INTERNATIONAL JOURNAL OF PEST MANAGEMENT, 2000, 46(3) 169–176

Photoprotection of Beauveria bassiana: testing simple formulations for control of the coffee berry borer

(*Keywords:* UV inactivation, entomopathogen, *Hypothemus hampei*, biological control, coffee, Mexico)

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Abstract. The entomopathogenic fungus, *Beauveria bassiana* is considered to be one of the few natural enemies available for use against the coffee berry borer. In an attempt to enhance the efficacy of this pathogen, a range of concentrations of 22 substances was tested in simple laboratory tests using natural sunlight or a UV light source. Unprotected *B. bassiana* spores were almost completely inactivated by exposure to 60 min of direct sunlight or 20 s of UV light of 302 nm wavelength. Seven of the 22 substances tested showed little or no photoprotective properties and eight of the substances appeared directly detrimental to spore germination. Of the remainder, sucrose, yeast, yeast extract, uric acid, casein, and molasses had limited photoprotective properties. The most effective substances tested were egg albumen and skimmed milk powder which could extend the persistence of *B. bassiana* spores by a factor of almost three. A mixture of 3% (w/v) albumen and 4% (w/v) milk powder gave the highest degree of spore protection per unit cost. Young coffee plants sprayed with this mixture did not suffer any significant phytotoxic effects. A field trial, involving two applications of spores with or without the milk and albumen mixture, failed to show that improved spore persistence resulted in increased coffee berry borer control. Very low levels of pest infestation observed in field plots together with unusual, unfavourable weather conditions may have accounted for this unexpected result.

1. Introduction

All microbial insecticides are inactivated by exposure to sunlight (Ignoffo *et al.*, 1977; Gaugler and Boush, 1979), and photoinactivation has emerged as the major environmental factor limiting their effectiveness (Cohen *et al.*, 1990). The UV-B portion of solar radiation (290–320 nm) represents the UV light of greatest biological interest, and whilst negative effects on microorganisms have been noted for wavelengths outside this range (Bullock *et al.*, 1970) it is within the UV-B range that most damage occurs (Roberts and Campbell, 1977; Killick, 1987; Fargues *et al.*, 1997). Exposure can have two effects: (i) direct damage to DNA, creating strand breaks or cross-linkages between bases, which can block the synthesis of normal DNA and create high levels of mutations; (ii) the production of highly reactive and deleterious radicals such as peroxides (Pearlman *et al.*, 1985; Ignoffo and Garcia, 1994). The result of both is a rapid reduction in the stability of the microorganism, and a limit to its insecticidal activity (Moore *et al.*, 1993; Inglis *et al.*, 1995).

Over the last three decades considerable work has been devoted to finding suitable materials which can inhibit or delay the photoinactivation of microorganisms, especially baculoviruses (Ignoffo and Batzer, 1971; Shapiro *et al.*, 1983; Martignoni and Iwai, 1985; Killick, 1990; Vail *et al.*, 1996). A variety of materials has been tested including commonly used sun-screens, clays, flour, charcoal, and fluorescent materials such as optical brighteners.

The coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae), is considered as the most serious pest of coffee in the world. In Mexico, the potential of *B. bassiana* to assist in berry borer control has been recognized and governmental and cooperative organizations of coffee growers presently produce and distribute the fungus on a local scale. However, *B. bassiana* applications rarely achieve more than 30–40% control in the field, leading to the need for additional control measures including periodic liberation of the exotic ectoparasitoid, *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae) (Barrera *et al.*, 1990; Murphy and Moore, 1990).

The objective of the present study was to identify photoprotectant substances that would increase the persistence of *B. bassiana* spores, resulting in an increased incidence of infection of the *H. hampei* population following each application. Particular emphasis was placed on cheap substances which would be accessible to growers in tropical coffee-producing regions. Also, when selecting materials for testing, preference was given to natural substances with minimal environmental impact, an issue of concern with certain synthetic photoprotectors (Margulies *et al.*, 1985; Cohen *et al.*, 1990; Patel *et al.*, 1996).

2. Materials and methods

All tests employed an isolate of *B. bassiana*, named 'strain 26', originally isolated from *H. hampei* in Chiapas and shown to have a high infectivity towards a local culture of this insect (De la Rosa *et al.*, 1997). The fungus was grown until sporulation on Sabouraud dextrose agar plus 0.5% yeast extract in sterile Petri dishes at ambient laboratory temperatures, 26–30°C. Spores were harvested from the plates and stored in a desiccator at 4°C. Batches of spores showing less than 80% germination in control tests at any time during testing were discarded. Potential protectants were selected by reviewing previously published

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ISSN 0967-0874 print/ISSN 1366-5863 online
http://www.tandf.co.uk/journals

work, mainly on photoprotection of baculoviruses. All work involving plants was performed using arabica coffee (*Coffea arabica*).

2.1. Laboratory tests on protectant materials

For testing, spores were suspended to a concentration of approximately 1×10^9 spores/ml in 0.1% Triton X-100. A 3 μl droplet of this suspension was smeared evenly across the length of a clean glass microscope slide using the edge of another slide. Spores were exposed to direct natural sunlight by placing test slides on the roof of the laboratory, with exposure time varying according to the criteria of the test. Tests were carried out on cloudless days between 11 a.m. and 1 p.m. when sun intensity was at a maximum.

Following exposure, a thin layer of 3% Sabouraud agar (+0.02% aureomycin) was added onto each slide and all were then placed in a dark humid box for 18 h at ambient laboratory temperatures (26–30°C). Spore germination was determined using a phase contrast microscope at ×600 magnification. The mean percent spore germination was calculated for each slide by counting the number of germinated and ungerminated spores in five fields of view. Between three and six replicate slides were used for each treatment. Treatments included spores with and without photoprotectant placed in the sun, spores with photoprotectant placed in the sun and wrapped in aluminium foil (to control for heating effects on spore viability), and spores with and without photoprotectant held in a dark box.

Potential photoprotectants were initially screened using one concentration and were exposed to natural sunlight for 60 min; a period observed to result in 94–100% inactivation for unprotected spores. For the substances that showed promise as photoprotectors further tests were performed using a single concentration over a range of exposure times from 0 to 240 min.

Repeat tests were made exposing spores to ultraviolet light emitted from a transilluminator, peak 302 nm (Hoefer UVTM-25). The lamp provided precise repeatable test conditions although with a very restricted wavelength spectrum and was used as a means of verifying results from the tests under natural sunlight. Test slides were located at a distance of 2 cm from the UV filter, and exposed for between 5 and 25 s. Spore germination was assessed as previously described.

To quantify the efficiency of each substance tested, the degree of spore inactivation was quantified using the formula for the percentage of original activity remaining (OAR)—a standard parameter used for quantifying UV inactivation of entomopathogens (Shapiro *et al.*, 1983; Martigoni and Iwai, 1985; Ignoffo and Garcia, 1994; Patel *et al.*, 1996) where:

$$\mathrm{OAR} = \frac{\text{Percentage germination of exposed spores}}{\text{Percentage germination of unexposed spores}} \times 100\%$$

For materials showing the greatest promise in laboratory tests the cost–benefit ratio was calculated for a range of concentrations by plotting percent spore germination after 60 min of solar exposure per unit cost of the additive against additive concentration and fitting a regression. The peak value was identified as being the concentration of the substance that offered the greatest protection per unit cost.

2.2. Testing for phytoinhibitory effects

A test was conducted to see whether the albumen–milk formulation was detrimental to the growth or development of the coffee plant. Two treatments were applied to groups of 15 young coffee plants: (i) water control plus 0.2% wetter-sticker (Agralplus, Zeneca) or (ii) a suspension of 4% milk and 3% albumen with 0.2% Agralplus.

The plants were watered daily at the base of the stem and arranged randomly over an area under partial shade. Prior to the experiment, four parameters were measured for each plant: height, number of branches, total number of leaves, and average leaf area for a random sample of three leaves per plant. Spray applications were made using a hand-held garden sprayer at a volume of 25 ml per plant, with a repeat application 2 weeks following the first. Thirty days following this second application the four parameters were measured again. The experiment was designed to detect only gross differences in plant development.

2.3. Field trial

Field trials were conducted during the months of June, July, and August 1997 at an organic coffee farm (Santa Anita) located at an altitude of approximately 900 m in the Nueva Alemania area of the Soconusco region of Chiapas (15°10′ N, 92°21′ W). A uniform area of approximately 1 hectare was selected within which 18 blocks were marked, each block consisting of five coffee plants surrounding a central plant of average height 2.3 ± 0.05 m and average width 1.73 ± 0.04 m (mean ± SE).

Prior to the experiment, a random sample of berries from each block was taken in the zone between knee and head height in order to estimate the infestation of the berry borer (Baker, 1984). These berries were also used to determine the natural incidence of *B. bassiana* infection; berries infested with *H. hampei* were placed in moist Petri dishes maintained at a temperature of 25°C for 5 days. The presence of *B. bassiana* infection was subsequently determined by examining the berry borer entry hole for sporulating mycelia, followed by microscopic examination of mycelia and spore morphology.

One of three treatments was randomly assigned to six replicate blocks:

(i) water + 0.2% Agralplus;
(ii) 1×10^9 spores/ml of *B. bassiana* in 0.2% Agralplus;
(iii) 1×10^9 spores/ml of *B. bassiana* in a suspension of 4% milk, 3% albumen and 0.2% Agralplus.

Application of spore suspension (360 ml per plant) was made with a manual backpack sprayer (Jacto X-15) typical of those used locally, and took place early in the morning in order to avoid spraying in the afternoon which is generally accompanied by rainfall and an increase in wind speed.

One week after spraying, 50 *H. hampei*-infested fruits were collected from the central plant of each block and the incidence of *B. bassiana* assessed as described above. A second assessment was conducted 3 weeks after spraying. The trial was performed twice; initially on 2 July 1997 and again on 7 August 1997.

Percent spore germination results obtained in natural sunlight or on the UV transilluminator were arcsine transformed and subjected to linear or quadratic regression analysis. Plant growth data were analysed by *t*-test comparison of plants sprayed with the milk–albumen mixture and the water + wetter-sticker controls. The prevalence of *B. bassiana* infection in coffee berry borer-infested berries collected in the field experiment was analysed by Kruskal–Wallis non-parametric ANOVA (Sokal and Rohlf, 1981).

3. Results

3.1. Sunlight exposure

There was a highly significant negative relationship between spore germination and duration of exposure to sunlight ($F_{1,30}$ = 264.0, $P < 0.0001$) (figure 1(a)). Unprotected spores were inactivated rapidly; after 60 min exposure spore germination had been reduced from an initial mean of 89.4% (range of SE 90.5–88.3) to just 2.8% (range of SE 3.4–2.3) (data in figure 1(a) appear different due to arcsine transformation).

Of the substances tested, eight were detrimental to spore germination at most or all of the concentrations tested, due to presumably toxic or inhibitory effects detected in the controls. These were methyl anthranilate (0.1–2.5%), *p*-amino cinamic acid (0.1–5%), Indian ink (2-10%), choline chloride (0.01–0.5%), titanium dioxide (0.1–5%), ethyl *p*-amino benzoate (0.1–5%), flavin mononucleotide (0.1–2.5%) and oxybenzone (0.1–5%).

Zinc oxide (1%), starch (5–20%) and ascorbic acid (0.1–5%), Tinopal LPW (0.1–3%) showed no photoprotective properties at the concentrations tested (OAR values <3.0). Of the remainder, brewer's yeast, molasses, sucrose, casein, and yeast extract all showed moderate levels of protection (table 1). Folic acid and uric acid showed some photoprotection properties, but at high concentrations (5% and 10% respectively), both were detrimental to spores exposed to UV.

The substances which gave the highest level of protection were milk and egg albumen. For spores formulated with 20% milk powder, 50% inactivation was seen after approximately 55 min, and after 30 min for unprotected spores. For 10% egg albumen, 50% spore inactivation occurred after 72 min when formulated in comparison to 30 min for unprotected spores. When spores were formulated with the combination of 4% milk and 3% egg albumen, 50% spore inactivation was observed after 50 min compared with 20 min for unprotected spores (figure 2).

Activated charcoal was tested but could not be assessed because spores were adsorbed onto charcoal particles making accurate counts of germination impossible.

3.2. Transilluminator exposure

Tests with the transilluminator lamp using the most effective materials from the sunlight tests verified the observations made in natural sunlight. Spore inactivation was extremely rapid and was linearly correlated to the duration of exposure ($F_{2,62}$ = 365.9, $P < 0.0001$) (figure 1(b)). After just 20 s of UV irradiation, mean spore germination had fallen from an original 95.3% (range of SE 94.9–95.7) to 0.74% (range of SE 0.92–0.59). Sucrose (20%) and casein (4%) gave very little protection in this test; after 20 s exposure spore germination was 3.4 and 0% respectively. Separate tests with milk (20%) and albumen (10%) confirmed the properties of these substances as potential photoprotectants. After 20 s exposure, the OAR value of spores with milk was 40.2% compared with 54.2% for spores with albumen (figure 3).

3.3. Cost-benefit analysis

From a spline plot of spore-enhancement/cost against concentration of protectant, the combined protectant of milk at 4% and egg albumen at 3% was the most cost-effective protectant substance. The cost of milk powder from a local supermarket was US$0.005 per gram whereas egg albumen from a local scientific supplier cost US$0.02 per gram. The total cost of incorporation of these two substances at 4% and 3% respectively was therefore US$0.875 per litre of spray suspension.

With 50% spore inactivation recorded after 30 min exposure to sunlight, this combination was as effective as high concentrations of either of the substances alone but was substantially cheaper. For example, if the two protectants were used separately, egg albumen at 10% (US$2.20 per litre) resulted in 50% inactivation recorded after 42 min, and milk at 20% (US$1.25 per litre) gave 50% inactivation after 24 min in full sunlight.

3.4. Phytoinhibitory tests

Student's *t*-tests conducted on the growth parameters of each group indicated no significant differences between treatments and controls. Branch number increased by 0.71 from 3.06 ± 0.22 to 3.77 ± 0.24; the number of leaves per plant increased by 6.3% from 35.67 ± 1.22 to 37.93 ± 1.42; plant height increased by 4.5% from 37.27 ± 0.69 to 38.96 ± 0.71 and leaf area increased by 11.7% from 29.84 ± 1.32 to 33.34 ± 1.10 (figures are means of all plants $\pm$ SE). Additional effects, such as deformations, discolouring, etc., were not observed. The formulation of milk and albumen did not appear detrimental to the growth or development of coffee plants.

3.5. Field trial

From the samples taken in the experimental area throughout the 2 months of field work, natural background coffee berry borer infestation was consistently very low. During the month of July, infestation levels varied from 2.1% (n = 602), 2.4% (n = 402) and 4.5% (n = 454) for samples taken immediately prior to spray application and at 1 week and 3 weeks after spraying respectively. In these samples, the natural incidence of *B. bassiana* infection was 0.0–5.0%. During August, berry borer infestation varied from 3.6% (n = 1010), 6.3% (n = 702) or 7.2% (n = 580) for samples taken immediately prioir to the second application, at 1 week or at 3 weeks following spraying respectively. Of these infested berries, the natural incidence of *B. bassiana* infection varied from 0.0 to 4.7%.

One week and 3 weeks following the initial treatment application, there was a higher incidence of *B. bassiana* infection in plots treated with spores compared with controls

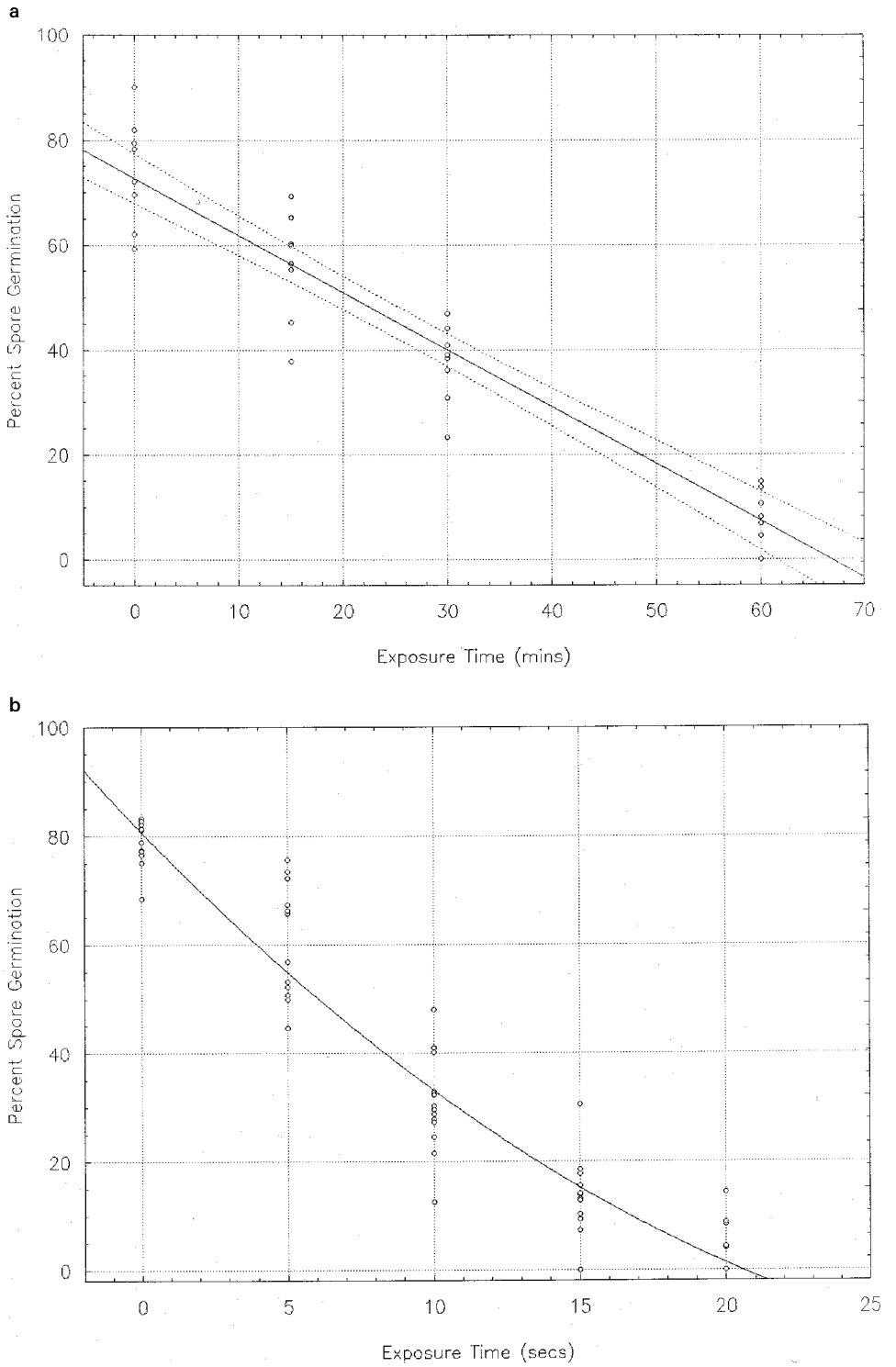

Figure 1. Inactivation of unprotected B. bassiana *spores exposed for various periods to (a) direct natural sunlight ($y = -1.09x + 72.71$, $R^2 = 0.898$, $F_{1,30} = 264.0$, $P < 0.0001$). Dotted lines indicate 95% CL. (b) UV light from a transilluminator (312 nm peak output) ($y = -5.536x + 0.0783x^2 + 80.69$, $R^2 = 0.922$, $F_{2,62} = 365.9$, $P < 0.0001$). The percentage of germination values have been arcsine transformed.*

but treatment effects were not significant (Kruskal–Wallis test, ($\chi^2_2 = 3.821$, $P = 0.148$ at 1 week post-spray and ($\chi^2_2 = 3.406$, $P = 0.182$ at 3 weeks post-spray) (table 2).

Following the second field application, at 1 week there was a significant treatment effect with higher incidence of *B. bassiana* infection in plots receiving spore treatments compared with controls (Kruskal–Wallis ($\chi^2_2 = 10.510$, $P = 0.005$). The incidence of disease, however, was not influenced by the presence of the photoprotective susbstances (11.4% in spore-sprayed plots vs 12.6% in spore + photoprotectant plots). At 3 weeks post-spray the treatment effect was no longer significant (Kruskal–Wallis ($\chi^2_2 = 3.472$, $P = 0.176$) (table 2).

Discussion

Rapid inactivation of *B. bassiana* spores occurred following exposure to UV light or natural sunlight. The rate of inactivation was reduced through the addition of protective substances. However, several substances proved ineffective even though photoprotective properties had been previously described in studies with baculoviruses: ascorbic acid, zinc oxide, folic acid and molasses for example (Shapiro *et al.*, 1980, 1983; Shapiro, 1985; Ignoffo and Garcia, 1994).

Of the substances tested, egg albumen and milk powder were the most effective at reducing the rate of spore inactivation. The probable reason is that their proteinaceous content absorbs UV-B radiation (Jaques, 1970) and acts as a protectant barrier over the surface of the spore. A formulation of 4% milk and 3% egg albumen provided the most cost-effective protection and these two substances appear to function well as UV protectants when combined. The results of tests in natural sunlight were confirmed using a UV transilluminator with peak output at 302 nm. No gross

Table 1. Percentage original activity remaining (OAR) for B. bassiana *spores exposed to 60 minutes direct sunlight and different concentrations of candidate photoprotective substances including blank control and control with highest concentration of substance (controls not exposed to sunlight)*

	Concentration of substance										
Substance	0.1%	0.5%	1%	2%	3%	5%	7%	10%	20%	Control (blank)	Control (max)
Ascorbic acid	0.0		0.0		0.0	0.0				85.7 (83.15–89.91)	0.0
Brewer's Yeast						7.2 (5.73–8.92)				90.1 (88.39–91.49)	90.3 (88.41–91.96)
Casein				13.6 (11.86–15.13)						92.4 (90.31–93.91)	93.0 (91.51–94.29)
Choline chloride	0.0	0.0								88.6 (86.19–90.12)	0.6 (0.21–1.31)
Egg albumen			1.5 (1.16–1.89)		15.7 (14.46–17.09)	16.1 (14.14–17.09)	26.1 (23.47–28.89)	37.9 (34.7–41.09)	38.7 (35.60–41.87)	87.8 (86.18–89.34)	93.1 (91.57–94.35)
Folic Acid	11.5 (10.78–13.73)	12.3 (11.69–12.91)		15.5 (14.71–16.31)		0.6 (0.58–0.71)				93.4 (92.05–94.66)	89.1 (87.56–90.53)
Milk Powder	2.4 (1.82–3.22)		6.7 (5.74–8.18)		12.5 (11.17–13.91)		13.8 (12.26–15.60)	16.6 (14.72–18.72)	32.7 (31.12–34.29)	84.2 (83.03–85.34)	91.9 (90.55–93.22)
Molasses						4.68 (3.24–5.69)		5.96 (4.55–7.78)	8.65 (6.98–10.70)	81.6 (79.37–83.87)	83.1 (81.77–85.04)
Naphthol black			0.0			0.0				95.1 (94.08–96.01)	61.2 (58.71–63.68)
Starch						0.4 (0.15–1.11)		0.0	0.3 (0.13–0.93)	93.4 (91.76–94.71)	86.5 (84.99–87.90)
Sucrose									18.3 (15.46–21.60)	80.4 (78.69–81.96)	85.0 (83.22–86.65)
Tinopal LPW	0.0		0.0		0.0					94.6 (93.25–95.74)	94.0 (92.41–95.12)
Titanium dioxide	0.0	0.0	0.0			0.0				85.2 (83.11–87.40)	40.2 (37.24–43.30)
Uric Acid	0.0		10.6 (9.66–11.68)			14.1 (12.93–15.42)		0.6 (0.34–1.07)		95.4 (94.76–96.04)	95.5 (94.62–96.31)
Yeast Extract	2.7 (1.76–3.19)		7.4 (5.84–9.31)			15.2 (13.36–17.30)		12.8 (10.74–15.10)		95.3 (94.12–96.39)	93.75 (92.33–94.92)
Zinc oxide			1.24 (0.61–2.49)							88.8 (86.96–90.40)	91.2 (89.50–92.68)

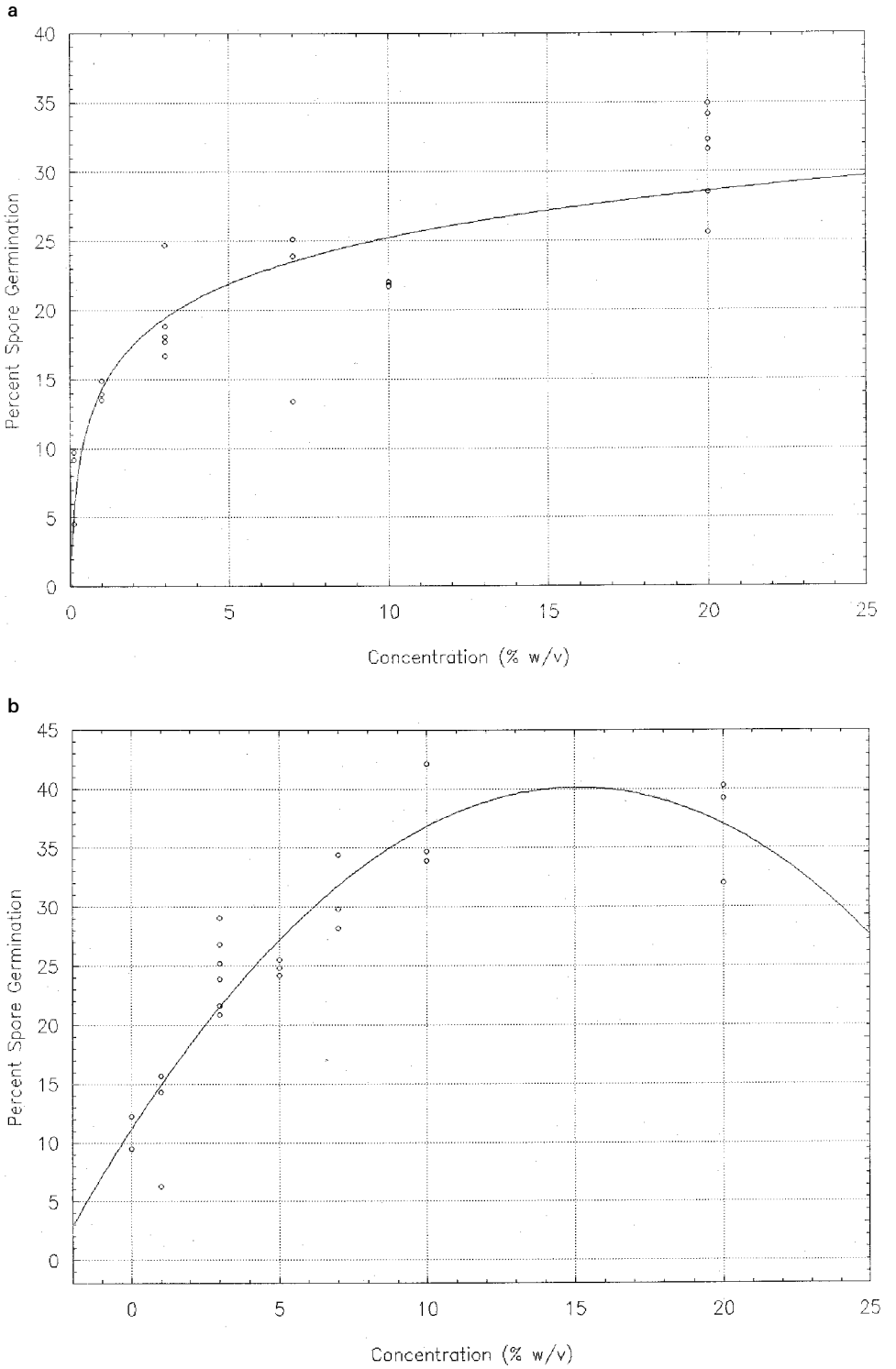

Figure 2. Inactivation of B. bassiana *spores at different concentrations of (a) skimmed milk powder (y = 4.882 1 ln [9.696x + 0.6803] + 2.8630,* $R^2 = 0.879$, $F_{4,19} = 22.56$, $P < 0.0001$*) or (b) egg albumen (y = 3.841x - 0.1273x2 + 11.151,* $R^2 = 0.852$, $F_{2,20} = 57.48$, $P < 0.0001$*) following a 20 s exposure to UV light from a transilluminator. The percentage of germination values have been arcsine transformed.*

phytotoxic effects were detected in simple spray tests on young coffee plants and we assumed that the environmental impact of spraying dilute proteinaceous mixtures in coffee plantations would be minimal.

The hypothesis tested in the present study, that photo-protective substances present in spray applications of fungal spores would result in a substantial increase in the incidence of *B. bassiana* infection, was not borne out in the field trail. This result may have been due to two factors. First, the unusual weather conditions which were on average 2.0°C hotter with 13.3% more hours of direct sunlight and 51.6% less rainfall than is typical for the experimental period. Second, the density of the *H. hampei* population was also very low. The level of infestation at 'Finca Santa Anita' varies from year to year but typically

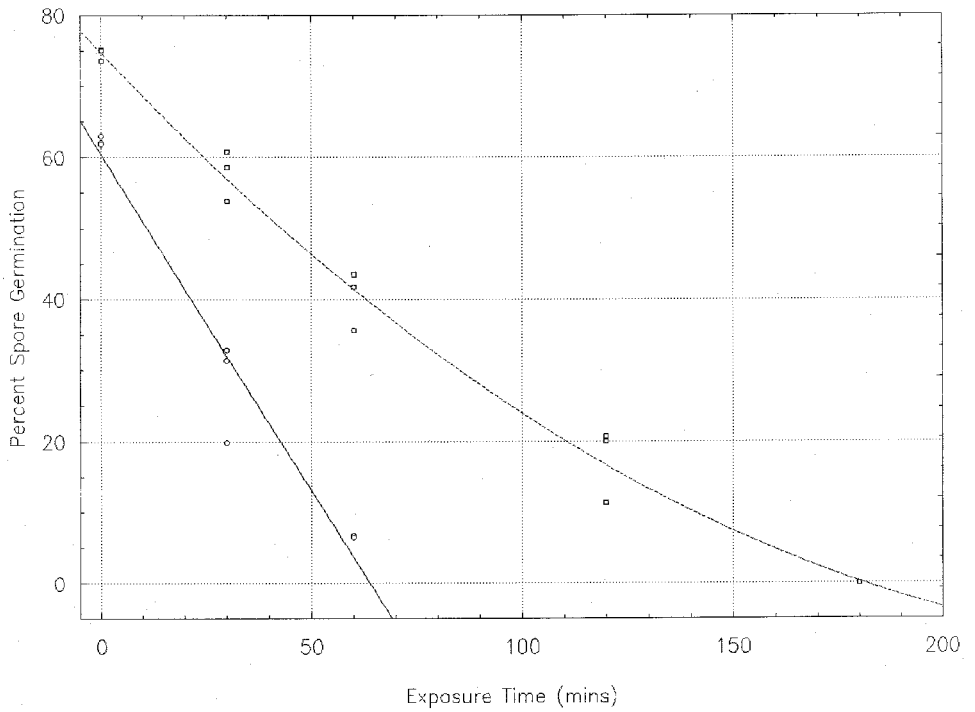

Figure 3. Effect of a combination of milk powder (4%) and egg albumen (3%) on spore inactivation in direct natural sunlight. Solid line and circles represent unprotected spores ($y = -0.945x + 60.344$, $R^2 = 0.956$, $F_{1,6} = 130.91$, $P < 0.0001$), dotted line and squares represent spores with egg and milk ($y = -0.6238x + 0.00117x^2 + 74.678$, $R^2 = 0.972$, $F_{2,13} = 1714.9$, $P < 0.0001$). The percentage of germination values have been arcsine transformed.

Table 2. Incidence of B. bassiana *infection in coffee berry borer following field application of spores with or without egg and milk suspension. Values are percentages based on a sample of 50 infested berries (figures in parentheses indicate range of SE)*

Date	Control (water)	*B. bassiana* spores	*B. bassiana* spores + albumen+ milk powder
First application			
Post-spray sample, 1 week	2.0 (1.33–2.99)	5.2 (4.18–6.79)	7.3 (5.96–8.98)
Post spray sample, 3 weeks	1.0 (0.56–1.77)	4.0 (3.01–53.29)	5.7 (4.47–7.16)
Second application			
Post-spray sample, 1 week	0.0	11.3 (9.63–13.29)	12.6 (10.86–14.71)
Post spray sample, 3 weeks	0.0	3.33 (2.44–4.54)	2.7 (1.88–3.77)

averages 25–30% (W. de la Rosa, unpublished data), which is considerably more than was observed in the present trial (maximum 7.2%).

Inglis *et al.* (1995) tested the effect of various sunscreens including optical brighteners, dyes and a clay based emulsion on *B. bassiana* spore survival on glass coverslips and plant foliage under artificial UV-B exposure. Subsequent field tests indicated that optical brightener 28 (Tinopal LPW) and the clay emulsion significantly increased the survival of spores whereas another optical brightener, congo red dye and oil soluble sunscreens were ineffective. Samples taken 16 days post-application indicated that spore viability had fallen by 3.5–4.0 logs in control plots compared with approximately 2.0–2.6 logs (in terms of colony forming units/cm^2 of plant area) in plots treated with optical brightener 28 or the clay emulsion, i.e. the photoprotectors increased spore persistence by about ×17–×28 (estimated from graphs in Inglis *et al.*, 1995). However, the result of enhanced spore persistence in terms of the incidence of *B. bassiana* infection was not tested.

Considerable work has been performed on UV protection of insect baculoviruses but relatively little on entomopathogenic fungi despite the fact that UV light is recognized as a key factor limiting the impact of these pathogens (Gardner *et al.*, 1977; Daoust and Pereira, 1986; Feng *et al.*, 1994; Inglis *et al.*, 1995, 1997). The present study confirmed the very high sensitivity of *B. bassiana* spores to UV radiation and demonstrated how cheap and simple substances could enhance spore persistence even under intense exposure of sunlight in a tropical region. Unfortunately improved spore persistence was not reflected in improved control of the coffee berry borer in the field, perhaps due to unfavourable environmental and/or test conditions.

Acknowledgements

We gratefully acknowledge the help of Javier Valle Mora for statistical assistance, Alfredo Castillo for coffee berry borer material, Gerardo Hernández for assistance in the field, and Finca Santa Anita for access to the field site. Financial support for the study came from SIBEJ (A-023-97) and an academic exchange grant to S. Edgington via the Secretaria de Relaciones Exteriores de México.

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