ABSTRACT

Spray dried *Bacillus thuringiensis* formulations, based on citric or lactic acid, pregelatinized corn flour, cornstarch, isopropyl alcohol, sugar, and corn oil, were used in leaf and diet incorporation bioassays, to determine the effects of solar radiation and rain on insecticidal activity. In diet incorporation tests against *Helicoverpa zea*, *Trichoplusia ni*, *Heliothis virescens* and *Spodoptera exigua*, insecticidal activity of spray-dried *B. thuringiensis* did not decrease when compared with non-spray dried material. Cotton leaf bioassay tests using *Ostrinia nubilalis* showed that insecticidal activity of formulations exposed to solar radiation was higher than technical *B. thuringiensis* exposed to solar radiation, suggesting the formulations provided protection against solar radiation. In cotton leaf bioassays, when five different starches were used in the formulations, insecticidal activity was reduced after exposure to solar radiation in only one case. Cotton leaf bioassays also showed a reduction in insecticidal activity due to exposure to solar radiation as the amount of active ingredient in the formulation increased. Throughout all tests, rainfastness did not improve over technical *B. thuringiensis* alone.

KEY WORDS: Formulation, cornstarch, corn flour, *Bacillus thuringiensis*, spray dry, microencapsulation

Commercially available pesticide formulations containing *Bacillus thuringiensis* are applied as dry granules or as sprayable preparations. Both types of formulations, however, suffer from a lack of residual insecticidal activity due to wash-off by rainfall or degradation by sunlight. In addition, most preparations of B. thuringiensis are not palatable to insects thus limiting their effectiveness. (Gillespie et al. 1994) . Previous research to address these problems has resulted in the development of adjuvants containing starch (McGuire and Shasha 1990, 1992), gluten (Behle et al. 1996b), casein (Behle et al.1996a), or lignin (McGuire et al. unpublished) as additives to spray tanks. These ingredients protect *B. thuringiensis* from wash-off by rainfall and/or degradation by sunlight. However, as adjuvants they require different amounts of material depending on the spray volume used. Therefore, a user applying 10 L/Ha spray volume would require much less material than a user applying 100 L/Ha. Thus potential for commercialization of these materials is limited because packaging for multiple uses is a problem. Development of granular formulations that are applied dry has also resulted in increased residual activity of microbial pesticides. Examples of these formulations include corn meal baits (Creighton et al. 1961, Cannerday et al. 1975) and granules formed with gelatinized starch or flour (Dunkle and Shasha 1988, Gillespie et al. 1994, Castro-Franco 1994), or gluten (Connick et al. 1991). However, the use of these large granules is restricted to corn or other plants that have a retaining device such as a whorl. This paper focuses on recent efforts conducted jointly by Mexican and U.S. scientists aimed at developing a sprayable granule that can resist wash-off and solar degradation of *B. thuringiensis*.

To overcome the limitations of adjuvant-based protectants, we wanted to develop a formulation in which the protectants maintained contact with the active ingredient throughout

the tank mixing and application process. One way to do this is to quickly dry materials onto small particles of active ingredient. Spray drying is a method commonly used in food and other industries to produce microcapsules of uniform quality containing multiple ingredients. The equipment is relatively inexpensive to purchase and operate, is commonly available and easy to use (Taylor 1983, Youngs 1986). The initial step in the spray drying process is to select a suitable coating material for the active ingredient. This material should have good rheological properties, disperse evenly, be non-reactive, and have the ability to hold the active ingredient (Shahidi and Han 1993). We have found that gelatinized starchy materials such as corn flour provide the properties described above. We now report on the use of spray drying as a method to prepare small (pass 60 mesh) granules containing *B. thuringiensis* that are sprayable and that extend the residual insecticidal activity of B. thuringiensis in response to simulated environmental factors.

Materials and Methods

***B. thuringiensis* isolate:** Technical *B. thuringiensis* provided by Abbott Laboratories (North Chicago, IL) was used in all formulations. The powder contained 64,000 IU/mg and was derived from the strain HD1 used commercially.

Spray drying conditions: A Niro atomizer (Niro Inc. Columbia MD) was used for all formulations. The settings of the atomizer were: inlet temperature 120-130°C, air pressure 4 KPS. outlet temperature 60-90°C and varied throughout the run depending on viscosity of the formulation mixture. Mixtures were pumped into the atomizer at the rate of 10 ml/min. As the formulations entered the atomizer, much of the material adhered to the sides of the tank. At the end of the run, the tank was opened and the material was scraped off the sides and into the collection vessel. Therefore, residence time for the material in each formulation varied from a few seconds to 30 min.

Formulations: The ingredients used in the formulations (Table 1) were selected based on availability, cost, and functionality. The combinations and designations for each of the formulations are shown in Table 2. The ingredients were mixed under precise conditions as follows: The oil and starches were first mixed by pressing the two together in a beaker; the mixture was then added to 280 ml warm (60°C) water to form a soft dough; sugar was added, then the alcohol, then the acid, then the malachite green, and then the active ingredient. In some cases the viscosity of the mixture was too high and the addition of more water was necessary to enable pumping of the material to the spray drier. A preliminary formulation utilized, among other ingredients, nixtamalized flour (used for food and industrial purposes and commonly available in Mexico), cornstarch, and formaldehyde (Tamez-Guerra et al.

1996). In the U.S., nixtamilized flour is not readily available and the use and presence of formaldehyde may raise concerns. Therefore, several types of formulations were developed. Formaldehyde was substituted with citric or lactic acids (1,2; Table 2), nixtamilized flour was substituted with other common flours or starches (3-7; Table 2), amount of active ingredient (initially 3% of the solids level) was increased up to 50% (8-10; Table 2). The ratio of nixtamalized flour to ungelatinized cornstarch was adjusted from the original 50:50 (11-12; Table 2) to determine if the starch content could be increased (thus decreasing overall cost of the formulation) without loss of protection or activity. Finally, malachite green, a potential sunscreen was removed from the formulation to determine if it played a role in solar stability (13).

Insect cultures: *Ostrinia nubilalis* was reared using procedures modified from Ortega et al. (1980) as reported by Bartelt et al. (1989). Neonates were used in all cotton leaf bioassays. *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera exigua*, and *Trichoplusia ni* were reared on artificial diet modified from Shorei and contained soy meal (1.0 g), corn meal (31.1 g), corn oil (10.6 g), SACAROSA (13.6 g), sorbic acid (1.0 g), methyl-p-hydroxybenzoate (1.6 g), sorbic acid (4.26 g), agar (15.7 g), formaldehyde at 10% (4.4 ml), clorox at 15% (7.3 ml), acetic acid at 25% (12 ml), vitamin solution (3.5 ml), and distilled water (1 liter). The vitamin solution contained calcium pantothenate (12 ml), niacin (6.0 ml), riboflavin (3.0 ml), folic acid (3.0 ml), thiamine (3.0 ml), pyridoxine (1.5 ml), biotin (0.12 ml), B12 (25 ml) and the volume was brought to 1 liter with distilled water.

Diet Incorporation Bioassays: In Monterrey, diet incorporation assays were done to determine the effect of spray drying on activity of the active agent. Selected formulations

were added to warm artificial diet, lacking the antibiotics, at a rate equivalent to 10, 25, 50, or 100 μg *B. thuringiensis* technical material per mg diet. The diet was then poured into cups (5 $\pm$1 ml per cup) and allowed to cool. One neonate was added to each of 75 cups per treatment and held for one week. Probit analysis (Finney 1971) was done to determine LC50 for each formulation for each insect species.

Leaf Bioassays: In Peoria, bioassays on cotton leaves were conducted with *O. nubilalis* and involved methods similar to those reported by Behle et al (1996a). Cotton plants were treated with spray dried formulations added to water. For assays involving rainfastness, formulations were applied over the tops of plants in a spray chamber with a single traveling flat fan nozzle (8002ss, Spraying Systems, Wheaton, IL) at 4.9 kg/cm^2 and a track speed setting of 3.0 km/h to apply 35 ml total volume. This rate is equivalent to 235 liters/ha. To account for differences in susceptibility to *B. thuringiensis* by laboratory-reared neonate insects, the relative amount of *B. thuringiensis* was reduced because field rates will kill all neonate larvae (MRM unpublished). The rate of 10 mg technical powder /50 ml water was used and generally killed about 85% of the test larvae. For all applications, an amount of spray dried material that contained the equivalent of 10 mg technical material was added to 50 ml water and held until use. For at least 5 min prior to use, suspensions were mixed thoroughly using a magnetic stirrer. The rainfastness assay was conducted in the same chamber as that used to apply formulations. The chamber was modified to provide continuous traversing of the nozzle assembly that was attached to a water source. As the assembly traversed, water was sprayed through a full cone nozzle until 5 cm of water was collected in a rain gauge (approximately 50 minutes of spraying). For solar stability assays, (10 cm^2) circles

were marked onto cotton leaves. This area was then treated with 0.033 ml of formulation that was spread evenly across the circle with a glass rod. Once dried, plants were then placed under a light source (CPS SunTest, Hereaus, Hanau, Germany), and exposed for 8 h. Regardless of assay type, leaf disks were cut from the plants after treatment and placed in plastic petri plates containing a filter paper disk. Ten larvae were placed in the dish and capped with a sealing lid. The dishes were held for three days in the dark at 28°C and then percentage mortality was obtained for each dish. Data were analyzed with analysis of variance and means were separated using a protected least significant difference test (Statistix 1994).

To determine if the process of spray drying was adding a benefit to the overall formulation, a test was done comparing spray dried and tank-mix formulations. Spray dried formulations made with 3% and 50% technical material (2 and 10 respectively; Table 2) were compared with a tank mix containing the same ingredients and levels of *B. thuringiensis*. The tank mix formulations had not been spray dried. Solar and rainfastness assays were done as described above.

Results

Diet Incorporation: Assays to determine LC_{50} for spray dried formulations were done for four insect species (Tables 3-6) to determine if the conditions inherent to spray drying (e.g. high temperatures, presence of alcohol) caused a loss of insecticidal activity. In all cases, activity did not decrease when compared with non-spray dried *B. thuringiensis* and in many cases, LC_{50} decreased. This effect could be due to increased feeding due to the large amount of sugar incorporated into the formulation.

Leaf Bioassays: Bioassays were conducted on cotton leaves against *O. nubilalis*. The plant leaf assays gave a good indication of how the formulations protect insecticidal activity of *B. thuringiensis* when exposed to simulated rainfall or simulated sunlight. Previous tests have indicated that these procedures provide results that will be applicable to field performance (Behle et al.1996a).

Effect of acid type and ratio of nixtamalized flour to unmodified cornstarch: Formulations with either lactic or citric acid caused high mortality of *O. nubilalis* after exposure of treated leaf surfaces to simulated sunlight compared with technical *B. thuringiensis* only (Table 7). No significant loss of activity occurred after exposure to sunlight. In addition, both formulations retained some activity after exposure to artificial rain. Significant loss of activity did occur upon exposure to sunlight when formulations were made with a ratio of nixtamalized flour to unmodified starch other than 50:50. However, significantly more activity was retained in these formulations than technical material only. Very little activity remained after simulated rainfall.

Effect of modified starch type: Five different starch types were incorporated into the

formulations and tested on cotton leaves for rainfastness and solar stability (Table 8). In this test, percent mortality before solar or rain simulation was similar for all formulations, including technical material only, except for the formulation made with oxidized starch. None of the formulations protected the insecticidal activity against 5 cm rainfall. However, several of the formulations provided solar protection; most notably, flour 961, a gelatinized corn flour available commercially.

Effect of amount of *B. thuringiensis* and malachite green: The initial formulation contained only 3% B. thuringiensis technical powder. While this rate produced some rather remarkable results with respect to solar stability, to be economically viable, the level of active ingredient must be increased. In leaf assays, a clear trend emerged with respect to solar stability and amount of active ingredient (Table 9). As the level of active ingredient increased, the solar protection decreased. Formulations with *B. thuringiensis* levels of 3 and 10% survived significantly better than formulations with 25 or 50% *B. thuringiensis*. However, formulations with 50% B. thuringiensis were significantly better than technical powder alone both before and after exposure to simulated sunlight. Perhaps the initial difference is due to the ingredients in the formulation acting as feeding stimulants. While this may affect overall results, original activity remaining after solar simulation for spray dried formulations with 50% active ingredient (44%) is still better than technical material by itself (39%). As above, none of the formulations provided protection from rainfall. There was no significant improvement in solar stability from the addition of malachite green when the amount of *B. thuringiensis* in the formulation was 3%. There was, however, an effect on rainfastness. The formulation made without malachite green adhered better during the simulated rainfall than

the formulation without malachite green. The formulation without malachite green was not more rainfast than technical *B. thuringiensis*.

Effect of spray drying: To test the effect of spray drying on resistance to wash-off and solar degradation, formulation ingredients were tank mixed using the same proportions and solids levels of two previously made spray dried formulations. While there was no effect on rainfastness, there were two other effects worth noting. First, spray dried formulations made with 50% *B. thuringiensis* gave higher mortality before exposure to sunlight. This effect could be due to the close adherence of potential feeding stimulants to the active agent. The second point is that spray dried formulations provided better solar protection than tank mixed formulations when 3% *B. thuringiensis* is used. The 50% *B. thuringiensis* spray dried formulation did not provide additional solar protection compared with technical material only.

Discussion

Some of the first formulations of *B. thuringiensis* involved some form of protection system and some of the formulations were considered to be encapsulated (Creighton et al. 1961, Angus and Luthy 1972). Angus and Luthy (1972) suggested the use of smudge proof carbon paper as a matrix and demonstrated that the matrix provided phagostimulation, solar protection and did not harm activity. Rainfastness has continued to plague formulations of *B. thuringiensis* and a search for ingredients with resistance to wash-off resulted in the use of pregelatinized starches and flours (McGuire and Shasha 1990). Since this discovery, gluten (Behle et al. 1996b) and casein (Behle et al. 1996a) have been shown to be more effective with smaller amounts of material necessary for resistance to wash-off. In addition, each of these materials provides solar stability to *B. thuringiensis* foliar deposits. However, all of these materials require addition to a spray tank in relatively specific amounts, i.e. flour requires 2 g per 100 ml (2% solids); gluten requires 1% solids and casein requires 0.5% solids.. These requirements place limitations on the commercial use of the adjuvants because end users may use different amounts of water for different applications. The spray dried formulation, however, removes this percent solids requirement. By tying the protective matrix directly to the active ingredient and maintaining the two together in the spray tank, the deposition on the leaf surface will survive in response to sunlight.

The spray dried formulations did provide adequate solar protection to *B. thuringiensis* when levels of *B. thuringiensis* were relatively low (3% or 10%) in the formulation. Increasing the amount of *B. thuringiensis* in the formulation is important because costs

associated with handling and processing large amounts of formulation ingredients could prohibit wide scale use of these formulations. Although costs of the ingredients are relatively low (approximately $0.50 U.S. dollars/kg for the flour), applying 500 g *B. thuringiensis* per ha in a 3% formulation would require 16.6 kg of formulated product. A 50% formulation would require only 2 kg/ha and is more representative of a commercial formulation.

Surprisingly, rainfastness was not consistently improved with the spray dried formulations and in certain cases performed worse than technical material only (Table 9). Possibly, the granules provide enough relief on the leaf surface to enable water to physically knock the formulation off the plant.

In summary, the formulations presented in this manuscript do provide a mechanism for tying protective ingredients to an active agent. These ingredients are inexpensive, most are commonly available and easy to use. Likewise, the spray drying process and equipment is well known and generally available. The formulations can be added to a spray tank in any ratio to the amount of water and sprayed onto a leaf surface. While these formulations do not render stability to rainfall, they do provide a high level of solar stability.

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Table 1: Ingredients used in the formulations

Ingredient	Source
Nixtamalized flour	Maseca, location
Cornstarch	CPC products, New Jersey
Miragel	Staley, Inc. Decatur, IL
Flour 961	Illinois Cereal Mills, Paris, IL
Oxidized Starch	National Starch, New Jersey
Potato Starch	National Starch, New Jersey
Enriched Bleached Wheat Flour	IGA Grocery store brand
Corn Oil	Mazola, CPC International, New Jersey
Powdered Sugar	IGA Grocery store brand
Citric acid	Eastman, Rochester, New York
Lactic Acid	Fisher Chemical, New Jersey
Isopropyl alcohol	Fisher Chemical, New Jersey

Table 2: Amounts of ingredients used for different tests.

	Formulation Number												
Ingredient	1	2	3	4	5	6	7	8	9	10	11	12	13
Nixtamalized flour (g)	50	50						50	50	50	25	75	50
Cornstarch (g)	50	50	50	50	50	50	50	50	50	50	75	25	50
Miragel (g)			50										
Flour 961 (g)				50									
Oxidized Starch (g)					50								
Potato Starch (g)						50							
Enriched Flour (g)							50						
Citric acid (ml)	0.5												
Lactic Acid (ml)		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Malachite green (mg)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
B. thuringiensis (mg)	6.6	6.6	6.6	6.6	6.6	6.6	6.6	22	55	110	6.6	6.6	6.6

All formulations also contained 100 g powdered sugar, 120 ml isopropyl alcohol, and 20 g oil.

Table 3. Effect of spray drying on activity of *B. thuringiensis* insecticidal activity in diet incorporation tests against *Helicoverpa zea*

Formulation(#)[1]	Slope (±SE)	LC_{50}	95% CI	X^2
Technical only[2]	2.195 (±0.3323)	7.77	10.266-4.951	0.441
Citric (1)	2.482 (±0.4243)	6.80	9.036-4.116	3.417
Lactic (2)	2.194 (±0.4588)	4.06	7.047-1.856	4.062
Miragel (3)	2.33 (±0.3622)	7.62	9.996-4.896	1.613
961 (4)	2.352 (±0.4141)	6.32	8.641-3.580	2.223
10% Bt (8)	1.979 (±0.4135)	4.22	6.746-1.513	1.334
Maseca 25 (11)	1.538 (±0.2653)	6.52	9.740-3.141	3.285
Maseca 75 (12)	1.467 (±0.2616)	5.82	9.065-2.527	2.639

[1]Refer to Table 2

[2]Technical Bt only

Table 4. Effect of spray drying on activity of *B. thuringiensis* insecticidal activity in diet incorporation tests against *Trichoplusia ni*

Formulation(#)[1]	Slope (±SE)	LC_{50}	95% CI	X^2
Technical only[2]	1.583 (±0.2015)	1.23	1.697-0.7984	0.643
Citric (1)	1.241 (±0.2121)	0.44	0.796-0.154	0.720
Lactic (2)	0.870 (±0.2245)	0.09	0.335-0.0022	5.329
Miragel (3)	1.461 (±0.2148)	0.71	1.085-0.365	1.919
961 (4)	1.416 (±0.2405)	0.45	9.778-0.173	0.138
10% Bt (5)	1.176 (±0.2410)	0.27	0.579-0.0545	3.257
Maseca 25 (11)	1.048 (±0.1775)	0.55	1.010-0.189	3.070
Maseca 75 (12)	1.185 (±0.2044)	0.51	0.905-0.184	1.753

[1]Refer to Table

[2]Technical Bt only

Table 5. Effect of spray drying on activity of *B. thuringiensis* insecticidal activity in diet incorporation tests against *Heliothis virescens*

Formulation(#)[1]	Slope (±SE)	LC_{50}	95% CI	X^2
Technical only[2]	2.319 (±0.3327)	8.72	11.165-5.968	0.545
Citric (1)	2.313 (±0.3848)	6.86	9.222-4.115	1.201
Lactic (2)	1.922 (±0.3993)	4.20	6.756-1.488	0.971
Miragel (3)	2.017 (±0.3043)	8.00	10.699-5.001	0.204
961 (4)	2.409 (±0.4046)	6.90	9.190-4.192	2.018
10% Bt (5)	1.997 (±0.3903)	4.83	7.354-2.0416	1.027
Maseca 25 (11)	1.711 (±0.3129)	5.01	7.789-2.129	0.272
Maseca 75 (12)	1.365 (±0.2927)	3.47	1.365 ±0.292	0.605

[1]Refer to Table

[2]Technical Bt only

Table 6. Effect of spray drying on activity of *B. thuringiensis* insecticidal activity in diet incorporation tests against *Spodoptera exigua*.

Formulation(#)[1]	Slope (+SE)	LC_{50}	95%CI	X^2
Technical only[2]	2.72 (0.311)	13.93	11.30-16.47	0.0025
Citric Acid (1)	2.68 (0.397)	8.74	6.22-8.74	3.75
Lactic Acid (2)	2.08 (0.362)	5.68	2.92-8.16	0.70
Miragel (3)	2.26 (0.325)	8.43	5.64-10.91	1.26
Flour 961 (4)	2.47 (0.407)	7.18	4.50-9.43	2.25
10% Bt (5)	2.75 (0.388)	9.12	6.67-11.24	2.25
Maseca 25% (11)	1.63 (0.237)	11.52	7.54-15.23	7.56
Maseca 75% (12)	1.82 (0.244)	12.81	9.07-16.31	6.19

[1]Refer to Table 2

[2]Technical Bt only

Table 7. Effect of acid type and ratio of nixtamilized flour to buffalo starch on rainfastness and solar stability of *Bacillus thuringiensis* as measured by percentage mortality of *Ostrinia nubilalis* placed on treated cotton leaves

	Exposure of Leaf Surfaces		
Formulation (#)[1]	None	Rain	Solar
Citric Acid (1)	100.0	64.4	92.5
Lactic Acid (2)	99.0	59.4	91.8
25:75 (11)	98.0	19.5	76.2
75:25 (12)	100.0	27.0	78.0
Technical Bt only	59.0	10.4	36.1

Cotton leaves were treated with formulation, then exposed to simulated rainfall or sunlight, then fed to neonate *O. nubilalis*. $F=29.08$, df=14,126, $P<0.001$; critical value (SE) for comparison 16.276 (8.225)

[1]See Table 2 for formulation composition.

Table 8. Effect of modified starch type on activity of *Bacillus thuringiensis* as measured by percentage mortality of *Ostrinia nubilalis* placed on treated cotton leaves

	Exposure of Leaf Surfaces		
Formulation (#)[1]	None	Rain	Solar
Miragel (3)	94.0	17	66
Flour 961 (4)	94	35	80
Oxidized starch (5)	59	23	41
Potato Starch (6)	78	17	74
Enriched flour (7)	86	29	71
Technical Bt only	84	27	40

Cotton leaves were treated with formulation, then exposed to simulated rainfall or sunlight, then fed to neonate *Ostrinia nubilalis*. $F=16.00$, df=20,180, $P<0.001$; critical value (SE) for comparison 18.544 (9.398)

[1]See Table 2 for formulation composition.

Table 9: Effect of relative amount of *Bacillus thuringiensis* and malachite green on insecticidal activity of formulations as measured by percentage mortality of *Ostrinia nubilalis* placed on treated cotton leaves

	Exposure of Leaf Surfaces		
Formulation (#)[1]	None	Rain	Solar
3% (2)	100.0	6.9	95.8
10% (8)	100.0	4.2	86.0
25% (9)	95.0	7.0	64.4
50% (10)	96.0	6.0	42.0
Without green (13)	100.0	20.5	91.0
Technical Bt only	49.3	15.1	19.2

Cotton leaves were treated with formulation, then exposed to simulated rainfall or sunlight, then fed to neonate *O. nubilalis*. $F=118.20$, df=20,177, $P<0.001$; critical value (SE) for comparison 10.23 (5.184)

[1]See Table 2 for formulation composition.

Table 10: Effect of spray drying on activity of *Bacillus thuringiensis* as measured by percentage mortality of *Ostrinia nubilalis* placed on treated cotton leaves

		Exposure of Leaf Surfaces		
Method[1]	Level of B. t	None	Rain	Solar
Spray dry (1)	3%	100.0	10.1	90.0
Spray dry (10)	50%	83.8	5.0	25.2
Tank mix	3%	97.0	2.0	71.0
Tank mix	50%	69.8	3.0	32.5
Technical Bt only		53.6	24.8	32.7

Cotton leaves were treated with formulation, then exposed to simulated rainfall or sunlight, then fed to neonate *O. nubilalis*. $F=68.7$, df=15,159, $P<0.001$. LSD Critical value for comparison = 12.28

[1]Refer to Table 2

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Bacillus thuringiensis microencapsulated formulation for control of the coleopteran *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae)

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ABSTRACT

The process for microencapsulating a strain *Bacillus thuringiensis* subsp. *kumamotoesis* (C-9), for the biocontrol of *Epilachna varivestis* Mulsant, a bean pest, is discussed. This strain was isolated from a dead *E. varivestis* from a bean (*Phaseolus vulgaries*) field in Durango, Mexico. The bioinsecticide activity of this microcapsulated strain had an LD_{50} of 397 μg/ml against coleopteran larvae in bioassays with plants, and an LD_{50} of 219.35 μg/ml against *Trichoplusia ni* larvae using artificial diets. The formulation was prepared with equal parts of corn starch and corn nixtamalized flour as a formulation matrix, as well as vegetable oil, powdered sugar, isopropanol, malachite green (photoprotector), and formaldehyde (antoxidant). This mixture was microencapsulated using the spray dry technique. Residual activity of the original formulation was observed 15 days after exposure to environmental conditions on plants, and after six mounths and two years in storage. A field test under dry conditions, using a sprayable formulation at 3% of the active ingredient, showed mortality of 75 and 16%, eight and fifteen days after application respectively of the C-9 microcapsules. If it rained after application, the activity fell to 14% in one week. This study shows the possibility of using *Bt* microencapsulated material for pest control in bean.

KEY WORDS: coleopteran, *Epilachna varivestis*, microencapsulated formulations, *Bacillus thuringiensis*.

Bacillus thuringiensis used against insects as a biocontrol agent has received considerable attention in recent years. The field experiments have been directed toward protection of *B. thuringiensis* spores and crystals from noxious environmental factors. Good results were obtained with granulated formulations employing corn meal bait (Creighton *et al*, 1961; Cannerday *et al*, 1975); and optimum conditions of dispersion, residuality and application in the field (Dunkle & Shasha, 1988). The research on formulation technology for *B. thuringiensis* principally includes UV-protectants, adherents, and phagostimulants (Angus & Lüthy, 1971; Dunkle & Shasha, 1989; Liu *et al*, 1993; McGuire *et al*, 1989, McGuire *et al*, 1990). Recently, different materials were used for preparing granulated matrices or microcapsules, depending on the type of crop and application. In general, the granulated formulations are used in crops where retention on the leaf is possible (corn, cotton, grass, etc.) (Dunkle & Shasha, 1988). In field crops most microbial applications, both experimentally and operationally, are foliar sprays (Couch, 1978). Currently, sprayable formulations are used more extensively than granular formulations (McGuire & Shasha, 1990).

Microencapsulated *B. thuringiensis* formulations have been used successfully against the Colorado potato beetle (*Leptinotarsa decemlineata*) (Zehnder *et al*, 1992). The Mexican bean beetle *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae), is an important pest in bean, soybean and lima bean crops (Kogan, 1971), causing serious economic loss, especially in irrigated crops (Valdez & Alvarez, 1991). This

pest is distributed from Panama to southern Canada (Kogan & Pitre, 1980). In Durango, Mexico, 10-100% of the bean foliage is consumed by this pest every year, especially in irrigated crops. Current control techniques include chemical insecticides and, more recently, occasional introductions of a parasitoid wasp *Pediobius foveolatus* Crawford (Hymenoptera: Eulophidae). However, the parasitoid just attacks pupae, and the damaging larvae are not controlled (Carrillo-Sanchez, 1971; Bernhardt & Shepard, 1978). Perhaps, *B. thuringiensis* could be used in several crops against *E. varivestis*, but a report indicates very low toxicity (Keller & Langenbruch, 1993).

This research is focused on the possibility of introducing *B. thuringiensis* formulated as microcapsules into bean crops as a bioinsecticide against *E. varivestis*.

Materials and Methods

Strain production. The strain used was C-9 (subsp. *kumamotoensis*) obtained from the International Collection of *Bacillis* Entomopathogens located at the Facultad de Ciencias Biologicas, Universidad Autonoma de Nuevo Leon. This strain was isolated from a dead *E. varivestis* in Durango, Mex. Spores and crystals were produced using a fermentation process in a fermentor (Microferm New Brunswick MF-214). The fermentation media contained: molasses, 20.0 g; soy flour, 20.0 g; corn liquor, 10.0 g; $CaCO_3$, 2.0 g; tap water, 1000 ml; at pH - 7.2 ± 0.2. The fermentation conditions were automatically maintained. They included agitation at 700 rpm; temperature, 30°C ±5°C; pH 7.0 ± 1.0. Oxygen saturation was 90-100% initially and oxygen was supplied at 1 v.v.m. (air volume/ media volume/minute). The process was followed with microscopic examinations every two hours until ca. 90% of spores and crystals were liberated. The spore-crystal complex was obtained following coprecipitation with lactose (Dulmage *et al*, 1970).

Bioassays. The strain was tested against first instar larvae of the coleopteran *E. varivestis* with bean leaves and the lepidopteran *Trichoplusia ni* with an artificial diet under laboratory conditions. For *E. varivestis*, bean leaves were inoculated with 100 µl of each concentration on a marked area of 33 cm^2 and allowed to dry. The concentrations employed were 10, 25, 50, 100, and 250 mg/50 ml of spore-crystal complex or active ingredient of the microencapsulated formulation. A disk was cut from

each leaf and put in a plastic dish. Each disk was infested with three larvae, using five repetitions by treatment, and one negative control. The dishes were maintained in a room at 25°C ± 3°C, and 65% ± 5% relative humidity (RH) for five days and supplied with one untreated leaf each third day. The LD_{50} was obtained using the Probit statistical method (SAS, 1989). Bioassays with the lepidopteran *T. ni* were done using first instar larvae and artificial media (modified Shorei diet) (Castro-Franco *et al*, 1995). The concentrations employed were 5, 10, 13, 15, 25, 50, and 100 µg/ml, with three repetitions and 25 larvae/repetition; one larva per container. Each treatment was maintained at 28°C ± 5°C, and 65% ± 5% of RH, for one week. The LD_{50} was obtained in the same way as for the coleopteran larvae.

Encapsulating agents. Two different materials were used for matrix encapsulation at three concentrations: 0.0, 50.0, and 100.0 g cornstarch (Maizena™) and (0.0, 50.0, and 100.0 g) nixtamalized corn flour (Maseca™). Vegetable oil (Capullo™) was also assayed in several portions (5.0, 10.0, 20.0, 40.0 and 50.0 ml) for polar coating. The other materials used were: isopropanol (Capullo™), 120.0 ml; powdered sugar (Valle Verde™) 100.0 g; malachite green and its oxalate salts (Merck™), 0.1 g; as a solar protectant, and formaldehyde (Baker™), 0.5 ml, as an antoxidant (Castro-Franco, 1994). The different mixtures were subjected to the following rheological assays: viscosity, shear rate, and fluid type, following the methodology of Rao (1992). The viscosimeter was a Brookfield RVF and employed a correction factor of a sucrose standard at 60%. The viscosity measurements were at 20°C, 30 min. and 2 h after initial mixing.

Microencapsulation procedures. The microencapsulating technique employed a spray dryer with an Apex SSE (Niro atomizer). The conditions for microencapsulation were: feed source, 10 ml/min; air temperature in, 130°C; air temperature out, 60°C; air pressure 4 Kilopounds (Kp); (Saucedo-Mendiola, 1990). Moisture content of the microencapsulated formulation has measured immediately, six months and two years after preparation. Moisture content was obtained by determining dry weight (Lynch *et al*, 1972). Texture, particle size, percent dissolved solids were also tested. The insecticidal activity (% mortality) was measured the same way as described above, but using only two concentrations of active ingredient in the formulate product: 500 and 50 µg/ml . This test also was done against *T. ni* larvae immediately after preparation and after six months and two years of storage, and with *E. varivestis* after six mounths and two years of storage.

Adherence of formulations to leaf surface. The different formulations were assayed on bean plant leaf surface grown in greenhouse conditions. The bean, *Phaseolus vulgaris* var *peruano*, was planted in plastic pots (25 cm diameter), with 25-30 plants per pot; and the plants were used approximately 3 wk later when 2-4 true leaves had expanded on each plant. The formulation was mixed with water (100.0 g in 500.0 ml) and was sprayed on bean plants with a plastic manual sprayer of 1000 ml, approximately 10 ml/plant. The plastic pots were placed in a greenhouse without rain. Other pots were left under environmental conditions, and were natural rained on two hour after spraying. Leaf surfaces were examined grossly and microscopically every 1-2 d to obtain the percent of original material remaining.

Persistence assays. Three different materials were assayed with the strain to determine residual insecticidal activity of *B. thuringiensis* on a bean crop naturally infested by *E. varivestis* in Vicente Guerrero, Dgo, Mex. Plot size was 3 m by 1.5 m with 15-20 bean plants/plot. The microencapsulated formulation containing 4% w/w of *B. thuringiensis* was sprayed at a rate of 100 liter/hectar, (2 kg/hectar of the formulation). Live and dead larvae were counted each week and taken to the laboratory for reisolation of *B. thuringiensis.* When no larvae were found, the crop was atificially infested twice with 60 first and second instar larvae.

Results

Strains production. The fermentation behavior was as follows: 200 ml antifoam consumed; 200 ml 20% HCl consumed; 20 ml 20% NaCl consumed; sporulation ocurred in about 18 h; total fermentation time was 30 h. The rate of active ingredient production (spore-crystal complex) was 13.1 g/l employing the lactose coprecipitation method. The results of bioassays were expressed as % mortality and they are shown in Table 1.

Rheological Properties of Encapsulating Materials. The first encapsulating material assayed was corn starch . One hundred grams was manually mixed with 20 ml of vegetable oil . This was added to 280 ml of warm water (60-70°C) and a dough formed, and 100 g pulverized sugar was then added. At this moment, the mixture behaved like a liquid as a result of the decrease in water activity giving a low rate of viscosity (11.264 centipoises (cp), Table 2). When the nixtamalized corn flour was substituted for the cornstarch, the viscosity value was greatly increased (1,434.353 cp). A third assay with equal parts of both (50 g each), obtained a viscosity value of 49.28 cp. The viscosity measurement was taken immediately and again two hours after mixing, with variable velocity, to determine the fluid type (Newtonian or non-Newtonian). The viscosimeter is most accurate between 20 and 500 cp. Non-Newtonian fluids give unequal values at different times and velocities. It was found that using only nixtamalized corn produced a non-Newtonian pseudoplastic fluid type (Table 2). There were also assays of different amounts of vegetable oil, whose function as a

phagostimulant and nonpolar coat of microcapsules is very important. Low quantities (5-10 ml), did not give a good coat and it appeared that the material dissolved very fast. Large amounts (40-50 ml) caused clumping of the microcapsules. The most suitable quantity was 20.0 ml. The viscosity values with starch and nixtamalized matrixes and variable quantities of vegetable oil were: 5.0 ml = 4.2 cp; 10.0 = 5.5 cp; 40.0 ml = 12.0 cp; and 50.0 ml =14.2 cp. The shear rate and fluid type is also shown in Table 2.

Microencapsulation characteristics. The microencapsulated formulations presented several characteristics. There were more large particles when only nixtamalized corn was added. The other formulations were suitable for spraying. The mixture of nixtamalized corn with corn starch and corn starch alone had lower solubilities and remained in suspension longer. There were no significant differences in moisture content. We found that the moisture content was approximately 4.5% after two years (Table 4).

Adherence to Leaves. The different formulations showed variable adherence to bean leaves. All of them displayed a certain degee of agglutination, and nixtamalized corn was most likely to obstruct the spray nozzle. The small particles were retained more efficiently than the larger sizes. 65% of the microcapsules were retained up to 12 days in dry conditions, but were easily washed off by rain. Microscopically, we observed the aggregation of materials after spraying on a glass slide, but the aggregates were easily washed off.

Persistence assays. Microcapsulates were applied to bean crops naturally *E. varivestis* infested. Larvae were in different instars at the time of application. Seven

days after applying the *B. thuringiensis*-microcapsules, we found only living pupae. No larvae were present. A few pupae showing softness or blackness were collected and re-isolation of *B. thuringiensis* was reisolated. Mortality rate after artificial infestation was 65% at 8 d and 16% at 15 d after application. When the pots were exposed to rain, the mortality fell to 13% after one week (Table 3).

Microcapsulation stability and shelf life. The moisture content and toxic activity results of the recently prepared and stored formulations are shown in Table 4. The moisture content was higher in the older formulations, but the value after two years of storage (4.5%) showed a good shelf life. Although the toxic activity after two years was low, it was within the range of most *B. thuringiensis* formulations.

Discussion

The toxicity of *B. thuringiensis* against coleopteran has been studied relatively recently (Krieg, 1989) by investigators using *B. thuringiensis* serovar. *tenebrionis*. Subsequently, a few strains active against these insects have been reported, but the toxicity of each strain is variable against different coleopteran species (Herrnstadt *et al*, 1986). Reports indicate slight toxicity against the *E. varivestis* by *B. thuringiensis* (Keeler & Langenbruch, 1993). Using bioassays we confirmed these findings. In the field test, the application of this formulation reduced the number of larvae, but the natural mortality of these larvae is 60% for first instar and 35% for the second instar (Bernhardt & Shepard, 1978). This work shows the same as was mentioned by Keeler and Langenbruch (1993) that the economic control of *E. varivestis* with *B. thuringiensis* seems unlikely. In addition to the δ-endotoxin, the β-exotoxin has been reported as toxic to *E. varivestis* (Cantwell *et al*, 1985, cited by Keeler & Langenbruch, 1993), and we observed a cytotoxic effect likely be caused by β-exotoxin in mice.

Since the 1960's, encapsulation as a protection system for *B. thuringiensis* formulations has been studied (Creighton *et al*, 1961; Angus & Lüthy, 1971). Angus and Lüthy, (1971) suggested encapsulation of spores and crystals of bacilli by a method similar to that used in producing smudge-proof carbon paper. They used a matrix that was a phagostimulant and less sensitive to sunlight while toxicity was unaffected. Zehnder *et al* (1992) found that the success of *B. thuringiensis* against

coleopterans depended on using a microencapsulated formulation. An interesting observation that should be followed up is that in the bioassays, the larvae would not eat the treated parts of the leaf. Instead they fed on the untreated edge of the disks. This lack of palatability may explain the seeming nonsusceptibility of coleopteran larvae to the effects of *B. thuringiensis* toxin.

When we tested the sprayable formulation of corn starch, we found increased water solubility (Chinachoti, 1993). Low water solubility is desirable to reduce the loss of active ingredients from the microcapsules. On the other hand, when nixtamalized corn flour was used as encapsulating materials it produced good results in large granule formulations (Castro-Franco, 1994). The nixtamalization process in Mexico includes both heat and alkaline treatment after which 80-90% of the molecules are pregelatinized forming a meshlike matrix. (Collison, 1968). Employing only nixtamalized corn as an encapsulating matrix, we found the particle size was too large and unsprayable.. Smaller particles also remain in suspension longer. We think that the matrix based on corn starch and nixtamalized flour is a material suitable for spraying. This formulation gives adequate solar protection to *B. thuringiensis*, but lacks the ability to adhere to the leaves. These results were comparable to others obtained with the same formulation prepared with the HD-1 strain in artificial conditions tested against *Ostrinia nubilalis* on cotton plants using a solar lamp and simulated rain a reported in a previous paper (Tamez-Guerra *et al*, unpublished).

Although we found no difference in bean yield between the control and treated plots, we feel it was because of a later attack by *Esigmene acrea* (Lepidoptera:

Arctiidae) on both treated and control plots in the first year. The second year we found that *E. varivestis* did not infest either the control plots or the treated plots in high numbers.

Our studies show that the toxic activity is conserved for two years in storage in conditions below 28°C. Although higher than the freshly prepared material, the moisture content of the stored formulation (4.5%) showed it has a good shelf life. With this formulation we have the possibility of using *B. thuringiensis* strains for *E. varivestis* pest control on beans. Moreover, we show that preparation of these materials is economical and accessible to the manufacturer and may be applied by spraying. It may be possible to use this methodology for bioinsecticide production, but to use this process successfully, we need to look for another adherent material suitable for use in the spray dry technique and that mantains the shelf life of the product.

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Table 1.Percent mortality of *Epilachna varivestis* and *Trichoplusia ni* larvae using leaves and artificial diet respectively treated with strain C-9.

Epilachna varivestis[1]		*Trichoplusia ni*[2]	
Dose (µg/50ml)	Percentage mortality	Dose (µg/ml)	Percentage mortality
25	0/15	5	3/75
50	1/15	10	17/75
100	4/15	13	17/75
250	7/15	15	22/75
500	10/15	25	31/75
1000	14/15	50	38/75
Control	0/15	100	42/75
		Control	1/75

1. Bioassays on bean leaves, LD_{50} = 397µg/ml. Slope 95% 264 - 2.3 x 10^5
2. Bioassays on artificial diet, LD_{50} = 219.35 µg/ml. Slope 95% 156-668.
Probit stadistic method (SAS, 1989).

Table 2. Rheological properties of encapsulating agents.[1]

	Corn flour	Corn Starch	Both (50-50)
Viscocity (cp)[2]	1434.179	11.264	49.28
Reading	43.0	3.2	8.0
rpm[3]	2	20	20
correction factor	33.353	3,52	6.25
Fluid type	non-Newtonian[4]	Newtonian	non-Newtonian
Share rate	high	low	medium

1. With 20 ml of vegetable oil
2. Centipoises
3. Revolutions per minute
4. Pseudoplastic

Table 3. Toxic activity persistency of a sprayable *B. thuringiensis* formulation in bean field infested both naturally and artificially with Mexican bean beetle.[1]

Number of larvae naturally infesting			Number of larvae artificially infested[2]		
	Initial	After 8 days	Initial	After 8 days	After 15 days
Without rain	80	>10 (88%)	60	21 (65%)	51 (16%)
With rain	80	72 (14%)	60	52 (13%)	N. D.

1. The number in the parenthesis indicates the mortality percent. Average of three replications.
2. Artificial infestation was done each week after the first application.

Table 4. Moisture content and toxic activity vs *E. varivestis* and *T.* ni, of microcapsulated C-9 *B. thuringiensis* strain

	Immediately	Six month later	Two years later
Moisture content	1.2	2.8	4.5
Insect	Percent mortality (500 µg/ml)		
E. varivestis[1]	64	48	52
T. ni[2]	88	72	66

1. Bioassays on bean leaves, average of five replications, three larvae per replication
2. Bioassays on artificial diet, average of three replications, 25 larvae per replication