Research Article

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Increased efficacy and extended shelf life of spinosad formulated in phagostimulant granules against *Spodoptera frugiperda*

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Abstract

BACKGROUND: Spinosad is recommended for control of *Spodoptera frugiperda* (J.E. Smith) larvae; its application with phagostimulants may reduce the quantity of active ingredient required for effective pest control. Spinosad (Tracer®) was formulated in maize flour matrix granules and three field tests compared 10–100 ppm a.i. granules (equivalent to 0.24–2.4 g a.i. ha^{-1}) with Tracer as an aqueous spray (200 ppm a.i.; 60 g a.i. ha^{-1}), and the recommended application rates of *Bacillus thuringiensis*, a chemical and an untreated controls were performed.

RESULTS: The 100 ppm spinosad granules resulted in similar *S. frugiperda* mortality compared with the chemical treatments in all three field trials, and resulted in a significantly higher maize grain yield compared with unformulated and control treatments (4141 vs. 2857 and 2407 kg ha^{-1}, respectively) that was similar to the chemical treatment (3778 kg ha^{-1}). Bioassays of granules stored at room and cold temperatures showed that after 5 years, ∼ 70% of the original activity remained (OAR) of spinosad when formulated as granules. Nevertheless, after 9 years, efficacy was reduced (26.2% and 48.5% OAR) at both room (25 °C) and refrigerated temperatures (4 °C).

CONCLUSION: Spinosad, in the granular phagostimulant formulations evaluated in this study, had advantages measured as high efficacy and long shelf life.

Supporting information may be found in the online version of this article.

Keywords: maize field trials; spinosad efficacy at lower rate; shelf-life; *Spodoptera exigua*; *Trichoplusia ni*

1 INTRODUCTION

In the search for safe and effective insect pest control products, biological insecticides and biorational compound products have become increasingly adopted in agricultural production over the past 20 years. An example is spinosad, a combination of metabolites (spinosyn A and D) produced by the soil actinomycete *Saccharopolyspora spinosa* Mertz & Yao during liquid fermentation.[1] Spinosad is highly effective against lepidopteran and dipteran pests, among others, and has proved to have a very favorable ecotoxicological profile. As such, it has been widely adopted (∼ 250 countries) in integrated pest management (IPM) programs worldwide.[2,3] In fact, spinosad-based products have been registered in more than 82 countries for the control of a broad range of foliar-feeding insect pests (http://msdssearch.dow.com/PublishedLiteratureDOWCOM/dh_091c/0901b8038091c93b.pdf?filepath=productsafety/pdfs/noreg/233-00381.pdf&fromPage=GetDoc). Spinosad is active by ingestion, and shows lower activity by contact.[4] The degradation of spinosad in the environment occurs primarily by photodegradation; the half-life by photolysis in soil is <10 days and ∼1–2 days in water.[5,6]

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) is distributed from the southern regions of the USA to Argentina. This pest was also recently introduced to West Africa, and is currently spreading rapidly across the continent.[7] *Spodoptera frugiperda* displays a wide host range that includes >80 plant species recorded, but prefers maize, sorghum, rice, Bermuda grass and other grasses. When larval populations are high, they can severely defoliate their preferred plants resulting in major reductions in yield.[8,9] In developing countries, the pest is usually controlled by application of organophosphate insecticides[10] or by planting Bt transgenic maize in countries where the use of transgenic plants is permitted.[11] Other plant

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metabolites and entomopathogens are under evaluation[10] (http://www.irac-online.org/documents/brazil-irm-recommendations-soybean-cotton-and-corn/?ext=pdf).

Granular phagostimulant formulations of nucleopolyhedrovirus, *Bacillus thuringiensis* and spinosad, using nixtamalized maize flour as matrix, have been tested for control of *S. frugiperda*, resulting in increased levels of pest control at lower doses than required for spray applications.[12,13] This was because the phagostimulant properties of the formulation increases feeding by the pest and protects the active ingredient (a.i.) from degradation by environmental factors, especially ultraviolet radiation.[14] In fact, by using ultra-low rates (≥ 10-fold lower than the recommended rate) of spinosad in granular formulations (i.e., testing 0.24, 0.8 and 2.4 g a.i. ha^{-1} vs the recommended dose of 60.0 g a.i. ha^{-1}), it was possible to control *S. frugiperda* in maize in southern Mexico as effectively as an organophosphate insecticide spray.[15]

The stability of a formulated product in storage and after application is an important consideration for commercialization. Products based on natural molecules tend to be less stable than synthetic compounds[16] and thus, shelf life and field stability are important issues to consider. In the present study, we examined the influence of granular formulation of spinosad with phagostimulant materials used as a granular matrix, on the efficacy of low rates of spinosad and the effect of formulation on the stability of spinosad during storage. We assessed the insecticidal activity of spinosad granular formulation in three field trials against natural infestations of *S. frugiperda* larvae on maize, and evaluated its insecticidal efficiency after storage for up to nine years at both ambient and refrigerated temperatures.

2 MATERIALS AND METHODS

2.1 Insect source

Insects were obtained from a laboratory colony of *S. frugiperda* that was started in 2000 using healthy larvae collected from maize planted in Linares, Nuevo Leon, northeastern Mexico. Larvae were reared individually on wheatgerm-based artificial diet at 25 ± 2 °C, 55 ± 5% RH, and 14: 10 h light/dark photoperiod.[17]

2.2 Bioassays

Bioassays were performed using insects from the laboratory colony. Both granular and commercial spinosad toxicity toward *Spodoptera exigua* (Hübner), *S. frugiperda* and *Trichoplusia ni* (Hübner) were evaluated because these species are common pests of maize crops. The insecticidal activity of spinosad (Tracer 480SC, Dow Agrosciences LLC, Indianapolis, IN, USA) against *S. frugiperda* was determined using a droplet feeding and a diet overlay bioassays.[18] For both assays, the highest concentration (100.0 ng a.i. ml^{-1}) after mixing with 2% w/v sucrose and 0.4% w/v of food coloring (ASIS, Monterrey, Mexico) was used to prepare five additional concentrations, 33.33, 11.11, 3.70, 1.23, and 0.41 μg a.i. ml^{-1} (equivalent to 33.33–0.41 ppm a.i. spinosad) in sucrose and dye solution. Neonates (< 24 h old) were placed in the center of a plastic Petri dish containing 1 μl droplets of each solution and allowed to drink the droplets. Groups of 45 larvae that consumed the droplets within 10 min were individually transferred to plastic cups containing diet and reared at 25 ± 2 °C, 55% ± 5% RH for 5 days, after which the number of dead larvae was scored. Groups of control larvae consumed sucrose and dye solution without spinosad.

For the diet overlay bioassay, a 35 μl volume of 3.33, 1.11, 0.37, 0.12, and 0.041 μg a.i. ml^{-1} was applied to the surface (3.3 cm^2) of artificial diet in a cup. Each concentration was applied to 24 cups and allowed to dry for 2 h. Two neonates were then placed in each cup and reared as described for the droplet bioassay larvae. Following 5 days of incubation, dead larvae were quantified.

Both types of bioassays were performed three times and the results were subjected to Probit analysis using the Polo program.[19] For comparison, identical bioassays were performed using neonate *T. ni* and *S. exigua*, testing concentrations of 11.11, 3.70, 1.23, 0.41, and 0.137 μg a.i. ml^{-1} for the droplet bioassay and 1.11, 0.37, 0.12, 0.041 and 0.014 μg a.i. ml^{-1} for the overlay bioassay. These concentrations were used as they provided a suitable range of mortality responses (~10–90%) in preliminary assays (data not shown).

2.3 Spinosad granular formulations

Experimental granular formulations were prepared at three concentrations, 10, 30, and 100 mg a.i. kg^{-1} spinosad (equivalent to 10–100 ppm), using Tracer 480SC. Granules were prepared by mixing 750 g nixtamalized maize flour (Mazeca®, Guadalupe, Nuevo León, Mexico), 250 g cornstarch (Maicena®, Unilever de México, Tultitlán, Edo. de México, Mexico), 5 g maize oil (Maceite®, Promotora de Productos y Mercados Mexicanos, Guadalajara, Mexico), 1000 ml of warm distilled water (55 ± 5 °C), and the selected amount of spinosad. The ingredients were mixed thoroughly to produce soft dough, which was allowed to stand for 30 min before being passed through a #20 sieve with 0.8 mm pore-size. Using this technique, irregular granules ~1 mm wide and 0.5–3 mm long were produced. The granules were scattered on waxed paper and allowed to air dry for 14 h at 25 ± 1 °C prior to use. Control granules without spinosad were also prepared.

2.4 Field tests on maize

Three field trials were performed to determine the efficacy of spinosad granule and spray treatments with chemical or Bt-based insecticide treatments for control of *S. frugiperda* in maize.

The first trial was performed in late July to early August 2003 in the experimental field site of the Universidad Autónoma de Nuevo Leon, Linares, Nuevo León, Mexico. Maize plants (var. Blanco Purísima, Semillas y Alimentos Santa Martha, San Nicolás de los Garza, Mexico) were planted at a density of ~ 20 000 plants ha^{-1} and were at the whorl stage (50–60 cm tall) at the start of the trial. During the experimental period the weather was hot (daily range 25–38 °C), no rainfall was recorded, and irrigation was not applied.

Experimental plots of 25 m^2 were surrounded by an untreated area of the same size. One week after *S. frugiperda* adults were detected in pheromone traps placed at the experimental site, one of each of the following treatments was applied to each plot: (1) control granules without spinosad; (2) 10 ppm spinosad granules; (3) 30 ppm spinosad granules; (4) 100 ppm spinosad granules; (5) spinosad (Tracer 480SC) spray application at 200 ppm, equivalent to 60.0 g a.i. ha^{-1}; (6) DiPel® 10G water dispersible granules (*Bacillus thuringiensis* subsp. *kustaki*) (Sumitomo Chemical Co., Valent de Mexico, Guadalajara, Jalisco, Mexico) at the recommended rate of 103 g a.i. ha; (7) cypermethrin 200 EC at the recommended rate of 250 ml ha^{-1}; and (8) untreated control. Spinosad granules were applied at a rate of 25 g per plot, equivalent to 10 kg ha^{-1}, resulting in applications of 0.24, 0.80 and 2.4 g a.i. ha^{-1} for the 10, 30 and 100 ppm treatments, respectively. Granule treatments were applied directly into the leaf whorl using a paper cup with a hole in the base. Spray applications of spinosad and cypermethrin were applied to maize plants using a hand-held, manual sprayer with

three hollow cone nozzles directed at the whorl, at 2.8 kg cm^{-2} spray pressure. The spray system was under continuous agitation during applications and was flushed with water following each treatment application. Each treatment was applied to five replicate plots arranged in a fully randomized plot design, over an area of 85×55 m^2 (4675 m^2 in total). The application volume was 0.8 L to each 20 m^2 area, equivalent to 400 L ha^{-1}.

Ten randomly selected maize plants from each plot were cut at the first node above the soil and collected at 1 h post application, and at 2, 5, 10 and 15 days after application. Plants were taken to the laboratory and dissected to find *S. frugiperda* larvae, which were individually reared on artificial diet at 25 ± 1 °C. The numbers of larvae that died from intoxication or due to the emergence of parasitoids were recorded at daily intervals until pupation.

The second field trial involved the same experimental design and treatments as the first, but was performed in mid-August 2004, at the Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM) experimental field site, Apodaca, Nuevo Leon, Mexico. Maize plants (var. NL V52, ITESM, Monterrey, Mexico) were planted at a density of ~ 35 000 plants ha^{-1} and were in the whorl stage (50–60 cm tall) at the start of the trial. One week after *S. frugiperda* adults were detected by pheromone lure, the experiment described in the first trial was repeated, but using irrigated maize that had not been treated previously with insecticides. Samples of 20 plants per plot were collected at random at 1 h, and 9 and 15 days post application. Larvae of *S. frugiperda* were reared and mortality was recorded. During the experimental period, the daily temperature range was 22–36 °C, heavy rainfall (131 mm m^{-2}) occurred on the second day post application and irrigation was not applied. Each treatment was applied to five replicate plots arranged in a randomized block design.

The third field trial was performed in August 2013 at Cueramaro, Guanajuato, Mexico, in a field of maize (var. Puma, Asgrow®), at the whorl stage (80–90 cm tall) at the start of the trial, planted at a density of 80 000 plants ha^{-1}. The experiment was laid out as a fully randomized plot design with 20 m^2 plots (2 m gap between plots) over an area of 700 m^2. To reduce the cost of granule applications, 1 kg batches of spinosad granules comprising concentrations of 400, 800 and 1600 ppm were each mixed with 7 kg of sand to give application rates of 50, 100 and 200 ppm (1.2, 2.4 and 4.8 g a.i. ha^{-1}), respectively. The following seven treatments were applied to experimental plots: (1) untreated control; (2) Palgus (Spinetoram, Dow Agrosciences LLC) at the recommended rate of 75 ml ha^{-1} in 400 L of water and 1 ml L^{-1} of wetting agent (Inex®, Cosmocel S.A., San Nicolas de los Garza, Mexico); (3) 400 ppm spinosad granules with sand; (4) 800 ppm spinosad granules with sand; (5) 1600 ppm spinosad granules with sand; (6) 250 ppm spinosad (Tracer 480SC) spray application equivalent to 65.0 g a.i. ha^{-1}; (7) spray application of Xentari® DF (*B. thuringiensis* subsp. *aizawai*) (Sumitomo Chemical Co.) at the recommended dose of 1.0 kg ha^{-1}, equivalent to 103.0 g a.i. ha^{-1}. Each treatment was applied to five replicate plots. Granules were applied directly to the whorl at a rate of 8 kg ha^{-1}, whereas spray applications were made using a manual sprayer fitter with a cone nozzle number 2 (Teejet Technologies, Spraying Systems Co., Agroservicios Nieto, S.A. de C.V., Celaya, Guanajuato, Mexico), directed at the top of the plants, at a pressure of ~ 2.1 kg cm^{-2}. All treatments were applied once between 16.00 and 18.00 h. At 1 h, and 4 and 9 days post application three randomly selected plants per plot were dissected and living and dead larvae counted.

2.5 Crop yield

To determine the influence of crop protection treatments on yield, the second trial was repeated with four replicate plots per treatment but without destructive sampling. The treatment of 10 ppm spinosad granules was not tested. At 162 days post planting, maize ears were harvested from all the remaining plants in each plot (72 m^2) and maize grain was removed from the cob using a manual grain collection device (Desgranadora de Maíz Torotrac, Campotencia S. *A. panama* City, Panama). Moisture content was calculated based on weight loss after drying in an oven at 54 °C for 72 h and subtracted from the final grain yield weight.

2.6 Shelf-life determination

The activity of the spinosad granular formulation following storage was determined by diet overlay bioassay. After drying for ~ 24 h at room temperature, granules from each spinosad-concentration treatment were divided into two subsamples that were placed in 500 ml plastic containers; one of the samples was stored in the dark in the laboratory at 25 ± 2 °C and the other was stored at 4 ± 1 °C in a laboratory refrigerator. The insecticidal activity of both subsamples was tested using a single dose bioassay for each formulation compared with 25 μg a.i. ml^{-1} spinosad (Tracer 480SC) stored at 4 ± 1 °C. To achieve this, 1 g of each granular sample (10, 30 and 100 μg a.i. g^{-1} spinosad) or spinosad solution (25 μg a.i. ml^{-1} spinosad) was mixed with 24 ml of 2% w/v sucrose and 0.4% w/v blue food color solution for 30 s. A volume of 35 μl was applied to the diet surface of each cup (~ 7 ml of artificial diet, 3.3 cm^2 surface area) in 25 ml plastic cups to give concentrations of 0.42, 1.27 and 4.2 μg a.i. cm^{-2}. The spinosad solution was expected to result in ~ 85% mortality, based on previous bioassay results.

Two *S. frugiperda* neonates were placed in each cup (12 cups per treatment) and incubated for 7 days under the conditions mentioned above. After this period, mortality was determined. Each cup received two larvae because cannibalism was observed among *S. frugiperda* neonates incubated at higher densities. Bioassays were performed at intervals of months during the first year, then once a year until insecticidal activity of the spinosad granules stored at room temperature resulted in <30% mortality against *S. frugiperda* neonates. For comparison purposes, the entire experiment was repeated using *T. ni* neonates. Original insecticidal activity remaining (OAR)[20] was calculated by dividing the mean percentage mortality of each stored sample per treatment by the mean percentage mortality from the suspension concentrate formulation of spinosad (Tracer) stored at 5 ± 2 °C.

2.7 Statistical analysis

To analyze the efficacy of *S. frugiperda* control by spinosad granules and other insecticides, the results of the first field trial were analyzed by fitting generalized linear models in GLIM (Generalized Linear Interactive Modeling, Numerical Algorithms Group 1993) with a binomial error structure, where the means of binomial data have asymmetrical standard errors. Where necessary, scaling of the error distribution was performed to account for minor over-dispersion. Results of scaled analyses were provided as *F* statistics. The accuracy of models was determined by examination of the distribution of observed and fitted values and residuals.[21]

The second field trial mortality results and maize grain yields were analyzed by ANOVA and means were separated using Tukey HSD.[22] Because of the high number of treatments, mortality due

Table 1. LC_{50} values for neonates of three Lepidoptera species exposed to spinosad (Tracer 480SC) in droplet and diet overlay bioassays[a]

	Droplet bioassay (μg ml^{-1})				Diet overlay bioassay (μg cm^{-2})			
Insect	LC_{50}	95% CI	χ^2	Slope ± SE	LC_{50}	95% CI	χ^2	Slope ± SE
Spodoptera exigua	2.56	1.96–3.73	3.7	1.54 ± 0.42	0.16	0.14–0.18	4.2	1.98 ± 0.29
S. frugiperda	11.10	7.03–36.33	3.7	1.95 ± 0.25	1.54	1.16–1.97	4.1	2.36 ± 0.38
Trichoplusia ni	4.46	2.52–11.10	3.9	1.60 ± 0.44	0.93	0.70–1.25	3.6	2.32 ± 0.15

[a] Groups of 45 or 48 neonates were treated with one of five concentrations and a control in a droplet bioassay and diet overlay bioassay, respectively. Concentration–mortality probit regression and estimation of LC_{50} values were performed using the POLO-plus program (LeOra, 2007).

to parasitoids or pupation, and shelf life bioassays were analyzed using the multivariate analysis procedure known as Multidimensional Scaling (MDS), where the distribution and relationship of data is represented on dimensional plots to facilitate the interpretation. Mortality percentages were normalized by arcsine transformation and dissimilarity matrixes were determined by Euclidean distance correlation for simultaneous comparison of all treatments.[23] To graph the distribution of these dissimilarities, two-dimensional MDS plots were generated using the Ordinal (1) model with 500 iterations with 0.00001 convergence were performed using XLSTAT 2016. High degrees of confidence were determined when the Kruskal's stress values were <0.05.[23] Data values grouped in clusters on MDS graphs were considered similar.

3 RESULTS

Spinosad efficacy was determined as the prevalence of larval mortality in naturally infested plants at intervals up to 15 days post application. In one trial, the prevalence of parasitism of *S. frugiperda* larvae was monitored, whereas in another the yield of grain was recorded, and in the last trial granular formulations were tested at 50, 100 and 200 ppm (1.2, 2.4 and 4.8 g a.i. ha^{-1}) on maize using 1/10 of maize flour matrix mixed with sand, to achieve a less expensive formulation.

3.1 Bioassays of spinosad activity

The insecticidal activity of spinosad (Tracer 480SC) against *S. exigua*, *S. frugiperda* and *T. ni* neonates was compared by the droplet and diet surface overlay bioassays (Table 1). Larvae were between 5- and 16-fold more sensitive to spinosad in the diet overlay compared with the droplet feeding bioassay, depending on species. Of the species tested, *S. exigua* was the most susceptible to spinosad, *S. frugiperda* the least susceptible and *T. ni* was of intermediate susceptibility in both types of bioassay (Table 1). Control mortality was <5%.

3.2 First field test

Samples obtained at time point zero (1 h post application) showed that maize plants were naturally infested with *S. frugiperda* with an average of 15–60 larvae per plot (Fig. 1). The levels of infestation of experimental plots differed significantly among treatments at the first time point. The highest populations of *S. frugiperda* were present in plots treated with granulated spinosad at 30 and 100 ppm ($F_{4,20=}$ 21.99, $P < 0.001$). However, larval mortality did not differ significantly at time point zero, with 2–18% of natural mortality among treatments ($F_{7,32} = 1.445$, $P = 0.222$) (Fig. 1B).

Mortality increased to 62–90% for the spinosad granular treated plots, which was similar to the mortality for the Tracer, Dipel and cypermethrin treatments (range 55–85%), and significantly higher than mortality observed in the control and granule treatment without spinosad ($F_{7,32} = 10.312$, $P < 0.001$). Larval mortality remained significantly higher in the spinosad granule, Tracer, Dipel and cypermethrin treatments, in the samples taken at 5 days ($F_{7,32} = 14.703$, $P < 0.001$) and 10 days ($F_{7,32} = 12.026$, $P < 0.001$) post application, in comparison with the control and granule treatment without spinosad (Fig. 1B). The highest prevalence of insect mortality was observed in the 100 ppm spinosad granule treatment (78–92%) and the cypermethrin treatment (63–83%), in samples taken at 2–10 days post application. The numbers of larvae recovered from plots was reduced for those treated with spinosad granules, Tracer, Dipel and cypermethrin, compared with the control and granules without spinosad when sampled 2–10 days post application (Fig. 1A). Post-application, the 100 ppm spinosad granule treatment was the only treatment in which larval mortality was significantly higher than observed in the control or granules without spinosad treatments ($F_{7,32} = 12.026$, $P = 0.004$).

Laboratory rearing of field collected larvae was used to identify the prevalence of larval mortality, natural parasitism and pupation of survivors (Fig. 2). Data were initially analyzed by using ANOVA and significant differences were observed. However, due to the number of treatments and variables compared, MDS graphs were presented in Fig. 2. Because plants in treated plots were naturally infested by *S. frugiperda* larvae, a dissimilarity matrix was first obtained by using Euclidean distance obtained to analyze the data distribution among the eight different treatments.[20] Percentages were calculated based on the total number of surviving larvae and normalization was achieved by arcsine transformation due to the binomial nature of these data. Dissimilarity matrices were plotted using MDS settings as mentioned in the Materials and Methods. In these graphs, the degree of confidence of the data being grouped into specific clusters was determined by the Kruskal's stress value of <0.05. Each cluster is considered different from any other cluster being distant within the 2D space of the graph. Low larval mortality was observed related to a separate cluster in which control and GC, SG10, SG30, D10G and chemical insecticide treatments were grouped. Furthermore, treatments SG100 and S200 were grouped in a separate cluster in which the observed larval mortality was higher (Fig. 2A) (Kruskal's stress = 0.001), in response to the high concentrations of spinosad. Regarding the effects on pupation, clear grouping in specific clusters was not observed, indicating a similar prevalence among all treatments except low pupation in the 100 ppm spinosad granule treatment. In general, high pupation was observed among all treatments (Fig. 2B) (Kruskal's stress = 0.003), particularly in larvae collected at 2 days post treatment. Mortality due to parasitoids was also analyzed (Fig. 2C) (Kruskal's stress = 0.004). Low mortality due to parasitoids was

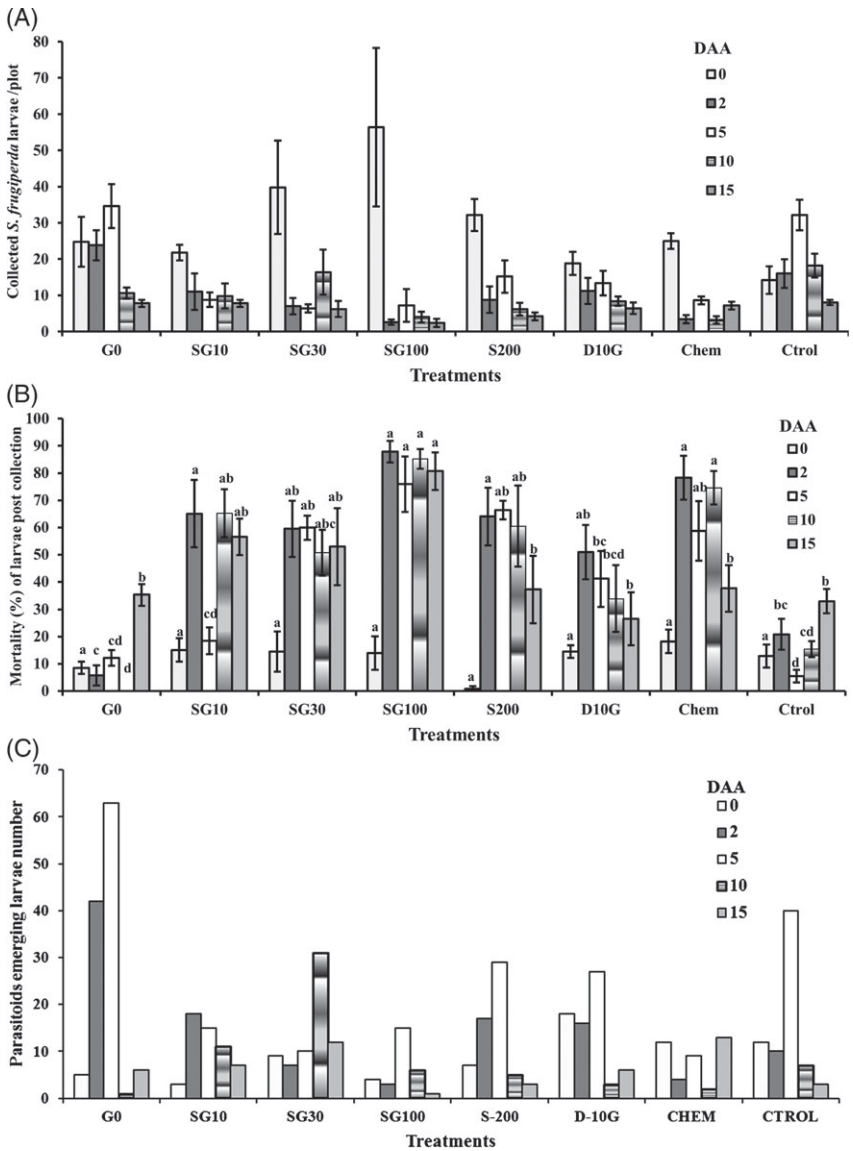

Figure 1. (A) Total number of collected *Spodoptera frugiperda* larvae per plot (20-plant sample; five replicates), recorded in the first field trial performed in Linares Nuevo Leon, Mexico. Statistical results of collected *S. frugiperda* larvae are shown in Fig. S1. (B) Larval mortality percentage per plot post collection during rearing on artificial diet in laboratory. (C) Parasitoids emerging from collected larvae per plot, after rearing on artificial diet in laboratory. Bars represent SEM. Columns headed by identical letters are not significantly different for comparisons among treatments within each time point (Tukey HDS, $P < 0.05$). G0 = maize flour granules with no spinosad; SG10, SG30 and SG100 = granulated spinosad at 10, 30 and 100 ppm, equivalent to 0.24, 0.8 and 2.4 g a.i. ha^{-1}, respectively; S200 = unformulated spinosad in aqueous solution at 200 ppm, equivalent to 60.0 g a.i. ha^{-1}; D10G = Dipel 10G at 3.42 kg ha^{-1}; Chem = cypermethrin at 250 ml ha^{-1}; Ctrol = untreated control; DAA = days after application.

observed in all treatments being highest in the 10 ppm spinosad granules, chemical and Dipel treatments. Obvious clustering was not observed based on treatment or collection time, thus treatments did not affect mortality due to parasitoids. High mortality was observed in the granulated spinosad at 100 ppm treatment, 200 ppm unformulated spinosad, chemical and Dipel treatments, which appeared as a separate cluster in Fig. 2A (Kruskal's stress = 0.001). Interestingly, similar mortality rates were observed at the different collection times. Low prevalence of pupation was observed in the 10, 100 and 200 ppm spinosad granules and Tracer (spray) treatments (Fig. 2B) (Kruskal's stress = 0.003) particularly in larvae collected at 2 days post treatment. Pupation increased in larvae collected at 5, 10 and 15 days after treatment, independent of the treatment. Mortality due to parasitoids was also analyzed (Fig. 2C) (Kruskal's stress = 0.004). A low prevalence of parasitoid mortality was observed in all treatments which was highest in the 10 ppm spinosad granules, chemical and Dipel treatments. Obvious clustering was not observed based on treatment or collection time.

3.3 Second field test

No significant differences in percentages of mortality among *S. frugiperda* larvae collected at time point zero (1 h post application) were observed (ANOVA: $F_{7,32} = 2.132$, $P = 0.068$) (Fig. 3A). Data analysis at 9 days post application, showed significant differences in *S. frugiperda* larval mortality ($F_{7,32} = 16.558$, $P < 0.001$). The 100 ppm spinosad granule and cypermethrin treatments resulted in significantly higher *S. frugiperda* larval mortality (64.5 and 51.7%, respectively), compared with other treatments, whereas

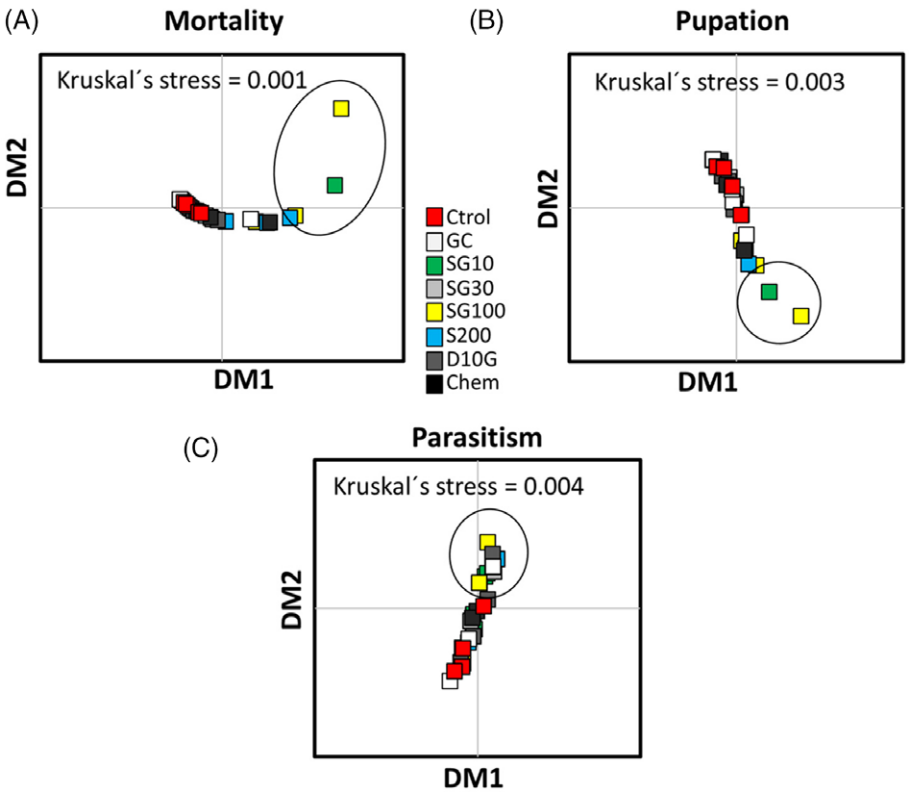

Figure 2. MDS analysis using averages from four replicate plots of prevalence of mortality, pupation and parasitism of *Spodoptera frugiperda* surviving larvae collected from 20 randomly selected maize plants per plot. Percentages of (A) mortality, (B) pupation and (C) parasitism. GC = maize flour granules without spinosad; SG10, SG30 and SG100 = spinosad granules at 10, 30 and 100 ppm, equivalent to 0.24, 0.8 and 2.4 g a.i. ha^{-1}, respectively; S200 = unformulated spinosad in aqueous solution at 200 ppm, equivalent to 60.0 g a.i. ha^{-1}; D10G = Dipel 10G at 3.42 kg ha^{-1}; Chem = cypermethrin at 250 ml ha^{-1}; Ctrol = untreated control.

the granular control (12.9%) showed significantly lower mortality compared with all other treatments (Fig. 3B). Sampling at 15 days post application also revealed significant differences among treatments ($F_{7,32} = 5.981$, $P < 0.001$). At 15 days post application, the 100 ppm spinosad granule treatment resulted in the highest larval mortality (50%) compared with the lowest mortality in the 10 and 30 ppm spinosad granule treatments, cypermethrin, untreated control and granular control (range 8–22%). The remaining treatments had an intermediate prevalence of mortality (Fig. 3B).

As for grain yield estimates, plots treated with 30 ppm spinosad granules or the chemical insecticide had significantly higher grain yield ($F_{6,21} = 22.612$, $P < 0.001$). Tukey analysis ($\alpha = 0.05$) identified two groups in which 100 ppm spinosad granules and chemical treatment had the highest yields, with 4171 and 3778 kg ha^{-1}, respectively. All remaining treatments had lower yields that were not significantly different from each other (spinosad granules at 10 or 100 ppm and the spray treatment of Tracer at 200 ppm, with 2883, 2793 and 2857 kg ha^{-1}, and Dipel and the untreated control with 2363 and 2407 kg ha^{-1}, respectively (Fig. 3C).

3.4 Third field test

In the 2013 trial, larvae were collected at 0, 4 and 9 days after application. Before application, natural *S. frugiperda* mortality was not significantly different among treatments ($F_{6,28} = 1.448$, $P = 0.232$) (Fig. 4A). At 4 days post application, larval mortality differed significantly among insects collected from different treatments ($F_{6,28} = 15.594$, $P < 0.001$). The highest mortality was observed for larvae collected from Tracer (spray) and Palgus treated plots (97% and 92%, respectively) By contrast, 49–79% mortality was observed for larvae collected from plots treated with mixtures of spinosad granules with sand, to give application rates of 50, 100 and 200 ppm (1.2, 2.4 and 4.8 g a.i. ha^{-1}) that resulted in 49–79% mortality. The 100 ppm spinosad granule treatment resulted in very high larval mortality (100%), as did the Tracer spray and Palgus treatments at 9 days post application, which was significantly higher than observed in other treatments ($F_{6,28} = 3.479$, $P = 0.011$) (Fig. 4B). Unexpectedly, 200 ppm spinosad granules were less effective because average mortality was 65%, similar to that of 50 ppm spinosad granules and Xentari (83% and 84%, respectively). Natural mortality in the untreated control was lower than all other treatments at 4 and 9 days after application, except the 200 ppm spinosad granule treatment sampled at 9 days (Fig. 4B).

3.5 Shelf life

To determine whether the shelf life of spinosad was improved in maize flour-based matrix granular formulation, the insecticidal activity of granules at the four concentrations were compared after storage at room (25 ± 2 °C) and cold (4 ± 1 °C) temperatures.

Mortality of *S. frugiperda* neonates was measured in bioassays using granules with 10, 30, and 100 ppm spinosad. For putative correlations of stored granule activity over time among treatments, multidimensional analysis was applied (Fig. 5) (Kruskal's stress = 0.002). Granules stored for 3 months showed positive correlation of concentrations and mortality as expected, independent of storage temperature. Granules stored at 25 °C resulted in 40%, 55% and 80% OAR, and granules stored at 4 °C had 67%, 73% and

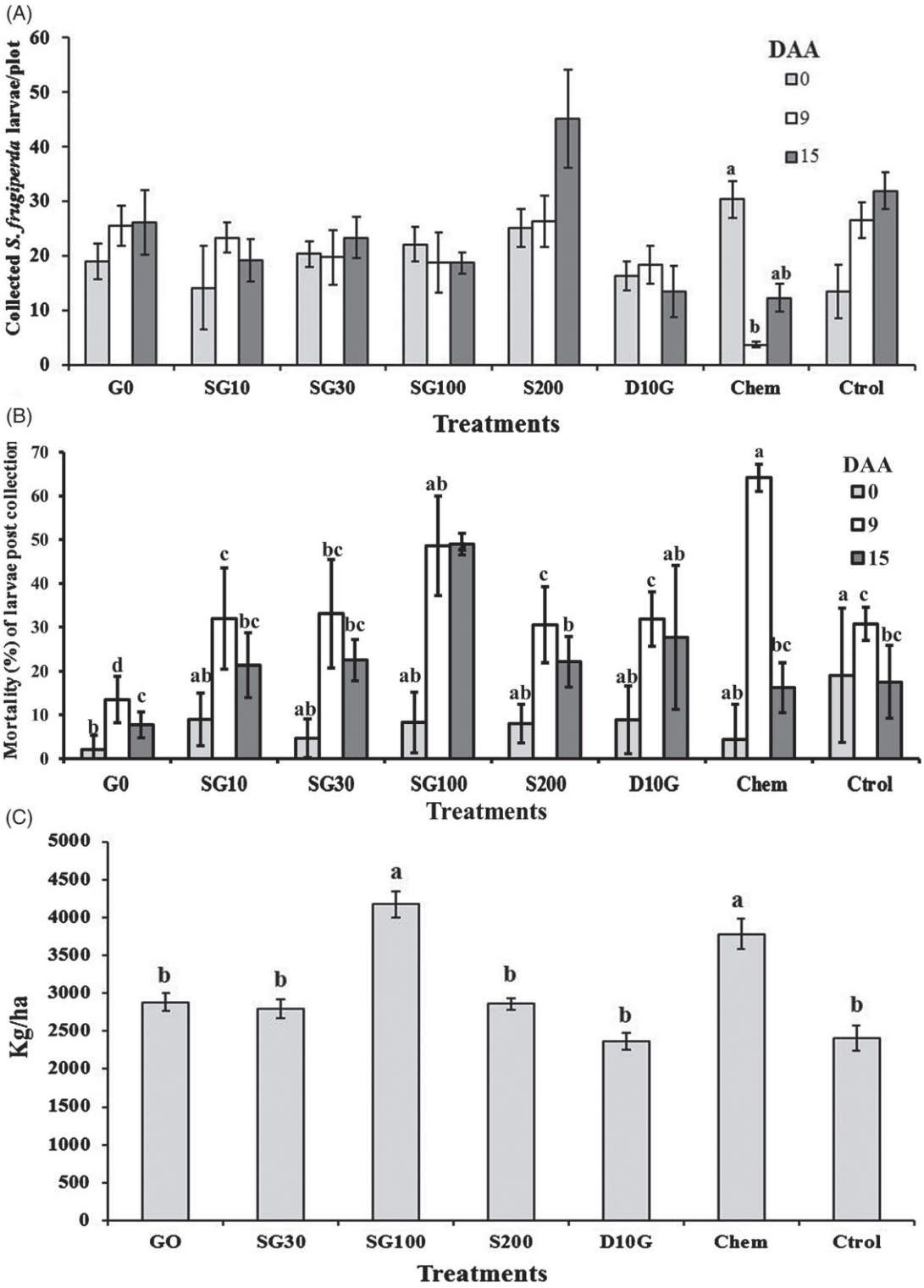

Figure 3. (A) Total number of collected *Spodoptera frugiperda* larvae per plot (20-plant sample; five replicates), recorded in the first field trial performed in Apodaca Nuevo Leon, Mexico. Bars represent SEM. Columns headed by different letters within time points in the same treatment are significantly different. (B) Larval mortality percentage per plot post collection during rearing on artificial diet in laboratory. Bars represent SEM. Columns headed by identical letters are not significantly different for comparisons among treatments within each time point (Tukey HDS, $\alpha = 0.05$). G0 = maize flour granules with no spinosad; SG10, SG30 and SG100 = granulated spinosad at 10, 30 and 100 ppm, equivalent to 0.24, 0.8 and 2.4 g a.i. ha^{-1}, respectively; S200 = unformulated spinosad in aqueous solution at 200 ppm, equivalent to 60.0 g a.i. ha^{-1}; D10G = Dipel 10G at 3.42 kg ha^{-1}; Chem = Cypermethrin at 250 ml ha^{-1}; Ctrol = untreated control. (C) Grain yield (kg ha^{-1}). Average of four blocks used as replications. Bars represent SEM. Different letters among treatments indicates significantly differences calculated by Tukey HSD ($\alpha = 0.05$) (SPSS, 2008). G0 = maize flour granules with no spinosad; SG30 and SG100 = granulated spinosad at 30 and 100 ppm, equivalent to 0.8 and 2.4 g a.i. ha^{-1}, respectively; S200 = spinosad in aqueous solution at 200 ppm, equivalent to 60.0 g a.i. ha^{-1}; D10G = Dipel 10G at 3.42 kg ha^{-1}; Chem = Cypermethrin at 250 ml ha^{-1}; Ctrol = untreated control; DAA = days after application.

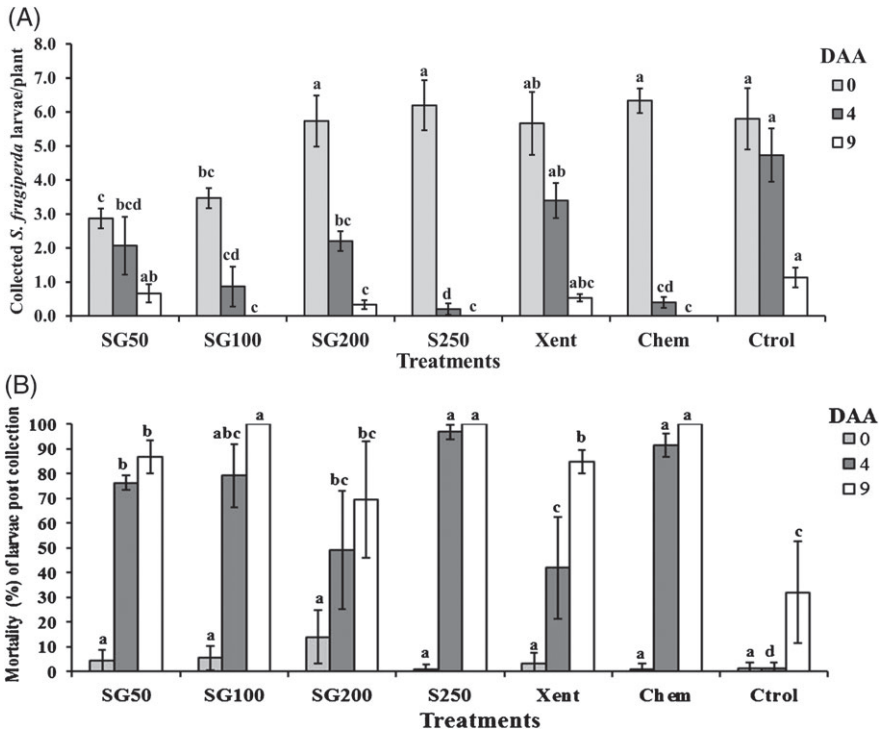

Figure 4. (A) Total number of *Spodoptera frugiperda* larvae collected from three-plant samples from five replicates, and (B) percentage of larval mortality post collection during rearing on artificial diet in the laboratory, recorded in the third field trial performed in El Salcillo, Cueramaro, Guanajuato, Mexico. Bars represent SEM. Columns headed by different letters in the same treatment are significantly different. (B) Percentage of larval mortality per plot post collection during rearing on artificial diet in the laboratory. Bars represent SEM. Columns headed by identical letters are not significantly different for comparisons among treatments within each time point (Tukey HDS, $P < 0.05$). G0 = maize flour granules without spinosad; SG10, SG30 and SG100 = granulated spinosad at 50, 100 and 200 ppm (equivalent to 1.2, 2.4 and 4.8 g a.i. ha^{-1}), respectively; S200 = unformulated spinosad in aqueous solution at 200 ppm, equivalent to 60.0 g a.i. ha^{-1}; D10G = Dipel 10G at 3.42 kg ha^{-1}; Chem = Palgus at 75 ml ha^{-1}; Ctrol = untreated control; DAA = days after application.

86% OAR for 10, 30, and 100 ppm spinosad, respectively (Fig. 5A). Similar results were observed using granules stored for 3 years, resulting in 40%, 51% and 64% OAR for granules stored at 25 °C, and in 41%, 64% and 88% OAR for granules stored at 4 °C with 10, 30, and 100 ppm spinosad, respectively (Fig. 5B). Similarly, after 5 years, analysis resulted in 28%, 37% and 80% OAR for granules stored at 25 °C, and in 67%, 76% and 87% OAR for granules stored at 4 °C, with 10, 30, and 100 ppm spinosad, respectively (Fig. 5C). However, when granules stored for 9 years were used, no correlation was observed but a cluster was identified in which analysis resulted in 2%, 8% and 21% OAR for granules stored at 25 °C, and in 2%, 8% and 48.5% OAR for granules stored at 4 °C, with 10, 30, and 100 ppm spinosad, respectively (Fig. 5D).

4 DISCUSSION

We hypothesized that spinosad formulated as granules comprising a maize flour-based matrix could be as effective in pest control as a standard spray application (Tracer), but at lower application rates of spinosad per hectare. In a previous study by us, a granular formulation of spinosad had proven to be effective for *S. frugiperda* control when applied at very low rates, due to the phagostimulant properties of the granules.[15] This previous study concluded that 30 and 100 ppm spinosad granule treatments were as effective as a chlorpyrifos spray applications for control of *S. frugiperda* larvae in maize. In the present study, three field evaluations were performed on maize in organic experimental fields, where no chemicals had been applied previously, and the maize plants were naturally infested with *S. frugiperda* larvae.

Because a standard spray application of Tracer was included in field trials, we evaluated the lethal concentration of spinosad (Tracer 480SC) not only towards *S. frugiperda*, but also against *S. exigua* and *T. ni* (Table 1). Our results suggest that spinosad applied as granules at low application rates may be effective for the control of lepidopteran pests in maize and possibly other crops. Overall, the toxicity of spinosad to these pests followed the pattern reported previously with *S. frugiperda* being slightly less sensitive than the other two species.[24] LC_{50} values were lower (1.5 ng a.i. cm^{-2}) than those previously reported by Méndez *et al.*[25] (LC_{50} value of 9.2 ng a.i. cm^{-2}), who tested *S. fugiperda* of a colony from Chiapas state in southern Mexico, whereas the colony tested in this study was from Nuevo Leon state, in northern Mexico. All three species were more sensitive to spinosad by diet surface overlay than by droplet feeding, presumably due to the prolonged contact and consumption of the toxicant when present on the diet surface.

Several studies have shown better insect control when the active ingredient is formulated into granules with phagostimulant ingredients as a granule matrix.[12,13,26] In the present study, granular spinosad formulated at very low concentrations in a phagostimulant matrix resulted in *S. frugiperda* control in two field trials. In the first field trial, spinosad granules comprising 10, 30 and 100 ppm, equivalent to 0.24, 0.8 and 2.4 g a.i. ha^{-1}, controlled *S. frugiperda* as effectively as an aqueous spray application at 200 ppm (Tracer), equivalent to 60.0 g a.i. ha^{-1} (Fig. 1A). When comparing all insecticide treatments (chemical, Dipel and spinosad) for *S. frugiperda*

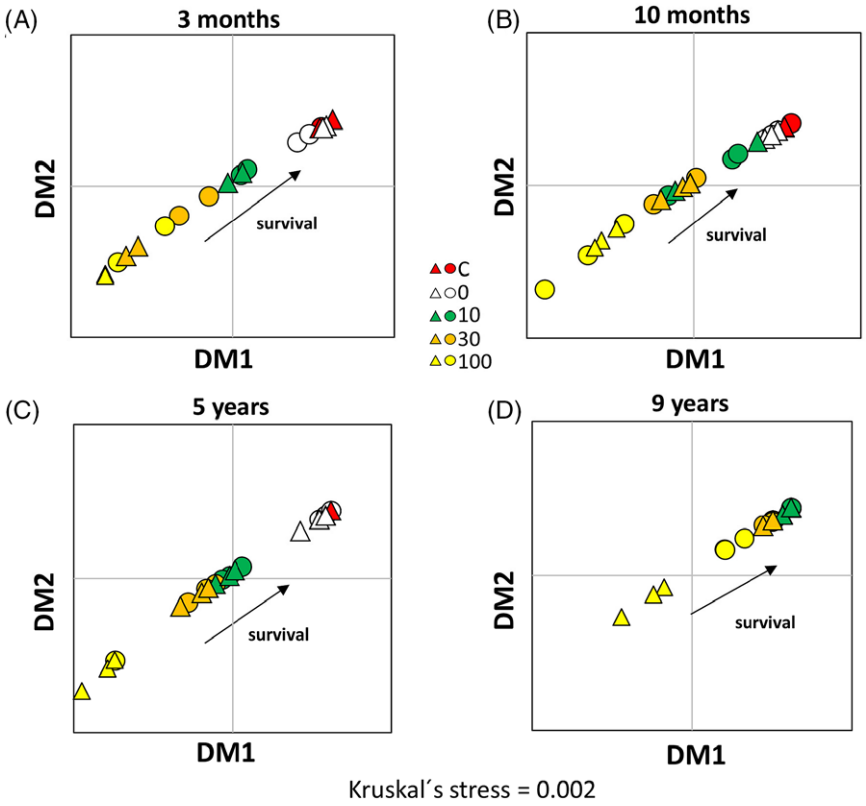

Figure 5. Shelf-life estimated as average percentage of mortality of *Spodoptera frugiperda* neonates exposed to spinosad in a maize flour matrix formulation in diet overlay bioassays performed following storage at room (25 ± 2 °C) (circles) or cold (4 ± 1 °C) (triangles) temperatures. MDS analysis using all data of three replicates per treatment. Mortality after (A) 3 months, (B) 10 months, (C) 5 years and (D) 9 years of storage. C = untreated control; 0, 10, 30 and 100 = maize flour granules with spinosad at 0, 10, 30 and 100 ppm, equivalent to 0.24, 0.8 and 2.4 g a.i. 10 kg^{-1}, respectively (for a 1 ha application).

control, only spinosad granules at 100 ppm showed higher larval mortality compared with the chemical treatment (cypermethrin 250 ml ha^{-1}), at 15 days post application (Fig. 1B). In a previous field trial using the same spinosad granular formulation and the same rates against a natural *S. frugiperda* population augmented with laboratory reared larvae released on to maize plants, 30 and 100 ppm spinosad granules showed similar insect control compared with chlorpyrifos.[15]

Mortality differed significantly in laboratory reared larvae collected at different intervals post application. To evaluate the integrated pest management approach, following the application of treatments, field-collected *S. frugiperda* larvae were reared under laboratory conditions, where parasitoid-induced mortality and larval development until the pupal stage were recorded. As expected, the highest prevalence of larval mortality was observed in larvae collected from the 100 ppm spinosad granule and 200 ppm spinosad spray treatments. Because larvae collected from the chemical treatment were those that were not killed by the treatment, the mortality of larvae during laboratory rearing was similar to that observed in the lower spinosad dosages and the controls. Larvae in the 10 and 100 ppm treatments died, but fewer than half were due to parasitoids, whereas most larvae that died during rearing in all other treatments died due to parasitism. Surviving larvae reached the pupal stage (Fig. 2). This suggests that the spinosad and *B. thuringiensis* treatments were compatible with the action of parasitoids, as part of an integrated pest management approach (Fig. 2).

Even though the high mortality observed among larvae collected from the 100 ppm spinosad granule treatment was not surprising, the mortality of larvae from the 10 ppm spinosad granule treatment was higher than expected (~ 30%). One explanation for this could be related to the fact that spinosad, although at a very low concentration, was formulated into phagostimulant matrices and any antifeedant properties of spinosad were likely reduced compared with the 30 and 100 ppm granules.[12]

Fermentation derived metabolites such as spinosad are susceptible to biodegradation, hydrolysis and photolysis. Estimated half-lives by hydrolysis exceed 30 days, by photolysis in aqueous systems and soil vary from 0.96 to 8.68 days, respectively, whereas half lives in aerobic and anaerobic soil metabolism are 17.3 and 161 days, respectively.[5] The stability of spinosad exposed to solar ultraviolet radiation is therefore much shorter than residues protected from sunlight. Moreover, both spinosad and *B. thuringiensis* can be washed off foliage by rain.[14] Because the second field test was performed in late September and heavy rain was present, it was not possible to collect samples 2 and 5 days after application to evaluate *S. frugiperda* infestation (living or dead larvae), and statistical analyses were only based on mortality at day zero (before application) and after 9 and 15 days post application. Before application, *S. frugiperda* natural infestation was statistically similar across the treatments in the maize field (Fig. 3). After 9 days the efficacy of *S. frugiperda* control among spinosad granule treatments was significantly higher in the 100 ppm treatment, but all spinosad granule treatments provided similar efficacy at 15 days post application (Fig. 3A), thus indicating that some granules

remained in the leaf whorls and continued to control larvae. Spinosad granules tested in this study were made with nixtamalized corn and cornstarch, both pregelatinized matrices with adherent and phagostimulant properties as previously observed by McGuire *et al.*[27] When comparing all insecticide treatments (chemical, Dipel and spinosad) for *S. frugiperda* control, after 9 days application the chemical treatment showed the highest percentage of mortality (62%), that was similar to that of the 100 ppm spinosad granule treatment (Fig. 3A), whereas after 15 days Dipel resulted in similar mortality compared with the chemical and control treatments (Fig. 3B). Regardless of the sampling periods, granulated control resulted in the lowest mortality.[15] By contrast, the 30% mortality observed in the untreated control may have been due to the high humidity after heavy rain prior to the sample time point.[28]

The highest grain yield was observed for plots treated with 100 ppm (2.4 a.i. ha^{-1}) spinosad granule treatment, similar to that of the chemical treatment, which represented 90.6% of the maximum yield. Lower yields were observed in unformulated spinosad treatment, which represented 68.5% of the yield in the 100 ppm spinosad granule treatment. Because maize yield reduction by *S. frugiperda* damage has been estimated at 15–73% when 55–100% of plants are infested by *S. frugiperda*,[9] only chemical and spinosad granules at 100 ppm showed the desired protection against this insect pest.

Establishing the product shelf life is a legal requirement for agrochemical registration in order to ensure that the product maintains a determined level of control efficacy or the active ingredient retains a minimal level of insecticidal activity. A concentration–mortality response of spinosad granules at 10, 30 and 100 ppm was observed in the evaluation of shelf life (Fig. 5). The Tracer 480SC product label indicates that this product stored at cool temperatures, retains suitable levels of insecticidal activity for up to 2 years. In previous shelf life tests on the maize flour granule formulation, but using *B. thuringiensis* as the active ingredient, the shelf life at room temperature was estimated at 2 years, after which time mean mortality in bioassays had fallen from 96% to 32%.[13] In the present study, bioassays revealed that all concentrations of spinosad in granules retained high levels of toxicity following 5 years of storage (Fig. 5C). After 9 years, the 100 ppm granules stored at cold temperature retained ~49% of the original activity (Fig. 5D). Our results showed that granular formulation extended the active ingredient shelf life at room temperature up to 5 years, and at cold temperatures by up to 9 years. One additional advantage is that by applying granules, most of active ingredient will stay within the structure of the leaf whorl,[29] reducing potential interactions with, and adverse effects on, beneficial insects such as predators and pollinators.[30,31]

In conclusion, the phagostimulant formulations of spinosad evaluated in this study provided multiple advantages based on efficacy and shelf life. The use of spinosad formulated in maize flour matrix granules at 100 ppm resulted in increased grain yield compared with unformulated spinosad and other biopesticides, and was equally effective as a spray application of cypermethrin or spinetoram (Palgus) for control of *S. frugiperda* on maize. The granular formulation of spinosad had an additional benefit in that it conferred a markedly extended shelf life to the formulated product.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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