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Studies on pollen function and pistil development: Effects of pesticides and pollen diluents
(Under the direction of HAZEL Y. WETZSTEIN and S. EDWARD LAW)

The studies 1) determined the potential detrimental impact of various pesticides on pollination, and 2) evaluated suitability of selected dry particulate materials as pollen diluents for use in artificial pollination. Almond pollen germination and tube growth were detrimentally impacted by incorporation of 10 fungicides under in vitro conditions. Fungicides differed in their effects on pollen, suggesting in vitro tests can be used to assess relative toxicity. When sprayed onto apple flowers, captan significantly inhibited pollen germination on the stigmatic surface. By contrast, none of the fungicides evaluated showed significant impact upon pollen function on the stigma and in the style of almond flowers, although, SEM showed they can cause stigmatic papillae collapse and increase exudate secretion. In the pollen diluent study, we identified two promising diluent powders: Rilsan ® ES and polyester resin which may be helpful facilitating artificial pollination by achieving accurate and uniformly metered applications.

INDEX WORDS: Pesticides, Fungicides, Pollination, Pollen diluent, Stigma, Style, SEM, Spray

STUDIES ON POLLEN FUNCTION AND PISTIL DEVELOPMENT: EFFECTS OF PESTICIDES AND POLLEN DILUENTS

by

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DEDICATION

To my father, Tengyun Yi, my mother, Xinlan Wang, my sister, Weixin Yi, and my wife, Xiaoli Su for their love . . .

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 The role of pollination in agriculture

Adequate pollination is critical in agricultural crops where fruit or seed is the final product. In addition to directly affecting crop yield (via effects on fruit number), the extent of pollination (i.e., the number of pollen grains deposited on the stigmatic surface during its receptive stage) and subsequent fertilization are important factors in determining crop quality. Fruit size and shape, sugar content, and storage ability are enhanced with sufficient pollination in a number of crops. Reports include apple, kiwifruit, cacao, pear, citrus, strawberry, and cucurbits (Volz et al., 1996; Costa et al., 1993; Gonzalez et al., 1998; Falque et al., 1995; Kovach et al., 2000). Honeybees are estimated to play a pivotal role in pollinating at least 50 U.S. crops valued at $47 billion annually (Morse and Calderone, 2000). The value of pollination to agriculture is even greater when crops pollinated by other means (e.g., other insects, wind, birds, mammals) are included.

Unfortunately, insufficient pollination is one of the important causative factors for low yield in many field and orchard species (Shivanna and Sawhney, 1997). Reproductive strategies (such as dichogamy, dioecy, monoecy, and sexual incompatibility) have evolved to promote out-crossing and genetic diversity. However, these same mechanism can contribute to inadequate pollination during crop production. Lack of pollination has been identified as a factor limiting yield and quality in a number

of crops, and has been exacerbated by the decimation of bee populations due to mite infestation, habitat destruction, excessive pesticide use, and unfavorable climate.

1.2 Fungicide effects on pollination

1.2.1 Fungicide application in agriculture

Applications of early- season fungicides are necessary in most fruit and vegetable crops to protect susceptible young foliage and/or floral parts. Fungicide sprays are often prescribed to control the onset of disease, particularly in seasons when weather conditions are conducive to disease development. In some fruit crops, fungicide applications are targeted specifically into the open bloom to control plant pathogens that cause flower blights or fruit infection (Ogawa and English, 1991; Ploetz et al., 1994). The timing of fungicide applications, thus, necessarily overlaps the time at which flowering and pollination occur. Fungicides are applied routinely during the bloom period to control diseases such as brown rot blossom blight (almond), gray mold (blueberry, strawberry), powdery mildew (mango, strawberry), anthracnose (strawberry), and mummy berry disease (blueberry).

1.2.2 Potential fungicide effects on pollen germination, tube growth, fruit set, and yield

Critical events are associated with pollination in higher plants and include the development of a receptive stigma and functional pollen, pollen transfer and attachment, pollen hydration and activation, pollen germination, and tube growth (Johri, 1984; Raghavan, 1997; Wetzstein, 1989; Wetzstein and Sparks, 1989). If these events occur successfully, fertilization and fruit set can proceed. However, it has been shown that certain fungicides can impact any one or several of these crucial steps.

Numerous studies report detrimental effects of agrochemical sprays on pollen germination, fruit set and/or yield in selected crops. Reports include those for apple and

pear (Hutcheon et al., 1986; Locke and Andrews, 1986; Eaton, 1963; Fell et al., 1983; Church and Williams, 1977; Church and Williams, 1978; Zuccherelli et al., 1996; Williams et al., 1987; Church et al., 1984; Marcucci and Filiti, 1984; Legge and Williams, 1975a), blueberry (Bristow and Windom, 1987; Bristow, 1981), raspberry (Redalen, 1980), cranberry (Bristow and Shawa, 1981), mango (Dag et al., 2002), strawberry (Eaton and Chen, 1969), and pecan (Wetzstein, 1990; He and Wetzstein, 1994). Using *Tradescantia* as a model system, immunolocalizations showed that benomyl and triazole fungicides inhibit pollen tube growth by altering microtubule organization and inhibiting tube elongation (He et al., 1995; He et al., 1996).

Certain chemical sprays have been reported to cause remarkably detrimental effects to fruit set and yield. Kovach et al. (2000) evaluated the effects of captan, benomyl, iprodione and vinclozolin, and reported that some of these fungicides applied at bloom reduced the number of seeds per berry and subsequently berry weight in field tests in strawberry. Eaton and Chen (1969) reported that captan decreased strawberry achene set and berry development in the greenhouse and increased the proportion of misshapen fruits when sprayed after anther dehiscence. In cranberry, triforine-EC resulted in the production of smaller and lighter berries and significantly reduced yield by 41% (Bristow and Shawa, 1981). In apple, Legge and Williams (1975a) reported that captan and binapacryl significantly decreased fruit set when sprayed 2 to 4 hours after pollination under orchard conditions. Church and Williams (1983) reported that dodine deceased fruit set in apple in one year of a 2-year study. Also in apple, Donoho (1964) conducted orchard tests for three consecutive years and assessed fruit set and yield compared with a captan + DDT standard. They found that trees consistently having the lowest fruit set and yields were treated with phenyl mercury acetate + DDT. 2, 3-

dichloro-1,4-Naphthoquinone + DDT and 0,0-Dimethyl S-(5-oxo-1,2,3,-benzotriozinyl-3-methyl) phosphorodithioate + captan also reduced set, but to a lesser extent. 1-naphthyl N-methyl-carbamate + captan reduced fruit set and yields only when the application began at petal fall. MacDaniels and Hildebrand (1940) found that 2-6-100 Bordeaux mixture, 20-80 copper-lime dust, and sulfur significantly reduced pollen germination on stigmata of apple. Elgetol at 0.25% altogether prevented pollen germination. Field trials indicated that this material significantly inhibited fruit set on apple trees.

Detrimental effects of fungicides on pollen germination and tube growth in the style have been reported in some crops. Lockhart (1967) evaluated the effects of ferbam, zineb, and ziram in lowbush blueberry. A fungicide-coated spatula was used to place pollen grains on the stigmata of lowbush blueberry flowers in the greenhouse. For dry conditions, the flowers were held in the greenhouse while for wet conditions, the flowers were sprayed immediately following pollination and then held in a mist chamber. Under wet conditions, all three fungicides inhibited germination, while under dry conditions only ferbam and ziram had some inhibitory effect. In highbush blueberry, triforine significantly inhibited pollen germination on the stigma and tube growth in the style when it was applied directly to the stigma at recommended rates (Bristow, 1981). In cranberry, captafol and cupric hydroxide decreased pollen germination by more than 50% when they were applied to the stigmatic surface 1 hr before pollination. Church and Williams (1977) found that captan, dinocap, binapacryl, and dithianon significantly reduced pollen germination on stigmata under greenhouse conditions. Church et al. (1983) found that captan and dinocap significantly inhibited pollen germination and tube

growth in intact apple flowers under orchard conditions when they were sprayed 2 hours after pollination.

The stigma is the receptive surface for pollen. It can provide nutrients to the pollen and direct pollen tube growth. A functional stigmatic surface provides a correct physiological condition for the pollen, i.e., a balanced osmolarity and a sufficient water supply (Johri, 1984). Any damage to the stigmatic surface by fungicide sprays may potentially cause the pollination process to fail. Published studies evaluating the effects of pesticides on stigma morphology are extremely limited. The only evaluation was in pecan. Pesticide sprays applied in pecan caused varying degrees of injury in the stigma, ranging from minor surface wrinkling with triphenyltin hydroxide to severe collapse and degeneration of stigmatic papillae with phosalone treatment (Wetzstein, 1990).

1.2.3 Potential fungicide effects on pollen germination, tube growth, fruit set, and yield in apple and almond

Almond (*Prunus dulcis*) is an important nut crop which blooms relatively early in the spring (February in California). The crop is self-incompatible and requires cross-pollination. Pollination can be limiting in production areas in part due to the cool, damp weather conditions during bloom which may limit insect activity. It has been reported that the percentage of fruit set in commercial orchards is commonly only 5 to 30% of that possible (Gary et al., 1976). This crop is heavily sprayed during the bloom period to control blossom blight. Unfortunately, little is known about the effects of fungicides on pollen germination and tube growth in almond. Interference of pollen germination and function by fungicide sprays during the pollination period would be of particular concern in almond production.

Apple *(Malus domestica)* is a major fruit crop worldwide and includes in its family a number of other economically important species. Insufficient pollination has been shown to limit yield, particularly in 'Red Delicious' (Stern et al., 2002). Fruit shape, size, and ability to stand long storage are affected by pollination and the number of seed set per fruit (Volz et al., 1996).

With apple, several researchers have reported the inhibitory effects of different fungicides on pollen germination in medium/agar. Reports include thiophanate-methyl, benomyl, sulfur, binapacryl, bupirimate, mancozeb, zineb, dinocap, triforine, captan, dithianon (Church and Williams, 1977); captan and binapacryl (Legge and Williams, 1975a); dinocap, binapacryl, ditalimfos, pallinal, bupirimate, fenarimol (Butt et al., 1985); captan, dithianon, mancozeb, sulfur (Marcucci and Filiti, 1984); hexythiazox 50 WP (Mayer and Lunden, 1986); captan, dikar (Fell et al., 1983); captan, dinocap, sulfur, triforine, dithianon (Church and Williams, 1978); captan (Eaton, 1963).

However, there are conflicting reports concerning effects of chemical sprays on fruit set even when similar compounds are tested. Legge and Williams (1975a) found that captan and binapacryl significantly inhibited fruit set when they were applied during bloom. In contrast, Church and Williams (1977) found no effects on fruit set with applications of captan, binapacryl, dinocap, dithianon, thiophanate-methyl, or benomyl. In another study, Church et al. (1983) found that dinocap applied during bloom significantly decreased initial fruit set, while captan did not. In field tests, Brown and Hendrix (1978) concluded that captan, benomyl, maneb, folpet, captafol, chlorothalonil, and thiophanate-methyl applied to apple trees in bloom did not significantly affect fruit set. MacDaniels and Burrell (1934) reported that sulfur applied either before or shortly after pollination significantly reduced the set of fruits. Rich (1957) in an evaluation of

several fungicides concluded that none of these compounds tested, including sulfur, reduced pollen germination or fruit set when applied to apple trees in bloom. Environmental conditions of studies can moderate the effects of fungicides and may contribute to these discrepancies. Church and Williams (1983) conducted tests in the orchard and greenhouse. They found that dodine significantly decreased fruit set under both conditions. Sulfur significantly decreased fruit set under greenhouse conditions, but increased fruit set under orchard conditions.

1.3 Artificial pollination

1.3.1 Agricultural significance

Artificial pollination can be an effective means to overcome pollen-related production problems. Hand pollination has been implemented in agricultural production for some time. MacDaniels (1930) reported hand pollination was reliable to increase fruit set in 'Delicious' and 'Oldenburg' apple. Artificial pollination of tree fruits on a commercial basis was initiated in the state of Washington in the 1920s, and the increase in yield was greatest where pollination by natural means was not satisfactory (Bullock and Overley, 1949). Large numbers of pollen grains deposited on the stigma can be beneficial to overall fruit set. Dennis (1979) found that a minimum of 50 pollen grains per flower is required for consistent fruit set in 'Delicious' apple even though fruits generally contain only ten ovules. Fewer grains resulted in poorer germination and slower tube growth. Orchard studies in kiwifruit showed that hand pollination produced maximal fruit size that was not obtainable with wind and bee pollination (Costa et al., 1993). In apple, Volz et al. (1996) documented that supplementary pollination increased initial and final set on spur and terminal sites, seed number, and final fruit calcium concentrations compared with bee-only pollination. Recently, electrostatically charged

pollen was applied supplementally to almond and increased pollination, fruit set, and yield (Law et al., 2000; Vaknin et al., 2001).

1.3.2 Selection of pollen diluent powders

In most crops, pollen acquisition is difficult and the high cost of pollen may prohibit the use of supplemental pollination techniques unless efficiently applied. Unfortunately, the efficient application of small volumes of pollen is difficult in achieving accurate and uniformly metered applications. The identification of dry particulates which could be used as pollen diluents would therefore be highly beneficial. This is particularly important in mass supplemental pollination of taxa which have pollen that is sticky and clumps. Incorporation of a diluent may improve the flow and provide more uniform grain distribution.

In addition, the identification of useful diluent powders would be helpful in seed production and breeding programs where controlled artificial pollinations are necessary to produce inbred lines for certain self-incompatible types and hybrids (Desai et al., 1997). In many cases, the number of pollen grains added to stigmata exceed that required for adequate seed set due to volumes dictated by application methods. Use of a pollen diluent would extend the use of collected pollen and decrease the costs required for pollen acquistion. This is of particular interest in species where pollen is very hard to collect. Also, diluents would be useful in reproductive studies evaluating pollen density effects and pollen competition. In many cases, the effects on pollen function have not been ascertained for substances used.

To be effective, a diluent must be nontoxic and have little or no inhibitory effects on pollen viability and germination. In addition, conditions in pollen-diluent mixtures must be such that pollen viability is not compromised by desiccation or other physical

degradation, and that the pollen is not mechanically damaged during mixing and handling due to angular surface configurations of particulates. The diluent must also be of a comparable size so that homogeneous mixtures of pollen and diluent can be consistently maintained during application.

The use of different powders as pollen diluents has been reported. These include *Lycopodium* spores in almond (Thorp et al., 1967); alder pollen (Legge and Williams, 1975b), diatomaceous earth, bentonite, magnesium oxide, wheat flour, cake flour, lycopodium spores, walnut shell meal, corn meal, charcoal, different grades of ground Douglas fir bark, dicolite, Fullers earth and corn starch (Overley and Bullock,1947), pine pollen and polyvinylchloride granules (Williams and Legge, 1979) in apple; and talc in raspberry (Jennings and Topham, 1971). However, reports about systematic comparisons of different kinds of pollen diluents are limited, as is information regarding their effects on pollen function and pollination.

1.4 Objectives and plan of research

The **objectives** in these studies were:

1. To evaluate the effects of selected fungicides on aspects of pollination in almond and apple.
2. To evaluate the suitability of selected dry particulate materials as pollen diluents in artificial pollination using petunia as a model system.

The **research plan** for achieving these objectives were divided into two parts:

1. Fungicide studies

a) *Develop in vitro germination assays to assess the relative toxicity of various fungicides on pollen germination and tube growth.*

These studies quantified the relative toxicity of various fungicidal agents toward almond pollen in vitro. This would provide a useful initial screen of agrochemicals for their impact on pollen germination and development.

b) *Determine the in vivo effects of fungicides on pollen germination and tube growth when applied as sprays to detached flowers.*

Fungicide effects on pollen function in vivo were affected by considerations such as the persistence of the chemical and how spray residues interact with the pistil. Pollen germination and tube growth through the style was followed microscopically to assess the effects of chemicals. Two fruit crops, almond and apple, were evaluated.

c) *Evaluate the effects of fungicide sprays on the development and receptivity of the stigmatic surface in flowers.*

Pesticide sprays may affect the viability and receptivity of the stigmatic surface (which serves as the site of pollen deposition and germination). Pesticides may interact with constituents of the stigmatic surface and affect pollination and receptivity. Structural studies of treated flower assessed if inhibition of pistil development occurred. Almond flowers were used in this study.

2. Pollen diluent studies

In preliminary assessments of potential diluents, Wetzstein and Law (1998) evaluated the size and morphological characteristics of several powders. Germination assays were conducted of pollen mixed with different diluents, and toxicity of compounds was determined in germination studies containing diluent extracts. No inhibition was observed with two Rilsan® resin formulations, polyester resin, and wheat

flour. The objectives of the current study were to further evaluate these powders as well as *Lycopodium* spores.

a) *Evaluate the effects of storage time with diluents on pollen germination.*

Pollen-diluent storage studies evaluated the viability of pollen held intermixed with diluents for durations up to 6 days.

b) *Assess pollen performance in the style after pollination with pollen-diluent mixtures on the stigma.*

Pollen tube growth studies quantified the number of tubes growing in the styles of petunia using fluorescence staining.

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CHAPTER II

AN IN VITRO STUDY OF FUNGICIDE EFFECTS ON POLLEN GERMINATION AND TUBE GROWTH IN ALMOND

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Abstract. In almond, fungicide sprays are required to prevent blossom blight which can infect open flowers. Numerious studies have reported detrimental effects of agrochemical sprays on pollination, fruit set and yield in fruit tree crops. However, effects of fungicides on pollen germination and growth in almond are little known, particularly with recently developed active ingredients. This study evaluated the effects of commercial formulations of ten fungicides on pollen germination and tube growth in almond using in vitro assays. Assays conducted at 1/100 recommended field rates (RFR) were effective in delineating differences in almond pollen sensitivity to the different fungicides. Captan and azoxystrobin were the most inhibitory, with germination percentages of less than 1% of the no-fungicide control. Germination was not significantly affected by propiconazole and benomyl. Intermediate inhibitory effects on pollen germination were observed with ziram, cyprodinil, maneb, thiophanate-methyl, iprodione, and myclobutanil. In contrast to germination, tube growth was less affected by the presence of fungicide. In pollen that germinated, tube elongation was the same as controls in half of the fungicides evaluated. Nonetheless, azoxystrobin and captan reduced tube elongation by ca. 90%. Some fungicide treatments also influenced tube morphology. In the absence of field evaluation studies, in vitro germination data may provide insight on how specific chemicals may impact pollination processes and further guide in vivo studies particularly in the case of new chemical formulations .

Introduction

Applications of early-season fungicides are necessary in most fruit crops to protect susceptible foliage and/or floral parts. In some cases, fungicide applications are targeted specifically into the open bloom to control plant pathogens that cause flower blights or fruit infection (Ogawa and English, 1991; Ploetz et al., 1994). In almond, fungicide sprays are required to prevent blossom blight caused by *Monilinia fructicola* or *M. laxa* which can infect various flower parts during bloom. Thus the timing of fungicide applications necessarily overlaps the time at which flowering and pollination occur.

Numerious studies report a detrimental effect of agrochemical sprays on pollen germination, fruit set and/or yield in fruit tree crops. Reports include those for apple (Church and Williams, 1977; Legge and Williams, 1975), mango (Dag et al., 2002), pear (Butt et al., 1985; Marcucci and Filiti, 1984; Mayer and Lunden, 1986), pecan (Wetzstein, 1990; He and Wetzstein, 1994), and sweet cherry (Eaton, 1961). Effects of fungicides on pollen germination and growth in almond are little known. Particularly with the advent of new active ingredients (e.g., strobilurin and azole products), comprehensive assessments regarding the effects of fungicides with different modes of action are lacking. Information on the relative inhibitory effects of fungicides would be valuable for the development of spray programs that would provide efficacy in disease control, yet have minimal inhibition on pollen function.

In vitro germination assays are routinely used to assess pollen viability (Kearns and Inouye, 1993), and have been used to determine relative toxicity of chemical substances on pollen function (Eaton and Chen, 1969). With apple pollen, in vitro assays were effective in assessing the relative toxicity of several commercial fungicides in that

good correlations were obtained between in vivo and in vitro results (Church and Williams, 1977). The objectives of the current study were to evaluate the effects of selected fungicides on pollen germination and tube growth in almond using in vitro assays. Results of this screening will be used in further laboratory and field studies to assess pollen function in vivo.

Materials and Methods

Almond [*Prunus dulcis* (Mill.) D. A. Webb] pollen was collected from 'Butte' trees in Bakersfield, California. To maintain viability, pollen was stored at -20 ^{o}C until use. Ten fungicides potentially applied to almond during the blooming season were selected for study: 1) myclobutanil, Rohm and Haas Co., Philadelphia; 2) propiconazole, Novartis Crop Protection, Inc., Greensboro, N.C.; 3) benomyl, DuPont, Wilmington, Del.; 4) thiophanate-methyl, Elf Atochem North America, Inc., Philadelphia; 5) iprodione, Aventis CS, Research Triangle Pk, N.C.; 6) captan, Micro Flo Company, Memphis, Tenn.; 7) maneb, Griffin L.L.C., Valdosta, Ga.; 8) ziram, Elf Atochem North America, Inc., Philadelphia; 9) cyprodinil, Novartis Crop Protection, Inc., Greensboro, N.C.; 10) azoxystrobin, Zeneca Agricultural Products, Wilmington, Del. The class, trade name, formulation and the recommended field rate for each of the compounds are shown in Table 2.1. Evaluations were conducted in medium at three concentrations: the recommended field rate (RFR), 1/10 RFR, and 1/100 RFR of each fungicides. In fungicides where a range of rates was prescribed, an average concentration of upper and lower values was used. Germination medium without fungicide served as controls.

Pollen germination was assessed using an in vitro assay. The germination medium consisted of a solution of 12% sucrose (w/v), 0.062% $CaNO_3$ (w/v) and 0.024% boric acid (w/v). Assays were conducted in tissue culture plates (Falcon Microtest III, Lincoln

Park, N.J.). For each assay, 200 µl of medium with or without fungicides was placed into each well. Approximately 400 pollen grains were inoculated uniformly into each well. To insure proper mixing of pollen and germination medium, the contents of each well were agitated by repeatedly drawing and expelling the contents through a 200 µl micropipette. Culture plates were placed into a dark incubator at 27 ºC. After 2.5 hrs, 20 µl of HistoChoice (Amresco Inc., Solon, Ohio) fixative was added into each assay well to arrest tube growth and preserve tube morphology. Culture plates were examined using a inverted microscope (Nikon, Garden City, N.Y.). Pollen was counted as germinated if tube extension was greater than the diameter of pollen grains. Tube lengths of germinated pollen grains were measured using a gridded ocular. Average tube lengths were calculated from measurements of randomly selected pollen grains.

A factorial design was used in this study. Each treatment (10 fungicides x 3 concentrations plus control) was replicated 5 times. Statistical analysis was conducted using Duncan's multiple range test within the general linear model procedure of SAS (SAS Institute Inc., Cary, N.C.). For germination percentage, data were transformed with arcsine to meet the equal variance assumption (Gomez and Gomez, 1984).

Results and Discussion

Germination. Relative germination percentages were calculated based on actual treatment germination percentages relative to the control which was expressed as 100% [i.e. (actual treatment % germination / control treatment % germination) x 100]. Relative germination percentages for pollen with different concentrations of fungicide incorporated into the germination medium are shown in Table 2.2. No pollen germination was observed in assays incorporating any of the fungicides at recommended field rates. This was in contrast to the control, which showed 53.7% actual germination.

Pollen grains typically exhibit high sensitivity to chemicals with in vitro germination assays where contact with chemicals is intense. Although germination also was limited severely at 1/10 RFR, the fungicides differential effects on pollen germination. No pollen germination was observed in the presence of maneb, cyprodinil, ziram, azoxystrobin, and captan. Germination rates in propiconazole and iprodione were only about 10% of the control. The highest relative germination occurred in the presence of myclobutanil, which was about 60% of the control. No data were taken for the two highest concentrations of benomyl and thiophanate-methyl, because particulate materials of the fungicide formulations obscured counting.

Assays conducted at 1/100 RFR were effective in delineating differences in almond pollen sensitivity to the different fungicides (Table 2.2). Captan and azoxystrobin remained extremely inhibitory, with germination less than 1% of the controls. Ziram and cyprodinil were also strongly inhibitory. In contrast, germination did not differ from the control when propiconazole or benomyl was incorporated into the medium. Pollen germination in the presence of myclobutanil exhibited slight but significant inhibition at 76% that of the control. Intermediate inhibitory effects on pollen germination were observed with maneb, thiophanate-methyl, and iprodione with relative germination ranging from 51 to 63% of the control.

Inhibition of pollen germination by some of the same fungicides has been reported in other species. The inhibitory effects of captan, benomyl, and thiophanate-methyl were observed in apple pollen germinated in vitro (Church and Williams, 1977). Likewise, captan was reported to inhibit pollen germination in pear (Butt et al., 1985). Ziram was also found to inhibit pollen germination in lowbush blueberry (Lockhart, 1967). In pecan, propiconazole, thiophanate-methyl, and benomyl were reported to

inhibit pollen germination (He and Wetzstein, 1994). From our test results, the two new azole products, propiconazole and myclobutanil, had little or no inhibition, while the other new product, azoxystrobin, had very toxic effects. This illustrates the potential usefulness of in vitro assays for evaluating new chemicals.

Tube Growth. In general, the fungicides that had the greatest inhibition on pollen germination also had the greatest inhibitory effects on tube growth (Table 2.3). Of the limited pollen grains that germinated with captan and azoxystrobin at 1/100 RFR, tube length was only 10% of the control. Tube lengths of pollen germinated in the presence of propiconazole, benomyl, myclobutanil, iprodione, or thiophanate-methyl at 1/100 RFR were not significantly different from that of the control. This was the case even though myclobutanil, iprodione, and thiophanate-methyl significantly inhibited pollen germination percentages (Table 2.2). Intermediate inhibitory effects on tube length were observed with maneb, cyprodinil, and ziram (Table 2.3), where pollen exhibited 32%, 32%, and 21% of the tube length of controls, respectively.

In contrast to pollen germination, pollen tube growth was less affected by the presence of fungicide. In pollen that did germinate, tube elongation was no different than controls in half of the fungicides evaluated even though pollen were in continuous contact with fungicides under in vitro assay conditions. Nonetheless, some chemicals reduced tube elongation by ca. 90%. Captan and azoxystrobin were extremely inhibitory to both pollen germination and tube growth.

Tube Morphology. In addition to inhibiting pollen germination and tube elongation, some fungicide treatments also influenced tube morphology. Pollen germinating in the absence of fungicides had tubes which were straight, long, and smooth with tapering ends (Fig. 2.1 A). Pollen tubes grown in the presence of benomyl

exhibited tubes of similar length to the control. However, some pollen tubes were characterized by swelling and rupture in the tip region (Fig. 2.1 B). In the presence of myclobutanil, pollen tubes exhibited abnormal growth with tubes changing direction during extension (Fig. 2.1 C). Pollen tubes that germinated and grew in the presence of propiconazole, had a sinuous, wavy configuration (Fig. 2.1 D).

Some fungicides have been shown previously to exhibit marked effects on plant developmental processes including effects on vegetative and reproductive structures (Wetzstein et al., 2002; Koller, 1987). Propiconazole has been reported to alter cell arrangement in leaves of pecan shoots (Wetzstein et al., 2002). Benomyl induced abnormal pollen tube morphology and microtubule organization. In *Tradescantia virginiana*, benomyl induced pollen tube rupture in the apical region (He et al., 1996), which is similar to the phenomenon observed in the current study. Also in *T. virginiana*, He et al. (1995) reported propiconazole inhibited cytoplasmic streaming, induced abnormal tube morphology and cytosketetal distribution, and affected both microfilaments and microtubules. In some situations, pollen tube growth and vigor may influence successful fertilization. If pollen exhibits divergent growth patterns in vivo, fungicides may impact successful fertilization.

A number of the compounds tested included fungicides in the same chemical class, i.e., two azoles, two benzimidazoles, two dicarboximides, and two dithiocarbamates were evaluated. Some generalities were observed related to chemical class. The azole compounds (myclobutanil and propiconazole) exhibited no or very limited inhibition of pollen germination and tube growth. The benamidazoles (benomyl and thiophanate-methyl) had no-to-intermediate effects. The dithiocarbamate compounds (maneb and ziram) and the pyrimidine (cyprodinil) more severely suppressed pollen

germination and growth. Azoxystrobin, a strobilurin, was highly inhibitory. In contrast, the two dicarboximides (iprodione and captan) were highly variable in their effect on pollen function. This indicates the necessity to evaluate how new fungicide products may effect pollen function.

This study provides data on the relative toxicity of different classes of fungicides on pollen germination and tube growth in almond. Several compounds were identified (i.e., propiconazole, benomyl, and myclobutanil) that had little or no inhibition on pollen function. Likewise, several compounds severely suppressed pollen germination (i.e., azoxystrobin and captan). It should be noted that effects of fungicides on pollen under in vivo conditions will be affected by additional considerations such as the persistence of the chemical, whether or not it is systemic, and how it may interact with the constituents of the stigmatic papillae. In the absence of field evaluations, particularly in the case of new chemical formulations, in vitro germination studies may provide insight in how specific chemicals may impact pollination processes and further guide in vivo studies. Conditions may arise where effective disease control can be obtained by a number of fungicides. Particularly under situations where pollination may be limiting, important benefits may accrue by selecting fungicides that have the least detrimental impact on pollen performance.

Figure 2.1. The effects of fungicides on almond pollen tube morphology in vitro. Pictures were taken 2.5 hours after pollen was inoculated into germination medium with or without fungicides. A. control (pollen tubes germinated in the absence of fungicides); B. pollen tubes germinated in medium with benomyl at 1/100 RFR; C. pollen tubes germinated in medium with myclobutanil at 1/100 RFR; D. pollen tubes germinated in medium with propiconazole at 1/100 RFR.

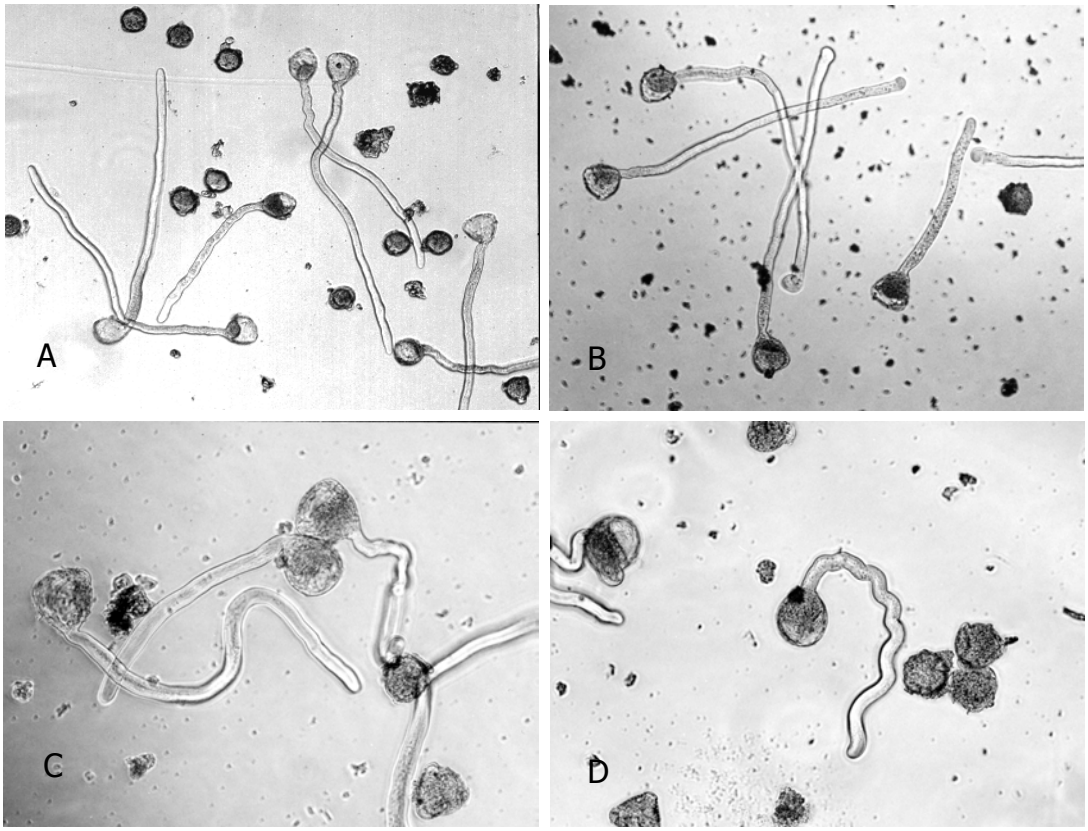
A
B
C
D

Table 2.1. Selected fungicides and their recommended field rates.

Active ingred.	Class	Trade name	Formulation [x]	Percentage of active ingred.	Recommended field rate used (per acre)[z]	Lab equivalent [y]
Myclobutanil	Azole	Rally	WSP	40.0	1.625 oz.	0.24 mg/ml
Propiconazole	Azole	Break	EC	41.8	4 fl. oz.	0.63 ml/l
Benomyl	Benzimidazole	Benlate	SP	50.0	20 oz.	3.0 mg/ml
Thiophanate-methyl	Benzimidazole	Topsin M	WSP	70.0	1.5 lb.	3.6 mg/ml
Iprodione	Dicarboximide	Rovral	powder	50.0	1 lb.	2.4 mg/ml
Captan	Dicarboximide	Captan	WP	48.9	6.5 lb.	15.6 mg/ml
Maneb	Dithiocarbamate	Manex	flowable	37.0	5.6 qt.	28.0 ml/l
Ziram	Dithiocarbamate	Ziram	WG	76.0	8.0 lb.	19.2 mg/ml
Cyprodinil	Pyrimidine	Vangard	WG	75.0	7.5 oz.	1.1 mg/ml
Azoxystrobin	Strobilurin	Abound	flowable	22.9	7.7 fl. oz.	1.2 ml/l

[z] Recommended field rates in the amount of commercial formulation of fungicides per acre.

[y] Fungicide concentrations in medium were calculated based on a 50 gallon spray volume per acre.

[x] WSP = water soluble pouches, EC = emulsifiable concentrate, SP = wettable powder in water soluble film, WP = wettable powder, WG = water dispersable granule.

Table 2.2. Germination [z] of almond pollen in the presence of selected fungicides.

Fungicide	Fungicide concentrations (RFR)		
	1	1/10	1/100
No fungicide control	100 a [y]	100 a	100 a
Propiconazole	0.0 b	8.0 cd	103.9 a
Benomyl	- [x]	-	96.3 a
Myclobutanil	0.0 b	58.3 b	76.4 b
Iprodione	0.0 b	11.2 c	62.9 c
Thiophanate-methyl	-	-	59.4 cd
Maneb	0.0 b	0.0 d	50.7 d
Cyprodinil	0.0 b	0.0 d	30.2 e
Ziram	0.0 b	0.0 d	18.8 e
Azoxystrobin	0.0 b	0.0 d	0.6 f
Captan	0.0 b	0.0 d	0.2 f

[z] Germination percentages shown are relative to the control which is expressed as 100%. Actual pollen germination in the no fungicide control was 53.7%.

[y] Mean values within a column followed by the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's multiple range test.

[x] Not counted because of interference of particulate materials from the fungicide.

RFR: Recommended Field Rate.

Table 2.3. Tube growth of almond pollen germinated in the presence of selected fungicides.

Fungicide	Tube length (μm) [z]		
	1 RFR	1/10 RFR	1/100 RFR
No fungicide control	504 a	504 a [y]	504 a
Propiconazole	0 b	93 d	422 a
Benomyl	- [x]	-	453 a
Myclobutanil	0 b	349 b	459 a
Iprodione	0 b	202 c	442 a
Thiophanate-methyl	-	-	403 a
Maneb	0 b	0 e	165 b
Cyprodinil	0 b	0 e	163 b
Ziram	0 b	0 e	107 c
Azoxystrobin	0 b	0 e	50 d
Captan	0 b	0 e	53 d

[z] Mean length of pollen tubes germinated in medium with fungicides at 1, 1/10, or 1/100 times recommended field rates. Length was measured 2.5 hours after inoculation.

[y] Mean values within a column followed by the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's multiple range test.

[x] Not measured because of interference of particulate materials from the fungicide.

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CHAPTER III

FUNGICIDE SPRAYS CAN INJURE THE STIGMATIC SURFACE DURING RECEPTIVITY IN ALMOND

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Abstract. Fungicides applied during flowering can be detrimental to flower development, pollen function, and/or fruit set in a number of crops. Almond is a nut crop which is self incompatible, requires cross pollination, and has a fruit set of only ca. 30% of the total flowers. Thus, interference of pollination and fertilization by fungicide sprays are of particular concern in almond production. Selection of chemicals having the least detrimental effects would be highly desirable. The objective of this study was to evaluate the effect of specific fungicide sprays on stigma morphology and function in almond using a mechanized spray apparatus to simulate field application conditions. Four fungicides (azoxystrobin, myclobutanil, iprodione, and cyprodinil) were applied to detached flowers in the laboratory. Fresh, unfixed stigmatic surfaces were observed by scanning electron microscopy at 4 or 24 hrs after spray. Increased exudate accumulation was induced by azoxystrobin at both 4 and 24 hrs after spray, and localized damage and collapse of stigmatic cells were observed at 24 hrs elapsed time. Damaged areas exhibited wrinkling, distortion of cell surfaces, or concave stigmatic papillae. Similar to azoxystrobin, myclobutanil caused significant damage and collapse to stigmatic papillae which were more extensive at later observations. Slight increase of exudate accumulation was observed 4 hrs after spray. Iprodione had no effects on exudate accumulation, but caused marked and severe collapse of stigmatic papillae which was more pronounced at 24 hrs vs 4 hrs after spray. Cyprodinil promoted a copious increase in exudate secretion and caused the most severe collapse of stigmatic cells of all the fungicides evaluated. Damage was somewhat localized at 4 hrs but more global at 24 hrs. In general, this study showed some fungicide sprays can have a direct detrimental effect on stigma morphology and enhance exudate production in almond flowers.

Introduction

Adequate pollination and fertilization are critical in agricultural crops where fruit or seed is the final product, and have been identified as factors limiting yield and crop quality (e.g., fruit size, shape, sugar content, and storage ability) in numerous economically important crops including apple, kiwifruit, cacao, and melon (Volz et al., 1996; Costa et al., 1993; Gonzalez et al., 1998; Falque et al., 1995; Dag and Eisikowitch, 1995). Critical events associated with pollination in higher plants include the development of a receptive stigma and functional pollen, pollen transfer and attachment, pollen hydration and activation, pollen germination, and tube growth (Johri, 1984; Raghavan, 1997). If these events occur successfully, fertilization and fruit set can proceed.

Almond (*Prunus dulcis*) is an important nut crop which blooms relatively early in the spring (February in California). The crop is self incompatible and requires cross pollination. Pollination can be limiting in certain production areas in part due to the cool, damp weather conditions during the blooming period which may limit insect activity. It has been reported that the percentage of fruit set in commercial orchards is commonly only 30% (Gary et al., 1976).

Almond is heavily sprayed during the bloom period for blossom blight caused by *Monilinia fructicola* and/or *M. laxa*. Previous studies have shown (chapter II) that fungicides can significantly inhibit pollen germination under in vitro conditions. Interference with pollen germination and function by fungicide sprays during the pollination period under in vivo condition would be of particular concern in almond production.

The stigma is the receptive surface for pollen. It can provide nutrients to the pollen and direct pollen tube growth. The stigmatic surface needs a correct physiological condition for the pollen, i.e., a balanced osmolarity and a sufficient water supply (Johri, 1984). Any damage to the stigmatic surface by fungicide sprays may potentially cause the pollination process to fail. A number of studies have reported negative effects of fungicide sprays on fruit set and/or yield in crops such as apple (Hutcheon et al., 1986), cranberry (Ozgen and Palta, 2001; Shawa et al., 1966; Bristow and Shawa, 1981), raspberry (Redalen, 1980), strawberry (Eaton and Chen, 1969; Kovach et al., 2000), and pecan (Wetzstein, 1990; He and Wetzstein, 1994). However, studies specifically evaluating the effects of fungicides on stigma morphology are extremely limited for fruit crops and absent in almond.

The objective of this study was to evaluate the effect of fungicide sprays on stigma morphology in almond at the ultrastructural level. Fungicide sprays were applied in the laboratory to detached flowers using a mechanized spray apparatus to simulate field application conditions. Stigmatic surfaces were observed in a fresh, living state to avoid loss of stigmatic exudate associated with fixation and critical point drying.

Materials and Methods

Plant material. Almond budwood cv. Nonpareil was collected from a commercial almond orchard (Paramount Farming Co., Bakersfield, California), packed in coolers, and air-shipped for next-day delivery. Upon delivery, shoots were recut and placed, with their bases in water, in a cold room at 7^{o}C. Buds were forced as needed and flowers were emasculated before anther dehiscence to remove sources of pollen contamination. Just prior to spraying, individual flowers were selected and removed with about a 1 cm portion of the shoot still attached, then transferred into the wells of tissue

culture plates (Costar Tissue Culture Clusters 12, Costar, Cambridge, MA) containing tap water. Flowers were evaluated and selected for normalcy of stigma development as well as similar style length and stigma orientation using a dissecting microscope. Flowers were carefully selected to be all at the open petal stage of development and within a day after anthesis.

Chemicals and spray application. Four fungicides that are widely applied during the bloom season to prevent blossom blight were tested: 1) azoxystrobin, Zeneca Agric. Products, Wilmington, Del.; 2) myclobutanil, Rohm and Haas Co., Philadelphia; 3) iprodione, Aventis CS, Research Triangle Park, N.C.; and 4) cyprodinil, Novartis Crop Protection, Inc., Greensboro, N.C. The formulated product name and classification of each fungicide is shown in Table 3.1. A water spray served as control. Just prior to spraying, eight flowers were attached (via a spring-clip to their woody stem) to a circular arc holder with their stigmas oriented facing the oncoming spray. Fungicides or water were then applied using a laboratory spray apparatus designed to simulate field application conditions (Fig 3.1). The spray apparatus was set to accurately provide application conditions equivalent to a spray volume of 100 US gallons/acre (935 liters/hectare), 2 mph (3.2 km/hr) tractor ground speed, tree spacing of 24 ft (7.3 m) between rows, and an application rate of 0.263 gal/min (16.6 ml/s) per nozzle (using TeeJet # 8003, Spraying Systems Co., Wheaton, Ill.) at 40 psi (276 kPa) for dual-pass by targets. Immediately after spraying, flowers were transferred back into the culture plates and kept under light conditions at 24 ^{o}C. In a separate experiment, a Rhodamine B stain solution was applied using the same spray conditions to evaluate the spray pattern on the stigmas.

Experimental design. A factorial design was used. Two factors were evaluated: the spray compounds and time periods for observation after spray. The design was: 2 time periods X 5 spray compounds (including water control) x 8 flowers. Five stigmas were used in observations without fixation; three stigmas were fixed, coated, and observed as described below.

SEM observations. Flowers were sampled at 4 or 24 hrs after spraying. Spray treatments were scheduled and staggered to allowed accurate sampling and observation time of flowers. Five flowers per treatment were used in fresh tissue observation without fixation. Pistils were dissected from flowers and the stigma and style were mounted on aluminum stubs using carbon paste. Each sample was observed immediately using a JEOL JSM-5800 scanning electron microscope at 5 kV. Images were captured digitally. Three other flowers from each treatment were dissected and fixed with 2% glutaldehyde in 0.1 M cacodylate buffer, pH 7.2. The tissues were dehydrated in a graded ethanol series, then critical point dried using a Samdri 780-A Critical Point Drier. The tissue was mounted in carbon paste on an aluminum stub and coated with gold using a SPI module sputter coater. Observation were conducted at 20 kV.

Results

Fixed and critical point dried tissue samples failed to preserve stigmatic structure effectively as compared with observations of fresh, living samples. Considerable amounts of stigmatic exudate were retained, but losses during fixation and/or critical point drying were evident when fresh and fixed tissues were compared. Surface secretions in critical point dried tissues appeared as dried residues that were often irregular, plate-like or granular. This was in contrast to the fluid-like secretions observed

in fresh tissue samples. Observations of fresh tissues were deemed more informative and accurate. Thus, the data summarized in Table 3.2 are based on fresh tissue observations.

Morphology of control stigmas. Pistil morphology in almond is characterized by a circular, bilobed stigmatic surface that expands slightly fan-like beyond an elongated and cylindrical style (Fig. 3.2A). Stigmatic surface cells are comprised of raised papillae (Fig. 3.2A, 3.2B). In flowers observed 4 hrs after water sprays (Table 3.2), papilla cells were bulbous, elevated, and intact. Electron-dense, raised regions were evident on papilla surfaces visualizing a mottled stigmatic exudate. In most cases, exudate was uniformly dispersed. However, some flowers exhibited regions on the stigma where slight fluid secretions accumulated in interstices at the base of papillae (Fig. 3.2C). Control flowers observed after 24 hrs spray had stigmas exhibiting a range of from minor (Fig. 3.2D) to more extensive (Fig. 3.2 E, 3.2F) exudate accumulation. Exudate production did not appear to be associated with collapse or distortion of papilla cells. Exudate accumulation (Fig. 3.2F) occured particularly at the central region of the stigma.

Types of spray-induced responses observed. Some of the fungicide spray treatments induced common morphological responses. Thus, the types of general cytological reactions will be described first, followed by a characterization of the specific responses observed for each of the fungicide treatments (Table 3.2).

Increased exudate accumulation was induced by some fungicide sprays and varied in extent (Figs. 3.3A to 3.3F). Exudate production could be very localized (Fig. 3.3B) with an accumulation observed between as few as four adjacent cells; neighboring cells could exhibit no or little accumulation. Unlike control stigmas where the greatest accumulation of stigmatic secretions occurred in the depression between lobes, fungicide-induced accumulation occurred in all regions of the stigmatic surface (Fig.

3.3A, 3.3C, 3.3E). Accumulation of fluid was observed between cells (Fig. 3.3D). Some fungicide sprays induced substantial and copious secretions which could inundate the whole stigmatic surface in some cases (Fig. 3.3E). Stigmatic papillae could be completely submerged or have only apical regions exposed (Fig. 3.3F).

Damage to and collapse of stigmatic cells were also observed with some fungicide sprays (Figs. 3.4A to 3.4D). Damaged areas could be localized (Fig. 3.4A), where severely collapsed papillae occurred adjacent to normal, undamaged areas. Damage could also be extensive with collapsed areas encompassing one third or more of the stigmatic surface (Fig. 3.4C). Damaged areas ranged from papillae cells exhibiting wrinkling and distortion of cell surfaces (Fig. 3.4B), to those that were concave or totally flattened (Fig. 3.4C). In addition to observations where enhanced stigmatic exudate and cell damage were observed independently, some fungicides induced a simultaneous occurrence (Fig. 3.4D). Copious exudate production was associated with collapse and/or inversion of exposed papilla tips.

The specific effects of sprays on almond stigmas are summarized in Table 3.2. Increased exudate accumulation was induced by azoxystrobin at both 4 hrs and 24 hrs after spray. Exudate production ranged from that occurring at very localized regions (with an accumulation between few-to-several adjacent cells) to very expansive regions that could inundate the entire stigmatic surface. Exudate accumulation was more extensive and copious at 24 hrs after spray than at 4 hrs after spray.

Although stigmatic papillae were generally intact at 4 hrs after spray with azoxystrobin, damage and collapse to stigmatic cells were observed 24 hrs after spray. Damaged areas were generally localized, where severely collapsed papillae occurred adjacent to normal, undamaged areas. Damaged areas exhibited wrinkling, distortion of

cell surfaces, or concave stigmatic papillae. Some stigmatic region had both damage and exudate accumulation.

Myclobutanil caused significant damage to and collapse of stigmatic papillae. Damaged areas exhibited wrinkling, distortion of cell surfaces, or concave stigmatic papillae as in the case of azoxystrobin. Damaged regions varied in area and location, and ranged from localized areas where severely collapsed papillae were adjacent to undamaged area, to extensive areas with collapsed papillae encompassing one third or more of the stigmatic surface. In some samples, exudate production was associated with collapse and/or inversion of exposed papilla tips. More extensive collapse was observed at 24 hrs after spray than at 4 hrs after spray. Compared with controls, a slight increase of exudate accumulation was noted at 4 hrs after spray with myclobutanil. Exudate accumulation 24 hrs after spray was similar to that observed at the same time in controls.

Iprodione did not affect exudate accumulation at 4 hrs or 24 hrs after spray. However, the chemical caused marked and severe collapse of stigmatic papillae. As with other sprays, damaged areas ranged from localized to extensive and varied in severity of damage. More severe damage and collapse occurred at 24 hrs after spray than at 4 hrs after spray. Stigmatic cell collapse was observed with and without exudate accumulation.

Cyprodinil promoted a copious increase in exudate secretion that was evident at 24 hrs after spray. Commonly, stigmatic papillae were totally submerged or had only apical regions exposed. Exudate accumulation was so extensive in some cases that the entire stigmatic surface was inundated. Cyprodinil also caused severe collapse of stigmatic cells. Damage associated with cyprodinil sprays was the most severe of all the

fungicides evaluated. Damage was somewhat localized at 4 hrs, but was more global at 24 hrs often covering an extensive region of the stigma.

Sprays with Rhodamine B dye. Spray applications of Rhodamine B staining solution visualized the distribution pattern of sprays. All stigmas were targeted but the area of the stigma showing dye deposition was variable among stigmas. Stain deposition ranged from localized to more extensive areas (Fig 3.5A and B).

Discussion

This study documented that fungicide sprays applied to flowers during anthesis can have a direct effect on stigma morphology and exudate production. All of the fungicides evaluated induced changes in almond stigmatic surfaces. Common morphological responses were collapse of surface cells and enhanced production of exudate. The current study was conducted under controlled laboratory conditions using a spray apparatus that closely simulates field application conditions. This allowed sprays to be directly targeted onto the stigmatic surfaces of flowers of the same developmental stage. An additional feature of this study was that SEM observations directly assessed spray effects on the stigmatic surface; that is, ultrastructural examinations were made of living tissues, which eliminated artifacts induced by fixation and critical point drying.

A marked collapse of stigmatic papillae was observed with some fungicide sprays. This loss of cellular integrity could degrade the function of these stigmatic cells and effectively result in a loss of stigmatic surface available to support pollen capture, hydration and germination. Large numbers of pollen grains deposited on the stigma can be beneficial to overall fruit set in some crops. Dennis (1979) found that a minimum of 50 pollen grains per flower is required for consistent fruit set in ‘Delicious’ apple even though fruits generally contain only ten ovules. Fewer pollen grains resulted in poorer

germination and slower tube growth. In the current study, the collapse of stigmatic papillae might result in lower numbers of pollen grains germinating. Therefore, although certain undamaged areas might still be receptive to pollen germination, fruit set may be detrimentally impacted.

Pollen activation and hydration is mediated by the uptake of water by colloidal imbibition and endosmosis. This hydrodynamical process strongly depends on the condition of the cytoplasm of the vegetative cell and the thickness of the intine because of its imbibition capacity (Johri, 1984). Wetzstein (1990) evaluated effects of pesticidal sprays on the stigma in pecan and found that some fungicides were detrimental to pollen function. Benomyl applied in combination with triphenyltin hydroxide, caused severe inhibition of pollen-stigma interactions. Pollen grains failed to hydrate and remained concave. Although no pollination was conducted in the current study, it is possible that fungicides may hinder almond pollen hydration, especially in the location of collapsed papillae without exudate, and thereby reduce pollen germination on the stigma.

The stigmatic surface is a critical component in post-pollination responses and plays a crucial role in pollen capture and adherence and germination. Any damage of fungicide sprays to the stigmatic surface could potentially affect stigma receptivity, decrease the effective pollination period (EPP), and thereby detrimentally impact fertilization. EPP, i.e., the period when the embryo sac remains functional for fertilization minus the time required for pollen to reach the egg apparatus (Williams, 1969), is a very important factor for successful fertilization. It can be limited by the time period in which the stigma retains its receptivity. In kiwifruit, stigma receptivity was found to be the main factor responsible for a short EPP (Gonzalez et al., 1995). In apricot (*Prunus Armeniaca)*, a close relative of almond, short duration of stigma receptivity was noted to

be the limiting factor of EPP (Egea and Burgos, 1992). Stigma receptivity is considered by many as fundamental in explaining fruit yield differences (Egea et al., 1991). In almond it has been documented that the percentage of pollinated flowers which set fruit was highest for newly opened flowers and was significantly lower at progressively later stages (Vezvaei and Jackson, 1995).

A common observation associated with some fungicide sprays was enhancement of exudate production. This was particularly marked in flowers sprayed with azoxystrobin and to a lesser extent with cyprodinil. Exudate accumulation was copious and increased with longer periods after spray. Increased exudate production is commonly associated with flower development. Control flowers in the current study exhibited some increases in exudate accumulation at later observation times. However, fungicide-induced increases were clearly greater than found in controls. Copious exudate formation is characteristic of receptive flowers in some species. However, this is not the case in almond. In association with flower development studies with almond, we have evaluated stigmatic surface changes at different flower stages. We found stigmatic papillae intact with minimal exudate production at anthesis when petals were open (data not shown). Cell collapse and copious exudate did not appear until petal fall and flower senescence. This indicates that the exudate formation caused by fungicide spray potentially may be the signature of senescence. Further studies are required to determine whether fungicide-induced exudates inhibit, or even prompt, pollen germination and tube growth. It is also possible that this is a senescence or stress response which may decrease the period of stigma receptivity, EPP, and thereby detrimentally impact fertilization.

Spray deposition patterns by Rhodamine B dye showed that the area of the stigma showing dye deposition was variable among stigmas. This could explain why observed damage after spray varied in area and location. In addition, this study found that extraction of stigmatic exudate occurred during fixation and/or critical point drying, which was evident compared to that observed with fresh tissue (data not shown). The dried surface secretions in fixed and critical point dried tissues sharply contrasted with the fluid-like secretions observed in fresh tissue samples. Likewise, Cresti et al. (1982) observed that part of the stigmatic exudate in citrus stigmas was removed as a result of fixation and critical point drying. This emphasizes the need to critically consider sample preparation methods and the potential introduction of artifacts in ultrastructural studies.

Table 3.1. Fungicides and rates applied to apple and almond flowers.

Active ingredient	Formulated product name	Class	Field rate per acre
Azoxystrobin	Abound	Strobilurin	12.8 oz.
Myclobutanil	Rally	Conazole	6.0 oz.
Iprodione	Rovral	Iprodione	1.0 lb.
Cyprodinil	Vangard	Pyrimidine	5 oz.

Note: Spray solutions were calculated based on spray application volumes of 100 gallons per acre.

Table 3.2. Effects of spray treatments on the morphology of almond stigmatic surfaces.

Fungicide	Time after spray (hrs)	Characteristic features [z]	
		Stigma papillae	Exudate production
Water control	4	No damage, cells raised and intact	None-to-slight accumulation
	24	No damage, cells raised & intact	Slight-to-extensive accumulation
Azoxystrobin	4	No damage	Enhanced production, intermediate-to-extensive accumulation
	24	Collapsed cells in localized areas	Enhanced production, extensive copious accumulation
Myclobutanil	4	Collapsed cells, damage varying in area & location	Slight increase in exudate
	24	Collapsed cells, damage more extensive than at 4 hr	Same as control
Iprodione	4	Collapsed cells, damage varying in area & location	Same as control
	24	Severe collapse and flattening of cells, damage varying in area & location	Same as control
Cyprodinil	4	Severe collapse of cells, damage varying in area & location	Same as control
	24	Severe collapse of cells over extensive regions of the stigma	Enhanced production, copious exudate engulfing papillae

[z] Based on observations of 5 flowers per treatment. Times of observation were 4 and 24 hrs after spraying.

Figure 3.1. Laboratory apparatus used for fungicide applications simulating field conditions. A conventional hydraulic-atomizing nozzle at operational pressure commonly used for fungicide applications provided the appropriate droplet-size spectrum, volumetric flow rate, and active ingredient concentration for each fungicide. An electronically controlled robotic arm swept the spray nozzle at controlled speed past test flowers positioned around a circular-arc holder.

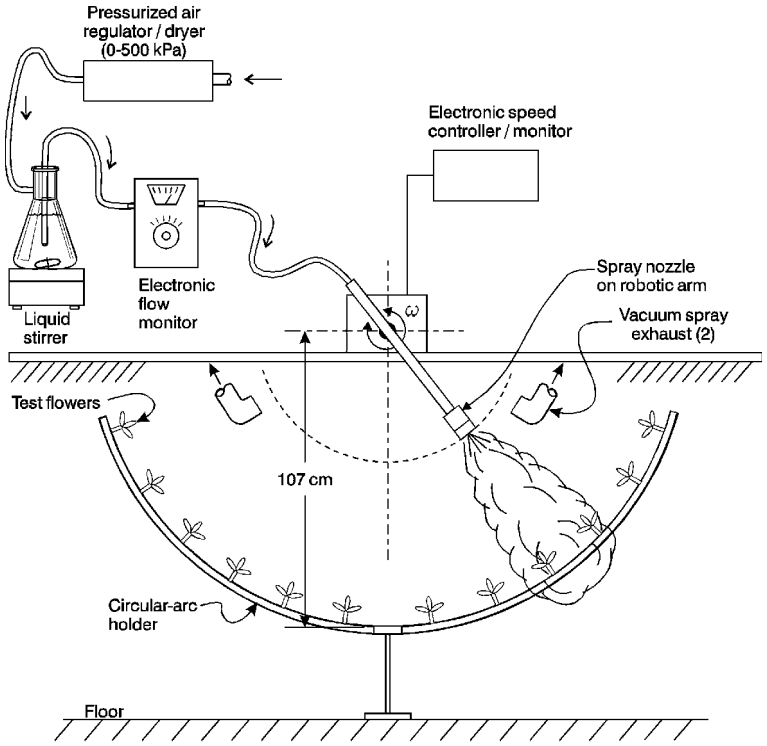
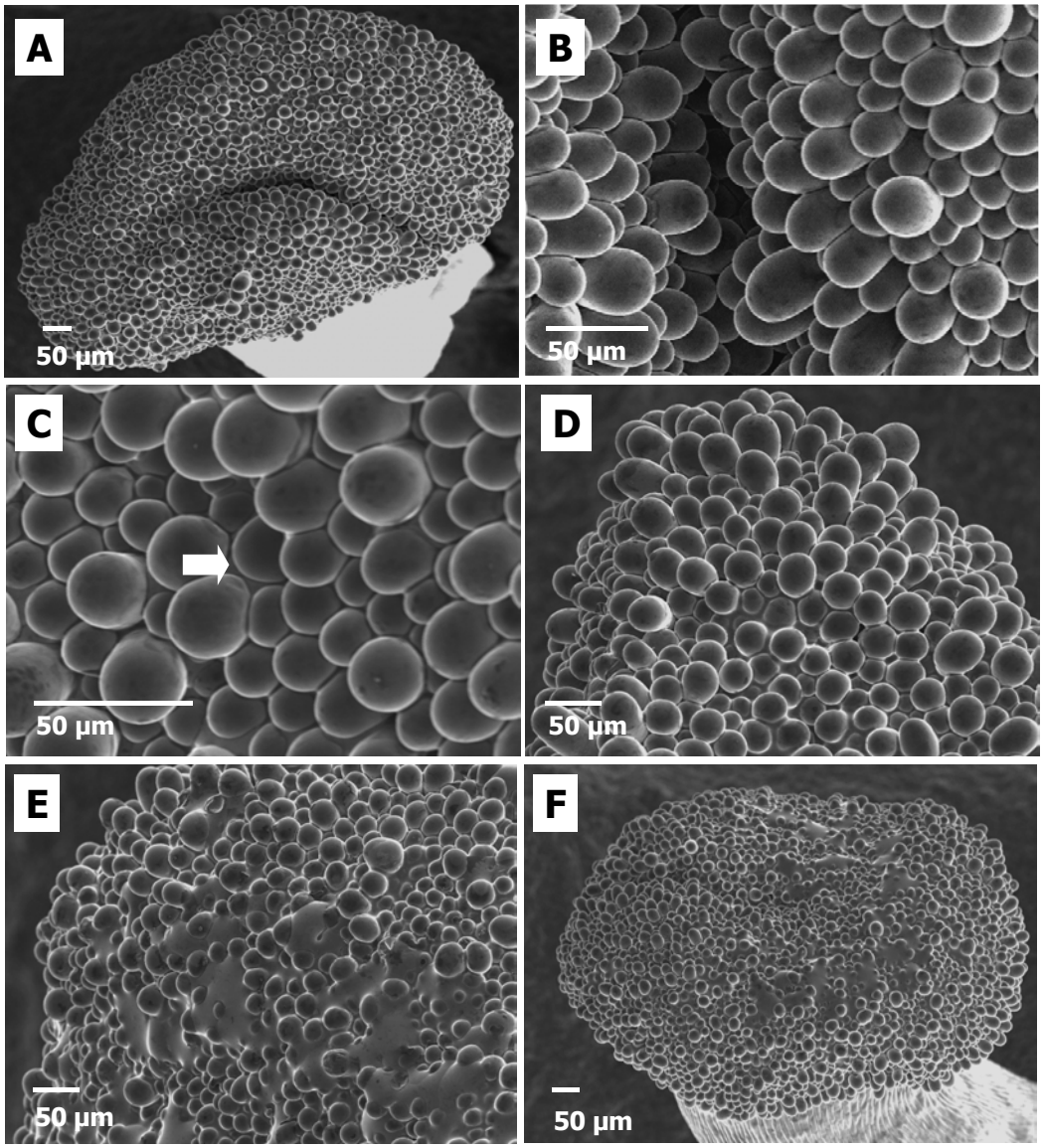

Pressurized air
regulator / dryer
(0-500 kPa)
Electronic speed
controller / monitor
Spray nozzle
on robotic arm
Vacuum spray
exhaust (2)
Electronic
flow
monitor
Liquid
stirrer
ω
Test flowers
107 cm
Circular-arc
holder
Floor

Fig. 3.2. Stigma morphology of almond flowers after water sprays. A-C) Stigmas 4 hrs after spray. A) Pistil morphology in almond is characterized by a circular, bilobed stigmatic surface that expands slightly fan-like beyond an elongated and cylindrical style. B) Stigmatic surface cells are comprised of raised papillae. C) Slight exudate secretions accumulated in interstices at the base of papillae. Arrow: exudate accumulation. D-F) Stigmas 24 hrs after spray. Exudate production was variable. D) Slight accumulation, similar to at 4 hrs. E) and F) More extensive exudate accumulation.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D
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Fig. 3.3. Increased exudate accumulation induced by some fungicide spray materials (Figs. A to F). A) Fungicide-induced accumulation occurred in any region of the stigmatic surface, not necessarily limited to the depression between lobes. Image taken 4 hrs after being sprayed with azoxystrobin. B) Exudate production could be very localized with an accumulation observed between as few as four adjacent cells; neighboring cells exhibited no or little accumulation. Image taken 4 hrs after being sprayed with azoxystrobin. C) Considerable exudate accumulation occurred in any region of the stigmatic surface. Image taken 4 hrs after being sprayed with azoxystrobin. D) Accumulation of fluid was observed bridging between cells. Image taken 4 hrs after being sprayed with azoxystrobin. E) Substantial and copious secretions inundated the whole stigmatic surface. Image taken 24 hrs after being sprayed with cyprodinil. F) Stigmatic papillae completely submerged or only apical regions exposed. Image taken 24 hrs after being sprayed with cyprodinil.

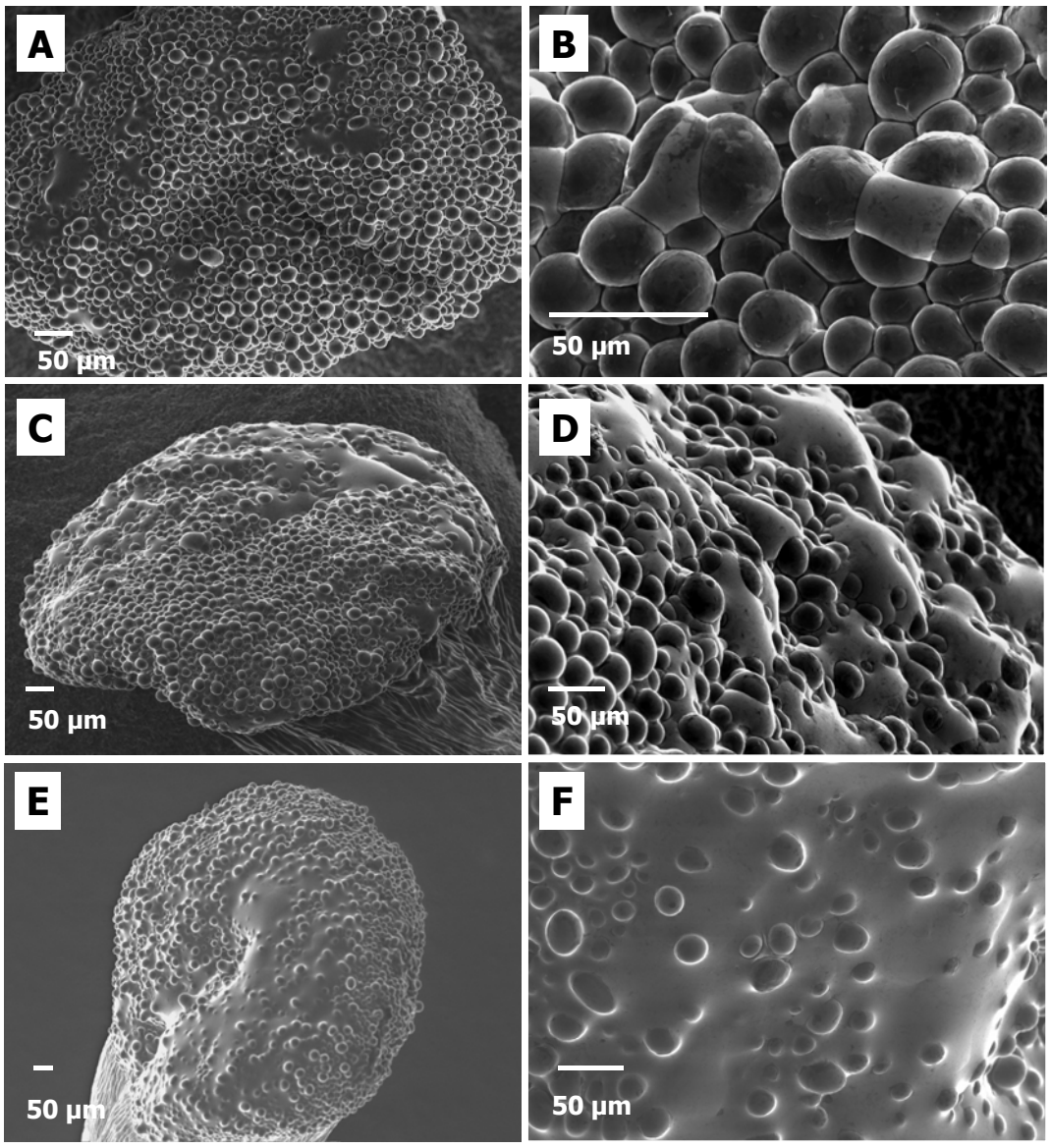
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Fig. 3.4. Fungicide-spray caused stigmatic papillae collapse. A) Localized fungicide-spray damage to stigmatic papillae, where severely collapsed papillae occurred adjacent to normal, undamaged areas. Image taken 4 hrs after being sprayed with myclobutanil. B) Damaged papillae cells exhibited wrinkling and distortion of cell surfaces. Image taken 4 hrs after being sprayed with cyprodinil. C) Extensive damage with collapsed areas encompassing one third or more of the stigmatic surface. Papillae concave or totally flattened. Image taken 24 hrs after being sprayed with cyprodinil. D) Enhanced stigmatic exudate and cell damage could occur simultaneously. Image taken 24 hrs after being sprayed with iprodione.

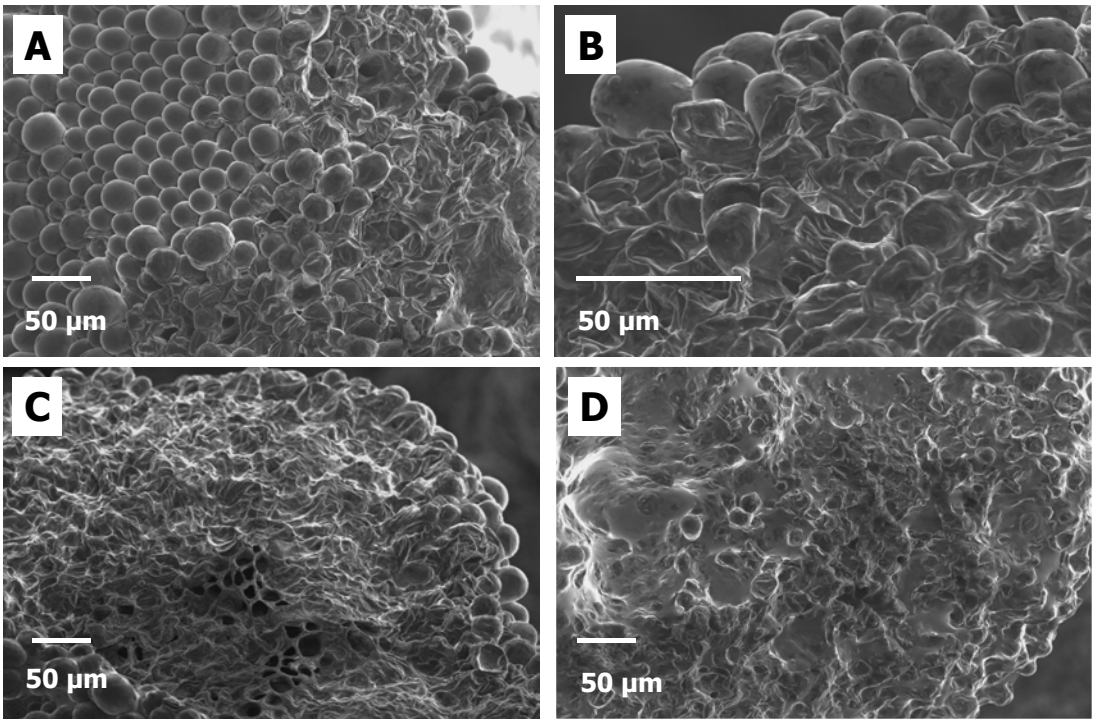
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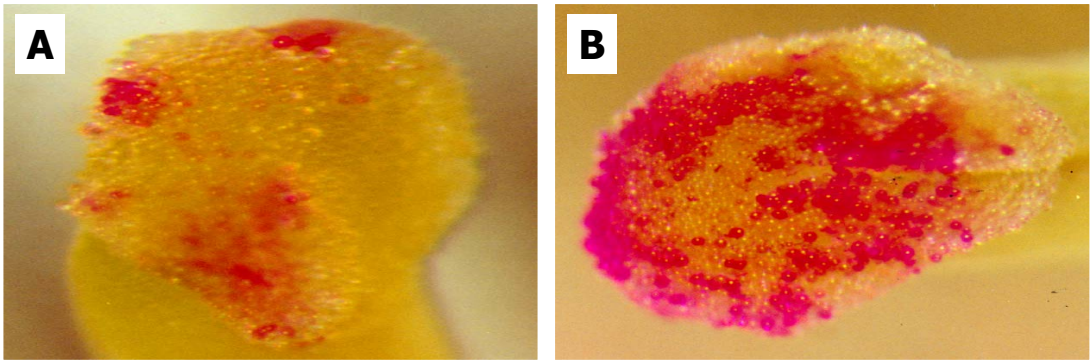
Fig. 3.5. Spray patterns on the stigmatic surface as evidenced in Rhodamine B dye sprays. A) Rhodamine B dye deposited in localized area of the stigmatic surface. B) a more extensive distribution pattern.


A
B

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CHAPTER IV

EFFECTS OF PESTICIDE SPRAYS ON POLLEN GERMINATION AND TUBE GROWTH ASSOCIATED WITH THE STIGMA AND STYLE OF APPLE AND ALMOND FLOWERS

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Abstract. The timing of fungicide and antibiotic applications in fruit crops often overlaps the time at which flowering and pollination occur. Numerous studies reported detrimental effects of fungicide applications on pollination, fruit set and yield. However, field results can be quite variable even when similar compounds are tested on the same crop. Problematic is that field variables unrelated to the effects of fungicides on reproductive activities can mask results. In the current study, we investigated the effects of selected chemical sprays on pollen germination and tube growth on and in the stigma and style. Using two model systems, apple and almond, sprays were applied to detached flowers under constant laboratory conditions using an electronically controlled robotic apparatus which simulated a field sprayer. Flowers were pollinated at specific times after spraying, and pollen tube numbers and growth assessed 20 or 24 hrs after pollination using fluorescence microscopy. The pesticides evaluated were for apple: captan, myclobutanil and streptomycin; and for almond: azoxystrobin, myclobutanil, iprodione and cyprodinil. In apple, captan significantly inhibited pollen germination on the stigmatic surface, reducing germination by 20% compared with water controls. The number of tubes that reached the basal part of the style within 20 hrs was not affected. In contrast, myclobutanil and streptomycin had no significant effect on pollen tube growth. With almond, none of the fungicide sprays evaluated had any significant effect on pollen germination or tube growth. Of the seven compounds evaluated in apple and almond, only captan detrimentally affected pollen germination on apple stigmas.

Introduction

Applications of early-season fungicides are necessary in most fruit and vegetable crops to protect susceptible foliage and/or floral parts. In addition, antibiotics are sprayed in some crops to prevent bacterial diseases. In some cases, chemical spray applications are targeted specifically into the open bloom to control plant pathogens that cause flower blights or fruit infection (Ogawa and English, 1991; Ploetz et al., 1994). Thus, the timing of spray applications necessarily overlaps the time at which flowering and pollination occur. In almond, fungicide sprays are required to prevent blossom blight which can infect and destroy the flowers during bloom. In apple, fungicides and antibiotics are widely applied to prevent scab and fire blight.

Numerous studies have reported detrimental effects of pesticide sprays on fruit set and/or yield in crops. Reports include cranberry (Shawa et al., 1966; Bristow and Shawa, 1981), raspberry (Redalen, 1980), and strawberry (Eaton and Chen, 1969; Kovach et al., 2000). However, there are conflicting reports concerning effects of chemical sprays on fruit set even when similar compounds are tested. For example with apple, Legge and Williams (1975) found that captan and binapacryl significantly inhibited fruit set when they were applied during bloom. In contrast, Church and Williams (1977) observed no effects on fruit set with applications of captan, binapacryl, dinocap, dithianon, thiophanate-methyl, or benomyl. In another study, Church et al. (1983) found that dinocap applied during bloom significantly decreased initial fruit set, while captan did not. In field tests, Brown and Hendrix (1978) concluded that captan, benomyl, maneb, folpet, captafol, chlorothalonil, and thiophanate-methyl applied to apple trees in bloom did not significantly affect fruit set. MacDaniels and Burrell (1934) reported that sulfur applied either before or shortly after pollination significantly reduced

the set of fruits. Rich (1957), in an evaluation of several fungicides, concluded that none of them, including sulfur, reduced pollen germination or fruit set when applied to apple trees in bloom. Environmental conditions of studies can alter effects of fungicides and may contribute to discrepancies. Church and Williams (1983) conducted tests in the orchard and glasshouse. They found that dodine significantly decreased fruit set under both conditions. Sulfur significantly decreased fruit set under glasshouse conditions, but increased fruit set under orchard conditions.

Assessments of the impacts of fungicides on fruit set, quality, and yield are problematic because control plots with no fungicide can exhibit high losses to disease, thereby masking any reductions in yields caused by fungicides. Kovach et al. (2000) were able to circumvent this problem in strawberry by using biocontrol agents to control disease in no-fungicide treatments. They found the number of seeds per berry and berry weight were significantly reduced by some fungicides in commercial field plots. Insufficient pollination not only affects fruit set, but also fruit size, shape, and fruit Ca concentration (Volz et al., 1996). In field studies with almond where the efficacy of disease control was considered, the application of several commercial fungicides was evaluated under orchard condition. Some fungicides differentially decreased fruit set (D. McCoy, personal com.).

Field studies to assess fruit set or yield can be impacted by a number of variables that are unrelated to the effects of fungicide on flowering, pollen function and/or fertilization events. These include pest control, weather, pollinator activity, and physiology of the plant. In contrast, an assessment of the effects that fungicides have on specific reproductive processes would provide direct information on if and how these pesticides impact fertility. The approach of this study was to evaluate the effect of

selected agricultural sprays on pollen germination on the stigma and tube growth in the style. Pesticides were applied to detached flowers in the laboratory using an electronically controlled robotic spray apparatus to simulate field-sprayer conditions. Pollen tube growth was assessed following controlled pollinations using fluorescence microscopy. Two species, almond (*Prunus dulcis*) and apple (*Malus domestica*), were used as models. Both are economically important fruit crops, and the two taxa represent divergent types of stigmatic surfaces.

Materials and Methods

Plant material. 'Red Delicious' apple trees grown at the University of Georgia Horticulture Farm near Watkinsville, Ga. were the source of apple flowers. The study was conducted in spring, 2000. Prior to anthesis, branches were bagged using Delnet Pollination Bags to prevent insect pollination. Flowers were emasculated before anther dehiscence to prevent pollen contamination. Flowers with about 1 cm stems attached were collected from the trees and transferred to the laboratory with their bases immersed into the wells of tissue culture plates (Costar Tissue Culture Clusters 12, Costar, Cambridge, Mass.) containing tap water. Prior to spray, flowers were evaluated for normalcy of stigma development, similar style length, and stigma orientation using a dissecting microscope. Since it was difficult to consistently obtain flowers with five stigmas meeting our selection criteria, one of the five stigmas in each flower was removed. Thus, flowers used in the study had four well-developed pistils, with stigmas oriented into the path of sprays. After spray applications, apple flowers were pollinated using 'Golden Delicious' pollen obtained from Antles Pollen Supplies, Inc. (Wenatchee, Wash.).

Almond studies were conducted in spring, 2001. Almond budwood cv. 'Nonpareil' was collected from a commercial almond orchard (Paramount Farming Co., Bakersfield, Calif.), packed in coolers and shipped by next-day mail to the laboratory. Upon delivery, shoots were recut under water, placed with their bases in water, and held in a cold room at 7ºC. Buds were forced as needed and flowers were emasculated before anther dehiscence. Flowers were evaluated using a dissection microscope for normalcy of stigma development and transferred into microwells as in the apple flower studies. After spays were applied, flowers were pollinated using 'Sonora' pollen obtained from Paramount Farming Co. (Bakersfield, Calif.).

In vitro germination tests were conducted on the pollen prior to use to verify viability. Pollen were inoculated into germination wells with 12% sucrose (w/v), 0.062% $CaNO_3$ (w/v) and 0.024% boric acid (w/v). Germination percentages were 82% and 51% for apple and almond pollen, respectively.

Pesticides and spray application. In experiments with apple, three pesticides commonly sprayed during bloom were tested. Two fungicides are used to control scab: captan (Micro Flo Company, Memphis, Tenn.), and myclobutanil (Rohm and Haas Co., Philadelphia). Streptomycin (Novartis Crop Protection, Inc., Greensboro, N.C.) is an antibiotic used to control fire blight. In almond, four fungicides that are widely applied during the bloom season to prevent blossom blight were tested: 1) azoxystrobin (Zeneca Agric. Products, Wilmington, Del.); 2) myclobutanil (Rohm and Haas Co., Philadelphia); 3) iprodione (Aventis CS, Research Triangle Park, N.C.); and 4) cyprodinil (Novartis Crop Protection, Inc., Greensboro, N.C.). A water spray served as the control. Table 4.1 lists the pesticides evaluated, their common name, and classification.

Pesticides or water were applied using a laboratory spray apparatus designed to simulate field application conditions (Fig. 4.1). Just prior to spraying, nine flowers were attached (via a spring-clip to their woody stems) to a circular- arc holder with their stigmas oriented in the direction of the approaching spray. The spray apparatus was set to provide application conditions equivalent to a spray volume of 100 US gallons/acre (935 liters/hectare), 2 mph (3.2 kilometer/hr) tractor ground speed, tree spacing of 24 ft (7.3 meter) between rows, and an application rate of 0.263 gal/min (16.6 ml/second) per nozzle (TeeJet # 8003, Spraying Systems Co., Wheaton, Ill.) at 40 psi for dual-pass by the targets. Immediately after spraying, flowers were transferred back into the culture plates and kept at room temperature under normal light condition.

Pollination and tube growth assessment. In apple, stigmas were hand pollinated using a small brush with the aid of a dissecting microscope at 1 hr or 18 hrs after spray applications. Approximately 350 pollen grains were applied to each stigma. In almond, pollination was similarly done, but at 24 hrs after spray. About 250 pollen grains were applied per flower. Evaluations of fresh, unfixed stigmas using a scanning electron microscope verified that uniform numbers of pollen grains were deposited on stigmas.

Flowers were dissected to remove pistils 20 hrs after pollination in apple and 24 hrs after pollination in almond. The stigmas and styles were fixed in ethanol: acetic acid (3:1 v/v). Tissues were softened and cleared by autoclaving at 120 ^{o}C for 20 min in 1% sodium sulfite solution, stained using aniline blue (0.01 % aniline blue in 0.1M K_3PO_4) for at least 4 hrs, then examined using a Zeiss Standard microscope (Carl Zeiss, Oberkochen, West Germany) under flourescent light (G365, LP420) (Currier, 1957). The numbers of pollen tubes and the extent of their growth through the length of the style were assessed.

Experimental design and statistical analysis. In apple, the experimental layout was a factorial design with 2 pollination times x 4 spray compounds x 2 trees x 6 replications. In almond, a completely randomized block design with 5 blocks and a single factor (5 spray compounds) was used. Sets of 9 flowers were used as experimental units. Statistical analysis was conducted by GLM followed by Duncan's multiple range test at α=0.05.

Results

These studies were conducted using a laboratory spray system that simulated field application conditions. The fabricated apparatus closely controlled the spray flow rate, volume, pressure, droplet size, travel speed, and orientation of the stigmas. This allowed us to obtain reproducibly-sprayed flowers with less variability than that characterizing conventional field studies.

Apple: The soonest that pollination was feasible was 1 hr after spraying under our laboratory condions. At this time, most of the spray-carrier liquid had evaporated. However, some moisture was still visible on most stigmatic surfaces. In flowers that were pollinated 1 hr after spraying (Fig 4.2 A), there was no significant difference among chemical sprays and water sprays in relation to the resultant number of pollen tubes either penetrating the stigmatic surface, or reaching regions farther down the style. In contrast, some differences were observed with flowers that were pollinated 18 hr after spraying (Fig 4.2 B). Significantly fewer pollen tubes germinated and penetrated the stigma after spraying with captan as compared to the water spray control. Pollen tube numbers were only 80% of the water-spray control. The number of pollen tubes that reached regions farther down the style was not affected. In contrast, myclobutanil and streptomycin had no significant effect on pollen germination and tube growth.

Almond: None of the fungicide treatments (azoxystrobin, myclobutanil, iprodione, or cyprodinil) showed any significant effects on pollen tube numbers that had penetrated into the stigmatic surface (Table 4.2). The maximum tube length (percent of length of style through which the tube passed) was also assessed. Statistical analysis showed no significant difference among any fungicide treatments and water control.

Discussion

A number of chemical sprays (including sprays with captan iprodione) have been reported to cause remarkably negative effects to fruit set and yield. Kovach et al. (2000) evaluated the effects of captan, benomyl, iprodione and vinclozolin, and reported that commercial fungicides applied at bloom reduced the number of seeds per berry and subsequently berry weight in field tests in strawberry. Church and Williams (1983) reported that dodine decreased fruit set in apple in one year of a 2-year study. Eaton and Chen (1969) reported that captan decreased strawberry achene set and berry development in the greenhouse and increased the proportion of misshapen fruits when sprayed after anther dehiscence. To evaluate pesticide effects, Donoho (1964) conducted tests on an apple orchard for three consecutive years and assessed fruit set and yield compared to captan + DDT. He found that trees consistently having the lowest fruit set and yields were treated with Phix + DDT. Phygon XL + DDT and Guthion + captan also reduced set, but to a lesser extent. Sevin + captan reduced fruit set and yields only when the application began at petal fall. In cranberry, Bristow and Shawa (1981) found that captafol and cupric hydroxide decrease pollen germination by more than 50% when they were applied to the stigmatic surface 1 hr before pollination. Triforine-EC resulted in the production of smaller and lighter berries and significantly reduced yield by 41% when it was applied twice during bloom.

In the current study, captan significantly inhibited pollen germination in apple. This concurs with previous studies in apple. Legge and Williams (1975) reported that captan and binapacryl significantly decreased fruit set when the fungicide was sprayed 2-4 hrs after pollination under orchard conditions. Church and Williams (1977) found that Captan, dinocap, binapacryl, and dithianon significantly reduced pollen germination on stigmas under greenhouse conditions. Church et al. (1983) found that captan and dinocap significantly inhibited pollen germination and tube growth in intact apple flowers under orchard condition when they were sprayed 2 hrs after pollination.

The timing of pollination in relation to when fungicides are applied may have an impact on how fungicides affect pollen function. In highbush blueberry, triforine was applied to stigmas at time periods ranging from 24 hr before pollination to 24 hr after pollination (Bristow, 1981). Results showed that the inhibition of pollen germination was greatest when the fungicide was applied to the stigma at the closest interval to pollination and decreased as the interval between fungicide application and pollination lengthened. Inhibitory effects related to the chemical toxicity of spray materials would be expected to be more severe with pollinations made soon after spray applications. However, in the current study, apple flowers exhibited no inhibition to pollen germination in flowers pollinated 1 hr after spray, although captan significantly inhibited pollen germination in flowers pollinated 18 hrs after spray. This indicated that chemical sprays may affect pollen function indirectly by modifying the development or physiology of the stigmatic surface. Fungicide sprays have been shown to damage stigmatic papillae when applied to receptive flowers in pecan (Wetzstein, 1990), and in almond (chapter III). In addition, there may be critical pollen-stigma interaction stages during which fungicides may be more damaging. Church and Williams (1977) detected inhibition of

pollen germination by captan only when spray was applied 2 hr after pollination. Spray applications 1 day before, 2 hrs before, 1 day after, or 2 days after pollination had no effect.

Although no inhibitory effects of streptomycin in apple was found in the current study, Polito et al. (personal communication) found that it significantly decreased pollen germination and tube growth on intact flowers, and increased nut drop in walnut. This indicated that the same chemical spray may cause different effects upon pollination in different species.

Apparently, no previous studies have evaluated pollen tube growth patterns within regions of the pistil. In the current study, pollen tube staining was conducted 20 hr after pollination to minimize the effects of potential stress associated with detaching flowers, since vigor of flower will impact pollen tube growth. At this time, almost 70% of the germinating pollen had reached a distance one third down the length of the style in control flowers. A smaller population of pollen grains, i.e., 19%, had tubes that reached 2/3 or more down the style. This faster growing, more vigorous population of pollen grains was not affected by the presence of chemical sprays. Whether inhibition of pollen germination will affect subsequent events such as fertilization, fruit set, and/or fruit quality, may depend upon the amount of pollen available to the stigma. If sufficient numbers of pollen tubes grow down the style, fruit set may not be impaired. This could be one of the reasons why studies have had variable results with some failing to find reductions in fruit set (Rich, 1957; Church et al. 1983; Church and Williams, 1977). Artificial pollinations were conducted in all of these experiments, but none of them mentioned the number of pollen grains applied. In contrast, if pollination is limiting

(which may be the case in orchard conditions), inhibitory effects of fungicides would be anticipated to have a greater potential impact on final fruit set and yield.

In almond, none of the fungicides evaluated showed significant inhibitory effects to pollen germination and tube growth, even though four fungicides representing different classes of compounds were evaluated. Fungicide effects on pollen are proposed to vary with genotype (Church and Williams, 1978). Whether captan has similar inhibitory effects on pollen tube growth in almond was not tested in the current study because it was of less interest than the newer compounds evaluated.

This study shows that a number of fungicides used under commercial conditions have no major inhibitory effects on pollen germination and tube growth. Of the seven compounds evaluated, only captan negatively affected pollen germination on the apple stigma. This is although test conditions provided sprays directly to the stigmatic surface. However, spray application were made only to receptive flowers and in periods prior to pollination. Whether fungicides have inhibitory effects when applied to flowers at different developmental stages or at different intervals pre- or post- pollination is unknown. Likewise, fungicides may influence other processes such as flower, fruit or vegetative development.

Fig. 4.1. Laboratory apparatus for spray applications to simulate field-sprayer conditions. A conventional hydraulic-atomizing nozzle at operational pressure commonly used for pesticide applications provided the appropriate droplet-size spectrum, volumetric flow rate, and active-ingredient concentration for each fungicide. An electronically controlled robotic arm swept the spray nozzle at controlled speeds past test flowers positioned around a circular-arc holder.

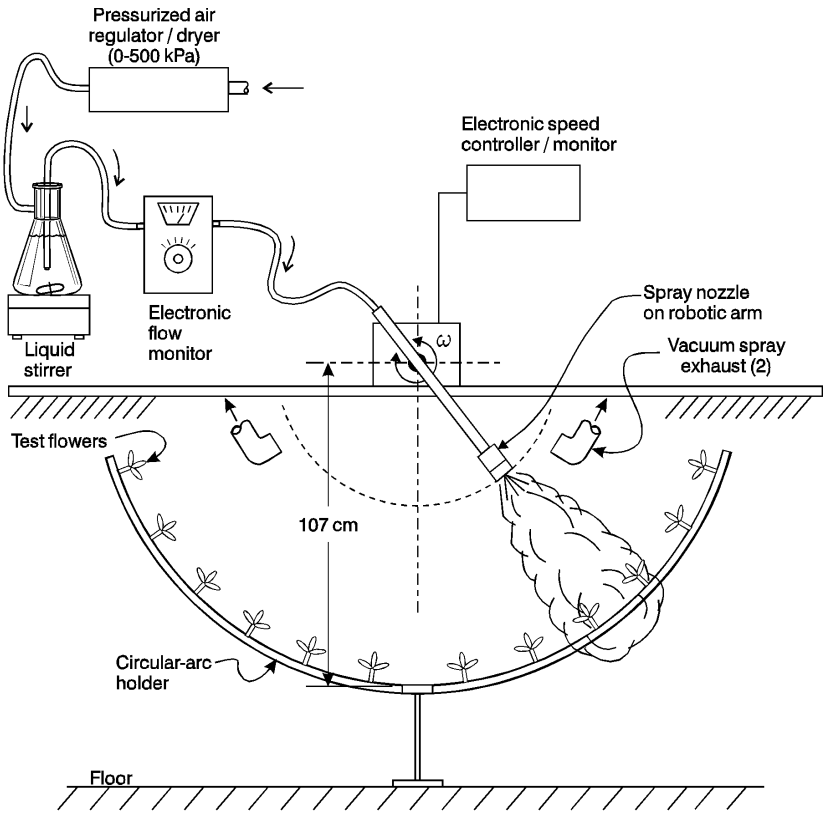
Pressurized air
regulator / dryer
(0-500 kPa)
Electronic speed
controller / monitor
Spray nozzle
on robotic arm
Vacuum spray
exhaust (2)
Electronic
flow
monitor
Liquid
stirrer
ω
Test flowers
107 cm
Circular-arc
holder
Floor

Table 4.1. Pesticides and rates applied to apple and almond flowers.

Crop	Active ingredient	Product name	Class	Field rate per acre[z]
Apple	Captan	Captan 4L	Dicarboximide	2 qts.
Apple	Myclobutanil	Nova 40W	Conazole	5-10 oz.
Apple	Streptomycin	Agri-mycin 17	Antibiotic	4.8-8 oz.
Almond	Azoxystrobin	Abound	Strobilurin	12.8 oz.
Almond	Myclobutanil	Rally	Conazole	6.0 oz.
Almond	Iprodione	Rovral	Dicarboximide	1.0 lb.
Almond	Cyprodinil	Vangard	Pyrimidine	5 oz.

[z] Spray solutions calculated based on application volumes of 100 gallons per acre.

Table 4.2. Fungicide effects on pollen germination and tube growth after treatment of detached almond flowers.

Fungicides	Numbers of tubes that penetrated the stigma and grew in the style	Maximum tube length [x] (%)
Azoxystrobin	14 a [y]	33 a
Myclobutanil	10 a	26 a
Iprodione	10 a	29 a
Cyprodinil	10 a	28 a
Water	13 a	33 a

[x] Max. tube length expressed as the percent of the whole length of the style.

[y] Values in columns followed by the same letter are not significantly different at " = 0.05 according to Duncan's multiple range test. Values are means of 45 observations.

Fig 4.2. The effect of chemical sprays in apple on the number of pollen tubes reaching different distances down the style 20 hr after pollination. Values are means and standard error of 12 observations. Means within the same group followed by the same letters are not significantly different at α=0.05.

A) Pollination made 1 hr after spray application.

B) Pollination made 18 hr after spray application.

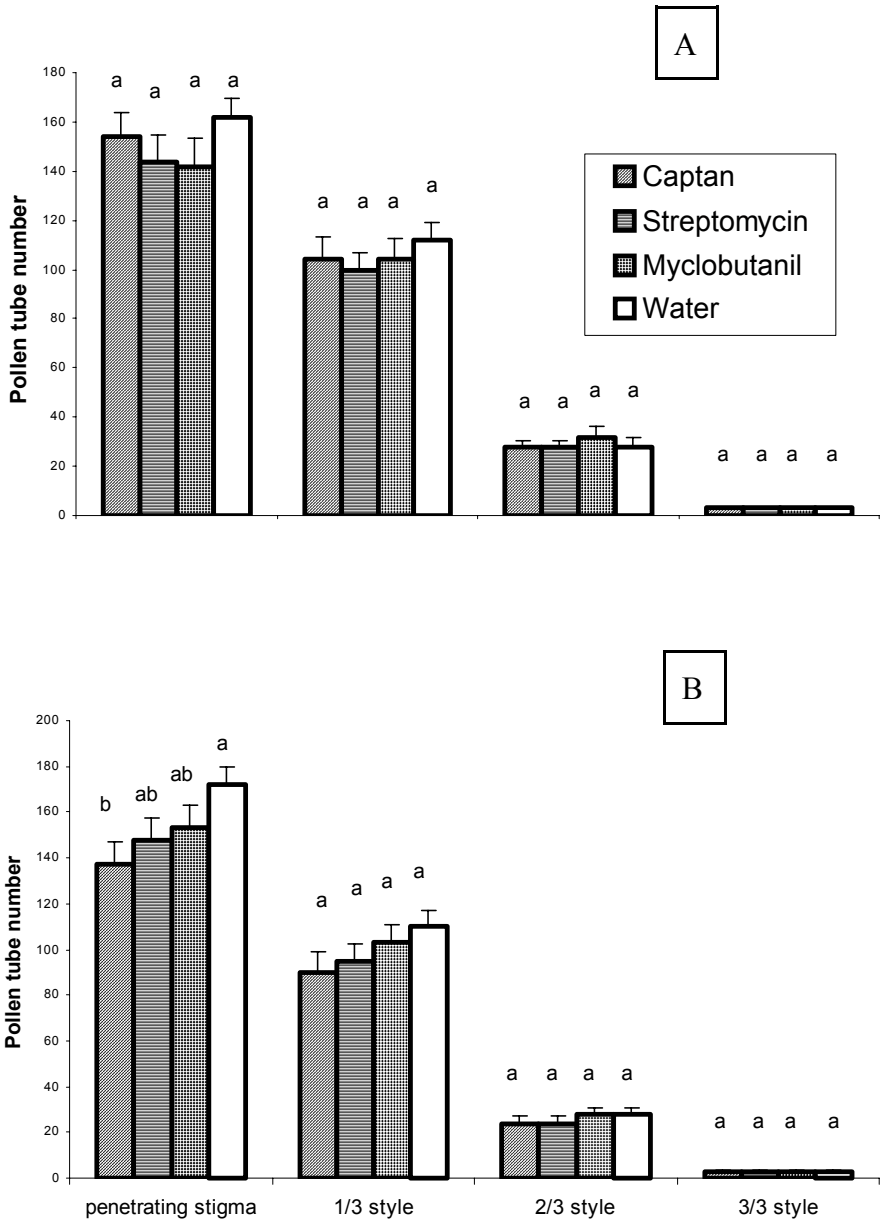
A
Pollen tube number
Captan
Streptomycin
Myclobutanil
Water
B
Pollen tube number
penetrating stigma
1/3 style
2/3 style
3/3 style
Distance of tube growth

Table 4.3. Pollen tube growth through the stigma and style in apple flowers.

Treatment	No. pollen tubes reaching different distances down the style [x]							
	Pollination 1 hour after spray				Pollination 18 hours after spray			
	Stigma	1/3	2/3	3/3	Stigma	1/3	2/3	3/3
Water	162 a [y]	112 a	28 a	3 a	172 a	110 a	28 a	3 a
Captan	154 a	104 a	28 a	3 a	137 b	90 a	24 a	3 a
streptomycin	144 a	100 a	28 a	3 a	148 ab	95 a	24 a	3 a
myclobutanil	142 a	104 a	32 a	3 a	153 ab	103 a	28 a	3 a

[x] Pollen tube numbers that had penetrated the stigmatic surface and 1/3, 2/3, or 3/3 the length of the style assessed at 20 hrs after pollination.

[y] Values followed by the same letter in the vertical columns are not significantly different at " = 0.05 according to Duncan's multiple range test. Values are the average of 12 observations.

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CHAPTER V

EVALUATION OF THE EFFECTS OF SELECTED POLLEN-DILUENT POWDERS ON POLLEN GERMINATION AND TUBE GROWTH IN THE PISTIL OF PETUNIA

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Abstract. In seed production and breeding programs, collection of pollen for controlled crosses can be costly and difficult. Pollen acquisition is also necessary for supplemental pollination used to overcome insufficient pollination, a cause of low yield in many crops. The identification of dry particulates for use as pollen diluents would be very beneficial to facilitate the use of limited amounts of pollen and to aid in accurate application and dispersion. In this study, four powders, Rilsan® ES resin, polyester resin, wheat flour, and *Lycopodium* spores, were evaluated for their potential use as pollen diluents using petunia as a model system. Diluents were combined with petunia pollen at a 5:1 (v/v) ratio. Two types of studies were conducted: 1) storage studies evaluated the viability of pollen combined and held with diluent for different durations; and 2) in vivo studies evaluated pollen tube growth in the styles of flowers pollinated with pollen-diluent mixtures. Pollen germination was not affected when stored as pollen-diluent mixtures for 2 days. However, slight but significant detrimental effects on pollen germination were observed after 4 and 6 days storage with Rilsan® ES resin and polyester resin, respectively. Pollination with all the pollen-diluent mixtures resulted in fewer pollen tubes growing in the style compared to controls that included heat-killed pollen instead of diluents. *Lycopodium*-pollen mixtures were the most inhibitory, providing only 8% of the tube numbers observed in controls. Wheat flour-pollen mixtures had intermediate numbers of tubes. Pollen mixed with polyester resin or Rilsan® ES resin showed the highest tube numbers in the style at a level of ca. 60% of the control; over 200 pollen tubes were observed which would be sufficient for high rates of seed set in petunia. This study identifies two promising diluent powders: Rilsan® ES and polyester resin. Both powders are inert and nontoxic. Their low hygroscopic nature and similar size to pollen facilitate homogeneous distribution and metering.

Introduction

Insufficient pollination is one of the important causative factors for low yield in many field and orchard species (Shivanna and Sawhney, 1997). Reproductive strategies (such as dichogamy, dioecy, monoecy, and sexual incompatibility) have evolved to promote out-crossing and genetic diversity. However, these same mechanism can contribute to inadequate pollination during crop production. Lack of pollination has been identified as a factor limiting yield and quality in a number of crops, and has been exacerbated by the decimation of bee populations due to mite infestations, habitat destruction, excessive pesticide use, and unfavorable climate. Supplemental pollination may be a means to overcome this situation.

Hand pollination has been implemented in agricultural production for some time. As early as 1930, MacDaniels reported that hand pollination reliably increased fruit set in apple. Also in apple, Volz et al. (1996) documented that supplementary pollination increased initial and final set on spur and terminal sites, seed number, and final fruit calcium concentrations compared to bee-only pollination. Orchard studies in kiwifruit showed that hand pollination produced maximal fruit size that was not obtainable with wind and bee pollination (Costa et al., 1993). Artificial pollination of tree fruits had the greatest increase in yield when pollination by natural means was not satisfactory (Bullock and Overley, 1949). Recently, electrostatically charged pollen was applied supplementally to almond and increased pollination, fruit set, and yield (Law et al., 2000; Vaknin et al., 2001).

In most crops, pollen acquisition is difficult, and the high cost of pollen may prohibit the use of supplemental pollination techniques unless efficiently applied. Problematic with the desire to efficiently apply small volumes of pollen are difficulties in

achieving accurate and uniform application. The identification of dry particulates which could be used as pollen diluents would therefore be highly beneficial. This is particularly important in mass-supplemental pollination of taxa which have pollen that is sticky and in clumps. Incorporation of a diluent can improve the flow and provide more uniform grain distribution.

In addition , the identification of useful diluent powders would be helpful in seed production and breeding programs where controlled artificial pollination is necessary to produce inbred lines and hybrids (Desai et al., 1997). In many cases, the number of pollen grains added to stigmas exceeds that required for adequate seed set due to volumes dictated by present application methods. Use of a pollen diluent would extend the use of collected pollen and decrease the amount required and cost for collection. This is of particular interest in species where pollen is difficult to collect. Also, diluents would be useful in reproductive studies evaluating pollen density effects and pollen competition. In many cases, the effect on pollen function has not been ascertained for substances used.

To be effective, a diluent must be nontoxic and have little or no inhibitory effects on pollen viability, germination or fertilization. In addition, conditions in pollen-diluent mixtures must be such that pollen viability is not compromised by desiccation or other physical degradation, and that the pollen is not mechanically damaged during mixing and handling due to angular surface configurations of particulates. The diluent must also be of a comparable size so that homogeneous mixtures of pollen and diluent can be consistently maintained during application.

The use of different powders as pollen diluents has been reported. These include *Lycopodium* spores in almond (Thorp et al., 1967); alder pollen (Legge and Williams, 1975), diatomaceous earth, bentonite, magnesium oxide, wheat flour, cake flour,

Lycopodium spores, walnut shell meal, corn meal, charcoal, different grades of ground Douglas fir bark, dicolite, Fullers earth and corn starch (Overley, 1947), pine pollen and polyvinylchloride granules (Williams and Legge, 1979) in apple; and talc in raspberry (Jennings and Topham, 1971). However, reports about systematic comparisons of different kinds of pollen diluents are limited, as is information regarding their effects on pollination and pollen function.

In preliminary assessments of potential diluents, Wetzstein and Law (1999) evaluated the size and morphological characteristics of several powders. Germination assays were conducted of pollen mixed with different diluents, and toxicity of compounds was determined in germination studies containing diluent extracts. No inhibition was observed with two Rilsan® resin formulations, polyester resin, and wheat flour. The objectives of the current study were to further evaluate these powders with the addition of *Lycopodium* spores. Factors assessed were: 1) the effects of storing pollen intermixed with diluents on subsequent germination. The viability of pollen held in mixture with diluents for durations up to 6 days was determined. 2) Pollen performance in the style after pollination with pollen-diluent mixture on the stigma. Pollen tube growth and the number of tubes growing in the styles were quantified using fluorescence staining of petunia flowers pollinated with these mixtures.

Materials and Methods

Plant material. Four-month-old petunia (*Petunia hybrida*) plants were maintained in the greenhouses in the Dept. of Horticulture at the University of Georgia, Athens, GA, during summer 2001. Plants were kept at 26 ºC, under natural light conditions. Cultivar 'Carpet Rose' was the source for pollen. Anthers were excised using small scissors and allowed to dehisce at room temperature. Pollen was cleaned of debris

by successively passing it through 380 ：m and 190 ：m stainless steel mesh screens. Petunia flowers of cultivar ‘White Dream’ were used as maternal plants in pollination studies.

Diluent powders. Four materials were evaluated as pollen diluents: 1) Rilsan® (Fr. Natural ES), a polyamide Nylon II powder (Elf Atochem N.A., Philadelphia, PA) manufactured from a biologically derived monomer of castor bean oil, 1.1 specific gravity, size range 20-80 ：m, nominal circularity ~0.8, water absorption 1-2%; 2) polyester resin (Polytech Coatings, Inc., Reading, PA) ground to ~50 ：m nominal diameter; 3) wheat flour (Pillsbury Flour, Minneapolis, MN) purchased from a local grocery store, size range 20-60 ：m; and 4) *Lycopodium* spores (EM Science, Inc., Cherry Hill, NJ), ~35 ：m diameter. When appropriate, dead petunia pollen was used for a control diluent. Pollen was heat-killed by keeping it in a 90 ºC oven overnight.

Pollen-diluent storage. Each of the first three diluents (Polyester resin, Rilsan® ES resin, wheat flour) was mixed with petunia pollen at a 5:1 (v/v) ratio. A fixed volume of each mixture was examined under a Nikon inverted microscope (Garden City, N.Y.) and the number of pollen grains counted to verify that mixtures were uniform and that the same numbers of pollen grains were present in an equivalent volume of each pollen-diluent mixture. Aliquots of pollen and pollen-diluent mixtures were sampled after storage for different durations (i.e., 0, 2, 4, or 6 days) at room temperature, at which time pollen germination assays were conducted. No germination test was made with *Lycopodium* spores, because petunia pollen could not be distinguished from the spores for accurate germination counts.

Pure pollen or pollen-diluent mixtures were first hydrated in a humidified petri dish with wet tissue paper for 4 hrs prior to inoculation. Uniform volumes of pollen-

diluent mixtures were inoculated into germination medium (12% sucrose, w/v; 0.01% boric acid, w/v). Pollen controls were inoculated into the medium maintaining the same number of pollen grains per well. Assays were conducted in tissue culture plates (Falcon Microtest III, Lincoln Park, N.J.). For each assay, 200 µl of medium was placed into each well. Approximately 400 pollen grains were inoculated uniformly into each well. After inoculation, culture plates were stored in darkness inside an incubator at 27 ºC. After 3 hrs, 20 µl of HistoChoice (Amresco Inc., Solon, Ohio) fixative was added into each assay well to arrest tube growth and preserve tube morphology. Germinated pollen grains were counted under a Nikon inverted microscope (Garden City, N.Y.). Pollen with tubes extending greater than the diameter of the pollen was deemed germinated.

Pollen tube growth in the style. Petunia flowers of cultivar 'White Dream' were emasculated before anther dehiscence to prevent self pollination. Flowers were collected for pollination when copious exudate was visible on the stigmatic surface. The cut ends of the flowers were placed into small containers filled with water. Rilsan® ES resin, polyester resin, wheat flour, or *Lycopodium* spores were combined with pollen at a 5:1 (v/v) ratio. Pollen-diluent mixtures were held at room temperature for 2 hrs before pollination. Uniform volumes of each mixture (containing about 600 viable petunia pollen grains) were applied under a dissection microscope to each stigmatic surface using a small handmade plastic spatula and a small brush. As a control, heat-killed pollen was mixed with viable petunia pollen at a 5:1 (v/v) ratio. This was so the controls had a similar volume of material inoculated onto each stigma as treatments, i.e., total volume of diluent: viable pollen or heat-killed pollen: viable pollen were equivalent. Flowers were kept at 26 ºC under continuous light conditions. Flowers were dissected to remove pistils 23 hrs after pollination. The pistils were then fixed in ethanol: acetic acid (3:1 v/v).

Tissues were softened and cleared by autoclaving at 120 ºC for 20 min in 1% sodium sulfite solution, and stained overnight using aniline blue (0.01 % aniline blue in 0.1M K_3PO_4). Portions of the style exhibited autofluorescence which interfered with accurate visualization of pollen tubes. Thus, the transmitting tract was dissected out, mounted in a drop of aniline blue on a slide, and gently squashed using a cover slip. The number of pollen tubes and the extent of their growth through the length of the style were assessed using a Zeiss Standard microscope under flourescent light (G365, LP420) (Currier, 1957).

Experimental design and statistical analysis. In pollen-diluent storage studies, a factorial design was used. Two factors were evaluated: the diluent mixture and storage period. The design was: 4 diluent mixture/control x 4 periods x 3 rep. In the pollen tube growth studies in the style, a completely randomized block design was used. The design was: 5 diluents x 5 blocks (times) x 5 rep. Experiments were repeated 5 times which were treated as blocks. Statistical analysis was conducted by using GLM followed by Duncan's multiple range test at $\alpha = 0.05$.

Results

Pollen-diluent storage. Table 5.1 shows germination percentages, after various storage times under room temperature, for pollen stored as a pure form or within pollen-diluent mixtures. Germination percentage decreased the longer the pollen samples were held at room temperature. Significant linear trends between exposure time and germination percentages existed for pure pollen and for all of the pollen-diluent mixtures (P values were 0.0086, 0.0113, 0.0006, and 0.0001 for pure pollen and mixtures of pollen with wheat flour, polyester resin, or Rilsan® ES resin, respectively). However, loss of viability was slight with storage. Pure pollen maintained 88% of its original viability

after 6 days at room temperature. On the day when pollen was collected and combined with diluents (0 day), there was no significant difference in germination between pure pollen and pollen mixed with any of the diluents evaluated. Pollen germination percentages ranged from 74% to 78%. Likewise, germination was the same for pure pollen and pollen-diluent mixtures when germination assays were conducted after 2 days storage. After 4 days storage, pollen mixed with Rilsan® ES resin showed significantly lower germination than pure pollen controls, although germination percentages were still 90% of that exhibited by the control. After 6 days storage, pollen mixed with polyester resin and Rilsan® ES resin had significantly lower germination than pure pollen controls. Germination percentages were 91% and 87% of the pure pollen control, respectively. Wheat flour had no detrimental effect on pollination even after 6 days.

Pollen tube growth in the style. Pollen mixed with all of the diluents evaluated had significantly lower numbers of pollen tubes growing in the style compared with the control of viable pollen mixed with dead pollen. The diluents had varied markedly in their effects on pollen tube growth. Table 5.2 shows the results of pollen tube growth in the style of petunia flowers pollinated with different types of pollen-diluent mixtures. In evaluation of tube numbers that passed ½ or 3/4 the length of the style, *Lycopodium* spores imposed the most severely inhibiting effects, with flowers exhibiting only 7 to 8% of the number of the tubes found in controls. Pollen mixed with wheat flour showed intermediate numbers of tubes (ca. 48% of the control). Among the four diluent powders, pollen mixed with polyester resin and Rilsan® ES resin showed the highest tube numbers growing down the style. The pollen tube numbers reaching 3/4 the length of the style were 61% and 53% respectively, of that found in control pollinations.

Discussion

In this study, mixing pollen with any of the diluent powders caused no detrimental effects in germination even when held for 2 days at room temperature. Longer-term storage was required before inhibitory effects on germination were observed for some powders, i.e., 4 days with Rilsan and 6 days with polyester resin. However, in all cases, germination percentages of pollen combined with Rilsan still maintained 90 and 87% germination of the control, respectively, after 4 and 6 days storage, while pollen-polyester resin mixtures maintained 91% germination rates of the control. A 2- to 4- day storage window should pose few constraints in the development of practical pollen-application protocols. This is in contrast to other reports where short-term contact with other pollen diluents severely inhibited pollen germination. In an effort to develop methods for supplemental pollination, Vaknin et al. (1999) evaluated the effects of dilution with talc and silica gel on the germinability of almond pollen. Both diluents improved pollen flow. However, germination in vitro was dramatically reduced to only 43% of pure pollen controls after only 30 minutes contact with 10% (w/w) of either talc or silica gel.

With in vivo pollination studies, the control was selected so that a standardization of the total amount of particulate material (whether it be diluent or pollen) applied to the stigma was obtained. Under these conditions, physical characteristics such as space on the stigma and/or pollen contact (which would affect subsequent hydration and activation) were similar. Likewise, it was important to apply the same number of pollen grains per stigma so that diluent effects on pollen-stigma interactions and pollen function could be directly compared. In that regard, the pure pollen control treatment used with in

vivo studies had a portion of the pollen heat-killed, so that the amount of "viable" pollen per pollination was consistent with the diluent treatments.

In some cases, higher pollen loads can cause a population or mentor pollen effect where additional pollen can provide a stimulus leading to enhanced germination and pollen tube growth. This can be the case even when pollen is killed chemically (Dayton, 1974; Visser, 1981) or by (-irradiation (Visser et al., 1983; Montalti and Filiti, 1984). Cruzan (1986) reported density-dependent responses by pollen populations growing in the pistils of *Nicotiana glauca*. Pollen extracts promoted in vitro pollen germination in almond whether from compatible or self-incompatible sources (Eisikowitch and Wetzstein, 1999). Brewbaker and Majumder (1961) reported in petunia that pollen killed by heating at 100 °C in water still contributed a population effect with in vitro germination assays. Thus, the germination percentages observed in our control treatments of viable plus dead pollen may be elevated over what would be expected if fewer total pollen grains were applied. The inhibitory effects observed with pollen-diluent mixtures may therefore be less consequential than indicated in the study. Although the number of tubes growing midway down the style with polyester and Rilsan diluents was ca. 60% of pure-pollen controls, over 200 pollen tubes were observed which would in any event be sufficient for high seed set in petunia.

Although pollen combined with wheat flour, polyester resin or Rilsan had the same in vitro germination percentages, pollen-wheat flour mixtures had significantly fewer tube numbers growing in the style compared with pollen-polyester (at " = 0.05) or pollen-Rilsan combinations (" = 0.1). Pollen-stigma interactions include pollen adherance and hydration, stigmatic secretion, pollen germination and stigma penetration (Johri, 1984). The water absorption of polyester resin and Rilsan ES resin is only ca. 1-

2% and considerably lower than for wheat flour (ca. 80%). The higher water absorption of wheat flour could impact pollen hydration on the stigma and inhibit pollen germination. In contrast to in vitro germination assays where pollen hydration readily occurs, pollen activation and hydration on the stigma is more limited and reliant on stigmatic cell exudate production.

Lycopodium spores used as a diluent severely inhibited the numbers of tubes penetrating the style. Pollen tube numbers were only about 8% of the controls and 14% of that obtained with polyester resin and Rilsan. Bullock and Overlay (1949) evaluated 37 different materials as pollen diluents in regard to the pollen maintaining its viability in storage. *Lycopodium* spores, powdered milk, and egg albumen were identified as promising agents according to in vitro germination tests. However, they did not evaluate the effects of diluents in vivo, which reinforces the need to consider pollen stigma interactions. We were not able to assess the effect of *Lycopodium* spores as a diluent for petunia pollen with in vitro studies because we could not distinguish between the spores and pollen which have very similar shape and size. The use of *Lycopodium* spores to adjust pollen load in pollination studies. Our work suggest that *Lycopodium* spores may introduce errors due to their inhibitory effect on pollen function, and that lack of inhibitory effects be verified before use.

Conclusion

This study identified two promising diluent powders, Rilsan® ES and polyester resin, that may be useful for pollination. Pollen germination was not affected when stored as pollen-diluent mixtures for 2 days with these powders. In vivo studies verified that although pollen tubes numbers were fewer than in pure pollen applications, large numbers of pollen tubes could be obtained with 5:1, powder:pollen dilutions. Both

powders are inert, nontoxic, and widely used in the food and medical industries. Their low hygroscopicity and similar size to pollen should facilitate homogeneous distribution and metering.

Table 5.1. In vitro germination percentages of petunia pollen stored for different durations as a pure form or in a pollen-diluent mixture

Pollen-diluent mixture [y]	% Germination after specified storage times			
	0 days	2 days	4 days	6 days
Pure pollen control	76 a [z]	68 a	67 a	67 a
Pollen + wheat flour	74 a	71 a	69 a	66 a
Pollen + polyester resin	78 a	66 a	65 a	61 b
Pollen + Rilsan® ES resin	77 a	70 a	60 b	58 b

[z] Values within columns followed by the same letters are not significantly different at " = 0.05 according to Duncan's multiple range test. Value is the average germination percentage of 3 replications.

[y] Mixtures are 5:1 (v/v) diluent-to-pollen ratio.

Table 5.2. Pollen tube growth in the style of petunia flowers pollinated with different types of pollen-diluent mixtures.

Diluent	Number of tubes reaching indicated location [z]	
	½ style length	3/4 style length
Dead pollen control	365 a [y]	249 a
Polyester resin	218 b	152 b
Rilsan® ES resin	211 b c	132 b c
Wheat flour	175 c	116 c
Lycopodium spores	30 d	18 d

[z] Pollen tube numbers that had passed ½ or 3/4 of the total length of the style 23 hrs after pollination with about 600 pollen grains.

[y] Values within columns followed by the same letters are not significantly different at " = 0.05 according to Duncan's multiple range test. Value is the average of 25 observations.

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SUMMARY AND CONCLUSIONS

Adequate pollination is critical in agricultural crops where fruit or seed is the final product. In addition to directly affecting crop yield, the extent of pollination and subsequent fertilization are important factors in determining crop quality. Unfortunately, insufficient pollination is one of the most important causative factors for low yield in many field and orchard crops.

Applications of early season fungicides are necessary in most fruit/nuts and vegetable crops to protect susceptible young foliage and/or floral parts. The timing of pesticide applications, often overlaps the time at which flowering and pollination occur. Numerous studies report detrimental effects of fungicide applications on pollination, fruit set and yield in selected crops. Unfortunately, information about the potential detrimental effect of fungicides on almond, an important nut crop, is lacking. While numerous reports have documented the detrimental effects of pesticides on apple under in vitro conditions, there are conflicting reports concerning effects of chemical sprays on fruit set even when similar compounds are tested.

Artificial pollination may provide a useful way to overcome pollen-related production problems. Artificial pollination is also required in seed production and breeding programs to make controlled crosses. However, collection of pollen can be costly and difficult. The identification of dry particulates for use as pollen diluents would be very beneficial to facilitate the use of limited amounts of pollen and to aid in accurate application and dispersion.

In this study, research was conducted in two parts: a) evaluate the potential detrimental effects of pesticides on pollination in almond and apple; b) evaluate the effects of selected dry particulate materials as pollen diluents in artificial pollination using petunia as a model system.

In order to screen the effects of selected fungicides on almond pollen function, in vitro germination assays were first conducted in ten fungicides at three concentrations of their recommended field rates (1x, 1/10x, and 1/100x). Assays conducted at 1/100 x were effective in delineating differences in almond pollen sensitivity to the different fungicides. Captan and azoxystrobin were the most inhibitory, with relative germination percentages of less than 1% of the control. Germination was not significantly affected by propiconazole and benomyl. Intermediate inhibitory effects on pollen germination were observed with ziram, cyprodinil, maneb, thiophanate-methyl, iprodione, and myclobutanil. In contrast to germination, pollen tube growth was less affected by the presence of fungicide. In pollen that germinated, tube elongation was the same as controls in half of the fungicides evaluated. Nonetheless, azoxystrobin and captan reduced tube elongation by ca. 90%. Some fungicide treatments also influenced tube morphology.

The effect of fungicide sprays on stigma morphology was also evaluated, since the stigma is the place where pollen is deposited and germinates, and any damage to it will impact pollen germination and subsequent fertilization. Four fungicides were sprayed onto intact almond flowers under constant laboratory conditions using an electronically controlled robotic apparatus which simulated a field sprayer. Stigmatic surfaces were observed in a fresh, unfixed state using a SEM. Stigmatic surfaces were observed at 4 or 24 hrs after spray. Azoxystrobin caused increase of exudate

accumulation on the stigma. Furthermore, localized damage and collapse to stigmatic cells were observed 24 hrs after spray. Similarly, myclobutanil caused significant damage and collapse to stigmatic papillae. Collapse was more extensive at later observations. Slight increase of exudate accumulation was observed 4 hrs after spray. Iprodione had no effect on exudate accumulation, but caused marked and severe collapse of stigmatic papillae which was more severe at 24 hrs after spray. Cyprodinil promoted a copious increase in exudate secretion and caused the most severe collapse of stigmatic cells of all the fungicides evaluated.

Direct effects of selected pesticide sprays on pollen germination on the stigma and tube growth in the style were studied in both almond and apple. Sprays were applied to detached flowers. Flowers were pollinated at specific times after spraying, and pollen tube numbers and growth assessed using fluorescence microscopy. In apple, captan sprays significantly inhibited pollen germination on the stigmatic surface, reducing germination by 20% compared with water controls. In contrast, myclobutanil and streptomycin had no significant effect on the pollen tube growth. With almond, none of the fungicide sprays evaluated had any significant effect on pollen germination or tube growth. Of the seven compounds evaluated in apple and almond, only captan detrimentally affected pollen germination on apple stigmas.

In the diluent study, four powders, Rilsan® ES resin, polyester resin, wheat flour, and *Lycopodium* spores, were evaluated for their potential use as pollen diluents. Two types of studies were conducted: 1) storage studies evaluated the viability of pollen combined and held with diluent for different durations; and 2) in vivo studies evaluated pollen tube growth in the styles of petunia flowers pollinated with pollen-diluent mixtures. Pollen germination was not affected when stored as pollen-diluent mixtures for

2 days. However, slight but statistically significant detrimental effects on pollen germination were observed after 4 and 6 days storage with Rilsan® ES resin and polyester resin, respectively. Pollinations with all the pollen-diluent mixtures resulted in fewer pollen tubes growing in the style compared with controls that included heat-killed pollen as diluent. *Lycopodium*-pollen mixtures were the most inhibitory, providing only 8% of the tube numbers observed in controls. Wheat flour-pollen mixtures had intermediate numbers of tubes. Pollen mixed with polyester resin or Rilsan® ES resin showed the highest tube numbers in the style with ca. 60% of the control; over 200 pollen tubes were observed which would be sufficient for high rates of seed set in petunia. This study thus identifies two promising diluent powders: Rilsan ® ES and polyester resin. Both powders are inert and nontoxic. Their low hygroscopic nature and similar size to pollen facilitate homogeneous distribution and metering.