# PRIMARY AND SECONDARY UPTAKE OF CONIDIA OF ENTOMOPATHOGENIC FUNGI AND THE EFFECT OF OXYBENZONE AS A CHEMICAL SUNLIGHT PROTECTOR

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**Abstract**

The importance of secondary pickup of infective conidia in a spray application of fungal bio-pesticide has been demonstrated. The effect produced by conidia picked up from leaf and soil surfaces together is higher than direct contact from the spray. Oxybenzone powder (2% w/v) as a spray additive to an oil formulation of *Metarhizium flavoviride* offered enhanced protection to conidia within the first 3 days of field application.

**Résumé**

ADU-MENSAH, J.: *Prise primaire et secondaire de la conidie de fongus entomopathogène et l'effet d'oxybenzone comme protecteur chimique contre le soleil.* L'importance de la prise secondaire de conidie infectant dans une application de pulvérisation de bio-pesticide fongique a été démontrée. L'effet produit par conidie pris de surfaces de feuille et de sol ensemble est plus élevé que le contact direct de la pulvérisation. Poudre d'oxybenzone (2% w/v) comme un additif de pulvérisation à la formulation d'huile de *Metarhizium flavoviride* donnait une protection améliorée contre la conidie pendant les trois premiers jours d'application sur le terrain.

## Introduction

Fungi belonging to Deuteromycota are considered the most promising group for locust and grasshopper control because they work by direct contact through the insect cuticle and can be mass produced (Prior & Greathead, 1989). However, conidia of entomopathogenic fungi as well as spores of bacteria, protozoa and viruses are extremely sensitive to sunlight (Roberts & Campbell, 1977; Cohen *et al.*, 1991). Research on formulation of entomopathogenic fungi in oil suitable for controlled ultra-low volume (ulv) application has reached an advanced stage (Bateman, 1992; Bateman *et al.*, 1993).

Conidia formulated in oil have enhanced more infection (36 times more) than those suspended in water (Prior, Jollands & Le Patourel, 1988). However, in ulv spray applications a large proportion of spray volume miss their intended target surfaces resulting in 'wastage'. Spray volumes which fail to make primary contact with their host insect may produce secondary effects if infective doses of viable conidia are picked up in the course of feeding and/or moving about (Johnson, Huang & Harpen, 1988). Methods to protect conidia from ultra-violet component of sunlight by the addition of protective chemical screens could extend the survival time of conidia in the field (Dunkle & Shasha, 1988). This may be especially important for myco-insecticides sprayed onto vegetation.

Many physical and chemical sunlight protectants such as the para-aminobenzoates, salicylates, cinnamates and benzo-phenones have been developed by the cosmetic industry (Shaath, 1990). Several attempts have been made to increase field persistence of microbial insecticides by including some of these in liquid formulations for spraying (Young & Yearing, 1986; Shapiro, Poh Agin & Bell, 1983). This approach generally results in a five to10-fold increase in field persistence (Ignoffo & Garcia, 1992). However, most attempts have been with viruses and bacteria

(Killick, 1990; Kreig *et al.*, 1980), little information being available on fungi.

Laboratory studies have indicated that the addition of 1% Oxybenzone (2-Hydroxy-4-Methoxybenzophenone) resulted in some protection of spores of *M. flavoviride* formulated in oil and exposed to artificial sunlight (Moore *et al.*, 1993). In this study carried out at IITA, Benin, the protection given by the chemical sunscreen Oxybenzone to field applied conidia of *M. flavoviride* in oil was investigated. Also, conidia picked up directly by variegated grasshoppers, secondary pickup from vegetation and soil, as well as horizontal transmission from cadavers were assessed separately for their contribution to the overall performance of a spray application in the field.

## Experimental

The isolate of *M. flavoviride* (IIBC 191-609) was isolated from a field-infected specimen of *Zonocerus variegatus*. Laboratory bioassays showed it to be the most virulent of three strains of the fungus against *Z. variegatus*, with a median-lethal-time ($LT_{50}$) of 5.4 days at a dose of $10^5$ spores/insect. The fungus was mass produced on rice by inoculating sterilized boiled rice with 3-day old blastospores produced in glucose and brewer's yeast at 28 °C on an orbital shaker at 60 rpm. Dry spores were harvested after 7 days and a formulation of $2.5 \times 10^9$ conidia $ml^{-1}$ in a 50 : 50 mixture of kerosene and vegetable oil was prepared. Three-month old cassava plots with no history of fungal pathogen application were assigned to four treatments and control with four replicates. Control plots were fixed upwind to prevent drift contamination, and treatment plots were randomized. Insects used for the study were young adults collected from the field and maintained for at least 5 days to detect infections, if any. *Z. variegatus* cadavers collected from a field sprayed with 2 l $ha^{-1}$ of $2.5 \times 10^9$ conidia $ml^{-1}$ of the fungus were placed on moist tissue paper singly in Petri-dishes at room temperature for external sporulation.

In Treatment 1, 100 adult *Z. variegatus* were released into the cassava canopy 3 h before an application of 2 l $ha^{-1}$ of $2.5 \times 10^9$ conidia $ml^{-1}$, delivered by a hand held ulv sprayer (Micron sprayer Ltd, Bromyard, U.K.). Sixty insects recovered immediately after spraying were incubated at 27 ± 2 °C. Dead insects were placed singly on moistened tissue paper in Petri-dishes at room temperature for possible fungal sporulation on cadavers. In Treatment 2, plots were sprayed as in Treatment 1 before field cages of dimensions 1.5 m × 1.5 m × 1.0 m were erected to cover four cassava plants. Insects were released into them and recovered after 24 h for similar treatment. In Treatment 3, bare patches of ground were created by removing all vegetation from the centres of Treatment 2. For Treatment 4, five sporulating cadavers were arranged in a lattice on the bare soil created in the centres of plots. Erection of field cages and subsequent procedure were as above. Sixty control insects were sprayed with a mixture of kerosene and vegetable oil at the same rate as above. They were kept in a separate room with similar conditions for observation.

### *Chemical protection of conidia from sunlight*

One per cent and 2% (w/v) Oxybenzone powder were added to $2.5 \times 10^9$ conidia $ml^{-1}$ of the mycopathogen suspended in a 50:50 mixture of kerosene and peanut oil. A suspension without the protectant and a control formulation of kerosene and vegetable oil with 2 per cent protectant were also used. Germination tests of conidia in each formulation were carried out on water agar. A conidium was considered germinated when the germ tube length was equal to or greater than the width of the conidium after 20 h. Four control plots (625 $m^2$ each) were fixed upwind (to avoid drift contamination), and treatment plots were randomized. Spray volumes were delivered by hand-held Micro-Ulva with red restrictor at an application rate of 2 l $ha^{-1}$. Field cages were erected immediately after spraying. Thirty insects from the laboratory culture were released into each cage and removed after 24 h for incubation in the

laboratory. The procedure was repeated 1, 2, 3 and 4 days after spraying. Cages were shifted each day to cover a new set of plants (to ensure maximum exposure to sunlight) before fresh insects were introduced. Median-lethal-times were computed by probit analysis. Exponential curves were fitted to estimate rates of loss of biological activity, half-lives and persistence of biological activity.

## Results

Infection through primary and secondary pickup of conidia from leaves and cadavers caused mortalities significantly higher than controls ($P \leq 0.05$) for the 11 days under observation (Table 1).

TABLE 1

*Percentage of grasshoppers killed by diferent sources of* M. flavoviride *(IIBC- 191-609) conidia under field conditions*

| *Source of Conidia* | *Days after incubation* 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|
| (a) | | | | | |
| Primary | 11.0a | 22.0a | 38.9a | 51.9a | 64.8ab |
| Leaves | 10.1a | 21.7a | 33.3a | 45.8a | 51.7ab |
| Soil | 03.7b | 07.4b | 16.7a | 25.9a | 35.2a |
| Cadavers | 49.1c | 85.5c | 90.9b | 94.5b | 100b |
| Control | 01.7b | 01.7b | 03.3c | 03.3c | 03.3c |
| (b) | | | | | |
| Primary | 7.3a | 18.2a | 34.5a | 45.5a | 56.4ab |
| Leaves | 10.1a | 21.7a | 33.3a | 45.8a | 51.7ab |
| Soil | 3.7a | 7.4a | 16.7a | 24.1a | 33.3a |
| Cadavers | 49.1b | 85.5b | 90.9b | 94.5b | 100b |
| Control | 0.0c | 0.0c | 1.7c | 1.7c | 1.7c |

(a) - data from mortalities; (b) - data from mycosing mortalities. Numbers in a column followed by same letters not significantly different ($P \geq 0.05$).

TABLE 2

*Proportion of total mortality and median-lethal- times for mortalities due to different sources of conidia of* M. flavoviride *(IIBC-191-609)*

| *Source of conidia* | *Infection (%)* | *Proportion of infection* | *Probit slope* | *$LT_{50}$ (Days)* |
|---|---|---|---|---|
| Primary | 25.8 (23.3) | 1.9 (1.7) | 1.31a | 9.4 ± 1.5a |
| Leaves | 20.3 (21.3) | 1.5 (1.6) | 1.38a | 10.2 ± 2.4ab |
| Soil | 13.4 (13.5) | 1.0 (1.0) | 1.32a | 12.3 ± 1.1bd |
| Cadavers | 40.5 (41.9) | 3.0 (3.1) | 1.25b | 7.2 ± 0.2c |

Numbers in a column followed by same letters not significantly different ($P \geq 0.05$). Data from mycosing mortalities given in parenthesis.

Up to 8 days of incubation, infection caused by ineffective conidia on the soil surface resulted in mortalities not significantly different from controls ($P \geq 0.05$). Conidia picked up from cadavers caused mortalities significantly higher than all other treatments up to 10 days after incubation.

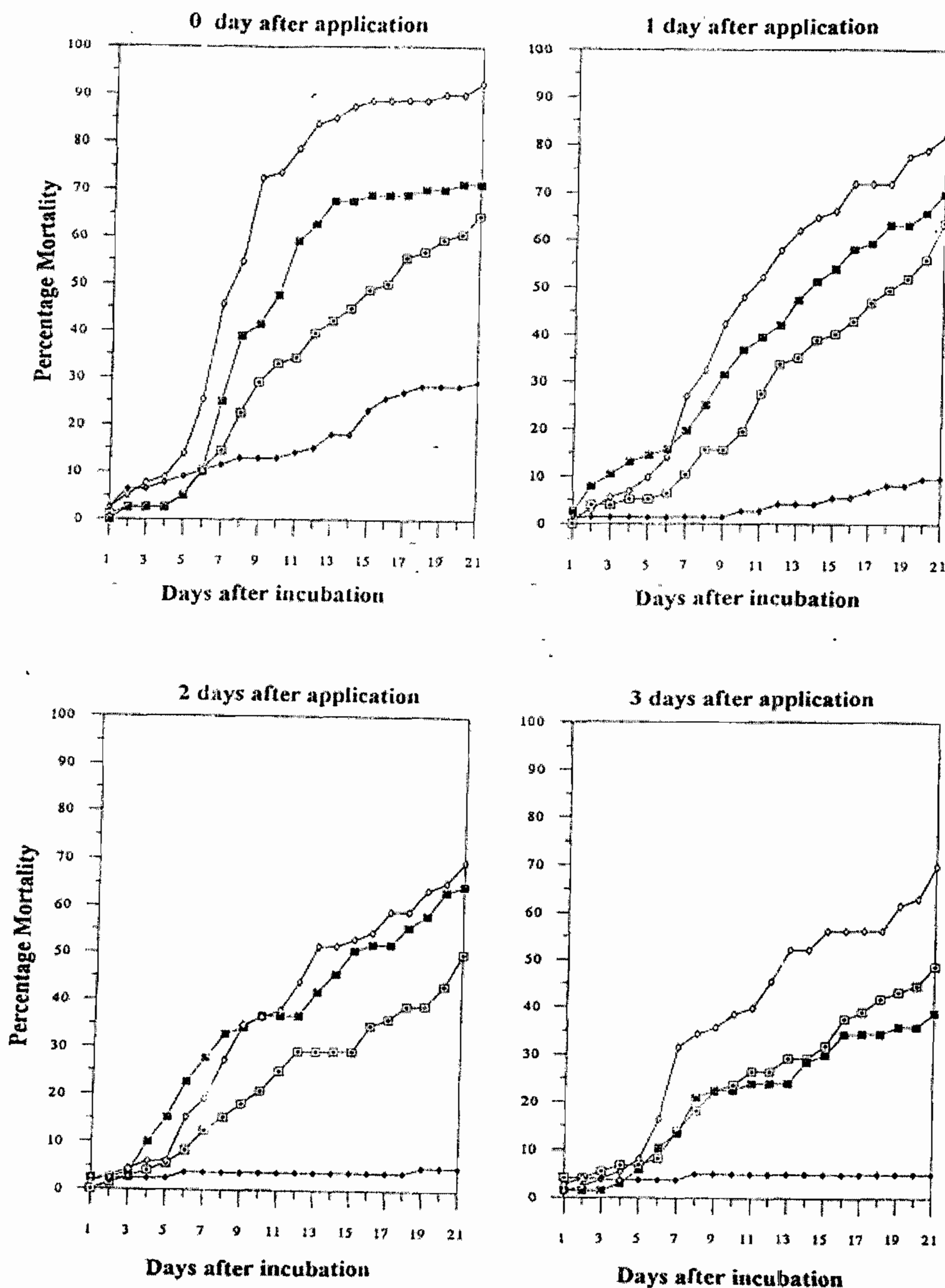


Fig. 1. Mortality-time curves for *Zonocerus variegatus* sprayed with oil formulations with or without oxybenzone; 2% oxybenzone (◇), 1% oxybenzone (▣), without oxybenzone (▣), control (◆)

TABLE 3

*Cumulative mortalities of* Z. variegatus *exposed to oil formulations of* M. flavoviride *(IIBC-191-609) with or without Oxybenzone from day 0 to 3*

| *Treatment formulation* | *Days after application* 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| (a) No Oxybenzone | 64.1 ± 7.2a | 63.7 ± 7.3a | 48.8 ± 6.0a | 39.1 ± 3.1a |
| 1% Oxybenzone | 77.6 ± 6.1a | 68.9 ± 6.2a | 64.7 ± 7.4b | 49.3 ± 7.8a |
| 2% Oxybenzone | 97.1 ± 5.5a | 82.1 ± 7.1a | 69.2 ± 3.1b | 63.4 ± 3.3b |
| Control | 8.5 ± 4.1b | 11.0 ± 5.2b | 5.0 ± 0.0c | 5.5 ± 0.0c |
| (b) No Oxybenzone | 14.2 ± 3.2a | 13.7 ± 5.3a | 11.6 ± 2.1a | 10.0 ± 2.1a |
| 1% Oxybenzone | 15.3 ± 7.2a | 14.8 ± 4.1a | 13.3 ± 3.4a | 12.1 ± 2.0a |
| 2% Oxybenzone | 20.6 ± 2.5a | 17.6 ± 2.1a | 15.2 ± 5.1a | 15.5 ± 5.1a |
| Control | 2.6 ± 2.1b | 0.7 ± 0.0b | 0.0b | 0.0c |

(a) - data from mortalities; (b) - data from mycoses on resulting cadavers. Numbers in a column followed by same letters are not significantly different ($P \geq 0.05$).

TABLE 4

*Median-lethal-times (days) of* Z. variegatus *exposed to oil formulations of* M. flavoviride *(IIBC-191-609) with or without Oxybenzone*

| *Treatment formulation* | *Days after application* 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| (b) No Oxybenzone | 15.6a | 17.8a | 24.0a | 22.7a |
| 1% Oxybenzone | 11.4a | 13.8ab | 15.1b | 31.5a |
| 2% Oxybenzone | 7.9b | 10.1b | 14.1b | 13.9b |

Numbers in a column followed by same letters are not significantly different ($P \geq 0.05$).

TABLE 5

*Effect of Oxybenzone on the biological activity of oil formulations of* M. flavoviride *(IIBC-191-609) against (*Z. variegatus *in the field*

| *Treatmment formulation* | *Rate of loss of biol. activity* | *Days to 50% loss of biol. activity* | *Persistence of biol. activity (days)* |
|---|---|---|---|
| (a) No.Oxybenzone | -0.228 | 1.2 ± 0.5 | 9.78 ± 2.4 |
| 1% Oxybenzone | -0.222 | 1.3 ± 0.6 | 10.0 ± 3.4 |
| 2% Oxybenzone | -0.166 | 2.0 ± 0.9 | 12.5 ± 2.8 |
| (b) No Oxybenzone | -0.198 | 1.7 ± 0.4 | 11.0 ± 1.7 |
| 1% Oxybenzone | -0.170 | 1.8 ± 0.4 | 11.8 ± 2.0 |
| 2% Oxybenzone | -0.138 | 2.4 ± 0.7 | 15.6 ± 2.5 |

(a) - data from mortalities, (b) - data from mycoses on resulting cadavers.

From the 12th day to the end of incubation (21 days), no significant differences were observed among treatments but all were significantly higher than controls. Similar results were obtained using sporulation on resultant cadavers which are proof of death due to mycosis.

Cadavers contributed 40.5 per cent of the total biological effect of infective conidia, primary uptake from direct spray making a slightly higher contribution than secondary uptake from leaves, and the soil the least (Table 2). Also the estimated median-lethal-time ($LT_{50}$) for the cadaver treatment was the shortest while the soil produced the slowest time for 50 per cent mortality. Physical contact between live and dead insects such as attempted mating and cannibalism were observed. Characteristic sigmoid response curves for mycoses mortalities were obtained for the first 4 days after treatment application (Fig. 1). Mycoses caused by formulation with 2% Oxybenzone after 21 days of incubation was significantly higher than formulation without the additive from the first to the third day after application (Table 3). There were no significant differences between treatments on the 4th day but all were signifcantly higher than the control. The speed of kill for 2 per cent protectant was significantly higher than formulation without the protectant (Table 4), which had the fastest rate of loss of biological activity and the shortest half-life and persistence (Table 5).

## Discussion

The importance of secondary uptake of infective *M. flavoviride* (IIBC 191-609) conidia after an initial spray application has been demonstrated. The contribution of infective conidia from leaf surfaces and the soil together amounted to more than 30 per cent of the overall biological effect which is higher than infection caused by direct contact of conidia. Using ultra-violet tracers in conidial suspensions, Lomer *et al.* (1993) reported considerable mortalities from secondary pickup of conidia of the same fungal strain from vegetation and/or soil 13 days after spray application. Secondary uptake of conidia from vegetation is important since the chances of a direct hit of the intended insect targets are low because *Z. variegatus* is lethargic, dropping to lower layers of vegetation at the least intrusion, and in response to temperature and time of day. Oil formulation constituents render the overall controlled ulv droplet density low while enhancing the retention efficiency, particularly on surfaces which are difficult to wet, such as leaves (Young, 1986).

Cadavers produced the highest single contribution to the total biological effect although, in practice, this would be a delayed effect and highly subject to environmental factors such as humidity and temperature. With a spore production potential of $2.8 \times 10^9$ per cadaver, pathogen inoculum density is similar to a spray application using $2.5 \times 10^9$ conidia/ml at 2 l $ha^{-1}$, but the extra protection from sunlight given by melanin in spore walls of mature dry spores in cadavers may explain the high levels of infection (mycosis) and short $LT_{50}$. Cannibalism among grasshoppers is common in nature (Henry, 1972) and was observed in the cadaver treatment in the present study. Contamination of mouthparts could have enhanced infection, however, whether *Z. variegatus* feeds on cadavers in the field when food is non-limiting has not been established. Working with *Verticillium lecannii* in a similar study, Johnson, Huang & Harpen (1988) did not observe cannibalism among grasshoppers confined with cadavers, with pathogen transmission lower than controls. Lack of direct contact with cadavers was cited for lack of pathogen transmission a situation which could have been enhanced by aeration in the plastic cages.

Infection caused by formulations with 2 per cent Oxybenzone was signficantly higher than formulations without the protectant 2 and 3 days after field application $LT_{50}$s were also shorter. $LT_{50}$s are a better indication of infectivity under the present circumstances because unless the fungal strain is non-pathogenic, given sufficient time it would cause a 100 per cent mortality in susceptible hosts which pickup viable conidia. This is because viable conidia multiply inside the host

after infection. *B. bassiana* bioassayed against *Spodoptera frugiperda* (Smith) larvae under natural sunlight produced a half-life of 4.2 days (Gardner, Sutton & Noblet, 1977). Estimates from such studies are influenced by environmental factors as well as intrinsic properties of the biological components most importantly virulence of the pathogen to the test host (Ignoffo *et al.*, 1977). Rainfall may have little influence on conidial stability in the field (Oliveira & Moscardi, 1984). Inactivation of conidia may be directly related to the amount of ultra-violet radiation actually penetrating the cell wall, the effect mediated through germination and growth.

It is concluded that the addition of 2 per cent Oxybenzone in oil formulation enhanced infection of the preparation up to three days after spray application. However, since secondary uptake continues beyond 3 days after a spray application the value of this degree of conidia protection would depend on specific aims and circumstances. For example, the benefit would be fully realised where dense vegetation would make it impossible for a large proportion to be hit directly but with increased chances of secondary pickup of infective spores from leaves and soil surface within 3 days. However, these benefits would have to be weighed against the extra cost of the spray addition.

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