

**BIOCHEMICAL CHANGES IN KEY NITROGEN COMPOUNDS
DURING INITIATION AND ONTOGENY
OF 'WASHINGTON' NAVEL ORANGE FLOWERS**

A Lecture Presented By

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Regulation of flowering has long been a goal of growers, horticulturalists, and plant physiologists. There are many practical benefits to be derived from the ability to regulate flowering. It would be of benefit to be able to increase floral intensity to achieve maximum yield or to decrease flower number to improve fruit size or quality. An ability to increase or decrease flower number would make it possible to correct alternate bearing. An ability to regulate flowering would make it possible to change the time of flowering - earlier, later, or to a completely different time of the year. Finally, regulation of flowering would permit crop production in marginal areas because we would be able to insure a good bloom.

The idea that nitrogen might play a key role in the regulation of flowering became popular within the context of the Kraus-Kraybill Hypothesis. The potential regulation of flower initiation by the balance of carbohydrate to nitrogen or by a calculated mathematical ratio of carbohydrate divided by nitrogen is internationally known as the Kraus-Kraybill Hypothesis.

The results of our research on the biochemical changes of key nitrogen compounds during flower initiation in *Citrus* species is presented in light of the Kraus-Kraybill Hypothesis. The first half of this lecture takes a new look at the Kraus-Kraybill Hypothesis and the regulation of flowering in *Citrus* species, especially in the 'Washington' navel orange. The second half of the lecture summarizes our most recent results on the potential roles of key nitrogen compounds in fruit development and fruit set of the 'Washington' navel orange.

In taking a new look at the Kraus-Kraybill Hypothesis and flowering in *Citrus*, three points must be made clear. (1) It must be recognized that the work of Kleb's attempting to establish a role for carbohydrate-nitrogen status as a causal agent in flowering preceded by 14 years the publication of Kraus-Kraybill's manuscript, entitled "Vegetation and Reproduction with Special Reference to the Tomato," which is cited as the source of this idea. (2) There is an excellent and provocative article by J. S. Cameron and F. G. Dennis, Jr. (1986, HortScience 21:1099-1102), which demonstrates that by today's standards, Kraus and Kraybill's experimental design and statistical analyses were inadequate to establish the hypotheses for which they are internationally known. (3) I wish to make it perfectly clear so that I shall not later be misquoted, that with the discovery of plant hormones and demonstration of their influence on flowering, carbohydrate-nitrogen status must be considered a factor contributing to, but not solely regulating, flower initiation. Still, the Kraus-Kraybill Hypothesis identifies a concept with which we are all familiar. It is a hypothesis which still remains to be proven or disproven.

Flowering in *Citrus* is recurrent under tropical and subtropical conditions unless synchronized into a well-defined period of concentrated bloom by external environment conditions. Flower formation in *Citrus* is promoted by drought and low

temperature stress followed by restoration of climatic conditions favorable for growth. Thus, stress applied in a quantitative manner provides a controlled system during which changes in the ratio of carbohydrate to nitrogen or hormone levels can be monitored in relation to flower initiation.

In this lecture, data is presented on the relationship between the concentration of carbohydrate and nitrogen species, in young, fully-expanded citrus leaves and floral intensity. The results are from experiments employing: (1) low temperature stress to induce flowering in 5-year-old rooted cuttings of the 'Washington' navel orange; and (2) water-deficit stress to induce flowering in commercially grown 16-year-old Frost Lisbon lemon trees in 'Troyer' citrange rootstock.

In the San Joaquin Valley of central California, Farm Advisors, Joseph Maranto and Kater Hake, are experimenting with the use of deficit irrigation to induce a second bloom in the fall which will produce a crop of summer or "verdelli" lemons. This is in addition to the winter crop which is harvested from the normal spring bloom. The summer crop of "verdelli" lemons is highly desirable because it has a five-to-ten-fold greater market value than the winter crop. The use of water-deficit stress to induce a summer lemon is commonly carried out in Israel.

Five-year-old rooted cuttings of the 'Washington' navel orange induced to flower by low temperature stress were subjected to an 8-h day ($500 \mu\text{E}/\text{m}^2\text{-sec}$) at 15 to 18° C, 16-h night at 10 to 13° C. After 4, 6, or 8 weeks of low temperature treatment, the trees were transferred to 12-h day ($500 \mu\text{E}/\text{m}^2\text{-sec}$) at 24° C, 12-h night at 19° C. Maximum flower number, or peak bloom, occurred four weeks after the trees were transferred to the warm temperature. Control trees maintained at the warm temperature throughout the experiment did not flower. Low temperature

stress did not cause water-deficit stress. The average predawn water potential of the trees during the 8 weeks of low temperature treatment was the same as for the warm temperature control trees: -0.7 ± 0.2 MPa.

There was no significant change in the glucose or starch content of the youngest, fully expanded leaves of the 'Washington' navel orange during 8 weeks of low-temperature stress. The correlation between the length of the cold treatment and starch content was negative but not significant at the 10% level ($r = -0.186$). Previously, it has been assumed that photosynthesis continues during low temperature stress but that carbohydrate utilization is reduced, causing starch accumulation during the stress. We found photosynthetic rates to be the same between warm-temperature control trees and low-temperature stress trees. Since starch remains constant during the stress period, carbohydrate utilization, like photosynthesis, must also be proceeding at the normal rate during the stress. The low temperature treatment did not cause leaf abscission. Therefore, the amounts of glucose and starch available to the tree for the flowering process were the same both before and after the stress. For glucose, this concentration was $1.25 \mu\text{g}$ glucose/g fr wt leaf tissue. Leaf starch concentrations were highly variable. For the more than 100 trees used in the many experiments conducted values ranged from 1.4 to 150 mg starch/g fr wt leaf tissue.

Despite the fact that leaf carbohydrate content did not change in response to low-temperature stress, nor in a manner that paralleled floral intensity within an experiment, linear regression analysis of all the trees from all the treatments provided evidence that starch content of the youngest, fully expanded leaves correlated significantly with number of floral shoots initiated ($p < 0.0001$; $r = 0.75$). Leaf starch content was not correlated with the number of vegetative shoots

produced. This is a significant finding. It says that starch is not simply serving as an energy source to increase bud break, but is actually influencing floral shoot development.

Neither total N nor the NO_3^- content of the leaves changed during or after the stress. Total N measured by Kjeldahl was 25.5–3.8 mg/g dry wt leaf tissue. Leaf NO_3^- content was 3% of the total N level both during and after the low temperature treatment. However, there was a significant increase in leaf $\text{NH}_3\text{-NH}_4^+$ content during the low-temperature stress. $\text{NH}_3\text{-NH}_4^+$ accumulated in a manner that paralleled the duration of the stress ($p < 0.01$; $r = 0.605$). In addition, the amount of $\text{NH}_3\text{-NH}_4^+$ that accumulated was directly proportional to the number of flowers produced per tree ($p < 0.0001$; $r = 0.803$). Leaf $\text{NH}_3\text{-NH}_4^+$ content had no effect on the number of vegetative shoots initiated per tree.

To convince ourselves that there was a cause and effect relationship between leaf $\text{NH}_3\text{-NH}_4^+$ content and flower initiation and floral intensity, we subjected trees to only 4 or 6 weeks of low temperature stress and artificially raised the $\text{NH}_3\text{-NH}_4^+$ status of the trees by foliar application of low biuret urea at the rate of 1.5 g/tree. For trees receiving only four or six weeks of low temperature stress, foliar application of low biuret urea increased the leaf $\text{NH}_3\text{-NH}_4^+$ content 166 and 215% of value without added urea and gave a concomitant and equal increase in flower number per tree of 194 and 230% of the value without urea, respectively. For the more than 100 trees used in the many experiments conducted, including trees subjected to 2, 4, 6, or 8 weeks of low temperature stress and trees treated with a foliar application of low biuret urea, leaf $\text{NH}_3\text{-NH}_4^+$ content ranged from 389 to 2636 $\mu\text{g/g}$ dry wt leaf tissue. The corresponding number of flowers for each tree was 4 to 3065. These results indicate that there is a threshold level of $\text{NH}_3\text{-NH}_4^+$ below

which flowering will not take place in *Citrus*. That value is approximately 400 $\mu\text{g/g}$ dry wt leaf tissue.

We also used water-deficit stress to induce flowering in 16-year-old Frost Lisbon lemon trees on 'Troyer' citrange rootstock under commercial production in the San Joaquin Valley, California. Trees were subjected to water-deficit stress during July and August, the hottest months of our summer, and rewatered at the end of August to give maximum bloom by the end of September. Trees were subjected to water-deficit stress treatments of differing severity. Well-watered control trees ($\psi > 1$ MPa) had the lowest leaf $\text{NH}_3\text{-NH}_4^+$ content (519 $\mu\text{g/g}$ dry wt) and produced only 14 flowers per tree. One set of trees was subjected to severe water stress of short duration. The trees were taken to $\psi = -3$ MPa in 30 days and rewatered. The leaf $\text{NH}_3\text{-NH}_4^+$ of these trees increased to 646 $\mu\text{g/g}$ dry wt and flower number per tree increased to 53. One set of trees received severe water stress followed by moderate water stress. The trees were taken to $\psi = -3$ MPa in 20 days and then maintained at $\psi = -2$ MPa for 40 days with deficit irrigation. These trees accumulated the highest endogenous level of $\text{NH}_3\text{-NH}_4^+$ (728 $\mu\text{g/g}$ dry wt leaf tissue) and produced the highest number of flowers (611 per tree). One set of trees was subjected to moderate water stress. The trees were maintained at $\psi = -2$ MPa for 50 days by deficit irrigation. Half of the trees received a foliar application of low biuret urea at the rate of 0.1 Kg N as urea per tree. Foliar application of low biuret urea raised the leaf $\text{NH}_3\text{-NH}_4^+$ content of the trees to 863 $\mu\text{g/g}$ dry wt and increased the number of flowers per tree 2.6-fold over trees not receiving a foliar application of urea. This degree of floral intensity was not significantly different at the 5% level from the number of flowers produced by trees receiving the maximum stress. For lemon trees induced to flower by water-deficit stress, the average $\text{NH}_3\text{-}$

NH_4^+ content of the leaves during the stress was significantly correlated with the number of flowers per tree ($p < 0.05$; $r = 0.56$).

From the results of our work, a simple linear regression model was developed to predict the influence of leaf starch and $\text{NH}_3\text{-NH}_4^+$ content on flower production in *Citrus*. The model predicts that 66 new floral shoots will be initiated for each increase in 10 mg starch per g fr wt youngest, fully expanded leaf tissue during the first week after transfer of the trees to the warmer temperature ($p < 0.0001$; $r = 0.75$). The model predicts 208 new flowers will be produced for each increase in 100 μg $\text{NH}_3\text{-NH}_4^+$ per g dry wt youngest, fully expanded leaf tissue during the first week after transfer to the warmer temperature ($p < 0.0001$; $r = 0.8$).

Consistent with the broadest interpretation of the Kraus-Kraybill Hypothesis, the results of our research provide evidence that carbohydrate, specifically starch, and nitrogen, specifically $\text{NH}_3\text{-NH}_4^+$, do influence the number of floral shoots and total number of flowers initiated. Our data, however, do not support a role for a carbohydrate-to-nitrogen ratio in flower initiation and demonstrate that only specific metabolic forms of carbohydrate (starch) and nitrogen ($\text{NH}_3\text{-NH}_4^+$) influence flower number, while other metabolic species do not. Glucose, total N, and NO_3^- do not influence flower initiation. Starch and $\text{NH}_3\text{-NH}_4^+$ appear to influence flowering independently of each other, except when one or the other is limiting to floral initiation.

I propose that starch and $\text{NH}_3\text{-NH}_4^+$ do not influence flower initiation directly, but serve as substrates for the synthesis of key metabolites that act alone or through plant hormones at the genetic level to initiate the flowering process. Studies to elucidate the pathways of starch and $\text{NH}_3\text{-NH}_4^+$ metabolism critical to flowering

are under way in my lab. We are focusing on metabolic pathways which exhibit accelerated activity in response to stress and, specifically, in response to the accumulation of $\text{NH}_3\text{-NH}_4^+$. Thus, at present we are focusing on arginine and polyamine metabolism. The activity of the pathway for the *de novo* biosynthesis of arginine measured in youngest, fully expanded leaves during the first week after transfer of the trees from the low-temperature treatment to the warm temperature increased with the length of the low temperature treatment as follows: 0 weeks (warm temperature control), 4, 6, and 8 weeks of low temperature treatment gave rates of arginine biosynthesis of 5, 7, 11, and 27 nmoles $\text{NaH}^{14}\text{CO}_3$ incorporated into the guanido carbon of arginine per g fr wt leaf tissue during a 3-h incubation period, respectively.

For convenience and for development of commercial analyses in the future, we have monitored changes in carbohydrate and nitrogen metabolism in leaves, specifically the youngest, fully expanded leaves to avoid the secondary effects of leaf senescence and mineral nutrient deficiencies. Previous work in my lab has demonstrated that senescence and mineral nutrient deficiencies cause $\text{NH}_3\text{-NH}_4^+$ to accumulate in leaves. Whether the leaves contribute to flower initiation or simply reflect parallel changes occurring in the buds remains to be determined and is the subject of current research in my lab. In either case, leaf starch and $\text{NH}_3\text{-NH}_4^+$ concentrations may prove useful in monitoring flower initiation in *Citrus*.

While the Kraus-Kraybill Hypothesis may require modification, as it did in the past, to accommodate the discovery of plant hormones, it is a concept that may still prove useful in the study of flowering in *Citrus*.

Finally, the results of this research demonstrate that foliarly applied low biuret urea can raise the tree $\text{NH}_3\text{-NH}_4^+$ status and increase flowering suggesting that foliar applied urea can be used as a tool to manipulate floral intensity in *Citrus* species.

For example, in areas or years where there is inadequate chilling or water-deficit stress prior to spring bloom, urea could be applied to raise the $\text{NH}_3\text{-NH}_4^+$ status of the orchard and improve flowering. We are doing this in California to augment low temperatures that precede spring bloom. A former student of mine, Etienne Rabe, is using foliar applied urea in South Africa to improve spring flowering in the navel orange and several cultivars of easy-peelers in areas where spring bloom is in response to water-deficit stress.

Second, as I described to you, in California Joe Maranto and Kater Hake are using deficit irrigation to produce a summer lemon. Previously, trees were stressed to -3 MPa. This meant that some trees were stressed to as much as -4 MPa which can cause them to collapse, especially if there is *Phytophthora* spp. present. Some soils just do not dry out well enough to take the trees -3 MPa or the orchard dries out unevenly. The use of deficit irrigation to maintain trees at a moderate stress of -2 MPa plus a foliar application of low biuret urea results in floral intensity equal to trees receiving the maximum stress of -3 MPa. Thus, deficit irrigation combined with foliar application of low biuret urea extends the types of soils and climates under which you can the *Verdelli* practice and gives much more uniform results through the field and from year to year.

In addition to influencing flowering, fruit set is another practical problem, especially with regard to shy bearing varieties, like the 'Washington' navel orange,

'Shamouti' orange, and varieties of mandarins, that we feel might be influenced by changes in $\text{NH}_3\text{-NH}_4^+$, arginine, and polyamine metabolism. Thus, we feel that fruit set could be improved by foliar applied low biuret urea. Our preliminary results suggest that $\text{NH}_3\text{-NH}_4^+$ or its metabolite(s) could influence fruit set in two ways.

The 'Washington' navel orange is considered a shy bearer. Under California conditions, fruit set in this variety is extremely low. Erickson and Brannaman in 1960 (The Citrus Industry, Vol. I, University of California Press) collected every flower and fruit that abscised from spring bloom through harvest of 'Washington' navel orange trees. They then counted the number of fruit harvested. Fruit set was 0.2%.

The 'Washington' navel orange produces inflorescences which have from one to many flowers with zero to many leaves. In a typical spring bloom in California, the number of flowers borne on leafy inflorescences, *i.e.*, those inflorescences with more than one leaf per flower, is a small percentage of the total number of flowers produced by the 'Washington' navel orange. Flowers borne on leafless inflorescences, *i.e.*, those with less than one leaf per flower, usually comprise 50% or more of the total flower population. Leafy inflorescences exhibit a higher percentage of fruit set and contribute a disproportionately high number of fruit to harvest. For a recent spring bloom in Riverside, California, flowers borne on leafy inflorescences represented only 16% of the total flower population on the trees, but contributed 60% of the harvested fruit. Flowers borne on leafless inflorescences comprised 60% of the total flower population, yet contributed only 15% of the harvested fruit.

Our preliminary results suggest that urea may prove beneficial in increasing the number of leafy inflorescences and number of leaves per inflorescence. When five-year-old rooted cuttings of the 'Washington' navel orange were induced to flower by 6 weeks of low temperature stress and were also given a foliar application of low biuret urea at the rate of 1.5 g per tree, the number of leafless inflorescences doubled. However, the number of leafy inflorescences increased four-fold bringing the number of leafy inflorescences from a value one-half the total flower population to two-thirds of the total population. Thus, foliar application of low biuret urea caused a disproportionately greater increase in leafy inflorescences than leafless inflorescences. In addition, the foliar application of low biuret urea increased the average number of leaves per inflorescence four-fold. This is the first way in which we believe foliar applied urea can improve fruit set in the 'Washington' navel orange and other shy-bearing varieties through increased production of leafy inflorescence and greater number of leaves per inflorescence.

Fruit that set and persist on the tree until harvest are faster-growing. Slower-growing fruit tend to abscise early in their development. Note that the fruit actually stop growing one week before they abscise. The diameters of the fruit are not significantly different at petal fall. But, one week after petal fall, growth rates of the fruit are significantly different at the 5% level.

Most studies on fruit set have emphasized changes in hormone content and carbohydrate allocation. Since we have just demonstrated that flower initiation is influenced by $\text{NH}_3\text{-NH}_4^+$ or one of its metabolite(s), our research examined changes in the metabolism of key nitrogen compounds as possible factors influencing flower development, including development of the ovary.

Of additional interest to us was the question of whether arginine might play a role in flower and ovary development in citrus. Previous work in my lab demonstrated that the activity of the pathway for the *de novo* biosynthesis of arginine was accelerated in response to increased $\text{NH}_3\text{-NH}_4^+$ content of leaves of several species and hybrids of citrus (Rabe and Lovatt, 1986, *Plant Physiol* 81:774-779). Arginine is the predominant precursor of polyamines synthesized in plants. Polyamines are a class of nitrogen compounds that accumulate during stress and a class of nitrogen compounds that are being recognized as important factors in cell division and organogenesis. For example, in tomato, fruit growth was reduced by inhibition of polyamine synthesis. Resupplying polyamines restored normal fruit development.

The current study was undertaken: (1) to determine if flower initiation by low temperature stress results in elevated levels of $\text{NH}_3\text{-NH}_4^+$ in developing flowers of the 'Washington' navel orange; (2) to define the relationship between the pool size of available $\text{NH}_3\text{-NH}_4^+$, the activity of the *de novo* arginine biosynthetic pathway, and tissue concentrations of polyamines; and (3) to determine whether there is a difference in the metabolism of these key nitrogen compounds in flowers borne on leafy versus leafless inflorescences and in flowers borne in apical versus subapical positions. The apical or terminal flower of an inflorescence is precocious and exerts dominance over the flower buds below, retarding their development. The flower in the subapical position, the one just below the terminal flower, is the most delayed in its development. There is a controversy in the literature as to which sets more fruit.

The results I will now present are those of Dr. Oded Sagee, who was a BARD Postdoctoral Fellow in my laboratory during 1987 to 1988.

In the apical flower borne on a leafy inflorescence, the highest concentrations of $\text{NH}_3\text{-NH}_4^+$ and putrescine and the greatest rate of *de novo* arginine biosynthesis are all found in the earliest (youngest) stage of flower development. This is consistent with the accumulation of $\text{NH}_3\text{-NH}_4^+$ in flower buds as well as leaves during the low temperature induction of flowers in *Citrus*. Flower $\text{NH}_3\text{-NH}_4^+$ content, *de novo* arginine biosynthesis, and putrescine content all decreased in parallel from Stage I of flower development to Stage V, petal fall. These changes are not the result of dilution as the flower increased in biomass as Stage II and Stage III are the same size and weight, one is a closed flower, the other is an open flower. However, Stage III flowers have significantly ($p < 0.05$) higher levels of $\text{NH}_3\text{-NH}_4^+$, arginine synthesis, and putrescine than Stage IV flowers. Parallel decreases in all three parameters through flower development were also recorded for subapical flowers and for flowers borne on leafless inflorescences.

To determine whether $\text{NH}_3\text{-NH}_4^+$ availability influences amino acid metabolism in general, or only the synthesis of specific amino acids, arginine in particular, Dr. Sagee also quantified changes in the activity of the shikimic acid pathway for the *de novo* biosynthesis of tyrosine. The activity of the tyrosine biosynthetic pathway in developing flowers changes in a manner that is totally different from that of the arginine biosynthetic pathway. This suggests that the parallel changes in flower $\text{NH}_3\text{-NH}_4^+$ content, arginine biosynthesis, and polyamine content are causally related.

Now, we will take a look at the influence of flower position and inflorescence type on the metabolism of these key nitrogen compounds. During early flower development, terminal flowers had higher levels of $\text{NH}_3\text{-NH}_4^+$ than subterminal flowers ($p < 0.05$). For each flower type, the flower borne on the leafy

inflorescence had the higher $\text{NH}_3\text{-NH}_4^+$ content ($p < 0.05$). One week after petal fall, these differences were insignificant at the 5% level. *De novo* arginine biosynthesis followed this same pattern ($p < 0.05$), except one week after petal fall, there was an increase in *de novo* arginine biosynthesis in the fruit. This increase in *de novo* arginine biosynthesis resulted in a marked increase in the putrescine content of the fruit one week after petal fall ($p < 0.05$), with the highest level of putrescine found in flowers borne on leafy inflorescences.

Putrescine is the first polyamine synthesized from arginine. It is subsequently converted to spermidine, which is converted to spermine. If we now look at the sum of these three polyamines, we see the same pattern of change with a dramatic increase in total polyamines at one week after petal fall. Fruit on leafy inflorescences exhibit the greater concentration of total polyamines. Remember that fruit borne on leafy inflorescences exhibit faster-growth rates and a greater potential to set and survive to harvest.

To borrow a concept from the plant pathologists, we must now in essence "prove Koch's postulates" with polyamine metabolism. If polyamines are essential for faster-growing fruit and/or fruit set, we should be able to increase fruit abscission by inhibiting polyamine biosynthesis. Conversely, we should be able to improve fruit set by supplying fruit with polyamines. Our research suggests that we may be able to improve fruit set by improving the polyamine content of the fruit by increasing fruit $\text{NH}_3\text{-NH}_4^+$ content by foliar application of low biuret urea. So, this is the second way we feel foliar application of urea can improve fruit set. While we still have much work ahead of us, our results do provide us with a testable, practical approach for improving fruit set in shy bearing *Citrus* varieties.