

A Novel Semiochemical Tool for Protecting *Pinus contorta* From Mortality Attributed to *Dendroctonus ponderosae* (Coleoptera: Curculionidae)

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ABSTRACT Verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) is an antiaggregant of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), a notable forest insect capable of causing extensive levels of tree mortality in western North America. Several formulations of verbenone are registered for tree protection, but failures in efficacy are not uncommon, particularly when applied during large infestations. A formulation of (–)-verbenone was developed (Specialized Pheromone & Lure Application Technology [SPLAT] Verb, ISCA Technologies Inc., Riverside, CA) and evaluated for protecting individual lodgepole pine, *Pinus contorta* Douglas ex Loudon, and small stands of *P. contorta* from mortality attributed to *D. ponderosae*. SPLAT Verb applied to individual *P. contorta* resulted in complete tree protection, while 93.3% mortality occurred in the untreated controls. Significantly fewer *P. contorta* were killed by *D. ponderosae* within 0.041-ha circular plots surrounding *P. contorta* treated with SPLAT Verb compared with the untreated control. In a second study, a smaller percentage of *P. contorta* were colonized and killed on 0.4-ha square plots treated with SPLAT Verb compared with the untreated control. No significant differences in levels of tree mortality were observed between the untreated control and another formulation of verbenone (7-g pouch) or between the 7-g pouch and SPLAT Verb. In a trapping bioassay, no significant differences were observed among captures in multiple-funnel traps at 1, 2, or 4 m from the point of release of SPLAT Verb. Significantly fewer *D. ponderosae* were collected at 1 and 2 m compared with 8 m. Significantly more *D. ponderosae* were captured at the farthest distance evaluated (16 m) than at any other distance. Our data indicate that SPLAT Verb is effective for protecting individual *P. contorta* and small stands of *P. contorta* from mortality attributed to *D. ponderosae* at moderate doses. The high levels of tree protection observed are attributed to the ability of applying release points (dollops) at high densities, and a larger zone of inhibition than reported for other formulations of verbenone. SPLAT Verb was registered by the U.S. Environmental Protection Agency for use on pines, *Pinus* spp., in 2013.

KEY WORDS lodgepole pine, mountain pine beetle, SPLAT Verb, tree protection, verbenone

Introduction

Mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), is a major disturbance in conifer forests of western North America, where it colonizes several tree species, most notably lodgepole pine, *Pinus contorta* Douglas ex Loudon, ponderosa pine, *Pinus ponderosa* Douglas ex Lawson, sugar pine, *Pinus lambertiana* Douglas, limber pine, *Pinus flexilis* E. James, western white pine, *Pinus monticola* Douglas ex D. Don, and whitebark pine, *Pinus albicaulis* Engelm (Gibson et al. 2009). While *D. ponderosae* is an important part of the ecology of

these forests, extensive levels of tree mortality associated with outbreaks may hasten succession, and may impact timber and fiber production, water quality and quantity, fish and wildlife populations, aesthetics, fire risk and severity, recreation, grazing capacity, real estate values, biodiversity, carbon storage, endangered species, and cultural resources, among other factors. There are two general approaches for reducing the negative impacts of *D. ponderosae* on forests. Direct control involves short-term tactics designed to address current infestations by manipulating beetle populations, and commonly includes the use of insecticides, semiochemicals (i.e., chemicals produced by one organism that elicit a behavioral response in another organism), sanitation harvests, or a combination of these treatments (Fettig et al. 2014). Indirect control is designed to reduce the probability and severity of future infestations within treated areas by manipulating stand, forest, and landscape conditions (Fettig et al. 2014). The use of

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semiochemicals has focused on inhibitors for protecting individual trees and small stands of trees (e.g., campgrounds; Progar et al. 2014).

Recent outbreaks of *D. ponderosae* have been severe, long lasting, and well documented, with >27 million hectares impacted (U.S. Department of Agriculture [USDA] Forest Service 2012, British Columbia Ministry of Forests, Lands, and Natural Resource Operations 2013). As a result, research needs have been prioritized in the United States, and include refinement or development of semiochemical-based tools to mitigate undesirable levels of tree mortality attributed to *D. ponderosae*, particularly in high-value areas (Negrón et al. 2008). Progar et al. (2014) provide a thorough review of the chemical ecology of *D. ponderosae* relevant to host finding, selection, colonization, and mating behaviors. In short, females initiate colonization of the lower tree bole in a behavioral sequence mediated by aggregation pheromones (Vité and Gara 1962, Vité and Pitman 1967, Pitman et al. 1968, Pitman and Vité 1969, Ryker and Libbey 1982) and host kairomones (Renwick and Vité 1970, Borden et al. 1987, Miller and Lindgren 2000). Females are later joined by males, and mass attack ensues (Pitman et al. 1968, Wood 1982a), enabling *D. ponderosae* to overcome host tree defenses. During latter stages of colonization, increasing amounts of verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) are produced, which inhibit additional *D. ponderosae* from infesting the target tree, presumably limiting the number of beetles to a density that increases the likelihood of brood survival (Progar et al. 2014). In nature, verbenone is produced in small amounts by autooxidation of the monoterpene α -pinene (Hunt et al. 1989), but the principal route of production is through metabolic conversion by bark beetles of the inhaled and ingested α -pinene to the terpene alcohols *cis*- and *trans*-verbenol, which are metabolized by yeasts in the alimentary system and beetle galleries to verbenone (Leufvén et al. 1984, Hunt and Borden 1990).

Verbenone has been evaluated as a tool for mitigating tree mortality attributed to *D. ponderosae* for several decades (Progar et al. 2014). Results have been favorable, but inconsistent over time (Shea et al. 1992, Amman 1994), geographic area (Gibson et al. 1991, Amman 1994), outbreak intensity (Progar 2003, 2005; Progar et al. 2013), dose (Borden and Lindgren 1988, Gibson et al. 1991), and tree species, with several studies indicating that verbenone is largely ineffective for reducing the levels of tree mortality attributed to *D. ponderosae* in *P. ponderosa* (Bentz et al. 1989, Gibson et al. 1991, Negrón et al. 2006). Lack of suitable levels of tree protection have been linked to, among other factors, photoisomerization of verbenone to behaviorally inactive chrysanthenone (Kostyk et al. 1993), inconsistent or inadequate release (Bentz et al. 1989), rapid dispersal (Gibson et al. 1991, Negrón et al. 2006), and limitations in the range of inhibition (Miller 2002). While several formulations of verbenone are registered in the United States (reviewed by Progar et al. 2014), pouches (several registrants) are most commonly used and stapled at maximum reach (~2 m in height) to individual trees, or applied in a grid pattern (50–148

pouches per hectare) when stand protection is the objective. More widespread use of verbenone has been hampered by the limitations identified above, among other factors, such as the substantial investment in research and development necessary to produce cost-effective formulations that yield sufficient efficacy (Progar et al. 2014).

In recent years, ISCA Technologies Inc. (Riverside, CA) developed a Specialized Pheromone & Lure Application Technology (SPLAT) composed of a wax emulsion matrix for dispensing pheromones (Mafra-Neto et al. 2013). The primary objective of our research was to evaluate SPLAT formulated with (–)-verbenone as a tool for tree protection. Specific objectives were 1) to determine efficacy for protecting individual *P. contorta* and small stands of *P. contorta* from mortality attributed to *D. ponderosae*; 2) to determine suitable doses and release rates; 3) to analyze the qualitative chemical content of field-exposed dollops; and 4) to determine the effective range of inhibition under field conditions.

Materials and Methods

Individual Tree Study. This study was conducted on the Greys River Ranger District, Bridger-Teton National Forest, Wyoming (43° 08.442' N, 110° 52.188' W; 1,890–1,951 m elevation) in 2012–2013. Site selection was based on aerial and ground surveys indicating that *D. ponderosae* infestations were active in the area. Stands had 20.7 m² of basal area per hectare (mean live tree, ≥ 12.9 cm diameter at breast height [dbh]; diameter at 1.37 m above ground level), of which 98.4% was *P. contorta* with a mean dbh of 26.0 cm. The remainder was represented by Engelmann spruce, *Picea engelmannii* Parry ex Engelmann, and subalpine fir, *Abies lasiocarpa* (Hooker) Nuttall. About 12.2% of *P. contorta* and 16.3% of *P. contorta* basal area had been killed by *D. ponderosae* during the previous 2 yr.

Sixty *P. contorta* were selected and 30 were randomly assigned to each of the two treatments: 1) SPLAT formulated with (–)-verbenone [SPLAT Verb, 10.0% (–)-verbenone by weight; EPA Reg. No. 80286-20 (August 2013); ISCA Technologies Inc.] applied at 7.0 g of (–)-verbenone per tree (70 g of SPLAT Verb per tree) on 19 June 2012 as four ~17.5-g dollops (~5.5 cm in diameter by 1.2 cm in height, Fig. 1) to the tree bole (at cardinal directions) at ~2.5 m in height using a caulking gun (model X-Lite, Newborn Brothers Co. Inc., Jessup, MD; mean dbh $\pm$ SEM = 27.1 $\pm$ 0.8 cm; and 2) an untreated control (mean dbh $\pm$ SEM = 32.3 $\pm$ 1.2 cm). Experimental trees were separated by > 100 m. There was a significant difference in tree dbh between treatments ($t = -3.53$; $df = 58$; $P < 0.001$), which is generally thought to influence susceptibility to attack by *D. ponderosae* (Fettig et al. 2014), but presumably was small enough (~5 cm) to exert little influence. One commercially available two-component tree bait [*trans*-verbenol (~1.2 mg/d) and *exo*-brevicomin (~0.3 mg/d); Contech Inc., Delta, BC] was stapled to the bole of each tree at ~2 m in height on the northern aspect on 19 June 2012. Baits were removed on 9–10 October 2012 after *D. ponderosae*



Fig. 1. A dollop of SPLAT Verb applied to the bole of *P. contorta*, Bridger-Teton National Forest, Wyoming, 2012.

flight had ceased. The manufacturer estimates the life expectancy of these baits is 100–150 d, depending on weather conditions, covering most of the flight activity period of *D. ponderosae* in this area.

Each experimental tree ($N=60$) and all live *P. contorta* (≥ 13.9 -cm dbh) within a 0.041-ha circular plot (11-m radius) surrounding each experimental tree were visually inspected for *D. ponderosae* attacks (none, unsuccessful, strip, and mass attacks; Gibson et al. 2009) on 9–10 October 2012. However, mortality was based on the presence (dead) or absence (live) of crown fade on 21–22 June 2013. Treatments were considered to have sufficient *D. ponderosae* “pressure” (i.e., a relative measure of population density based on recent levels of tree mortality) if $\geq 60\%$ of the untreated, baited control trees died from colonization by *D. ponderosae*. Treatments were considered efficacious when less than seven trees died as a result of *D. ponderosae* colonization, while $\geq 60\%$ of the untreated, baited control trees died (see Shea et al. 1984 for a complete description of this experimental design and underlying statistical assumptions). A one-way analysis of variance (treatment) was performed on the proportion of *P. contorta* killed by *D. ponderosae* within 0.041-ha circular plots surrounding each experimental tree using $\alpha = 0.05$ (SigmaStat version 12.0, Systat Software Inc., San Jose, CA). Data were not normally distributed (Shapiro–Wilk test, $P < 0.05$), and therefore analyzed with nonparametric statistics (SigmaStat Version 12.0).

Small Stand Study. This study was conducted on the Montpelier Ranger District, Caribou-Targhee National Forest, Idaho, in 2012–2013 (Table 1). Site selection was based on aerial and ground surveys indicating that *D. ponderosae* infestations were active in the area. The experimental design was completely randomized with three treatments and five replicates (0.4-ha square plots) per treatment. *P. contorta* was the dominant tree species on all plots, except Plot 14

(SPLAT Verb), where *A. lasiocarpa* was dominant (54.2%). Other tree species included trembling aspen, *Populus tremuloides* Michaux, Douglas-fir, *Pseudotsuga menziesii* (Mirbel) Franco, and to a much lesser extent, *Pi. engelmannii*.

Treatments included: 1) SPLAT formulated with (–)-verbenone (SPLAT Verb) applied at 350.0 g of (–)-verbenone per plot (3,500 g of SPLAT Verb per plot) to tree boles (both host and nonhosts) at 2 m in height on the northern aspect in appropriate dollop sizes (5.5 cm in diameter by 1.2 cm in height, 17.5 g) to achieve as uniform coverage as possible (i.e., a dollop every 5–6 m, depending on tree distributions) using a caulking gun (model X-Lite); 2) 7-g (–)-verbenone pouches (50 mg/d at 20°C; Contech Inc.) stapled to the tree bole at 2 m in height on the northern aspect along a 9.1- by 9.1-m grid (125 U/ha; 350.0 g of (–)-verbenone per plot); and 3) an untreated control. All treatments were applied on 18–22 June 2012. One commercially available two-component tree bait (Contech Inc.) was attached to the nearest tree to the center of each plot (regardless of the species or size) at 2 m in height on the northern aspect immediately after treatment. Baits were removed on 10–12 October 2012, after *D. ponderosae* flight had ceased.

Prior to treatment, stand structure and composition were determined by the census of live trees (≥ 12.7 -cm dbh) on each plot on 12–15 June 2012. At the same time, all currently infested and recently killed trees ≥ 12.7 -cm dbh were tagged, and the causal agent of mortality and time of tree death (Klutsch et al. 2009) was determined. On occasion, a 625-cm² section of the bark was removed to locate any bark beetle galleries in the phloem or cambium. The shape, distribution, and orientation of galleries, and host species are commonly used to distinguish among bark beetle species (Furniss and Carolin 1977). All currently infested and dead trees were excluded from statistical analyses (i.e., they were colonized and killed prior to

Table 1. Plot locations and numbers of *P. contorta* colonized and killed during the small stand study, Caribou-Targhee National Forest, Idaho, 2012–2013

Plot	Treatment	Coordinates	Elevation (m)	Slope (%)	<i>P. contorta</i> attacked/ killed per plot
1	SPLAT Verb	42°35.037'N 111°14.135'W	2,424.3	15	0/0
2	Untreated control	42°35.094'N 111°14.165'W	2,426.7	17	9/5
3	Untreated control	42°34.933'N 111°14.005'W	2,440.1	11	34/16
4	Verbenone pouch	42°34.918'N 111°14.147'W	2,436.2	30	12/7
5	Verbenone pouch	42°35.158'N 111°13.942'W	2,420.0	26	7/1
6	Verbenone pouch	42°35.377'N 111°13.855'W	2,384.0	26	7/3
7	SPLAT Verb	42°35.026'N 111°14.430'W	2,489.5	15	6/2
8	Untreated control	42°35.371'N 111°13.437'W	2,368.2	23	17/15
9	Verbenone pouch	42°37.141'N 111°12.482'W	2,369.7	17	0/0
10	SPLAT Verb	42°37.294'N 111°12.369'W	2,387.1	30	2/1
11	Verbenone pouch	42°21.836'N 111°33.647'W	2,396.5	0	3/1
12	Untreated control	42°20.720'N 111°35.622'W	2,118.3	9	3/1
13	SPLAT Verb	42°21.392'N 111°33.447'W	2,364.5	28	8/4
14	SPLAT Verb	42°21.218'N 111°33.512'W	2,381.9	30	0/0
15	Untreated control	42°20.636'N 111°34.056'W	2,493.1	34	13/12

implementation of treatments). The presence of currently infested trees was considered desirable to help ensure adequate levels of beetle “pressure” to challenge treatments, and represents conditions under which operational use of verbenone is considered.

A census was conducted on each plot for trees colonized by bark beetles on 10–12 October 2012. However, tree mortality was based on the presence (dead) or absence (live) of crown fade on 23–25 July 2013. A one-way analysis of variance (treatment) was performed on the proportion of trees successfully colonized (strip and mass attacks) and killed by *D. ponderosae* using $\alpha = 0.05$ (SigmaStat Version 12.0). Data were tested for normality using the Shapiro-Wilk test (SigmaStat Version 12.0).

Laboratory Analyses of Release Rate and Chemical Composition. Dollops containing 17.5 g of SPLAT Verb were analyzed by gas chromatography (GC) to estimate release rates, and GC and gas chromatography–mass spectrometry (GC-MS) to determine the chemical identity and composition. Each dollop was created using caulking guns (model X-Lite) in the same manner and configuration (5.5 cm in diameter by 1.2 cm in height) as in the field studies (Fig. 1), and placed in a forested environment for 30 d in Riverside, CA ($18.7 \pm 1.5^\circ\text{C}$ minimum, $33.1 \pm 2.2^\circ\text{C}$ maximum, 0.2 mm precipitation). At 1, 10, and 30 d, a cohort of five randomly selected dollops was removed. Each dollop was then placed in a 100-ml scintillation vial, and stored at -20°C until extraction.

Samples were extracted using a procedure modified from Meissner et al. (2000). Ten milliliters of an internal standard solution (232 $\mu\text{l/liter}$ methyl myristate [$>99\%$, Fluka Chemika, Buchs, Switzerland] in acetonitrile) was added to each scintillation vial. Samples were then placed in a shaker water bath (model YB-531, American Scientific Products, Columbus, OH) at $80\text{--}85^\circ\text{C}$ for 3 min; and shaken for 7 min, after which the rack was removed and agitated by hand for 0.5 min and then returned to bath and shaken for an additional 3 min; and then agitated by hand for a final 0.5 min before removal. Samples were then placed in a

freezer at -20°C for at least 8 h, during which time the wax solidified and settled to the bottom of the vials. The vials were then removed from the freezer and allowed to thaw. Vials were vortexed and 1 ml of the solution was removed with a disposable glass Pasteur pipette and filtered through another disposable glass Pasteur pipette fitted with a 4.5- by 6.5- by 8-cm triangular piece of Kimwipe (Kimberly-Clark Corp., Roswell, GA) folded to form a plug at the tapered end. The solution was then deposited into a 1.5-ml GC vial (Supelco, Bellefonte, PA). Compounds in the sample were then quantified using a GC (GC7890A, Agilent Technologies Inc., Santa Clara, CA) equipped with a 30-m HP DBWAXETR polar column. Helium was used as a carrier gas and passed through the column at a constant pressure of 137.9 kPa. The oven was initially set to 50°C for 5 min, ramped ($25^\circ\text{C}/\text{min}$) to 150°C , then ramped ($40^\circ\text{C}/\text{min}$) to a final temperature of 250°C . The presence and identity of compounds were determined using GC-MS (GC7890A/5975C, Agilent Technologies Inc.) equipped with a 30-m HP DBWAXETR polar column, programmed with the same conditions as the GC. Chemical content was calculated using the internal standard method (Miller and Haynes 1998) to determine the amount of verbenone remaining, and the presence and quantity of the breakdown molecule chrysanthenone (Kostyk et al. 1993).

Range of Inhibition Trapping Bioassay. A trapping bioassay was conducted on the Ogden Ranger District, Uinta-Wasatch-Cache National Forest, Utah ($41^\circ\text{--}40.780^\circ\text{N}$, $111^\circ\text{--}25.160^\circ\text{W}$; 2,313–2,478 m elevation) during 31 July–16 August 2013. Site selection was based on observations indicating that *D. ponderosae* infestations were active in the area. Stands had 19.1 m^2 of basal area per hectare (mean live tree, $\geq 13.9\text{-cm dbh}$), of which 78.7% was *P. contorta* with a mean dbh of 28.9 cm. The remainder was represented by *Pi. engelmannii*, *A. lasiocarpa*, and *Po. tremuloides*. Numerous *P. contorta* $<13.9\text{-cm dbh}$ were also present in the understory and midstory. About 25.8% of *P. contorta* and 27.0% of *P. contorta* basal area had been killed by *D. ponderosae* during the previous 2 yr.

Seventy grams of SPLAT Verb [7 g of (–)-verbenone] were applied as four 17.5-g dollops (5.5 cm in diameter by 1.2 cm in height; at cardinal directions) at 2.5 m in height using a caulking gun (model X-Lite) to the bole of one *P. contorta* (mean dbh $\pm$ SEM = 36.7 ± 1.2 cm) at the center of each of eight blocks on 31 July 2013. One 16-unit multiple-funnel trap baited with a commercially available trap lure (*exo*-brevicommin [0.3 mg/d], *trans*-verbenol [1.0 mg/d], and myrcene [270 mg/d] at 20–25°C; Contech Inc.) was placed at 1, 2, 4, 8, and 16 m from the SPLAT Verb-treated *P. contorta* at a randomly selected bearing of 0, 72, 144, 216, or 288°. Traps were hung on 3-m metal poles with collection cups 1 m above the ground. A 3 by 3-cm time-released insecticidal Prozap Pest Strip (2,2-dichlorovinyl dimethyl phosphate, Loveland Industries Inc., Greeley, CO) was placed in the collection cup to kill arriving insects and reduce damage or loss to predacious insects. Samples were collected and each treatment re-randomized daily on six dates (Fettig et al. 2009). Specimens were tallied and identified using available keys (Wood 1982b) and voucher specimens. Gender was determined through examination of the seventh abdominal tergite (Lyon 1958).

The experimental design was a randomized complete block with 48 replicates per distance. A test of normality was performed and square root transformations were used when data deviated significantly from a normal distribution. A three-way analysis of variance (block, distance, and gender; $\alpha = 0.05$) was performed on the number of *D. ponderosae* caught per trap per day (SigmaStat Version 2.0). The Tukey's multiple comparison test (Tukey's honestly significant difference [HSD]) was used for separation of treatment means.

Results and Discussion

Individual Tree Study. The application of SPLAT Verb resulted in complete tree protection, while 100% of the untreated controls were colonized and 93.3% killed (Table 2). This is encouraging given this experimental design is used for evaluating the efficacy of insecticides for tree protection, and provides a very conservative test of efficacy (Fettig et al. 2013). As such, it is not often used for evaluating semiochemicals, which are generally expected to provide lower levels of tree protection than insecticides (Progar et al. 2014). Significantly fewer *P. contorta* (percentage of trees) were killed on 0.041-ha circular plots surrounding *P. contorta* treated with SPLAT Verb ($U = 46$, $P < 0.001$, Mann–Whitney; Fig. 2), suggesting attraction was disrupted at levels sufficient to cause tree protection within 11 m of the point of release.

Several authors have speculated that the efficacy of verbenone could be enhanced by applying verbenone around the circumference of the tree assuming that a circumferential release from several point sources provides better protection than fewer or a single point source per tree as provided by pouches (Progar et al. 2014). For example, Gillette et al. (2006) reported near complete protection of *P. contorta* from *D. ponderosae*

in California using a plastic flake formulation (15 g of verbenone per tree) consisting of 3-mm² laminated polyvinyl chloride layers surrounding a plastisol middle layer that released verbenone. Alternatively, Kegley et al. (2010) reported flakes were more costly and laborious to apply than pouches, and provided similar levels of tree protection. Being able to apply multiple release points per tree may help explain the high level of protection provided by SPLAT Verb in our study.

On an operational basis, two verbenone pouches are recommended for protecting individual *P. contorta* from mortality attributed to *D. ponderosae* (Kegley and Gibson 2009, Kegley et al. 2010) and generally provide $\geq 80\%$ tree protection. For larger trees (> 61 -cm dbh), three to four pouches may be used. One registrant suggests treating individual trees with two pouches at 1–1.5 and 2.5 m on the northern aspect in addition to placing pouches at 4–5 m intervals on vertical objects within 5 m of the tree being treated. Alternatively, SPLAT Verb provided suitable levels of tree protection at much lower doses.

Small Stand Study. In total, 121 *P. contorta* were colonized by *D. ponderosae*, 68 at densities sufficient to cause tree mortality within 1 yr (Table 1). The untreated control had the highest number of *P. contorta* colonized and killed (76 and 49, respectively), while SPLAT Verb had the fewest (16 and 7, respectively; Table 1). Significantly fewer *P. contorta* (percentage of trees) were colonized ($F_{2,12} = 5.2$; $P = 0.024$) and killed ($F_{2,12} = 4.9$; $P = 0.028$) on plots treated with SPLAT Verb than untreated control plots (Fig. 3). No other significant differences were observed (Fig. 3), despite relatively large differences in the numbers of trees colonized and killed, specifically between the verbenone pouch and untreated control (Table 1).

Factors such as tree diameter, stand density and host density are often positively correlated with the severity of bark beetle infestations (Fettig et al. 2007). Large *P. contorta* generally have a thick phloem, which affords the offspring of *D. ponderosae* a higher reproductive potential and probability of survival, factors shown to influence the overall reproductive success (Amman 1972). Stand density may impact host vigor, microclimate, pheromone plumes, and inter-tree spacing, among other factors, which influence the population dynamics of *D. ponderosae* and associated levels of tree mortality (Fettig et al. 2007, 2014). Although stand density metrics varied among plots, there were no significant differences in dbh ($F_{2,12} = 0.07$, $P = 0.94$), trees per hectare ($F_{2,12} = 0.21$; $P = 0.81$), basal area per hectare (m²/ha; $F_{2,12} = 0.48$; $P = 0.63$), or percentage *P. contorta* ($F_{2,12} = 0.75$; $P = 0.49$) among treatments (Table 3).

No significant correlations were found between trees per hectare and trees colonized ($r = 0.265$; $P = 0.34$; $n = 15$), trees per hectare and trees killed ($r = 0.117$; $P = 0.71$; $n = 15$), basal area per hectare (m²/ha) and trees colonized ($r = 0.037$; $P = 0.90$; $n = 15$) or basal area per hectare (m²/ha) and trees killed ($r = -0.072$; $P = 0.80$; $n = 15$) on a plot-level basis. Similar results were observed for SPLAT Verb ($P > 0.07$, all cases; $n = 5$), the verbenone pouch ($P > 0.61$, all cases; $n = 5$)

Table 2. Efficacy of SPLAT Verb for protecting individual *P. contorta* from mortality attributed to *D. ponderosae*, Bridger-Teton National Forest, Wyoming, 2012–2013

Treatment	Mean dbh $\pm$ SEM ^a	Dose ^b	Percentage of <i>P. contorta</i> colonized by <i>D. ponderosae</i> ^c	Percentage of <i>P. contorta</i> killed by <i>D. ponderosae</i>
SPLAT Verb	27.1 $\pm$ 0.8	7	0.0%	0.0%
Untreated control	32.3 $\pm$ 1.2	0	100.0%	93.3%

^a dbh, diameter at 1.37 m above ground level; $n = 30$.

^b Values are grams of (–)-verbenone.

^c Based on strip and mass attacks (Gibson et al. 2009).

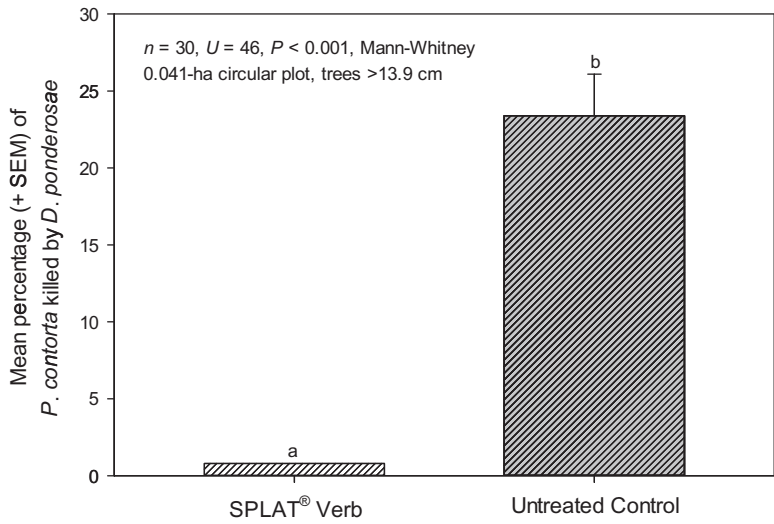


Fig. 2. Mean percentages (+SEM) of *P. contorta* killed by *D. ponderosae* on 0.041-ha circular plots surrounding each SPLAT Verb-treated and untreated control tree, Bridger-Teton National Forest, Wyoming, 2012–2013. Means (+SEM) followed by the same letter are not significantly different ($P > 0.05$).

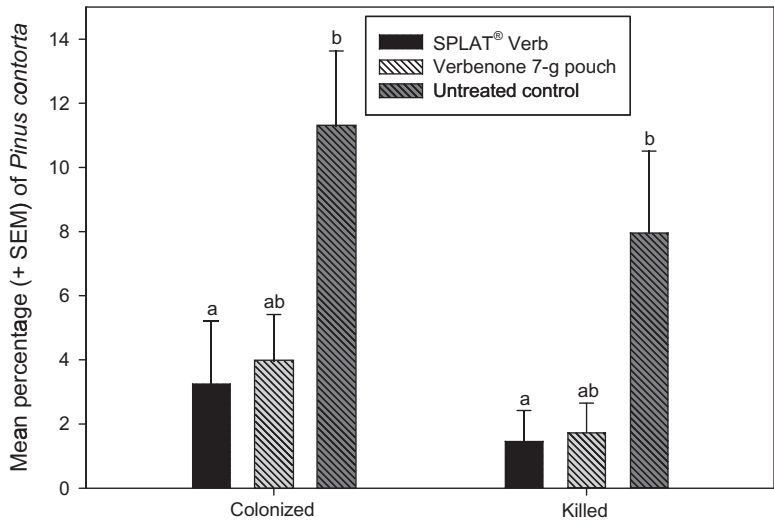


Fig. 3. Mean percentages (+SEM) of *P. contorta* colonized and killed by *D. ponderosae* on 0.4-ha square plots, Caribou-Targhee National Forest, Idaho, 2012–2013. Means (+SEM) followed by the same letter within group are not significantly different (Tukey's HSD; $P > 0.05$).

Table 3. Conditions within 0.4-ha square plots at the beginning of the small stand study, Caribou-Targhee National Forest, Idaho, 2012–2013

Treatment	dbh ^{a,b}	Trees per hectare ^a	Basal area per hectare ^a	Percentage of <i>P. contorta</i> ^a	Currently infested <i>P. contorta</i> per plot ^{a,c}
SPLAT Verb	25.6 ± 1.5 a	448.4 ± 41.7a	26.5 ± 1.8a	72.3 ± 10.8a	25.8 ± 6.1a
Verbenone pouch	25.9 ± 0.9 a	422.4 ± 44.1a	24.5 ± 3.1a	87.4 ± 3.3a	10.6 ± 3.8a
Untreated control	25.2 ± 1.8 a	400.4 ± 67.7a	22.2 ± 4.1a	78.9 ± 9.9a	13.8 ± 3.8a

^a Means ± SEM followed by the same letter are not significantly different ($P > 0.05$).

^b dbh, diameter at 1.37 m above ground level.

^c Present prior to treatment.

and both verbenone treatments combined ($P > 0.29$, all cases; $n = 10$) when analyzed separately. Thistle et al. (2004) examined near-field canopy dispersion of a tracer gas (SF_6) used as a surrogate for bark beetle pheromones. They showed that when surface layers of the air are stable (e.g., during low wind velocities), the tracer plume remained concentrated and directional because of suppression of turbulent mixing by the forest canopy. Lower density stands result in unstable layers of air and multi-directional traces (eddies) that diluted “pheromone” concentrations (Thistle et al. 2004) and presumably reduce beetle aggregation, thus influencing host finding and colonization successes. In the case of verbenone, these effects would negatively impact the performance of synthetic verbenone plumes (Progar et al. 2014). Despite this, stand density did not appear to influence treatment efficacy in our study.

Because of previous failures in the efficacy of verbenone (Progar et al. 2014), research has concentrated on combining verbenone with other inhibitors (e.g., non-host volatiles); combining verbenone with other methods of control (e.g., sanitation); and developing novel formulations to increase levels of tree protection. To date, the latter has focused on flake formulations. For example, Disrupt Micro-Flake VBN was demonstrated to be effective when applied at 1,101 g of verbenone per hectare by reducing the portion of infested *P. contorta* by 37% (Gillette et al. 2012). In an earlier study, a smaller proportion of *P. contorta* was colonized on plots treated with flakes (370 g of verbenone per hectare) than in control plots in California and Idaho; however, beetle “pressure” was somewhat limited (means = 5.1 and 4.6 trees per hectare were colonized in the untreated controls, respectively; Gillette et al. 2009). In our study, SPLAT Verb reduced the number of trees colonized by 79% during relatively high beetle “pressure” (mean = 37.5 trees per hectare were colonized in the untreated control), and represents an effective alternative to other formulations of verbenone for small stand protection.

Laboratory Analyses of Release Rate and Chemical Composition. SPLAT is a “matrix-type” diffusion-controlled release device. As such, the release of active ingredients is determined by Fick’s First Law of Diffusion, which states that molecules move from regions of high concentration to regions of low concentration at a rate that is directly proportional to its concentration gradient (Mafra-Neto et al. 2013). Dollops containing 17.5 g of SPLAT Verb had $1,963.5 \pm 7.5$ mg, $1,398.3 \pm 0.8$ mg, and $1,104.3 \pm 2.5$ mg (mean ± SEM)

of verbenone on days 1, 10, and 30, respectively (Table 4). The average release during the 30-d period was 28.6 mg/d, but, as expected, was greater during the first 10 d (56 mg/d) than the following 20 d (14.7 mg/d). This is also commonly observed with other formulations of verbenone (Progar et al. 2014). For example, Fettig et al. (2009) reported the mean release (± SEM) of (–)-verbenone from 7-g pouches (i.e., the same formulation as used in our study) analyzed gravimetrically at 30°C was 132.5 ± 5.3 mg/d, 105.4 ± 6.9 mg/d, and 65.3 ± 3.3 mg/d at days 3, 9, and 30, respectively. The mean release of pouches declined to 21.3 ± 1.0 mg/d on day 56 (Fettig et al. 2009).

Dollops of SPLAT Verb contained 56.2% of verbenone on day 30. Assuming the release of verbenone plateaus at 15 mg/d (i.e., mean release was 14.7 mg/d for days 10–30, as shown above) and continues at this rate from then on, a 30-d dollop should last another 75 d before exhausting the reservoir of verbenone. This period (105 d) is sufficient to cover most of the flight activity period of *D. ponderosae* in *P. contorta* forests. Each *P. contorta* treated with four 17.5-g dollops of SPLAT Verb cumulatively released 224 mg/d of (–)-verbenone during the first 10 d, and 60 mg/d during the next 20 d. As previously mentioned, two verbenone pouches are commonly recommended for protecting individual *P. contorta* from mortality attributed to *D. ponderosae* (Kegley and Gibson 2004, 2007), which would result in a much higher release of verbenone (e.g., 131 mg/d after 30 d) compared with our study. If desired, larger dollops could be used to increase the reservoir of verbenone and longevity of release (A.M.-N., unpublished data).

As mentioned, verbenone is known to photodegrade to behaviorally inactive chrysanthenone (Kostyk et al. 1993). This was considered enough of a concern that the addition of a cyasorb UV stabilizer that scavenges UV-generated radicals was added to verbenone pouches. Fettig et al. (2009) reported chrysanthenone was not detected in any volatile extracts obtained from 7-g verbenone pouches that had been deployed in *P. ponderosa* forests in California for several weeks, but that trace amounts of filifolone, a thermal- or photorearrangement product of (+)-chrysanthenone, were present. In our study, only after dollops of SPLAT Verb were aged in the field for >12 mo were trace amounts of chrysanthenone detected, which is inconsequential as biological activity is desired for only a few months (i.e., to cover the primary flight activity period of *D. ponderosae* during a single field season).

Table 4. Verbenone remaining in 17.5-g dollops of SPLAT Verb at 1, 10, and 30 d following field deployment, Riverside, CA, 2013

Verbenone	Day 1	Day 10	Day 30
Mass (mg)	1,963.5 ± 7.5	1,398.3 ± 0.8	1,104.3 ± 2.5
Percentage of formulation ^a	11.2 ± 0.4	8.0 ± 0.1	6.3 ± 0.2
Percentage of initial load remaining	100.0	71.2	56.2

^a SPLAT Verb is reported to be 10% a.i.

However, it is important to note that these and similar analyses do not address changes in the chemical stability of verbenone once released into the active airspace, which may also influence levels of inhibition, but over which we have no control.

Range of Inhibition Trapping Bioassay. In total, 5,105 *D. ponderosae* were captured in baited multiple-funnel traps. Overall, the ratio of males to females was 0.85. There was no significant distance X gender interaction ($F_{4, 419} = 0.91$; $P = 0.46$) and, therefore, results pertain equally to both male and female responses. This is of practical importance as females initiate host selection and, therefore, inhibition of females is critical to preventing colonization and tree mortality. A significant treatment effect was observed ($F_{4, 210} = 14.91$; $P < 0.001$). No differences were observed among captures at 1, 2, or 4 m from the point of release of SPLAT Verb, but significantly fewer *D. ponderosae* were collected at 1 and 2 m compared with 8 m (Table 5). One limitation of the experimental design is that the placement of traps is not continuous and, therefore, it is impossible to determine where the actual break in maximum inhibition occurs between 4 and 8 m. Significantly fewer *D. ponderosae* were captured at 8 m than at 16 m (Table 5), indicating that the range of inhibition was at least 8 m. This agrees with results from the individual tree protection study as few *P. contorta* were killed by *D. ponderosae* within 11 m of *P. contorta* treated with SPLAT Verb (Fig. 2).

Limited research has been carried out examining the zone of inhibition provided by different formulations of verbenone. Furthermore, the response of *D. ponderosae* to verbenone in the presence of aggregation pheromones and host kairomones varies by, among other factors, the ratio of verbenone to attractants (Pureswaran et al. 2000), population density (Progar 2003, 2005; Progar et al. 2013), and among individuals within a population (Borden et al. 1986), making comparisons among different studies tenuous. In British Columbia, Canada, Miller (2002) demonstrated that verbenone bubble caps (14 mg of verbenone per day at 24–28°C) inhibited *D. ponderosae* attraction to baited (*exobrevicomin* [0.01 mg/d], 13:87 mixture of *cis*- and *trans*-verbenol [1.7 mg/d], and myrcene [281 mg/d]) multiple-funnel traps at a distance of <4 m in *P. contorta* forests, and that maximum inhibition occurred at <1 m (i.e., captures were significantly lower at the point of release [0 m] compared with 1 m). While the constituents, doses, and release rates of baits used in

Table 5. Disruption of *D. ponderosae* attraction to baited multiple-funnel traps at five distances from 70 g of SPLAT Verb applied as four 17.5-g dollops to the bole of individual *P. contorta*, Uinta-Wasatch-Cache National Forest, Utah, 2013

Distance (m)	Mean daily trap catch ± SEM ^a
1	15.1 ± 1.6a
2	13.3 ± 1.5a
4	18.4 ± 2.2ab
8	25.1 ± 3.3b
16	34.5 ± 3.8c

^a Means ± SEM followed by the same letter are not significantly different ($P > 0.05$; Tukey's HSD).

our study were similar to Miller (2002), trap catches in our study were much higher (e.g., 5.1 vs. 25.1 per trap per day at 8 m, Table 5), as were levels of tree mortality in surrounding stands (10–20 vs. 26%), suggesting a more rigorous examination of the zone of inhibition observed. However, a larger release of verbenone was used in our study (Table 4), presumably increasing levels of inhibition. Fettig et al. (2009) reported similar results to those of Miller (2002) for western pine beetle, *Dendroctonus brevicomis* LeConte, in *P. ponderosa* forests in California when analyzing responses to a 5-g verbenone pouch (50 mg of verbenone per d at 30°C). In their study, maximum inhibition occurred at <4 m (i.e., no significant differences were observed among captures at 0.5, 1, or 2 m, but a significant increase occurred at 4 m; Fettig et al. 2009). In both studies (Miller 2002, Fettig et al. 2009), the authors stated that to achieve maximum efficacy for stand protection, substantial increases in the density of release devices would be required.

In conclusion, SPLAT Verb provided high levels of tree protection in both individual tree and small stand studies during high levels of beetle “pressure” (Table 2; Figs. 2–3), which we attribute to the large zone of inhibition provided and the ability of applying dollops at high densities. Based on our results, we recommend applying four 17.5-g dollops (5.5 cm in diameter by 1.2 cm in height) per *P. contorta* (i.e., one dollop placed at maximum reach at each cardinal direction) when individual tree protection is the objective, and applying one 17.5-g dollop per tree at the northern aspect in as uniform a distribution as possible (see label for dose restrictions), when stand protection is desired. Lower doses may yield suitable efficacy, and are currently being evaluated. Significant research and development remains including: 1) refinement of application methods, doses, and distribution patterns in *P. contorta*; 2) determination of efficacy in other hosts, specifically *P. albicaulis*, a high-elevation keystone species being considered for listing as a threatened and endangered species in the United States under the Endangered Species Act; and 3) determination of efficacy in other bark beetle-host systems. Relatedly, preliminary results (data not shown) from ongoing studies indicate SPLAT Verb is also effective for protecting individual *P. ponderosa* from mortality attributed to *D. ponderosae* and *D. brevicomis*.

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