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Relationship of polyamines to low-temperature stress-induced flowering of the 'Washington' navel orange (*Citrus sinensis* L. Osbeck)

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SUMMARY

The objectives of the present study were to quantify the relationship between flowering and leaf polyamine content at the initiation of, and during, a low-temperature floral-induction treatment and to test the ability of canopy sprays of L-arginine (50 mM), putrescine (10 and 20 mM), and spermidine (10 and 20 mM) to enhance the flowering response of the 'Washington' navel orange (*Citrus sinensis* L. Osbeck). Five year old container-grown 'Washington' navel orange trees were subjected to four weeks of low-temperature treatment of 8 h days at PFD of 500 μmol m^{-2} s^{-1} and 10°C and 16 h nights at 7°C. Leaf NH_3–NH_4^+, putrescine and spermidine concentrations were significantly greater at the end of, or one day after, the low temperature floral-induction treatment, while spermine content decreased. Trees receiving putrescine (20 mM) or spermidine (10 mM) had significantly greater leaf concentrations of spermidine at the initiation of the low-temperature floral-induction treatment. Flower number per tree was positively correlated with leaf spermidine concentration at the initiation of the induction treatment. Arginine and polyamines applied to the foliage at the end of the four 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 resulted in significantly greater leaf concentrations of spermidine one week after application and large, but statistically nonsignificant, increases in flower number per tree of 42% and 87%, respectively. The results provide evidence that spermidine availability at the time of flower initiation and organogenesis might be a factor affecting floral intensity in the 'Washington' navel orange.

FLOWERING in *Citrus* is promoted by low-temperature or water-deficit stress followed by restoration of climatic conditions favourable for growth (Monselise and Goren, 1969; Moss, 1969; Monselise, 1985; Southwick and Davenport, 1986; Lovatt *et al.*, 1988a). It is well documented that the degree of flowering in *Citrus* is directly proportional to the duration or severity of either stress (Moss, 1969; Southwick and Davenport, 1986; Barbera and Carimi, 1988; Lovatt *et al.*, 1988a, b). It was subsequently demonstrated that NH_3-NH_4^+ accumulating in the leaves of five year old rooted cuttings of 'Washington' navel orange induced to flower by low-temperature stress and of 16 year old 'Frost Lisbon' lemon trees induced to flower by water-deficit stress was significantly correlated with the length or severity of the stress, respectively, and with floral intensity. Lovatt *et al.* (1988a) proposed that accumulating NH_3-NH_4^+ accelerated arginine *de novo* biosynthesis and provided increased arginine for the synthesis of polyamines. Consistent with this proposal, arginine, not ornithine, has been demonstrated to serve as the precursor of putrescine synthesized during abiotic stresses (Flores and Galston, 1984a, b). Ammonium fertilization of apple enhanced flower production over nitrate alone

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(Gramanis and Leeper, 1967; Gramanis and Edwards, 1974; Edwards, 1986). Subsequently, it was demonstrated that ammonium raised the level of arginine and that arginine or putrescine supplied exogenously increased apple flower formation (Edwards, 1986; Rohozonski *et al.*, 1986).

In cultures of thin layers of tobacco epidermis, floral buds had greater concentrations of spermidine than vegetative buds, flowering was promoted by spermidine and inhibition of spermidine synthesis prevented flowering (Kuar-Sawhney *et al.*, 1988). With intact tobacco plants, hydroxycinnamoyl putrescine was demonstrated to influence flowering (Martin-Tanguy, 1985) and polyamine mutants exhibited abnormal flower organogenesis (Malmberg and McIndoo, 1983).

These results and others provide evidence strongly suggesting one or more polyamine species may play a role in regulating flower formation or development (Malmberg *et al.*, 1985; Dai and Wang, 1987; Bendeck and Kandler, 1989). For *Citrus*, changes in concentrations of polyamine species during flower development have been quantified (Kushad *et al.*, 1990; Sagee and Lovatt, 1991), but evidence of an effect of polyamines on the flowering process is lacking. Thus, the four objectives of the study were to determine: (i) which polyamine, if any, influences flower organogenesis; (ii) at what critical time is the polyamine(s) exerting the effect; (iii) does polyamine content change in response to a low-temperature floral-induction treatment; and (iv) does artificially raising the polyamine status of 'Washington' navel orange trees by foliar application of L-arginine, putrescine or spermidine at the end of the floral-induction treatment increase flowering.

MATERIALS AND METHODS

Chemicals

Trichloroacetic acid (TCA) was purchased from Fisher Scientific, Pittsburgh, PA. All other chemicals used in this study were purchased from Sigma Chemical Company, St. Louis, MO.

Plant material

Five year old 'Washington' navel orange trees (*Citrus sinensis* L. Osbeck) on Cuban shaddock rootstock (*C. limon*) grown in plastic pots containing 19 l 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 μmol m^{-2} s^{-1}) at 10°C and 16 h nights at 7°C for four 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 four 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, at the end of the low-temperature treatment but prior to arginine, putrescine or spermidine application and one, four and seven days after application of arginine, putrescine and spermidine to the canopy.

Two weeks after the trees were transferred to the warmer temperature conditions the following 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 NH_3-NH_4^+ and polyamine content

Mature leaves were washed with mild detergent and rinsed thoroughly with distilled water. Leaves (1 g fresh weight, mid-vein removed) were immediately homogenized in 5 ml 10% TCA using a Polytron tissue homogenizer (PCU, Brinkmann Instruments, Westbury, NY). 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 and then filtered through Whatman No. 1 filter paper. For ammonia analysis, the supernatant was filtered through nylon and glass fibre filters. The ammonia content of the acid soluble supernatant fraction, containing the combined pool of NH_3-NH_4^+ as NH_4^+ was determined with an Alltech Inorganic Nitrogen Analyzer

TABLE I

Leaf NH_3–NH_4^+ concentration(nmol g^{-1} fresh wt) at the beginning and at the end of low-temperature stress treatment, and 1, and 4 d after foliar application of arginine, putrescine or spermidine[w]

Treatment	Sampling Date[x]				
	Leaf NH_3–NH_4^+ concentrations				
	T_0	T_1	T_2	T_3	Significance[y]
	nmol g^{-1} fresh wt				
Control	$1450^{c}{}_{a}$	2648^{a}	2305^{b}	2092^{b}	****
Arginine					
50 mM	$1078^{c}{}_{b}$	2583^{a}	1984^{b}	1842^{b}	****
Putrescine					
10 mM	$1161^{c}{}_{b}$	2412^{a}	2100^{ab}	1919^{b}	****
20 mM	$1202^{c}{}_{b}$	2348^{a}	2165^{ab}	1914^{b}	****
Spermidine					
10 mM	$1159^{b}{}_{b}$	2281^{a}	2461^{a}	2224^{a}	**
20 mM	$1202^{b}{}_{b}$	2491^{a}	2225^{a}	2175^{a}	***
Mean	1209	2460	2207	2028	
Significance[z]	**	n.s.	n.s.	n.s.	

[w] Low temperature conditions used in this experiment were 8 h day (PFD of 500 μmol m^{-2} S^{-1}) at 10°C and 16 h night at 7°C for four weeks.

[x] Leaf samples for analysis were taken at the start of (T_0) and after four weeks of low-temperature stress (T_1), and 1 day (T_2), and 4 d (T_3) after foliar application of arginine, putrescine or spermidine.

[y] **, ***, **** represent significance at $P = 0.01$, 0.001 and 0.0001, respectively. Means within a horizontal row with the same superscript letter are not significantly different by Duncan's Multiple range test at $P = 0.05$.

[z] ** represent significance at $P = 0.01$. Means within a column with the same subscript letter are not significantly different by Duncan's Multiple range test at $P = 0.05$. n.s. = Non-significant at $P = 0.05$ and subscripts are not presented.

according to the method previously described by Carlson (1978). The assay was linear for NH_3-NH_4^+ concentrations from 0 to 100 μg ml^{-1}. Samples were diluted to give values in this range.

The free polyamine content of the supernatant fraction was determined after benzoylation according to the method of Flores and Galston (1982). Benzoylated free polyamines were separated by reverse phase high performance liquid chromatography through a 3.9 × 150 mm, 4 μm particle size C_{18} column (Nova Pak, Waters) eluted with 60% methanol at a flow rate of 1 ml min^{-1}. The benzoylpolyamines were detected at an absorbance wavelength of 254 nm at a sensitivity of 0.04 absorbance units full scale. According to Slocum *et al.* (1989), this method is unable to separate agmatine from putrescine. In the present study, comparisons of the 3.9 × 150 mm C_{18} column (Nova Pak, Waters) to a longer C_{18} column (3.9 × 300 mm, Bondapak, Alltech) revealed that neither was able to separate agmatine from putrescine. Therefore, the data we report in this communication are the combined pool of agmatine plus putrescine. The assays were linear for concentrations of each polyamine from 0.01 to 1 nmol. Benzoylation permitted recovery of 76% ± 11% of known amounts of putrescine, spermidine, or spermine added to the extracts (Sagee and Lovatt, 1991).

RESULTS

At the initiation of low-temperature floral-induction treatment, the control trees had a significantly greater concentration of NH_3-NH_4^+ than trees assigned to receive other treatments (Table I). The low-temperature floral-induction treatment resulted in a two-fold increase in leaf NH_3-NH_4^+ content at the end of the four week stress period for all trees (Table I). For the control trees and those treated with arginine, leaf NH_3-NH_4^+ content decreased significantly one day after transfer to the warmer conditions (Table I). For the putrescine treated trees, this decrease was observed after 4 d; while the leaf NH_3-NH_4^+ concentration of the spermidine-treated trees did not decrease during this period (Table I). Despite the above changes, leaf NH_3-NH_4^+ concentration for all trees subjected to the low-temperature treatment remained significantly greater than the pretreatment level for the 4 d following the stress treatment (Table I).

At the start of the low-temperature floral-induction treatment, leaves of trees assigned to receive 10 mM putrescine had a significantly greater concentration of agmatine-putrescine than the rest of the trees including the control

TABLE II

Concentration (nmol g^{-1} fresh wt) of the combined pool of agmatine and putrescine at the beginning and at the end of low-temperature stress treatment, and 1, 4 and 7 d after foliar application of arginine, putrescine or spermidine[w]

	Sampling Date[x]					
	Leaf agmatine-putrescine concentrations					
Treatment	T_0	T_1	T_2	T_3	T_4	Significance[y]
Control	$70.1^{b}{}_{bc}$	114.9^{ab}	128.7^{a}	99.2^{b}	81.4^{b}	*
Arginine						
50 mM	$55.3^{c}{}_{bc}$	119.6^{b}	170.9^{a}	92.3^{bc}	125.9^{b}	***
Putrescine						
10 mM	$93.1^{b}{}_{a}$	122.9^{ab}	151.1^{a}	95.2^{b}	99.9^{b}	*
20 mM	$48.1^{d}{}_{c}$	135.3^{b}	191.0^{a}	93.4^{c}	127.0^{b}	****
Spermidine						
10 mM	$75.7^{b}{}_{ab}$	103.8^{b}	182.6^{a}	97.7^{b}	96.9^{b}	****
20 mM	$55.8^{b}{}_{bc}$	104.2^{a}	136.4^{a}	106.7^{a}	127.7^{a}	***
Mean	66.4	116.8	160.1	97.4	109.8	
Significance[z]	***		n.s.	n.s.	n.s.	n.s.

[w] Low temperature conditions used in this experiment were 8 h day (PFD of 500 μmol m^{-2} s^{-1}) at 10°C and 16 h night at 7°C for four weeks.

[x] Leaf samples for analysis were taken at the start (T_0) and after four weeks of low-temperature stress (T_1), and 1 d (T_2), 4 d (T_3) and 7 d (T_4) after foliar application of arginine, putrescine or spermidine.

[y] *, ***, **** represent significance at $P = 0.05$, 0.001, and 0.0001, respectively. Means within a horizontal row with the same superscript letter are not significantly different by Duncan's Multiple range test at $P = 0.05$.

[z] *** represent significance at $P = 0.001$. Means within a column with the same subscript letter are not significantly different by Duncan's Multiple range test at $P = 0.05$. n.s. = Non-significant at $P = 0.05$ and subscripts are not presented.

trees (Table II). Leaf agmatine-putrescine concentration significantly increased during the low-temperature induction treatment for trees assigned to receive 50 mM arginine, 20 mM putrescine or 20 mM spermidine (Table II). Agmatine-putrescine concentration of leaves was significantly greater than the pretreatment values for all trees, including the control trees, one day after transfer from the low-temperature treatment to the warmer conditions. Application of arginine, putrescine or spermidine did not significantly increase the leaf agmatine-putrescine concentration over that of untreated control trees (Table II). Due to the continued increase in the leaf concentration of agmatine-putrescine in the control trees one day after transfer from the low-temperature to warm conditions, the contribu-

TABLE III

Concentration (nmol g^{-1} fresh wt) of polyamine spermidine at the beginning and at the end of low-temperature stress treatment, and 1, 4 and 7 d after foliar application of arginine, putrescine or spermidine[w]

	Sampling Date[x]					
	Leaf spermidine concentration					
Treatment	T_0	T_1	T_2	T_3	T_4	Significance[y]
Control	$119.0^{c}{}_{b}$	163.4^{a}	161.4^{ab}	124.9^{bc}	$144.2^{abc}{}_{c}$	*
Arginine						
50 mM	$110.5^{b}{}_{b}$	168.4^{a}	162.6^{a}	152.6^{a}	$179.5^{a}{}_{ab}$	**
Putrescine						
10 mM	$85.5^{b}{}_{b}$	168.9^{a}	167.1^{a}	154.3^{a}	$160.4^{a}{}_{bc}$	****
20 mM	$130.3^{a}{}_{a}$	160.7^{a}	152.0^{a}	133.5^{a}	$164.0^{a}{}_{bc}$	n.s.
Spermidine						
10 mM	$133.6^{c}{}_{a}$	143.2^{bc}	188.2^{a}	171.4^{ab}	$167.8^{abc}{}_{bc}$	*
20 mM	$85.0^{b}{}_{b}$	175.4^{a}	165.6^{a}	155.4^{a}	$200.5^{a}{}_{a}$	***
Mean	110.6	163.3	166.1	148.7	169.4	
Significance[z]	***	n.s.	n.s.	n.s.	*	

[w] Low temperature conditions used in this experiment were 8 h day (PFD of 500 μmol m^{-2} s^{-1}) at 10°C and 16 h night at 7°C for four weeks.

[x] Leaf samples for analysis were taken at the start of (T_0) and after four weeks of low-temperature stress (T_1), and 1 d (T_2), 4 d (T_3) and 7 d (T_4) after foliar application of arginine, putrescine and spermidine.

[y] *, **, ***, **** represent significance at $P = 0.05$, 0.01, 0.001 and 0.0001, respectively. Means within a horizontal row with the same letter are not significantly different by Duncan's Multiple range test at $P = 0.05$.

[z] *, *** represent significance at $P = 0.01$ and 0.001, respectively. Means within a column with the same subscript letter are not significantly different by Duncan's Multiple range test at $P = 0.05$. n.s. = Non-significant at $P = 0.05$ and subscripts are not presented.

TABLE IV

Concentration (nmol g^{-1} fresh wt) of polyamine spermine at the beginning and at the end of low-temperature stress treatment, and 1, 4 and 7 d after foliar application of arginine, putrescine or spermidine[w]

	Sampling Date[x]					
	Leaf spermine concentration					
Treatment	T_0	T_1	T_2	T_3	T_4	Significance[y]
Control	$47.4^{a}{}_{b}$	38.4^{b}	45.4^{ab}	46.4^{ab}	19.6^{c}	****
Arginine						
50 mM	$53.4^{a}{}_{ab}$	34.4^{c}	38.7^{bc}	42.6^{b}	19.8^{d}	****
Putrescine						
10 mM	$46.4^{ab}{}_{b}$	36.4^{c}	41.0^{bc}	49.6^{a}	19.6^{d}	****
20 mM	$62.6^{a}{}_{a}$	35.3^{c}	42.4^{bc}	50.4^{b}	19.7^{d}	****
Spermidine						
10 mM	$56.6^{a}{}_{ab}$	32.8^{c}	42.1^{b}	44.6^{d}	19.8^{d}	****
20 mM	$46.9^{a}{}_{b}$	38.5^{a}	38.1^{a}	43.8^{a}	19.8^{b}	****
Mean	52.2	36.0	41.3	46.2	19.7	
Significance[z]	**	n.s.	n.s.	n.s.	n.s.	

[w] Low temperature conditions used in this experiment were 8 h day (PFD of 500 μmol m^{-2} s^{-1}) at 10°C and 16 h night at 7°C for four weeks.

[x] Leaf samples for analysis were taken at the start of (T_0) and after four weeks of low-temperature stress (T_1), and 1 d (T_2), 4 d (T_3) and 7 d (T_4) after foliar application of arginine, putrescine and spermidine.

[y] *, **, ***, **** represent significance at $P = 0.05$, 0.01, 0.001 and 0.0001, respectively. Means within a horizontal row with the same letter are not significantly different by Duncan's Multiple range test at $P = 0.05$.

[z] ** represent significance at $P = 0.01$. Means within a column with the same subscript letter are not significantly different by Duncan's Multiple range test at $P = 0.05$. n.s. = Non-significant at $P = 0.05$ and subscripts are not presented.

tion of foliar-applied arginine, putrescine or spermidine to the level of agmatine-putrescine present in the treated trees at that time and subsequently is unclear.

Trees receiving putrescine (20 mM) or spermidine (10 mM) had sigificantly greater leaf concentrations of spermidine at the initiation of the low-temperature floral-induction treatment (Table III). One week after foliar application of polyamines, trees receiving 20 mM spermidine had significantly greater leaf spermidine concentrations than the rest of the treatments. The low-temperature floral-induction treatment resulted in a significant increase in leaf spermidine content for the control trees and trees assigned to be treated with 50 mM arginine, 10 mM putrescine or 20 mM spermidine (Table III). Leaf spermidine concentrations remained greater than the time zero value for one week after transfer to the warmer conditions for all trees except the control trees and the trees treated with 10 mM spermidine.

At time zero, trees assigned to receive 20 mM putrescine, 50 mM arginine or 10 mM spermidine had significantly greater leaf concentrations of spermine. Low-temperature stress significantly reduced leaf concentrations of spermine except for trees assigned to be treated with 20 mM spermidine (Table IV). Leaf spermine concentrations remained below the time zero value for four days for trees treated with 50 mM arginine, 20 mM putrescine and 10 mM spermidine.

The number of flowers and number of leafless inflorescences (floral shoots with less than one leaf per flower) for trees receiving putrescine (20 mM) or spermidine (10 mM) were increased more than 2-fold ($P = 0.05$) compared with the control trees sprayed with water (Table V). Interestingly, the trees included in these two treatments had significantly greater leaf concentrations of spermidine at the initiation of floral-induction treatment. The number of flowers per tree was positively correlated ($r = 0.37$, $P = 0.005$) only with leaf spermidine concentration at the initiation of the floral-induction treatment. There was no correlation between floral intensity, inflorescence types or vegetative shoots produced and leaf concentrations of NH_3-NH_4^+, putrescine or spermine, for any sampling date. The number of vegetative and leafy inflorescences were not significantly affected by any treatment (Table V).

DISCUSSION

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

TABLE V

The effect of foliar application of arginine, putrescine or spermidine on number of flowers, percent and number of floral shoots, percent and number of leafless, percent and number of leafy inflorescences and percent and number of vegetative shoots per tree.

Treatment	Flower number	No. of floral shoots	Leafless inflorescence (no.)	Leafless inflorescence (%)[y]	Leafy inflorescence (no.)	Leafy inflorescence (%)	Vegetative shoots (no.)	Vegetative shoots (%)
Control	107b	51a	22b	20c	31a	25a	59a	55a
Arginine								
50 mM	152ab	72a	35ab	25bc	36a	28a	55a	47a
Putrescine								
10 mM	163ab	72a	37ab	26bc	36a	23a	83a	51a
20 mM	229a	91a	60a	43a	32a	23a	47a	34a
Spermidine								
10 mM	234a	86a	52a	38ab	34a	25a	50a	37a
20 mM	201ab	78a	41ab	31abc	35a	26a	58a	43a
Significance[z]	*	n.s.	*	*	n.s.	n.s.	n.s.	n.s.

[y] Expressed as percent of all shoots produced.

[z] *, n.s. represents significance and non-significance at $P = 0.05$, respectively. Means within a column with the same letter are not significantly different by Duncan's Multiple range test at $P = 0.05$.

shoot within a resting bud to become 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, 1985, 1987). If the apex is floral, the laterals will form flowers; if the apex is vegetative, the laterals will be diverted to thorns. Early-developing floral shoots produce predominantly leafless inflorescences at anthesis (floral shoots with fewer than one leaf per flower). In contrast, due to abortion of lateral floral buds and increased development of leaf primordia along the shoot axis, later-developing inflorescences are leafy (floral shoots with more than one leaf per flower) (Lovatt *et al.*, 1987).

Logically, floral intensity would be enhanced by conditions that convert vegetative shoot apices to floral apices, thereby committing lateral mainstems to flowers, prevent the reversion of floral shoots to vegetative shoots or minimize abortion of lateral flower buds in later-developing inflorescences. Conditions leading to the irreversible commitment to flowering in *Citrus* would probably be those present at the initiation of, and/or during the floral-induction treatment, while those that reduced abortion of later-developing lateral floral primordia would more probably be those present toward the end of or after the induction treatment. The observation that leaf spermidine concentration at the start of the low-temperature floral-induction treatment was positively correlated with floral intensity suggests several lines that warrant further investigation: (i) that spermidine is more important than putrescine or spermine to the flowering process in *Citrus;* (ii) that spermidine affects floral initiation early in the induction process; and (iii) that treatments that improve tree spermidine status at the onset of the floral organogenesis should enhance flowering. These interpretations are consistent with the evidence accumulating in the literature which more strongly supports a relationship between flowering and spermidine as opposed to other polyamines (Bendeck and Kandler, 1989; Dai and Wang, 1987; Kuar-Sawhney *et al.*, 1988; Malmberg *et al.*, 1985); and with the observation that application of polyamines to apple trees during the fruit set period (which occurs concurrently with floral initiation) promoted flowering in the following year (Costa and Bagni, 1983). Applications of 50 mM arginine or 20 mM spermidine, which significantly increased leaf spermidine concentration one week after foliar application, also resulted in 42% and 87% increases in flower number per tree, respectively. These increases are physiologically significant although not statistically significant. These results provide further evidence that spermidine has a positive effect on the flowering process in *Citrus.* The increase in flowering response was accompanied by decreased vegetative shoot number, suggesting that conversion of vegetative shoot apices to floral apices continues to some degree after the induction treatment is completed.

The significant increase in leaf NH_3-NH_4^+

content observed in this study in trees subjected to low-temperature stress was reported previously by Lovatt *et al.* (1988a). In addition, the present study demonstrates that leaf agmatine-putrescine and spermidine content also increased in response to the low-temperature floral-induction treatment. It should be noted that leaf spermine concentration decreased during the low-temperature treatment. The decrease in spermine concentration one week after the low-temperature floral-induction treatment was previously observed by Sagee and Lovatt (1991).

Virtually nothing is known about the role of spermidine, or any other polyamine, in citrus floral ontogeny. Direct assay of buds during the floral-induction treatment, while difficult, might provide more clear evidence for or against the putative role of polyamines in flowering in general and in *Citrus* in particular.

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