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Putrescine extends effective pollination period in 'Comice' pear (*Pyrus communis* L.) irrespective of post-anthesis ethylene levels*

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ABSTRACT

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Putrescine (PUT) 10^{-3} M, a polyamine applied at bloom, enhanced pollen tube ovule penetration and delayed ovule senescence, extending the effective pollination period of cultivar 'Comice' pear (*Pyrus communis* L.) by at least 2 days and 4 days in 1985 and 1986, respectively, without reducing post-pollination flower ethylene levels. Evolved flower ethylene peaked at pollination and again at petal senescence in vivo while vacuum-extracted ethylene peaked only during fertilization. Endogenous polyamine levels were not related to ethylene levels during the post-bloom period in any flowers. After pollination, putrescine-treated flowers exhibited lower levels of evolving ethylene than untreated flowers. Hand-pollinated flowers had greater [illegible] than unpollinated flowers. PUT-treated flowers had lower endogenous polyamine levels and higher nitrogen contents than untreated flowers. Earlier pollen tube penetration, delayed ovule longevity and extended 'Comice' effective pollination period from a PUT application were not associated with reduced post-anthesis, ethylene levels.

Keywords: endogenous polyamines; evolved ethylene; flower culture in vitro; ovule senescence; pollen tube growth; vacuum extracted ethylene.

Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid; EPP = effective pollination period, FPA = formaldehyde/propionic acid/95% ethanol; OLP = ovule longevity period; PUT = putrescine; SAM = *S*-adenosyl-methionine; SPD = spermidine; SPM = spermine; TMF = time of maximum fertilization.

INTRODUCTION

Polyamines are low molecular weight aliphatic amine compounds ubiquitous in plants, animals, and bacteria. They have been associated with growth and tissue differentiation (Bagni et al., 1982; Galston, 1983).

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Polyamines delay senescence in some systems (Smith, 1985) in a manner similar to cytokinins. These compounds promote the incorporation of [^{3}H] uridine into RNA and decrease ribonuclease activity of oat leaf protoplasts (Shir et al., 1982) and *Helianthus tuberosus* protoplasts (Bagni et al., 1982). Altman and Bachrach (1981) found an increase in polyamine levels after plant hormone applications and suggested the possibility that polyamines might act as secondary messengers controlling nucleic acid and protein synthesis. Polyamines and polyamine biosynthetic enzyme levels have been associated with fertilization and growth in the reproductive organs of tobacco and tomato (Bagni et al., 1982), citrus (Nathan et al., 1984), and beans (Palavan and Galston, 1982).

On the other hand, the anti-senescent effects of polyamines may be related to inhibition of ethylene production, possibly via competition for *S*-adenosylmethionine (SAM). SAM is the precursor for both polyamines and 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor for ethylene biosynthesis (Galston and Kaur-Sawhney, 1980). This has been reported in senescing *Tradescantia* petals (Altman et al., 1977), tobacco leaf (Apelbaum et al., 1977), soybean hypocotyls (Sutter, 1981), and pear leaf and fruit discs (Toumadje and Richardson, 1988). Putrescine (PUT), spermine (SPM), and spermidine (SPD) inhibited incorporation of [1-^{14}C] methionine into ethylene, and reduced endogenous levels of ACC in pear (Toumadje and Richardson, 1988) and apple (Galston and Kaur-Sawhney, 1980) tissue. Furthermore, it has been suggested that polyamines may alter membrane properties inhibiting conversion of ACC to ethylene (Smith, 1985).

Recently, the polyamines PUT, SPM, and SPD were tested for their effect on fruit set and yield increase in apple (Costa and Bagni, 1983), olive (Rugini and Mencucciai, 1985), and pear (Crisosto et al., 1986). However, its mode of action on increasing fruit set and yield has not been studied in detail.

Crisosto et al. determined that under Oregon conditions the effective pollination period (EPP) (the remaining period of ovule viability beyond the time required for the first pollen tube to reach and fertilize the ovules) of cultivar 'Comice' was around 5 days. Ovule senescence was delayed and fruit set and yield were increased following floral application of 10^{-3} M putrescine during the 3 years of this study (Crisosto et al., 1986, 1988a,b). A similar situation occurred in pear cultivar 'Packam's Triumph' growing under Chilean conditions (data not shown).

This study continues our earlier work in 'Comice' (Crisosto et al., 1986, 1988a,b) to clarify the positive effect and mode of action of PUT on improving fruit set and yield.

MATERIALS AND METHODS

Putrescine at 10^{-3} M was foliarly applied to runoff in 1985 and 1986 2 days before anthesis to 12 'Comice' pear trees in a completely randomized design

(Crisosto et al., 1986, 1988a,b). Flowers were collected every 2 days to quantify evolved ethylene and polyamines (1985 only), and to analyze nitrogen content, ovule longevity, and fertilization time (number of days after hand-pollination). Selected flowers from untreated and treated trees were hand-pollinated at anthesis with cultivar 'Bartlett' pollen extracted from surface sterilized flowers previously forced under growth chamber conditions. 'Bartlett' branches were collected, and surface sterilized in 0.25% (w/v) sodium hypochlorite for 15 min and then rinsed three times with sterile distilled water. Branches were placed in a glass container with water and forced to grow under sterile conditions at a room temperature of 20°C.

Ovule longevity period (OLP) and fertilization time were determined as described by Crisosto et al. (1988b) while nitrogen was quantified by the modified Kjeldahl method according to Schuman et al. (1973). To measure ovule longevity at anthesis, flowers were emasculated and bagged. To quantify time of maximum fertilization (TMF), at anthesis, flowers were emasculated, hand pollinated with 'Bartlett' pollen and bagged. 'Bartlett' pollen was previously collected and stored under sterile conditions. Samples of 20 emasculated flowers and 20 emasculated and hand-pollinated flowers from the bagged branches were collected and fixed in formaldehyde/propionic acid/95% ethanol (FPA) in a proportion of 5:5:90 every 2 days for 12 days following anthesis in 1985 and 1986. Flowers were removed from the FPA, washed in distilled water for 30 min, soaked in 1% sodium carbonate for 1 h, and rinsed three times in distilled water. The pistil and ovary samples were softened by autoclaving in 1% sodium sulfite for 2 min. The ovaries were rinsed in water, split longitudinally, and the ovules were removed with fine forceps. Ovules and styles were then mounted on slides, squashed directly in 0.05% aniline blue in 0.15 M K_3PO_4, and observed in a Zeiss fluorescence microscope equipped for epi-illumination using near-UV excitation. Ovule longevity was based on the differential intensity of ovule fluorescence after staining with aniline blue, with ovules showing very intense fluorescence considered non-viable or senescent. Time of fertilization was determined as the number of days from hand pollination to the time when the pollen tubes could be seen entering the micropyle. Maximum fertilization was defined as occurring after the highest number of ovules were penetrated by 'Bartlett' pollen tubes (Crisosto et al., 1988b). Based on these measurements, EPP was calculated as the difference in number of days between OLP and TMF for each treatment.

The effect of 10^{-3} M PUT on ethylene production during the bloom period was determined on non-pollinated and hand-pollinated 'Comice' flowers growing under field and laboratory conditions.

For in vitro samples, gynoecium cultures of 'Comice' flowers were prepared. Buds were collected at the first open bud stage and surface sterilized in 0.25% (w/v) sodium hypochlorite for 15 min and then rinsed three times with sterile distilled water. Petals, sepals, stamens, and the basal part of the

flower were excised under sterile conditions. Sterile nutrient medium (Lane, 1979) was used for the gynoecium culture, with pH adjusted to 5.2. Ten gynoecia were planted to each container (12 cm × 10 cm) with 10 replicates for each sampling time. The gynoecium cultures were maintained at 20°C, under 45 μmol $m^{-2} s^{-1}$ of photosynthetically active photon flux density (cool white fluorescent lamps) with a 16 h photoperiod.

To determine evolved ethylene, eight replicate flower samples were excised (about 5 g fresh wt.) and held for 20 min, to release wound ethylene, then placed in 25 ml vials capped with serum stoppers. After 5 h, a 1 ml gas sample was withdrawn from each vial and assayed for ethylene by gas chromatography (Carle Analytical 311, 2 m × 4 mm, activated alumina column, 55°C). Vacuum-extracted ethylene was withdrawn from eight replicate freshly excised flower samples (about 10 g fresh wt.) under water in a vacuum desiccator. Sampling was completed within 10 min after excision, before wound ethylene production began. An inverted funnel was placed over the tissue and sealed with a serum stopper. The desiccator was evacuated (about −100 KPa) until gas bubbles from the tissue no longer appeared. Internal gas was collected from the funnel and assayed for ethylene by gas chromatography. A preliminary time course experiment on wounded and unwounded flower samples (data not shown) determined the holding time used for evolved and vacuum-exhausted ethylene determinations during this study.

Vacuum-extracted ethylene measurements were compared to ethylene evolved from excised flowers growing in the field and also from cultured gynoecia.

Polyamines were analyzed from 1985 flower samples. For this, polyamines from the gynoecium flower tissue were extracted in 3% perchloric acid, separated, and quantified (Flores and Galston, 1982) by isocratic HPLC analysis in 60% methanol at a flow rate of 1.0 ml min^{-1} (Beckman-Alltech Model 110A pump with a Econosil 5 μm reverse phase C-18 column (250 mm × 4.6 mm), 40°C). Polyamines were quantified at 254 nm using an Altex 155-00 flow cell in a Hitachi Model 100-10 spectrophotometer using authentic standards taken through the same isolation procedure.

The data were subjected to analysis of variance prior to being subjected to the Least Significant Means Separation Test using the Number Cruncher Statistical Package (Leroy Hintze, 856 East 400th, North Kaysville, Utah).

RESULTS

By 4 days after hand-pollination, PUT-treated ovules presented a significantly higher number of ovules penetrated than untreated ones. Thus, pollen tube penetration was observed 2 days earlier in ovules of PUT-treated flowers than in the untreated ones following hand pollination, although, during both

seasons, time of maximum fertilization was reached on the same day in both treatments (Table 1).

Putrescine at 10^{-3} M delayed 'Comice' pear ovule senescence by 2 days and 4 days in 1985 and 1986, respectively (Crisosto et al., 1988b). In the 1985 season, at the time of maximum pollen tube penetration (Day 6 after hand-pollination), only 28% of the untreated and 95% of the putrescine-treated ovules were viable resulting in 11% and 64% penetrated viable ovules, respectively (Table 1). Thus PUT-treated flowers had a higher percentage of fertilized viable ovules prior to complete ovule senescence. The difference in the number of viable ovules at fertilization was subsequently confirmed by greater seed number and fruit set in the PUT treatment (Crisosto et al., 1988a).

PUT treatment delayed OLP without affecting the TMF, and extended the EPP by 2 days and 4 days during the 1985 and 1986 seasons, respectively (Table 2).

In both seasons, PUT-treated flowers presented higher post-pollination flower nitrogen contents than untreated flowers (data not shown). This data confirm our previous work reporting an increase of nitrogen only in 'Comice' reproductive tissues (Crisosto et al., 1988b) and 'Packam's Triumph' gynoecium after putrescine application. In the 1985 season, gynoecium nitrogen levels became significantly higher in PUT-treated than untreated flowers 6 days after anthesis (maximum fertilization time) and remained higher during the post fertilization period (Fig. 1).

In the 1985 season, more ethylene evolved from hand-pollinated flowers than from not-pollinated flowers (Fig. 2). Evolved ethylene from flowers

TABLE 1

Effect of putrescine applied at anthesis on the rate of 'Bartlett' pollen tube penetration in 'Comice' ovules

Treatments	Percent of ovules penetrated by 'Bartlett' pollen tubes[1]				
	Days after anthesis				
	(0)	(2)	(4)	(6[2])	(8)
Control	0	0	2222	40 (11)	45
Putrescine (10^{-3} M)	0	5	30	65 (64)	60
Significance[3]	NS	NS	*	*	*

[1]Ovule penetration determined by aniline-blue stain method, 200 ovules examined at each period. Numbers between parentheses indicate percentage of viable ovules examined in the putrescine-treated and control flowers.

[2]Mean separation between treatments in same column by analysis of variance ($P=0.05$),

[3]NS, not significant; *, significant.

TABLE 2

Influence of putrescine application at anthesis on the time of maximum fertilization (TMF), ovule life period (OLP), and effective pollination period (EPP)

Treatment	Number of days		
	TMF	OLP	EPP
1985 season			
Untreated	6	8	2
PUT-treated	6	10	4
Significance	NS	*	*
1986 season			
Untreated	7	12	5
PUT-treated	7	16	9
Significance	NS	*	*

Mean separation between treatments in same year by analysis of variance ($P=0.05$). NS, *, Non-significant or significant by analysis, respectively.

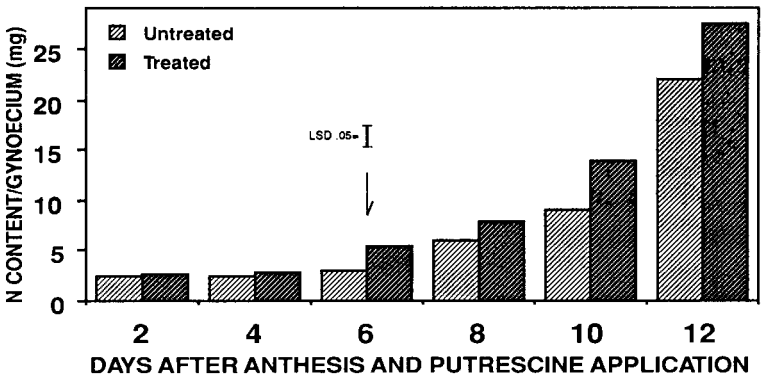

Fig. 1. Effect of 10^{-3} M putrescine at anthesis on N content of hand-pollinated 'Comice' flowers during bloom period. Arrow indicates time of maximum fertilization. Each value is an average of six replications.

growing in the field presented two peaks: the first, during hand-pollination and a second, large peak of evolved ethylene was observed in the pollinated treatments during petal fall and during fertilization time (Fig. 3). Petal abscission occurred about 4–8 days after anthesis in 1985 and 7–12 days after anthesis in 1986 (Fig. 3).

Putrescine applied at anthesis did not reduce the levels of ethylene evolved from flowers during the post-anthesis period in 1985 and 1986. Furthermore, PUT-treated flowers evolved more post-anthesis ethylene than untreated flowers during the 1986 season. PUT-treated flowers exhibited a lower level of ethylene evolving than untreated-flowers only during the first peak (just

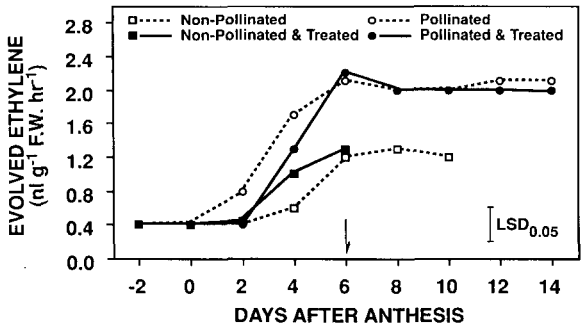

Fig. 2. Evolved ethylene levels of non-pollinated and handpollinated 'Comice' flowers with and without an application of 10^{-3} M putrescine at anthesis. Arrow indicates time of maximum fertilization. Bar indicates LSD at 5% level, each mean represents an average of five replications.

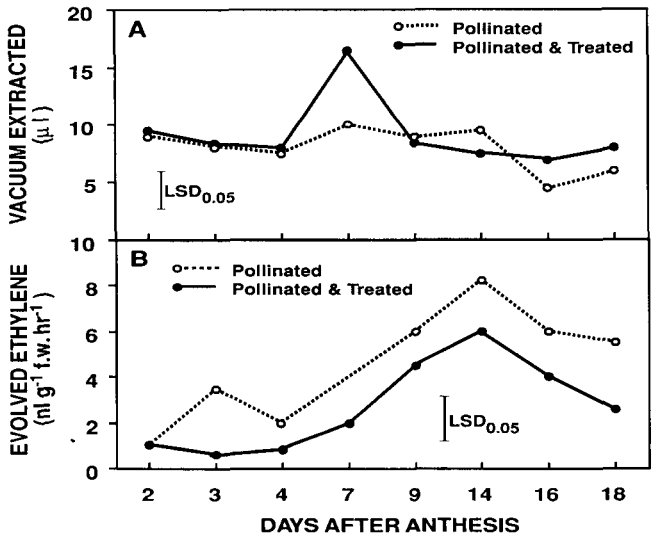

Fig. 3. Evolved and internal ethylene levels from hand-pollinated 'Comice' flowers with and without application of 10^{-3} M putrescine at anthesis. (A) vacuum extracted ethylene in hand-pollinated flowers and (B) evolved ethylene in hand pollinated flowers. Arrow indicates time of maximum fertilization. Bar indicates LSD at 5% level. Each mean represents an average of five replications.

after pollination). Halevy et al. (1984) determined that about 90% of the ethylene evolved from carnation flowers was derived from the petals. During the two seasons, petal abscission coincided with the time of maximum pollen penetration. The burst of ethylene produced during petal abscission could have masked the internal ethylene produced during the fertilization process.

Vacuum extracted internal ethylene peaked only once, coinciding with the

maximum fertilization time for PUT-treated and untreated flowers (Fig. 3). At the time of maximum fertilization, internal ethylene was higher in PUT-treated flowers than untreated.

Evolved ethylene from the gynoecium culture showed only one peak of ethylene during fertilization (Fig. 4). Even though fertilization was delayed 6 or 7 days in vitro for both treatments, evolved ethylene still coincided with the maximum fertilization time. PUT treatment yielded almost 250% more ethylene than the untreated one (Fig. 5). Internal flower ethylene levels from the field and evolved ethylene levels from the gyneocium culture had similar peaks. This suggests that ethylene was produced as a consequence of fertilization and its rate of production may be associated with high fertilization. The fact that ethylene evolved from the gynoecium culture and vacuum extracted internal ethylene presented a similar pattern suggests that the vacuum extracted internal ethylene method avoids the masking effect of evolved ethylene produced during petal abscission.

In the 1985 and 1986 samples, SPD and PUT were the most abundant polyamines, while SPM in both hand-pollinated and non-pollinated 'Comice' flowers occurred at levels only about one fifth as high. Similar data were reported for *Phaseolus vulgaris* flowers after pollination (Palavan and Galston, 1982). Hand-pollinated flowers contained higher polyamine levels after fertilization. During 1985, PUT treatment reduced endogenous polyamine levels (PUT, SPD and SPM) after fertilization in pollinated as well as in non-pollinated flowers, suggesting a possible relationship to an increase in nitrogen content in the treated flowers. A similar situation occurred in the 1986 season (data not shown).

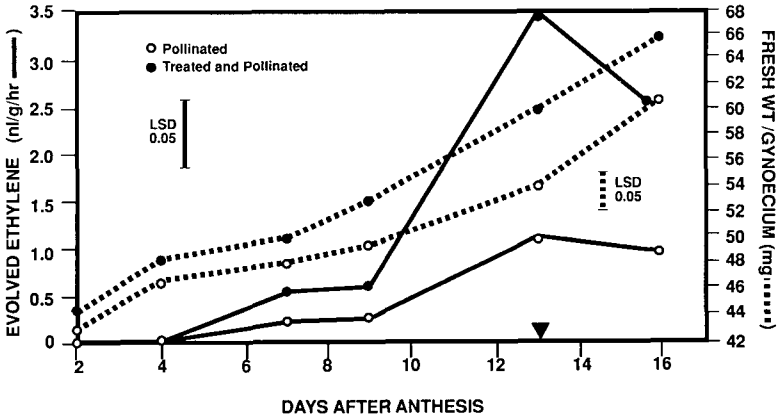

Fig. 4. Evolved ethylene level (solid line) and fresh weight (broken line) from hand-pollinated 'Comice' gynoecium growing in vitro with and without an application of 10^{-3} M putrescine at anthesis. Bar indicates LSD at 5% level. Arrow indicates time of maximum fertilization. Each mean represents an average of 10 replications.

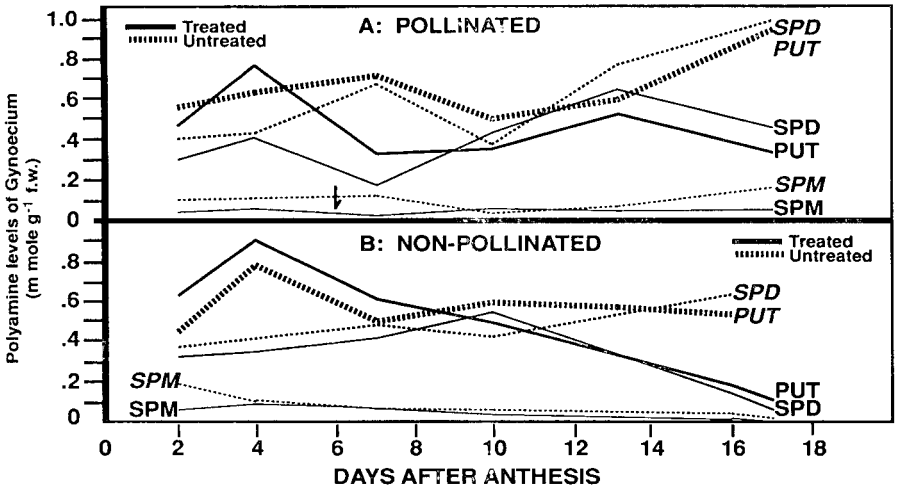

Fig. 5. Three endogenous polyamine levels of 'Comice' flowers with and without an application of 10^{-3} M putrescine at anthesis. Polyamines analyzed were PUT, SPM, and SPD. Arrow indicates time of maximum fertilization. Each mean represents an average of three replications. (A) polyamines in hand-pollinated flowers and (B) polyamines in non-pollinated flowers.

DISCUSSION

Delay of ovule senescence, a high rate of pollen tube growth and high fruit set obtained with PUT treatment was associated with a high nitrogen flower content and reduction in the endogenous polyamine levels. Bagni et al. (1982) reported that putrescine could act as a nitrogen source in *Helianthus tuberosus* growing in culture. However, in our case, the 0.001 M PUT solution applied at bloom contained only 0.09% (w/v) of nitrogen, which cannot account for the increased flower nitrogen occurring after fertilization. The pathway of PUT metabolism has been reported by several authors (Altman et al., 1977; Galston, 1983; Smith, 1985), showing that PUT biosynthesis occurs from ornithine and arginine via ornithine decarboxylase and arginine decarboxylase. The formation of SPM and SPD from PUT involves the use of SAM as a methyl donor and ethylene precursor. Polyamine degradation and conjugate formation in plant tissues has not been studied in detail (Smith, 1985). Holtta (1977), working on rat liver cells reported that radioactive exogenously applied polyamines were metabolized to arginine by a novel enzyme (polyamine oxidase) capable of forming PUT from SPD and SPM.

Our data showed a reduction in gynoecium polyamine levels and a greater production of ethylene and higher nitrogen content in treated flowers as a result of the exogenous PUT application. Based on this, we may speculate that exogenous PUT application stimulated polyamine oxidase which could

catabolize SPD, SPM, and PUT into arginine. However, further data with emphasis on conjugated polyamines is needed to verify whether the high nitrogen content in the flowers was a result of translocated nitrogen as a response to endogenous PUT and SPD catabolism or PUT may have delayed senescence reactions and/or stabilized membranes in pollen/ovular tissues as a primary reaction.

Evolved ethylene production during the bloom period showed two peaks. In relation to this first peak, Nichols et al. (1983) reported that pollination stimulated a sequential increase in ACC and evolved ethylene by stigmas, ovaries, receptacles, and petals of carnation flowers. Reid et al. (1984) hypothesized that ACC may be acting as a signal to prepare the ovules for fertilization. ACC in the stigma is translocated to petals and induces movement of nutrients, hormones, and carbohydrates from surrounding tissues toward ovules thus, delaying ovule senescence.

In our study, where ethylene production for PUT-treated flowers was lower than for untreated ones just after pollination a large amount of ACC (signal) may have been translocated to other flower tissues.

These data negate the hypothesis that the extension of ovule longevity, early pollen tube penetration and high fruit set in 'Comice' pear may be due to a reduction of the ethylene levels during the post-pollination period by the putrescine application. However, these data encourage further research to study the possible role of ACC as a pollen signal. In addition, the action of ethylene inhibitors at pollination time should be pursued to understand the role of ethylene in pollination and fertilization of fruit trees.

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