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STUDIES ON THE SUSCEPTIBILITY OF *ORYCTES RHINOCEROS* TO SOME ENTOMOGENOUS FUNGI

G.C.M. LATCH

Plant Diseases Division, D.S.I.R., Palmerston North, New Zealand

Cultures of *Metarhizium anisopliae* (METSCH.) SOR., *Beauveria bassiana* (BALS.) VUILL., *B. tenella* (DELAC.) SIEM., *Aspergillus fumigatus* FRES. and *Paecilomyces farinosus* (DICKS, ex FR.) BROWN & SMITH were tested for pathogenicity to *Oryctes rhinoceros* L. All the long spored cultures of *M. anisopliae* isolated from *Oryctes* spp. were equally pathogenic to *O. rhinoceros*. Most of the 23 short spored cultures from other insects caused lesions on *Oryctes* larvae but only 5 isolates were lethal. Some *Beauveria* isolates caused brown lesions on larvae but generally these larvae developed into normal adults. One isolate of *B. bassiana* from *Carpocapsa pomonella* L. killed larvae at the time of pupation. Cultures of *A. fumigatus* and *P. farinosus* were not pathogenic. A method for growing *M. anisopliae* on oats for large scale field use is described.

A study was initiated with a view to using fungi as "biological insecticides" for controlling the coconut rhinoceros beetle (*Oryctes rhinoceros* L.). This beetle is one of the most important pests of the coconut palm and is found in most of the coconut growing areas of Asia and the Indian and Pacific Oceans (CATLEY, 1969). The insect breeds in decaying vegetable matter such as coconut logs and stumps, sawdust, compost and animal dung.

The entomogenous fungus *Metarhizium anisopliae* (METSCH.) SOR. was first seen attacking *O. rhinoceros* in Western Samoa by FRIEDERICHS (1920) in 1912 and attempts have been made by FRIEDERICHS and others to control the insect with this fungus. CATLEY (1969) stated that *M. anisopliae* probably has a significant effect in reducing the population of *O. rhinoceros*. More recently *Rhabdionvirus oryctes* has been released on some Pacific Islands and control of the insect has been improved greatly (ZELAZNY, 1973). However, *O. rhinoceros* still causes economic loss in some Pacific Islands, and it seemed worthwhile that use of fungi to control the insect should be explored further.

The species *M. anisopliae* comprises 2 forms; a short spored form (conidia generally 5-8 μ long) found on a variety of insects, and a long spored form (conidia generally 9-14 μ) found attacking *O. rhinoceros* and a few other insect species. Cultures of *M. anisopliae* initially isolated from species of *Oryctes*, and short spored forms of the fungus isolated from a number of other insects, were obtained from various parts of the world. Isolates of *Beauvaria bassiana* (BALS.) VUILL., *B. tenella* (DELAC.) SIEM., *Aspergillus fumigatus* FRES. and *Paecilomyces farinosus* (DICKS, ex FR.) BROWN & SMITH were also collected to test their pathogenicity to *O. rhinoceros*.

Attempts to control *O. rhinoceros* with *M. anisopliae* in the Pacific basin have been made by growing the fungus on agar cultures and spraying a suspension of the spores in water on to *Oryctes* breeding sites (MARSCHALL, 1969). Growing the fungus

by this method is expensive in terms of labour and materials, and the control has not been satisfactory (YOUNG, 1971). It was necessary, therefore, to seek methods of producing inoculum simply and cheaply, and in a form in which the fungus would remain viable for some considerable time after it had been distributed in the field. A method that meets these requirements for bulk production is described in an Appendix to this paper. It seemed appropriate, also, to seek a strain of the fungus which would kill larvae slowly, prolonging the supply of spores on cadavers.

This paper describes laboratory work to compare pathogenicity of *M. anisopliae* isolates and other entomogenous fungi to *O. rhinoceros*. The effect of substrate moisture on infection of larvae by *M. anisopliae* was also investigated, and attempts were made to increase the pathogenicity of short spored forms of the fungus. Suitable culture media for laboratory growth and sporulation of the fungi were investigated, and a method for large scale production of *M. anisopliae* for field use was developed.

MATERIALS AND METHODS

REARING OF *Oryctes rhinoceros* LARVAE

A mixture of rotten kapok wood and cow dung is used for rearing *O. rhinoceros* in Western Samoa. This wood is not available in New Zealand. Tests showed that larvae would grow well in media made up by mixing old sawdust of *Pinus radiata* D. DON with sheep dung, with cattle dung, or with sawdust impregnated with dung and urine from a cattle yard.

The best medium for larval growth and for fungal infection was found to be a mixture of 4 parts by weight of autoclaved dried sawdust with 1 part of dried, ground, sheep dung, with water added to give a moisture content of 50 %. The medium had a pH of 6.7-7.0. The sawdust/cattle yard mixture had a pH of 9 and was too alkaline for infection of larvae by *M. anisopliae*, but it was on this account a good medium for holding 3rd instar larvae until needed for infection experiments.

Eggs and larvae were air freighted from the insect rearing unit in Western Samoa to our laboratory in New Zealand. All insect rearing and experimental work was carried out in 2 quarantine rooms held at 27°C. Contamination of the healthy *Oryctes* larval stocks by *M. anisopliae* seldom occurred and presented no problem.

MEDIA FOR FUNGAL GROWTH AND SPORULATION

Growth and sporulation of the fungi were tested on Sabouraud's medium, on potato dextrose agar (PDA), and on modifications of these media. *Aspergillus fumigatus* and *Paecilomyces farinosus* grew well and sporulated well on nearly all media tested, but the *Metarhizium* and *Beauveria* isolates were discriminatory. Media finally chosen for these fungi were : *M. anisopliae*. Buttermilk Agar, consisting of 2 % buttermilk powder, 2 % Difco bacto-peptone, 4 % dextrose, 1.2 % agar. *Beauveria* species. PDA, to which was added 1 % buttermilk powder.

PATHOGENICITY TESTING

Thirty-six isolates of *M. anisopliae*, 27 isolates of *B. bassiana*, 7 of *B. tenella* and 1 isolate each of *A. fumigatus* and *P. farinosus* were tested. Spores from 2-3 weeks old Petri plate cultures were used to inoculate healthy *O. rhinoceros* larvae.

In the initial test, spores were mixed into soft butter and spread all over the larvae with a spatula. Inoculated larvae were placed in a sawdust/dung mixture in plastic containers and held at 27°C. Larvae were examined daily, and the dead ones removed and kept under humid conditions until the fungus sporulated.

If an isolate tested in this manner was pathogenic or caused severe lesioning on the larvae, it was retested by dipping healthy larvae in a suspension of spores in water.

SUSCEPTIBILITY OF THE LIFE STAGES OF *O. rhinoceros* TO *M. anisopliae*

The stages in the life cycle which were tested were 1st instar. 2nd instar, young 3rd instar, old 3rd instar, young pupae, old pupae and adults. They were infected with isolates of *M. anisopliae* which were originally obtained from *O. rhinoceros* larvae. Inoculation was carried out by dipping the insects in a suspension of 0.05 g (1×10^9) spores in 100 ml of distilled water. Between 20 and 60 individuals at each stage were inoculated and the insects were placed in a sawdust/dung substrate and kept at 27°C. Dead insects were removed daily and held until spores of *M. anisopliae* developed on the cadavers, confirming that the fungus had killed them.

The relative pathogenicity of various isolates of *M. anisopliae* was compared by dipping larvae into a suspension of 0.02 g (4×10^8) spores in 100 ml of distilled water. Thirty 3rd instar larvae were successively dipped into the spore suspension and then placed in individual containers. Because the spores tended to float on the surface of the water the spore concentration became weaker with the removal of each larva until the last few did not become infected.

ENHANCEMENT OF PATHOGENICITY

Metarhizium anisopliae

An attempt was made to enhance the pathogenicity to *O. rhinoceros* of 2 short spored forms of *M. anisopliae*, one isolated from *Conoderus* sp. and one from *Costelytra zealandica* WHITE.

Spores of each isolate were mixed with sterile water and 0.25 ml doses of the spore suspension were injected with a hypodermic syringe into the second anterior segment of 3rd instar larvae. Control larvae were injected with sterile water only. Infected larvae usually died 2-6 days after injection and spores formed on the cadavers several days after death. These spores were used to inject a fresh series of healthy larvae. The sequence was repeated 5 times and then pathogenicity of the original isolate and of the spores which formed on cadavers after the fifth passage of the fungus was compared.

Beauveria bassiana

The isolate from *Carpocapsa pomonella* L. was passaged 3 times through *O. rhinoceros* larvae in a similar manner to that for *M. anisopliae* and compared for pathogenicity with the original isolate.

EFFECT OF MOISTURE ON DEATH RATE OF INFECTED LARVAE

A dried sawdust/dung mixture containing 4 % moisture was prepared, and 500 g was put into each of 16 plastic containers. Moisture level of the mixture in each container was then adjusted by adding distilled water as follows : 1 250 ml for 73 % moisture; 500 ml, 52 %; 300 ml, 40 %; 200 ml, 31 %. Four containers were adjusted to each moisture level. The mixture at 73 % moisture appeared wet, that at 31 % was almost dusty. The pH varied 6.7-7.0.

Three isolates of *M. anisopliae* obtained from *O. rhinoceros* were used in the trial. Each isolate was used for 1 box of larvae at each of the 4 moisture levels. The fourth series of boxes contained uninoculated control larvae. Nine 3rd instar larvae were put in each box after they had been dipped in a suspension of 0.05 g *M. anisopliae* spores in 100 ml distilled water. Control larvae were dipped in distilled water.

Boxes complete with larvae were weighed and held at 27°C. The larvae were inspected daily, dead ones were removed and weighed, and then water was added to replace that lost by evaporation.

Toxicity of *M. anisopliae* to mammals

Freshly harvested spores from oat grain cultures were fed to 10 weanling white mice and to 4, 3 week old guinea pigs for 28 days. The mice were fed a ration of wholemeal flour/buttermilk powder (2:1) and the guinea pigs received a commercially prepared ration consisting mainly of lucerne meal. Spores were fed at the rate of 10 % of the daily ration. A similar number of control animals received the same ration but without the fungal spores.

RESULTS

Pathogenicity of the fungi

Short spored isolates of *M. anisopliae*, and both species of *Beauveria* induced fewer lesions when the larvae were dipped in a spore suspension than when spores were buttered on. Larvae dipped in an aqueous spore suspension of the long spored form of *M. anisopliae* were just as severely lesioned as when spores were buttered on them.

Metarhizium anisopliae inoculations

(a) Isolates from *O. rhinoceros*. Long spored isolates of *M. anisopliae* from *O. rhinoceros* larvae collected in Western Samoa, American Samoa, Tonga, Fiji, India, and Mauritius all killed larvae within 7-16 days of inoculation. Black lesions often developed on the integument 3-4 days after inoculation, but in some instances larvae were unmarked when they died. Differences in pathogenicity between the isolates were small.

A short spored isolate of *M. anisopliae* from a dead *O. rhinoceros* larva collected on Vomo Island, Fiji, did not infect healthy *O. rhinoceros* larvae.

(b) Isolates from other *Oryctes* species. Long spored isolates of *M. anisopliae* from *Oryctes boas* F., *O. elegans* Prell, *O. monoceros* Ol., and *O. nasicornis* L. infected *O. rhinoceros* larvae. Lesioning was similar to that caused by isolates from *O. rhinoceros*, and larvae died 8-13 days after inoculation. There was no apparent difference in pathogenicity between these isolates and those from *O. rhinoceros*.

(c) Isolates from other insect genera. Twenty-three short spored isolates of *M. anisopliae* from insect genera other than *Oryctes* all caused black lesions on *O. rhinoceros* larvae. Most did not kill the larvae, which eventually developed into adults, but infection prolonged the third instar stage, in one instance to twice normal length.

Four short spored isolates of *M. anisopliae* from *Thaumetopoea wilkinsoni* (Tams), *Melolontha melolontha* L., an ambrosia beetle, and from an unknown insect sent from Japan killed *O. rhinoceros* larvae. Death generally occurred when 3rd instar larvae were turning into pupae.

A short spored isolate from a *Conoderus* sp. larva killed *O. rhinoceros* larvae, but the infected larvae could often survive until the pre-pupal, or pupal stage. Infected larvae were heavily lesioned, and many deaths occurred 12-40 days after inoculation.

Beauveria species inoculations

Of a total of 34 isolates of *B. bassiana* and *B. tenella* inoculated on to *O. rhinoceros* larvae, 17 caused lesions on the integument similar to those caused by *M. anisopliae*. One culture of *B. bassiana* isolated from *Carpocapsa pomonella* L. caused very severe lesioning, and usually the larvae died at the beginning of pupation some 2-4 months after inoculation. However, some heavily lesioned larvae developed into apparently normal beetles.

Aspergillus fumigatus and Paecilomyces farinosus

Neither of these fungi was pathogenic to larvae of *O. rhinoceros*.

SUSCEPTIBILITY OF LIFE STAGES OF *O. rhinoceros* TO *M. anisopliae*

Infection occurred at all stages in the life cycle tested. Mortality was 100 %, except for beetles where mortality varied between trials but averaged 38 %. First instar larvae died more quickly than did other larval stages; in all trials they were dead within 11 days of inoculation. The 2nd instar larvae, early and late stages 3rd instar larvae, and pupae, died 8-17 days after inoculation. In one trial 10 pupae were inoculated, and within 8 days all had turned into adults. These adults died between the 9 th and 15th day after inoculation.

ENHANCEMENT OF PATHOGENICITY

No difference in pathogenicity could be detected between the original cultures of the 2 short-spored *M. anisopliae* isolates and the cultures obtained after 3 or 5 passages through larvae. It was observed however, that the *Conoderus* isolate produced fewer spores in culture with succesive passages through larvae.

The culture of *B. bassiana* did not appear to be enhanced in pathogenicity to *O. rhinoceros* after 3 passages through larvae.

All control larvae remained healthy.

EFFECT OF MOISTURE ON DEATH RATE OF INFECTED LARVAE

There was no difference in the performance of the 3 *M. anisopliae* isolates. Results have been combined and are presented in figure 1. Larvae at the 3 lower moisture levels died 8-15 days after inoculation, the majority dying between days 8-11. Larvae held in substrate with 73 % moisture died 9-25 days after inoculation, with mortality fairly evenly spread during this period. All larvae in the control boxes remained healthy.

TOXICITY OF *M. anisopliae* TO MAMMALS

Animals fed spores of *M. anisopliae* in their diet showed similar weight gains to the control animals and were normal in their behaviour. When the animals were sacrificed at the end of the trial post mortem examination discovered no organ or tissue abnormalities.

DISCUSSION

The entomogenous fungus *M. anisopliae* has been reported on a great many insects. Studies by DIOMANDÉ (1969), FERRON *et al.* (1972), and LATCH (1965) showed that isolates of the fungus were generally more pathogenic to the species from which they were originally obtained than to other species of insects. DIOMANDÉ (1969) and FERRON

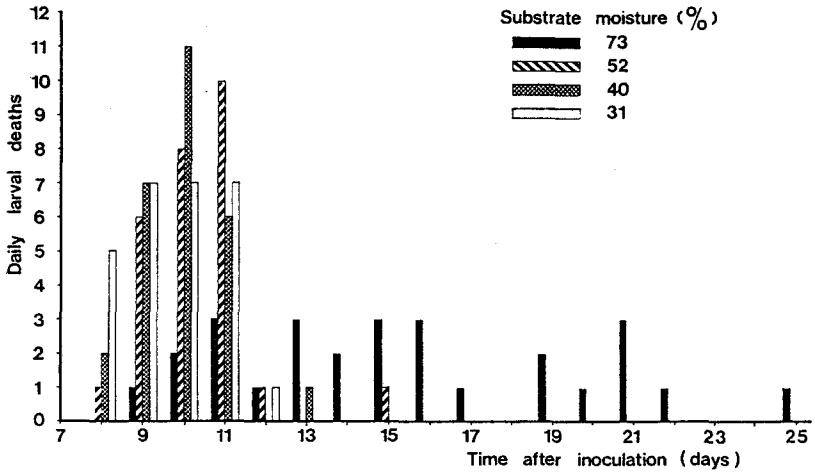

FIG. 1. Effect of substrate moisture level on the death rate of *O. rhinoceros* arvae inoculated with *M. anisopliae*.

et al. (1972) found that it was predominantly the long spored forms of *M. anisopliae*, isolated from *Oryctes* spp., that attacked *Oryctes* larvae and FERRON *et al.* (1975) have shown that *O. rhinoceros* adults were susceptible to strains of the major type only. In the present study, all of the long spored isolates from *Oryctes* spp. proved pathogenic to *O. rhinoceros*, infection being fatal. Short spored isolates taken from a variety of genera caused lesions on *O. rhinoceros* larvae, but few of these isolates caused death.

No isolates of *Beauveria* caused complete kill, and *Aspergillus* and *Paecilomyces* were ineffective.

The long spored forms of *M. anisopliae* tested in the present work were of similar pathogenicity to *O. rhinoceros*. Hence, an isolate introduced into an area in the field is unlikely to be more pathogenic than any long spored strains of the fungus on *Oryctes* that may be present there already. Introduced isolates or local strains might differ in ability to survive in soil in the absence of suitable host insects, but this could only be determined from field trials.

Virulence of the short spored isolate from *Conoderus* was not increased by passage through *O. rhinoceros*, but this isolate might have a place in the biological control of *O. rhinoceros*. Larvae infected with the isolate do not die until after a relatively long period has elapsed. Hence, the inoculum would tend to be persistent in the substrate.

In India, NIRULA *et al.* (1955) found that infection of *O. rhinoceros* with *M. anisopliae* was widespread during the monsoon season but restricted in the drier months. Moisture level of the substrate could be important in determining incidence of infection. DIOMANDÉ (1969) found that infection of *O. monoceros* in the laboratory was similar in substrates ranging in moisture level 25.0-56.6 %. In our experiments, infection of *O. rhinoceros* and the time taken for larvae to be killed were similar in substrates ranging 31-52 % moisture, but larvae took longer to die in a wetter (73 % moisture) substrate. It is likely that moisture levels in *O. rhinoceros* breeding sites in the Pacific Islands would normally be sufficient for infection of larvae by *M. anisopliae*.

Our finding, in agreement with SCHAERFFENBERG (1968), that *M. anisopliae* was not pathogenic or toxic to warm blooded animals indicates that the fungus could safely be grown and distributed on a large scale on the Pacific Islands.

Field trials to test selected isolates of *M. anisopliae* for controlling *O. rhinoceros* and to compare the longevity of dried and wet oat/fungus inoculum in breeding site material have been undertaken on the Kingdom of Tonga. The results of these studies will be presented in a further paper.

APPENDIX

BULK PRODUCTION OF *M. anisopliae* INOCULUM FOR FIELD USE

An acceptable field inoculum should be relatively simple and cheap to produce, and it should remain viable for a reasonable period after it has been produced. Cereal grain was chosen as a suitable substrate. Several cereals were tested, and oats (*Avena sativa* L.), were selected for producing best growth and sporulation of *M. anisopliae*. Supplements of buttermilk powder, skimmilk powder, yeast extract or bactopeptone did not increase spore yield appreciably.

Oats and tapwater in the ratio 1.4 ml water/1.0 g grain were put in flasks and autoclaved. Flasks were inoculated with a suspension of *M. anisopliae* spores, and incubated in darkness for 21 days at 25°C. Spore yield at the end of this period was 16-18 g conidia/100 g dry weight of oats.

Effects of different storage conditions on oat inoculum viability were checked. Samples of 21-day cultures were held in Petri plates for 11 months, and the spores were then tested for germination and the oat grains for growth of mycelium on to agar. In samples held at room temperature, 15-24°C, there was no germination or mycelial growth. Samples held at 4°C, and at —15°C gave good spore germination and mycelial growth, the deep frozen samples needing 6 days incubation for vigorous growth. Inoculum dried 3 days at 40°C and held 6 months at 15-24°C had no viable spores, but when the dry grain was moistened by placing it on agar at 25°C a fresh crop of spores developed on the surface within a week.

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RÉSUMÉ

Études sur la sensibilité de *Oryctes rhinoceros* à certains champignons entomopathogènes

Des cultures de *Metarhizium anisopliae*, de *Beauveria bassiana*, de *B. tenella*, d'*Aspergillus fumigatus* et de *Paecilomyces farinosus* ont été examinées en fonction de leurs effets pathogènes sur *Oryctes rhinoceros*. Toutes les cultures à spores longues de *M. anisopliae* isolées des *Oryctes* spp. ont été également pathogènes pour *O. rhinoceros*. La plupart des 23 cultures à spores courtes obtenues à partir d'autres insectes ont causé des lésions sur les larves d'*Oryctes* mais cinq seulement ont été mortelles. Certaines souches de *Beauveria* ont causé des lésions brunes sur les larves mais, en général, ces larves ont donné des adultes normaux. Une culture de *B. bassiana* isolée de *Carpocapsa pomonella* a tué les larves au moment de leur métamorphose en chrysalides. Les cultures d'*A. fumigatus* et de *P. farinosus* n'ont pas eu d'effet pathogène. Une méthode de culture de *M. anisopliae* sur avoine pour l'utiliser sur de grandes surfaces est décrite.

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