Effects of Some Polyamine Biosynthesis Inhibitors and Calcium Chloride on In Vitro Growth and Decay Development in Apples Caused by *Botrytis cinerea* and *Penicillium expansum*

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ABSTRACT. Three polyamine biosynthesis inhibitors, α -difluoromethylornithine (DFMO), α -difluoromethylarginine (DFMA), and α -methylornithine (MeOrn), alone and in combination with CaCl_2 , were tested for their ability to reduce in vitro growth and soft rot development in apple (*Malus domestica* Borkh.) fruit caused by *Botrytis cinerea* Pers.:Fr. and *Penicillium expansum* Link. All three inhibitors reduced the in vitro growth of the pathogens. Calcium had no effect on fungal growth in vitro. Pressure infiltration of millimolar concentrations of DFMO or DFMA or 25 g L^{-1} CaCl_2 solutions into apples reduced subsequent soft rot development by *B. cinerea* and *P. expansum* >40%. A combination treatment of Ca and DFMO or DFMA reduced decay >67%. Treatment of apples with MeOrn was less effective at inhibiting decay development. None of the inhibitors affected polyamine levels in apple cortical tissues. Some injury to the fruit surface was observed with Ca treatments. Fruit treated with Ca and any of the inhibitors were less firm than those treated with Ca alone. Specific polyamine biosynthesis inhibitors in combination with Ca may prove useful in reducing postharvest decay in apples.

Economic losses caused by postharvest pathogens can be considerable, and the losses potentially can be avoided, or at least reduced, through postharvest chemical treatments. The two most important postharvest pathogens causing decay in apples are *Penicillium expansum* Link and *Botrytis cinerea* Pers.:Fr. (Rosenberger, 1991). Both postharvest wound pathogens are effectively controlled by benzimidazole fungicides, and the commonly used postharvest antioxidant diphenylamine (Rosenberger, 1991). However, benzimidazole-resistant isolates of the pathogens have been found in many packinghouses (Rosenberger, 1991) and continued postharvest use of diphenylamine is being questioned as it is currently under review for recertification. Alternative mechanisms of resistance to postharvest pathogens need to be identified to protect apples in storage.

Postharvest pressure infiltration of CaCl_2 solutions reduces decay in apples caused by *P. expansum* and *B. cinerea*, but optimal concentrations of CaCl_2 for decay control sometimes cause commercially unacceptable levels of injury to the fruit surface (Conway et al., 1991, 1992, 1994). Lower concentrations of CaCl_2 substantially reduce the risk of surface injury to the fruit but do not provide as much resistance to pathogenic infections. While the mechanism of Ca-induced resistance to pathogens is not fully understood, it is

partially attributable to improved structural integrity of host cell walls, which reduces maceration by fungal polygalacturonases (Conway et al., 1988; Tobias et al., 1993). Although Ca treatments alone are unlikely to totally replace benzimidazole fungicides, they could be highly effective along with other new and conventional techniques for integrated control of postharvest decay pathogens.

Polyamine biosynthesis inhibitors are a diverse group of chemicals that have been effectively used to control a number of fungal plant diseases (Galston and Weinstein, 1988; Rajam, 1993; Rajam et al., 1985). The effectiveness of polyamine biosynthesis inhibitors at inhibiting fungal pathogens and the diseases they cause is based on the general principles that 1) polyamines are required by all cells for normal growth and differentiation, 2) more actively growing and differentiating cells require higher levels of polyamine biosynthesis than mature fully differentiated cells, and 3) higher plants have two pathways for polyamine biosynthesis whereas many fungal pathogens have only one pathway (Rajam, 1993). In regard to the latter principle, the essential diamine, putrescine, is synthesized directly by the decarboxylation of ornithine via ornithine decarboxylase (ODC) or indirectly by decarboxylation of arginine via arginine decarboxylase (ADC) (Rajam, 1993). Both pathways operate in higher plant cells, while only the ODC pathway operates in most fungal pathogens (Slocum and Galston, 1987). The polyamines, spermidine and spermine, are further synthesized from putrescine (Smith, 1985).

Two of the most potent and specific inhibitors of fungal polyamine biosynthesis are D,L- α -difluoromethylornithine (DFMO) and D,L- α -difluoromethylarginine (DFMA) (Rajam, 1993). Both inhibitors are highly specific, irreversible and enzyme activated (substrate based) [i.e., DFMO and DFMA inhibit ODC (Metcalf et al., 1978) and ADC (Kallio et al., 1981), respectively]. Growth

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inhibition of fungal pathogens caused by DFMA may be due to the existence of the ADC pathway in the affected fungi or the conversion of DFMA to urea plus DFMO by arginase (Slocum and Galston, 1985; Slocum and Galston, 1987; Slocum et al., 1988). DFMO at millimolar concentrations have inhibited the in vitro growth of *B. cinerea*; DFMA likewise inhibited *B. cinerea*, but this result was probably due to intracellular conversion of DFMA to DFMO (Slocum and Galston, 1985). At the same concentrations, DFMO and DFMA have no apparent effect on growth and polyamine biosynthesis of higher plants (Rajam, 1993). α -Methylornithine (MeOrn) is a less specific competitive (Slocum and Galston, 1985) inhibitor of polyamine biosynthesis, but it is available commercially at low cost.

Irreversible inhibitors like DFMO essentially are not cytotoxic (Sjoerdsma, 1981) and are well tolerated (Sunkara and Prakash, 1984; Tabor and Tabor, 1985) by animals and humans at levels well above those used to control preharvest fungal diseases (Rajam, 1993; Rajam et al., 1985). In addition, the inhibitors should not affect beneficial microorganisms in the soil as inhibitor concentrations would get diluted significantly in the soil water (Rajam et al., 1993). Alternatively, strategies to inactivate waste solutions of inhibitors before disposal could be developed. To our knowledge, postharvest use of polyamine biosynthesis inhibitors as a new strategy to reduce diseases caused by fungal pathogens has not been reported.

Our study examines the efficacy of these polyamine biosynthesis inhibitors, alone and in combination with Ca, to reduce in vitro fungal growth and postharvest soft rot development in apple fruit caused by *B. cinerea* and *P. expansum*. A second objective was to examine the effect of inhibitor treatments on fruit firmness, injury potential to the fruit surface, and polyamine levels in cortical tissue of apple fruit.

Materials and Methods

'Golden Delicious' apples were harvested in the preclimacteric stage (ethylene production $<0.1 \mu\text{L}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ and the climacteric rise in CO_2 production had not yet begun) from a commercial orchard and randomized. Fruit were rinsed with water to remove surface residual material from preharvest sprays and dried.

The apples were then treated in the following manner. Fruit were dipped five times for 15 s in a $2\text{-g}\cdot\text{L}^{-1}$ solution of the surfactant, Silwet L-77 (OSi Specialties, Tarrytown, N.Y.). The fruit were allowed to dry and then were pressure infiltrated (48 kPa for 3 min at $20 \pm 1^\circ\text{C}$) in the following solutions: distilled water, a $5.0 \text{ mmol}\cdot\text{L}^{-1}$ solution of DFMO, DFMA, or MeOrn; a $25 \text{ g}\cdot\text{L}^{-1}$ solution of $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$; or inhibitor plus Ca solutions. In addition, one lot of fruit was left untreated. The fruit then were stored at 0°C .

After 3 months, the fruit were removed from 0°C storage, warmed to $20 \pm 1^\circ\text{C}$, then wound inoculated with *B. cinerea* or *P. expansum*. For pathogen inoculation, 60 fruit from each treatment were wounded on two sides to a depth of 2 mm by pressing them down on a nail head (2 mm in diameter). The wounded fruit from each treatment were divided into two subsamples and were inoculated by dipping for 15 s in a freshly prepared conidial suspension (spores of either *B. cinerea* or *P. expansum* at $1\cdot 10^5\cdot\text{mL}^{-1}$). Both pathogens were isolated from naturally infected apple fruit and maintained on potato dextrose agar. Apples were rated for decay severity by measuring the surface diameter of the decayed area as the mean of its width and length after 5 to 7 d at $20 \pm 1^\circ\text{C}$.

In another set of experiments, chemically untreated fruit were stored at 0°C . After 3 months, the fruit were removed from cold storage, warmed to $20 \pm 1^\circ\text{C}$, then sequentially chemically treated and pathogen inoculated as previously described. The fruit were stored an additional 2 to 3 months at 0°C before rating the apples for decay severity.

The firmness of 20 fruit from each treatment was measured with a manually controlled digital penetrometer (EPT-1; Lake City Technical Products, Kelowna, B.C., Canada). The EPT-1 was set in the Magness-Taylor mode and interfaced to a personal computer. Firmness was measured at two opposite points on the equator of each fruit after removal of a 2-mm slice with a fixed-blade slicer.

Fruits treated with chemicals at harvest were rated for injury to the fruit surface immediately after removal from cold storage. Injury was assessed on a five-point scale based on the percentage area of fruit surface injured and on the presence or absence of brown, sunken lesions on the fruit surface. The injury ratings were as follows: 1 = presence of brown, sunken lesions, 2 = $>50\%$ of fruit surface injured, 3 = 25% to 50% of fruit surface injured, 4 = fruit surface injury localized and not exceeding 25% of fruit surface, and 5 = no fruit surface injury. Forty fruit per treatment were examined. On our scale, an injury rating below four is unmarketable as fresh fruit due to surface discoloration, superficial lenticel pitting, or both. However, only fruit with a rating of one would be unsuitable for processing. Injury rating indicates the severity of injury on individual fruit, whereas percent fruit injured indicates the percentage of the fruit showing any indication of injury.

Polyamine levels in the cortex of apple fruit were determined by dansylation and HPLC separation according to the method of Saftner and Baldi (1990). The identity of the dansyl polyamines was determined by retention times relative to an internal standard of 1,6-diaminohexane, by coinjection of known standards, and by mass spectrometric verification of HPLC-eluting peaks. Four polyamine samples from each treatment were collected by remov-

Table 1. The effect of DFMO, DFMA, Ca, and Spd, alone and in various combinations, on in vitro growth of *B. cinerea* and *P. expansum* mycelia.

Treatment	Concn. (mmol·L ⁻¹)	Mycelial length (μm; % inhibition)	
		<i>B. cinerea</i>	<i>P. expansum</i>
Control		518 a ²	613 a ²
DFMO	0.1	430 ab (17)	590 a (4)
	0.3	330 b (36)	470 a (23)
	1.0	235 c (55)	312 b (49)
	2.0	165 d (68)	270 b (56)
	5.0	90 e (83)	153 c (75)
DFMA	0.1	448 a (14)	600 a (2)
	0.3	358 b (31)	490 a (20)
	1.0	228 c (56)	299 b (51)
	2.0	146 d (72)	248 b (60)
	5.0	78 e (85)	111 c (82)
MeOrn	5.0	247 c (52)	233 b (62)
Spd	1.0	488 a (6)	614 a (0)
DFMO + Spd	5.0 + 1.0	469 a (9)	594 a (3)
DFMA + Spd	5.0 + 1.0	460 a (11)	618 a (0)
Ca	5.0	561 a (-8)	628 a (-2)
Ca + DFMO	5.0 + 5.0	99 e (81)	121 c (80)
Ca + DFMA	5.0 + 5.0	81 e (84)	142 c (77)
Ca + MeOrn	5.0 + 5.0	271 bc (48)	258 b (58)

²Means followed by the same letter within a column are not significantly different at $P \leq 0.05$.

ing the peel and outer flesh (2 mm deep) with a mechanical peeler and discarding. The next 2 mm was similarly removed, and 3 g portions of this tissue from each of five fruit were combined for analysis.

The Ca content of fruit treated at harvest with CaCl_2 solutions was determined from 28-fruit lots. Calcium content analysis was conducted by removing the peel and outer flesh (2 mm deep) with a hand peeler and discarding. The next 2 mm of flesh was similarly removed, freeze dried, and ground. Dried tissue samples were then dry-ashed, dissolved 1:25 in 1 mol·L⁻¹ HCl, and analyzed for Ca content by inductively coupled plasma emission spectrometry (model 61E; Thermo Jarrell Ash, Franklin, Mass.). Calcium content is reported on a dry mass basis (in mg·kg⁻¹). Each sample consisted of the flesh from seven apples, and four samples were obtained from each treatment.

For in vitro growth studies, pure cultures of *B. cinerea* and *P. expansum* were grown on potato-dextrose agar. Filter-sterilized inhibitor and CaCl_2 solutions were added to the sterilized agar medium cooled to 42 to 44 °C to obtain final concentrations at 0.1 to 5.0 mmol·L⁻¹ of inhibitor, Ca at 5.0 mmol·L⁻¹, or both. Each solution then was dispensed aseptically into 12 wells of a 24-well sterile tissue culture dish, 0.5 mL per well, each well having a culture area of 1.9 cm², and allowed to solidify. Control plates comprised the previously mentioned agar medium with no amendments. For experiments involving reversal of inhibition, a 5.0 mmol·L⁻¹ concentration of an inhibitor was used along with a 1.0 mmol·L⁻¹ concentration of the polyamine, spermidine (Spd), to counteract the inhibitions. To initiate growth, 100 µL of a spore suspension (1·10⁵·mL⁻¹) was aseptically added to each well. The *B. cinerea* and *P. expansum* cultures were incubated under fluorescent lighting at 20 ± 1 °C until mycelial lengths in control plates ranged between 500 and 700 µm (1 to 2 d). Mycelial length was measured

Fig. 1. Effect of some polyamine biosynthesis inhibitors and Ca, alone and in combination, on decay development after 5 or 7 d of incubation at 20 ± 1 °C in 'Golden Delicious' apples. Except for nontreated fruit, apples were pretreated with a 2-g·L⁻¹ solution of Silwet L-77 and then pressure infiltrated (48 kPa for 3 min at 20 ± 1 °C) in distilled water, a 25-g·L⁻¹ solution of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, a 5-mmol·L⁻¹ solution of an inhibitor of polyamine biosynthesis, or both. Pressure infiltration treatments are 1 = nontreated, 2 = distilled water, 3 = DFMO, 4 = DFMA, 5 = MeOrn, 6 = Ca, 7 = Ca + DFMO, 8 = Ca + DFMA, and 9 = Ca + MeOrn. Apples were rated for decay severity by measuring the surface diameter of the decayed area as the mean of its width and length. Mean separation pertaining to each pathogen individually is by Duncan's multiple range test at $P \leq 0.05$.

Fig. 2. Effect of some polyamine biosynthesis inhibitors and CaCl_2 treatments, alone and in combination, on decay development after 2 to 3 months of incubation at 0 °C in 'Golden Delicious' apples. Fruit treatments are as identified in the legend to Fig. 1. Apples were rated for decay severity by measuring the surface diameter of the decayed area as the mean of its width and length. Mean separation pertaining to each pathogen individually is by Duncan's multiple range test at $P \leq 0.05$.

using a manually operated, video digitizing analysis system (Bioquant II, R&M Biometrics Corp., Nashville) connected to a microscope with a long working distance objective.

The decay data was analyzed as a two factor general linear models procedure (SAS Institute, 1989). The assumptions of the general linear model were checked, and no transformations were needed. The treatment means were separated at the 5% significance level using Duncan's multiple range test. All experiments were repeated at least once and results are presented from individual experiments. Unless stated otherwise, only results significant at the 5% level are discussed.

Results

IN VITRO GROWTH. DFMO, DFMA, MeOrn, Ca, and Spd, alone and in various combinations, affected the in vitro elongation growth of *B. cinerea* and *P. expansum* mycelia (Table 1). DFMO and DFMA at 0.1, 0.3, 1.0, 2.0, and 5.0 mmol·L⁻¹ produced successively greater inhibition of mycelial growth of both fungi, with *B. cinerea* being inhibited somewhat more than *P. expansum*. DFMO, DFMA, and MeOrn at millimolar concentrations inhibited the in vitro growth of *B. cinerea* and *P. expansum* in the absence and presence of a 5.0 mmol·L⁻¹ concentration of CaCl₂. The specific, irreversible inhibitors, DFMO and DFMA, were more inhibitory than the less specific, competitive inhibitor, MeOrn. A 1.0 mmol·L⁻¹ concentration of Spd effectively counteracted the inhibitory effect of 5.0 mmol·L⁻¹ DFMO and DFMA on pathogen growth.

POSTHARVEST DISEASE RESISTANCE. The diameters of decay of fruit treated with either a 25-g·L⁻¹ solution of CaCl₂ or a 5.0-mmol·L⁻¹ solution of DFMO or DFMA and subsequently inoculated with either *B. cinerea* or *P. expansum* were reduced at least 40% whether decay development occurred at 20 °C (Fig. 1) or during cold storage at 0 °C (Fig. 2). The Ca content of cortical tissue from CaCl₂-treated fruit ranged between 1360 and 1610 mg·kg⁻¹ dry mass vs. <250 mg·kg⁻¹ dry mass in tissue from fruit not treated with Ca. Combination treatments with Ca and either inhibitor were more effective at reducing decay than treatments with Ca, DFMO, or DFMA alone. The combination treatments inhibited decay development between 67% and 88%. When the

concentrations of DFMO and DFMA were reduced from 5.0 to 2.0 mmol·L⁻¹, combination treatments of apple fruit with Ca and DFMO or DFMA remained more effective at reducing decay than treatment with Ca or either inhibitor alone, but the differences between such treatments were not statistically significant (data not shown). DFMO and DFMA had a stronger inhibitory effect on in vitro elongation growth of *B. cinerea* and *P. expansum* than on in vivo resistance to decay caused by the same pathogens (Table 1 and Figs. 1 and 2). Treatment of apple fruit with the reversible inhibitor, MeOrn, did not reduce decay caused by either pathogen, whether supplied alone or in combination with Ca (Figs. 1 and 2).

FRUIT INJURY. There was fruit injury in the form of skin discoloration, superficial pitting, or both in the lenticel regions of the fruit after treating the fruit with Ca (Table 2). Although not statistically significant, MeOrn also may have injured the surface of the fruit. However, all treated fruit would be commercially acceptable for processing because there was no internal fruit injury (R.A.S., unpublished data).

FIRMNESS. As expected, fruit infiltrated with Ca were firmer than water-treated and nontreated fruit (Fig. 3). In the absence of Ca, inhibitor-treated fruit were as firm as control (water-treated) fruit.

POLYAMINE CONTENT. The spermidine and putrescine concentrations in the cortical tissue of nontreated apple fruit were 27 ± 5 and 6 ± 2 nmol·g⁻¹ fresh mass, respectively, and were not reduced in fruit treated with any inhibitor used in this study (data not shown). The spermine levels in tissue from nontreated and treated fruit were too low (<0.5 nmol·g⁻¹ fresh mass) to make any comparisons.

Discussion

DFMO and DFMA were potent inhibitors of *B. cinerea* and *P. expansum* mycelia growth (Table 1) and associated decay under the conditions of this study (Figs. 1 and 2). Since the in vitro effect of either DFMO or DFMA could be reversed by the polyamine, spermidine, growth inhibition of *B. cinerea* and *P. expansum* mycelia appeared to result from the specific inhibition of polyamine biosynthesis. Several fungal diseases can be controlled through specific inhibition of polyamine biosynthesis (Rajam, 1993). The same mechanism could account for the increased decay resistance in apples treated with either DFMO or DFMA (Figs. 1 and 2). The inhibitors did not have nonspecific injurious effects on apple fruit (Table 2). The specific inhibition caused by DFMA may be due to either the existence of the ADC pathway in *B. cinerea* and *P. expansum* or the more likely conversion of DFMA to DFMO by arginase (Slocum and Galston, 1985; Rajam, 1993). MeOrn, a reversible inhibitor of polyamine biosynthesis, also inhibited the in vitro growth of the two pathogens tested though to a lesser degree than the irreversible inhibitors DFMO and DFMA. In vivo, however, it was not potent enough to consistently reduce decay (Figs. 1 and 2) and may not be sufficiently selective to be devoid of untoward biological effects (Table 2). Unlike the inhibitors, Ca did not inhibit the in vitro growth of either fungal pathogen (Table 1). Calcium also did not counteract the growth inhibition caused by a 5 mmol·L⁻¹ concentration of the inhibitors (Table 1). This result is in accord with the known inability of Ca to form strong complexes with amino acids and analogs (Larsen, 1980), a chemical class that would include the inhibitors used in this study.

Postharvest treatment of apple fruit with Ca reduced decay caused by *B. cinerea* and *P. expansum* to the same degree as postharvest treatment of fruit with a 5.0 mmol·L⁻¹ concentration of either DFMO or DFMA (Figs. 1 and 2). Combination treatments

Table 2. Fruit surface injury of 'Golden Delicious' apples pressure infiltrated (3 min; 48 kPa) with distilled water or a solution of CaCl₂ and/or an inhibitor of polyamine biosynthesis.^z

Pressure treatment	Injury rating + SD ^y	Fruit injured (%) ^x
1. Nontreated	5.0 ± 0.0	0
2. Distilled water	4.9 ± 0.3	8
3. DFMO	4.8 ± 0.5	15
4. DFMA	4.9 ± 0.4	8
5. MeOrn	4.7 ± 0.7	20
6. Ca	4.2 ± 0.7	68
7. Ca + DFMO	4.5 ± 0.5	50
8. Ca + DFMA	4.6 ± 0.5	43
9. Ca + MeOrn	3.7 ± 0.8	83

^zExcept for nontreated fruit, the fruit used in the experiment were dipped for 15 s in a 2 g·L⁻¹ solution of Silwet L-77. Inhibitors were supplied at 5.0 mmol·L⁻¹, Ca as a 25 g·L⁻¹ solution of CaCl₂ · 2H₂O.

^yFruit were rated for injury after 3 months of storage at 0 °C. Injury rating: 5 = no fruit surface injury; 3 = 25% to 50% of fruit surface injured; 1 = severe injury with brown sunken lesions.

^xPercent fruit injured indicates the percent of the 40 fruit evaluated showing any sign of injury.

with Ca and DFMO or DFMA reduced decay more than treatment with an inhibitor or Ca alone. The efficacy of the combination treatment is in accord with the finding that Ca and the inhibitors affect fungal pathogen growth and development by different mechanisms. While Ca caused some injury to the surface of the fruit (Table 2) as previously noted (Conway et al., 1992), neither Ca nor inhibitors affected polyamine levels of the fruit. This result may be of importance because polyamine levels have been associated with maintenance of postharvest quality of apple fruit during cold storage (Wang et al., 1993).

The marked sensitivity of the fungal pathogens tested, and the apparent insensitivity of apple fruit, to DFMO and DFMA underscores the efficacy of these treatments. Based on the inhibitor content of fruit estimated by gain in fruit fresh mass (<3 mg per fruit; R.A.S., unpublished data) from inhibitor solutions at 5 mmol·L⁻¹, it is estimated that <10 g of inhibitor would be adequate to protect a ton of fruit from major postharvest diseases, especially if used in combination with Ca. Unless the risk of superficial injury associated with Ca treatment is reduced, the combination treatment could only be considered for apples intended for processing. The postharvest protection against decay afforded by the postharvest inhibitor treatments are in accord with DFMO and other inhibitors of polyamine biosynthesis being effective protectants against various kinds of preharvest fungal plant diseases (Rajam, 1993). While metabolism of DFMO and DFMA is not well established (Rajam, 1993), it is interesting that inhibitor treatments of apple fruit at harvest reduced fungal decay in the fruit after 3 months of cold storage at 0 °C (Fig. 1). The persistence of the DFMO inhibitory effect on fungal plant infections was previously observed in preharvest studies (Rajam et al., 1985).

The prospects of using irreversible polyamine biosynthesis inhibitors for postharvest control of major broad-range fungal pathogens of apple fruit appears to have significant potential. In addition to the marked sensitivity of the fungal pathogens tested, the apparent insensitivity of apple fruit itself, the persistence of the inhibitory effect and the combined efficacy of inhibitors and Ca already discussed, irreversible inhibitors like DFMO are essentially nontoxic to man and other animals and should not have an adverse impact on beneficial microorganisms (Rajam, 1993; Sunkara and Prakash, 1984; Tabor and Tabor, 1985). However, the inhibitors are not approved and should not be considered for postharvest horticultural use at this time. Using irreversible polyamine biosynthesis inhibitors as protectants against fungal plant pathogens remain experimental due to their limited availability and to the associated costs of registering them as fruit protectants. Once these shortcomings are resolved, irreversible polyamine biosynthesis inhibitors combined with Ca may provide an interesting alternative to traditional fungicides in postharvest technologies.

Fig. 3. Effect of some polyamine biosynthesis inhibitor and Ca treatments, alone and in combination, on fruit firmness. Fruit treatments are as identified in the legend to Fig. 1. Mean separation is by Duncan's multiple range test at $P \leq 0.05$.

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