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A Novel Pheromone Dispenser for Mating Disruption of the Leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae)

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ABSTRACT The sex pheromone of the leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) was deployed in a Florida citrus (*Citrus* spp.) grove by using a novel deployment device (IFM-413) containing SPLAT, a flowable formulation of an emulsified wax compound designed to provide slow release of semiochemicals. The device consisted of two disks connected by string. Each disk was loaded with 1 g of SPLAT containing either 0.15% (Z,Z,*E*)-7,11,13-hexadecatrienal (triene) or 2% (Z,Z)-7,11-hexadecadienal (diene). The devices were deployed using a two-dimensional multivariate design to determine the optimal rate of pheromone per unit area and degree of aggregation of the deployment devices (number of trees treated per unit area). The IFM-413 device proved effective at becoming securely entangled in tree branches. Furthermore, the devices effectively delivered pheromone-loaded SPLAT that resulted in disruption of trap catch of male *P. citrella*. Response surfaces showed a quadratic response of trap catch disruption to both total amount of pheromone per unit area and the degree of aggregation of the deployed devices (number of treated trees per unit area). The response surfaces for 0.15% triene or 2.0% diene were similar. The diene produced an effect similar to that of the triene at ≈13 times the rate of the triene. The greatest disruption of trap catch occurred when the number of treated trees per unit area was greatest (no aggregation of deployment devices). Manufacturing, packaging, and mechanical deployment of the devices remain to be investigated.

KEY WORDS sex pheromone; (Z,Z,*E*)-7,11,13-hexadecatrienal; (Z,Z)-7,11-hexadecadienal; citrus leafminer; SPLAT

The leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) is a pest of citrus (*Citrus* spp.) crops throughout the world (Heppner 1993). Larvae feed within leaves, producing serpentine mines that result in distortion and loss of photosynthetic capacity, thereby reducing yield (Peña et al. 2002). The larvae are a particular concern where the citrus bacterial canker, *Xanthomonas axonopodis* pv. citri, occurs. In addition to direct feeding damage that results in reduced yield, larval feeding increases the susceptibility of leaves to the canker pathogen (Graham et al. 2004, Gottwald et al. 2007). Control of *P. citrella* larvae with insecticides is often ineffective due to the location of larvae within the leaf and the loss of natural enemies (Peña et al. 2002).

The sex pheromone of *P. citrella* was determined to contain three components (Leal et al. 2006, Moreira et al. 2006). Of these components, two—(Z,Z,*E*)-7,11,13-hexadecatrienal (triene) and (Z,Z)-7,11-hexadecadienal (diene)—in a 3:1 ratio were shown to be required for attraction in the field (Lapointe et al. 2006). Neither component is attractive by itself (Lapointe et al. 2009). Control of *P. citrella* by mating disruption has proven effective at very low rates of pheromone deployment (Stelinski et al. 2008). Furthermore, either pheromone compound alone is capable of disrupting mating (Lapointe et al. 2009). Field trials used an emulsified wax formulation (SPLAT, ISCA Technologies, Riverside CA) for slow-release of *P. citrella* pheromone applied to citrus trees. The triene was ≈13 times more effective compared with the diene alone and was as effective or more effective compared with the natural 3:1 blend (Lapointe et al. 2009).

SPLAT is a flowable formulation of an emulsified wax compound designed to provide slow release of semiochemicals and capability for mechanical application. Various devices are being developed to apply semiochemical-containing SPLAT in orchard crops. Our objective was two-fold: to test a novel delivery device that allows for precise location of uniform droplets of pheromone-loaded SPLAT and to determine the effect of distribution of such devices on trap catch disruption for control of *P. citrella* in commercial citrus groves.

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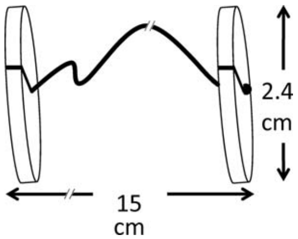

Fig. 1. Deployment device for delivery of semiochemicals in tree crops. Cardboard disks (2.4 cm in diameter) connected with 15 cm of nylon string were loaded with 1 g of SPLAT containing either (*Z,Z*)-7,11-hexadecadienal or (*Z,Z,E*)-7,11,13-hexadecatrienal. Devices were thrown into the canopy and became entangled in the foliage or branches of orange trees.

Materials and Methods

Deployment Device. We designed and manufactured ≈10,000 IFM-413 devices. Each device (Fig. 1) consisted of two cardboard disks with a slit from the center to the periphery and connected by a 15-cm length of nylon string. For the field experiment, each disk was loaded with 1.0 g of SPLAT (ISCA Technologies) containing either 0.15% triene or 2.0% diene. Lapointe et al. (2009) demonstrated that these rates of the individual pheromone components are equivalent in terms of trap catch disruption. The SPLAT dollops were allowed to dry on lab benches for 2–4 h until dry to the touch. The loaded devices were stored in a cooler at 20°C until the day of deployment. In the field, the IFM-413 devices were thrown by hand into the canopy of orange trees at a height of 1.5–2.0 m. When more than one device was placed in a single tree, we tried to evenly distribute the devices through the canopy.

Field Trial. The field trial was conducted in commercial citrus groves near Lake Placid, FL (27° N, 81° W). The devices were deployed in two adjacent groves of *Citrus sinensis* L. 'Hamlin' or 'Valencia'. Tree spacing in the triene experiment was 4.6 m between trees within rows and 7.6 m between rows and 3.8 m between trees within rows and 7.6 m between rows for the diene experiment. Treatment plots were nine rows (68.6 m) × 15 trees (68.6 m) for the triene experiment and nine rows × 18 trees (68.6 m) for the diene experiment. In both experiments, treatment plots were 0.5 ha. The arrangement of treatment plots was completely randomized within each experiment. Treatments consisted of varying rates expressed as g/ha of pheromone and varying levels of aggregation of IFM-413 devices, calculated as the *n*th tree treated, e.g., where $n = 1$, every tree received ≥1 device; where $n = 30$, every 30th tree received ≥1 device, and so on. The amount of pheromone per ha was varied by attaching a variable number of devices to the trees. Control plots received no devices. Treated trees received amounts varying from 1.0 g (one of the two disks comprising the IFM-413 device loaded with 1.0 g of SPLAT) to 80 g (40 devices) in a single tree depending on the rate and degree of aggregation specified by the experimental design (Table 1) equivalent to 245–988 g/ha of 0.15% triene SPLAT and 245-1134 g/ha of 2% diene SPLAT. All plots were separated by buffer trees and rows consisting of at least 10 trees (46 and 38 m in the triene and diene experiments, respectively) within rows and 10 rows (76 m) across rows. The total area of the experiment including buffers was 55 ha of which 11 ha were treated and 2.3 ha were included as control plots.

Within each plot, four traps, each baited with 0.103 mg of a 3:1 blend of the sex pheromone of *P. citrella*, were placed in trees equidistant from treated trees (Fig. 2). Traps were placed at the canopy edge at a height of 1.5–1.8 m. Sticky trap liners were collected and counted weekly. Control plots consisted of untreated trees. Counts of male *P. citrella* trapped on the sticky liners were expressed as a percentage of the number of males caught in traps in the control plots. For projection of response surfaces (see below), re-

Table 1. Treatment combinations giving the total amount of formulated product (SPLAT) and pheromone (A.I.) per ha, the amount of SPLAT per tree, and the number of trees treated per 0.5-ha treatment plot

Plot	Triene exp			Diene exp		
	g/ha (AI)	g/tree	No. treated trees/plot	g/ha (AI)	g/tree	No. treated trees/plot
1	0 (0)	0	0	0 (0)	0	0
2	245 (0.37)	11	9	245 (4.90)	11	9
3	245 (0.37)	11	9	245 (4.90)	11	9
4	334 (0.50)	1	135	494 (9.88)	40	5
5	494 (0.74)	40	5	494 (9.88)	40	5
6	494 (0.74)	40	5	519 (10.38)	10	21
7	519 (0.78)	10	21	734 (14.68)	33	9
8	667 (1.00)	2	135	734 (14.68)	33	9
9	667 (1.00)	2	135	756 (15.12)	2	153
10	734 (1.10)	33	9	979 (19.58)	33	12
11	734 (1.10)	33	9	979 (19.58)	33	12
12	979 (1.47)	33	12	988 (19.76)	80	5
13	979 (1.47)	33	12	988 (19.76)	80	5
14	988 (1.48)	80	5	1,134 (22.68)	3	153
15	988 (1.48)	80	5	1,134 (22.68)	3	153

Where the design called for an odd number of grams per tree, only one disk of the IFM-413 device was loaded with SPLAT. Six control plots were included for comparison.

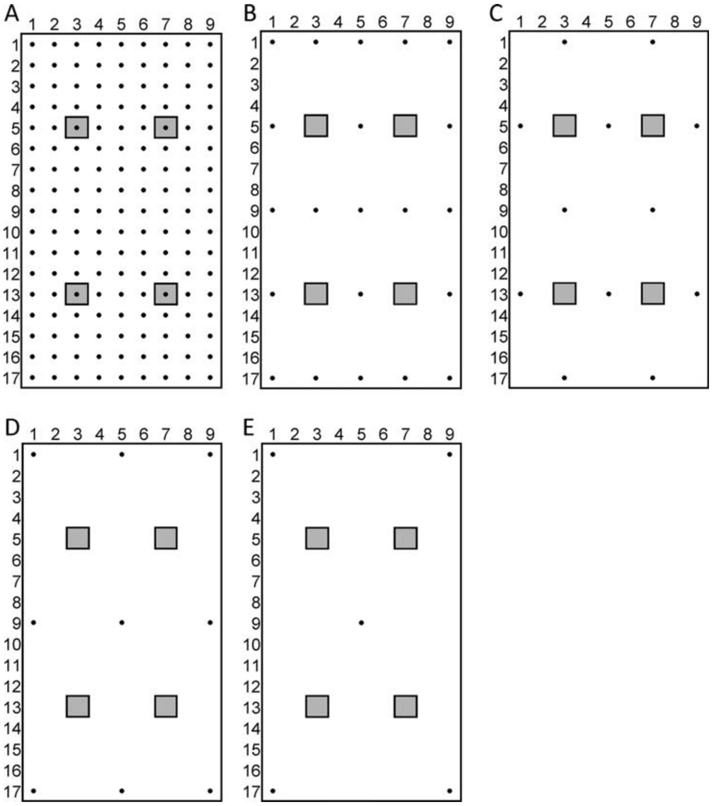

Fig. 2. Design of field plots (0.5 ha) used to vary amount and aggregation of pheromone dispensers. Every *n*th tree received one or more pheromone dispensers. The *n*th tree values were 1.0, 7.3, 12.8, 17.0, and 30.0 for A through E, respectively. Dots indicate trees that received at least one pheromone dispenser; shaded squares indicate location of sticky traps baited with 3:1 sex pheromone attractant. Dimensions of plots are not proportional.

sults from 14 July (8 d after deployment of IFM-413 devices) were selected. Results from other weeks were similar in the shape of the response surfaces.

To analyze longevity of the trap catch disruption effect, results from plots where every tree received an IFM-413 device (plots eight and nine in the triene experiment and plots 14 and 15 in the diene experiment) were plotted over time and compared with previously published data on the longevity of trap catch disruption obtained with manual application of SPLAT containing *P. citrella* pheromone components (Lapointe et al. 2009). Linear regression was used to describe the percentage of disruption of trap catch obtained in the treated plots compared with the control plots.

Experiment Design. A modified D-optimal geometric design was created with Design-Expert, version 8 (State-Ease, Inc., Minneapolis, MN) sufficient to satisfy a quadratic response surface model. The model was used to sample the experiment space defined by amount and degree of aggregation (Fig. 3). In addition to those needed to satisfy model terms, points were added to estimate lack of fit (LOF). Several points were duplicated to attain sufficient degrees of freedom to estimate pure error across the design space and to attain near uniform leverage for all points (Weisberg 1985). The resulting quadratic design (Fig. 3) had five model, five LOF, and eight pure error degrees of freedom (Myers and Montgomery 2002). The LOF design points were chosen so that they could be used to satisfy higher order model coefficients if necessary. The selection of design points was constrained by the limited availability of distribution patterns (Fig. 2) that resulted in a uniform array of treated trees and allowed for equidistant positioning of traps. The design was modified to accommodate this constraint. However, the resulting design was not aliased and no single point had unreasonably high leverage, i.e., close to 1 (Anderson and Whitcomb 2007).

Statistical Analysis. For each trial, all possible models from the mean to cubic polynomial were calculated with Design-Expert (Stat-Ease, Inc.). Initial model selection was based on a lack of any aliased terms; low residuals; a low *P* value; nonsignificant lack of fit; a low SD; high R^2, R^2_{adj}, and R^2_{pred}; close agreement between R^2_{adj} and R^2_{pred}; and a low PRESS value in relation to the other models. If two or more models were satisfactory then the most parsimonious model was chosen. The selected model was then further evaluated according to a battery of adequacy tests as described by Anderson and Whitcomb (2005). Normality was determined by examining a normal probability plot of the internally studentized residuals and

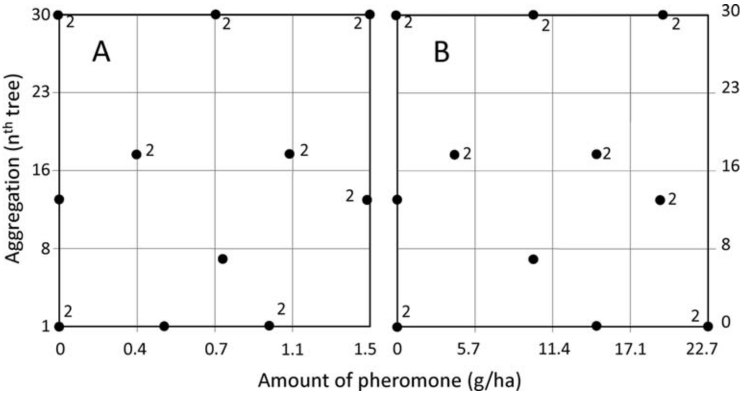

Fig. 3. Coordinates of experimental treatments within the design space to generate response surface models for (A) (Z,Z)-7,11-hexadecadienal or (B) (Z,Z,*E*)-7,11,13-hexadecatrienal of the interaction between amount of pheromone per unit area and the degree of aggregation of pheromone dispensers as measured by disruption of trap catch. Points labeled with 2 were replicated.

assuring that the residuals fit closely to a straight line. Constant variance was determined by plotting the internally studentized residuals versus the predicted responses. If the points fell within an interval of ±3SDs (σ) and exhibited a constant range of residuals across the graph then constant variance was assumed. A Box–Cox plot for selecting the correct power law transformation was created by generating a curve of the natural log of the sum of squares of the residuals (Box and Cox 1964); a transformation was deemed necessary based on the best lambda value, which is the nadir of the generated curve. Adequate precision of the model was determined by comparing the range of the predicted values at the design points ($\hat{y}$) to the average variance (V-bar) of the prediction (Anderson and Whitcomb 2005). Potential outlier points were checked with externally studentized "outlier-t" (Weisberg 1985, Myers 1990) and Cook's distance (Cook and Weisberg 1982) graphical plots.

R^2 is reported as a measure of the amount of variation around the mean explained by the response surface model. The adjusted-R^2 (R^2_{adj}), which decreases as the number of terms in the model increases if those additional terms do not increase the precision of the model, was calculated as follows:

$$R^2_{\text{adj}} = 1 - ((n - 1)/(n - p)) \times (1 - R^2) \quad [1]$$

where n is the sample size and p is the number of model terms. Predicted-R_2 (R^2_{pred}), a measure of the amount of variation in new data explained by the model, was calculated as follows:

$$R^2_{\text{pred}} = 1 - (\text{PRESS}/\text{SS}_{\text{Total}}) \quad [2]$$

where PRESS is the prediction error sum of squares (Allen 1971). PRESS is calculated by removing a single observation from the response surface model, predicting that response point with the remaining n-1 observations, repeating this process for all observations, and then summing the squares of the n PRESS residuals (cf. Myers and Montgomery, 2002).

Results

There was a highly significant ($P < 0.01$) two-factor interaction response surface model (RSM) for the number of male moths captured in pheromone-baited traps (Table 2) for both the triene and diene experiments. Data were transformed (natural log) based on the best lambda value from the Box–Cox plot [see Lapointe et al. (2008) for complete statistical methods]. The three R^2 statistics (R^2, R^2_{adj}, and R^2_{pred}) (Table 2) were in reasonable agreement (Anderson and Whitcomb 2005).

Less than 1% of devices thrown into trees by hand fell to the ground. The device (Fig. 1) was easily tossed and became entangled in tree branches. Disruption of trap catch ranged from 0 to 98% for the triene (Fig. 4) and from 0 to 86% for the diene (Fig. 5) at 8 d after device deployment. Tree spacing within rows of the groves selected for the two trials (triene and diene) varied and resulted in a different value for amount of SPLAT product per unit area (Fig. 3). The triene trial

Table 2. ANOVA and model diagnostic data of polynomial models developed for trap disruption of *P. citrella* in field plots treated with varying amounts of two *P. citrella* sex pheromone components and degree of aggregation of deployment devices

Source	Triene			Diene		
	F	*P*	RC	*F*	*P*	RC
Model	10.26	**0.0008**		8.54	**0.0015**	
Intercept			3.36			3.93
Amount (X1)	13.43	**0.0026**	−0.76	9.03	**0.0089**	−0.47
Aggregation (X2)	17.15	**0.0010**	0.86	8.09	**0.0123**	0.39
X1 × X2	7.39	**0.0167**	0.78	5.26	**0.0367**	0.42
Lack of fit	1.24	0.3909		1.39	0.3254	
R^2	0.69			0.63		
R^2_{adj}	0.62			0.56		
R^2_{pred}	0.43			0.36		

Both models are two factor interaction models with log transformation determined by Box–Cox plot analysis (*P* values in bold are significant, $\alpha = 0.05$; RC = polynomial regression coefficient; *P* and RC values presented in coded form by placing values between −1 and +1 to allow direct comparison).

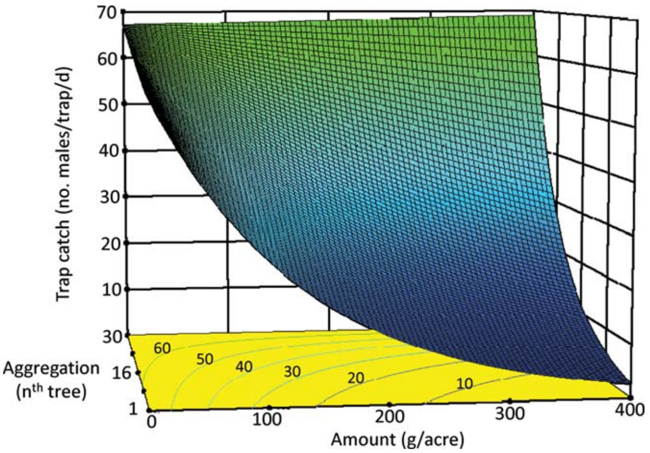

Fig. 4. Predicted three-dimensional surface response plot for trap catch in plots treated with (*Z*,*Z*,*E*)-7,11,13-hexadecatrienal in varying amount and distribution of dispensers. Aggregation of dispensers is measured as the *n*th tree receiving one or more dispensers; e.g., at the lowest level of aggregation ($n = 1$), each tree received at least one dispenser bearing SPLAT containing 0.15% (*Z*,*Z*,*E*)-7,11,13-hexadecatrienal. Contour lines correspond to projected values from the vertical y-axis.

tested amounts up to 988 g/ha of SPLAT containing the triene corresponding to 1–1.5 g/ha of triene (**AI**). The diene trial tested up to 1,137 g/ha of SPLAT containing the diene corresponding to 0–22.7 g/ha of diene (**AI**). The overall shapes of the response surfaces (Figs. 4 and 5) were similar and demonstrated similar interactions between amount and degree of dispenser aggregation within the grove. Trap catch disruption declined with decreasing amount of pheromone per unit area and with increasing aggregation. When aggregation of deployment devices was high (12.4 treated trees per ha or every 30th tree), trap catch was equivalent to trap catch in the control plots.

The plots that received a deployment device in every tree were selected for comparison with previously published results on hand application of SPLAT directly to tree limbs (Lapointe et al. 2009). In these plots, the amount of the diene component was applied at 22.7 times the rate of the triene component. Nonetheless, the triene was more effective at the rates tested compared with the diene (Fig. 6). The percentage of disruption of trap catch reached 50% at 44 d in the diene experiment, and by extrapolation of the linear regression (Fig. 6), would have reached 50% at 75 d in the triene experiment, assuming a linear decline. The slopes of the two linear regressions were very similar suggesting that the relative rate of emis-

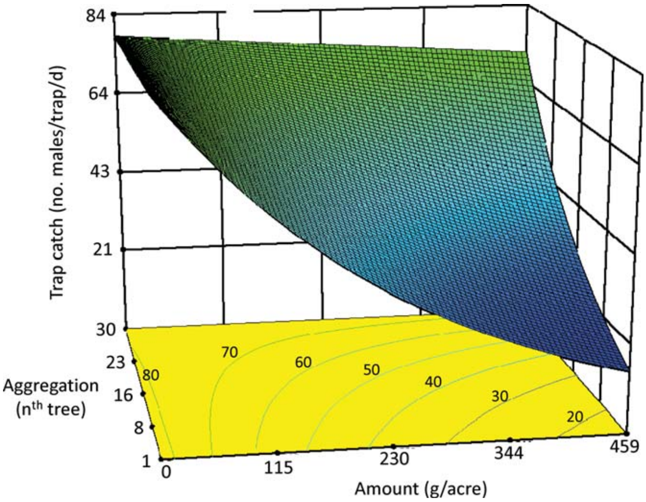

Fig. 5. Predicted three-dimensional surface response plot for trap catch in plots treated with (*Z*,*Z*)-7,11-hexadecadienal in varying amount and distribution of dispensers. Aggregation of dispensers is measured as the nth tree receiving one or more dispensers. Contour lines correspond to projected values from the vertical y-axis.

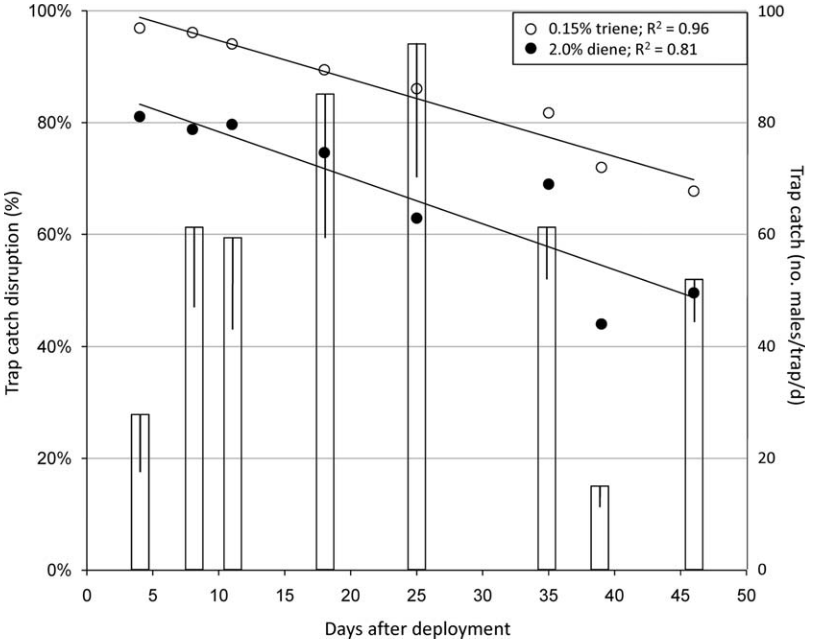

Fig. 6. Decay of trap catch disruption (percentage) over time of two SPLAT formulations deployed on IFM-413 devices containing single pheromone components: (Z,Z,E)-7,11,13-hexadecatrienal (triene) or (Z,Z)-7,11-hexadecadienal (diene) calculated as percentage of the number of *P. citrella* males caught in traps in untreated plots. Traps were baited with a septum loaded with 103 μg of a 3:1 triene:diene blend. Columns are trap catch in untreated plots (number of male *P. citrella* captured per trap per day; error bars are SEM, $n = 6$).

sion of the volatile compounds from the SPLAT matrix was similar in both cases.

Discussion

The IFM-413 device proved effective at becoming securely entangled in tree branches. Furthermore, the devices effectively delivered pheromone-loaded SPLAT that resulted in disruption of trap catch of male *P. citrella* similar to that reported in field trials where SPLAT containing *P. citrella* pheromone was applied directly to tree branches (Lapointe et al. 2009). Our response surfaces (Figs. 4 and 5) show that trap catch disruption, and presumably mating disruption, declined exponentially as the degree of aggregation and distance between pheromone sources increased. This result is similar to the model of Byers (2007), wherein an exponential increase in pheromone release rate was required to maintain mating disruption as the effective attraction radius (EAR) increased linearly. EAR is analogous to the amount of pheromone varied by applying multiple release devices to a single tree in our empirical study (Byer's model increased EAR by increasing the amount of pheromone in a single release device). Byers' model suggested that more dispensers of smaller EAR would provide better disruption and be more economical compared with fewer dispensers of greater EAR in those cases were the cost of pheromone synthesis is high, as it is for *P. citrella.*

Epstein et al. (2006) also observed increased trap catch disruption of codling moths when the number of pheromone point sources per unit area was increased. Both Epstein et al. (2006) and Byers (2007) assumed competitive attraction to pheromone point sources. In the case of *P. citrella*, however, we applied an unnatural blend consisting of a single pheromone component of the natural binary (or tertiary) blend (Lapointe et al. 2006, 2009). Stelinski et al. (2010) observed no orientation of male *P. citrella* moths to the SPLAT droplets containing the triene component only.

The response surfaces for 0.15% triene or 2.0% diene were congruous. This confirms prior observations that the two sex pheromone components operate to disrupt trap catch in similar fashion albeit at different concentrations; the diene produced an effect similar to that of the triene at ≈13 times the rate of the triene (Lapointe et al. 2009). Nevertheless, the triene was more effective at reducing the catch of males at pheromone-baited traps at the rates tested compared with the diene (Figs. 4 and 5). For both, the trap catch disruption response seemed to be asymptotic implying that the rate of 494 g/ha (200 g/acre) of formulated product may be sufficient for effective control when every tree receives a SPLAT dollop. An equivalent degree of trap catch disruption could be achieved at a higher rate with a modest level of aggregation (Figs. 4 and 5). Our results demonstrated that the best trap catch disruption occurred when the number of treated trees was greatest (no aggregation of deployment devices).

The longevity of the disruptant effect of the IFM-413 devices compared very favorably with that of hand-applied SPLAT (Lapointe et al. 2009). In our previous study, SPLAT was applied by hand directly to citrus tree branches. This is necessarily a somewhat imprecise method and results in dollops of widely varying shapes and dimensions. In contrast, dollops of SPLAT were applied to the IFM-413 devices under controlled conditions resulting in dollops of a relatively consistent three-dimensional shape that we believe contributed to sustained release of pheromone over a longer period of time.

As in previous trials, SPLAT containing the triene performed better than SPLAT containing the diene at the rates of pheromone content tested. The relative cost of synthesis of the diene and the triene will determine which of the two compounds will be most cost effective in a commercial product. The relative costs of synthesis of the two compounds are almost exactly the inverse of their relative effectiveness (Lapointe et al. 2009). The IFM-413 device would seem to be most appropriate for use in small plots, so-called dooryard citrus, or other situations where manual distribution of the devices would not be overly expensive. Issues associated with manufacturing, packaging and mechanical deployment of the device for larger scale applications remain to be investigated.

We provide the first account of a novel pheromone deployment device that operates by entanglement in tree branches and foliage analogous to a "bolas" or throwing device with weights positioned at both ends. This device was easy to apply to fruit trees with minimal loss of dispensers and thus active pheromone ingredient. The SPLAT wax-based matrix was highly compatible with this technology and allowed precise and reliable location of the pheromone within citrus tree canopies. The 'bolas' design can consist of any two reservoir-type dispensers connected by a string and is amenable to machine application. Although citrus was the system in which the technology was tested, similar application success should occur in any temperate tree fruit crop as well as small fruit crops such as grapes and berries.

Our results verify previous investigations that disruption of *P. citrella* operates by a noncompetitive mechanism (Stelinski et al. 2008) and that efficacy of disruption is equivalent between individual triene and diene pheromone components of *P. citrella* when ≈13 times more of the latter component is deployed than the former (Lapointe et al. 2009). Finally, we used response surface modeling to investigate the effect of pheromone dispenser distribution on disruption of moth orientation to pheromone sources. For *P. citrella,* our results indicated that disruption efficacy increased with increasing pheromone distribution and thus clumping of pheromone point sources reduced efficacy despite a noncompetitive mode of action for *P. citrella* disruption. These results indicate that optimal disruption of *P. citrella* can be obtained when pheromone source distribution is dense, suggesting that such distribution creates the most thorough dispersion of synthetic background pheromone. Response surface modeling should be a useful tool generally for investigating the effect of pheromone source distribution pattern on mating disruption of insects as well as vertebrates (Johnson et al. 2006).

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