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Toxicity/Pathogenicity Evaluation of *Metarhizium anisopliae* LMA-06 by Means of Oral and Intranasal Dosing

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Abstract: Entomopathogenic fungus *Metarhizium anisopliae* has been employed on a large scale in Brazil, Asia and Eastern Europe and is registered in the United Kingdom and United States for the control of various insect pests. In order to evaluate the toxicity/pathogenicity of *M. anisopliae* strain LMA-06 to Sprague Dawley rats, it was given a single dose of $2.2x10^8$ colony-forming units by oral route and $1.1x10^8$ colony-forming units by pulmonary administration. Clinical examinations of animals were performed daily and body weight was weekly measured. For infectivity evaluation, *M. anisopliae* was enumerated from organs and corporal fluids of three treated animals per sex, sacrificed 3 days after and at one week intervals after dosing. Clearance was estimated by means of faeces collection (oral assay) and lungs examination (pulmonary assay). Gross necropsy was performed on all animals at interim and final sacrifice. There were no deaths, no significant infection of test animals and no evidence of pathogenicity or treatment-related toxicity. It was concluded that, at the tested doses, *Metarhizium anisopliae* strain LMA-06 is not pathogenic following oral or pulmonary administration.

Key words: *Metarhizium anisopliae,* toxicity, pathogenicity, infectivity, clearance

INTRODUCTION

Environmental concerns and health risks associated with the use of synthetic chemical insecticides have stimulated efforts to develop biological control agents for integrated pest management. There are a number of microbial agents including fungus, protozoa, virus and bacteria which are at present considered for this purpose. Fungi are of special relevance for biological control. Because most fungi invade the host insect through the exoskeleton, they provide the only practical means of microbial control of insects which feed by sucking plant or animal fluids[1] and for many coleopteran pests which have no known viral or bacterial diseases[2]. Among fungi, great efforts had been made in the development of entomopathogenic Hyphomycetes[3] .

The Hyphomycetes have simple life cycles and lack sexual reproduction and many of them have considerably broader insect host ranges[4]. *Metarhizium anisopliae* has been employed on a large scale in Brazil, Asia and Eastern Europe and is registered in the UK and USA for control of various insect pest[5,6]. It is used at biosafety level 1 as it is a well-characterized agent of minimum potential hazard to laboratory personnel and the environment *M. anisopliae* has proved excellent for biological control research against insect pests since has a clonal population structure (strains persist over time and space), thus, gene exchange in nature is a very rare event *M. anisopliae* is a cosmopolitan pathogen of innumerable insect species, but contains a diverse assemblage of genotypes and individual isolates or pathotypes exhibit a substantially restricted host range[3] and demonstrate low environmental impact[7,8]. On the basis of weight of product required to achieve LD50, *Metarhizium* spores comprise a very potent insecticide as compared with chemical agents, but the speed of kill by the fungal product is slower[9].

With the aim of the toxicological evaluation of *M. anisopliae* strain LMA-06, oral and pulmonary toxicity-pathogenicity studies were carried out. These assays are included in the TIER 1 of the Microbial Pesticide Test Guidelines of the EPA, which has the overall purpose of providing a toxicological evaluation of a microbial pest control agent preparation with respect to pathogenicity and infectivity/unusual persistence[10].

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MATERIALS AND METHODS

Studies were accomplished according to the international principles of Good Laboratory Practice[11] and the principles of care and use of laboratory animals[12,13].

M. anisopliae strain LMA-06 was obtained from LAVERLAM Laboratories (Cali, Colombia).

Sprague Dawley rats were obtained from CENPALAB (Havana, Cuba). The animals were approximately 7 weeks of age when the study began and housed three per cage. Water and feed (EMO 1002, ALYco®, CENPALAB) were sterilized and available *ad libitum*. Eighteen rats/sex were used for oral toxicity assay, with a mean body weight of 204 g for females and 276 g for males. Twenty one rats/sex were used in the pulmonary toxicity assay, with a mean body weight of 177 g for females and 230 g for males. Room environment was 25-28°C, 75-85% relative humidity and 12 h photoperiod.

Animals were randomly distributed into three groups. Treated rats in the acute oral toxicity/pathogenicity assay (12/sex) received $2.2x10^8$ Colony Forming Units (CFU) by intragastric administration, while treated animals in acute pulmonary toxicity/pathogenicity study (15/sex) were inoculated with $1.1x10^8$ CFU through intranasal instillation. A untreated control group of six animals per sex was established.

The observation period was 21 days after dosing. A careful clinical examination was made daily. Observations included evaluation of skin and fur, eyes and mucous membranes and also respiratory, circulatory, autonomic and central nervous system, somatomotor activity and behaviour pattern. Particular attention was directed to observation of tremors, convulsions, diarrhea, lethargy, salivation, sleep and coma. Weights of individual animals were determined shortly before *M. anisopliae* was administered, weekly thereafter and at interim or final sacrifice.

For evaluating infectivity, *M. anisopliae* was enumerated from kidney, brain, liver, spleen, blood, mesenteric ganglion and lungs or caecum content (according to the given assay) of three treated animals per sex, sacrificed at 3 days after and at one week intervals after dosing. Sacrifice days post-dose were established taken into account the infective process of fungus into his insect host.

Feces and lungs from test animals were collected soon after dosing and at 3, 7, 14 and 21 days and examined for the presence of *M. anisopliae* to estimate clearance after oral or pulmonary administration. Quantification of *M. anisopliae* (viable count) was performed by means of culture of serial dilutions of samples in Sabouraud dextrosebased medium. Count of colonies was made 3 days after, taken into account the typical colonies (green color); the result of this count was multiplied by the milliliter factor (5).

At interim or final sacrifice, the animals were bleed through a femoral vein and applied a cervical dislocation maneuver. Gross examination of the external body surface, orifices, cranial, thoracic and abdominal cavities and all organs was conducted.

The statistical significance of differences between groups was determined using Student's t test (Statistical Package Scientific System, SPSS for Windows, Copyright SPSS Inc., 1997). Statistical significance was assessed at the $p<0.05$ level.

RESULTS

Both assays finished with a 100% survival. The animals did not show clinical alterations, maintaining normal behavior of the species[13], except two control males who showed injuries at testis, reaching to the rupture of the scrotum and subsequent healing of the wounds. In order to establish the presence of infectious agents or the inoculated fungus, an exude was made. It was found the presence of *E. coli* and *Proteus* sp. Also, two treated animals (7 day sacrifice) showed some macroscopic alterations. One of them showed brown-yellowish liver and kidneys, being kidneys swollen. The other rat showed hemorrhagic mesenteric ganglion. All alterations were microscopically studied and unspecific hemorrhage was corroborated.

Statistical analysis of body weight behavior did not reveal significant differences between treated and control groups (Fig. 1).

Clearance of *M. anisopliae* was limited and insignificant, being fungus poorly recovered from feces during experimental period (Table 1). Fungus enumeration in tissues, organs and body fluids of animals from interim sacrifice was also limited, being isolated only 3 days after administration of *M. anisopliae* (Table 2). Control animals failed to show infection.

Animals did not show clinical alterations, maintaining normal behaviour of the species[13]. One animal sacrificed on day 14 showed a limited congestion at the median lobule of the liver. Microscopic study of the affected area evidences a hemorrhagic zone, neither inflammatory reactions nor any change indicating fungus pathogenicity. *M. Anisopliae* clearance was immediate, obtaining a isolation of fungus 3 h after inoculation (Table 1). Infectivity study showed positive isolation only on 3 and 7 days (Table 3). Control animals failed to reveal infection. Statistical analysis of body weight behavior did not reveal significant differences between treated and control groups (Fig. 2).

Table 1: Quantitative recovery of *Metarhizium anisopliae* LMA-06 from feces and lungs of treated rats

		Time of sampling				
Assay	Animal	3 h	3 days	7 days	14 days	21 days
Oral	F	0		0	0	0
		$2x10^3$	0	0	0	0
		25	0	0	25	0
	M	0	0	0	0	0
		0	0	0	0	0
		0	25	$2.5x10^2$	0	0
Pulmonary						
	F	$2.5x10^2$	25	0	0	0
		0	25	0	0	0
		$1x10^2$	0	0	0	0
	M	25	0	0	0	0
		75	0	0	0	0
		25	0	0	0	0

F: Female, M: Male

Table 2: Quantitative recovery (colony-forming units) of *Metarhizium anisopliae* LMA-06 from tissues, organs and body fluids of treated rats on 3 days in acute oral toxicity/pathogenicity test

Animal		Kidneys	Brain	Liver	Spleen	Lungs	Blood	Lymph nodes
Females	1	0	25	0	0	0	50	25
	2	0	0	0	0	25	0	0
	3	0	0	0	0	0	0	0
Males	1	0	0	25	25	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0

Table 3: Quantitative recovery (colony-forming units) of *Metarhizium anisopliae* LMA-06 from tissues, organs and body fluids of treated rats on 3 and 7 days in acute pulmonary toxicity/pathogenicity test

Days	Animal	Kidneys	Brain	Liver	Spleen	Blood	Mesenteric ganglion	Caecum content
3	F	25	250	25	25	25	0	250
		0	0	0	50	25	25	50
		0	0	25	0	25	0	0
	M	0	0	0	0	0	0	125
		0	0	0	0	0	0	0
		0	0	0	0	0	0	0
7	F	0	0	0	0	0	0	25
		0	0	0	0	0	0	0
		0	0	25	0	0	0	0
	M	0	0	0	0	0	0	0
		0	0	0	0	0	0	250
		0	0	0	0	0	0	2500

F: Female, M: Male

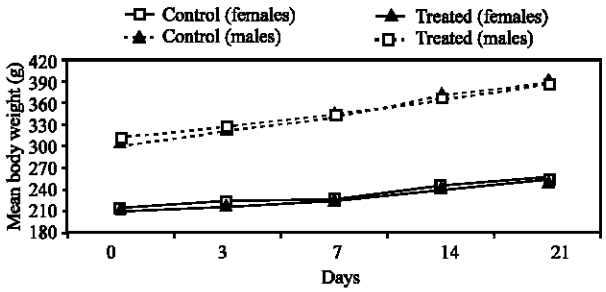

Fig. 1: Changes in body weights of Cenp:SPRD rats treated with *Metarhizium anisopliae* LMA-06 in acute oral toxicity/pathogenicity test

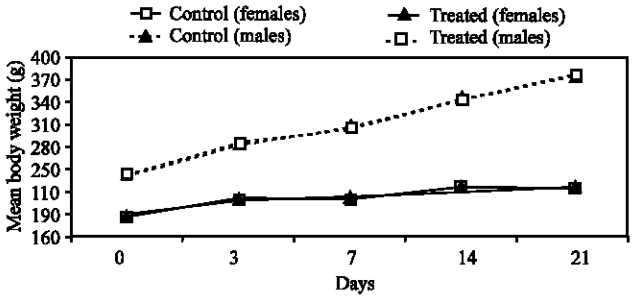

Fig. 2: Changes in body weights of Cenp:SPRD rats treated with *Metarhizium anisopliae* LMA-06 in acute pulmonary toxicity/pathogenicity test

DISCUSSION

There has been considerable interest in the use of fungi as microbial control agents of pests and some have been developed as commercial biocontrol products. However, fungi also contain many species that are pests themselves. Consequently, the development and use of fungi as biocontrol agents requires an assessment of unintended effects associated with their use. Thus, pathogenicity towards the target host is usually the desired effect. However, pathogenicity towards non-target organisms could be an unintended effect. Fungi, including species intended for biological control, can infect a variety of hosts, which sometimes include mammals.

In the acute oral toxicity test, the opportunistic nature of the microorganisms found at testis, besides the characteristics of the injuries, allowed to associate the clinical manifestations with a skin ulceration plus secondary infection, possibly due to the friction with the bottom grille of the cage. Gross pathology findings on 7 days were not positive for the presence of the fungus and the characteristics of the lesions were not typical of fungal infection. Likewise, microbiological analysis of the alterations found at the liver of one rat at the pulmonary toxicity test, failed to show infection by *M. anisopliae*, not being possible to associate liver change to the administered fungus.

Clearance and infectivity results agree with entomopathogenic fungus characteristics. Entomogenous fungi are not thermophilic in nature and do not possess mechanisms such as lipid solubilization, rapid resynthesis of essential metabolites, molecular thermostability and ultrastructural thermostability[14]. The optimum temperature for entomopathogenic Hyphomycetes is between 20 and 25°C, but infection and disease can occur at temperatures ranging between 15 and 30°C. Above 30°C, vegetative growth is inhibited and growth ceases at

37°C[3,15]. Thus, the fungus does not cause adverse effects in mammals and cannot grow at mammalian body temperatures[3]. The results of the temperature growth study show that *M. anisopliae* strain F52 cannot grow at mammalian body temperatures and should not be able to grow in the organs or tissues of humans[7]. Similar results were obtained with *M. anisopliae* strain ESC1[16].

Obtained results are in accordance with the specific for insect mechanism of action of the *M. anisopliae*. This mechanism requires adhesion, pre-penetration growth, penetration into the host and establishment of the pathogen in the host[17]. The life cycle begins with a conidium attaching to the host cuticle, forming an appressorium, followed by a penetration peg to enter the cuticle. After entering the hemocoel, hyphae are formed that produce and release toxins, killing the host 4-16 days (depending mainly on the host species) after contamination[18]. For penetration, *Metarhizium* use cuticle-degrading trypsins and subtilisin-like proteases. Aside from the proteolytic degradation of the cuticular barrier, other possible roles include the utilization of host proteins for nutrition, the destruction of antifungal proteins of the host and the release of amino acids for amine production to elevate the pH[19]. When the insect dies, the fungus takes over the cadaver and grows back out through the body wall and sporulates on the surface. The dead insect is then enveloped with a mat of green conidias.

Extensive pathogenicity/toxicity testing with other strains of *M. anisopliae* for many years with no observable human health hazards suggest that there are few if any health risks[20]. No deleterious effects have been reported from humans handling cultures or administering *M. anisopliae*[21]. This fungus has undergone extensive mammalian safety testing including inhalation and intraperitoneal injection in mice and rats, intraocular injection in rabbits, oral dosing of frogs and long-term feeding studies in rats and birds[22]. Comparative studies with the most widely used biocontrol agent, *Bacillus thuringiensis*, showed that *M. anisopliae* is less persistent when injected into animal tissues, or when administered into eyes, being rapidly cleared from animal tissues without replication[23]. The results of the toxicity/pathogenicity studies of *M. anisopliae* strain ESF1 show no toxic, pathogenic, or adverse effects. These studies demonstrated that rodents can effectively clear the fungus from their bodies even after it is injected at high amounts[24]. However, Mycotech observed extreme toxicity to mice by an isolate of *M. anisopliae* and one of *M. anisopliae* var *acridum*[25].

It's has been stated that certain strains of *M. anisopliae* produce a class of mycotoxins called destruxins (insecticidal cyclodepsipeptides)[26]. Over 23 variants have been identified in cultures of this fungus; all comprise five amino acids and a hydroxyacid[26]. There are no known reports of destruxins causing adverse mammalian health effects[27]; however, other mycotoxins are known to adversely affect human health[24]. Based on these studies, it is believe that the tested *M. anisopliae* did not contain destruxins, or it posed no significant adverse effects to human health if destruxins were present. This conclusion is made according to the EPA assumption that mycotoxins that may adversely affect human health would show acute adverse effects in toxicity/pathogenicity studies[24].

It could be concluded that, at the tested doses and under our experimental conditions, *Metarhizium anisopliae* strain LMA-06 is not pathogenic following oral or pulmonary administration.

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