FINAL REPORT
PROJECT NO. US-1587-88

Involvement of Different Gibberellins in Flowering of Biennial Bearing Olive Trees

Y. Ben-Tal

1993
DATE: 24.11.1993

BARD project No. US-1587-88

Title:
Involvement of Different Gibberellins in Flowering of Biennial Bearing Olive Trees.

Investigators' Names
(Principal listed first)

Y. Ben-Tal

Maria Wodner

Investigators' Institutions

A.R.O. The Volcani Center

A.R.O. The Volcani Center

Project's starting date: October 1988

Type of Report: 1st Annual _____ 2nd Annual _____ Final √

Signature
Principal Investigator

Signature
Institution's Authorizing Official
<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Objectives of the project</td>
<td>2</td>
</tr>
<tr>
<td>Scientific report:</td>
<td></td>
</tr>
<tr>
<td>Introduction</td>
<td>3</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>5</td>
</tr>
<tr>
<td>Results</td>
<td>10</td>
</tr>
<tr>
<td>Discussion</td>
<td>27</td>
</tr>
<tr>
<td>References</td>
<td>31</td>
</tr>
<tr>
<td>Cooperation</td>
<td>37</td>
</tr>
<tr>
<td>Project Achievements</td>
<td>37</td>
</tr>
</tbody>
</table>
Involvement of different gibberellins in flowering of biennial bearing olive trees

Abstract

Biennial bearing is a severe obstacle in olive production for both the producer and the consumer. It causes large fluctuations in growers income and in market prices from one year to the next. As in most species, biennial bearing in olive is characterized by poor blossoming during "off" years followed by enormous flowering the next year ("on" years). In many plants, plant hormones, especially gibberellins (GAs), have been shown to be involved in the flowering process. In this project olive buds taken from "on" and "off" trees, during their differentiation season, were tested for their endogenous GAs. Using a combination of biological and current biochemical and physico-chemical instrumental techniques, enabled us to identify 20 different GAs in these buds. It was found that the buds taken from trees after "off" years, contained more GAs and larger quantities of these GAs than did buds after "on" years. Furthermore, the buds after "off" years, that were suppose to flower during the following spring, contained large amounts of GAs from the GA4 route, which are known to be associated with flowering, during the early stages of the differentiation. However, during January and February, toward inflorescences evocation, the majority of GAs in these buds ("off") were from the GA1 route, which are known to be associated with growth and elongation. The extracts from "on" buds contained small GA-like activity in January and February which coincided with their delayed development on the tree as compared with flowering buds.
Objectives of the project:

1. To quantitatively determine the endogenous GAs of apple and olive buds in the course of their development during both "on" and "off" years.

2. To determine if shoots of apple and olive, that are either in the "on" or "off" condition, synthesize GAs that differentially accumulate in their respective buds (axillary and spur buds of apple, and axillary buds of olive).

3. To determine if there is a difference in the biological activity of GA₄ compared to GA₅ on return bloom of apple and olive trees when treated either in the "on" or "off" condition.

4. To determine if there is a difference in the metabolism of GA₄ compared to GA₅ in spur buds of apple and axillary buds of olive in "on" and "off" years.

All 4 objectives presented in the original proposal have been investigated in the olive. Reasonable understanding was achieved regarding biennial productivity of the olive tree. It is not clear whether these objectives have been achieved for apple too.
Introduction

Biennial bearing in olive is a severe drawback of the entire olive industry. Both products, olive oil and pickled fruits are stored up to two years in order to meet market demands during "OFF" years. Fruit prices are low and labour costs are high during plentiful seasons, and the opposite occurs during "OFF" years.

The phenomenon of biennial bearing in fruit trees is quite common, and is well known in apple, citrus, avocado, nuts and many other crops (Hennerty and Forshey 1971, Williams and Edgerton 1974, Hoad 1978, Jonkers 1979, Monselise and Goldschmidt 1982). But the extent of alternate bearing in these crops is never as severe as in olive (Lavee 1985, Lavee et al 1986, Stutte and Martin 1986). Many investigations were performed and reviews were published trying to explain the reasons for biennial bearing, but no single prime cause was ever found (Hennerty and Forshey 1971, Williams and Edgerton 1974, Hoad 1978, Monselise and Goldschmidt 1982). Therefore, a thorough investigation is required to study all the factors which are known to be involved in biennial bearing, and establish their relative influence, and their exact role in the sequence of events leading to biennial bearing.

Olive trees bear their fruits on one year old branches. These twigs begin their development in the spring, and grow during summer and fall. By winter they reach length of ca. 30 - 40 cm.

Reproductive differentiation in the buds of these twigs occurs probably during winter months (Hartmann 1951, Lavee 1985, Ben-Tal unpublished data). It was established mainly by circumstantial evidence, but its validity is quite strong. The facts that support this conclusion are that warm winters result in poor flowering even after "OFF" years; that girdlings performed during December were most effective in increasing flowering of olive trees (Lavee et al 1983, Ben-Tal and Lavee 1984,1985); that a number of chilling hours requirements for flower differentiation of several olive cultivars were determined (Hartmann and Prolongis 1958, Hackett and Hartmann 1963, Badr and Hartmann 1971, Hartmann and Wisler 1975); and that olive trees grown under tropical conditions never flower.
In the following spring most of the axillary buds develop into inflorescences and set fruits if the winter requirements were fulfilled. During the summer and fall the fruits develop, and in late fall or early winter the crop is harvested. During "OFF" years a large number of twigs develop on the trees due to lack of fruits and therefore intensive vegetative growth. After "ON" years the opposite occurs. Many fruits were developed, the vegetative growth was limited to only a small number of twigs due to severe competition with the fruit load. Yet, comparisons between the percentage of inflorescences developed on similar twigs that had been grown during the two types of years revealed that after an "OFF" year 70 - 80% of the buds had developed inflorescences while only less than 15% of the buds did so after "ON" years (Hartmann and Wisler 1975, Ben-Tal unpublished data). This means that although some twigs did grow and develop during "ON" years, their buds were not capable of producing inflorescences. A few of these buds, that could not flower, did grow vegetatively while the rest of the buds remained dormant. Furthermore, reproductively differentiated buds begin to develop earlier than the vegetative ones. Toward the end of February the reproductive buds begin to swell and change their shape while vegetative buds still look dormant. These facts ruled out the possibility that competition for assimilation products or being stronger sinks advantage vegetative growth over reproductive development and becomes the primary reason for biennial bearing.

Our assumption was that some other factors may control the ability of the buds to flower. We thought that the presence or absence of an endogenous growth regulator might be the major control mechanism. Gibberellins (GAs) were chosen because they are known to be involved in flowering processes in many plants (Hoad 1978, Dunberg 1980, Marino and Greene 1981, Goldschmidt 1984, Pharis and king 1985, Pharis and Ross 1986, Bonnet-Masimbert and Zaerr 1987, Metzger 1988, Takahashi et al 1991). Badr and Hartmann (1972) Pinney and Polito (1986) and Lavee (1989) suggested that endogenous growth regulators were involved in alternate bearing in olive.

In order to study the role of endogenous gibberellins' involvement in flowering ability of olive trees, buds were taken from "OFF" and "ON" trees during fall and winter months. The aim of
this study was to determine and identify differences among endogenous GAs during the differentiation period in buds taken from olive trees after "ON" and "OFF" years.

**Materials and methods**

Twelve year old "Manzanillo" olive trees (*Olea europeae* L.) located in the experimental olive orchard at the Volcani Research Center in Bet Dagan, were the source of plant material for the entire project. The yield of these trees has been recorded since their first production, and their biennial production pattern was well established (Table 1).

Eight twigs of 30 - 40 cm long were collected from each of 6 olive trees that were after their "ON" year and from 6 trees that were after their "OFF" year, a total of 96 twigs. The twigs were taken from these trees around the middle of the month from October through February in both years 1988/9 and 1989/90. The number of inflorescences was counted on another 8 twigs on each of these trees during the following flowering season (late April - early May of each of these years), and the ratio between the number of inflorescences and the total bud number on these twigs was calculated (Table 2). Upon harvesting the yield of these trees was determined (Table 3).

**Table 3. The actual average yields of the trees that were expected to be either "ON" or "OFF".**

After recording the yields of 1988, these trees were marked and used for buds analysis throughout the entire project. The actual yields were recorded in both years, 1989 and 1990, in order to validate whether our predictions were correct.

<table>
<thead>
<tr>
<th>Type of year predicted for 1989 and 1990</th>
<th>1988 Yield (kg) ±SE</th>
<th>1989 Yield (kg) ±SE</th>
<th>1990 Yield (kg) ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;ON&quot;</td>
<td>14.75 ± 2.62</td>
<td>85.17 ± 3.87</td>
<td>12.5 ± 1.04</td>
</tr>
<tr>
<td>&quot;OFF&quot;</td>
<td>69.83 ± 4.89</td>
<td>20.33 ± 2.19</td>
<td>63.5 ± 3.43</td>
</tr>
</tbody>
</table>
Table 1. Yields (kg) of the selected trees in the olive grove of which buds were taken

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>18</td>
<td>65</td>
<td>15</td>
<td>74</td>
<td>15</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>21</td>
<td>60</td>
<td>14.5</td>
<td>86</td>
<td>10</td>
<td>88</td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>12</td>
<td>63</td>
<td>6</td>
<td>82</td>
<td>9</td>
<td>72</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>23</td>
<td>93</td>
<td>25</td>
<td>96</td>
<td>14</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>14</td>
<td>57</td>
<td>18</td>
<td>86</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>56</td>
<td>18</td>
<td>78</td>
<td>10</td>
<td>87</td>
<td>12</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>44</td>
<td>10</td>
<td>81</td>
<td>23</td>
<td>72</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>21</td>
<td>51</td>
<td>12</td>
<td>58</td>
<td>16</td>
<td>69</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>54</td>
<td>2</td>
<td>69</td>
<td>22</td>
<td>48</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>28</td>
<td>78</td>
<td>21</td>
<td>86</td>
<td>18</td>
<td>66</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>14</td>
<td>59</td>
<td>0</td>
<td>55</td>
<td>29</td>
<td>60</td>
<td>36</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
<td>85</td>
<td>12</td>
<td>70</td>
<td>14</td>
<td>66</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 2. Percent of flowering on twigs that grew on the olive trees after "ON" or

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;ON&quot;</td>
<td>41.35 ±0.96</td>
<td>6.69 ±0.68</td>
<td>16.3 ±1.85</td>
<td>29.6 ±1.72</td>
<td>3.37 ±0.57</td>
<td>11.39 ±1.91</td>
</tr>
<tr>
<td>&quot;OFF&quot;</td>
<td>32.35 ±1.17</td>
<td>23.3 ±0.59</td>
<td>72.3 ±1.91</td>
<td>22.87 ±1.03</td>
<td>15.29 ±0.85</td>
<td>66.86 ±2.04</td>
</tr>
</tbody>
</table>
These counts, measurements and calculations were made to validate that our predictions concerning the biennial pattern of these olive trees was maintained throughout the duration of this project.

The twigs that were collected for buds’ analysis were frozen in the orchard by liquid nitrogen and freeze dried by lyophilization. After drying, approximately 1000 buds were removed from the entire group of twigs of the same kind ("ON" or "OFF") of trees every month. The buds were counted and weighed. The dry weight of each group of buds was ca. 800 - 900 mg. Then, the buds were ground in 80% MeOH for GAs extraction. Two tritiated marker GAs, usually 3H-GA1 and 3H-GA4, at known quantities, were added into each extract and were monitored after each and every separation and purification step in order to account for possible loses of endogenous GAs during the purification and separation process. Separation between conjugated and free GAs, and purification of the extracted GAs was made according Koshioka et al (1983a) with minor changes (Fig. 1). The resulted dry extract was collected in 100% MeOH and was injected into a C18 reverse phase column on a 'Varian' 5000 high performance liquid chromatography (HPLC) equipped with a Merck Hibar LiChrosorb RP-18 (250 x 10 mm), 7μm column, for analytical separation of the GAs. The range of retention times (Rt) where GAs were suppose to appear was established by injecting a group of standard GAs, including GA8, 1, 4, 7, and GA9 which account for a wide range of retention times of GAs on this column with a running phase of increased MeOH percentage program (Fig. 2). This procedure was adopted from Koshioka et al (1983b) with our minor changes. Twenty five (46 - 91% program, Fig. 2a) or thirty five (10 - 73% program, Fig. 2b) 2 min fractions at 2 ml/min were collected from every injected sample after HPLC separation. These 4 ml fractions were dried completely by vaccum and N2 stream.

For a 'Tan ginbozu' dwarf rice bioassay (Koshioka et al 1983a). The dried fractions were dissolved in 100 μl of 100% ethanol. Either 1 μl or 0.5 μl from each dissolved fraction was applied on the dwarf rice seedlings to test the various fractions for their ability to promote gibberellin like activity (GA-like activity). Groups of fractions that caused significant seedlings'
Separation and purification flow chart

Dry buds extracted in 80% MeOH

Separation by Buchner funnel

- Pulp discarded
- pH adjusted to 6.7, radioactivity count
- C18 bondapak column rinse with 80% MeOH
  2nd rinse 50 ml of 100% MeOH

100% MeOH rinse
radioactivity count (pigments)

- 80% MeOH soluble fraction, radioactivity count
  (GAs, precursors, conjugated GAs)
  H2O added, dilution to 40% MeOH
  rinse with 40% MeOH
  2nd run through C18 bondapak column 2nd rinse with 50 ml of 100% MeOH

100% MeOH rinse
radioactivity count (precursors)

- 40% MeOH soluble fraction, radioactivity count
  (GAs, conjugated GAs)
  dry completely, transfer to celite and run through SiO2 column
  of EtOAc:Hexane. 2nd rinse with 100 ml of 100% MeOH

100% MeOH rinse
radioactivity count (conjugated GAs)

- 95.5 EtOAc:Hexane soluble fraction radioactivity count (GAs)
  dry, HPLC
  C18 column gradient
dwarf rice bioassay

- Fractions containing GA-like activity
  2nd run in HPLC
  C18 column 10 - 73% MeOH
  2nd dwarf rice bioassay

- Sharp peaks with GA-like activity
derivatized, GC-MS

- Wide peaks with GA-like activity, 3rd run in HPLC, nucleosil N(CH3)2 column,
isocratic 99.9% MeOH:0.1% acetic acid
  3rd dwarf rice bioassay

- Sharp peaks with GA-like activity
derivatized, GC-MS
Fig. 2b. Running phase program for the 2nd HPLC run including several standards.

Fig. 2a. Running phase program for the 1st HPLC run including several standards.
elongation were combined together, dried by N₂ and re-injected into the HPLC using a different MeOH running phase program (Fig. 2b). Then, all fractions of the 2nd HPLC run were subjected to a 'Tan ginbozu' dwarf rice bioassay. Some of the fractions that caused significant GA-like activity were methylated and trimethylsulphonated (Steffens et al 1991) and injected into a 'Hewlett Packard' 5890 gas-chromatograph combined with a 5791A mass-spectroscopy detector (GC-MSD) equipped with DB1 (0.25μm, 30m x 0.25mm) column for GAs analysis (Gaskin and MacMillan 1991). Other fractions that promoted GA-like activity in 2-4 adjacent tubes were combined together and were injected again for further separation and purification into HPLC, equipped, this time, with a HiChrom NC100-10, Nucleosil [N(CH₃)₂] column (250 x 4.6mm) with isocratic running phase of 99.9% MeOH: 0.1% Acet. Ac. 1ml/min (Yamaguchi et al 1982). This 3rd HPLC separation had produced 25 fractions, of 2 minutes each, which were subjected to another 'Tan ginbozu' dwarf rice bioassay. The fractions that promoted GA-like act. were derivatized as described above for GC-MSD analysis. GAs identification was based on comparisons with authentic GA standards (purchased from L.N. Mander, Research School of Chemistry, The Australian University, G.P.O. Box 4, Canberra A.C.T. 2601, Australia). These standards included GA₅, GA₆, GA₇, GA₈, GA₉, GA₁₀, GA₁₁ and GA₁₂. For other GAs comparisons were made with literature published identifications (Koshioka et al 1988, Nakayama et al 1989, 1990, Talon et al 1990, Beale and Willis 1991, Gaskin and MacMillan 1991, Steffens et al 1992) that contained lists of Kovatt retention index (KRI) values and ion abundance.

**Results**

The data in table 1 indicate that the olive trees used for the experimental work were actually representing trees in their "ON" and "OFF" years. The recorded yields of these trees during the two years in which buds were collected, indicated that the predicted production of these trees was maintained (Table 3), and that their "ON" and "OFF" years occurred in opposite years.
The low flowering rate after "ON" years (Table 2) is another indication that although twigs and buds were present they did not develop inflorescences as did buds on trees after their "OFF" years.

Bioassays of buds' extracts were made from the buds taken from both kinds of twigs during the 5 consecutive months in both years (1989, 1990). GA-like activity was determined by measuring elongation of the dwarf rice seedlings. October results revealed relatively small quantitative differences between "ON" and "OFF" buds (Fig. 3), and that the major peaks of activity had appeared at the same retention times in both types of buds. It means that they were probably the same GAs. The few qualitative differences resulted probably from the fruit load that was not harvested yet on the "ON" trees. In November, a large quantitative difference was observed in the bioassay at fractions 19 and 27 in "OFF" buds and possible qualitative difference at fractions 25 and 30 in "OFF" buds (Fig. 4). A similar picture was observed in December (Fig. 5). But in January the quantitative difference in fractions 25-28 almost disappeared while a new peak at fractions 17-19 appeared in the "OFF" extracts (Fig. 6). This new peak in fractions 17-19 grew even larger in February in the "OFF" buds, while the peak in fractions 25-28 almost disappeared in both kinds of extracts (Fig. 7).

These results (described in Figs. 3-7) exposed fractions that had promoted rice seedlings elongation, therefore containing GA-like activity. In figure 8, the sequence of HPLC and bioassay steps are presented.

These following HPLC runs were required to achieve satisfactory separation of the final fraction that was derivatized for GC-MSD analysis.

In table 4 the list of GAs identified in both kinds of buds is given.

Table 4. A list of all GAs identified in the olive buds during this project. In brackets the KRI values.

<table>
<thead>
<tr>
<th>GA</th>
<th>(KRI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA1</td>
<td>(2667)</td>
</tr>
<tr>
<td>GA3</td>
<td>(2695)</td>
</tr>
<tr>
<td>GA4</td>
<td>(2500)</td>
</tr>
<tr>
<td>3-epi-GA4</td>
<td>(2621)</td>
</tr>
<tr>
<td>GA5</td>
<td>(2477)</td>
</tr>
<tr>
<td>GA7</td>
<td>(2522)</td>
</tr>
<tr>
<td>GA8</td>
<td>(2825)</td>
</tr>
<tr>
<td>GA9</td>
<td>(2368)</td>
</tr>
<tr>
<td>GA15</td>
<td>(2604)</td>
</tr>
<tr>
<td>GA17</td>
<td>(2575)</td>
</tr>
<tr>
<td>GA19</td>
<td>(2598)</td>
</tr>
<tr>
<td>GA24</td>
<td>(2445)</td>
</tr>
<tr>
<td>GA30</td>
<td>(2760)</td>
</tr>
<tr>
<td>GA35</td>
<td>(2648)</td>
</tr>
<tr>
<td>GA37</td>
<td>(2765)</td>
</tr>
<tr>
<td>GA44</td>
<td>(2787)</td>
</tr>
<tr>
<td>GA51</td>
<td>(2522)</td>
</tr>
<tr>
<td>GA53</td>
<td>(2495)</td>
</tr>
<tr>
<td>GA61</td>
<td>(2490)</td>
</tr>
<tr>
<td>GA63</td>
<td>(2709)</td>
</tr>
</tbody>
</table>
Fig. 3. GA-like activity in rice seedlings as a result of buds' extracts. After HPLC separation samples from every fraction were applied on 'Tan-ginbozu' rice seedlings. Elongated seedlings are an indication of endogenous GAs. Peaks at different fractions are an indication of different GAs. The height of the peak is an indication for the amount of this endogenous GA.
Fig. 4. GA-like activity in rice seedlings as a result of buds' extracts. After HPLC separation samples from every fraction were applied on 'Tan-ginbozu' rice seedlings. Elongated seedlings are an indication of endogenous GAs. Peaks at different fractions are an indication of different GAs. The height of the peak is an indication for the amount of this endogenous GA.
Fig. 5. GA-like activity in rice seedlings as a result of buds' extracts. After HPLC separation samples from every fraction were applied on 'Tan-ginbozu' rice seedlings. Elongated seedlings are an indication of endogenous GAs. Peaks at different fractions are an indication of different GAs. The height of the peak is an indication for the amount of this endogenous GA.
Fig. 6. GA-like activity in rice seedlings as a result of buds' extracts. After HPLC separation samples from every fraction were applied on 'Tan-ginbozu' rice seedlings. Elongated seedlings are an indication of endogenous GAs. Peaks at different fractions are an indication of different GAs. The height of the peak is an indication for the amount of this endogenous GA.
Fig. 7. GA-like activity in rice seedlings as a result of buds' extracts. After HPLC separation samples from every fraction were applied on Tan-ginbozu' rice seedlings. Elongated seedlings are an indication of endogenous GAs. Peaks at different fractions are an indication of different GAs. The height of the peak is an indication for the amount of this endogenous GA.
Fig. 8. Sequence of HPLC and bioassay steps leading to GC-MS identification. A demonstration of steps required for identifications of GAs in extracts made of buds collected during October.
The identification procedure is shown for one of the GAs. It appeared as a peak at Rt of 26.14 after a full scan analysis and was expected to be GA₄ (Fig. 9). In the next test the same compound was tested for selected ions monitored (SIM) with hydrocarbons (ion 85) to determine the exact KRI and the fact that all the relevant ions appeared at exactly the same Rt (Fig. 10). The results indicated that it was GA₄ based on the ions ratio and the KRI calculated. The next step compared it with a scan of a standard GA₄ which is stored in the library of the GC-MSD. This comparison established that it actually was GA₄ at 86% validity (Fig. 11). Not all the identified GAs were validated by comparisons with standards. We did not have standards for all the GAs identified. Therefore we had to establish their authenticity only by comparisons with GAs' data in the literature based on their KRI values, the appearance of all the ions at the same Rt, the ions abundance and the relative ratios of these ions (Gaskin and MacMillan 1991). This validation is not as solid as the one described above, but it is accepted by all scientific journals because nobody has standards for all 86 known GAs. In all the comparisons made when authentic standards were available, it was found that KRI values, ions' masses and the relative ratios of these fractures, were enough for accurate GAs identifications. The comparisons with standards were only an additional validation which never contradicted the identifications based on the other 3 parameters.

It was found that "OFF" buds contained a large quantity of GA₄ during October (Fig. 12) which gradually decreased during November (Fig. 13), December (Fig. 14) and January (Fig. 15), and completely disappeared in February (Fig. 16). While in extracts of "ON" buds it was mainly epi-GA₄ in October and November (Figs 12 and 13) which decreased during December and January (Figs 14 and 15) and disappeared in February (fig. 16). The "ON" extracts also contained minute quantities of GA₄ in October and November (Figs 12 and 13). Another consistent gibberellin was GA₃₇ which prevailed in most of the extracts no matter whether originated from "ON" or "OFF" buds. GA₁ was present in October, decreased in November and completely disappeared in December of both "ON" and "OFF" buds (Figs 12, 13 and 14). It had reappeared in January and grew even larger in "OFF" buds from February (Figs 15 and 16) but
Fig. 9. Total scan of an October "OFF" fraction that contained a large peak of GA-like activity.
A restricted region of a GC-MS total scan of a fraction that included the Rt of a standard GA₄ marker. The scan indicates the ions 289, 284, 224 and 129 at KRI of 2499. Typical of GA₄.
Fig. 10. SIM check of the same fraction including hydrocarbons. The characteristic GA₄ ions plus ion 85 (indication of hydrocarbons) were selected and the same sample was re-injected.
Fig. 11. Comparison of the total scan results with a library standard of GA₄.
Fig. 12. GAs identified by GC-MS in olive buds' extracts. Buds' extracts were subjected to 2 - 3 different HPLC separations. However, the fractions marked above as containing the GAs are always the first HPLC run of which further separations were made as indicated in figure 8.
Fig. 13. GAs identified by GC-MS in olive buds' extracts.
Buds' extracts were subjected to 2 - 3 different HPLC separations. However, the fractions marked above as containing the GAs are always the first HPLC run of which further separations were made as indicated in figure 8.
Fig. 14. GAs identified by GC-MS in olive buds' extracts. Buds' extracts were subjected to 2 - 3 different HPLC separations. However, the fractions marked above as containing the GAs are always the first HPLC run of which further separations were made as indicated in figure 8.
Fig. 15. GAs identified by GC-MS in olive buds' extracts.
Buds' extracts were subjected to 2 - 3 different HPLC separations. However, the fractions marked above as containing the GAs are always the first HPLC run of which further separations were made as indicated in figure 8.
Fig. 16. GAs identified by GC-MS in olive buds' extracts. Buds' extracts were subjected to 2 - 3 different HPLC separations. However, the fractions marked above as containing the GAs are always the first HPLC run of which further separations were made as indicated in figure 8.
was relatively small in January and almost did not exist in February in extracts taken from "OFF" buds (Figs 15 and 16).

**Discussion**

The results of this investigation clearly indicated that there were significant differences between endogenous GAs in olive buds during the differentiation period depending whether these buds were originated from growth occurred following "ON" or "OFF" years. These differences already existed in October both quantitatively and qualitatively. They might have resulted from two separate reasons. The first; "ON" trees were still loaded with fruits and their seeds might have been a source of different GAs which did not exist in trees without fruits. The second; due to an "OFF" year the vegetative growth of these trees was more vigorous and therefore might have resulted in larger quantities of GAs which are known to be associated with vegetative growth (Koshioka *et al* 1985, Fujioka *et al* 1988, Juntila and Jensen 1988, MacMillan 1990, Talon and Zeevaart 1990) and as we actually found. In November and December vegetative growth ceased and differentiation occurred. The "vegetative" GAs decreased and new GAs appeared. These new GAs were probably associated with flowering processes, like GA₄ and its precursors (Bonnet-Masimbert and Zaerr 1987, King *et al* 1987, Lin and Stafford 1987, Pharis *et al* 1987). They appeared almost only in the "OFF" buds which were supposed to differentiate into inflorescences during the winter. GA₄ was found to be associated with flowering in other plants like apple (Looney *et al* 1985), *Lolium temulentum* (Pharis *et al* 1987, Evans *et al* 1990), *Pharbitis* (King *et al* 1987) and others. In January, and more so in February, the GA₄ disappeared from extracts of "OFF" buds and a new GA appeared - GA₁ - due to the beginning of vegetative development of these buds (Phinney 1985, Fujioka *et al* 1988).

The fact that GAs are involved in flowering processes is not new (Zeevaart 1976). In many fruit trees GA₃ is known to inhibit flowering (Sachs and Hackett 1969, Jackson and Sweet 1972).
Yet, during the last decade, since specific GAs became available, it was found that there is a wide range of GAs' influences on physiological processes which are controlled by different GAs. Looney et al (1985) demonstrated that GA₄ increased flowering in apple, while GA₃ inhibited it (Luckwill and Silva 1979). Moore et al (1986) extracted GA₁, GA₃ and iso-GA₃ from flowering apices of sugarcane while GA₁₆ and GA₁₉ were present only in vegetative apices. Dunberg (1980) showed that GA₄₇ promoted female flowering in Picea abies while GA₃ had no effect. Ingram et al (1985) listed 5 crops from which they isolated different GAs from vegetative parts as compared with GAs isolated from developing seeds. Lin and Stafford (1987) found in flowering (vernalized) wheat less C-20-GAs (GA₅₃, GA₄₄, GA₁₉) and more C-19-GAs (GA₁, GA₃, GA₂₀) and the opposite in non-vernalized wheat. It was suggested that conversion from C-20-GAs to C-19-GAs in wheat was controlled by vernalization, and the flowers development in this system is controlled by C-19--GAs. A single long day treatment induced flowering in the long day plant Lilium temulentum. As a result, extracted polar GAs were increased 3-5 fold 24 h after the completion of the single long day exposure. Rood et al (1989) demonstrated that different GAs were extracted from Brassica napus whether it was vegetative or not. Exogenous treatments with GA₃₂ (a very polar GA) caused intensive flowering and almost no elongation, while GA₁ (much less polar) caused mainly elongation.

Both treatments were applied under non-inductive conditions (Pharis et al 1987). On the other hand the same investigators found that in Pharbitis nil less polar GAs (GA₄ and GA₁) promoted flowering when applied before the inductive photoperiod (King et al 1987).

All these information support the hypothesis that specific GAs are required to promote flowering in different plants. Sometimes, the unique GA that promote flowering in one plant is inhibitory in another. Both promotion and/or inhibition might be controlled by the same GA in two different plant systems. The theory that less polar GAs promote flowering as was suggested by Pharis and Ross (1986) was proven not general by Pharis and co-workers in Pharbitis (King et al 1987). It is more likely that under different evolutionary conditions - day length, temperature, draught, different gases ratios in the atmosphere, anaerobic conditions etc. - plants had
developed different "right" GAs for controlling flowering. The biosynthesis pathway of the GAs had probably been "stopped" at different oxidation levels, or was diverted to other routes under different evolutionary growing conditions, which had affected rates and levels of oxidation along the biosynthesis pathway.

Our results indicated larger quantitative expression of GA-like activity in buds after "OFF" years. This difference was associated mainly with GAs of the GA4 pathway like GA37, GA15, GA24 and GA51. Later in the winter, January and February, the stronger GA-like activity was expressed mainly as GA1 and others of its route (GA53, 44, 19, 8 and GA3).

It is likely that during the early stages of the winter, when differentiation occurs, the GA4 route is dominating in the buds that have the potential to differentiate into inflorescences and possibly enables them to materialize as flowering buds. After differentiation was completed and the buds were ready to develop, the GA1 route took over. In the "ON" buds, although the total GA-like activity was almost equal in October and slightly smaller in November, the major GA identified was 3-epi-GA4 which probably interfered with the differentiation process during these months. Later in the winter, both the quantity and the number of GAs identified in "ON" buds, were reduced dramatically. This might be an indication that all these GAs in the "OFF" buds, resulted from differentiation which began earlier in the winter or even during the previous summer. The large number and quantities of GAs in the "OFF" buds toward the end of the winter also fits the fact that differentiated buds start to develop earlier than vegetative buds. The GAs that were identified in these buds at the end of winter belonged to the GA1 pathway which is another indication of growth. These vegetative GAs had vanished from both kinds of buds in the early winter when growth ceases and differentiation was suppose to occur, and reappeared after differentiation, when vegetative growth was resumed. While GAs from the GA4 route were dominant in buds during their differentiation, it was also observed that applications of GA4 early in the winter on "OFF" trees, were much less inhibitive to flowering than GA3 treatments (Ben-Tal, unpublished data). Figure 17 presents a scheme of the possible biosynthesis route of GAs in plants. The GAs that were identified in this investigation are marked. GAs 30, 35, 61 and 63
were not included because we were not sure how they were connected into this scheme. It is based on a scheme presented by Rebers et al in a poster in the 14th international conference of the International Association of Plant Growth Substances (1991), and other partial pathways suggested by Koshioka et al (1988), Juntila and Jensen (1988), Nakayama et al (1989,1990) and Talon et al (1990).

![Diagram](image)

**Fig. 17. Possible metabolic connections among GAs in plants.**
Adopted from Rebers et al (1991) with some additions suggested by others (see text). GAs in circles were identified in the olive buds.

In conclusion we assume that this is the order of events:

1) In the fall, buds after "OFF" season, contain large quantities of GAs from the GA₄ route. The "ON" buds contain mostly 3-epi-GA₄ which is interfering with the differentiation process. Both kinds of buds still contain GAs of the GA₁ route that are left overs from the end of the last growing season. This GA₁ is probably responsible for the major part of the GA-like activity observed in the bioassys of October and November.

2) In the early winter, the GAs of the GA₁ route disappear in both kinds of buds and only GAs from the GA₄ route are present. But these are "right" GAs required for the differentiating buds ("OFF"), while the 3-epi-GA₄ in the non-differentiating buds ("ON") is interfering with the ability of these buds to differentiate.
3) In late winter the GAs of the GA₄ route start to subside in both kinds of buds. But large quantities of GAs from the GA₁ route start to accumulate in the differentiated buds ("OFF"), while the "ON" buds are still dormant and hardly any GAs can be detected. This GA₁ accumulation probably pushes forward the early development of the differentiated buds (after "OFF" years).

References

Badr SA and Hartmann HT, 1971, Effect of diurnally fluctuating and constant temperatures on flower induction and sex expression in the olive (Olea europeae L.). Physiol. Plant. 24: 40-45


Ben-Tal Y, 1979, unpublished data.


Evans LT, King RW, Chu A, Mander LN and Pharis RP, 1990, Gibberellin structure and


Goldschmidt EE, 1984, Endogenous abscisic acid and 2-trans-abscisic acid in alternate bearing 'Wilking' mandarin trees. Plant Growth Regul. 2:9-13


Hartmann HT and Prollingis I, 1958, Effects of different amounts of winter chilling on fruitfulness of several olive varieties. Bot. Gaz. 119:102-104

Hartmann HT and Whisler JE, 1975, Flower production in olive as influenced by various chilling temperature regimes. J. Am. Soc. Hort. Sci. 100:679-674


Jackson DI and Sweet GB, 1972, Flower initiation in temperate woody plants. Hortic. abst. 42: 9-24


Junttila O and Jensen F, 1988, Gibberellins and photoperiodic control of shoot elongation in
Salix. Physiol. Plant. 74:371-376


Luckwill LC and Silva JM, 1979, The effect of daminozide and gibberellic acid on flower
initiation, growth and fruiting of apple cv. 'Golden Delicious'. J. Hort. Sci. 54:217-223

MacMillan J, 1990, Metabolism of gibberellin A<sub>20</sub> and A<sub>9</sub> in plants: Pathways and
Enzymology. In: Plant Growth Substances 1988, RP Pharis and SB Rood (eds),
Springer-Verlag, Berlin, Heidelberg, pp 307-313

Marino F and Greene DW, 1981, Involvement of gibberellins in biennial bearing of 'Early

Metzger JD, 1988, The role of gibberellins in reproductive development. Bul. Plant Growth
Regul. Soc. Amer. 16(2): 13-19

4:128-173

Moore PH, Pharis RP and Koshioka M, 1986, Gibberellins in apical shoot meristems of
flowering and vegetative sugarcane. J. Plant Growth Regul. 5:101-109

Nakayama M, Yamane H, Yamaguchi I, Murofushi N, Takahashi N and Katsumi M, 1989,
Endogenous gibberellins in the shoots of normal and bush-type Cucumis sativus L. J.
Plant Growth Regul. 8: 237-247

Nakayama M, Yamane H, Yokota T, Yamaguchi I, Murofushi N, Takahashi N, Nishijima T,
Endogenous gibberellins in mature seeds of Raphanus sativus L. cv. Taibyo-sobutori.
Agric. Biol. Chem. 54: 837-840

Rev. Plant Physiol. 38:517-568


Pharis RP, Evans LT, King RW and Mander LN, 1987, Gibberellins, endogenous and applied,
84:1132-1138


Sachs RM and Hackett WP, 1969, Control of vegetative and reproductive development in seed plants. HortSci. 4:103-107


Steffens GL, Lin JT, Stafford AE, Metzger JD and Hazebroek JP, 1992, Gibberellin content of immature apple seeds from paclobutrazol-treated trees over three seasons. J. Plant Growth Regul. 11: 165-170


Yamaguchi I, Fujisarva S and Takahashi N, 1982, Qualitative and semi-quantitative analysis of gibberellins. Phytochemistry 21: 2049-2055

Cooperation:

During the first year there was some information exchange and both principal
investigators have presented a joint annual report. In the first year (1989)
Ben-Tal visited Brenner's laboratory for a few weeks and used Brenner's GC-MS
for identification of certain olive gibberellins. Further cooperation did not
develop although Ben-Tal did send his 2nd year results for a joint annual
report. The final report is related only to the Israeli project and is limited to
biennial bearing in olive trees.

Project Achievements:

This project enlightened the role of GAs in the biennial bearing of olive. It was
found that GA₃ is inhibitory to inflorescence differentiation when applied
exogenously. But, it was found that GA₁ was necessary for inflorescence
evocation and development. In "on" buds the total quantity of GAs was smaller
and bud differentiation was delayed. However, it is not clear whether vegetative
differentiation of buds after "on" years was due to lack of GAs of the GA₁
route that were supposed to be responsible for buds' evocation and development,
or the fact that differentiation itself was inhibited because GA₄ type GAs were
missing during the early phase of the winter.