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ARTICLE

Characterization of Microencapsulated Pear Ester, (2*E*,4*Z*)-Ethyl-2,4-decadienoate, a Kairomonal Spray Adjuvant against Neonate Codling Moth Larvae

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Codling moth (CM), *Cydia pomonella* (Lepidoptera: Tortricidae), is the key pest of apples, pears, and walnuts worldwide. The pear-derived kairomone, ethyl (2*E*,4*Z*)-2,4-decadienoate, the pear ester (PE), evokes attraction and arrestment of CM larvae. Microencapsulated PE formulation (PE-MEC) enhances the control efficacy of insecticides when used as a spray adjuvant. Characterization of the microencapsulated kairomone, including microcapsule size, concentrations, emission rates, and larval response, was performed. Microcapsule diameter ranged from 2 to 14 μm, with 68% of capsules being 2−3 μm, and the concentration of microcapsules averaged 25.9×10^4 capsules per mL of field spray solution. Headspace collections showed emission of PE was related to PE-MEC concentration and was best described as first-order power decay. Neonate larvae responded to PE-MEC applications aged through 14 days. These results demonstrated that application of PE-MEC concurrent with insecticides may increase neonate foliar wandering, thereby disrupting host location and enhancing mortality by prolonging its exposure to insecticide.

KEYWORDS: Codling moth; *Cydia pomonella*; pear ester; ethyl (2*E*,4*Z*)-2,4-decadienoate; microcapsules; kairomone; SPME; volatile emission

INTRODUCTION

Microencapsulation is a common formulation process for slow controlled-release of agrochemicals, including pesticides and insect pheromones applied for mating disruption (*1*, *2*). Another insect semiochemical of recent interest has been host−plant odor kairomones, which aid newly hatched neonate insect larvae in crawling to and locating their host fruit/nuts (*3*−*6*). Fruit-volatile kairomones have been demonstrated to attract neonate insect larvae and arrest their locomotion, which as been suggested to be a wandering behavior (*3*−*5*). This evoked attraction and arrestment behavior upon leaf surfaces has been postulated as a potential control mechanism through a host location disruption phenomenon, wherein crawling neonate larvae are enticed and diverted by foliar-applied kairomones. The neonate larvae thereby fail to find their way from the leaves, upon which eggs are commonly laid, to their target host fruit to infest (*4*). Moreover, such kairomones that disrupt host finding would promote longer temporal and spatial exposure to insecticides applied on plant surfaces, thus their potential utility as additives or adjuvants in enhancing efficacy and promoting the lower usage of insecticides, e.g., attracticides (*7*). To properly disrupt host-finding behaviors and enhance temporal and spatial exposure to insecticides, kairomone spray adjuvants should be present and active at low emission rates from point sources covering leaves for periods coinciding with the insecticides' residual activity, usually longer than two weeks. Therefore, kairomones that are moderately to highly volatile would require a slow-release formulation, e.g., microencapsulation, to fulfill a spray adjuvant role providing small but numerous capsules, releasing subtle but prolonged amounts of kairomone.

Codling moth (CM), *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), a key pest of apples, pears, and walnuts throughout the world, has been a focus of such fruit-odor based kairomones for over 35 years. Recently, a kairomone, derived from ripening pears, was identified as the pear ester (PE), ethyl (2*E*,4*Z*)-2,4-decadienoate (**Figure 1**). PE possesses a high degree of species specificity for CM (*8*) and is attractive to both female and male CM adults (*9*, *10*), as well as neonate CM larvae (*3*). Knight and Light (*3*) demonstrated in laboratory bioassays that neat PE stimulates increased rates of both crawling and turning by neonate larvae and stimulates their orientation to and arrestment at point sources of PE. Recently, PE was the first larval-targeted insect kairomone to be microencapsulated (PE-MEC) (*11*). In preliminary laboratory studies, doses of PE-MEC diluted in water evoked attraction, arrestment, and wandering by neonate CM larvae (*11*, *12*). Furthermore, PE-MEC has recently been tested in field trials as a tank-mixed spray-adjuvant to study its effect on control efficacy of various comixed insecticides against CM infestation. The addition of PE-MEC adjuvant to insecticide applications enhanced the insecticides' efficacy on average by 35% in trials spanning 16 various insecticides, including organophosphates, pyrethroids, insect growth regulators, botanicals, microbials, and other insecticides with lower environmental

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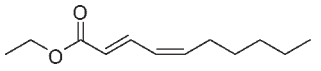

Figure 1. Chemical structure of ethyl (2*E*,4*Z*)-2,4-decadienoate, pear ester (PE).

risks (*13*−*16*). PE-MEC adjuvant has also been demonstrated in field tests to improve control tactics targeting adult CM. PE-MEC improved the mating disruption efficacy of sprayable microencapsulated pheromone when tank-mixed and coapplied (*17*). Additionally, when applied alone PE-MEC spray applications disrupted female oviposition behavior by causing the placement of eggs to be disassociated with fruit location (*18*). Thus, PE-MEC could potentially be used in both larval and adult targeted tactics and have simultaneous, parsimonious control activities.

PE is a good candidate for use in attracticide tactics as a microencapsulated spray adjuvant with insecticides due not only to its behavioral activity against the targeted pest but also to its minimal risk to nontargets. PE is the key flavor/aroma indicative of Bartlett pears (*10*) (CAS #3025-30-7, FEMA #3148, JECFA #1192, and Kosher) and has been classified as a "generally recognized as safe" (GRAS #3148 and FEMA 1970) food ingredient. Additionally, PE has been approved by U.S. Food and Drug Administration for 33 years as a food additive that is nontoxic and nonsensitizing to humans (*19*). PE has yet to be registered as biological pesticide or adjuvant by the U.S. Environmental Protection Agency while being studied under an experimental use permit. Because of its moderate volatility, PE has required a slow-release MEC formulation in order to study its attracticide potential in field spray trials (*20*, *21*).

Reported is the characterization of PE-MEC formulation, including the size-distribution and concentration of microcapsules present in the specified field application rate, and the relative evaporative rate characteristics of PE emission. As with other previously studied pheromone formulations, both as solid substrates as well as microcapsules, it was originally assumed that PE emission from PE-MEC applications would best be approximated by an exponential decay model (*20*, *21*). Last, preferential orientation and taxis responses of newly hatched CM neonate larvae to dilute applications of PE-MEC subject to aging for periods of weeks corroborated the characterization results.

MATERIALS AND METHODS

Microencapsulation and Field Application Rate. Purified PE was microencapsulated by Trécé, Inc. (Adair, OK) following established chemistry and procedures for encapsulation of similar aliphatic esters, e.g., methoprene, an insect growth regulator insecticide (Zoecon "Altosid" products, Wellmark International, Schaumburg, IL). Encapsulation was by interfacial polymerization under high-shear homogenization to create polyamide-walled rigid, semipermeable spherical microcapsules (*22*). The neat PE-filled microcapsules, PE-MEC, were then suspended in an aqueous solution having 5% PE active ingredient (AI) and given the concentrated formulation name DA-MEC (experimental formulation TRE #9489, Trécé, Inc., Adair, OK), having an encapsulation of approximately 50 mg PE AI/mL formulation. Samples (corresponding batch #) of DA-MEC were attained over a six year period (2004, #4440; 2005, #4753; 2006, #4921; 2007, # 5176, 5179, and #5202; 2008, #5436; and 2009, #5620) and stored in a cold room at 4 °C prior to use. The manufacturer's specified field application dose per hectare is 30 mL of DA-MEC formulation tank-mixed in usually 9.4 hectoliters (hL) of water, a vol/vol dilution of the formulation of ca. 1/32,000, or a field-application-rate of 1.5 g/ha PE (equivalent to 12 mL of DA-MEC in 100 gal/acre or 0.6 g/acre PE).

Microcapsule Size and Concentration. *Laboratory Mixed Samples*. Measurements of the diameter size (μm O.D.) and determination of titer (capsules/mL) of PE microcapsules were conducted in the laboratory on aqueous solutions of the manufacturer's specified field application rate of DA-MEC formulation diluted at 1/32,000 using 3.2 μL of PE-MEC in

Table 1. Diameter (μm) and Average Concentration (± SEM) of PE-MEC Microcapsules per Milliliter in Aqueous Solutions Diluted to the Field Application Rate (30 mL PE-MEC/9.4 hL water/ha) of Experimental Adjuvant

	mean (±SEM) concentration ($\times 10^4$) microcapsules/mL										
	microcapsule size classes (μm diameter)										
year PE-MEC sample produced	2 μm	3 μm	4 μm	5 μm	6 μm	8 μm	9 μm	11 μm	12.5 μm	14 μm	total (±SEM)[a]
2004	15.58 ± 1.39 b	8.58 ± 0.73 c	3.58 ± 0.33 b	3.25 ± 0.38 b	2.08 ± 0.22 a	1.25 ± 0.43 a	0.08 ± 0.08 a	0.17 ± 0.08 a	0	0	34.57 ± 3.64 b
2005	10.58 ± 0.35 a,b	7.68 ± 0.30 c	3.50 ± 0.23 b	3.03 ± 0.19 b	0.83 ± 0.10 a	0	0	0	0	0	25.62 ± 1.17 a
2006	10.38 ± 0.88 a	3.13 ± 0.31 a	1.92 ± 0.22 a	1.71 ± 0.22 a	1.33 ± 0.19 a	1.04 ± 0.14 a	0.08 ± 0.08 a	0	0.08 ± 0.05 a	0.04 ± 0.04 a	19.71 ± 2.11 a
2007(a)	13.10 ± 1.77 a,b	5.10 ± 0.77 b	2.45 ± 0.49 a,b	2.90 ± 0.54 a,b	1.55 ± 0.31 a	1.10 ± 0.32 a	0	0.08 ± 0.08 a	0	0.25 ± 0.14 a	26.53 ± 4.42 a
2007(b)	12.92 ± 1.97 a,b	4.67 ± 0.22 a,b	2.50 ± 0.14 a,b	2.53 ± 0.43 a,b	1.75 ± 0.14 a	1.83 ± 0.96 a	0.08 ± 0.08 a	0.25 ± 0.10 a	0	0	26.50 ± 3.94 a
2008	9.67 ± 0.59 a	4.92 ± 0.37 b	2.66 ± 0.26 a,b	2.38 ± 0.25 a,b	1.71 ± 0.11 a	1.05 ± 0.12 a	0.35 ± 0.09 b	0.09 ± 0.04 a	0.02 ± 0.02 a	0.06 ± 0.03 a	22.91 ± 1.88 a
average	12.04 ± 0.91	5.68 ± 0.83	2.77 ± 0.26	2.63 ± 0.23	1.54 ± 0.17	1.05 ± 0.24	0.10 ± 0.05	0.10 ± 0.04	0.02 ± 0.01	0.06 ± 0.04	25.97 ± 2.03
percentage	46.56 ± 1.82	21.50 ± 2.12	10.67 ± 0.69	10.11 ± 0.47	5.99 ± 0.60	4.09 ± 0.94	0.41 ± 0.23	0.35 ± 0.14	0.08 ± 0.07	0.23 ± 0.15	
ANOVA	$F = 4.8$	$F = 13.2$	$F = 2.8$	$F = 2.9$	$F = 2.1$	$F = 0.9$	$F = 2.8$	$F = 1.1$	$F = 1.2$	$F = 0.93$	$F = 8.6$
df = 5,38	$P = 0.005$	$P < 0.001$	$P = 0.048$	$P = 0.043$	$P = 0.111$	$P = 0.439$	$P = 0.047$	$P = 0.36$	$P = 0.34$	$P = 0.46$	$P < 0.001$

[a] One-way ANOVA, with significant *F*-ratio means separated with the SNK method multiple comparison test; different letters for values in columns are significantly different; $P < 0.05$.

Table 2. Diameter Size and Average Concentration (± SEM) of PE-MEC[a] Microcapsules Present in Dilute Aqueous Solutions Directly Used in Field Spray Trials, with the Samples Taken from the Mixed Spray Tank and the Unpressurized Spray Gun Nozzle

	mean (±SEM) concentration ($\times 10^4$) microcapsules/mL							
	microcapsule size classes (μm diameter)							
	2 μm	3 μm	4 μm	5 μm	6 μm	8 μm	>8 μm	total (±SEM)
mixed-tank	10.08 ± 0.49	3.47 ± 0.53	2.15 ± 0.26	1.69 ± 0.28	1.20 ± 0.21	0.85 ± 0.23	0	19.44 ± 3.16
spray-gun nozzle	10.23 ± 0.35	3.15 ± 0.22	2.98 ± 0.33	2.32 ± 0.25	1.03 ± 0.36	0.53 ± 0.13	0	20.24 ± 2.87
average	10.16	3.31	2.57	2.01	1.12	0.69	0	19.84 ± 2.94
percentage	51.2	16.7	12.9	10.1	5.6	3.5	0	

[a] PE-MEC applied at an equivalent rate of 1.5 g PE/ha (0.6 g PE/acre) PE A.I., i.e., 30 mL of DA-MEC applied in a spray volume of 9.5 hL/ha of water (12 mL of DA-MEC in 100 gal water/acre; equivalent to a dilution rate of 1/32,000 or 0.00316% vol/vol).

100 mL of distilled water. Note, for ease of discussion and to avoid confusion, the term PE-MEC will be used primarily throughout the text and is distinguished as a diluted version of the commercial concentrated mixture DA-MEC. Samples of six batch and yearly productions of the DA-MEC formulation were evaluated (**Table 1**). DA-MEC formulations were removed from cold storage, warmed to room temperature, and then mechanically shaken prior to dilution mixing, as were the mixed aqueous diluted PE-MEC suspensions shaken prior to microscopic measurements. Measurements of microcapsule diameter and numbers were conducted at 630× magnification using a compound microscope (Zeiss Inc., Thornwood, NY), a 10× ocular with radicel scale (with a scale division of 1.6 μm), a 63× objective, and a stage-held hemacytometer, blood-cell counting-chamber glass slide, with a 9 mm^2 precision etched lined-grid pattern (Spencer−Neubauer Bright-Line Counting-Chamber, American Optic Co., Buffalo, NY). For each of the six samples, 3 to 12 replicate 0.1 μL fillings of the counting-chamber were conducted, with diameter measurements (number of ocular scale lines converted to μm) and counting of all microcapsules present in four 1 mm^2 corner quadrants etched on the slide (0.1 mm^3 fluid volume per quadrant). The Shapiro−Wilk normality test was used to determine whether the production year data sets were normally distributed. One-way univariate analyses of variance (ANOVA) were used to compare the concentration of capsules (SigmaStat, Systat Software, Inc., Point Richmond, CA). Significant *F*-ratio means were further separated with the Student−Newman−Keuls (SNK) method multiple comparison test; $P < 0.05$.

Field Tank-Mixed Samples. Measurements of the diameter size (μm O.D.) and determination of titer (capsules/mL) of PE microcapsules were conducted on dilute aqueous solutions sampled directly from a field application trial conducted in 2006 (*14*) (**Table 2**). An all terrain vehicle-mounted, gasoline-engine powered, diaphragm-pump sprayer (PBM Supply & Manufacturing, Inc., Fowler, CA) with a 94.6 L (25 gal) polyethylene tank was used to apply by handgun (Spraying Systems Co., Wheaton, IL) foliar sprays of dilute aqueous PE-MEC solution to walnut tree canopies as an experimental treatment. Three milliliters of PE-MEC formulation (batch #4753) was mixed in 94.6 L of water. During the field spray trials (7 June, 2006), twelve 20 mL samples of the tank-mixed PE-MEC alone treatment (without insecticide) were taken from both the spray-tank directly and from the unpressurized spray-gun nozzle (after pressurization through the spray pump). To ensure full mixing, samples were taken half way through the test spraying, after the fourth of the eight treatment trees had been sprayed. Measurements of microcapsule diameter and numbers were conducted in the laboratory as previously described.

Headspace Analysis of Release Rate. *Determination of Calibration Line for PE*. Ethyl (*E*2,*Z*4)-2,4-decadienoate (PE) was obtained from Trécé, Inc. and used without further purification. PE was diluted in hexanes to 0.0125, 0.0250, 0.0500, and 0.1000 molar solutions and transferred to autosampler vials. Injections of 1.0 μL of the PE solutions were analyzed using an Agilent Technologies 6890N GC coupled to a 5975B inert MS (Santa Clara, CA) with a DB-Wax GC column (60 m, 0.320 mm ID, 0.25 μm film; Agilent J&W Scientific, Santa Clara, CA). GC-MS method: injector temp, 200 °C; split (2:1) injection setting; inlet pressure, 11.71 psi; total flow, 6.2 mL/min; split-flow, 2.4 mL/min; helium flow, 1.2 mL/min; average velocity, 29 cm/s; constant flow; initial temp, 150 °C; hold time, 0 min; ramp 1, 2 °C/min; final temp, 160 °C; hold time, 0 min; ramp 2, 20 °C/min; final temp, 200 °C; hold time, 3 min; post time, 210 °C, 2 min. Retention time of PE was 8.49 min. Injections were performed in triplicate for each concentration and the averages used to graph relative peak area versus concentration of PE in nanograms. Regression analysis using a linear trend line provided the equation $y = 3 \times 10^7 x - 3 \times 10^6$ ($R^2 = 0.9999$) with S/N = 20 for the lowest concentration. The limit of detection was estimated to be 0.100 ng (*23*). This limit was confirmed by injection (integration corresponded to 0.105 ng) of a diluted standard sample at the estimated limit-of-detection concentration and using *m*/*z* of 196, 151, and 125 (S/N = 5). The regression equations, emission decay curve models, and statistical analyses for all emission experiments were computed using standard regression software (Excel, Microsoft Inc.). The kinetic orders of emissions were determined by the linearity plots of the logarithmic transformations of emission rates.

SPME Headspace Collections. Volatilized PE was collected onto solid-phase microextraction (SPME) 100 μm polydimethylsiloxane fibers (Supelco, Bellefonte, PA). PE was desorbed, separated, and identified using a GC-MS program similar to that described above but with a splitless injection setting (total flow 8.6 mL/min). Individual SPME analyses of volatilized PE were kept consistent by using standardized PEST method parameters (*24*): *P*, permeation time, the length of time the chamber is closed prior to volatile collection; *E*, exposure time, the time the SPME fiber is exposed to the permeated volatiles; *S*, storage time, the time the volatiles are stored on the fiber prior to injection onto the GC; and *T*, thermal desorption, amount of time the fiber and SPME are kept in the GC injector port. The headspace collection containers for analyses of PE volatilization were short, clear glass, wide-mouth septa-jars with a 70 mL volume and 4.6 cm I.D. (Thomas Scientific, Swedesboro, NJ). The emission quantities determined are considered instantaneous measurements of rate over the period of SPME sampling (1 h).

Determination of the PE-MEC Evaporation Rate. DA-MEC formulation (June 2008, batch #5436) (concentration of 50 mg/mL PE formulation) was removed from cold storage, thoroughly shaken, then diluted in distilled water to two concentrations: 1/1,000 and 1/3,200 representing approximately 50 μg/mL and 15.6 μg/mL. Treatments were 200 μL doses of the 1/1,000 and 1/3,200 dilutions of DA-MEC pipetted, in 10 μL aliquots, onto filter papers (Whatman No. 1) of 55 mm diameter (23.8 cm^2) and placed in the glass headspace collection jars. A volume of 200 μL of water was previously determined to wet the surface of an average pear leaf (16.1 cm^2) to runoff or dripping. The estimated amounts of encapsulated PE initially present in the transferred microcapsules for the 1/1,000 dilution treatment was 10 μg and 3.19 μg for the 1/3,200 dilution. Similarly, a control treatment, comprising 200 μL doses of a 1/100 dilution of a nonfilled, blank MEC formulation, was tested. Each treatment had three replicate jars placed in a vented drying oven (50 × 70 cm) at 32 °C with a constant 1.0 L min^{-1} flow of charcoal-filtered air. The jar lids remained unattached during the evaporation/aging period for up to two weeks. The volatilized PE analyses were performed at intervals of ca. 24, 48, 72, 96, 168, and 336 h. Emission amounts of PE were measured by sealing the jar lids and the PEST method conducted while in the oven at 32 °C ($P = 0$ min; $E = 60$ min; $S = 2$ min; $T = 5$ min) and the adsorbed PE thermally desorbed onto the GC-MS injector port. Upon completion of PE analysis, the jar lids were removed and aging resumed. All analyses were performed in triplicate and reported as the average (**Figure 2**). An evaluation of the change in slope of the emission curves, from dynamic to static, was used to define a hypothetical but practical emission end-point extrapolated beyond the limit of detection of the instrument. The change in slope equation

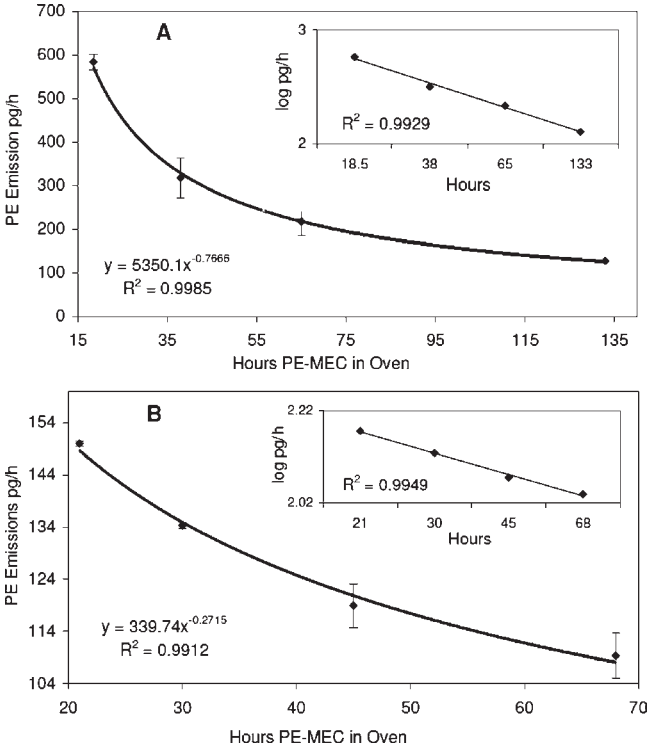

Figure 2. Emission profiles for PE-MEC at 1/1,000 (**A**) and 1/3,200 concentrations (**B**) at 32 °C. Y error-bars show standard deviations. Insets: log pg/h vs hours demonstrating linear correlation to first-order kinetics.

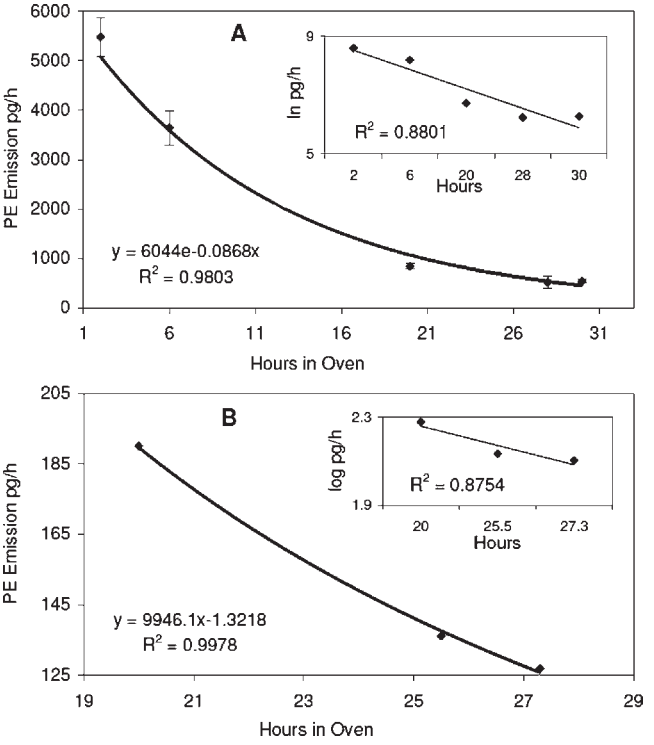

Figure 3. Emission profiles for unformulated PE at 470 μg in water (**A**) and at 320 ng in pentane (**B**) at 32 °C. Y error-bars show standard deviations. Insets: ln pg/h vs hours and log pg/h vs hours, respectively, demonstrating linear correlation to first-order kinetics.

used was simply: Δ-slope = slope2/slope1, where slope1 is 2 h before the time in question, and slope2 is 2 h after the time in question. When this change in slope of the emission trend line reached an arbitrary value of 0.980, indicating a relatively static, albeit diminutive, theoretical volatilized PE emission, the release of PE was deemed complete or at end-point in amount and duration. However, it should be noted that the sum of volatized PE, estimated by use of the trend line equations for each experiments, did not fully correspond to the total PE loaded onto the filter paper, most likely due to the adsorption equilibrium of SPME.

Determination of Evaporation Rate of Encapsulated PE. Since the commercial DA-MEC aqueous formulation has both dissolved free PE and microencapsulated PE, the capsules were isolated for headspace collection by filtration, using 0.2 μm pore, 50 mm diameter polycarbonate filter-discs (G.E. Water and Process Technologies). A solution of 1/1,000 dilution of DA-MEC formulation (2008, batch #5436) in distilled water was mixed. Aliquots of 200 μL of the solutions were pipetted as droplets (ca. 20 μL) onto the central (ca. 60%) area of the filter-discs in Buchner funnels, placed under slight vacuum, and then 10× rinsed with 20 applications of 100 μL of distilled water. Filter-discs were inserted into headspace collection jars and placed for evaporative aging in a drying oven. Three replicate jars were analyzed for the 10 μg treatment (1/1,000 dilution) dose of microencapsulated PE. The filter-discs containing the microcapsules were evaluated for volatilized PE in the same manner as that previously described.

Determination of the Evaporation Rate of Aqueous Free PE and Field-Application-Rate Neat PE. Neat PE was diluted in distilled water to obtain a 2.35 mg/mL solution, and 200 μL was placed onto a filter paper (a 470 μg dose). The volatilized PE was analyzed while at 32 °C and air flow of 1.0 L/min in the same manner previously described, but at intervals between 4 and 24 h (**Figure 3A**). The amount of solvated, non-MEC, PE was determined by extraction of DA-MEC (100 μL) diluted in water (2 mL total), extracted with 5 mL of pentane, and analyzed by GC-MS. Similarly, pear ester diluted in pentane at the field-applications rate (319 ng; 0.130 mL of a 0.0125 mM solution in pentane) was loaded onto a filter paper (Whatman No. 1). The pentane was allowed to evaporate, and the volatilization of PE was analyzed while at 32 °C and air flow of 1.0 L/min in the same manner previously described, but at intervals between 20 and 30 h (**Figure 3B**).

Determination of Batch Variation and Effect of Storage. Aliquots of DA-MEC (batch #5436, #5179, and #5620; received June 4, 2008, December 3, 2008, and July 6, 2009, respectively), stored at 4 °C, were diluted to 1/1,000. PE volatilization analysis was performed in the same manner as that previously described, in triplicate, after 18 h at 32 °C and an air flow of 1.0 L/min.

Neonate-Larval Bioassays. Petri-dish arenas were used to observe the orientation, taxis, and arrestment behaviors of neonate CM larvae in preference, dual-choice bioassays using either water or PE microcapsules applied to a half region of filter paper discs (**Table 4**). A solution of 1/32,000 dilution (approximate field application rate) of DA-MEC formulation (2008, batch #5436) was mixed in 6 mL of distilled water with 10 μL of yellow food coloring added (FD&C Yellow 5; McCormick Inc., Hunt Valley, MD). A control solution of distilled water and yellow dye was also mixed. Filter papers of 9 cm diameter (Ahlstrom Inc., Mt. Holly Springs, PA) were entirely wetted with distilled water and allowed to dry. Aliquots of 200 μL of the dilute PE microcapsules and water treatment solutions were pipetted, as 20 droplets of 10 μL each, onto ca. 60% of the area of one hemisphere side of the filter papers (n = 4), creating a faint yellow-colored treatment zone (outlined/delineated with a faint No. 3 pencil line) with the opposite hemisphere side of the filter paper remaining untreated as the blank untreated zone. The PE treatment level on the filter paper was equivalent to a loading of approximately 317 ng of encapsulated PE upon the ca. 16 cm^2 treatment zones or ca. 20 ng/cm^2. Treated filter papers were placed in the open lid of 10 cm diameter Petri dishes and placed in a fume hood (30 °C) for a 4 h drying time and then aged for a three week interval. For testing, single filter paper treatments were placed upon a rotatable stand (rotated 90° every 20 s), positioned 1 m below a single 60 W frosted-white light bulb (powered at 80 V DC rectified) in a warm (30 °C), darkened interior room without windows. CM eggs were supplied from an established mass-rearing colony at USDA-ARS San Joaquin Agricultural Center, Parlier, CA. Single neonate CM larvae (0−1 d old) were placed, via a fine tipped, sable-hair brush, at the center point of the filter paper at the interface of the two zone sides. Active, crawling neonates were each observed for a 5 min period while recording the number of crossing entries and exits of the zones and time spent crawling within a zone. Larvae that reached the filter paper edge and

Table 3. Statistical Values (R^2) for Linear, Exponential, and Power Trendline Regression Equations Approximating Emission Dynamics from Neat Pear Ester (PE) and Microencapsulated Pear Ester (PE-MEC) Applications[a]

treatment on filter paper	linear	exponential	power
PE-MEC, 1/1,000 dilution[b] (**Figure 2A**)	$y = -3.3778x + 526.82$ $R^2 = 0.7316$	$y = 581.01e^{-0.0122x}$ $R^2 = 0.9023$	**$y = 5350.1x^{-0.7666}$** **$R^2 = 0.9985$**
PE-MEC, 1/3,200 dilution[b] (**Figure 2B**)	$y = -0.8286x + 162.11$ $R^2 = 0.9118$	$y = 166.16e^{-0.0065x}$ $R^2 = 0.9341$	**$y = 339.74x^{-0.2715}$** **$R^2 = 0.9912$**
filtered PE-MEC, 1/1,000 dilution[c]	$y = -9.666x + 477.13$ $R^2 = 0.6289$	$y = 439.39e^{-0.036x}$ $R^2 = 0.7857$	**$y = 500.69x^{-0.419}$** **$R^2 = 0.9919$**
PE aqueous, 470 ng dose[b](**Figure 3A**)	$y = -169.55x + 5118.6$ $R^2 = 0.9128$	**$y = 6044e^{-0.0868x}$** **$R^2 = 0.9803$**	$y = 13098x^{-0.9278}$ $R^2 = 0.9553$
PE pentane, 320 ng dose[b](**Figure 3B**)	$y = -8.9445x + 368.02$ $R^2 = 0.9893$	$y = 588.7e^{-0.0567x}$ $R^2 = 0.9942$	**$y = 9946.1x^{-1.3218}$** **$R^2 = 0.9978$**

[a] Highest R^2 values are in bold. [b] On filter paper. [c] On polycarbonate filter discs.

climbed upon the Petri dish were repositioned to the center point of the filter paper, while inactive noncrawling larvae were discarded. Bioassays were conducted on the treated filter papers that had first dried for 4 h, and then after progressive aging for 7, 14, and 20 days in a fume hood, with 7 replicate runs for each treatment and control. The Shapiro−Wilk normality test was used to determine whether the data sets were normally distributed. One-way univariate analyses of variance (ANOVA) were used to compare treatment effects with significant F-ratio means separated with the Fisher LSD method pairwise multiple comparison test;, $P < 0.05$ (SigmaStat, 2007).

RESULTS AND DISCUSSION

Microcapsule Size and Concentration. The concentrated PE microencapsulated formulation, DA-MEC, consistently comprises a relatively high concentration of small diameter microcapsules, as has been utilized for the encapsulation of many agrochemicals, e.g., pesticides (*2*, *21*). The DA-MEC formulation comprised thin-walled (0.2 μm) microcapsules ranging in diameter from 2 to 14 μm, with the majority (68.1%) of capsules being 2 μm (46.6%) and 3 μm (21.5%) in diameter, followed by moderate levels (10.7 and 10.1%) of 4 and 5 μm capsules and lower levels (5.8 and 4.1%) of 6 and 8 μm capsules (**Table 1**). At the manufacturer's specified field application rate of DA-MEC formulation in spray water (ca. 1/32,000), the concentration of microcapsules averaged (mean ± SEM) $25.97 \pm 2.03 \times 10^4$ capsules/mL spray solution (**Table 1**). Over the five years of DA-MEC production analyzed, the concentration of microcapsules present at the dilute spray rate was fairly consistent, ranging from $19.71 \pm 2.11 \times 10^4$ to $34.57 \pm 3.64 \times 10^4$ capsules/mL, for the respective 2004 and 2006 production years, with the sample batch from 2004 being significantly different ($P < 0.001$) from the concentration of capsules determined for the other years (**Table 1**). Furthermore, the capsule sizes and capsule size distribution were generally similar and uniform over the five year production, with only the 2004 and 2005 production batches varying significantly ($P < 0.05$) in the proportion of capsules in size classes between 2 and 5 μm diameter (**Table 1**). Similar capsule sizes, capsule size distribution, and concentration of microcapsules (average of $19.84 \pm 2.87 \times 10^4$ capsules/mL) were found in samples taken from the mixed spray-tank and the spray-gun nozzle (after passing through a pressurized spray pump) used in the 2006 field application trials of the DA-MEC adjuvant (*14*) (**Table 2**). The maintenance of capsule integrity under pump pressurization suggests that this PE-MEC formulation is suitable for spray applications.

PE-Microcapsule Emission: Release-Rate Characteristics. The calculated PE emission decay curves were surprisingly consistent between the varying concentrations and profiles analyzed, with trend lines generated for three emission models (**Table 3**). Over the series of experiments, trend line correlations of the decay curves were generally high, with a linear model providing the lowest overall correlation values (R^2 = 0.6289 to 0.9893), followed by higher correlations attained with the exponential model (R^2 = 0.7857 to 0.9942). In general, the highest correlation values, albeit unpredictably, were found by using the power model (R^2 = 0.9553 to 0.9985) (**Table 3**). The linearity and high correlation values of the calculated logarithmic plots of the emission rate established that the decay emissions followed first-order kinetics (**Figures 2** and **3**, inserts) (*25*). Discussion of emission dynamics will focus on attributes of the emission decay model that provided the highest regression values for the trend line fit. PE-MEC was evaluated for emission rates of volatilized PE at 32 °C, the approximate average of daily summer orchard temperature.

Numerous attempts to analyze the field application rate, 1/32,000 concentration, were unsuccessful despite increased fiber exposure times to volatilized PE within the containers. At best, levels of ca. 103 pg were detected after 4 h of drying/aging in an oven for the field application rate of PE-MEC and exposure of the SPME fiber for up to 3 h; however, attempts to obtain reproducible results after further aging were untenable. Analysis of the relatively concentrated rate of the 1/1,000 dilution provided the highest correlation curve fit with an R^2 of 0.9985 for a power decay emission trend line (**Table 3**) and detectable volatilized PE to 133 h corresponding to 127 pg/h (**Figure 2A**). The release-rate profile for the 1/1,000 PE-MEC concentration showed PE being emitted at 468 pg/h after 24 h and with a slope of −15.523 corroborating a rapid release, then slowing to 201.6 pg/h after 72 h, and a slope of −2.173. As previously stated, a hypothetical emission end-point beyond the limit of detection was defined through an evaluation of the slope of the emission curves. Such an extrapolation of the 1/1,000 trend line indicated theoretical emission to 14.3 days (342 h) (slope = −0.137) and ending with an emission rate of ca. 61.1 pg/h. The graph of the more dilute rate of 1/3,200, yet 10 times more concentrated than the field-application-rate, showed very good correlation to the power trend line as well (**Figure 2B**). After 24 h, PE was being emitted at 143.4 pg/h but at a substantially lower slope of −1.666. The PE emission rate fell below the limit of detection at the 68 h analysis (109.4 pg/h). Extrapolation of the 1/3,200 trend line equation to an end-point (slope = −0.084) indicated the theoretical emission to be 10.3 days (247 h) and ending with an emission rate of 76.1 pg/h. Calculated logarithmic plots of emission rate verses time resulted in linear trend lines suggesting first-order emission kinetics for both the 1/1,000 and 1/3,200 concentration profiles (**Figure 2**, inserts). The relative discrepancy between ending rates of emission between the 1/1,000 and 1/3,200 concentrations may be due in part to the amount of free PE in the aqueous MEC medium, PE that is not encapsulated but rather solvated in water, as has been documented in other pheromone-based MEC studies (*26*).

To explore the effects of this free PE in the water surrounding the microcapsules on the initial release rate, the emission profile of the microcapsules loaded with PE and filtered from the PE-MEC dilution of 1/1,000 was studied at 32 °C. The emission trend line for the filtered capsules resembled the asymptotic curve of PE-MEC at the same concentration (**Figure 2A**) and was again best approximated by the power decay model (**Table 3**).

The release-rate profile for the filtered PE-MEC (1/1,000 dilution) showed PE being emitted at 132.2 pg/h after 24 h, with a slope of −2.379. The PE emission rate fell below the limit of detection at the 46 h analysis (106.9 pg/h). Extrapolation of the 1/1,000 trend line to the projected end-point (slope = −0.073) indicated theoretical emission to 11.5 days (275 h) and ending with a rate of 47.6 pg/h. When comparing the unfiltered PE-MEC, which includes solvated PE, to that of the filtered capsules, the experimental evidence suggests that the filtered capsules showed lower emission rates and shallower slopes as well as a lower extrapolated end-point rate and shorter duration.

Comparison of the emission profiles provides evidence supporting a hypothesis that free solvated PE affects the initial emission rate, as anticipated, and possibly the end-point emission. To further explore this phenomenon, experiments were performed to determine the emission profiles for PE dissolved in water at the estimated DA-MEC concentration (free PE) and also PE diluted in pentane and the solvent allowed to concentrate to dryness. The release-rate profile for the solvated free PE, at 2.35 mg/mL, 200 μL on filter paper (total of 470 μg) (**Figure 3**A), showed that the emission from the filter paper of PE suspended in water was the best approximated by an exponential trend line with an R^2 value of 0.9803. This exponential trend line showed free PE emission of ca. 752.7 pg/h at 24 h, and a slope of −68.3, indicating a rapid release in the early exposure-aging process. The 30 h analysis provided an observed high emission rate of 447.1 pg/h and a slope of −40.5, and reached the limit of detection at 47 h. When 319 ng of PE diluted in pentane (0.13 mL of a 0.0125 mM solution) was analyzed via the same protocol (**Figure 3B**), the PE emission fell below the instrument's limit of detection after the 27.3 h analysis (126.8 pg/h). Interestingly, the data points for this experiment still provided high correlation to a power emission decay curve relative to the exponential emission decay curve for the aqueous PE (**Table 3**). These experiments demonstrated that unformulated PE undergoes rapid dissipation and that microencapsulation provides a controlled release formulation for PE.

The results from the PE-MEC and isolated filtered capsule PE release rate experiments provided evidence of a power emission decay curve for microencapsulated PE. This was interesting given the known complexity of release rates of semiochemicals from microcapsules and microparticles (*26*), and is further demonstrated by the wide variety of reported emission decay curves of microencapsulated pheromones, which include all types, linear, polynomial, power, logarithmic, and exponential release dynamics (*26*−*29*).

Finally, to test production batch reproducibility from the PE volatilization perspective, three batches of DA-MEC (2007, 2008, and 2009) were analyzed at the 1/1,000 dilution dose after an arbitrary 18 h interval of aging, during the dynamic and high release phase, and were found to be very similar in emission rates (pg/h: 442.9 ± 43.4, 480.5 ± 42.8, and 449.1 ± 41.2). Furthermore, over the period of analysis of PE-MEC evaporative emission, volatilized PE was found to be stable in its geometric configuration, with no detectable increase in isomerization of PE (*E*,*Z*) content occurring over the low levels found in the initial DA-MEC formulation (*30*).

Neonate Larval Bioassays. Newly hatched neonate codling moth larvae spent significantly more time crawling within the region of filter papers having been treated with PE microcapsules than within the untreated region. For each of the four aging intervals tested (1/8 to 20 days), there were no significant differences ($P > 0.05$) observed between the time larvae spent on the two sides of the control water-alone treated filter papers over the 300 s test periods, averaging (mean ± SEM) 149.0 ± 15.7 s (**Table 4**). For the four testing periods (spanning 20 days of aging), neonate larvae expressed no preference for residing on the control water-alone treated side, with the observed percentage preference being consistent and ranging from 48 to 52%, in comparison a purely unbiased (50:50) expectation of 150 s duration on each side. In contrast, the PE microcapsule treatment evoked a significantly greater accumulative time spent crawling by larvae on the PE-treated zone of the filter paper than the untreated zone for the 1/8, 7, and 14 day aging interval tests, averaging 205.6 ± 15.4 s for these first three test periods (**Table 4**). Neonate larvae expressed a distinct and consistent preference for crawling within and residing on the PE-treated zone of the filter paper over the untreated side, with the observed evoked percentage preference ranging from 67 to 71%. Through the filter paper aging PE emission periods of 1/8 to 14 days, the accumulative time spent by larvae on the PE microcapsule-treated zones was significantly greater ($P < 0.05$) in comparison to the accumulative time larvae spent on the control water-alone-treated filter papers (**Table 4**). By 20 days of decremental evaporative aging there was no observed difference in time spent by larvae on control or PE-treated zones.

Table 4. Mean Time (s, ± SEM) Codling Moth Neonate Larvae Spent Crawling within Filter Paper Zones Treated with Either Water vs Water or Water vs DA-MEC Diluted at the Field Application Rate (FAR)[a]

	seconds in treated zone of filter papers, progressive aged (days)[b]			
treatment	day 0	day 7	day 14	day 20
water control	155.6 ± 12.2 b	148.9 ± 17.4 b	147.5 ± 9.9 b	143.9 ± 23.2 b
DA-MEC, FAR	203.8 ± 13.2 a	212.3 ± 18.9 a	200.6 ± 14.1 a	152.8 ± 19.7 b
	$P = 0.046$	**$P = 0.010$**	**$P = 0.028$**	NS, $P = 0.706$

[a] Individual larvae released at the center of filter papers and observed for 5 min; treatments were pipetted onto filter papers and then dried and aged in a fume hood for 4 h prior to the initial day 0 of testing, then allowed to evaporate volatiles, and age for 7, 14, and 20 days before retesting; $n = 7$. [b] One-way ANOVA, $F_{7,55} = 3.105$, $P = 0.009$; means followed by a different letter are significantly different based on an all pair-wise multiple comparison procedure using the Fisher LSD method.

Extrapolation of the 1/3,200 PE-MEC emission curves to 14 days provided an emission end-point emission rate of approximately 70 pg/h. The response of the neonate larvae to PE-MEC applied filter papers aged to 14 days at a 10-fold lower dose of 1/32,000 suggests that a behavioral threshold of the larvae could be in the tens of pg range and needs further investigation. Thus, these behavioral bioassays establish that neonate CM larvae have distinct sensitivities for PE that allows a chemoreception limit of detection exceeding that of the instrumentation. Additional bioassay experiments are needed to investigate dose-activity and longevity aspects of the CM larval sensitivity and responsiveness to PE microcapsule applications, in addition to pertinent experiments to resolve its activity when applied to host plant leaf and fruit/nut surfaces.

Small microcapsules, similar to the PE capsules, are common in formulations of agrochemicals, pesticides, and pheromones (*1*, *2*). However, unlike the kairomonal DA-MEC formulation, many pheromone-MEC formulations utilize large diameter capsules ranging from 50 to 150 μm diameter (*2*, *20*, *21*, *31*) with relatively high rates of emission per capsule (*26*−*29*). Pheromone-MECs used for mating disruption control of lepidopterous moths, which requires the emission of pheromone three-dimensionally

throughout large-volume crop canopies, have specifications for application in the range of 12 to 70 g/ha pheromone, with spray applications having observed capsule densities on sprayed leaves of <1.0 to >12.5 microcapsules/cm leaf surface (*31*−*33*). In contrast, this larval-targeted PE kairomone MEC is specified for application at 3.0 g/ha PE, with microcapsules ranging from 2 to 8 μm and estimated capsule densities of up to 3.31×10^3 microcapsules/cm of sprayed surface and emission levels in the picogram range. The target of PE-MEC applications comixed with insecticides is to influence and disrupt the host finding behaviors of tiny (1.4 mm long) neonate larvae. These crawling neonates must move and function in virtually a two-dimensional leaf-surface environment, with boundary layer influences affecting odor movement. Thus, the semiochemical MEC formulation and application properties for PE kairomone would be unique and fundamentally different from those required for pheromone-MEC-based mating disruption of flying adult males.

We demonstrated that microencapsulated PE provided a relatively long duration of emission at behaviorally effective rates. The sensitivity, attraction, and preference of CM larvae for PE showed that the microencapsulated formulation should function as an effective spray adjuvant over the expected residual activity duration of insecticides, often no longer than two weeks. Applications of the sprayable PE-MEC formulation might evoke an increase in the time neonate larvae spend crawling and wandering upon foliage prior to boring into fruits or nuts. This therefore could enhance larval mortality by increasing the temporal and spatial exposure to conventional insecticides and/or biotic factors.

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