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Regulation of Flowering for Improved Yield and Profit in Citrus

Y. Erner, C.J. Lovatt, A. Goel

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P.O. Box 6

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בית דגן

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REGULATION OF FLOWERING FOR IMPROVED YIELD AND PROFIT IN CITRUS

Y. Erner, C.J. Lovatt, and Ari Goell

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Methodology, data, results and discussion.

Israeli portion - Y. Erner and A. Goell

Abstract

Regulation of flowering is an important phenomenon in fruit production since it is the first step towards the reproductive processes. Carbohydrates as well as PGR's have long been postulated as related to flowering. These two major compounds were examined for their influence on flower formation. While different carbohydrate content in leaves found "On" and "Off" years from biennial yielding 'Murcott' tangerine, no such differences could be found in buds of the same trees. Moreover, the separation (from the buds) of the soluble sugars to constituents did not show any differences between "On" and "Off" trees. Exogenous sugar injection into branches in order to increase flower formation showed that the leaves were important to this process. The sugars were found to have minor influence on flowering although glucose tended to give best results. If it is accepted that carbohydrates are the major factor in flowering we assumed that soluble sugar would be at a higher level in buds which normally would flower. Our data do not confirm this assumption, since buds located along a branch which will differentiate to flower, or remain vegetative, had the same sugar content. Moreover, the vegetative branches (that will not flower at all) were found to contain higher levels of the sugars than those found in buds from reproductive branches. The grafting experiments emphasize the theory that some growth inhibitor which is produced by the fruit and translocated basipetally might inhibit flower formation. The plant growth inhibitor might be one of the gibberellins found in higher significant amounts in "On" buds than found in "Off" buds. On the other hand, artificial gibberellins has been found to increase flower formation. We found that gibberellins which are produced by the fruit serve as a major factor controlling flower formation. In addition, our data clarified the importance of gibberellins versus carbohydrate for flower formation and opened a new concept that some gibberellins might also induce flower formation and not as accepted for many years that they act as flower-inhibitor.

Original Objectives.

Gaining more basic knowledge as to the promotion and inhibition of the flowering process in Citrus trees. Such knowledge is needed to make possible the manipulation of both the intensity and timing of flowering in specific cultivars in the orchard, in order to raise yield and profits.

This will be addressed;

1. By studying the sequence of biochemical events leading to the irreversible commitment to flowering in Citrus.

2. By determining whether reduced floral intensity, such as that in an "Off" year of an alternate-bearing cycle, is due to (i) competition between sinks, i.e., between the buds, which have the potential to become floral, and the fruit of the "On"-year crop, or (ii) inhibition of floral bud development due to the action of PGRs produced by the fruit.

3. By confirming a cause-and-effect relationship for the factors identified by manipulating bloom in a predictable manner by either an exogenous supply of the putative causal agents or by inhibition of their endogenous synthesis or activity.

4. Based on the findings in this project, we will develop and test in a preliminary way practical, field-applicable methods for improving floral intensity, for shifting the time of bloom and for "evening out" alternate bearing.

Carbohydrate Content Associated With Bud Differentiation.

Deficiency of carbohydrate has long been held to be a factor contributing to poor return bloom when fruits are stored on the tree for a prolonged period or after an "On" year crop.

We conducted an experiment on mature 'Shamouti' trees to evaluate the differences between carbohydrate content in buds of a) reproductive branches - those that bloom in the spring having gone through flower differentiation in the autumn and expression in the next spring. b) vegetative branches those that flush during the summer (almost like water sprouts on two-year-old branches) and normally would not flower. It has been well documented that flower induction in citrus normally occurs during November and December and bud differentiation in January. Moreover, we divided each branch into 3 parts: apex, central and basal portion. Since it is known that flowering does not occur on buds at the base of the branch even in an "On" year. Four replicates of buds from the two branch types were sampled at two-week intervals, from November to March replicates. The buds in each sample branch were counted and fresh weight was determined before their being homogenized in 10 ml of Methanol:Chloroform:Water 12:5:3. Extraction was performed at 55°C for 2 h, then centrifuged and re-extracted with 5 ml for 2 more hours. The chloroform was separated by adding water and chloroform.

The fraction containing the soluble constituent was evaporated to 2 ml, passed through a Sep-Pak C₁₈ Cartridge, and washed 3 times with water. The 4 resultant fractions were evaporated to dryness and redissolved in 1 ml water.

An aliquot 50-100µl was injected onto an HPLC cation exchange column, in the calcium form, connected to refractive index detector.

We could not find any differences in bud sucrose contents neither at induction or evocation time, nor in reproductive branches. Sucrose tended to increase, at induction

time in the apex of vegetative branches. The reduction of sucrose content towards the end of the evocation period of the buds (buds were sprouted to 2-5 mm in apex) indicate that the sugars are consumed by the new growth either for flower formation or vegetative growth (Table 1). In reproductive branches, glucose content at induction time was almost undetectable, and only traces have been found (Table 2). However, some increase in glucose content was found during bud differentiation-till the last sample. Glucose, as well as sucrose were found in smaller amounts in the apex although this difference was not significantly different from that found in the center section and at the base section of the branch. Fructose was found to be at higher levels in base buds during the induction period in branches reproductive, from these which are known not to go through flowering (Table 3). On the other hand, vegetative branches accumulate significant amount of fructose in apex buds. It was found that during the differentiation period (towards flowering) there was an increase in fructose content in the buds from the 3 zones of the reproductive branch only. It can be seen that on average (for the whole season) fructose basis, a significant change was found in all zones of the two branch types. In the spring flush of reproductive branches, we found that terminal buds will differentiate normally to leafy inflorescences (LY-shoot bearing leaves and flowers) and bud apex will differentiate to leafless inflorescences (LS - bearing only flowers), while base buds will always sprout as vegetative branches. Comparing the total soluble sugars (Table 4) from buds of vegetative branches with reproductive ones, we found that they contained higher amounts and will not go through flowering process.

Table 1. Sucrose content (mg.g⁻¹F. wt) in buds of the base, center and apical sector of Reproductive and Vegetative branches at induction and differentiation

Bud Position	1989			Seasonal			1990			Seasonal	
	12 Nov.	26 Nov.	10 Dec.	24 Dec.	Average	8 Jan.	22 Jan.	4 Feb.	18 Feb.	21 March	Average
Reproductive branches											
Base	12.98 a	13.34 a	23.27 a	12.95 b	15.63 a	13.33 b	18.72 ab	14.88 ab	5.39 b	12.19 a	12.95 b
Center	13.08 a	16.44 a	14.81 a	13.84 b	14.54 a	24.67 a	14.06 ab	10.65 b	4.85 b	5.61 a	11.97 b
Apex	15.07 a	12.54 a	12.48 a	19.71 b	14.95 a	14.86 b	16.31 ab	9.37 b	5.17 b	2.99 *	9.74 b
Vegetative branches											
Base	12.71 a	14.18 a	14.75 a	16.41 b	14.51 a	17.68 b	20.34 a	23.13 a	12.86 ab	10.35 a	16.88 a
Center	13.47 a	11.23 a	19.49 a	15.23 b	14.85 a	13.99 b	10.02 b	21.75 a	14.67 a	7.68 *	13.61 b
Apex	13.58 a	11.95 a	17.90 a	28.76 a	18.05 a	14.59 b	14.54 ab	16.77 ab	9.17 ab	4.64 *	11.94 b

Means separation within columns by Duncan's multiple range test at $P \leq 0.05$

* Bud sprouts to 2-5 mm

Table 2. Glucose content (mg.g^{-1} F. wt) in buds of the base, center and apical sector of Reproductive and Vegetative branches at induction and differentiation.

Bud Position	1989			Seasonal			1990			Seasonal	
	12 Nov.	26 Nov.	10 Dec.	24 Dec.	Average	8 Jan.	22 Jan.	4 Feb.	18 Feb.	21 March	Average
Reproductive branches											
Base	0.70 b	traces	traces	traces	--	traces	0.49 b	traces	2.08 b	3.48 b	1.21 c
Center	0.65 b	"	"	"	--	"	1.42 b	"	1.29 b	7.29 b	2.00 c
Apex	0.31 b	"	"	"	--	"	traces	"	1.62 b	6.03 b*	1.53 c
Vegetative branches											
Base	2.34 ab	2.52 b	3.15 b	1.76 b	2.44 b	3.12 ab	6.64 a	7.75 a	5.37 a	5.19 b	5.61 a
Center	3.98 a	3.31 a	7.79 a	2.93 b	4.50 a	4.23 a	3.82 b	6.04 ab	5.99 a	10.30 a*	6.07 a
Apex	2.83 ab	2.17 ab	6.36 a	6.41 a	4.56 a	2.60 ab	3.08 b	3.34 b	2.39 b	6.86 b*	3.66 b

Means separation within columns by Duncan's multiple range test at $P \leq 0.05$

* Bud sprouts to 2-5 mm

Table 3. Fructose content ($\text{mg}\cdot\text{g}^{-1}$ F. wt) in buds of the base, center and apical sector of Reproductive and Vegetative branches at induction and differentiation

Bud	1989				1990				Seasonal	
	12 Nov.	26 Nov.	10 Dec.	24 Dec.	Average	8 Jan.	22 Jan.	4 Feb.	18 Feb.	21 March
Position	12 Nov.	26 Nov.	10 Dec.	24 Dec.	Average	8 Jan.	22 Jan.	4 Feb.	18 Feb.	21 March
Reproductive branches										
Base	1.35 b	0.63 b	1.17 b	2.015 bc	1.32 c	0.89 a	1.64 c	2.06 b	2.08 b	3.48 b
Center	1.14 b	0.74 b	0.98 b	0.55 c	0.85 c	1.02 a	2.78 bc	1.49 b	1.31 b	4.18 b*
Apex	1.10 b	0.38 b	0.49 b	1.07 c	0.76 c	0.74 a	1.25 c	1.78 b	1.85 b	3.35 b*
Vegetative branches										
Base	3.00 a	3.37 a	3.78 a	3.24 b	3.35 b	4.07 a	6.00 a	5.88 a	5.90 a	4.43 b
Center	3.63 a	3.39 a	4.58 a	3.21 b	3.70 ab	3.76 a	6.07 a	6.05 a	5.81 a	8.58 *
Apex	3.90 a	3.32 a	3.99 a	6.05 a	4.37 a	3.44 a	4.94 b	4.91 a	4.57 a	4.40 b*

Means separation within columns by Duncan's multiple range test at $P \leq 0.05$

* Bud sprouts to 2-5 mm

Table 4. Total soluble sugars in buds in 3 positions of the two branches type. (Average for the period).

Bud Position	<u>Reproductive</u>		<u>Vegetative</u>	
	Induction	Induction	Induction	Induction
mg.g ⁻¹ F. Wt				
Base	17.08 a	18.19 a	20.38 a	27.74 a
Center	15.55 a	16.12 a	23.05 a	25.75 a
Apex	17.69 a	12.96 a	27.10 a	19.83 a

Means separation within columns by Duncan's multiple range test at $P \leq 0.05$

The reduction in sucrose contents, as the reproductive bud progresses towards flowering, may be the result of the energy needed for the processes of flower formation, or for the breakdown to glucose and fructose. This might explain the increase in glucose and fructose in the last sample from center and apex buds of the 2 branch types. From the above we think that carbohydrates by themselves cannot be the key to bud differentiation from the vegetative to the reproductive state.

As already mentioned, carbohydrates were considered to play an important role in flowering. In order to have as complete as possible a picture about the influence of "On" and "Off" tree production on flowering, we monitored the carbohydrate fraction - soluble sugars and starch - from reproductive branches of "On" and "Off" crop loads in 'Shamouti' orange and 'Murcott' tangerine. These two varieties were selected because they exhibit the full range of tendencies for alternate bearing: 'Shamouti' shows relatively-little while 'Murcott' exhibits a tendency to extreme alternate bearing.

The well-known documentation as to different starch contents in "On" and "Off" varieties were always related to its content in the **leaves** and not in the buds. Our data confirm earlier reports that sugar and starch contents in the **leaves** were higher in "Off" trees (Table 5). Starch content in leaves of 'Murcott' in "Off" year status was almost triple that in the "On" year.

'Shamouti' leaves showed no differences either in sugars or starch in "On" or "Off" years.

Table 5. Sugar and starch concentration in leaves of 'Shamouti' orange and 'Murcott' tangerine from fruitless (-) and fruiting (+) branches (average during the period July - January).

BRANCH	SUGAR	STARCH
TYPE	mg.g ⁻¹ Fr. Wt.	
Shamouti + fruit	61.3 b	12.9 b
Shamouti - fruit	56.6 b	17.7 b
Murcott + fruit	49.0 b	28.3 b
Murcott - fruit	87.7 a	75.7 a

We could not find any differences in the content of sugar in **buds** of reproductive branches (4-11 months) either from "On" or "Off" year from 'Shamouti' or 'Murcott'. Similarly, no differences were found in starch content (Table 6).

Table 6. Sugar and starch concentration in buds of 'Shamouti' orange and 'Murcott' tangerine from fruitless (-) and fruiting (+) branches (average during the period July - January).

BRANCH	SUGAR	STARCH
TYPE	mg.g ⁻¹ Fr. Wt.	
Shamouti + fruit	61.8 a	26.7 a
Shamouti - fruit	70.1 a	36.8 a
Murcott + fruit	94.7 a	24.3 a
Murcott - fruit	81.6 a	27.3 a

The separation of the total soluble sugars to constituents did not show any differences between "On" and "Off". However 'Murcott' buds seem to have had higher amount of sucrose than 'Shamouti' while glucose contents varied (Table 7).

Table 7. Sucrose, glucose and fructose in buds of 'Shamouti' orange and 'Murcott' tangerine from fruitless (-) and fruiting (+) branches (average during the period July-January).

BRANCH TYPE	SUCROSE	GLUCOSE	FRUCTOSE
	mg·g ⁻¹ Fresh weight		
Shamouti + fruit	45.1	7.8	9.6
Shamouti - fruit	59.0	2.6	8.6
Murcott + fruit	84.0	1.3	10.0
Murcott - fruit	68.0	4.2	10.0

Seasonal changes in sucrose, glucose and fructose in buds, as related to fruit growth of 'Shamouti', are presented in figure 1. It is very interesting that glucose could not be detected during fruit growth. Moreover, glucose was not detected in buds in the induction period, although sucrose and fructose were found in considerable amounts.

Figure 1.

The data on carbohydrate contents in the buds of "On" and "Off" trees did not agree with reports of many other scientists on the content in leaves. During our work with the buds, we observed differences in fresh weight between buds from "Off" and "On" year from 'Murcott' but none was observed with 'Shamouti' (Table 8). Moreover, the fresh weight of buds from "On" year from 'Shamouti' was found to be twice as high as from 'Murcott' from 'Off' year, although the tendency for 'Shamouti' buds is for higher weight than 'Murcott', but the differences were not significant

Table 8. Weight of buds of 'Shamouti' orange and 'Murcott' Tangerine from fruitless (-) and fruiting (+) branches (average during the period July - January).

Branch type	Bud
	mg Fresh weight
Shamouti + fruit	1.58 ± 0.12
Shamouti - fruit	1.61 ± 0.20
Murcott + fruit	0.77 ± 0.05
Murcott - fruit	1.28 ± 0.11

It has been reported that fruit growth at stage II and III, both for linear fruit growth and ripening exhibit strong sinks which indirectly will cause lack of carbohydrate in other plant parts. Our data in buds did not confirm this assumption for sucrose. Although the decline in sucrose level in buds towards as we approached fruit maturation was found to be very strong, an equal content of carbohydrate being found in "Off" and "On" trees. Tables 6 and 7 point to a reduction in the importance of the influence of this decline on bud differentiation. Therefore, we assume that fruit growth might influence the carbohydrate content in plant parts other than buds, which normally do not accumulate a high level of carbohydrates.

Injection of Exogenous Sugars Into Branches.

Considering the fact that carbohydrates are considered to play an important role in flowering and, as a result of our endogenous carbohydrate measurements, we conducted experiments to evaluate the influence of sugar injection into branches. Layout of the experiment is shown in scheme 1. Leaf removal and girdling treatments were expected to help evaluate the effects of the movement of sugars (or other factors associated with flowering) from the leaf to the target buds. Injection of 100 ml of 5% concentration of the sugar into a branch of about 40 leaves had previously been found suitable for such experiments, being of a size sufficient for flowering measurements. The experiments were carried out on field-grown mature 'Murcott' and 'Shamouti' trees. Sucrose, fructose and glucose were injected into reproductive branches, girdled and non-girdled, defoliated and non defoliated, in late October.

Scheme 1. Layout for sugar injection experiment.

TREATMENT	INJECTION			
	Control	Sucrose	Glucose	Fructose
Control with leaves	+	+	+	+
Control without leaves	+	+	+	+
Girdling with leaves	+	+	+	+
Girdling without leaves	+	+	+	+

It has been shown, that the leaves play an important role in the inductive signal to the buds, The assumption being that some factors move from the leaves to the bud, inducing processes that will lead to the change of a bud from a vegetative to a reproductive stage.

Our data confirm that the leaves are important for flower induction (Table 9 and 10).

Table 9. Effect of injection of sugars into branches of 'Shamouti' orange on spring flush differentiation (sugars application in October 1991, flower counting April 1992).

	Flush (%)			LS (%)	LY (%)
Treatment	V	LY	LS	one flower	one flower
Non-Girdled with Leaves					
Control	52	21	27	49	23
Sucrose	30	31	39	45	32
Glucose	38	31	31	41	32
Fructose	53	20	27	45	52
Non-Girdled without Leaves					
Control	57	26	17	25	53
Sucrose	69	9	22	46	42
Glucose	63	19	18	33	36
Fructose	71	22	7	19	73
Girdled with Leaves					
Control	42	25	33	32	21
Sucrose	46	31	23	21	38
Glucose	42	18	40	56	13
Fructose	25	25	50	16	6
Girdled without Leaves					
Control	74	9	17	65	33
Sucrose	61	2	37	67	17
Glucose	17	15	68	62	13
Fructose	46	24	30	55	24

Table 10. Effect of injection of sugars into branches of 'Murcott' tangerine on spring flush differentiation (sugars application in October 1991, flower counting April 1992).

Treatment	Flush (%)			LS (%)	LY (%)
	V	LY	LS	one flower	one flower
Non-Girdled with Leaves					
Control	27	48	25	29	61
Sucrose	27	38	35	37	79
Glucose	22	32	46	40	49
Fructose	38	52	10	27	62
Non-Girdled without Leaves					
Control	74	22	4	8	90
Sucrose	72	26	2	6	90
Glucose	64	28	8	15	79
Fructose	66	26	8	14	74
Girdled with Leaves					
Control	7	36	57	49	18
Sucrose	7	42	51	46	16
Glucose	9	33	58	47	15
Fructose	12	38	50	45	16
Girdled without Leaves					
Control	78	13	9	13	85
Sucrose	50	31	19	29	68
Glucose	36	28	36	48	44
Fructose	63	7	30	53	39

The comparison between control, 'non-girdled + leaves' and 'non-girdled no leaves' showed a shift of flowering towards the vegetative branches. On the other hand, when 'non-girdled + leaves' control, were compared with 'girdled + leaves' or 'without leaves', emphasize the effect of girdling and the lack of leaves on flowering. Sugars injected into 'non-girdled + leaves' (natural branch) did not change the flowering habit significantly. We did not expect to get any increase in flowering from the treatment of 'girdling + leaves', since the girdling by itself raises the carbohydrate level significantly (Table 5), especially in 'Murcott'. Control, (non-girdled without leaves) in 'Murcott' was found to be similar to that in control, 'girdled without leaves' in 'Shamouti' with some increase in the number of vegetative branches. It means that preventing carbohydrate mobilization to the branch with no leaves will inhibit flowering, with the major effect being that of leaf removal. Glucose was found to increase flowering by means of a shift towards the leafless inflorescences (LS) compare with the control and the effect of other sugars, especially in 'girdled with no leaves'.

We counted the numbers of flowers in the LS and leafy inflorescences (LY) types. The number of flowers per inflorescence was found to be important for fruit set in accordance with the stem diameter of the flower. No consistent pattern was found for the influence of the sugar injection on LS or LY with one flower. The major effect was found in the branch 'with leaves' and the girdling manipulation. In 'Murcott', in 'non-girdled without leaves', a significant increase in the percent of LY with one flower was found and a decrease in 'girdled with leaves'. 'Girdled with leaves' increased the percent of LS with one flower and decreased the percent of LY. This means that the change to better fruit set occurred when more LY and LS with several flowers were favored. 'Shamouti' data from the injection of sugars showed a similar, but less pronounced, pattern as in 'Murcott'.

In summary, this part of the work emphasizes the importance of the leaves for flower induction. The sugars were found to have a minor influence on flowering induction, with glucose tending to give best results.

Grafting experiments

The hypothesis that the primary flowering stimulus is a leaf-mediated light reaction expressed at the bud level, and that a flowering-inhibiting effect, such as a flow at PGR from the fruit, or the depletion of carbohydrate by the fruit, were studied by reciprocal grafting using alternate-bearing trees and seedlings as source of flowering and non-flowering budwood and rootstocks.

The experiment consisted of 4 grafting treatments 1) "On" scion budded on "On" stock (On/On). 2) "On" scion on "Off" stock (On/Off) 3) "Off" scion on "On" stock (Off/On) 4) "Off" scion on "Off" stock (Off/Off). The field experiment failed as a result of low temperatures at the time of grafting. On the other hand, concomitant grafting experiments in a greenhouse showed dramatic and significant differences between the treatments. Four-year-old potted seedlings of 'Murcott' from showing "On" and "Off" symptoms were transferred in November 1992 to a controlled temperature greenhouse in which the temp. was held constant at $24^{\circ}\text{C} \pm 1.5$. This transfer was made in order to get cambial activity at the time of grafting (January 27, 1993). Scions were brought from a field where "On" and "Off" 'Murcott' trees had been chosen. Seedlings bearing fruit were chosen as "On" plants, while those in a vegetative stage (all set fruit were removed) were tagged as "Off" plants. Five seedlings of each were grafted with "On" scion material and 5 with "Off". Five to seven graftings were made on each seedling replicate. The grafted branches were covered with plastic and paper bags for a month, and thereafter no special treatment was needed. The take of the grafts was counted twice; 1) immediately after the plastic bag was removed and 2) at the date of the final measurements, as shown in table 11. Grafting of "Off" scion branches resulted in highly significant percent take at the time of plastic bag removal. During the experimental period (from grafting to flower count) an unexplained phenomenon of die-back was observed in some of the grafted stock branches. This ranged from 40% of the grafts in "On/On" to 11-15% in the others (Table 11).

Table 11. Effect of reciprocal grafting of "On" and "Off" branches on flowering.

Scion/stock Treatment	Number of grafting branches	Percent take at bag removal	Percent of stock die-back	Percent of flowering
"On"/"On"	32	3 c	40 a	--
"On"/"Off"	29	17 b	15 b	--
"Off"/"On"	23	70 a	15 b	30 a
"Off"/"Off"	26	80 a	11 b	32 a

This experiment emphasizes the influence of existing fruit on bud differentiation. "On" scions grafted on "Off" stock resulted in higher percent of vitality branches than in "On"/"On" but no flowers were induced. "Off" as a scion was the best, both as to take and flowering. Scion "Off"/"On" was similar to that from "Off"/"Off".

If lack of carbohydrate were the major factor inhibiting flower formation, the grafting of "On" on "Off" or "Off" on "On" would not result as shown in table 11, since, these combinations are supposed to release the buds either from lack of carbohydrate or a decrease in its source. It seems that some component(s) translocate basipetally and inhibit the induction of a vegetative bud to the reproductive stage.

Endogenous gibberellins in buds

Routinely, we operated according to the procedure developed by Prof. Pharis for the separation and identification of gibberellins using the dwarf rice bioassay. GC - MS was used for the final identification/determination of the specific gibberellin. We modified the procedure and evaluated it for the extraction of the gibberellins from citrus buds.

Frozen (-20°C) buds of about 500-1000 mg F. Wt. were ground in 10 ml of 80% Methanol (MeOH) using a mortar and pestle. The material was transferred to a centrifuge tube and separated at 10000 rpm after the addition of 50000 dpm of

$^3\text{HGA}_{20}$ as standard. The pellet was resuspended with 10 ml MeOH and the supernatants were combined. The procedure of extraction was repeated 3 times. Three aliquots of 250 μl were taken to count the radioactivity after measuring the total volume. The extract was then adjusted to pH 6.5-6.7 and gently added to the column of C_{18} μ -bondapak. The column was washed x2 with 10 ml MeOH 80%. The sample was collected in measuring cylinder and 2 aliquot samples were taken for scintillation counts. The column was washed with 30 ml of 100% MeOH in order to drive off pigments from column. An aliquot was taken for scintillation counts. The 80% MeOH extract was diluted to 40% with distilled water, bringing the pH to 6.5 - 6.7, and was loaded onto a second C_{18} column. The column was washed with 20 ml of 40% MeOH and the eluants were measured. Aliquots (x3) for scintillation counting were taken. The solvents were dried with rotary evaporator and kept in the freezer overnight. This extract contained the free GAs and GA conjugates. Precursors were eluted from the column by 60 ml of 100% MeOH. An aliquot was taken after measuring the volume. The dried 40% MeOH was redissolved in 2x 1ml of 40% MeOH and 2x 1ml 80% MeOH and added to 0.5 g of celite. When the celite sample mix was dry, it was added onto SiO_2 column and washed with ethyl acetate and hexane solution (95:5). The eluant was measured and aliquot was taken. The solvent was immediately evaporated to dryness and frozen in order not to degrade the GAs. Conjugate were eluted from the column by 100 ml of 100% MeOH. An aliquot was taken and the volume was reduced and neutralized to pH 7.0 since conjugates are unstable under acidic conditions. The neutralized solution was brought to dryness and kept in the freezer. The purpose of taking aliquots for scintillation counting after every step of extraction was to monitor the loss and efficiency of the separation. Scheme 2 illustrates the separation as a flow chart.

Scheme 2. Flow chart of gibberellin extraction from citrus buds.

FLOW CHART

The free GA samples were taken up by 5 x 1 ml of EtOAc:MeOH and 3 x 2 ml 100% MeOH to rotary evaporator flask, dried and redissolved with the initial conditions being used for the HPLC program (15% MeOH containing 0.05% acetic acid). HPLC column was a reversed phase C₁₈ (25 cm x 4.6 mm I.D., 5µm Ultraspher ODS). The injector was Rheodyne 100µl. Eluant, linear gradient from 15% MeOH to 100% MeOH (see scheme 3 of HPLC conditions), initial pressure 120at., final pressure 60at.

Scheme 3. HPLC pump separation conditions

Time min.	Pump A % water	Pump B % MeOH	Flow ml/min.
0 (injection)	85	15	0.75
5	85	15	0.75
65	0	100	0.75
75	85	15	0.75
80	85	15	0.75

Aliquots of the fractions collected from HPLC were counted by scintillation counter for identifying radioactive peak. The rest was immediately dried by centrifuge freeze drier in order to avoid the break down of GAs since the fraction were acidic. The samples were redissolved in 50µl of 95% Ethanol (ETOH) just prior to the application of 1µl and 0.5µl to dwarf rice bioassay (Tan-ginbozu dwarf rice were the generous gift of Dr. M. Koshioka).

Fractions that had been found to contain GA in the rice bioassay were injected into GC-MS after being methylated with diazomethan and silylated with trimethylsilane. The GC 'Hewlett Packard' 5890 combined with a 5791A Mass-spectroscopy detector

(GC-MSD) equipped with DB1 (0.25 μ m, 30 m x 0.25 mm) column were used for GA identification.

Gibberellins were tested with dwarf rice bioassay at two concentrations; 10 mg F. Wt and 20 mg F. Wt. of buds in order to identify the peaks without artifact. The separation of gibberellins by the HPLC revealed 35 fractions as illustrated by figure 2. Standards of 4 GAs used for identification. The GA₄ standard revealed two peaks, first one being identified as GA₇ and the second as GA₄. On the other hand, there is no good separation under these conditions to differentiate between GA₁ and GA₃. The identification of these two gibberellins can be easily made by the GC-MS. A sample of GC-MS separation of GA₄ is illustrated in fig. 3. The known hydrocarbons, C-23 through C-29, with the already installed GAs' library in the computer, enable us to identify the gibberellins.

Monitoring the GAs' in buds during the period when fruit were on the tree revealed significant specific gibberellin. Figure 4 shows GA-like activity in dwarf rice seedlings as a result of applying bud extracts from 'Shamouti' branches with fruit on June 30, 1991. Fractions numbers 23, 24, 25 showed high activity and therefore were combined for identification with GC-MS (fig. 5 and 6). The identification of the extract was made by specific ion emission and KRI and was found to be GA₉.

Rice bioassay of extract from buds of 'Shamouti' with fruit from September 1991 showed several peaks (fig. 7). We identified by the GC-MS gibberellin number 80 from fractions 20 and 21 (figs. 8 and 9). Although other peaks in the bioassay were high enough and indicate that some GA - like activity exists, we had difficulties in identifying the GA(s) with the GC-MS. It is possible that the initial amount of plant material in the sample was too small (because of the long time needed for bud sampling we were limited to a maximum humble of buds).

Buds extracted for gibberellin identification from 'Murcott' with fruit in October 1991 showed several peaks with the dwarf rice bioassay (fig. 10). Fraction number 18 of this separation was found to be GA₁₀ by the GC-MS (figs. 11 and 12). Extraction of buds from 'Murcott' with fruit from December 1991 showed a significant peak in

the dwarf rice bioassay (fig. 13) which has been identified with Rt of GA₁ and GA₃. Gibberellin A₇ has been found in buds from 'Murcott' with fruits on December 1992 by the dwarf rice bioassay and the GC-MS (figs. 14, 15 and 16).

Beside those gibberellins described above, we have been able to identify the gibberellins 53, 37 and 35 which are considered to be close to the beginning of the gibberellin synthesis pathway (scheme 4).

Calculating the GA content (ng GA₃-like activity bud⁻¹) in buds from "On" and "Off" years from 'Shamouti' showed that they differed in pattern (fig. 17). The buds from the non-fruiting branches had high levels from July to October but dropped almost to zero at the time of flower induction, while fruiting branches showed the opposite direction. A similar pattern was found with 'Murcott' (fig. 18). Although the total amount of GA found in buds were higher than that in 'Shamouti'.

Summarizing this chapter we can conclude that the gibberellins found in buds from "On" trees of 'Shamouti' or 'Murcott', although identically in behavior, were very close to the sequence of the biosynthetic pattern. Most of the gibberellins identified were from "On" trees buds while "Off" trees showed only small peaks of GA - like activity. These small peaks were mostly impossible to identify.

The increase of gibberellins such as A₃, A₁, A₉, and A₇ towards the induction period in "On" trees might interfere with the flowering process, inhibiting it, as found long ago by many scientists.

Fig. 2 Retention times of gibberellins in gradient C18 HPLC

```
File: E:\CHEMPC\GA4.D
Operator: Felix
Date Acquired: 16 Jun 93 1:08 pm
Method File: YOSSI.M
Sample Name:
Misc Info: standart GA4 + markers
ALS vial: 1
```

Fig. 3 Scan of GA₄ standard with known hydrocarbons.

Fig. 4 GA - like activity in dwarf rice seedlings as a result of bud extract from 'Shamouti' branches with fruit on June 30, 1991. Fractions No. 23, 24, 25 identified as GA-9 by the GC-MS.

File: A:\1SIM1.D

Operator:

Date Acquired: 12 Jun 92 9:41 am

Method File: FELIX1.M

Sample Name: SHAMOUTI+FRUITS,tubes 23,24,25

Misc Info:

ALS vial: 1

Fig. 5 Total scan of fraction No. 23, 24, 25 from 'Shamouti' branches with fruit on June 30, 1991 that contained a high peak of GA-like activity with the dwarf rice seedling. A restricted region of GC-MS included the Rt. of a standard marker and a scan at KRI that indicate a typical scan of GA-9.

File: A:\1SIM1.D
Operator:
Date Acquired: 12 Jun 92 9:41 am
Method File: FELIX1.M
Sample Name: SHAMOUTI+FRUITS,tubes 23,24,25
Misc Info:
ALS vial: 1

Fig. 6 SIM check of fractions No. 23; 24, 25 from 'Shamouti' branches with fruit on June 30, 1991 including hydrocarbons. GA-9 was identified with different ions.

Fig. 7 GA - like activity in dwarf rice seedlings as a result of application of bud extract from 'Shamouti' branches with fruit on Sept. 1, 1991. Fractions No. 20, 21 identified as GA-80.

Sample Name:
Misc Info:
ALS vial: 1

bioassay 9, tubes 20,21

Fig. 8 Total scan of fractions No. 20, 21 from 'Shamouti' branches with fruit on Sept. 1, 1992 that contained a high peak of GA-like activity with the dwarf rice seedling. A restricted region of GC-MS included the Rt. of a standard marker and a scan at KRI that indicate a typical of GA-80.

File: E:\CHEMPC\FELIX14.D
Operator:
Date Acquired: 5 Jul 93 8:51 am
Method File: GASKIN.M
Sample Name: bioassay 9, tubes 20, 21
Misc Info:
ALS vial: 1

Fig. 9 SIM check of fractions No. 20, 21 from 'Shamouti' branches with fruit on Sept. 1, 1992 including hydrocarbons GA-80 was identified with different ions.

Fig. 10 GA - like activity in dwarf rice seedlings as a result of bud extract from 'Murcott' branches with fruit on Oct. 29, 1991. Fraction No. 18 identified as GA-10 by the GC-MS.

Method File: GASKIN.M
 Sample Name:
 Misc Info:
 ALS vial: 1

bioassay 19, tube18

Fig. 11 Total scan of fraction No. 18 from 'Murcott' branches with fruit on Oct. 29, 1991 that contained a high peak of GA-like activity with the dwarf rice seedling. A restricted region of GC-MS included the Rt. of a standard marker and a scan at KRI that indicate a typical of GA-10.

File: E:\CHEMPC\FELIX40.D
Operator:
Date Acquired: 22 Aug 93 12:29 pm
Method File: GASKIN.M
Sample Name: bioassay 19, tube18
Misc Info:
ALS vial: 1

Fig. 12 SIM check of fraction No. 18 from 'Murcott' branches with fruit on Oct. 29, 1991 including hydrocarbons. GA-10 was identified with differentions.

Fig. 13 GA-like activity in dwarf rice seedlings resulting from application of bud extract from 'Murcott' branches with fruit on Dec. 2, 1991. Fractions No. 13, 14, 15 identified as GA-1 or GA-3.

Fig.14 GA-like activity in dwarf rice seedlings resulting from application of bud extract from 'Murcott' branches with fruit on Dec. 14, 1992. Fractions No. 23, 24, 25 identified as GA-7.

Operator:
 Date Acquired: 20 Jun 93 12:12 pm
 Method File: GASKIN.M
 Sample Name: bioassay 111, sample 81
 Misc Info:
 ALS vial: 1

Fig.15 Total scan of fractions No. 23, 24, 25 from 'Murcott' branches with fruit on Dec.14, 1992. These contained a high peak of GA-like activity with the dwarf rice seedlings. A restricted region of GC-MS included the Rt. of a standard marker and a scan at KRI that indicate a typical GA₇.

File: E:\CHEMPC\FELIX81.D
 Operator:
 Date Acquired: 20 Jun 93 12:12 pm
 Method File: GASKIN.M
 Sample Name: bioassay 111, sample 81
 Misc Info:
 ALS vial: 1

Fig.16 SIM check of fractions No. 23, 24, 25 from 'Murcott' branches with fruit on Dec. 14, 1992 including hydrocarbons. GA7 was identified with different ions.

Scheme 4. Possible metabolic connections among GAs in plants
(adopted from Rebers et al 1991)

fig.17 Seasonal changes in GAs-like activity from fruiting and non-fruiting 'Shamouti' orang buds

fig.18 Seasonal changes in GAs-like activity from fruiting and non-fruiting Murcott tangerine buds

Flower Induction by Exogenous Application of Artificial Gibberellin

(Collaboration with Dr. Yossi Ben-Tal and Prof. Pharis)

Research employing PGRs and anti-metabolites has established the inhibiting effects of GA on flowering in citrus species. Summing up an early portion of such work, Goldschmidt and Monselise proposed that all citrus buds are determined to flower from their inception in the adult phase, but that the prescience of GA, or any other inhibition, may inhibit flowering. In previous work, we found that it was possible to alter the inflorescence branch habitat from leafless towards vegetative by using promalin (GA_{4+7} + Kinetin) or GA_3 . This agrees with data published by many scientists. On the other hand, few publication have claimed that some native or artificial GA induced flowering.

We tried to induce flower formation in 'Shamouti' with several artificial gibberellins that were sent to Dr. Ben-Tal by Prof. Pharis. In the first year we treated 4- and 9- month-old spring flush branches on mature bearing 'Shamouti' trees. Several concentrations (3.3; 10; 33; 100 and 330 ppm) of 5 μ l of GA, were applied to each bud on tagged branches.

Surprisingly, the Endo 16, 17 and dihydro GA_5 increased, on 9-month-old branches, subtending leafy and leafless inflorescences with a peak 10 ppm concentration. Following upon the results of the first year, a second year of treatment with 5 gibberellins (A_3 , A_4 , A_5 , Exo and Endo 16,17 dihydro GA_5 using at the same concentration was applied at flower induction (according to our previous experience, the best time for application was around December 25). The GA_5 endo 16,17 dihydro increased the percentage of leafy and leafless inflorescences and decrease that of vegetative ones (Table 12), as found in the first year with the same peak of concentration This GA at 330 ppm had increased the percent of vegetative branches upon the inflorescences. The GA_5 exo 16,17 dihydro was not as effective as the endo

configuration, but there was some indication that at a concentration of 3 ppm some increment in the percent of flowering was seen. GA₄, especially mixed with GA₃, showed that the higher the concentration, the higher the percent of vegetative branches. GA₃ at 330 ppm inhibited flowering completely, as reported years ago by Monselise and Halevy. On the 3rd year of experimentation we tried several GAs' of those which had shown activity in the previous years. Since, only 8 treatments could be included this year, only 4 concentration of GAs' were examined (Table 13). The effect of GA₅ endo 16,17 dihydro as found previously, did not give results this year, while GA₄ epy dihydro at 10 and 33 ppm increased the percent of LY and LS inflorescences. Neither the other gibberellins, nor their combinations, increased the flowering. So, we tried spraying several branches (in the above experiments, the gibberellins were applied to the buds by micro pipette) with Endo 16,17 dihydro GA₅. These showed some increase in the percent of flowering at concentrations of 3.3; 10 and 33 ppm (Table 14).

The experiments done during the project indicate that some artificial gibberellins might act as a flowering promoters. The commercially-available GA₃ which is known to inhibit flowering and promote vegetative growth was found, to act similarly, in this work.

Table 12. Effect of various gibberellins and some combinations between them on 'Shamouti' buds on 9-month-old branches (application Dec. 93; flower counting April 94).

GA Treatment	Concentration (ppm)	Branch type (%)			% LS*		% LY*	
		V	LY	LS	1 fl.	1 fl.<	1 fl.	1 fl.<
GA ₅ , Endo 16,17 - dihydro								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	79.17	10.42	10.42	50.00	50.00	50.00	50.00
	10	76.60	23.40	0	0	0	8.30	91.70
	33	53.80	32.71	13.48	48.15	51.85	56.67	43.33
	100	60.80	5.71	31.92	19.44	80.56	33.33	66.67
GA ₅ , Exo 16,17 - dihydro								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	64.50	24.79	7.71	62.50	37.50	37.50	62.50
	10	81.50	6.48	12.02	55.60	44.40	26.20	73.80
	33	70.00	23.75	6.25	60.00	40.00	75.00	25.00
	100	75.00	16.67	8.33	50.00	50.00	0	100.00
GA ₅								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	74.10	9.62	16.28	75.00	25.00	33.30	66.70
	10	100.00	0	0	0	0	0	0
	33	83.60	16.40	0	0	0	87.50	12.50
	100	61.10	25.93	12.96	0	100.00	37.50	62.50
GA ₄ , Dihydro								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	-	-	-	-	-	-	-
	10	93.75	6.25	0	0	0	0	100.00
	33	100.00	0	0	0	0	0	0
	100	73.10	22.09	4.76	66.70	33.30	31.25	68.75

* Percent LS or LY inflorescence with one or more flower.

Table 12 continues on the next page.

Table 12, continued

Table 12, continued

GA Treatment	Concentration (ppm)	% Flush			% LS		% LY	
		V	LY	LS	0:1	0:2<	Fl 1	Fl2<
GA ₄ , Epy - dihydro								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	-	-	-	-	-	-	-
	10	36.94	46.39	16.67	50.00	50.00	64.60	35.40
	33	50.00	20.40	29.60	70.83	29.17	25.00	75.00
	100	80.90	10.60	8.50	25.00	75.00	33.33	66.67
GA ₄								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	91.67	8.33	0	0	0	0	100.00
	10	78.80	8.75	0	0	0	87.50	12.50
	33	83.00	7.91	9.09	50.00	50.00	33.33	66.67
	100	94.00	6.00	0	0	0	33.33	66.67
GA ₄ + GA ₅ Exo - dihydro								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	70.21	22.50	7.29	100.00	0	66.70	33.30
	10	83.80	13.70	2.50	100.00	0	66.70	33.30
	33	61.10	29.63	9.26	62.50	37.50	50.00	50.00
	100	77.80	13.33	8.89	0	100.00	25.00	75.00
GA ₄ + GA ₅								
	Control	65.83	30.83	3.33	100.00	0	45.83	54.17
	1	67.81	30.63	1.56	0	100.00	30.00	70.00
	10	88.90	7.41	3.70	100.00	0	50.00	50.00
	33	71.30	21.67	3.33	0	0	66.67	33.33
	100	85.70	14.29	0	0	0	75.00	25.00

* Percent LS or LY inflorescence with one or more flower

Table 13. Effect of various gibberellins on 'Shamouti' buds 9-month-old branches.

(application Dec. 25; 27, 1992 flower counting April 1993).

GA Treatment	Concentration (ppm)	Branch type (%)			% LS*		% LY*	
		V	LY	LS	1 fl.	1 fl.<	1 fl.	1 fl.<
Endo - GA - 5								
	Control	56.00	20.47	23.53	34.63	7.08	45.22	13.07
	3	45.18	15.95	38.87	52.08	20.08	16.44	11.40
	10	22.89	31.38	45.73	43.10	8.33	35.23	13.34
	33	46.56	21.63	31.81	48.07	7.74	20.84	23.35
	100	46.84	25.33	27.82	35.68	8.37	30.78	25.16
	330	76.67	14.21	9.12	24.55	5.22	57.14	13.10
Endo GA - 4								
	Control	56.00	20.47	23.53	34.63	7.08	45.22	13.07
	3	32.90	30.60	36.90	41.20	14.80	15.60	28.50
	10	62.90	14.60	12.40	26.10	7.10	52.00	14.70
	33	57.50	28.70	13.90	22.30	13.20	43.40	21.10
	100	28.60	32.80	38.60	26.30	22.20	25.10	26.30
	330	58.30	13.10	23.60	45.40	14.80	16.10	23.80
GA - 3								
	Control	56.00	20.47	23.53	34.63	7.08	45.22	13.07
	3	80.70	7.70	11.60	43.20	21.30	32.60	2.90
	10	82.90	7.60	9.50	40.00	8.30	39.20	12.50
	33	88.80	7.50	3.80	27.80	0	72.00	0
	100	70.00	20.00	10.00	33.30	0	55.60	11.10
	330	100.00	0	0	100.00	0	0	0

* Percent LS or LY inflorescence with one or more flower.

Table 13 continues on the next page

Table 13, continued

GA Treatment	Concentration (ppm)	Branch type (%)			% LS*		% LY*	
		V	LY	LS	1 fl.	1 fl.<	1 fl.	1 fl.<
GA - 4								
	Control	56.00	20.47	23.53	34.63	7.08	45.22	13.07
	3	57.50	26.00	16.50	16.80	14.80	45.40	23.00
	10	40.00	32.70	27.30	35.60	9.00	29.90	25.50
	33	60.70	21.00	18.30	35.00	0	50.00	15.00
	100	98.00	2.00	0	0	0	100.00	0
	330	76.50	21.60	2.00	8.30	0	58.30	33.30
GA - 5								
	Control	56.00	20.47	23.53	34.63	7.08	45.22	13.07
	3	38.10	29.00	32.90	34.70	16.30	15.70	33.30
	10	52.10	22.60	15.40	34.30	6.00	37.60	27.50
	33	44.60	37.70	15.50	24.40	2.70	69.20	3.70
	100	80.90	10.60	8.50	19.00	0	75.20	5.70
	330	77.30	13.60	9.10	14.30	19.40	40.50	25.80

* Percent LS or LY inflorescence with one or over one flower.

Table 14. Effect of Endo-16,17-dihydro GA₅ spray on branches of 'Shamouti' (Spray Dec. 93; flower counting April 94).

Concentration ppm	Branch type (%)		
	V	LS	LY
1	66.2	28.6	5.1
3.3	46.2	35.7	18.1
10	69.4	21.1	9.5
33	53.9	23.4	22.6
100	89.1	2.1	8.7
333	88.2	8.4	3.4
1000	76.5	15.5	8.0

Discussion

Low levels of carbohydrate has been held to be a factor contributing to poor return when fruit are "stored" on the tree for a prolonged period or after an "On" year crop. This conclusion has been drawn from the high correlation which was found between tree carbohydrate status i.e. starch availability and floral intensity, presented as a cause and effect relationship. Moreover, it is well documented no bloom occurs in the following season in a number of nodes sited along a fruiting branch basipetal to a growing fruit. In varieties bearing more than 2000 fruits per tree (many mandarin types), the above effect is so pronounced that almost no floral shoots are formed in the return bloom. Therefore, our proposal was directed in order to address the question whether these effects result from lack of carbohydrate or from basipetal transport of higher concentrations of flower inhibiting substance(s).

Our results clearly indicate that the distribution of carbohydrate found in buds, either as soluble sugars or as starch, differed significantly from the data published on the content of carbohydrate in leaves. Correlation found in the past between flowering and carbohydrate levels was not found to be similar when buds were tested instead of leaves. Neither sucrose nor glucose and fructose have been found to differ in content at induction and differentiation of reproductive or vegetative branches. If carbohydrate are the major factor in flowering we assumed that soluble sugar would be at a higher level in buds which normally would flower. Our data do not confirm this assumption, since buds located along a branch which will differentiate to flower, or remain vegetative, had the same sugar content (Tables 1, 2 and 3). Moreover, the buds on the vegetative branches (that will not flower at all) were found to contain higher levels of the sugars than those found in buds from reproductive branches (Table 4).

As already mentioned, trees will not flower after an "On" year and therefore carbohydrates in buds basipetal from the fruit were examined for their carbohydrate content. No differences were found in carbohydrate content in buds from "On" or "Off" years in 'Shamouti' orange or 'Murcott' tangerine. This means, again, that bud

differentiation is not a consequence of the carbohydrate level. We have no explanation why buds of 'Murcott' branches with fruit (Table 8) weighed less than buds from branches without fruit.

Exogenous application of sugars, by injection, was not found to significantly increase flower formation, except for glucose, which showed some variation in flower formation and changing the percent of LS and LY branches as compared with the control. It has been shown that girdling and defoliation are important for the induction. Reciprocal grafting, i.e. grafting a branch from "On" onto an "Off" plant and vice versa (Table 11) emphasize the theory that some growth inhibitor which is produced by the fruit and translocated basipetally might inhibit flower formation. This higher plant growth inhibitor might be one of the gibberellins found in significantly amounts in "On" than in "Off" buds (Fig 17 and 18). Gibberellin A₁, A₃, A₉ and A₇ have been known for long time to inhibit flowering. Although we were able to identify the gibberellins in small amounts of fresh bud materials collected from different branches (by GC-MS) especially, those that were assayed from "On" tree branches. It might be possible that a special gibberellin which we could not identify from "Off" buds will be involved in one of the sequences that lead to flower formation. This has been shown partially by the fact that artificial gibberellin increased flower formation over the control. Moreover, lately some scientists theorized that a balance between gibberellins that might inhibit, and those that might induce flower formation should occur.

Our data indicate that plant growth regulators (PGR) that are produced by the fruit serve as a major factor in controlling flower formation. Although, the carbohydrates are important as energy source for many processes in the plant, they were not found in buds of generative to vegetative at different levels. We think that carbohydrate might influence flower induction only by signal transmission and not as an energy source. As long as the possibility of signal transmission is not proved, PGRs can be regarded as the major factor inhibiting and/or promoting flowering.

Acknowledgments

We Thank Mrs. Bracha Artzi for her technical assistance in most part of the project, Shaya F. for the gibberellins work, and Hemou M. for the field experiments.

Publications resulting from the research

Erner, Y. 1992. Carbohydrate content associated with bud differentiation. 89th Annual Meeting of the Amer. Soc. Hort. Sci. Honolulu. Hawaii.

Erner, Y., Artzi, B., and Kaplan, Y. 1992. Exogenous applications of growth regulators affect carbohydrate content and fruit set of 'Shamouti' orange. Proc. Int. Soc. Citriculture Congress. Acireale, Italy. 1992.

Erner, Y. 1994. Morphology and anatomy of stems and pedicels of spring flush shoots associated with citrus fruit set. Submitted for publication, Annals of Botany.

Erner, Y., Felix, S., and Artzi, B. 1994. Alternate bearing affects carbohydrate and gibberellin content in citrus buds. Abst. O-17-5, XXIV Int. Hort. Congress. Japan.

Ben-Tal, Y., Erner, Y., Pharis, K. P., and Mander, L. N. 1994. Flower induction by artificial gibberellins. Abst. O-20-4, XXIV Int. Hort. Congress. Japan.

Achievement and benefit to Agriculture

The results of our research has been the first to show that correlation between the accumulation of carbohydrate in leaves and flower formation was not the same when measurements were done on buds of similar leaves. Experiments done to evaluate the endogenous carbohydrate in leaves and buds, the exogenous application of soluble sugar together with grafting experiments emphasize the role of existing fruit which control flower formation through the possible high content of "gibberellin inhibitors". The buds along fruiting branches contain higher amounts of gibberellins than those from non-fruiting branches.

We were able to show that the gibberellin accumulation pattern in buds indicates that in order to have flower formation the content should drop to a maximum level. This conform with the possibility to induce flowering with anti-gibberellin synthesis chemical such of Paclo butrazole by spraying during the time of flower formation. Artificial gibberellin which had been first applied to citrus bud showed the possible potential, of the application of such gibberellin in order to induce flowering.

Our data explain the alternate bearing habit of some citrus varieties which might be controlled primarily by thinning.

The development of special gibberellin which might induce flower formation and especially those of the leafy inflorescence will increase productivity. We think that the potential of the tree to carry more fruit without reducing fruit size exists on most seedless varieties and therefore the use of a flower induction chemical, such as artificial gibberellin, will increase production and growers' profit.

This work suggest a new technology to increase fruit production with the understanding of the importance of the carbohydrate in the process of flower formation.

Methodology, data, results and discussion

U.S.A portion - C. J. Lovatt

Abstract

Basic Research was undertaken to identify and quantify biotic and abiotic factors which initiate and/or regulate the expression of the flowering process in *Citrus* species. The abiotic factors investigated were low-temperature and water-deficit stress; biotic factors included endogenous concentrations of plant growth regulators, key nitrogenous compounds, and carbohydrates. The relationship of these factors to the initiation and expression (floral intensity) of flowering was investigated in experiments employing the 'Washington' navel orange (seedless), the 'Shamouti' orange (1-2 seeds per fruit), 'Murcott' mandarin (6-20 seeds per fruit), and 'Frost Lisbon' lemon (6-12 seeds per fruit).

While concentrations of PGRs changed in response to low-temperature treatment and in a manner related to shoot and root flushes, no significant relationship between changes in PGR levels and flowering could be demonstrated. In contrast, flower number per tree was positively correlated with leaf spermidine concentration at the initiation of low-temperature floral-induction treatment. In addition, trees sprayed with 50 mM arginine or 20 mM spermidine at the end of the low-temperature treatment had greater leaf concentrations of spermidine 1 week after application and 42 and 87% more flowers, respectively. *p*-Coumaroylputrescine, feruloylputrescine, and coffeoylputrescine were present in very low amounts in leaves of mature nonflowering 'Washington' navel orange and 'Frost Lisbon' lemon trees, but increased during low-temperature flower induction, with the greatest concentration in young flower buds just after transfer to the warmer temperature. Concentration of the phenolic amides decreased during floral development through petal fall.

Fruit with the faster growth rate and greater potential to set and survive to harvest were borne on leafy versus leafless inflorescences. These fruit were characterized by

significantly greater concentrations of total polyamines (putrescine, spermidine, and spermine). Foliar applications of low-biuret urea or putrescine during early stages of flower development increased ovary putrescine concentrations and fruit set.

Results of our basic research were developed into practical field-applicable management strategies to enhance flowering, fruit set, and yield of the 'Washington' navel orange without reducing fruit size and to enhance the yield of summer (off-season) 'Frost Lisbon' lemons. Our research results demonstrated that a properly timed winter prebloom application of low-biuret urea to the foliage of the 'Washington' navel orange at a rate of 0.16 kg N per tree increased yield. Moreover, the number of larger commercially-valuable fruit with diameters 6.1 to 8.0 cm increased as yield increased ($r^2 = 0.88$, $P \leq 0.05$). The cost benefit analysis indicated that this management strategy was economically viable each year of the 3-year study. In addition, it is also environmentally prudent since it reduces the amount of N applied to the soil.

We also demonstrated that deficit irrigation can be used in commercial lemon groves to initiate the flowering process and maximize floral intensity by withholding water until trees are stressed to a level between -2.4 and -3.2 MPa predawn xylem pressure potential and then initiating deficit irrigation at approximately 25% of the evapotranspiration for well-watered trees for 20 to 40 days, after which trees should be rewatered to field capacity.

Original Objectives

Citrus production in California, Arizona, Texas, and Israel is directed toward the fresh fruit market. A considerable proportion of Israeli citrus is exported to Europe; an increasing percentage of California-Arizona citrus is going to Japan. Competition in the domestic and worldwide marketplace is severe, causing a gradual reduction in grower income. A significant advantage is won by the grower or country that can

deliver its fruit even a few weeks earlier, or delay the harvest by a few weeks, to fill a niche in the market place when fruit is scarce and prices are high. An example of this is the early (autumn) grapefruit marketed in Europe by Israeli growers, or the South African equivalent, which reaches Europe in early summer. Such fruit comes from trees grown in specific areas in which early flowering occurs (Patt and Har-Even, 1962). A more extreme but economically viable example is the process of *forzatura* for producing the *verdelli* (light green) lemon or lime harvested in the summer. These fruit earn net returns 10 times greater than fruit from the spring bloom harvested in the winter. As currently practiced, results are often variable and the number of fruit produced is low, even in the limited areas considered edaphically and climatically suited for it. *Forzatura* is a harsh treatment involving long periods of water stress, which sometimes cause trees to collapse. Further knowledge of the regulation of flowering is necessary in order to make the practice agriculturally and economically viable.

Prolonged storage of fruit on the tree, an unusually heavy yield, or yield-reducing factors, such as frost, warm winter temperatures of hot "hamseen" or "Santa Ana" winds during a period critical to bloom or fruit set can cause trees to enter an alternate bearing cycle. Up to now, reducing flower and fruit number in the "On" year by mechanical or chemical thinning has met with variable results. The need to minimize alternate-bearing will become greater in the future. Israel has already initiated, and the US is beginning to develop, an easy peeler industry. A high-yielding mandarin of suitable fruit quality, good size, and seedlessness is needed to retain consumer interest in this fruit. Mandarin varieties are notorious for their susceptibility to alternate bearing.

Manipulation of flowering can be achieved by selecting or breeding for earlier or later flowering varieties, a method which is being utilized by a few specialized laboratories in long-term, expensive research programs. "Storing" fruit on the tree as a means to postpone harvest has already been optimized by the efforts of Coggins (1981). Understanding the regulation of flowering in citrus in order to manipulate the

intensity and timing of bloom in existing varieties is a reasonable approach to be optimized (Erner, 1989; Wright, 1989; Mullins *et al.*, 1989). To meet the need to broaden the period of marketability of existing commercial cultivars to increase the percentage of the fruit marketed at premium prices, the overall goal of the research was to develop effective, reliable agro-techniques to manipulate the intensity and the time of flowering in citrus. Three of the four specific objectives were designed to identify and quantify biotic and abiotic factors which initiate and/or regulate the expression of the flowering process. The abiotic factors investigated were low-temperature and water-deficit stress. Endogenous concentrations of plant growth regulators (PGRs), key nitrogenous compounds, and carbohydrates comprised the biotic factors. The relationship of these factors to the initiation and expression of flowering was investigated in experiments employing the 'Washington' navel orange (seedless), the 'Shamouti' orange (1-2 seeds per fruit), and 'Murcott' mandarin (6-20 seeds per fruit).

The results obtained in meeting these three specific objectives were used to develop field-applicable methods for improving floral intensity, for shifting the time of bloom, or for "evening out" alternate bearing. These production management strategies were tested in commercial orchards in fulfillment of the fourth specific objective and the overall goal of the project.

Results

As part of our research to determine the sequence of biochemical events leading to the irreversible commitment to flowering in *Citrus*, we conducted two sets of experiments: (i) one to identify the one or more plant growth regulators (PGR) that control floral shoot development and its (their) site(s) of production (shoot apex, leaves or roots); and (ii) another to identify the one or more PGR that control(s) vegetative shoot growth and its (their) site(s) of production (shoot apex, leaves or roots). Ultimately, we will need to understand the role of endogenous PGRs in regulating both vegetative and floral shoot development if we are to have control over these processes in the field.

Flower formation in *Citrus* species is promoted by drought or low temperature, followed by restoration of climatic conditions favorable to growth (Monselise and Halevy, 1964; Monselise and Goren, 1969; Monselise, 1978, 1985; Southwick and Davenport, 1986; Lovatt *et al.*, 1988a, b). Thus, stress applied in a quantitative manner provides a controlled system during which changes in tree carbohydrate status, nitrogen metabolism, or plant growth regulator levels can be monitored in relation to flower initiation.

(i) Our research employed low-temperature stress to induce flowering in 5-year-old rooted cuttings of the 'Washington' navel orange (*Citrus sinensis* L. Osbeck).

Since leaves are believed to be the site of reception of the environmental stimuli causing flowering, we removed leaves from trees during each successive week of the low-temperature induction treatment described previously (Lovatt *et al.*, 1988a) and monitored changes in ABA, GA_{1,3}, and 20, zeatin, zeatinriboside, dihydrozeatin, dihydrozeatinriboside, isopentenyladenine, isopentenyladenosine, and IAA in the leaves during stress. Cultural practices that alter the synthesis, metabolism or transport of PGRs by roots, e.g., girdling (Monselise, 1985), graft incompatibilities resulting in weak rootstocks (Mosse, 1962), confining roots to small pots (Furr *et al.*, 1947) or root pruning (Monselise, 1985), support the idea that changes in the level of PGRs moving from root to shoot may regulate vegetative versus floral shoot development. Thus, we

also measured changes in ABA, GAs, cytokinins, and IAA in roots and in the xylem sap during each successive week of the low-temperature induction treatment.

(ii) We removed all the shoots from 'Lisbon' lemon trees 2 years from the bud by pruning the tree just below the crown. We monitored the PGRs moving from the detached shoot which normally inhibit adventitious shoot development and collected xylem sap to confirm that these hormones were no longer present in the trunk. In addition, we monitored the PGRs produced by the roots to determine if the loss of leaves and resulting reduction in transpiration reduced the movement of root-produced PGRs in the xylem. We analyzed for ABA, GA_{1,3}, and 20, zeatin, zeatinriboside, dihydrozeatin, dihydrozeatinriboside, isopentenyladenine, isopentenyladenosine, and IAA. A considerable portion of our efforts in meeting Objective 1 was toward developing and standardizing both HPLC and radioimmunoassay for measuring plant growth regulators.

HPLC

The following conditions for isolation and quantitation of PGRs were established. PGRs were separated on a Zorbax ODS column, 250 x 9.4 mm equipped with a precolumn. The PGRs were eluted by a gradient solvent system. The initial conditions were 95% solvent A: 0.1 M acetic acid in 10% aqueous methanol adjusted to pH 3.4 with triethylamine and 5% solvent B: 100% methanol. The linear gradient shifts from 5% solvent B to 50% solvent B in 50 min. at a flow rate of 1 ml/min. Retention times for the PGRs were as follows:

Zeatin	18 - 19 min.
Zeatinriboside	20 - 21 min.
indole-3-acetic acid	31 - 32 min.
gibberelic acid	30 - 32 min.
abscisic acid	42 - 43 min.
isopentyladenosine	44 - 45 min.

RIA

The PI gratefully acknowledges Dr. Jonathan Cutting, University of Natal, for his generosity in supplying all antibodies needed for the RIAs.

Cytokinins. [^3H]Isopentyladenine, [^3H]isopentyladenosine, [^3H]zeatin, and [^3H]zeatinriboside were made from [^3H]adenine and [^3H]adenosine, respectively, by reaction with bromo-methyl butene and dimethylamine to form, respectively, [^3H]Isopentyladenine and [^3H]isopentyladenosine. These were reacted with tertiary butylhydroxiperoxide and dichloromethane to yield [^3H]zeatin and [^3H]zeatinriboside. [^3H]zeatin, [^3H]zeatinriboside, [^3H]Isopentyladenine, and [^3H]isopentyladenosine were isolated from the reaction mixture and purified by HPLC.

The antibody to zeatinriboside was tested against its substrate to determine the dilution necessary for 50% binding (50% binding is desired to give a range of values over which the standard curve is accurate). Optimal dilution for the antibody to zeatinriboside was found to be 1:2750.

Similarly, the optimal dilution of horse serum to be used in the assays was determined. Horse serum provides the extra protein necessary for precipitation of the antibody and the antigen. Optimal conditions are the lowest amount (based on dilution) of horse serum needed in an assay to still maintain 50% binding. This value was determined for each antibody.

[^3H]Isopentenyladenosine made from [^3H]adenosine, as well as [^3H]isopentenyladenine made from [^3H]adenine, were purified twice by HPLC. These fractions were tested for maximum binding with the antibody. Thereafter, the optimal dilution of the antibody for isopentenyladenosine was established. This antibody can also detect isopentenyladenine. The cross-reactivity was 85%.

GA. [^3H]GA₃ was partially purified and provided to us by Dr. Jonathan Cutting. GA₃ was further purified by HPLC.

To increase the sensitivity and the stability of the acid PGRs (GA_3 , ABA, IAA), the tracers, as well as the standards, were methylated. This was done with diazomethane which was produced by reaction of N-nitroso-N-methylurea. The optimal dilution of the antibody (50% binding of tracer) was determined to be a dilution of 1:1000.

ABA. The tracer was purchased from Amersham; no further purification was required. Antibody dilution of 1:500 yielded 50% binding.

IAA. [^3H]IAA was purchased from American Radiolabeled Chemicals; no further purification was necessary. The dilution of the antibody for IAA was 1:1000. The cross-reactivity of the antibody with tryptophan was tested and found to be less than 1%.

Standard curves were set up for all the hormones. Sensitivity of the assays for the following PGRs is:

Z/Zr	0.05-20 ng
IAA	0.25 - ng
GA_3	0.025 - 5 ng
ABA	0.01 - 10 ng
IPA	0.075 - 20 ng
IAde(2iP)	0.1 - 25 ng (with IPA antibody)

Sample Purification for RIA

A column system was set up for hormone purification. The system consists of a 5ml polyvinylpyrrolidone column with a 5-ml DEAE ion exchange column below it. Equilibration of the system was done by washing it with 15 ml 0.01 M ammonium acetate, followed by 15 ml 1.0 M ammonium acetate. Therefore, a SepPak cartridge (C18) (preconditioned by flushing with 10 ml methanol) was attached to the DEAE column. These three columns were washed with 15 ml ammonium acetate before the sample was added. The sample was the supernatant fraction (adjusted to pH 7.0) of

the original plant sample extracted in 80 % methanol, dried, redissolved in 6 ml 0.01 M ammonium acetate, and centrifuged at 12.000 g. The system was eluted with 30 ml 0.01 M ammonium acetate, the SepPak cartridge which contained the cytokinins was removed and saved, and the PVP column discarded. Another SepPak cartridge was preconditioned by flushing with 10 ml methanol followed by 10 ml 0.1 M acetic acid and attached to the sephadex column. This system was eluted with 25 ml 1.5 M acetic acid and the SepPak containing the acidic hormones removed and saved.

Bpth SepPak columns were washed with water and eluted with increasing concentrations of methanol in water. the elution of the SepPak columns was done in the following way:

	% Methanol	Hormone Eluted
SepPak I		
(cytokinins)	10 (4 ml)	discard
	30 (4 ml)	Z+ZR
	60 (4 ml)	IPA+2iP
SepPak II		
(acidic hormones)	20 (4 ml)	discard
	60 (4 ml)	GAs, ABA, IAA

Recovery of the different hormones was tested by running a sample of citrus leaf tissue with a radioactive standard for each hormone. The recovery for reach PGR was as Follows:

Z/ZR	85% recovery
IPA	85% recovery
GA ₃	78-90% recovery
IAA	40-60% recovery
ABA	70% recovery

The low-temperature stress caused a significant reduction in the concentration of IAA in both young and old leaves. Root concentrations of GA and ABA increased in

trees subjected to low-temperature stress. Many of the changes in the concentrations of PGRs were more closely related to shoot and root flushes, which occurred at different times in the warm-temperature control trees versus those subjected to low-temperature stress. For warm-temperature control trees, trees subjected to low-temperature stress and detopped trees, vegetative flushes were associated with dramatic increases in GA, IAA, zeatinriboside, and dihydrozeatinriboside.

There were, however, no dramatic changes in the levels of any of the PGRs we measured brought about by transferring the plants to the low-temperature floral induction treatment or subsequent transfer to the warm-temperature conditions that was significantly correlated with flowering.

These results are in agreement with those obtained by Southwick and Davenport(1986, 1987) with 'Tahiti' lime. While Southwick and Davenport (1986) also demonstrated that the degree of flowering in *Citrus latifolia* Tan. was directly proportional to the severity or duration of low-temperature or water-deficit stress, they found no correlation between the endogenous level of any of several plant growth regulators and flower number (Davenport, 1990). Davenport (1990) concluded in his thorough review that, at present, there are no specific candidates for a PGR inducer of flowering in *Citrus*.

In contrast, the aliphatic free di- and polyamines putrescine, spermidine, and spermine have been designated as plant growth regulator substances (Bagni *et al.*, 1982) and, along with their phenolic amides, have been correlated with the flowering process in apple (Edwards, 1986; Rohozinski *et al.*, 1986) and a number of annual plant species (Martin-Tanguy *et al.*, 1982; Malmberg *et al.*, 1985; Kuar-Sawhney *et al.*, 1988; Martin-Tanguy, 1985). Consistent with the potential role of these compounds in the regulation of flowering in *Citrus*, we (Lovatt *et al.*, 1992b) demonstrated that p-coumaroylputrescine, feruloylputrescine, and caffeoylputrescine were present in very low amounts in leaves of mature nonflowering trees of 'Washington' navel orange and 'Frost Lisbon' lemon. The three phenolic amides increased during the low-temperature stress induction period and were of greatest

concentration in flower buds at the time of transfer to the warm temperature condition. Concentrations of the phenolic amides decreased during development through petal fall, a developmental pattern that we (Sagee and Lovatt, 1991) previously reported for flower $\text{NH}_3\text{-NH}_4^+$ content and putrescine synthesis via arginine. *p*-Coumaroylputrescine was the dominant phenolic amide in flowers and leaves from flowering trees. We also established that the catecholamine dopamine and its precursors, phenylethylamine and tyramine were present in leaves of both nonflowering and flowering trees. The results of our research also provide evidence suggesting a relationship between the levels of key specific polyamine compounds in branches that give rise to floral shoots versus those that give rise to vegetative shoots. It is of special interest that leaf putrescine concentration can be increased by elevating the endogenous ammonia concentration of the leaf. Detached leaves of the 'Washington' navel orange were immersed at their base in 50 mM NH_4Cl for 12, 24, and 48 h. The $\text{NH}_3\text{-NH}_4^+$ content of the leaves increased with time, resulting in parallel increases in leaf arginine and putrescine content.

We conducted experiments to determine whether a cause and effect relationship exists between putrescine, spermidine, or spermine and flowering in citrus.

Five-year-old 'Washington' navel orange trees (*Citrus sinensis* L. Osbeck) on Cuban shaddock rootstock (*C. limon*) grown in plastic pots containing 19 liters of University of California soil mix were induced to flower by subjecting the trees to a low-temperature treatment of 8-h days (PFD of $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$) at 10°C and 16-h nights at 7°C for 4 weeks, and then transferring the trees to 12-h days at 24°C /12-h nights at 18°C (Moss, 1969). Aqueous solutions (adjusted to pH 7.0) of L-arginine (50 mM), putrescine (10 or 20 mM), and spermidine (10 or 20 mM) were sprayed on the canopy to the point of run-off immediately at the end of the 4-week, low-temperature treatment. The control trees were sprayed with distilled water. For each treatment, nine trees were used as single tree replicates in a completely randomized design.

Mature leaves were collected at the initiation of the low-temperature treatment, again at the end of the low-temperature treatment but prior to arginine, putrescine or

spermidine application, and successively 1, 4 and 7 days after the application of arginine, putrescine and spermidine to the canopy. Two weeks after the trees were transferred to the warmer temperature conditions, the following parameters were determined: (i) number of floral shoots versus vegetative shoots per tree; (ii) number of leafy versus leafless inflorescences per tree; and (iii) number of flowers produced per tree.

Leaf $\text{NH}_3\text{-NH}_4^+$, putrescine, and spermidine concentrations were significantly greater at the end of or 1 day after the low-temperature floral-induction treatment, while spermine content decreased. Trees receiving putrescine (20 mM) or spermidine (10 mM) were unique in having significantly greater leaf concentrations of spermidine at the initiation of floral-induction treatment ($P \leq 0.05$). Flower number per tree was positively correlated with leaf spermidine concentration at the initiation of the low-temperature floral-induction treatment ($r = 0.37$, $P \leq 0.05$). Arginine and polyamines applied to the foliage at the end of the 4-week, low-temperature treatment generally did not result in a significant increase in leaf polyamine content over control trees, two exceptions were trees receiving 50 mM arginine or 20 mM spermidine which exhibited significantly greater leaf concentrations of spermidine 1 week after application and statistically nonsignificant increases in flower number per tree of 42% and 87%, respectively. The results provided evidence that spermidine availability prior to and/or during flower organogenesis might be a factor affecting floral intensity in the 'Washington' navel orange.

As a corollary to our previous research which demonstrated that flower initiation is influenced by $\text{NH}_3\text{-NH}_4^+$ and to our current research testing whether there is a cause and effect relationship between flowering and polyamines, we tested our hypothesis that key nitrogen compounds that influence flower initiation may be important in the formation, development, and subsequent set of the ovary.

Early in their development, apical flowers borne on leafy inflorescences had higher tissue levels of polyamines (Table 1) and $\text{NH}_3\text{-NH}_4^+$ and higher rates of *de novo* arginine biosynthesis (data not shown) than apical flowers borne on leafless

inflorescences. In addition, navel orange fruit exhibiting faster growth rates during the first weeks after petal fall were borne on leafy inflorescences and higher levels of polyamines than slower-growing fruit borne on leafless inflorescences (Table 2).

Table 1. Polyamine content of Stage I apical flowers of leafy versus leafless inflorescences^Z

	Leafy	Leafless
	mg · flower ⁻¹	
Flower fresh weight	99 a	74 b
	nmol · g ⁻¹ fresh weight	
Putrescine	472	231
Spermidine	170	149
Spermine	31	19
Σ	673 a	401 b
	mg · g ⁻¹ fresh weight	
Flower protein content	19 a	12 a
	nmol · mg ⁻¹ protein	
Putrescine	26	14
Spermidine	9	8
Spermine	1	1
Σ	36 a	23 b

^ZData are the mean of four replicates from two separate experiments inducing flowering by low temperature stress in 5-year-old rooted cuttings of the 'Washington' navel orange and of four replicates from spring bloom of 30-year-old 'Washington' navel orange trees under commercial production. Mean separation was by Duncan's Multiple Range Test. Values within a horizontal row followed by different letters are significantly different at $p \leq 0.05\%$.

From Lovatt *et al.* (1992a).

Table 2. Characteristics of fruit borne on leafy versus leafless inflorescences during the second week after petal fall².

	Leafy	Leafless
	mm · day ⁻¹	
Fruit growth rate	0.130 a	0.067 b
	mg · fruit ⁻¹	
Fruit fresh weight	382 a	259 b
	mg · g ⁻¹ fresh weight	
Fruit protein content	6.4 a	6.8 a
	nmol · g ⁻¹ fresh weight	
Putrescine	388	189
Spermidine	132	81
Spermine	61	40
Σ	581 a	307 b
	nmol · g ⁻¹ mg protein	
Putrescine	62	32
Spermidine	21	15
Spermine	10	6
Σ	93 a	53 b
	nmol · organ	
Putrescine	144	55
Spermidine	49	22
Spermine	23	12
Σ	216 a	89 b

²Data are the mean of four replicates from two separate experiments inducing flowering by low temperature stress in 5-year-old rooted cuttings of the 'Washington' navel orange and of four replicates from spring bloom of 30-year-old 'Washington' navel orange trees under commercial production. Mean separation was by Duncan's Multiple Range Test. Values within a horizontal row followed by different letters are significantly different at $p \leq 0.05\%$.

From Lovatt *et al.* (1992a).

The ability of urea, arginine, and putrescine to increase set was tested in the field. Thirty-year-old 'Washington' navel orange trees were sprayed with 200 mM low-biuret urea, 100 mM arginine, or 20 mM putrescine at early bloom (flowers were the size of the head of a pin). Both urea and putrescine increased ovary putrescine content and increased fruit set ($P \leq 0.05$). The urea spray maintained the putrescine content of the fruit at the higher level for a longer time than the putrescine spray. The 100 mM arginine spray was too concentrated and crystallized on the leaves and buds.

Given that our research has demonstrated that ammonia and/or its metabolites increase flowering (Lovatt *et al.*, 1988a,b), that ammonia and the synthesis of arginine and putrescine are metabolically linked (Sagee and Lovat, 1991), and that greater putrescine and total polyamine concentrations are associated with fruit with a greater potential to set (Lovatt *et al.*, 1992a), we established a field experiment, the objective of which was to test whether a single winter prebloom foliar application of low-biuret urea would increase the yield of 30-year-old 'Washington' navel orange trees [*Citrus sinensis* (L.) Osbeck] on Troyer citrange rootstock [*C. sinensis* cv. Washington navel orange x *Poncirus trifoliata* (L.) Raf.] (Ali and Lovatt, 1994). All trees received a winter (November-January) soil application of urea (0.5 kg N per tree). Trees were maintained under irrigation or irrigation was withheld from 1 October to 1 March. To determine the optimal time for foliar urea application, trees in both irrigation main plots received a single application of low-biuret urea in mid-November, mid-December, mid-January or mid-February applied to the foliage at a rate of 0.16 kg N per tree. There was a set of control trees which only received the soil application of urea. Trees receiving foliar-applied urea in mid-January or mid-February, independent of the irrigation treatments, had significantly greater yield and fruit number per tree each year than the control trees for three consecutive years. The number of fruit with diameters 6.1 to 8.0 cm increased significantly as yield increased ($r^2=0.88$). Withholding irrigation from 1 October to 1 March had a negative impact on yield. Annual winter application of low-biuret urea to the foliage did not cause a statistically significant increase in leaf total N at the end of 3 years.

The unique feature of the approach employed in this research was the winter application of a relatively high rate of urea to the foliage (0.16 kg N per tree)-approximately 25 to 35% of the annual nitrogen required by the navel orange for maximum yield (Embleton and Jones, 1974). The winter application of foliar low-biuret urea was intended to replace an equivalent amount of N applied to the soil. However, the arrangements of trees along the irrigation furrows compromised our original intent, and nitrogen (0.5 kg N per tree) was applied in winter to all trees via the soil. Nitrogen is applied to the soil in the winter in California to coincide with the winter rains, but there has been considerable speculation on the amount of this nitrogen that is actually utilized by the citrus tree. Results of the 3-year-long study reported here provide evidence consistent with the interpretation that the yield increases obtained were dependent on the timing of the prebloom application of urea to the foliage. Only the mid-January and the mid-February applications of low-biuret urea significantly increased yield per tree above the control trees receiving soil-applied urea in every year of the 3-year study. With regard to fruit number per tree, there was an effect of the timing of the foliar application of low-biuret urea. Only the mid-January foliar application of low biuret urea significantly increased the number of fruit per tree over the control trees for each of the 3 years. Mid-February or mid-December foliar applications of low-biuret urea significantly increased the number of fruit per tree in 2 years of the study, while the mid-November application of low-biuret urea significantly increased the number of fruit per tree over that of the control trees receiving only soil-applied urea in only 1 year of the study. Thus, the yield increases obtained in this study are not likely the results of improved nitrogen status of the tree by the additional nitrogen supplied to the foliage, or all trees receiving a foliar application of low-biuret urea would have exhibited an increase in yield and fruit number per tree. In addition, all trees were nitrogen-sufficient at the start of the experiment, since they had an average leaf total N level of 2.68%. Total nitrogen concentration of the leaves did not increase significantly in response to the three annual foliar applications of low-biuret urea compared to that of the control trees

receiving only soil-applied urea. At the end of the 3-year study, leaf total nitrogen was not different from that at the start of the experiment and remained at a level considered optimum for navel oranges for California (Embleton *et al.*, 1973).

In Tables 3 and 4, we (Ali and Lovatt, 1992) report the results of a cost-benefit analysis which used the following values: (i) The average price of \$3.20 per carton; (ii) 15 gallons Unocal PLUS®, low-biuret urea per acre at \$1.10 per gallon; (iii) spray rig at \$25.00 per acre for the highest cost of application; and (iv) airplane at \$10.00 per acre for the lowest cost of application to give the lower and upper end of the range reported as net return in Tables 3 and 4. Our analysis underestimates total and net income because it does not take into account the increase in the number of fruit of carton sizes 72 and 88, which have a greater dollar value. Also keep in mind that yields were greatly reduced in the 1990-91 season due to the freeze in December of 1990.

In each year of this study, the winter foliar application of low-biuret urea (LBU) more than paid for itself (Table 3 and 4).

Table 3. Three-year net cumulative increase in packing cartons and in total and net dollar return per acre for trees receiving foliar LBU versus soil LBU.

	Date LBU applied to the foliage			
	November	December	January	February
Packing cartons	288	319	452	400
Total return	\$924	\$1024	\$1451	\$1284
Net return	\$814-845	\$914-945	\$1341-1372	\$1174-1205

Table 4. Average net dollar return per acre per year for trees receiving foliar LBU versus soil LBU.

Date LBU applied to the foliage			
November	December	January	February
\$271-282	\$305-315	\$447-457	\$391-402

Whether the yield response observed in this study is the results of improving the nitrogen status of the trees at a critical time in their phenology or to the specific effect of providing ammonia at the appropriate time to stimulate flowering and/or enhance fruit set as suggested by previous research with apples (Edwards, 1986; Grasmains and Edwards, 1974) and previous results with citrus (Lovatt *et al.*, 1988a, b; Sagee and Lovatt 1991; Lovatt *et al.*, 1992a) remains to be determined. From the results of the current field experiment, it cannot be ascertained whether or not the mid-January or mid-February foliar applications of low-biuret urea increased flowering; it is clear that they increased fruit set. In a previous study, a single foliar application of low-biuret urea to 'Frost Lisbon' lemon trees induced to flower by water-deficit stress (approx. -2.5 MPa predawn water potential maintained by deficit irrigation) increased flowering, fruit set, and yield compared to the trees equivalently stressed but not receiving a foliar application of low-biuret urea (Lovatt *et al.*, 1988a,b).

It is well documented (Barbera and Carimi, 1988; Barbera *et al.*, 1981; Lovatt *et al.*, 1988a,b; Southwick and Davenport, 1986, 1987) that water-deficit stress promotes flowering in *Citrus* species. However, withholding irrigation from 1 October through 1 March generally had a negative impact on yield in all 3 years of this study. Even in the first year of the study in which no significant differences in tree predawn water potential were detected and no significant differences in yield per tree or fruit number per tree were obtained between irrigation treatments, there was a significant reduction in the number of fruit with diameters 7.0 to 8.0 cm and 6.1 to 6.9 cm due to

withholding irrigation. In years two and three, withholding irrigation from 1 October to 1 March resulted in substantial water-deficit stress in December and February. In Both years, predawn water potentials were approx. -2.5 MPa. These trees suffered a greater loss (83%) of their harvestable crop due to the freeze of December 1990, and produced 50 % less fruit the following year than irrigated trees. Further experimentation is necessary to determine whether the use of water-deficit stress in October to reduce the fall flush of foliage and increase carbohydrate storage is a viable method for improving flowering, fruit set, and yield, The use of water deficit-stress beyond December is unlikely to prove of any commercial value.

It must be noted that there were no nitrogen-x-irrigation treatment interactions with regard to both yield and fruit number, and that the mid-January and mid-February foliar applications of low-biuret urea increased yield over that of the control trees receiving only soil-applied urea whether trees were irrigated or not.

The results of this study demonstrated that a properly-timed winter prebloom application of low-biuret urea to the foliage of the 'Washington' navel orange increased yield without decreasing the number of fruit with diameters 7.0 to 8.0 or 6.1 to 6.9 or increasing the number of small fruit with diameter from 5.2 to 6.0 cm. Cost benefit analysis indicated that this management strategy is economically viable (Ali and Lovatt, 1992) and because it provides, via the foliage, 25 to 35% of the nitrogen required annually by sweet oranges (Embleton and Jones, 1974), it is environmentally sound. Whether or not the yield of other citrus varieties might be increased by a properly-timed prebloom spray of low-biuret urea will be addressed in future research.

Results of our earlier research with 16-year-old trees of 'Frost Lisbon lemon' (*Citrus limon* [L.] Burm. f.) on 'Troyer' citrange rootstocks (*C. sinensis* x *Poncirus trifoliata* [L.] Raf.) under commercial production in the San Valley, CA (Lovatt *et al.*, 1988a), provided evidence that floral initiation and expression were not simply a function of the severity of the stress achieved. A short period of water-deficit stress resulting in severe stress (-3.0 MPa for 30 days) was not as effective as a longer

period of gradual water deficit (-2.0 MPa for 50 days), which resulted in the three times as many flowers per tree.

A large scale field experiment was established with the objective of comparing the efficacy of using deficit irrigation to maintain a prolonged moderate level of water-deficit stress with the conventional *forzatura* method of withholding all irrigation during the summer, to produce *verdelli* lemons for harvest the following summer. A commercial orchard of 20-year-old 'Frost Lisbon' Lemons on Troyer citrange rootstock was selected as the experimental site due to its coarse-textured soil, which permitted rapid tree response to decreases in irrigation volume and frequency.

Withholding irrigation during summer until trees reached a minimum predawn xylem pressure potential of -3.5 MPa, which resulted in a range of predawn xylem pressure potentials from -0.8 to 4.0 MPa in trees throughout the orchard prior to rewatering, did not produce consistent yields due to the variability in soil water availability and final stress achieved. Summer yields, but not winter yields, were weakly correlated with the final predawn xylem pressure potential at the time of rewatering.

In the contrasting system, individual trees were irrigated using a trickle/drip system consisting of 2 to 4 emitters per tree with a flow capacity of 2 to 8 liters per hour per tree. The emitters were placed 1 m away from the trunk of the tree and used to apply water to control the level of stress reached throughout the experiment.

The yield of *verdelli* fruit per tree was significantly correlated to the number of flowers per tree. The number of flowers per tree was significantly correlated with both the minimum predawn water potential achieved and the average water potential during the water-deficit period. The data indicated a water-deficit stress threshold for flower induction of -2.0 MPa average predawn xylem pressure potential. The number of flowers and yield per tree were also significantly correlated with the duration of stress following the attainment of -2.4 MPa predawn xylem pressure potential. After reaching this level of stress, mean flower number increased by 6% for every day that the stress was maintained beyond a 10-days threshold. Thus, using daily controlled

irrigation, we were able to manipulate the water stress experienced by individual lemon trees to investigate the pattern of predawn xylem pressure potential that maximizes flowering/ Regardless of the method of *forzatura* employed, summer and winter yields were not correlated with each other positively or negatively over the several years of the study.

Publication resulting from the research

- Ali, A.G. and C.J. Lovatt. 1992. Scientific rationale and economic incentives:
Winter application of foliar urea. *Citrograph* 78:7-9.
- Ali, A.G. and C.J. Lovatt. 1993. Foliar urea increases citrus yields. *Solution Sheet* 9:1-6.
- Ali, A.G. and C.J. Lovatt. 1993. Foliar urea improves yields. *Calif. Grower* 17:12-20.
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- Ali, A.G. and C.J. Lovatt. 1993. Leaf ahead. *In* L. Richardson (ed.), *Calif. Farmer* 276:8-9.
- Ali, A.G. and C.J. Lovatt. 1994. Winter application of low-biuret urea to the foliage of 'Washington' navel orange increased yield. *J. Amer. Soc. Hort. Sci.* 119:(accepted June 14, 1994, to appear in the November issue).
- Ali, A.G. and C.J. Lovatt. Evaluation of analytical procedures for quantifying ammonium in leaf tissue. (Submitted May 1994 to *J. Amer. Soc. Hort. Sci.*; 26 manuscript pages).
- Ali, A.G. and C.J. Lovatt. Relationship of polyamines to low-temperature stress-induced flowering of the 'Washington' navel oranges (*Citrus sinensis* L. Osbeck). (Submitted May 1994 to *J. Hort. Sci.*; 18 manuscript pages).
- Lovatt, C.J., O.Sagee, and A.G. Ali. 1992. Ammonia and/or its metabolites influence flowering, fruit set and yield of the 'Washington' navel orange. *Proc. Intl. Soc. Citriculture* 1:412-416.
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Benefits to Agriculture

The results of this research quantified the degree of influence that specific abiotic and biotic factors exert on flower initiation and the degree to which the flowering response is expressed (floral intensity) in *Citrus* species. The research provided evidence suggesting that species of polyamines are related to floral intensity and to the subsequent development and potential of the ovary to set and survive to harvest. The research also demonstrated that increasing the endogenous ammonia concentration of citrus resulted in a dramatic and significant increase in polyamines (putrescine especially). The results of the basic research were utilized to develop practical, field-applicable production management strategies to increase flowering, fruit set, and yield of the 'Washington' navel orange (*Citrus sinensis* L. Osbeck) without reducing fruit size and to shift the time of bloom from spring to fall and to maximize the summer yield of 'Frost Lisbon' lemons (*Citrus limon* L. Burm. f.).

Our research results demonstrated that a properly timed winter prebloom application of low-biuret urea to the foliage of the 'Washington' navel orange at a rate of 0.16 kg N per tree increased yield and increased the number of commercially valuable fruit with diameters 7.0 to 8.0 or 6.1 to 6.9 cm, but did not increase the number of small fruit with diameters from 5.2 to 6.0 cm. The cost benefit analysis indicated that this management strategy was economically viable each year of the 3-year study. In addition, it is also environmentally prudent as it reduces the amount of N applied to the soil.

Using daily controlled deficit irrigation, we were able to manipulate the water stress experienced by individual lemon trees to initiate the flowering process and to maximize floral intensity. In commercial lemon groves, this was accomplished by withholding water until trees were stressed to a level between -2.4 to -3.2 MPa predawn xylem pressure potential (a level at which leaves remained wilted in the early morning) and then initiating deficit irrigation at approximately 25% of the evapotranspiration for well-watered trees. Deficit irrigation was continued for 20 to 40 days, at which time, the trees were rewatered thoroughly to field capacity.