

BIOLOGICAL CONTROL

Effects of Different Formulations on Viability and Medium-Term Storage of *Metarhizium anisopliae* Conidia

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Efeitos de Diferentes Formulações na Viabilidade e no Armazenamento de Conídios de *Metarhizium anisopliae* a Médio Prazo

RESUMO – O objetivo deste trabalho foi avaliar os efeitos de diferentes formulações na viabilidade de conídios de *Metarhizium anisopliae*. No experimento 1, a viabilidade de conídios misturados com oito óleos adjuvantes emulsionáveis (OAE), sete espalhantes adesivos, três óleos vegetais e quatro óleos minerais foi avaliada 24h e 48h depois de espalhados sobre o meio de SDA. Formulações que não apresentaram nenhum efeito adverso sobre a viabilidade dos conídios foram utilizadas no experimento sobre armazenamento. No experimento 2, avaliaram-se os efeitos do armazenamento a 10°C e 27°C durante 40 semanas de conídios de *M. anisopliae* var. *acridum* formulados em cinco OAE, em um óleo vegetal e em uma mistura de dois óleos minerais, além de conídios puros e secos. No experimento 1, as formulações em óleo não causaram nenhum efeito negativo na germinação dos conídios. Os tratamentos com os óleos adjuvantes Actipron®, Ashlade® e Codacide® proporcionaram níveis de germinação semelhantes aos obtidos com óleos minerais e vegetais, 24h após a incubação. O espalhante Ethoken® C12 foi letal para os conídios. No experimento 2, a viabilidade conidial de uma mesma formulação declinou com o passar do tempo. A viabilidade foi mantida acima de 90% a 10°C em todas as formulantes testados. A formulação com Ashlade® (OAE) proporcionou níveis de germinação semelhantes aos obtidos com a mistura Shellsol e Ondina, a 10°C e 27°C durante 40 semanas. Logo, OAE podem ser usados para formular conídios de *M. anisopliae* sem nenhum efeito adverso na viabilidade dos mesmos.

PALAVRAS-CHAVE: Óleo adjuvante, formulação em óleo emulsionável, micoinseticida.

ABSTRACT – The aim of this work was to evaluate the effects of different formulations on the viability of *Metarhizium anisopliae* conidia. In a first experiment, the viability of conidia mixed with eight emulsifiable adjuvant oils (EAO), seven wetter/spreaders, three vegetable oils and four mineral oils was evaluated 24h and 48h after spreading over SDA medium surface. Some formulations, which did not present any adverse effect on conidial viability in the first 24h of incubation, were recommended for the medium-term storage experiment. In a second experiment, the effects of different formulations on medium-term storage (40 weeks) of *M. anisopliae* var. *acridum* conidia were evaluated at 10°C and 27°C. Five EAO, one vegetable oil, a mixture of mineral oils and pure dry conidia were tested. In the experiment 1, the oil formulations did not cause any negative effect on conidial germination. The treatments with the adjuvant oils Actipron®, Ashlade® and Codacide® gave germination levels equal to mineral and vegetable oils after 24h of incubation. The wetter/spreader Ethoken® C12 was lethal to conidia. All tested emulsifiable adjuvant oil-based formulations were compatible to the conidia as the oil-based formulations after 48h of incubation. In the experiment 2, conidial viability within the same formulation declined over time. Conidial viability was maintained above 90% at 10°C in all tested formulants. The conidial suspension with Ashlade® gave equal germination levels to Shellsol plus Ondina, when stored at 10°C and 27°C for 40 weeks. Thus EAO can be used to formulate *M. anisopliae* conidia without adverse effects on viability.

KEY WORDS: Adjuvant oil, emulsifiable oil-based formulation, mycoinsecticide.

The development of a suitable formulation is essential to the successful utilisation of commercial mycoinsecticides (Daoust *et al.* 1983). For example, many formulations can affect the conidial viability resulting in a short shelf life (Moore & Prior 1993). There is a need for careful assessment of the compatibility of formulation components with conidia prior to their use in formulations (Daoust *et al.* 1983). Therefore, one of the first steps in developing a mycoinsecticide formulation is to evaluate the effects of its components on conidial viability to select products compatible with fungal conidia.

Formulating pathogens in oil enhances their infectivity compared to conventional water-based formulations (Agudelo & Falcon 1983, Prior *et al.* 1988, Bateman *et al.* 1993). After that, studies on medium and long-term storage of the entomopathogenic fungi *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) formulated in mineral or vegetable oils are usually carried out (Stathers *et al.* 1993, McClatchie *et al.* 1994, Hedgecock *et al.* 1995, Moore *et al.* 1995, Moore *et al.* 1996). These works were mainly carried out using *M. anisopliae* var. *acridum*, previously known as *M. flavoviride* Gams and Rozsypal.

Views can differ on how long a mycoinsecticide shelf life is required, but estimates range from three to 18 months or even longer (Moore & Prior 1993). The same authors say that it is desirable to maintain the viability to cover two cropping seasons, and long term storage is more for the convenience of the manufacturers than of the farmer and should not be allowed to remain an obstacle.

In general, temperature and moisture content, or the humidity of the storage atmosphere are the major factors which influence conidial longevity (Hong *et al.* 1997). Hedgecock *et al.* (1995) studied the influence of moisture content on temperature tolerance and storage of *M. anisopliae* var. *acridum* in oil formulation and the results demonstrated that viability declined due to high temperatures and high moisture contents. Drying the conidia with silica gel greatly improved high temperature tolerance (McClatchie *et al.* 1994). The optimal moisture content for dried conidia storage was found to be 4-5% and a range of mineral oils proved satisfactory for dried conidia storage (Moore *et al.* 1996). Less moisture content than 4-5% may give better results but it is difficult to achieve.

Suspoemulsions can be defined as heterogeneous formulations consisting of a stable dispersion of active ingredients in the form of solid particles and of fine globules in a continuous water phase combinations (GCPF 1994). They are relatively new to the agricultural market and have a great potential for formulation and application of mycoinsecticides for pest control. They can be sprayed by very low volume/controlled droplet application techniques and still allow the use of conventional hydraulic sprayers and nozzles and water - the cheapest and most readily available carrier liquid for pesticides (Alves *et al.* 1998). A suspoemulsion, containing an emulsifiable adjuvant oil plus conidia of *M. anisopliae* and water, was as infective as oil-based fungal formulations and more infective than conventional water-based fungal formulations against the yellow mealworm, *Tenebrio molitor*, larvae (Alves *et al.* 1998).

Little information is available about the effects of different

commercially available products, which could be used in water-based formulations, on conidial viability and shelf life. Hence, further studies are required. The aims of this investigation were: 1) to evaluate the effects of different formulations on the viability of *M. anisopliae* conidia after 24h and 48h incubation. Some formulations which do not present any adverse effect on conidial viability in the first 24h of incubation, will be recommended for the medium-term storage experiment; 2) to evaluate the effects of a range of commercially available formulations on medium-term storage (40 weeks or 9.3 months) of *M. anisopliae* var. *acridum* conidia, at two different temperatures. Results on emulsifiable adjuvant oil-based conidial formulations were emphasised in this work, because they have a great potential to be sprayed in broad scale agriculture for pest control, where water-based formulations are predominant.

Material and Methods

Experiment 1. Effects of Different Formulations on *M. anisopliae* Conidial Viability. Conidia of *M. anisopliae* isolate 299984 originally obtained from sugarcane froghopper, *Aeneolamia varia saccharina* (Homoptera: Cercopidae), in Trinidad, were used in this experiment because they were highly virulent against the yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), a model insect used in previous bioassays (Alves unpublished).

Aerial conidia were grown on Sabouraud-Dextrose-Agar medium (SDA) in a petri dish of 5 cm diameter and 1 cm deep at 25±0.5°C under darkness. After 10 days, conidia were harvested using a spatula and then suspended in a total volume of 10 ml of 22 different formulations.

Conidia were formulated in different emulsifiable adjuvant oil, wetter/spreader and oil formulations for viability comparison. The water-based and oil-based formulations tested in this study are presented in Tables 1 and 2, respectively.

Conidia were mixed with emulsifiable adjuvant oils and wetter/spreader agents prior to the addition of distilled water to obtain homogeneous suspensions. The stock formulation of each replicate was filtered using a sterilized muslin cloth then mixed using a Whirli Mixer (FSA Laboratory, U.K.) for 3 min. to break down conidial chains and to reduce clumping. All conidial formulations were then calibrated at a concentration of 10⁶ conidia ml⁻¹ using an improved Neubauer's chamber. The resultant formulations remained resting for 2h, to allow the conidia to be sensitised to any adverse effect caused by the formulants. After that, they were thoroughly agitated for 10 seconds using the Whirli Mixer. Aliquots of 0.1 ml from each formulation were then pipetted by an Eppendorf Research piston-stroke pipette (Eppendorf-Nethler, Germany) and thinly spread over the SDA surface in a 5 cm diameter petri dish 1cm deep. The plates were incubated at 25±0.5°C.

Conidial viability tests were carried out after 24h and 48 h of incubation to allow time for conidia recovery from any adverse effect caused by the formulations. Conidia were examined at 400x magnification and germination was recorded when the germ tube was visible. All the conidia in each field of view were counted to obtain at least a total of

Table 1. Water-based formulations, compositions and suppliers.

Formulation	Composition	Supplier
10% Actipron®	Adjuvant oil containing 97% highly refined mineral oil plus emulsifiers	Bayer Ag., Germany
10% Ashlade®	Adjuvant oil containing 99% highly refined paraffinic oil plus emulsifiers	Ashlade Formulations Ltd., U.K.
10% Codacide®	Adjuvant oil containing 95% rapeseed oil and 5% emulsifiers	Microcide Ltd., U.K.
10% Cropspray® 11E	Adjuvant oil containing 99% highly refined paraffinic oil and 1% surfactant	Newman Agrochemicals Ltd., U.K.
10% Cutinol®	Adjuvant oil containing 95% emulsifiable rapeseed oil plus emulsifiers	Techsol Ltd., U.K.
10% Emoleo® R2	Adjuvant oil containing a blend of refined vegetable and emulsifying agent	John L. Seaton & Co. Ltd., U.K.
10% Natur'oil®	Adjuvant oil containing 94% pure rapeseed oil plus emulsifiers	Stoller Chemical Ltd., U.K.
10% Output®	Adjuvant oil containing 60% mineral oil and 40% surfactants	Zeneca Crop Protection, U.K.
0.05% Tween 80	Laboratory wetting agent made of polyoxyethylene-sorbitan monooleate	Sigma® Chemicals, U.K.
0.1% Agral	Non-ionic wetter/spreader containing 948 g/l alkyl phenol ethylene oxide	Zeneca Crop Protection, U.K.
0.1% Enhance®	Non-ionic wetter/spreader containing 900 g/l phenol ethylene oxide condensate	Techsol Ltd., U.K.
0.1% Ethoken®	Cationic surfactant containing 870 g/l polyoxyethylene tallow amine	Techsol Ltd., U.K.
0.1% Ethoken® C12	Cationic surfactant containing bis-2 hydroxyethyl coco-amine	Techsol Ltd., U.K.
0.1% Silwet® L77	Wetter organosilicone co-polymer containing 100% active liquid with a minimum of 80% polyalkylene oxide modified heptamethyltrisiloxane and a minimum of 20% alkyloxypolyethylene glycol methyl ether	Newman Agrochemicals Ltd., U.K.
0.1% Spreader®	Non-ionic wetter/spreader containing nonylphenol ethylene oxide condensate	Pan Britannica Industries Ltd., U.K.

Table 2. Oil-based formulations, compositions and suppliers.

Formulation	Composition	Supplier
Shellsol T	Refined paraffinic oil	Alcohols Ltd., U.K.
Ondina EL	Refined paraffinic oil	Shell Oil Co., U.K.
50% Shellsol T + 50% Ondina EL	Mixture of refined paraffinic oils	Alcohols Ltd. and Shell Oil Co., U.K.
Isopar M	Refined paraffinic oil	Exxon Company, U.S.A.
Peanut oil	Vegetable oil with high viscosity and low volatility	Sigma® Chemicals, U.K.
Soyabean oil	Vegetable oil with high viscosity and low volatility	Sigma® Chemicals, U.K.
Sunflower oil	Vegetable oil with high viscosity and low volatility	Sigma® Chemicals, U.K.

300 conidia in a range between 300 and 400, for each replicate (Moore *et al.* 1993).

The experiment had a factorial design with two main factors (formulations with twenty-two levels and incubation time with two levels) and three replicates. Factorial analysis of variance (ANOVA) on conidial viability data was performed using the statistical package SPSS® for Windows™ (Norusis & SPSS® 1993), after transforming the data to $\text{Arcsin} \sqrt{(\%/100)}$ to meet the requirements of ANOVA for a normal data distribution and homogeneity of variances. The results are presented untransformed in tables.

Experiment 2. Effects of Different Formulations on Medium-term Storage of *M. anisopliae* var. *acidum* Conidia. The isolate IMI 330189 of *M. anisopliae* var. *acidum*, originally isolated from a grasshopper *Ornithacris cavroisi* (Orthoptera: Acrididae) in Niger, was used in this experiment because it has been applied in several bioassays and field trials against locusts and grasshoppers in Africa and Australia (Lomer *et al.* 1993, Bateman *et al.* 1994, Milner *et al.* 1994, Price *et al.* 1997). This isolate is commercially available in the South African market (Bateman *et al.* 1998).

Aerial conidia were produced using a standard two-phase production system (Jenkins *et al.* 1998), which consists of a submerged liquid culture followed by conidiation on a solid substrate. The submerged liquid culture uses a simple liquid medium containing 20 g l⁻¹ of dried yeast, 20 g l⁻¹ of glucose and 1000 ml of tap water and generates hyphal bodies and mycelium for inoculation into the second phase. Autoclaved broken white rice in polypropylene bags was used as solid substrate in the second phase. The bags were incubated for 12 days at 25±1°C for fungal development and sporulation. The plastic bags were then opened, the rice with conidia was spread out in clean plastic trays and placed inside a Fisons drying cabinet (Fisons Scientific Equipments, U.K.) at 20±3°C. After five days, the moisture content was around 20%. Conidia were then extracted from the rice by sieving through brass laboratory sieves (300 mm mesh). After sieving, conidia were dried further in an auto-desiccator cabinet with a built-in hygrometer (Whatman International Ltd., U.K.) for four days to reduce the moisture content to around 5%, because a suitable optimal moisture content for long term dried conidia storage was found to be 4-5% (Moore *et al.* 1996).

The initial moisture content of six randomised 0.5 g samples of conidia, from the same batch which was used in the experiment, was assessed by a Mettler-Toledo HG53 Halogen Moisture Content Analyser (Mettler-Toledo, Switzerland). The analyser detected a mean moisture content of 4.93%.

One gram of conidia was weighed and mixed with 200 ml of distilled water plus 0.05% Tween 80 using a Whirli Mixer for 3 min. to break down conidial chains and to reduce clumping. The number of conidia/ml was counted using an improved Neubauer's chamber and the number of conidia g⁻¹ was calculated. This procedure was repeated six times. The mean result was 4.2 x 10¹⁰ conidia g⁻¹. The appropriate number of grams of conidia to be added to the formulants and to facilitate the germination countings later, was calculated. Then, 0.01 g of pure conidia containing approximately 4.2 x

10⁸ conidia were mixed with 15 ml of eight formulations selected from the previous experiment (with conidial viability above 90% after 24h of incubation), resulting in a suspension with approximately 2.8 x 10⁷ conidia ml⁻¹. The formulations were the following: pure Ashlade®, pure Codacide®, pure Cropspray® 11E, pure Emoleo® R2, pure Natur'1 oil®, pure peanut oil and in a mixture of Shellsol T plus Ondina EL.

There was also a treatment with 0.8 g of pure dry conidia (technical material) as a standard for general comparisons. Fresh dry non-indicating silica gel beads (20%) were added to 15 ml of each formulation and to the pure dry conidia treatment, to absorb any remaining moisture content from the formulations and to maintain the moisture content between 4 and 5% (Moore *et al.* 1996). The 20 ml Universal bottles were sealed with parafilm and stored at 10 °C and 27°C.

Viability of stored conidia from different formulations was assessed one day after formulation to obtain the initial germination level and at intervals of five weeks for 40 weeks. To facilitate the germination counting, 0.1 ml aliquots (containing approximately 2.8 x 10⁶ conidia) from each stored conidial suspension were pipetted by an Eppendorf pipette and mixed with 5 ml of distilled water for the adjuvant oil treatments, 5 ml of pure Shellsol T for the peanut oil and for Shellsol plus Ondina treatments (resulting in a suspension with approximately 5.6 x 10⁵ conidia ml⁻¹).

For the pure dry conidia treatment, a small amount of conidia weighing approximately 0.007 g was mixed with 5 ml of water plus 0.05% Tween 80 treatments (resulting in a suspension with approximately 5.88 x 10⁷ conidia ml⁻¹). It was diluted 100 times to obtain approximately 5.88 x 10⁵ conidia ml⁻¹.

All resultant diluted conidial formulations were then mixed using a Whirli Mixer for 3 min. to homogenize the suspensions. Finally, a new aliquot of 0.1 ml (containing approximately 5.6 x 10⁴ conidia) was pipetted from each treatment and thinly spread over the SDA surface in a 5 cm diameter petri dish 1cm deep. The plates were incubated at 25±0.5°C. Conidial viability tests were carried out after 24h of incubation using the same methodology of the previous experiment (Moore *et al.* 1993).

To check that the moisture content after 40 weeks from the three replicates with pure dry conidia stored at the two different temperatures was similar to the initial values, new assessments were carried out using the same equipment and methodology.

This experiment had a factorial design with three main factors (formulations with eight levels, temperature with two levels and storage time with nine levels) and three replicates. A three-way ANOVA on conidial viability data was performed using the same statistical package used in the previous experiment. The data were transformed to $\text{Arcsin} \sqrt{(\%/100)}$ to meet the requirements of ANOVA for a normal data distribution and homogeneity of variances.

Results

Experiment 1. Effects of Different Formulations on *M. anisopliae* Conidial Viability. There were significant differences between formulations on conidial viability (df = 21, 131, F = 278.31, P<0.05) and between incubation time

(df = 1, 131, F = 496.79, P < 0.05). There was also a significant interaction between the main factors (df = 21, 131, F = 26.26, P < 0.05). These results are shown in Table 3.

Conidial germination on SDA after 24h of incubation presented more significant differences between formulations than after 48h of incubation. None of the oil-based formulations caused any negative effect on conidial germination. Only soyabean oil was significantly different from the other oils after 24h, but not after 48h. The oils Shellsol and Ondina, in the mixture or individually, gave the highest conidial germination values after 24h of incubation. Peanut oil, Tween 80 and Agral also gave high germination values after 24 and 48h (above 99%). The emulsifiable adjuvant oils Actipron®, Ashlade® and Codacide® were equally compatible to the conidia such as Tween 80, Agral and the oil-based formulations. Cropspray®, Emoleo®, Cutinol® and Natur'l oil® gave germination levels above 92% after 24h. Conidia formulated with all tested adjuvant oils gave germination values between 99 and 100% after 48h.

Conidia formulated with the wetter/spreader Enhance® were also as viable as conidia formulated with Tween and Agral, after 24h and 48h. Silwet® caused a slight inhibition

on conidial germination in the first 24h, but there was a recovery after 48h. Spreader®, Ethoken® and Ethoken® C12 and the emulsifiable adjuvant oil Output® affected the germination of conidia after 24h of incubation. A high germination level was observed after 48h of incubation for the treatments with Spreader® and Output®. The treatment with Ethoken® C12 was lethal to conidia.

Experiment 2. Effects of Different Formulations on Medium-term Storage of *M. anisopliae* var. *acridum* Conidia. The viability of conidia stored in eight different formulations at two different temperatures during 40 weeks was significantly affected by formulations (df = 7, 431, F = 36.91, P < 0.05), temperature (df = 1, 431, F = 386.73, P < 0.05) and time of storage (df = 8, 431, F = 339.30, P < 0.05) (Table 4). There were also significant interactions between the main factors formulations x temperature (df = 7, 431, F = 15.26, P < 0.05), formulations x time of storage (df = 56, 431, F = 4.84, P < 0.05), temperature x time of storage (df = 8, 431, F = 86.10, P < 0.05) and formulations x temperature x time of storage (df = 56, 431, F = 4.08, P < 0.05). There were no significant differences between periods of storage,

Table 3. Conidial viability of *M. anisopliae* formulations, 24h and 48h after incubation at 25±0.5°C.

Formulation (plus conidia)	Conidial viability with standard error (%)					
	24h			48h		
Shellsol T plus Ondina EL	99.7 ± 0.16	a	A	100.0 ± 0.00	a	A
Ondina EL	99.7 ± 0.16	ab	A	100.0 ± 0.00	a	A
Shellsol T	99.7 ± 0.01	ab	A	100.0 ± 0.00	a	A
Peanut oil	99.4 ± 0.35	ab	A	100.0 ± 0.00	a	A
Water plus 10% Actipron®	99.4 ± 0.30	ab	A	100.0 ± 0.00	a	A
Water plus 0.05% Tween 80	99.4 ± 0.34	ab	A	100.0 ± 0.00	a	A
Water plus 10% Ashlade®	99.4 ± 0.24	abc	A	100.0 ± 0.00	a	A
Isopar M	98.9 ± 0.97	abc	A	100.0 ± 0.00	a	A
Water plus 0.1% Agral	98.1 ± 0.24	abcd	A	100.0 ± 0.00	a	B
Water plus 10% Codacide®	98.1 ± 0.90	abcd	A	100.0 ± 0.00	a	B
Water plus 0.1% Enhance®	97.7 ± 0.30	bcd	A	100.0 ± 0.00	a	B
Water plus 10% Cropspray®	96.8 ± 0.42	cde	A	100.0 ± 0.00	a	B
Water plus 10% Emoleo®	94.6 ± 2.29	de	A	99.7 ± 0.31	a	B
Water plus 10% Cutinol®	94.6 ± 2.56	de	A	100.0 ± 0.00	a	A
Water plus 10% Natur'l oil®	92.1 ± 1.38	ef	A	99.8 ± 0.17	a	B
Sunflower oil	91.2 ± 3.83	ef	A	100.0 ± 0.00	a	B
Water plus 0.1% Silwet® L77	88.3 ± 1.07	fg	A	94.9 ± 1.37	c	B
Soyabean oil	82.0 ± 0.58	g	A	100.0 ± 0.00	a	B
Water plus 0.1% Spreader®	44.1 ± 3.76	h	A	96.9 ± 1.32	b	B
Water plus 10% Output®	35.5 ± 8.43	hi	A	99.7 ± 0.31	a	B
Water plus 0.1% Ethoken®	28.8 ± 1.34	i	A	64.5 ± 5.38	d	B
Water plus 0.1% Ethoken® C12	0.0 ± 0.00	j	A	0.0 ± 0.00	e	A

Means followed by the same small letter within the same column, and by the same capital letter within the same row are not significantly different (P < 0.05).

temperatures and formulations over the first 15 weeks. Only the conidial viability results of the initial week and from the week 20 until 40 are shown in Table 4.

Conidial viability within the same formulation significantly declined over time, at different rates depending on the composition of the formulation. This effect was more pronounced between 35 and 40 weeks of storage, mainly at 27°C.

Viability of conidia in medium-term storage was better at 10°C than 27°C for all tested formulations and there were more significant differences between formulations stored at 27°C than formulations stored at 10°C. Conidia from different formulations stored at 10°C for 40 weeks did not give any significant difference in viability and they remained above 91%.

Pure dry conidia and peanut oil were the only treatments that maintained conidial viability higher than 90% in both temperatures after 40 weeks of storage. The treatment with the emulsifiable adjuvant oil Ashlade® gave equal germination levels to Shellsol plus Ondina, when stored at 27°C for 40 weeks. The emulsifiable adjuvant oils Codacide®, Cropspray®, Natur'l oil® and Emoleo® did not maintain the conidial viability higher than 70% after 40 weeks at 27°C. Results on mean moisture content of pure dry conidia

stored for 40 weeks at 10°C was 4.3% and 4.3% at 27°C. These numbers were very close to the mean initial value (4.9%).

Discussion

Compatible formulations with fungal conidia were selected in the first experiment. In practical terms, it was useful to carry out conidial viability tests after 24 and 48h of incubation for the purpose of this experiment, because conidia were capable of recovery from adverse effects caused by some tested formulations and gave high germination levels after 48h, which could explain the significant interaction between formulations and incubation time.

The mixture containing Shellsol plus Ondina has been successfully tested in Africa to control locusts and grasshoppers (Bateman 1997, Price *et al.* 1997). The wetting agent Tween 80 has been used in laboratory bioassays to facilitate suspension of hydrophobic conidia (Marques *et al.* 1981, Alves 1986, Prior *et al.* 1988). The wetter/spreader Agral has been used in Brazil to spray conidia of *M. anisopliae* to control the sugarcane froghopper, *Mahanarva posticata* (Stal), and the pasture land froghoppers, *Aeneolamia selecta selecta* (Walker), *Deois flavopicta* (Stal)

Table 4. Conidial viability ($X \pm s.e.$) of *M. anisopliae* var. *acidum* formulations stored at two different temperatures over 40 weeks, 24h after incubation at $25 \pm 0.5^\circ\text{C}$.

Formulants plus conidia	Conidial viability (%)					
	0 week (initial)		20 weeks		25 weeks	
	10°C	27°C	10°C	27°C	10°C	27°C
Conidia TC	97.2±0.06 a A	98.7±0.06 a A	97.1±0.31 a A	96.9±0.50 ab A	96.9±0.35 a A	96.2±0.76 a A
Ashlade®	99.3±0.11 a A	99.4±0.18 a A	95.0±1.09 a B	95.4±1.73 ab B	94.0±1.09 a B	89.3±1.99 b CD
Codacide®	99.6±0.11 a A	99.6±0.10 a A	97.9±0.15 a AB	96.9±0.38 ab AB	97.3±0.18 a AB	85.7±1.13 c C
Cropspray®	98.9±0.27 a A	99.4±0.18 a A	96.4±0.98 a AB	95.0±1.11 b B	94.4±0.11 a B	91.5±0.92 b BC
Emoleo®	99.5±0.11 a A	99.6±0.22 a A	96.9±0.06 a AB	97.2±0.43 ab AB	96.6±0.10 a AB	90.7±2.82 b CD
Natur'l oil®	99.6±0.10 a A	99.4±0.18 a AB	95.5±0.40 a C	95.8±0.97 ab BC	95.2±0.57 a C	85.6±4.89 c D
Peanut oil	99.7±0.19 a A	99.9±0.11 a A	97.8±0.22 a ABC	98.8±0.62 a AB	97.1±0.18 a ABC	97.5±0.63 a ABC
Shellsol plus Ondina	99.2±0.22 a AB	99.6±0.11 a A	95.8±1.12 a BC	94.8±0.78 b CDE	95.2±1.04 a CDE	91.7±1.20 b DE
	30 weeks		35 weeks		40 weeks	
	10°C	27°C	10°C	27°C	10°C	27°C
Conidia TC	96.5±0.62 a A	94.0±0.17 ab B	95.6±0.56 ab AB	92.3±1.05 a BC	95.4±0.53 a AB	90.2±1.31 a C
Ashlade®	92.7±1.49 b B	89.3±1.97 c CD	92.1±1.60 b B	86.2±2.20 bc D	91.8±1.79 a BC	72.9±2.99 b E
Codacide®	96.0±0.53 ab AB	85.6±1.30 d C	95.6±0.58 ab B	82.5±1.78 cd C	95.1±0.58 a B	56.8±0.67 d D
Cropspray®	94.1±0.10 ab B	90.7±0.97 bc C	93.7±0.10 ab BC	84.8±1.00 bc D	93.4±0.33 a BC	57.3±1.61 d E
Emoleo®	94.9±0.46 ab B	90.0±2.72 c D	94.7±0.57 ab B	83.7±3.82 bc E	94.1±0.74 a BC	62.5±7.84 c F
Natur'l oil®	95.3±0.40 ab C	85.0±4.52 d D	94.8±0.33 ab C	79.4±3.15 d E	93.6±0.15 a C	58.6±2.39 d F
Peanut oil	96.7±0.08 a ABC	96.8±0.38 a ABC	96.2±0.18 a ABC	95.0±1.21 a CD	95.2±0.47 a BC	91.4±0.99 a D
Shellsol plus Ondina	95.1±0.94 ab DE	91.3±1.34 bc E	94.6±0.84 ab CDE	87.0±2.07 b F	94.0±0.68 a CDE	74.7±4.28 b G

Means followed by the same small letter within the same column, and means followed by the same capital letter within the same row are not significantly different ($P < 0.05$).

TC = Technical material

and *Zulia entreriana* (Berg.) (Alves 1986). These three formulations proved to be highly compatible with conidia and to be appropriate standards for making comparisons with the emulsifiable adjuvant oils and the other wetter/spreaders.

Emulsifiable adjuvant oils contain emulsifiers. In the case of suspoemulsions, the emulsifiers (which can be surfactants) are the agents capable of forming stable oil-in-water emulsions, thereby providing a basis for dispersion of the active ingredient in suspension (Field & Dastgheib 1996). In this study, all emulsifiable adjuvant oils gave conidial germination levels above 92% after 24h and above 99% after 48h, except Output which was the only adjuvant oil that caused an adverse effect on conidial viability. This product has a high level of surfactants (40%) which could be the cause for that initial adverse effect on conidial viability, but it was not too severe to cause a permanent inhibition on germination after 48h of incubation. All emulsifiable adjuvant oils such as Tween 80, Agral and the oil-based formulations were equally compatible with the conidia after 48h of incubation. These results mean that the tested emulsifiable adjuvant oils can be used to formulate *M. anisopliae* conidia without permanent adverse effects on conidial viability.

The wetter/spreader Enhance® did not cause any adverse effect on conidial viability after 24h. However, Silwet® and Spreader® caused adverse effects in the first 24h, but there was great recovery after 48h. Only Ethoken® and Ethoken® C12 caused severe adverse effects on conidial viability without a good recovery and without any recovery, respectively. The cationic surfactants in their composition could explain this effect. The other wetter/spreaders have only non-ionic surfactants, including Agral.

Non-ionic surfactants are the most common type of surface active agents, deriving their hydrophilic characteristics from nonionizable groups such as phenolic and alcoholic hydroxyls, carbonyl oxygens of esters and amides, ether oxygens, and analogous sulphur-containing configurations. Their nonionic nature is often advantageous in formulations because of their lack of reactivity with ions present in hard water (e.g., calcium, magnesium, or ferric ions) and their chemical compatibility with many other chemicals (Field & Dastgheib 1996). Cationic surfactants ionise in water such that the hydrophilic group becomes positively charged. Primary, secondary, tertiary, and quaternary amino groups and ammonium cations are the most common types of cations formed by these surfactants (Field & Dastgheib 1996). The two cationic wetter/spreaders possibly ionised in water and formed amino groups and ammonium cations, which were toxic to the fungal conidia. It is possible that these types of products are more appropriate to be added to chemical pesticides and not to biological pesticides.

In the experiment on medium-term storage of conidia, viability was better maintained at 10°C than 27°C for all tested formulations. The conidial viability within the same formulation significantly declined over storage time. This effect was noticed only after 20 weeks (4.7 months) of storage for both temperatures (mainly at 27°C).

Practical requirements for field applications are minimal loss of viability after at least three months of storage, at 30°C. If at least twelve months of storage are required, cooled

storage would be necessary (Moore *et al.* 1996). In this work, conidia formulated in all types of oils and emulsifiable adjuvant oils retained >80% viability after seven months of storage at 27°C. Under cooled conditions (10°C), conidia retained >90% viability after 40 weeks in all formulations and they probably could retain high viability for more than twelve months. These results explain the significant interaction between temperature and formulants and between temperature and time of storage.

The mean moisture content of pure dry conidia was 4.93% before the beginning of the experiment and it decreased to 4.25% after 40 weeks of storage. The difference could be caused by the addition of dry non-indicating silica gel beads, which absorbed a small amount of moisture, but the important criterion was that the moisture was maintained between 4 and 5% (Moore *et al.* 1996) over the 40 weeks of the experiment.

Stathers *et al.* (1993) obtained no low viability when conidia were stored in Codacide®, peanut oil and Shellsol for more than one week at 25°C. This was possibly due to the fact that conidia with high moisture content were scraped directly from agar slopes without drying. In the present work, conidia were dried to 4-5% moisture before they were placed in all formulations, and the conidial viability in these formulations remained above 80% when stored at 27°C for at least 35 weeks. These results confirm that drying conidia greatly improved high temperature tolerance and in a large scale system, conidia need to be dried before being placed in oil (McClatchie *et al.* 1994). The drying of conidia, their final moisture content and the temperature of storage are important factors in conidial longevity (Hong *et al.* 1997).

The reason why the conidial viability declined more in the emulsifiable adjuvant oils than in peanut oil, Shellsol plus Ondina and as pure dry conidia, after 25 weeks stored at 27°C, could be explained by the presence of emulsifiers in their composition as mentioned above (Field & Dastgheib 1996). The emulsifiers did not cause any pronounced adverse effect during the first 20 weeks, but after that, the conidial viability could be affected at different rates (Table 4), depending on the composition of the formulations. Peanut oil and Shellsol plus Ondina do not have emulsifiers, consequently there would be no emulsifier expected effects from these. This could explain the significant interaction between formulations and time of storage and between formulations, temperature and time of storage.

The viability of conidia mixed with Ashlade® (which contains approximately 1% emulsifiers) declined more rapidly than pure dry conidia or conidia mixed with Peanut oil, but not more rapidly than Shellsol plus Ondina after 40 weeks of storage at the temperature of 27°C.

In practical terms, differences between 99% (initial) and 90% (40 weeks) that occurred with conidia formulated in all emulsifiable adjuvant oils and stored at 10°C, for example, may not be particularly significant because the effectiveness of conidial formulations are not significantly affected with germination levels $\geq 80\%$, when applied against the insect pest (Moore *et al.* 1995). Conidial virulence was not tested in this work, but results from a study carried out by Moore *et al.* (1995), where the conidial viability declined over 37 months in storage, showed that the virulence of the

formulations was not reduced after 30 months.

Results from the present work showed that emulsifiable adjuvant oil fungal formulations can be used to formulate and store conidia for medium-term and probably for long-term under cooled conditions similarly to oil-based formulations. In addition, they can also be sprayed with the existing delivery systems and used in broad scale agriculture where water-based formulations are predominant.

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