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INTRODUCTION

Locusts constitute one of the major concerns of agriculture in the developing countries from Africa through the mid-East to the Indian subcontinent. Realistic attempts to control these insects, however, began only when chemical pesticides became available. The international locust control program has been notably strengthened by the addition of accurate satellite monitoring to search out egg beds and to scout the movements of locust bands. However, the actual control strategies used still depend exclusively on the use of massive quantities of potent insecticides. Among these are Dieldrin and BHC, both of which are banned by EPA for use in the United States.

Despite the successes of insecticides in preventing the build-up of locust swarms, this approach presents major problems. The costs of chemical pesticides are directly linked to the spiralling costs of the petroleum from which they are made. Also, the high mammalian toxicities and detrimental residual effects in the ecosystem of numerous insecticides has caused the continued usage of these chemicals to come under increasingly vociferous criticism from the more enlightened public. The increasing costs of locust control with insecticides and the increasing call for moderation in the use of these chemicals has effectively increased both the urgency and the economic practicality to study pathogens and parasites as alternative agents for locust control. In fact, the 24th Report of the FAO Desert Locust Control Committee (1980) specifically recognized a need to study and to develop alternative means of pest control to supplement or to replace the use of chemical pesticides.

One of the most promising candidates for use in the microbial control of locusts and grasshoppers is the fungus Entomophaga grylli. This fungus, which is

better known as Empusa or Entomophthora grylli, appears to be the most effective naturally occurring pathogen of the Orthoptera. This pathogen is known from nearly every part of the world affected by locusts and grasshoppers, and is widely reputed for its occasionally high levels of natural control.

Among many other publications to note the effectiveness of E. grylli, the FAO Locust Handbook (Anon. 1966) indicates "that whole (locust) swarms have been wiped out by fungus disease" due to this species. Serious interest in E. grylli and wishes for a careful evaluation of this fungus' potential for use against locusts was expressed in locust control conferences as long ago as the 1930's (Anon. 1934; Hopkins 1934). Unfortunately, the often repeated clarion call for further studies of this fungus have been unheeded until the initiation last year in the United States of intensive studies of E. grylli for use against grasshoppers. The results of these studies (as noted below) have been very encouraging.

In addition to the reduction of pesticide usage, the adoption of biological control by agents such as E. grylli involves levels of technology which are appropriate for developing countries. The production and formulation of many entomopathogenic fungi involves a low level of technology and low capital investment. Currently, for example, Beauveria bassiana is being used on approximately one million hectares in the People's Republic of China, where most of the material is produced on demand at individual communes with rather unsophisticated techniques. In Brazil, Metarhizium anisopliae is produced commercially in plastic bags using rice as the sole growth medium. In other instances, deep fermentation facilities are used for producing these fungi, as is the case for Beauveria bassiana in the Soviet Union, Verticillium lecanii in Great Britain, and Hirsutella thompsonii: the latter fungus has been registered by the U.S. Environmental Protection Agency for use against citrus rust mites. Regardless of

the means of production, it seems likely that if a mycoinsecticide can be developed for use against locusts, it should be produced and formulated wherever possible within the countries where it is to be used. This would create employment and save export capital that would otherwise be spent on chemical pesticides manufactured from petrochemicals in fully developed countries. The commercial-scale application of fungal entomopathogens utilizes conventional spray technology.

BACKGROUND ON ENTOMOPHAGA GRYLLI

Disease development, specificity, and safety

For the first 4-8 days after infection by E. gryili, an insect shows no outward sign of infection. The fungus is then growing vegetatively as wall-less cells nearly indistinguishable from the host's own blood cells. As the body cavity fills with these amoeboid cells, the host's internal organs are attacked, and the fungus acquires cell walls and its more familiar filamentous form. The then moribund insect stops feeding and its movements slow. In its last hours, it climbs upwards to the top of a grass stem or onto other vegetation and dies in the late afternoon with its forelegs clasped around the stem and its jumping legs splayed characteristically outwards in a pose which makes infected grasshoppers or locusts easy to locate and to collect. During the night, the fungus usually forms a dense greenish-gray or cream-colored mat of conidium-bearing cells on the outside of the host's body. Conidia (spores) are forcibly discharged from this mat during the morning, fall on healthy grasshoppers below, and, within a few hours, germinate, produce a short germ tube which penetrates the body wall, and thus begins another round of infection. Sporulation will cease as the cadaver dries out, but begins again the next day, several days, or even weeks later when the cadaver becomes sufficiently moistened by dew or rainfall. If no mat is formed on the outside of the dead host, the body is

filled with thousands of golden brown, thick-walled spheres. These resting spores are the overwintering stage of the fungus, but they also appear to be involved in active disease transmission during dry climatic conditions.

Like many other biological control agents, E. grylli has the additional advantage over chemical pesticides of being completely specific for the Orthoptera. Despite some taxonomic disagreement regarding the specificity of E. grylli, no fungus from grasshoppers or locusts has been demonstrated to affect any other insects in the 125 years since this fungus was described. No beneficial insects such as bees or the insect parasites of the Orthoptera are affected by the fungus, nor is there any evidence that E. grylli presents any hazard for fish, birds, cattle, humans, or any other vertebrates.

E. grylli as a natural mycoinsecticide

A survey of the literature on Entomophaga grylli discloses several important points which form the basis for this research. This fungus is known from a very wide spectrum of grasshoppers, crickets, and locusts throughout the world. However, with relatively few exceptions, the literature only notes the presence of E. grylli and, often, speculates on this fungus' possible utility for microbial control.

The fungus has gained a respectable reputation for being able to destroy large populations of insects under the very best of conditions. This phenomenon has been noted in North America (Walton and Fenton 1939; Pickford and Riegert 1964; Henry and Onsager, 1982), Thailand (Roffey 1968), Australia (Milner 1978), and Africa (Skaife 1925a-b; Anon. 1934; Hopkins 1934; Faure 1935; Chapman and Page 1979). Gyllenberg (1974) found that his computer model for grasshopper populations did not function properly until the addition of a term for mortality due to E. grylli.

At least five locust species are known to be affected by Entomophaga grylli. Among these, the most significant fungal attacks seem to be in Africa against the red locust (Nomadacris septemfasciata) (Anon. 1934; Faure 1935), brown locust (Locustana pardalina) (Anon. 1934), and African migratory locust (Locusta migratoria migratorioides) (Remaudiere, personal communication; Soper and Humber, unpublished). Other locusts known to be affected by the fungus are the Bombay locust (Patanga succincta) (Roffey 1968) and the South American "langosta" (Schistocerca paranense) (Marchionatto 1934).

Recent advances with E. grylli

The one advance which enabled serious research on E. grylli was the culturing of the fungus in liquid insect tissue culture medium (MacLeod et al. 1980). Both in culture and in the host, this fungus grows vegetatively as wall-less amoeboid protoplasts much like those described for Entomophthora egressa (Tyrrell and MacLeod 1972; Tyrrell 1977).

Our involvement with E. grylli began in July 1979, and our rapid and encouraging progress—now in manuscript—is summarized below:

Several highly reliable means for isolating strains of E. grylli in culture from either vegetative protoplasts or conidia have since been developed. Grasshopper extracts have never been required as was suggested by MacLeod et al. (1980). We currently maintain 37 strains of this fungus from 6 species in 3 genera of North American grasshoppers. Extensive experimentation with the culture medium has lead to the development of a number of simplified media which are both better and less expensive for long-term maintenance of the protoplasts than the original medium first used to isolate E. egressa and, later, E. grylli.

Field and lab data indicate that there are two taxonomically distinct forms of Entomophaga grylli affecting grasshoppers and locusts. Pathotype I is known from a very broad spectrum of orthopterans and produces both forcibly discharged conidia and thick-walled resting spores. It is possible that E. grylli from other parts of the world also belong to Pathotype I, but isozyme analysis must be used to confirm this. Pathotype II is known only from Melanoplus species in North America and usually produces only resting spores. These distinct pathotypes (Soper, May and Martinell 1984, Environ. Entomol., in press), and can be interpreted as taxonomically distinct at either the specific or subspecific level. (Humber et al., 1982)

Evidence has accumulated for both forms of E. grylli that significant disease transmission can occur during much drier climatic conditions than most scientists feel can support fungal epizootics. Significant outbreaks of E. grylli in several parts of the western United States were observed where no substantial precipitation had fallen in the recent past. Similarly, R. A. Milner (personal communication) recently reported an epizootic of E. grylli decimating grasshopper populations at Alice Springs in the middle of the exceptionally dry Australian central highlands. Also, Chapman and co-workers describe ongoing epizootics of E. grylli causing substantial reductions of Zonocerus variegatus populations during the dry season in Nigeria (Chapman et al. 1979; Chapman and Page 1979). It is clear that the night-time conditions of temperature and humidity are far more important for the transmission of E. grylli than are any daytime extremes of heat and dryness.

Most research programs on the Entomophthorales for insect biocontrol depend on the thick-walled resting spore as the desired fungal unit for formulation, storage, and dispersal on the target population. However, the germination of these

resting spores has often been a serious obstacle to progress. We found a method with which nearly 100% of the relatively fresh resting spores tested germinated. Further, it has been possible to obtain infection of grasshoppers by injecting them with germinating resting spores, by spraying resting spores in the field, or by confining hoppers over soil containing resting spores (Nelson, Valovage and Fry 1982; personal communication).

A particularly important discovery was the copious production of infective conidia from infected cadavers which had dried, been stored, and were rehydrated several months later. This not only has significant epizootiological implications, but these conidial showers have also been used to carry out a series of host-range experiments. The most important result from these studies was that a sample of male Locusta migratoria migratorioides (allowed into the United States from a Canadian rearing facility) was found to be susceptible to E. grylli conidia discharged from a Carolina grasshopper (Dissosteira carolina) collected in the field at Ithaca, New York, and--on a routine basis--from Camnula pellucida cadavers collected at Alpine, Arizona. The infected L. migratoria produced conidia which should have been infective for other locusts; unfortunately, because of quarantine regulations, we did not have enough L. migratoria to perform the confirming experiments. This finding suggests, however, that it might be possible to initiate epizootics in African locusts using fungal strains isolated from North American grasshoppers.

STUDY OBJECTIVES

The purpose of this study was to investigate the feasibility of developing Entomophaga grylli for locust control. Significant changes in the original objectives had to be made because locusts were not present in Kenya during the study period. Therefore the research was centered on laboratory investigations of pathogenicity.

Infectivity and virulence of E. grylli pathotypes I and II were determined for the following locust species.

Africa

Locusta migratoria - Migratory Locust

Locustana pardalina - Brown Locust

Schistocerca gregaria - Desert Locust

Latin America

Schistocerca cancellata - South American Locust

Schistocerca piciefrons piciefrons - Central American Locust

Schistocerca piciefrons peruviana - Peruvian Locust

METHODS AND MATERIALS

Quarantine regulations prohibit the importation of locust species into the United States. Therefore, this research was conducted at the International Center for Insect Physiology and Ecology, Mombasa, Kenya, and at the Rothamsted Agricultural Experiment Station, Harpenden, Herts., England. In the latter instance, the Center for Overseas Pest Research, London, provided the locusts.

Pathogen Preparation: Conidia from E. grylli pathotype I were obtained from rehydration of Camnula pellucida cadavers. The insects were collected from a naturally occurring epizootic at Alpine, Arizona. Twelve to fifteen C. pellucida cadavers were soaked in sterile distilled water containing 0.2 ml gentamicin/100 ml for 15 to 20 minutes. After blotting off the excess water, the rehydrated insects were placed on filter paper in 1.5% water agar. They were then incubated at 100% relative humidity and 22°-26°C. Conidial formation began after 2-3 hours incubation and lasted up to 48 hours. When conidia production began, the mycotized

insects were held over an aqueous solution of Atmos 300/Tween 80 (0.10%/0.25%) to which 0.2 ml gentamicin/100 ml had been added. Conidia were pipetted from the collection container every 2 hours and placed under refrigeration to prevent premature germination. When sufficient quantities of conidia had been collected, they were concentrated by centrifugation and resuspended in 10 ml of Atmos 300/Tween 80 solution. The spores were then passed several times through a 200 mesh screen to break up the spore clumps. Spore counts were made using a haemocytometer. A suspension of 5×10^5 conidia/ml yields about 50 spores/mm^2 when 0.5 ml is applied using our spray tower. Dosages were thus controlled by using appropriate spore concentrations.

Pathotype II infections result in resting spore production. Spore preparation and inoculation are necessarily different from those for conidia. Melanoplus bivittatus grasshoppers killed by pathotype II were collected at Alpine, Arizona, and Fargo, North Dakota. The cadavers were soaked in distilled water for approximately 20 minutes. Needles were used to remove the resting spores from within the insects while submerged in water, and washed through a 200 mesh screen to remove insect parts and to break up spore clumps. The spores were then concentrated by centrifugation, and resuspended in 5 ml of water, plus 5 ml of full strength Clorox^R (5.25% sodium hypochlorite) with Tween 20 (3 drops/20 ml) resulting in a concentration of 50% clorox, and total volume of 10 ml. Spores were agitated in this hypochlorite solution for 65 seconds, quickly diluted with 40 ml of sterile distilled water, and centrifuged for 6 minutes at 15,000 rpm (0°C) in an angle rotor. The clorox solution was decanted and the spores resuspended in 50 ml sterile distilled water, and centrifuged again for 10 minutes at 15,000 rpm (0°C). The spores were then resuspended in 20 ml of sterile distilled water and stored at

4°C until needed. A small sample of the surface sterilized resting spores were placed in liquid culture medium (M199 + 5% fetal bovine serum) to check for contamination. Germination was tested by placing an aliquot of spore suspension on water agar and incubated for 5 days at 25°C. Percent germination was determined by counting germinated spores using a Wild dissecting microscope at 50x with transmitted light.

In order to obtain the germ conidia which are produced by germinating resting spores, approximately 0.5 — 1.0 ml of the resting spore suspension was dispensed into a 60 mm diameter petri dish, 1.5 ml of molten (50°C) sterile water was added, and the dish gently agitated to mix the spores. After the agar solidified, the dishes were incubated at high relative humidity and 25°C. The germinated resting spores formed a layer of conidiophores on the surface of the agar after six days, and the forcibly discharged germ conidia were collected using the same method described for primary conidia.

Spore Application: The spray tower was designed to be portable, with air pressure provided by a 23.5 liter Coleman[R] tank that could be pressurized with a tire pump, or by the outflow from a vacuum cleaner. The spray was provided by a number JL nickel-plated brass nozzle adjusted for a 3mm JJ round spray (Spraying Systems Co., Wheaton, IL), and modified to accept small volumes of spore suspensions (0.1 — 1.0ml). The normal spray inlet was sealed and a small hole was drilled in the top of the nozzle holder to accept a 1 cc tuberculin syringe. Air pressure was adjusted to 25 liters/minute as measured by an in-line flow meter. The nozzle was positioned 50 cm from the insects being treated. A plexiglass box (17 cm x 17 cm x 6.5 cm) was constructed with a 100 mm opening which was covered by a fine mesh screen. The opening was placed in the center of the spray

path. A vacuum was applied using a household vacuum cleaner connected by a tube on the side of the box.

Treatment Procedure: Treatments consisted of conidia injection and conidial sprays for pathotype I and injection of germinating resting spores and germ conidial sprays for pathotype II. The locusts used for experimental controls were injected with Grace's tissue culture medium containing 0.2 ml gentamicin/100 ml which was the carrier for the injected spores. Controls for the spray treatments were locusts sprayed with Atmos 300—Tween 80 solution. In general, 20 locusts were used for each treatment. Dosage calibration for conidial sprays was determined by counting the number of spores/mm^2 on cover slips treated at the same time the locusts were sprayed. Conidia were injected at the rate of $3.0 \pm 1.8 \times 10^3$ ($x \pm s$) spores contained in 0.1 ml/locust. Germination tests showed these spores were $95.7 \pm 3.82\%$ ($x \pm s$) viable. Resting spore injection was 6.5×10^4 in 0.1 ml of sterile distilled water. It was not possible to determine resting spore germination accurately, but probably was around 35%. Germ conidia were sprayed at 1.36 ± 0.47 ($x \pm s$)/mm^2 and had a viability of $97.6 \pm 0.55\%$. Primary conidia (discharged from cadavers) were sprayed at 5.59 ± 1.27 ($x \pm s$)/mm^2 and were from the same lot used for injection experiments. The locusts were held at 7°C for approximately 30 minutes to facilitate handling during the spray operation. Five locusts were placed on the spray tower at one time and treated. The locusts were reared individually in 120 ml specimen cups following treatment. A 25 mm diameter hole was cut in the lid and covered with screening. The insects were fed wheat bran and Romaine lettuce. Those cups containing insects which were sprayed were covered immediately following treatment with damp paper toweling for 14 to 18 hrs to provide a humid environment. All dead insects were checked for presence of

disease using phase microscopy. The bioassays were terminated at 16 days and all living locusts were also examined for disease development.

Similar experiments were conducted at Alpine, Arizona, on Camnula pellucida the host for pathotype I, Melanoplus bivittatus the host for pathotype II, and Melanoplus packardii, a known host for both pathotypes. The treatments in this experiment were injection of conidia or protoplasts of pathotype I.

RESULTS

All locust species tested were susceptible to pathotype I E. grylli (Table 1) when sprayed with conidia. This simulated the normal route of infection for pathotype I in which the insect body wall is penetrated by the fungal germ tube. In those cases were survival time (ST) could be calculated, the ST_{50} ranged between 12.58 and 14.27 days. The germ conidial sprays of pathotype II, resulted in only two species becoming infected; L. migratoria and S. p. peruviana. This test cannot be considered definitive since the infection was extremely low, probably because of a low dosage rate (1.36 germ conidia/mm^2). The injection of germinating resting spores resulted in higher mortality and decreased ST_{50}. This was not unexpected since the normal penetration process was circumvented. Comparison of the two methods of infecting S. cancellata with pathotype II gave surprising results. With 70% infection via spore injection, it was supposed that conidial sprays should have resulted in some infection. Since such was not the case, either pathotype II germ conidia cannot produce germ tubes which can penetrate this insect or the dosage of conidia was too low to cause infection. It is probable that the latter situation is correct and S. cancellata is susceptible to both pathotypes. This would have to be confirmed by repeating the experiments using a higher germ conidia dose.

Results of the study of M. bivittatus (a pathotype II host), C. pellucida (a pathotype I host), and M. packardii (which is susceptible to both pathotypes under field conditions) are presented in Table 2. In these trials, infection attempts were confined to injection of conidia or protoplasts (4×10^3/insect) of pathotype I. In the experiments with conidia, development was much faster in the normal hosts (ST_{50} = 7.35 - 9.34) than in M. bivittatus (ST_{50} = 14.62). When protoplasts were injected a similar result occurred. C. pellucida had a mortality range of 9--14 days while in M. bivittatus mortality was not seen until the 15th day with ST_{50} = 18.64. In this experiment M. packardii was not infected by protoplast infection.

Conclusion

The following general conclusions can be drawn from this research.

1. All African and Latin American locusts tested are susceptible to Entomophaga grylli pathotype I under laboratory conditions.

2. Only Locusta migratoria and Schistocerca piciefrons peruviana are susceptible to pathotype II when exposed to the normal infection route. The negative responses of the other locust species remain to be confirmed by further testing. It should be noted, however, that pathotype II protoplasts may proliferate extensively in the blood of many of the species tested but fails to cause mortality or to sporulate.

3. Locusta migratoria and other locust species may be excellent culture vessels for some pathotype II strains of E. grylli, and could serve as model systems

for studies of the mechanisms of fungal specificity and lost resistance. These studies could not be conducted in the United States, however, because of the quarantine regulations which prevent the maintenance of locust colonies.

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TABLE 1: Bioassay results of Entomophaga grylli tested against locusts[1]

Host	Pathotype I				II			
	Injection		Spray		Injection		Spray	
	% INF	ST_{50}/Mortality[2] Period	% INF	ST_{50}/Mortality Period	% INF	ST_{50}/Mortality Period	% INF	ST_{50}/Mortality Period
AFRICAN								
Locusta migratoria	40	8.13 ± 0.40	10	12 — 18	5	11	7	11
Locustana pardalina	45	8 — 9	30	11.58 ± 0.65	0		0	
Schistocerca gregaria	Not Tested		10	10 — 14	0		0	
LATIN AMERICAN								
Schistocerca cancellata	50	6.94 ± 0.68	15	15 — 18	70	8.55 ± 0.39	0	
S. piciefrons piciefrons	45	8.88 ± 0.28	20	14.27 ± 1.12	0		0	
S. piciefrons peruviana	45	10.22 ± 0.61	30	12.58 ± 1.03	0		7	11

[1] No infection occurred in any controls.

[2] Survival time expressed in days for 50% mortality with standard error ($ST_{50} \pm S_{\bar{x}}$) calculated only when sufficient data was collected; otherwise, period during which mortality was observed is presented.

TABLE 2: Bioassay results of Entomophaga grylli pathotype I against grasshoppers

Host	Conidial Infection %	Injection $ST_{50} \pm S_{\bar{x}}$	Protoplast Infection %	Injection $ST_{50} \pm S_{\bar{x}}$
Melanoplus bivittatus	40	14.62 ± 1.42	20	18.64 ± 1.34
Melanoplus packardii	50	9.34 ± 0.83	0	
Camnula pellucida	50	7.35 ± 0.27	25	(9—14 days)

[1] Dosage 4 x 10^3/conidia or protoplast/insect.

[2] Survival (days) for 50% (ST_{50}) of the test insects with standard error ($S_{\bar{x}}$).