their relation to growth of the young fruit.

Fruit growth, on a basis of either mean weight or mean volume (calculated as the cube of mean diameter), followed the expected sigmoid growth curve of citrus fruits as described in previous research (1, 2) (Fig. 4). Semi-logarithmic plots of mean weight and volume (Fig. 5) showed 2 stages of fruit growth during the sampling period. From full bloom until 6 weeks after petal fall, fruit growth was rapid, but variable, and increased exponentially. After this time, fruit growth-rate declined. Growth of fruits to the size of those at PF + 5 has been considered primarily due to cell division (stage I of fruit growth) with subsequent weight and volume increases primarily due to cell enlargement (stage II of fruit growth) (1, 2, 19).

Two relationships between growth of the young fruit and GA-like and GA₁-like gibberellins in the samples were examined. These were the effect of changes in gibberellin concentration on fruit growth during the stage of fruit growth when growth is primarily a result of cell division, and the effect of total amounts of gibberellins per fruit on initiation of the stage of fruit growth when growth is primarily by cell enlargement.

For the comparisons of fruit growth-rates and gibberellin concentrations during stage I of exponential fruit growth, weekly % increase in weight and weekly % increase in volume were used as indicators of weekly growth-rate. These growth rates were determined by calculating weekly weight or volume increments as percentages of weight or volume at the beginning of the week. In these comparisons (Fig. 6), significant changes in gibberellin concentration preceded significant changes in % rate of growth. The time-delay interval between increases and decreases in gibberellin concentration and corresponding changes in per cent-rate of growth was 2 weeks in the first 2 sampling periods and 1 week in subsequent sampling periods, due possibly to conditions more favorable for rapid growth response during the latter part of the sampling period.

When the time-delay between changes in gibberellin concentration and changes in per cent growth by weight was taken into account, and pairings were made of the weekly change in gibberellin concentration...
and the weekly change in per cent growth by weight during stage I of fruit growth, a very high correlation was found (Fig. 7). These paired values were taken from the individual data for the 4 experimental trees during the period from full bloom until 6 weeks after petal fall. Correlation analysis of the 24 pairs of values gave a highly significant correlation coefficient ($r = 0.703$). A similar comparison between gibberellin concentration and per cent growth by volume gave an almost identical result.

Changes in total amounts of gibberellins per fruit appeared to affect the cell enlargement phase of fruit growth. The first significant increase in total gibberellins per fruit occurred 4 weeks after petal fall while the first significant increase in mean weight occurred 5 weeks after petal fall (Fig. 8). Mean sample volumes and total gibberellins per fruit showed an identical relationship. Thus, at a time when young fruit growth primarily due to cell enlargement (stage II) was being initiated, total gibberellins per fruit increased significantly 1 week before the first significant increase in fruit weight or volume.

A time-delay between stimulus and fruit-drop has been described in citrus (3) and gibberellin concentration change and growth response in other plants (20, 21). The data from this experiment suggest that a cause and effect relationship exists between certain gibberellins and growth of young navel orange fruit.

The significant changes in gibberellin concentration and the rate of fruit growth may be a possible cause for the variable and sometimes damaging responses obtained when GA was applied to navel oranges for promotion of fruit-set (12, 18). If GA were applied directly to individual blossoms the ability to measure both endogenous, growth-promoting gibberellins and GA added to navel orange tissue makes feasible further studies of absorption and translocation of GA and the effects of gibberellins on fruit-set and fruit growth.

### Literature Cited


The Simultaneous Measurement of Apparent Photosynthesis and Transpiration of Citrus Seedlings at Different Soil Moisture Levels

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Abstract. Diurnal rates of photosynthesis and transpiration of sour orange and sweet lime seedlings were measured continuously and simultaneously under 4 different soil moisture regimes, using an infra-red gas analyzer and hygrosensors. Photosynthesis and transpiration rates were found to decrease gradually as the soil moisture dropped from 17% to 9% (equivalent to tensions of 0.2 to 3 atm). A sharp reduction in both processes was observed for both rootstocks when the soil moisture was in the wilting range. At the high moisture content, photosynthesis and transpiration rates of sweet lime were higher than those for sour orange plants. Conversely, at the low moisture content (in the wilting range), the rates were greater for the sour orange seedlings. The high soil moisture tensions had a relatively greater effect on photosynthesis than on transpiration.

T he effect of soil moisture on transpiration and photosynthesis has been the subject of many investigations. Some of the investigators have concluded that these processes are affected only when the soil moisture reaches the wilting point (9, 10). On the other hand, there are those who claim that transpiration and photosynthesis rates decrease before the wilting point is reached (7). This contrasting approach can be attributed somewhat to the different environmental conditions under which the various studies were conducted, and perhaps also to the fact that in most cases the 2 processes were measured separately, and with destructive methods.

If it is desired to compare the response of 2 plant varieties, it is necessary to conduct the research under identical conditions and to measure the pertinent physiological processes continuously and simultaneously, without destroying the plants.

The purpose of the present research was to study the response of 2 citrus rootstocks, sour orange and sweet lime, commonly used in the citrus groves of Israel, to different soil moisture regimes by measuring photosynthesis and transpiration. These 2 processes were measured simultaneously and continuously on intact plants of each of the 2 rootstocks by means of infrared-absorption and hygrosensors.

Materials and Methods

Sour orange, Citrus aurantium L., and sweet lime, Citrus limettoides Tanaka, seedlings were grown in a greenhouse in pots containing 1 kg of regosol loess soil. They were fertilized with a nutrient solution according to Hewitt (4). Five to 6 month old plants, having similar leaf numbers and areas, were selected for the experiments.

The water content of the soil at "pot capacity" was 20%, and at wilting percentage it was 7%, on a dry weight basis. Diurnal measurements of transpiration and photosynthesis were made simultaneously as the soil moisture content decreased from pot capacity to wilting percentage. The containers were irrigated when the water content reached 17%, 11%, 9%, or 7%, giving 4 different moisture treatments. These moisture levels were equivalent to soil moisture tensions of 0.2, 1.2, 3 and 15 atmospheres, respectively.

Soil moisture extraction was followed by weighing with a Mettler balance, and the soil moisture tensions were determined according to the moisture retention curve for the experimental soil. Photosynthesis and transpiration were measured using the method suggested by Bravdo (2).

The plants were enclosed in assimilation chambers consisting of 3 parts: A - a 0.02 mm thick polyethylene bag; B - a base; and C - a stand (Fig. 1, 2). In the base were 2 openings, one (AE) for air entry and the other (AO) for air withdrawal, and thermistor (T) for temperature measurement, and a fan (F). A fan produced air turbulence within the chamber to maintain a