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Ammonia and/or Its Metabolites Influence Flowering, Fruit Set, and Yield of the 'Washington' Navel Orange

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Abstract. **The hypothesis that key nitrogen compounds that influence flower initiation in *Citrus sinensis* (L.) Osbeck may be important in the formation, development, and subsequent set of the ovary is supported by our past and current research. Flower formation in *Citrus* is promoted by drought or low temperature. For 5-year-old rooted cuttings of the 'Washington' navel orange subjected to low temperature, leaf ammonia content increased in a manner that paralleled the duration of the stress and was significantly correlated with flower number. A cause and effect relationship between tree ammonia status and floral intensity was established. Artificially raising the ammonia content of the trees by foliar application of low-biuret urea at the end of a minimal stress treatment, increased leaf ammonia content, and both the number of floral shoots and flowers per shoot but did not influence the number of vegetative shoots produced. Maximum leaf ammonia content and activity of the de novo arginine biosynthetic pathway occurred during the first week after the stress treatment. Apical flowers initiated in response to stress also exhibited maximum tissue concentrations of ammonia and putrescine and maximum activity of the de novo arginine biosynthetic pathway 1 week after the end of the stress treatment. All three criteria decreased in parallel as flowers developed through petal fall. All three criteria were greater in flowers borne in the terminal position of leafy inflorescences than on leafless inflorescences. During the second week after petal fall, fruit borne in the terminal position of leafy inflorescences had significantly greater total polyamine content, faster growth rates, and exhibited a greater percent fruit set than fruit borne in the terminal position of leafless inflorescences. Winter applications of foliar urea to commercially-producing, nitrogen-sufficient 'Washington' navel orange trees just prior to or during flower initiation increased yield ($p \leq 0.05$) in three successive years without a reduction in fruit size.**

Citrus growers are not unique among the producers of tree crops in desiring to increase profits by increasing yield per tree without reducing fruit size to one of little economic value. However, increasing fruit number while maintaining a marketable fruit size is not a trivial pursuit.

Fruit number can be increased indirectly by increasing floral intensity to augment total set, or directly by improving percent set. In *Citrus* species, only a fraction of the shoot apices flower; others continue the vegetative growth of the tree. Thus, to increase flower number, it is necessary to shift the apical meristem of a vegetative shoot within a resting bud to a floral apex or to prevent the redirection of the floral apex of an inflorescence resting bud to vegetative growth. The shoot apex determines the fate of the lateral meristems, which always show retarded expression with respect to the apex (Lord and Eckard, 1987). If the apex is floral, the laterals will form flowers; if the apex is vegetative, the laterals will be diverted to thorns.

In our laboratory, we are investigating the hypothesis that endogenous conditions optimal for the promotion of flower formation, which includes formation of the ovary, are also optimal for early ovary growth and, thus, are prerequisite for fruit set and maximum fruit size. Our investigation includes examination of the roles of nitrogen compounds, carbohydrates, and hormones in flower initiation, fruit set, and fruit growth.

In this communication, results of past and current research on nitrogen metabolism are presented demonstrating that ammonia and/or its metabolites increase flowering, fruit set, and yield of the 'Washington' navel orange in a manner fully consistent with our hypothesis.

Materials and Methods

Chemicals. All radiolabeled chemicals were purchased from ICN Pharmaceuticals, Irvine, CA. Liquiscint (liquid scintillation cocktail) was purchased from National Diagnostics, Somerville, NJ. Mineral salts for Hoagland's and Shive's nutrient solutions were of analytical reagent quality from Fisher Scientific, Pittsburgh, PA. All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Plant material. Five-year-old rooted cuttings of the 'Washington' navel orange grown in pots containing about 19 liters of University of California soil mix were induced to flower by subjecting the trees to low temperature, 8-hr days (500 $\mu mol\text{-}s^{-1}\text{-}m^{-2}$) at 15 to 18°C/16-hr nights at 10 to 13°C for 8 weeks, and then transferring the trees to 12-hr days (500 $\mu mol\text{-}s^{-1}\text{-}m^{-2}$) at 24°C/12-hr nights at 19°C (Moss, 1969). Trees were watered once a week with half-strength Hoagland's nutrient solution and as needed with H_2O. Flowers at various stages of development borne at the apex of inflorescences were harvested weekly according to the criteria of Goldschmidt et al. (1980) and Sagee and Lovatt (1991). In addition, to reduce biological variation, a size criterion was also employed in selecting flowers at each developmental stage (Sagee and Lovatt, 1991).

Developing flowers were collected from Stage I (7 x 5 mm, length x width) through Stage V, petal fall and every 3 days thereafter for two weeks. At each harvest, fresh weight and protein content (Bradford, 1976) were determined for the flowers

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used in the assays described below.

Thirty-year-old 'Washington' navel orange trees on Troyer citrange rootstock under commercial production at the Agricultural Experiment Station of the University of California, Riverside, received a foliar-application of nitrogen on 14 November, 14 December, 14 January, or 14 February. The foliar-application of nitrogen consisted of 0.17 kg N as urea per tree (Unocal PLUS® zero-biuret urea donated by the Unocal Corporation) applied at the rate of 2.1 kg N/378.5 liters sprayed to the drip point (approx. 28.4 liters per tree) to 12 individual tree replicates per treatment to make it possible to detect differences in yield at the 5% level if such a difference existed (Jones et al., 1957). All trees received 0.5 kg N per tree as urea applied to the soil each year in November or December.

Fruit were harvested 23-25 January, 1990, 4-5 February, 1991, and 25-27 February, 1992. Yield as total fruit weight per tree and total fruit number per tree was determined. Fruit size distribution per tree was determined on the basis of 75 randomly chosen fruit per tree (Hamid et al., 1988).

Total nitrogen, NH_3-NH_4^+ and polyamine content. Five-month-old, spring-cycle leaves were collected from non-fruiting terminals in September, washed with soapy water and rinsed thoroughly with distilled H_2O. The leaves were oven dried at 60°C and ground with a Wiley mill to a size fine enough to pass through a 40-mesh screen. Total N was determined for a 25-mg sample using the conventional micro-Kjeldahl method.

Young, fully expanded leaves were immediately frozen in liquid N, lyophilized, and ground with a Wiley mill to a size fine enough to pass through a 40-mesh screen. Leaf NH_3-NH_4^+ per g dry weight was determined for a 200-mg sample extracted in 50 ml of 1 N KCl using a technicon autoanalyzer (Technicon, 1978).

Excised young, fully expanded leaves or flowers (1 g fresh weight) were immediately homogenized in 5 ml 10% trichloroacetic acid (TCA) using a Polytron tissue homogenizer (PCU-2, Brinkman Instruments) at speed 6. The probe was rinsed with 5 ml 10% TCA, which was added to the homogenate. The homogenate was centrifuged at 10,000*g* at 4°C for 10 min. The NH_4^+ content of the acid soluble supernatant fraction, containing the combined pool of NH_3-NH_4^+ as NH_4^+, was determined using a Wescan Ammonia Analyzer (Carlson, 1978). The assay was linear for NH_4^+ concentrations from 0 to 100 $\mu g \cdot ml^{-1}$. Samples were diluted to give values in this range.

The free polyamine content of the supernatant fraction was determined after benzoylation by reverse phase HPLC at room temperature through a 4.6 x 250 mm, 5-µm particle size C18 column (octadecylsilane from Alltech, Deerfield, IL) eluted with 60% methanol at a flow rate of 1 $ml \cdot min^{-1}$ (Flores and Galston, 1982). The benzoylpolyamines were detected at 254 nm at a sensitivity of 0.04 absorbance units full scale. The assay was linear for concentrations of putrescine, spermidine and spermine from 0.01 to 1 nmol. Benzoylation permitted recovery of 76%±11% of known amounts of putrescine, spermidine or spermine added to the citrus flower extracts.

Incorporation of $NaH^{14}CO_3$ into the combined pool of arginine plus urea. Activity of the *de novo* arginine biosynthetic pathway was assessed in the intact cells of leaves or developing flowers (500 mg fresh weight) by measuring the incorporation of radiolabeled carbon supplied as $NaH^{14}CO_3$ [5 mM, 37.5 µCi (1 Ci = 37 GBq)] into the combined pool of arginine plus urea during a 3-hr incubation period in Shive's nutrient solution. The amounts of [guanido-^{14}C]arginine and [^{14}C]urea synthesized from $NaH^{14}CO_3$ by leaves or flowers were determined using commercial arginase and urease as described by Lovatt and Cheng (1984).

Results and Discussion

Evidence that flower formation in *Citrus sinensis* is regulated by ammonia and/or its metabolites was provided by experiments employing low temperature stress to induce flowering in 5-year-old rooted cuttings of the 'Washington' navel orange. The results of these experiments demonstrated that: (i) NH_3-NH_4^+ accumulated in young, fully expanded leaves in a manner that paralleled the duration of stress ($p \leq 0.01$) (Lovatt et al., 1988a, 1988b) (Table 1); (ii) inflorescence number was significantly correlated with the concentration of NH_3-NH_4^+ in young, fully expanded leaves during the first week after transfer of the trees from the low-temperature stress treatment to the warm-temperature control conditions ($p \leq 0.01$) (Lovatt et al., 1988b) (Table 1); (iii) the number of lateral flowers per inflorescence increased with tree ammonia status ($p \leq 0.0001$) (Lovatt et al., 1988b) (Table 1); (iv) vegetative shoot number was not influenced by ammonia accumulation during stress at the 5% significance level (Lovatt et al., 1988b) (Table 1); and (v) foliar application of low biuret urea at the end of the low-temperature stress treatment increased leaf NH_3-NH_4^+ content with a concomitant and equal increase in floral intensity but had no consistent effect on vegetative shoot production (Lovatt et al., 1988a, 1988b) (Table 1). Taken together, the results suggest that ammonia, or its metabolite(s), may be a factor regulating flower formation in *C. sinensis*.

Flower induction by low-temperature stress resulted in paral-

Table 1. Effect of low temperature stress and foliar application of low biuret urea on leaf NH_3-NH_4^+ content, flowering and vegetative shoot production of the 'Washington' navel orange.

Duration of low temperature stress (weeks)	Leaf NH_3-NH_4^+ content[z] ($\mu g \cdot g^{-1}$ dry weight)		Average number of flowers per tree		Average number of vegetative shoots produced per tree	
	without urea[y]	with urea[x]	without urea[y]	with urea[x]	without urea[y]	with urea[x]
0	456 a	-	6 a	-		
4	559 b	166%	117 b	194%	31 a	49%
6	583 b	215%	131 b	230%	112 b	46%
8	672 c	134%	347 c	126%	55 a	112%

[z]Determined during the first week after transfer to the warm-temperature control conditions.

[y]Values within a column followed by different letters are significantly different at $p \leq 0.05$ by Duncan's Multiple Range Test.

[x]Low biuret urea was applied at the rate of 1.5 g per tree at the end of the low-temperature stress treatment. Figures represent percentages of the values recorded for trees to which no urea was applied. Derived from Lovatt et al. (1988a, 1988b).

lel accumulation of ammonia in developing flower buds and citrus leaves. The highest concentration of NH_3-NH_4^+ was observed for flowers at the youngest stage of development (Stage I) (356±27 $\mu g \cdot g^{-1}$ fresh weight) ($p \leq 0.05$) and for young, fully expanded leaves (867±166 $\mu g \cdot g^{-1}$ fresh weight) both collected one week after transfer of the trees from the low-temperature stress treatment to the warm-temperature control conditions (Sagee and Lovatt, 1991) (Table 2). Thereafter, for both flowers and leaves, tissue concentrations of NH_3-NH_4^+ declined with length of time in the warm-temperature control conditions (Table 2).

Evidence that flower NH_3-NH_4^+ content and putrescine synthesis via arginine are metabolically linked during navel orange flower development was provided by the results of experiments demonstrating that these three parameters decreased in parallel from Stage I through Stage V of flower development in a manner that was not related to changes in flower fresh weight (Table 2) or protein content (data not shown) (Sagee and Lovatt, 1991).

In *Citrus*, it is well known that leafy inflorescences (shoots bearing leaves and flowers) set more fruit than leafless inflorescences (for a recent review, see Erner, 1989). In addition, young fruit that are faster-growing have a greater potential to set and survive to harvest than slower-growing fruit (Zucconi et al., 1978).

Table 2. Content of ammonia and its metabolites in leaves and flowers after transfer of 'Washington' navel orange trees from the low-temperature stress treatment to the warm-temperature control conditions.

	Weeks after transfer[zx]			
Leaves	1	2	3	4
	μg NH_3-$NH_4^+ \cdot g^{-1}$ fresh weight			
	867 a	808 a	657 b	500 c
	nmol $NaH^{14}CO_3$ incorporated into arginine + urea $\cdot g^{-1}$ fresh weight $\cdot$ 3 h			
	28 a	24 a	11 b	7 c
	Stage of flower development[y,x]			
Flowers	I	II	III	IV	V
	μg NH_3-$NH_4^+ \cdot g^{-1}$ fresh weight				
	356 a	285 b	232 c	155 d	87 e
	nmol $NaH^{14}CO_3$ incorporated into arginine + urea $\cdot g^{-1}$ fresh weight $\cdot$ 3 h				
	22 a	20 b	17 c	12 d	7 e
	nmol putrescine $\cdot g^{-1}$ fresh weight				
	466 a	334 b	240 c	182 cd	134 d
	mg fresh weight $\cdot$ flower^{-1}				
	106 e	174 d	382 a	267 b	217 c

[z]Data are the mean for three separate experiments that induced flowering by low-temperature stress.

[y]Data are the mean for four replicates from two separate experiments inducing flowering by low-temperature stress. Stage I flowers were collected one week after transfer of the trees from the low-temperature stress treatment to the warm-temperature control conditions.

[x]Mean separation was by Duncan's Multiple Range Test. Values within a horizontal row followed by different letters are significantly different at $p \leq 0.05$. Derived from Sagee and Lovatt (1991).

Early in their development (Stage I), apical flowers borne on leafy inflorescences had higher tissue levels of polyamines (Table 3) and NH_3-NH_4^+ and higher rates of de novo arginine biosynthesis (data not shown) than apical flowers borne on leafless inflorescences. Navel orange fruit exhibiting faster growth rates during the first weeks after petal fall were borne on leafy inflorescences and had higher levels of polyamines than slower-growing fruit borne on leafless inflorescences (Table 4).

We tested our hypothesis in a field experiment employing 30-year-old 'Washington' navel orange trees on Troyer citrange rootstock to determine whether a winter foliar-application of low biuret urea at the rate of 0.17 kg per tree prior to or during the normal period of floral initiation preceding spring bloom of *Citrus* in California would increase floral intensity and result in increased fruit set and yield.

The trees employed in this study have a history of average fruit yields ranging from 500 to 700 fruit per tree in an "off" year to 1,300 fruit per tree in an "on" year. These yields are equal to the state average. During the first year of our study, the trees yielded close to the average for Southern California. The 1989-90 season shipments of navel oranges from Southern California averaged 655 cartons per tree (Source: Navel Orange Administrative Committee). The control trees in our experiment, i.e. those under standard commercial management and receiving soil-applied urea, averaged 689 cartons per acre for the 1989-90 season. All trees were nitrogen-sufficient according to September leaf analyses conducted at the start of the experiment.

In year one, all trees receiving a winter foliar application of low biuret urea had significantly higher yield per tree than trees receiving soil-applied urea ($p \leq 0.05$) (Table 5). The increase in yield was just over one packing carton (17 kg) per tree.

The results of the second year of our research were compromised by the freeze which occurred in December, 1990. Yield of

Table 3. Polyamine content of Stage I apical flowers of leafy versus leafless inflorescences[z].

	Leafy	Leafless
	mg $\cdot$ flower^{-1}	
Flower fresh weight	99 a	74 b
	nmol $\cdot g^{-1}$ fresh weight	
Putrescine	472	231
Spermidine	170	149
Spermine	31	19
Σ	673 a	401 b
	mg $\cdot g^{-1}$ fresh weight	
Flower protein content	19 a	12 a
	nmol $\cdot$ mg^{-1} protein	
Putrescine	26	14
Spermidine	9	8
Spermine	1	1
Σ	36 a	23 b

[z]Data 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 for 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 $p \leq 0.05$.

Table 4. Characteristics of fruit borne on leafy versus leafless inflorescences during the second week after petal fall.[z]

	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	186
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^{-1}	
Putrescine	144	55
Spermidine	49	22
Spermine	23	12
Σ	216 a	89 b

[z]Data 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 $p \leq 0.05$.

the control trees, i.e. those under standard commercial management and receiving only soil-applied urea, was reduced 50%: compare 333 cartons per acre in 1990-91 to 689 cartons for 1989-90. In addition, this level of production was 25% lower than the average for Southern California for 1990-91 (Source: Navel Orange Administrative Committee).

For 1990-91, there was a statistically significant effect due to the time of foliar urea application ($p \leq 0.10$). Trees receiving foliar applications of urea in January or February had greater yield than the control trees ($p \leq .05$) (Table 5). Trees receiving a foliar application of urea in January or February averaged 29 and 26 kg more fruit per tree, respectively, than trees receiving soil-applied urea.

Table 5. Effect of a January or February foliar application of low biuret urea on yield of the 'Washington' navel orange.

	Kg fruit · tree^{-1}			
	Date urea applied to the foliage			
Year	Control (none)	January	February	Significance level[z]
1	109 a	134 b	131 b	$p \leq 0.05$
2	49 a	78 b	75 b	$P \leq 0.05$
3	131 a	189 b	182 b	$p \leq 0.01$

[z]Data are the mean of 12 individual tree replicates per treatment. Mean separation was by Duncan's Multiple Range Test.

For the year following the freeze, 1991-92, all trees receiving a winter foliar application of low biuret urea had significantly higher yield per tree than trees receiving soil-applied urea ($p \leq 0.001$), approximately three packing cartons per tree (Table 5).

For all three years, the increase in yield resulting from the winter foliar application of low biuret urea did not change the number of large or medium-sized fruit that were harvested, i.e. fruit with diameters from 7.0 to 8.0 cm (box sizes 88 and 72) or from 6.1 to 6.9 cm (box sizes 138 and 113), respectively ($p \leq .05$).

The increase in yield resulting from the winter foliar application of low biuret urea was not a result of improved nitrogen status of the trees. At the end of the three-year experiment, leaf total nitrogen content of the 1991 spring flush leaves collected in September was not significantly different at the 5% level for control trees receiving soil-applied urea versus trees receiving a foliar application of low biuret urea. Leaf total nitrogen content was between 2.5 to 2.6 percent.

Conclusion

Results of our basic research demonstrated (i) that stress-induced flowering was causally related to tree ammonia status; (ii) that accumulated ammonia was metabolically linked to the synthesis of polyamines via arginine in developing flowers; and (iii) that fruit with a greater potential to set had significantly higher levels of Σ putrescine + spermidine + spermine. Results of our field experiment demonstrated that a winter application of low biuret urea in January or February significantly increased yield by more than one packing carton (17 kg) per tree for three consecutive years, without reducing fruit size or significantly increasing tree total nitrogen status, compared to control trees receiving a winter soil application of urea.

The results are consistent with and strongly support the interpretation that application of urea to the foliage of the 'Washington' navel orange provides sufficient ammonia to accelerate de novo arginine biosynthesis and lead to an increase in one or more species of polyamine that promotes flower initiation and ovary growth by cell division resulting in increased fruit set and yield. Results of experiments with apple provide additional support for this proposed sequence of metabolic events. Ammonia, arginine, and putrescine have all been shown to increase flowering in apple (Edwards, 1986), and exogenous application of putrescine to apple trees during early bloom increased fruit growth during the cell division phase with a concomitant increase in fruit set and yield (Costa et al., 1986).

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