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A New Look at the Kraus-Kraybill Hypothesis and Flowering in *Citrus*[1]

CAROL J. LOVATT, YUSHENG ZHENG AND KATER D. HAKE

Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA

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Abstract

Five-year-old rooted cuttings of the 'Washington' navel orange (*Citrus sinensis*) were induced to flower by subjecting the trees to 2, 4, 6, and 8 weeks of low-temperature, 8–h day (500 $\mu\Sigma/m^2$ 16–h sec) at 15–18°C/16–h night at 10–13°C, and then transferring the trees to 12–h day (500 $\mu\Sigma/m^2 \cdot sec$) at 24°C/12–h night at 19°C. In addition, 16-year-old Frost Lisbon lemon trees (*C. limon*) on 'Troyer' citrange (*C. sinensis* x *Poncirus trifoliata*) under commercial production were induced to flower by subjecting the trees to 60 days of water-deficit stress of increasing severity and then re-irrigating. Maximum bloom occurred 4 weeks after removal of either stress. Changes in photosynthesis and leaf concentrations of glucose, starch, nitrate, and ammonia (as the combined pool of NH_3–NH_4^+) were monitored prior to, during, and after the stress treatment. The results provide strong evidence that floral intensity is dependent on both starch and ammonia content, which are easily monitored in leaves. Threshold values for starch and ammonia, critical to maximum flowering in the systems studied, are reported. Evidence demonstrating increased floral intensity by artificially increasing leaf ammonia content by foliar application of low biuret urea is presented. The results are discussed in light of the Kraus-Kraybill hypothesis.

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Introduction

The potential regulation of flower initiation by the balance of carbohydrate to nitrogen or by a precise mathematical ratio of carbohydrate/nitrogen is recognized internationally as the Kraus-Kraybill hypothesis. In taking a new look at the Kraus-Kraybill hypothesis and flowering in *Citrus*, the authors of the present manuscript recognize that the work of Klebs attempting to establish a role for carbohydrate-nitrogen status as a causal agent in flowering preceded, by 14 years, the publication of Kraus and Kraybill's *Vegetation and Reproduction with Special Reference to the Tomato*, which is cited as the source of this idea (1). We also take note of the fact that by today's standards, Kraus and Kraybill's experimental design and analyses were inadequate to establish the hypothesis for which they are internationally known (1). Finally, the authors emphasize 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 and which remains to be proven or disproven.

Flowering in *Citrus* is recurrent under tropical and subtropical conditions (6), unless synchronized into a well-defined period of concentrated bloom by external conditions (2, 5, 7). Flower formation in *Citrus* species is promoted by drought or low temperature, followed by restoration of climatic conditions favourable for growth (4–8, 12). Thus, stress applied in a quantitative manner provides a controlled system during which changes in carbohydrate/nitrogen ratio can be monitored in relation to flower initiation.

In this communication, we present data on the relationships between the concentrations of carbohydrate and nitrogen species in young, fully expanded leaves and floral intensity 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 growth 16–year–old Frost Lisbon lemon trees on 'Troyer' citrange rootstock. Some of the data on stress-induced flowering and NH_3–NH_4^+ accumulation has been published previously (4), but is included in this manuscript to provide the basic information necessary to justify a new look at the Kraus-Kraybill hypothesis.

Materials and Methods

Low-temperature stress

Five-year-old rooted cuttings of the 'Washington' navel orange (*Citrus sinensis* L. Osbeck) grown in pots containing ca. 19 litres of University of California soil mix were induced to flower by subjecting the trees to low temperature, 8–h day (500 $\mu\Sigma/m^2 \cdot sec$) at 15–18°C/16–h night at 10–13°C for 4, 6, or 8 weeks, and then transferring the trees to 12–h day (500 $\mu\Sigma/m^2 \cdot sec$) at 24°C/12–h night at 19°C (9).

Control trees were maintained under the warmer conditions described above for the total length of the experiment. Ten trees comprised each treatment. Trees were watered once a week with half-strength Hoagland's nutrient solution, and as needed with H_2O. The average predawn water potential of the 5–year–old rooted cuttings of the 'Washington' navel orange over 8 weeks low-temperature treatment was the same as for the warm-temperature control trees: –0.7 ± 0.2 MPa. Urea-treated trees received one foliar application of low biuret urea at the rate of 1.5 g urea per tree at the time of transfer to the warmer temperature. Maximum bloom occurred 4 weeks after transfer to the warmer temperature.

Water-deficit stress

Sixteen-year-old trees of Frost Lisbon lemon (*Citrus limon* L. Burm. f.) on 'Troyer' citrange rootstock (*C. sinensis* x *Poncirus trifoliata* L. Raf.) under commercial production in the San Joaquin Valley, California, were treated as follows beginning mid-June: (1) well-watered control (–0.5 MPa); (2) water was withheld, trees were stressed to less than –3 MPa over 20 days and maintained at less than –2 MPa for 40 additional days, and then rewatered quickly; (3) water was withheld, trees were stressed to ca. –3 MPa over 30 days and rewatered quickly; (4) water was withheld, trees were stressed to ca. –2 MPa in 10 days and then irrigated for 50 days at 25% the rate of the well-watered control trees and then rewatered quickly; and (5) same as treatment 4, with foliar application of the low biuret urea at the rate of 0.1 kg N per tree at the end of the 10 days without H_2O. The experiment employed single tree replicates, four trees per treatment. Maximum bloom occurred 4 weeks after removal of the stress.

Physiological parameters

Leaf glucose and starch concentrations were monitored weekly according to the method of Hamid *et al.* (3). Leaf total N, NO_3^-, and NH_3–NH_4^+ concentrations were monitored weekly according to the method of Rabe and Lovatt (11); tree water status was monitored as predawn water potential by pressure bomb; total flower number per tree was determined by counting every flower on each tree. For the 16–year–old trees, counts were made in parallel vertical sectors from the ground to a height of 2 m. This number was confirmed by calculating total flower number from the percentage of fruit set per total flowers borne on tagged branches (10 per tree) and the total number of fruit harvested per tree.

Results and Discussion

Results of experiments employing low-temperature stress to induce flowering in 5-year-old rooted cuttings of the 'Washington' navel orange demonstrated that floral intensity (flower number) increased with the increased duration of the low-

temperature treatment ($p < 0.01$) (4; Table 1). A positive correlation between the duration of the low-temperature treatment and the number of floral shoots initiated by 1– to 2-year-old 'Tahiti' lime trees (*Citrus latifolia* Tan.) was reported previously (12).

Table 1: Effect of low temperature, 8–h day (500 $\mu\Sigma/m^2$-sec) at 15–18°C/16–h night at 10–13°C, on leaf NH_3–NH_4^+ content and flowering of the 'Washington' navel orange

Duration of low-temperature stress (weeks)	Leaf NH_3–NH_4^+ content at the end of the low-temperature treatment (µg/g dry wt)	Flower number
0 – warm temperature control	456 a	6 ± 9 a
4	559 b	117 ± 70 b
6	583 b	131 ± 42 b
8	672 c	347 ±147 c

Values within a column followed by a different letter are significantly different at $p < 0.05$. Derived from Lovatt *et al.* (4).

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 of the leaves was negative, but not significant (Table 2). 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 induction. For glucose, this concentration was approximately 1.25 µg per g fr wt leaf tissue. Leaf starch concentration for the more than 100 trees used in the experiments were highly variable. Values ranged from 1.4 to 150 mg per g fr wt 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 trees from all treatments provided evidence that the starch content of the youngest, fully expanded leaves during the first week after the trees were transferred from low-temperature induction to the warmer temperature correlated significantly with the number of floral shoots initiated ($p < 0.0001$) (Table 2). This observation, taken in light of the observation that there is no correlation between leaf starch content at any point in the induction period or during the subsequent bloom period and the number of vegetative shoots initiated (Table 2), provides strong evidence that leaf starch content is not simply increasing budbreak and total shoot production, but is, instead, an important factor influencing flower initiation. If starch was required simply to provide an energy source for shoot

production, then the number of vegetative shoots would also have increased as leaf starch content increased; it did not.

Table 2: Linear regression analyses of stress, starch, NH_3–NH_4^+, and flowering in *Citrus* spp.

Stress	Independent variable x	Dependent variable y	Probability p	Coefficient of linear correlation r
Low-temperature[z]	Duration of stress	Starch	NS[y]	–0.186
	Starch	Floral shoots per tree	$p < 0.0001$	0.750
	Starch	Vegetative shoots per tree	NS[y]	0.212
	Duration of stress	NH_3–NH_4^+	$p < 0.01$	0.605
	NH_3–NH_4^+	Flowers per tree	$p < 0.0001$	0.803
	NH_3–NH_4^+	Floral shoots per tree	$p < 0.01$	0.413
	NH_3–NH_4^+	Vegetative shoots per tree	NS[y]	0.077
Water-deficit[x]	NH_3–NH_4^+	Flowers per tree	$p < 0.05$	0.560

[z] = Starch (mg/g fr wt) and NH_3–NH_4^+ (µg/g dry wt) leaf tissue collected at the end of the first week in the warmer temperature after completion of the low-temperature treatment.
[y] = Not significant at $p < 0.10$.
[x] = Average leaf NH_3–NH_4^+ concentration (µg/g dry wt leaf tissue) during the stress period.

Total N in the youngest, fully expanded leaves of the 'Washington' navel orange did not change during 8 weeks of low-temperature stress. Total N averaged 25.5 ± 3.8 mg per g dry wt leaf tissue (x ± STD. DEV, N = 15) from time of transfer to the lower temperature through 8 weeks of low-temperature treatment and through a subsequent 7 weeks at the warmer temperature. Leaf NO_3^- concentration was not affected by low-temperature stress and remained at a level ca. 3% of total N level over the same period.

In contrast, NH_3–NH_4^+ accumulated in the youngest, fully expanded leaves of the 5-year-old rooted cuttings of 'Washington' navel orange in a manner that paralleled the duration of the stress ($p < 0.01$) (Table 2). Thus, of all the parameters measured, only the leaf concentration of NH_3–NH_4^+ changed in a manner that paralleled both the duration of the stress and flower number ($p < 0.0001$) (Table 2).

To provide additional evidence supporting a role for ammonia in flower initiation in the 'Washington' navel orange, the rooted cuttings were subjected to minimal low-temperature treatments of only 4 or 6 weeks. For half of the trees, the leaf NH_3–NH_4^+ content was increased artificially by foliar application of low biuret urea. The application of urea significantly increased the leaf concentration of NH_3–NH_4^+ and the number of flowers produced by the shorter induction treatments (4; Table 3).

Table 3: Effect of foliar urea (1.5 g per tree) applied at the end of the low-temperature treatment (see Table 1) on leaf NH_3–NH_4^+ content and klowering of the 'Washington' navel orange[z]

Duration of low temperature stress (weeks)	Increase in leaf NH_3–NH_4^+ content during the first week after transfer to the warm temperature as a percent of the value without urea for each treatment presented in Table 1	Increase in flower number as a percent of the value without urea for each treatment presented in Table 1
4	166%	194%
6	215%	230%
8	134%	126%

[z] = Derived from Lovatt et al. (4).

Floral shoot number was significantly correlated with the concentration of NH_3–NH_4^+ in the youngest, fully expanded leaves during the first week after transfer to the warmer-temperature treatment ($p < 0.01$) (Table 2). Total flower number and flower number per floral shoot were also significantly correlated with leaf NH_3–NH_4^+ content of the youngest, fully expanded leaves during the first week after transfer to the warmer temperature ($p < 0.0001$) (Table 2). The number of vegetative shoots was not influenced by NH_3–NH_4^+ accumulation (Table 2). NH_3–NH_4^+ concentrations, at this time, varied from 389 to 2636 µg per g dry wt for more than 100 trees used in the experiments (includes trees receiving urea sprays). For these same trees, flower number per tree ranged from 4 to 3065.

Results of experiments employing water-deficit stress to induce flowering in 16-year-old Frost Lisbon lemons, under commercial production, provided additional evidence supporting a role for ammonia-ammonium in flower initiation in *Citrus*

species. The NH_3–NH_4^+ content of leaves of the commercial lemon trees increased 209 µg per g dry wt leaf tissue for those trees subjected to the most severe stress treatment. These trees had predawn water potentials as low as –3 MPa within 20 days of withholding H_2O, followed by 40 days at ca. –2 MPa. Trees subjected to severe stress, but only for a short duration, i.e., stressed to –3 MPa within 30 days and rewatered, and trees subjected to moderate stress (–2 MPa) over the 60-day period, had leaf levels of NH_3–NH_4^+ that exceeded the NH_3–NH_4^+ content of the well-watered control trees by 127 and 164 µg per g dry weight leaf tissue, respectively (4; Table 4). The average leaf concentration of NH_3–NH_4^+ over the stress period was significantly correlated with the number of flowers per tree ($p < 0.05$) (Table 2).

Table 4: Effect of water-deficit stress and foliar urea on leaf NH_3–NH_4^+ content and flowering of lemon[z]

Treatment	Number of flowers	Average leaf NH_3–NH_4^+ content during stress (µg/g dry wt)
Control —no water stress ($\Psi > -1$ MPa)	14 b	519 c
Severe water stress of short duration ($\Psi < -3$ MPa in 30 days)	53 b	646 b
Severe water stress ($\Psi < -3$MPa in 20 days) followed by moderate water stress ($\Psi < -2$ MPa for 40 days)	611 a	728 b
Moderate water stress ($\Psi < -2$ MPa for 50 days)	165 b	683 b
Moderate water stress ($\Psi < -2$ MPa for 50 days) with foliar urea (0.1 kg nitrogen per tree)	426 a	863 a

[z] = Values within a column followed by a different letter are significantly different at $p < 0.05$ by Duncan's Multiple Range Test. Reproduced from Lovatt et al. (4).

In addition, foliar application of low biuret urea to trees maintained under minimal water-deficit stress (–2 MPa) by deficit irrigation increased the number of flowers per tree 2.6-fold ($p < 0.05$) (4; Table 4).

Conclusions

Consistent with the broadest interpretation of the Kraus-Kraybill hypothesis, the results of our research provide evidence that the carbohydrate (starch) and nitrogen (NH_3–NH_4^+) status of the tree does 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 (NH_3–NH_4^+) influence flower number, while other metabolic species do not (glucose, total N, and NO_3^-). Starch and NH_3–NH_4^+ appear to influence flowering independently of each other, except when one or the other is limiting to floral initiation.

We propose that starch and NH_3–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 NH_3–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 ammonia-ammonium.

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 (10, 11). 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 future research in my lab. In either case, leaf starch and NH_3–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*.

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