Refining Fungicide Spray Timing, Extending Tests of Fungicide Residual Effects on Fertilization through Stigma Receptivity, Pollen Germination and Tube Growth

Project No.: **15-POLL3-Williams**

Project Leader: Neal Williams
Associate Professor of Entomology
Department of Entomology and Nematology
UC Davis
One Shields Avenue
Davis, CA 95616-8584
530.752.9358
nmwilliams@ucdavis.edu

Project Cooperators and Personnel:

Kimiora Ward, Research Associate III, Heather Spaulding, Lab Assistant, and Claire Brittain, Postdoctoral Scholar, Department of Entomology, UC Davis

Objectives:

The objective of the study was to inform timing of fungicide sprays in almond for minimum impact on fertilization of almond flowers. Specifically, we investigated how pollen viability, stigma receptivity and pollen tube growth in almond were affected by the timing of fungicide application. We expanded on past years' exploration of two fungicide FRAC groups, and tested flowers exposed in the field to gain a more accurate reflection of how real field-based exposure levels impact fertilization. We quantified whether coarse differences in the timing of spray affected successful fertilization, specifically whether there were differences in the impact of fungicide on fertilization of flowers that were open when the fungicide was applied, versus those that had yet to open when the fungicide was applied. Localized systemic action of fungicides raises the potential for in-bud application to have impacts, although our previous trials of two FRAC groups suggested no clear impact.

Interpretive Summary:

Timing of fungicide spray according to label rates and best application practices appears to have no negative impact on post-pollination stages of fertilization (which leads to nut set). Our comparison increased the number of fungicide action groups (FRAC groups) tested for exposure to flowers that had already been pollinated and flowers pollinated post-spray exposure in open field trials. Our results showed inconsistent effects on pollen germination among exposure to pollen stigmas or both, but indicated a slight positive effect of fungicides on pollen tube growth. Our methods could be applied to test other fungicides with the same and different modes of action to further explore best timing of application and to reassure growers that the fungicides they are applying through best management practices are not detrimental to yield through direct effects on pollen germination or pollen tube growth in almond flowers.

Materials and Methods:

Spray 1: Exposure of unopened flowers
In a UC Davis orchard, almond branches were covered with mesh pollinator exclusion bags before bloom to prevent access to the flowers by bees but allow air flow. The exclusion bags were placed on trees of two varieties: Nonpareil (stigma variety) and Carmel (pollen donor), half of which were to be sprayed with a FRAC (Fungicide Resistance Action Committee) group 9 fungicide (a methionine biosynthesis inhibitor) and the other half with a FRAC group 11 fungicide (a quinone outside inhibitor). Previous work investigated impacts of FRAC groups 3 and 7.

Immediately before (20 min-1 hour) the first fungicide application on February 12, 2016 three quarters of the pollinator exclusion bags were replaced with fungicide exclusion bags. The remaining one quarter of the branches were left unbagged and any opened flowers were removed so that buds could be exposed to fungicide before anthesis. Half of the unbagged branches were on trees being sprayed with the FRAC 9 fungicide and the other half were on the trees sprayed with the FRAC 11 fungicide.

Immediately following the first fungicide application, the fungicide exclusion bags were switched back to pollinator exclusion bags to allow for better air flow around the branches. Branches with buds that were exposed to the fungicide application and a set of bagged no-spray control branches with open flowers removed were cut from the trees; their stems placed into water-filled vases to maintain their freshness, and brought to the laboratory for hand pollination. Daily thereafter, branches in the lab were checked so that any of the exposed or control buds that had opened could be hand pollinated. All hand pollination was done by crossing Carmel pollen onto Nonpareil stigma flowers. Hand pollinations were performed using dried honey bee thoraxes mounted on tooth picks as pollen brushes. Pollen was collected from Carmel flowers of a particular treatment group by brushing the thorax over the anthers and then deposited on the target Nonpareil stigma by touching the stigma with the pollen-coated thorax. Anthers were removed from receptive Nonpareil flowers beforehand pollination to avoid deposition of self-pollen. Bee brushes were cleaned before use with compressed air to avoid contamination between pollen treatment groups. For each of the two fungicides, four types of hand pollination crosses were performed (see **Table 1**). Crosses were not performed between the different fungicide types.

Table 1. Experimental design of pollination crosses.

	Control Drake pollen	Fungicide exposed Drake pollen
Control Nonpareil stigma	*Hand pollination*	*Hand pollination*
Fungicide exposed Nonpareil stigma	*Hand pollination*	*Hand pollination*

After hand pollination, flowers were placed in microcentrifuge tubes containing 0.5 ml tap-water, so the pedicle was in water, but the flower remained above and there was no contact of the stigma with the centrifuge-tube wall or water surface. The flowers remained in the tubes for three days at room temperature to allow for pollen tube development. After the three days, the

flowers were fixed in FAA (10:7:2:1 ethanol (95 %), H_2O, formalin, acetic acid, stored at 4°C) until further processing.

Spray 2: Exposure of opened flowers before and after pollination
The second fungicide application was performed on February 16, 2016 when the orchard was in 80% bloom and many opened flowers were available. The day prior to the second spray, we conducted hand pollinations in the field on branches that had been bagged to prevent pollination, and had protected from the first spray to test effects of fungicide application on flowers that had already been pollinated. We hand pollinated previously unexposed flowers (protected with fungicide exclusion bags during the first spray) using honey bee thoraxes as described above. Because timing of exposure was the focus here rather than routes of exposure through pollen, all Nonpareil stigmas were pollinated with unsprayed Carmel pollen. Half of the flowers pollinated were on trees to be sprayed with FRAC 9, half on trees to be sprayed with FRAC 11. In each cased equal numbers of branches were rebagged with fungicide exclusion bags to serve as field-pollinated controls. Field-pollinated branches were marked, any extraneous buds or un-pollinated flowers removed, and rebagged with pollinator exclusion bags.

The next day just prior to the second fungicide application, approximately 1/3 of the remaining branches (split between FRAC 9 and FRAC 11 trees) were covered with fungicide exclusion bags to act as no-spray controls and the rest were unbagged to be sprayed with either FRAC 9 or FRAC 11. Within each treatment group, approximately half of the flowers had been hand pollinated in the field the previous day.

After the spray, all branches were cut from the tree after rebagging with pollinator exclusion bags to keep pollen from treatment groups separate, and brought to the lab. Branches with flowers that had been hand pollinated in the field were set aside to allow for pollen tube germination and growth over a three-day period before fixation with FAA. All remaining exposed and control open flowers were then hand-pollinated in the lab using the same crosses and procedures as described above and in **Table 1**.

Examination of pollen tube development
Once all the flowers had been fixed they were processed in batches of twenty. The flower pistils were boiled in 5% sodium sulfite (Na_2SO_3) to soften the tissue and soaked in water for 20 minutes before staining. The pistils were incubated for 12-24 hours in a decolorized staining solution of 0.1% aniline blue dye dissolved for 1 hour in 0.1N K_3PO_4. The stained pistils were squashed onto a microscope slide to reveal the pollen tubes. The slides were examined using a fluorescent microscope (Nikon Eclipse 80i with a CFL-FITC filter). For each slide, the numbers of pollen grains, the number of pollen tubes initiating growth at top of the style and the number of pollen tubes reaching the base of the style were counted. ‘Pollen germination’ was calculated as the number of pollen tubes initiating growth divided by the number of pollen grains and ‘pollen tube development’ was calculated as the number of pollen tubes reaching the base of the style divided by the number of pollen tubes initiated at the top of the style.

Statistical Analysis
The flowers that were hand pollinated in the laboratory and those that were hand pollinated in the field were analyzed separately. For the flowers hand pollinated in the laboratory, pollen

germination, pollen tube development and the number of pollen tubes reaching the base of the style were tested to see if they differed between treatments. The four treatments were: CC (control stigma, control pollen), CE (control stigma, fungicide-exposed pollen), EC (exposed stigma, control pollen) and EE (exposed stigma and exposed pollen). Analysis of variance (ANOVA) followed by paired-comparisons (Tukey HSD (honest significant difference) among treatments were used to test for significant differences between the treatment groups. The tests were done separately for flowers exposed to the FRAC 9 fungicide and flowers exposed to the FRAC 11 fungicide (the same control data were used for both). ANOVA assumes homogeneity of variance and where necessary the data were log transformed to conform to this assumption.

For the flowers that were pollinated in the field ANOVA was used when possible, and the Wilcoxon-Mann-Whitney test was used when unequal variances could not be corrected with transformation, to test whether there was a difference in pollen germination, pollen tube development or the number of pollen tubes reaching the base of the style in flowers that were exposed to fungicide following hand pollination and flowers that were not (exposed vs. control).

Results and Discussion:

In total, 671 Nonpareil flowers were hand pollinated in the laboratory and successfully stained for pollen tube growth. In the field, 216 Nonpareil flowers were hand pollinated and subsequently stained to visualize pollen tube growth. The results from the analysis of the flowers hand pollinated in the laboratory are given in **Table 2**.

Table 2. Results (*P* values) of ANOVA tests, testing if pollen germination, pollen tube development or the number of pollen tubes reaching the base of the style differed with fungicide exposure. Significant results (*P*<0.05) given in bold. Differences between treatments shown in **Figure 1** and **Figure 2**.

Stigma variety	Chemical	Exposure	Pollen germination	Pollen tube development	No. pollen tubes end
Nonpareil	FRAC 9	bud	0.318	0.301	0.420
Nonpareil	FRAC 9	flower	0.353	[1]**0.002**	[2]**0.006**
Nonpareil	FRAC 11	bud	0.083	0.234	0.143
Nonpareil	FRAC 11	flower	**0.003**	0.276	[†]0.241

1. Data square-root transformed for homogeneity of variance.
2. Data log(x+1) transformed for homogeneity of variance.

No effect of treatment was found on pollen germination, pollen tube development or the number of pollen tubes reaching the base of the style when exposed to either FRAC 9 or FRAC 11 fungicide as buds. There was an effect of treatment on pollen tube development and the number of pollen tubes reaching the base of the style in Nonpareil stigmas exposed to the FRAC 9 as flowers (**Figure 1**). Although all treatment groups had limited pollen tube development and small numbers of pollen tubes reaching the base of the style, FRAC 9 exposed stigmas pollinated with FRAC 9 exposed pollen had significantly greater pollen

development (**Figure 1a**) and higher numbers of pollen tubes reaching the base of the style **(Figure 1b**) than no-spray control flowers.

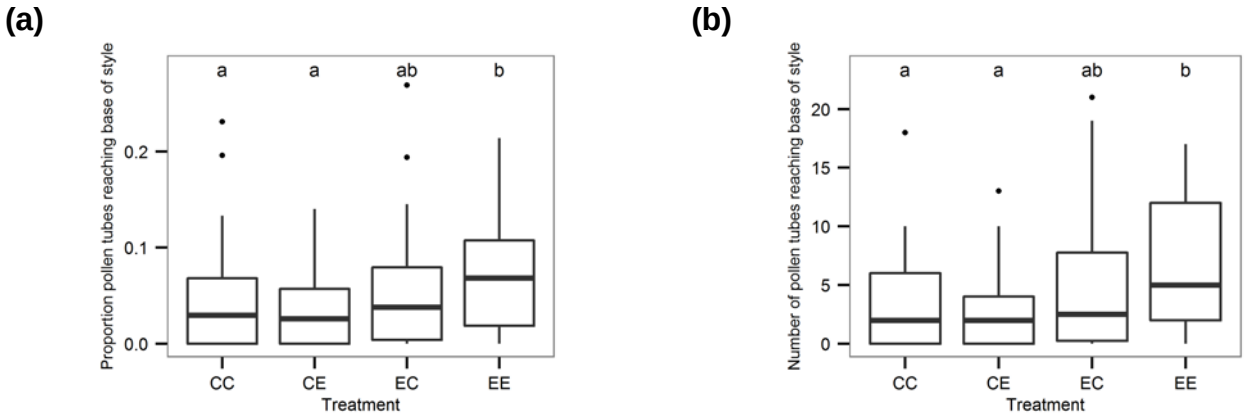

Figure 1. The differences in measures of pollination for the two cases where a significant effect of treatment with a FRAC 9 fungicide was found (**Table 2**). (a) pollen development and (b) number of pollen tubes reaching the base of the style in Nonpareil stigmas and Carmel pollen exposed to a FRAC 9 fungicide as flowers. The treatment letters represent the following: CC=control stigma, control pollen, CE=control stigma, exposed pollen, EC=exposed stigma, control pollen, EE=exposed stigma, exposed pollen. The letters a and b above treatment represent significant differences between those treatments if no letter is shared. The heavy lines within each box represent the median value and the upper and lower parts of the box the upper and lower quartile respectively.

There was also an effect of treatment on pollen germination in Nonpareil flowers exposed to the FRAC 11 as flowers (**Figure 2**). The combination of a control Nonpareil stigma and exposed Carmel pollen had higher pollen germination than the other three treatments (**Figure 2**). This effect of FRAC 11 on open flower-exposed Nonpareil did not persist through to differences between treatments in pollen tube development or in the numbers of pollen tubes reaching the base of the style.

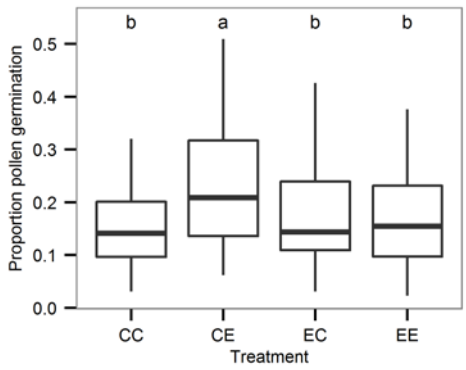

Figure 2. The differences in pollen germination for the only case where a significant effect of treatment with a FRAC 11 fungicide was found (**Table 2**). Pollen germination in Nonpareil stigmas and Carmel pollen exposed to a FRAC 11 fungicide as flowers. The treatment letters represent the following: CC=control stigma, control pollen, CE=control stigma, exposed pollen, EC=exposed stigma, control pollen, EE=exposed stigma, exposed pollen. The letters a and b above each treatment represent significant differences between those treatments if no letter is shared. The heavy lines within each box represent the median value and the upper and lower parts of the box the upper and lower quartile respectively.

The results from the analysis of flowers hand pollinated in the field are given in **Table 3.** For Nonpareil stigmas pollinated in the field prior to exposure, both FRAC 9 and FRAC 11 fungicides significantly affected pollination for all response variables except the number of pollen tubes reaching the end of the style in FRAC 9-exposed flowers. Pollen germination was significantly lower in flowers that were exposed to FRAC 9 fungicide after pollination, but pollen tube development was significantly higher with this same treatment (**Figure 3a, b**). All measures of pollination from pollen germination through to the number of pollen tubes reaching the base of the style, were significantly higher in the flowers exposed to the FRAC 11 fungicide following pollination than they were in the no spray control flowers (**Figure 3c, d, e**).

Table 3. The results (*P* values) of analysis of variance (or Wilcoxon-Mann_Whitney) tests, investigating if fungicide exposure following hand pollination affected pollination of the flowers. Significant results (*P*<0.05) given in bold and the differences between treatments shown in **Figure 3**.

Stigma variety	Chemical	Pollen germination	Pollen tube development	No. pollen tubes end
Nonpareil	FRAC 9	**0.040**	‡**<0.001**	‡0.271
Nonpareil	FRAC 11	**0.012**	**0.018**	**<0.001**

‡ Wilcoxon-Mann-Whitney test applied when no transformations improved unequal variances.

We found no consistent negative effect of exposure to FRAC 9 or FRAC 11 fungicide on the pollination of almond flowers. Rather, in the case of exposure to fungicides prior to pollination, the few significant effects of exposure to fungicide indicated slightly positive effects: flowers that were exposed to FRAC 9 fungicide before pollination had slightly higher pollen development and greater numbers of pollen tubes reaching the base of the style, and the combination of control stigma and exposed pollen donor showed higher pollen germination, though this did not translate to effects on later stages of pollination. It is important to note that in each case the magnitude of the effect was modest (a median of approximately 2 versus 4 grains reaching the base of the style). No effect on any measure of pollination was detected when the flowers were exposed as buds. Flowers exposed to fungicide after pollination showed inconsistent effects of fungicide exposure. Pollen germination was reduced with exposure to FRAC 9 after pollination, but exposure had the opposite effect on pollen development and no effect on the number of tubes reaching the base of the style. It is possible that some of the other ingredients in the FRAC 9 and FRAC 11 mixtures applied to the flowers are have physiological effects that slightly enhance pollen tube growth. Our previous work suggested a possible similar effect in flowers exposed to FRAC 7, but not FRAC 3, after pollination, but this finding was not consistent with results seen in flowers exposed before pollination. Our cumulative results show no consistent effect of the application of fungicides from four FRAC groups to the pollination of almond flowers. Within each FRAC group there are a range of chemicals and surfactants and we do not suggest that our results represent the effects of all fungicides that fall within these groups. In any case our method could be applied more widely to test for the potential effects of other fungicides on the post pollination impacts on almond fertilization and inform almond fungicide best management practices.

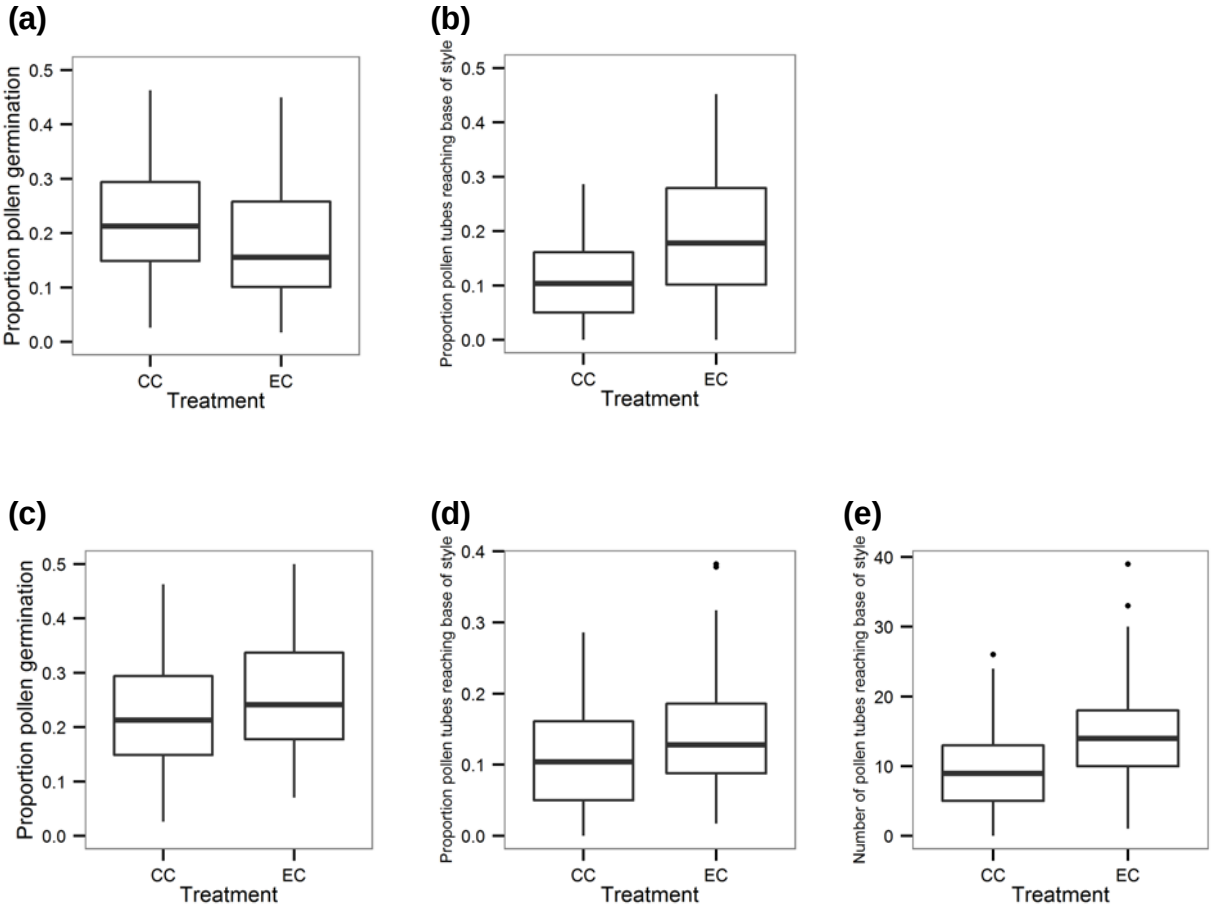

Figure 3. The cases where flowers were pollinated before fungicide exposure (**Table 3**). (a) Pollen tube development and (b) the number of pollen tubes reaching the base of the style in pre-pollinated flowers exposed to FRAC 9 fungicide, (c) pollen germination, (d) pollen tube development, and (e) the number of pollen tubes reaching the base of the style in pre-pollinated flowers exposed to FRAC 11. CC= control flowers that were hand pollinated in the field and then bagged to prevent fungicide exposure. EC= flowers that were hand pollinated in the field prior to the application of fungicide All pollen donors were control.

Research Effort Recent Publications:

none to date

References Cited:

none cited