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Insect Pest Management with Fungi:
A Mass-Production Technique for Farmers

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A. Executive Summary

The goal of this project was to promote the use of insect-killing fungi for pest management in Ethiopia by enhancing and supporting local capacity to conduct research on microbial control and develop procedures appropriate to Ethiopia for production of beneficial fungi. Four 1-month training programs were held at the Univ. of Vermont Entomology Research Laboratory (UVM) for scientists from the Plant Protection Research Center (PPRC), Ambo, Ethiopia. These scientists learned critical techniques needed to mass produce fungi and conduct research to evaluate efficacy. An insect pathology laboratory was established at the Ambo PPRC, which was supplied with the equipment and materials necessary to sustain the research. Over the duration of the project, UVM scientists visited Ethiopia on three occasions hosted by the Ethiopian Agricultural Research Organization. These visits provided essential interaction among the cooperators to ensure that the research proceeded smoothly and that problems encountered by the Ethiopian scientists could be resolved. A UVM scientist participated in two conferences to disseminate information to Ethiopian scientists and pest specialists about biological control, focusing on insect-killing fungi and results from this project. Research was conducted collaboratively among the Ethiopian, Israeli and US scientists to evaluate methods for fungal mass production and test the efficacy of indigenous fungal isolates against key target insects. At the start of this project, there was a general lack of in-country microbiological expertise and facilities to develop fungi into viable biological control agents. There was also little appreciation of integrated pest management and how fungi could be utilized within existing pest management strategies. As a result of this project, several Ethiopian scientists and technicians are trained in basic insect pathology techniques and have a facility in which to carry out research to further develop insect-killing fungi. There is also increased awareness among the research and extension community of Ethiopia of the potential for fungi in pest management.

B. Research Objectives

In the developing world, farmers in many countries, including Ethiopia, are largely dependent on agricultural chemicals for management of insect pests. In some places farmers are so poor they cannot afford to buy anything for pest management. New pest management solutions that are cost effective are desperately needed for both of these cases. One viable option is the use of indigenous biological control agents which can be produced locally to provide an inexpensive, non-toxic and self-sustaining means of pest management. Entomopathogenic (insect-killing) fungi have been largely neglected in the past as biological control agents, yet they represent a promising alternative to chemical insecticides. New discoveries in their growth characteristics and methods of formulation have stimulated a renewed interest in their development. To promote the use of fungi in developing nations, suitable in-country mass-production techniques must be devised that deliver viable, stable inoculum, are simple to implement and maintain, and rely on local ingredients. Production must be cost-effective and tailored to the economics of the region. Because of the perceived smaller potential dollar return compared with agrochemicals, most private companies are reluctant to consider development of fungi for pest management.

The overall aim of this project was to combine the scientific expertise of insect pathologists and entomologists from Tel Aviv University in Israel, the UVM Entomology Research Laboratory in the US and the Ethiopian Agricultural Research Center in Ethiopia to develop a technique for mass production of insect-killing fungi that would be suitable for use in farmer cooperatives. At the initiation of this project the scientific team of the UVM Entomology Research Laboratory (ERL) had been working for several years on developing fungi for management of thrips in forests. The Laboratory maintained an extensive collection of fungi from throughout the northeastern US, which has since then expanded to include most countries in West and Central Asia, Taiwan and California. ERL personnel had worked on developing fungi in forest and greenhouse settings, and had expertise in bioassay procedures and mass-production techniques. The research focus of the Israeli cooperators had been of a more fundamental nature. They had conducted trials to develop fungi for management of silverleaf whitefly through in-depth analysis of the morphological and physiological characters of different isolates. When this project was first conceived, the projected Ethiopian cooperator was a former graduate student of the ERL. This scientist had returned to Ethiopia where he was establishing a research program focusing on insect-killing for management of whitefly. The vision of this project was to build on in-country and international expertise in insect pathology and in the process develop a practical method for testing and producing viable fungal-based biological materials.

The specific objectives to achieve the project goal were as follows:

1. Develop a simple, cost-effective mass-production technique for insect-pathogenic fungi
2. Assess the suitability of this technique in farmer cooperatives in Israel and Ethiopia and evaluate the quality and quantity of fungal material produced.
3. Present a practical comprehensive educational program on the basics of entomopathogenic fungi to Ethiopian technical personnel.

The original vision of this project was to draw on the unique expertise of each cooperator. Adjustments to the original work plan were necessary because of personnel changes in Ethiopia,

and political unrest in Israel. The project was to be administered by Prof. Barash, of Tel Aviv Univ. In addition, Dr. G. Gindin from the Volcani Center was assigned the responsibility of conducting quality control testing of the fungal mass-production method. The mass-production techniques were developed by the US cooperators at the ERL, drawing on information generated by Dr. Gindin. The first phase of training of Ethiopian scientists in insect pathology and mass-production technology was completed by personnel from the ERL Testing of the mass-production method was to be carried out at a kibbutz in Israel, but because of political insecurity, the plan was revised and the testing was completed at the ERL. Two other training programs were organized at the ERL to adapt to personnel attrition in Ethiopia. This ensured that trained individuals in-country were available to carry on the work at the end of the project. In addition, ERL personnel organized and took part in training programs in Ethiopia to transfer the technology.

C. Methods and Results

Objective 1. Develop a simple, cost-effective mass-production technique for insect-pathogenic fungi.

Although fungal isolates will vary in their specific requirements for optimal growth and sporulation, the objective of the initial phases of this research was to develop a basic mass-production technique and protocols for production of a range of fungi for control of crop pests in Ethiopia. Several grain-based substrates and methods of preparation were included in this assessment, together with an evaluation of effects of different initial inoculum levels on production. In addition to measuring effects on spore production (yield), we assessed the effects on insect activity and spore stability (i.e., shelf life) at different storage temperatures.

i. Source of fungi. Two strains of *Metarhizium anisopliae* were used in the study: *M. anisopliae* var. *acridum* IMI 330189, the strain used in Green Muscle™ (CABI BioSciences, UK), which was included as a comparative control; and *M. anisopliae* PPRC-29, a strain originally isolated in Ethiopia and shown to have superior growth and sporulation characteristics, important traits for mass production. Two strains of *Beauveria bassiana* were also included: *B. bassiana* 726, a commercial strain used in BotaniGard™ and Mycotrol™ (Mycotech Corp., Butte, MT), included for comparative purposes; and *B. bassiana* PPRC-56, isolated in Ethiopia and shown to have good growth and sporulation characteristics.

Once defined, the efficacy of the mass production methods were tested on two additional fungal strains: *M. anisopliae* 1080 and *B. bassiana* 082. Both strains were originally isolated in North America. Material from these trials was then used to test effects of two drying processes on conidial viability.

ii. Growth substrates and preparation. Sorghum is widely grown in Ethiopia and would be readily available as an inexpensive substrate for mass production of fungi. It was used as the basal medium in all of the trials. Spore production was compared on two substrates: sorghum only and sorghum plus bran (ratio 1:0.5, respectively). Bran was mixed with the sorghum to enhance aeration (and thus growth) throughout the substrate, and to increase the surface area on which the fungi could grow. Trials were first carried out on a small scale with the substrate contained in screw-topped autoclavable glass jars (canning jars). Methods were then tested on a larger scale,

holding the growth medium in plastic autoclavable bags (60x30 mm). Methods and results presented herein are from the large-scale production trials.

Both substrates were mixed with hot water at a ratio (vol:vol) of 1:1 or 1:0.75. The grains needed to be cooked or autoclaved before use in mass production. The volume of water used in the processing of the substrate greatly influenced the final characteristics of the material. Too much water and the substrate became waterlogged and growth only occurred on the surface; too little and the grains remained too hard for fungi to grow on them.

To prepare the material for inoculation, the bags plus water were first placed in a water bath at 90-100°C for 1.5-2 h, taking care not to allow water from the bath to enter the bags. This 'cooking' caused the sorghum and bran to swell. The bags were then transferred to an autoclave, where they were processed at 121°C for 1 h. After sterilization, the bags were removed from the autoclave and allowed to cool completely prior to inoculation.

iii. Inoculation and growth. Substrates were inoculated with conidia (spores). These were harvested from 12-d old cultures grown on a sorghum substrate in glass jars (Fig. 1). Each bag was inoculated with 10 ml of suspension containing either 10^7 or 10^8 conidia/ml 0.01% Tween. Inoculation was carried out using a sterile syringe. Use of a high concentration of inoculum promoted colonization of the medium by the entomopathogen rather than any potential contaminants. This is an important consideration that ensures minimal occurrence of contaminants in the final product. Once inoculated, the bags were closed around a plastic collar with two layers of sterile paper or dense cloth over the mouth of the bags, allowing free exchange of oxygen to the growing culture while preventing entry of contaminants (Fig. 2 and 3). The substrate and inoculum were gently mixed by hand and the bags held at room temperature (22-28°C). After 3-4 d, the substrate in the bags was again gently mixed to prevent clumping of the medium as the fungi grew vegetatively. At this time the fungus was fully colonizing the growth medium. This process was repeated twice over the first 9 d. The fungi were allowed to grow for 15 d, at which time sporulation was complete (Fig. 2.3). The bags were then opened to allow the conidia and substrate to dry. The build-up of moisture in the bags at this stage can rapidly inactivate the conidia. The conidia were stored on the substrate under three temperature regimes (24-28; 10-12; and 4-6°C) and viability was assessed every 15 d for 60 d. Insecticidal activity was confirmed using standard bioassay procedures against WFT.

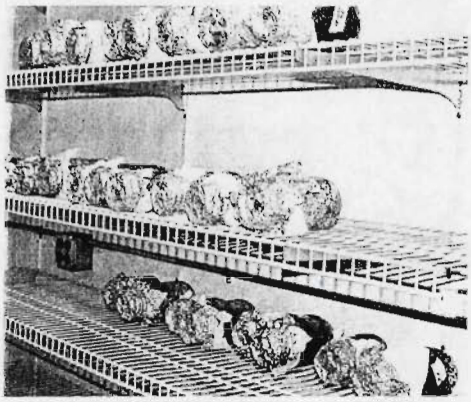

Figure 1. Production of inoculum in autoclavable glass jars.

Figure 2. Plastic 'collar' for closure of plastic culture bags.

iv. Harvesting conidia. For spray application, conidia must first be separated from the growth substrate. Conidia can be harvested in two ways. First, if the conidia are to be used immediately, they can be harvested 'fresh' by placing the substrate in water plus a wetting agent (e.g., 0.001% Tween 80) at a ratio of 1 part biomass: 10 parts water, agitating the mixture and then

filtering through several layers of cheesecloth. One kg of substrate yields 1 liter of suspension containing ~ 10^{10} conidia/ml. Kept cool, conidia in the suspension remain viable for several hours but are rapidly inactivated at room temperature and higher. This means that suspensions must be applied within 1-2 h of preparation.

Conidia remain viable for a longer period, i.e., they have a greater shelf-life, if dried. We tested two drying method: air drying and using a dehumidifier. Moisture content and viability were assessed at different stages of the drying process to quantify effects on the conidia. Once dry, spores can be easily separated from the substrate by sieving. By passing through a series of progressively smaller mesh sizes, conidial powders can be obtained containing minimal grain residue. This provides a clean powder that can be suspended for spraying and will not clog up nozzles during application. Tests were done to document the long-term stability of the dried conidia when held at room temperature (22-28°C), under cool conditions (10-12°C) and under refrigeration (4-6°C).

v. Results and Discussion. Results of the mass production trials are summarized in Tables 1, 2 and 3. Table 1 shows, by fungal isolate, the relative number of conidia produced/g of dry substrate and virulence to second instar WFT according to the growth medium used, ratio of substrate:water, and initial concentration of inoculum. Each of these variables affects productivity. Overall, greater numbers of conidia were obtained from the *B. bassiana* strains than *M. anisopliae*, although some intra-strain variation was also evident. Production of conidia was higher on the sorghum/bran substrate than the sorghum-only substrate for all of the isolates tested. Initial inoculum levels had little observable influence on conidial yield, although this would be more important if the growth medium was not completely sterile. In the current trials, we used an autoclave to completely sterilize the media. If such equipment were not available, then the media could be partially sterilized by boiling. Use of a higher concentration of inoculum would then improve colonization of the medium by the fungi rather than microbes (contaminants) surviving the sterilization process.

Figure 3. Production of *M. anisopliae*; note green color indicating heavy conidiation on the sorghum/bran substrate.

There was some variation in virulence to WFT by isolate, although there seemed to be no effect of substrate, water ratio or concentration of inoculum. At the standard test concentration used (5.10^6 conidia/ml 0.01% Tween), WFT infection and mortality ranged from 75-95%.

Spore viability decreased over time for all strains and at each storage temperature (Table 2). Viability declined most rapidly at room temperature. Viability was highest in material stored under refrigeration. Overall, viability of *B. bassiana* conidia was higher after 60 d than for *M. anisopliae*, although there were marked differences between the two *M. anisopliae* strains tested. The growth substrate did not appear to influence conidial viability over time within each of the test temperatures. The amount of water used in the preparation of the substrate did appear to affect conidial stability, however. The viability of conidia produced on substrates prepared at a substrate:water ratio of 1:0.75 was higher irrespective of the growth substrate for all sampling times and test temperatures. Use of a larger volume of water could have influenced the rate at which the medium dried, with subsequent negative effects on the viability of the conidia.

Table 1. Spore production and virulence on different grain media

Ratio of Substrate:Water	Concentration of Inoculum	Spore Production/g Dry Grain (x 10^8)	Virulence: % WFT mortality (5.10^6/ml)
Metarhizium anisopliae PPRC-29 on sorghum			
1 : 0.75	1.10^7	6	95
1 : 0.75	1.10^8	6	95
1 : 1	1.10^7	5	95
1 : 1	1.10^8	5	95
Metarhizium anisopliae PPRC-29 on sorghum/bran			
1 : 0.75	1.10^7	8	95
1 : 0.75	1.10^8	8	95
1 : 1	1.10^7	8	95
1 : 1	1.10^8	8	95
Metarhizium anisopliae var. *acridum* IMI-330189 on sorghum			
1 : 0.75	1.10^7	9	75
1 : 0.75	1.10^8	9	75
1 : 1	1.10^7	9	75
1 : 1	1.10^8	9	75
Metarhizium anisopliae var. *acridum* IMI-330189 on sorghum/bran			
1 : 0.75	1.10^7	10	75
1 : 0.75	1.10^8	10	75
1 : 1	1.10^7	9	75
1 : 1	1.10^8	9	75
Beauveria bassiana PPRC-56 on sorghum			
1 : 0.75	1.10^7	13	85
1 : 0.75	1.10^8	13	85
1 : 1	1.10^7	6	85
1 : 1	1.10^8	6	85
Beauveria bassiana PPRC-56 on sorghum/bran			
1 : 0.75	1.10^7	15	85
1 : 0.75	1.10^8	15	85
1 : 1	1.10^7	9	85
1 : 1	1.10^8	9	85
Beauveria bassiana 726 on sorghum			
1 : 0.75	1.10^7	10	85
1 : 0.75	1.10^8	10	85
1 : 1	1.10^7	7	85
1 : 1	1.10^8	7	85
Beauveria bassiana 726 on sorghum/bran			
1 : 0.75	1.10^7	13	85
1 : 0.75	1.10^8	15	85
1 : 1	1.10^7	8	85
1 : 1	1.10^8	8	85

Table 2. Viability of conidia produced on different substrates over time at three temperatures

Ratio Substrate: Water	Conc. of inoculum	Viability of Conidia (%) Stored at 4-6° C				Viability of Conidia (%) Stored at 10-12° C			Viability of Conidia (%) Stored at 24-28° C		
		1 d	15 d	30 d	60 d	15 d	30 d	60 d	15 d	30 d	60 d
Metarhizium anisopliae PPRC-29 on sorghum											
1 : 0.75	1.10^7	98	85	75	60	65	60	40	35	25	18
1 : 0.75	1.10^8	98	85	75	60	65	60	40	35	25	18
1 : 1	1.10^7	98	80	68	30	55	45	18	30	15	8
1 : 1	1.10^8	98	80	68	30	55	45	18	30	15	8
Metarhizium anisopliae PPRC-29 on sorghum/bran											
1 : 0.75	1.10^7	98	85	78	60	65	62	40	35	28	20
1 : 0.75	1.10^8	98	85	78	60	65	62	40	35	28	20
1 : 1	1.10^7	98	80	68	32	55	48	20	30	18	8
1 : 1	1.10^8	98	80	68	32	55	48	20	30	18	8
Metarhizium anisopliae var. *acridum* IMI-330189 on sorghum											
1 : 0.75	1.10^7	98	90	85	68	82	78	60	65	50	30
1 : 0.75	1.10^8	98	90	85	68	82	78	60	65	50	30
1 : 1	1.10^7	98	85	78	65	72	65	42	50	25	15
1 : 1	1.10^8	98	85	78	65	72	65	42	50	25	15
Metarhizium anisopliae var. *acridum* IMI-330189 on sorghum/bran											
1 : 0.75	1.10^7	98	90	88	72	82	80	62	65	58	32
1 : 0.75	1.10^8	98	90	88	72	82	80	62	65	58	32
1 : 1	1.10^7	98	85	80	68	72	68	48	50	28	18
1 : 1	1.10^8	98	85	80	68	72	68	48	50	28	18
Beauveria bassiana PPRC-56 on sorghum											
1 : 0.75	1.10^7	98	95	92	80	85	78	65	70	65	50
1 : 0.75	1.10^8	98	95	92	80	85	78	65	70	65	50
1 : 1	1.10^7	98	90	85	75	75	70	55	65	50	40
1 : 1	1.10^8	98	90	85	75	75	70	55	65	50	40
Beauveria bassiana PPRC-56 on sorghum/bran											
1 : 0.75	1.10^7	98	95	94	82	85	80	68	70	68	55
1 : 0.75	1.10^8	98	95	94	82	85	80	68	70	68	55
1 : 1	1.10^7	98	90	88	80	75	70	55	65	60	42
1 : 1	1.10^8	98	90	88	80	75	70	55	65	60	42
Beauveria bassiana 726 on sorghum											
1 : 0.75	1.10^7	98	95	90	80	92	78	60	66	58	40
1 : 0.75	1.10^8	98	95	90	80	92	78	60	66	58	40
1 : 1	1.10^7	98	90	80	70	86	70	58	50	40	35
1 : 1	1.10^8	98	90	80	70	86	70	58	50	40	35
Beauveria bassiana 726 on sorghum/bran											
1 : 0.75	1.10^7	98	95	82	78	92	80	68	66	60	50
1 : 0.75	1.10^8	98	95	82	78	92	80	68	66	60	50
1 : 1	1.10^7	98	90	82	78	86	70	58	50	50	40
1 : 1	1.10^8	98	90	82	78	86	70	58	50	50	40

On the basis of these data, the sorghum/bran substrate was selected as the most suitable medium for mass production. This was prepared for inoculation by mixing with water in a 1:0.75 (substrate:water) ratio and inoculating with a suspension containing 10^7 conidia/ml. Three strains were inoculated on the substrate: *B. bassiana* 726 and 082, and *M. anisopliae* 1080. All of these fungi readily grew and sporulated on the medium (Table 3). Conidia remained viable both through the air drying process (drying time 96 h) and when dried more rapidly with the aid of a de-humidifier (drying time 24 h) (Table 3). Viability of the dry conidia over time and at different storage temperatures was also assessed. Generally, dry conidia remained viable for several months, especially when stored under cool (<12°C) conditions. This would be of obvious benefit for farmers, as fungi could be produced ahead of the growing season and stored until needed, or could be produced at a more central location and shipped to areas where they were needed. If spores are harvested wet, the material must be used immediately. As it is difficult to accurately forecast exact dates when pests need to be controlled, use of a dry formulation offers much greater flexibility to the producer and farmer. These trials demonstrated the importance of defining protocols for a mass production system. Individual parameters can significantly impact spore yield, as well as shelf life. Furthermore, it is important to select strains that show good growth characteristics for mass production, i.e., minimal vegetative growth, dense and ready sporulation. Some strains which were highly virulent caused clumping of the grain substrate due to extensive mycelial growth. This not only limits sporulation, but makes it very difficult to harvest conidia. This should be one of the key characteristics included in the selection criteria for fungal strains in addition to virulence and ability to germinate and grow at temperatures that occur in the field at the time of pest infestation.

Table 3. Test of mass production protocols using two No. American isolates with *B. bassiana* 726 as the standard control: yield, moisture content and viability of conidia after drying

Fungal Strain	Yield/g Substrate	Moisture Content (%) After Drying		Conidial Viability (%) Before and After Drying		
		96 h air dry	24 h de-humidifier	0 h	96 h air dry	24 h de-humidifier
B. bassiana 726	$2.0x10^{10}$	9.2±1.3	19.6±3.4	99.0±0.4	97.0±3.5	98.7±0.5
B. bassiana 082	$6.7x10^{9}$	11.2±0.4	28.6±2.1	97.2±0.9	93.7±0.8	98.3±1.4
M. anisopliae 1080	$4.5x10^{10}$	17.9±5.2	25.9±2.4	98.8±2.0	97.4±1.5	96.9±0.6

Other Supporting Research Activities.

Because the ultimate goal of this project was to promote the use of indigenous isolates for biological control, research was completed to determine the most promising isolates from a collection made by Mr. S. Ayesheshim. The following work was conducted for several Ethiopian fungi: 1) bioassays to determine their relative pathogenicity to western flower thrips; 2) laboratory trials to assess their growth and sporulation characteristics and, 3) comparative tests to evaluate the suitability of different solid substrates for growing the selected Ethiopian fungi. In addition, fungal bioassays were conducted in Ethiopia against the sorghum chafer.

Pathogenicity assays. Single dose bioassays with western flower thrips (WFT) were conducted on 33 fungal isolates from Ethiopia. The fungi were grown on Sabouraud dextrose agar containing 0.25% w/v yeast extract (SDAY). Suspensions of each isolate were prepared in 0.01% Tween 80, adjusting the concentration to 5 x 10^6 conidia/ml. About 0.7 ml of the fungal suspension

was applied to two 7 cm-diam filter paper disks. Twenty thrips were placed on a filter paper, covered with a bean leaf disk and the second filter paper disk, after which all were clamped within a Munger cell. The Munger cells were placed in plastic bags to prevent dessication, held at 22°C, and mortality was read 7 d after treatment. Mortality among the 33 isolates ranged from around 65-98%, whereas mortality among the controls averaged <10%. Results from these assays were used to select the most promising isolates for further research and development.

Fungal growth and sporulation assessment. The growth rates of 33 isolates from Ethiopia were measured at four temperatures (20, 25, 30 and 35°C) on SDAY. A suspension of each isolate was prepared, adjusting the concentration to 1 x 10^6 conidia/ml. A circular disc of filter paper was placed in the center of each dish and inoculated with 10µl of each suspension. Each isolate and test temperature was replicated four times. The rate of radial growth was measured every 5 d for 20 d (Table 4).

Table 4. Fungal growth rate at different temperatures

Genera	Growth after 20 d		
	20°C	25°C	30°C
Beauveria	0-41.0	29.8-62.8	31.3->80.0
Metarhizium	0-40.0	37.5-72.8	28.3->80.0

Using plates prepared for the growth studies described above, four circular discs were taken from each plate with a cork borer along the radial growth of the colony at 5, 10, 15 and 20 d after inoculation. The discs were put in test tubes with 10 ml of 0.6% Greenshield, 0.1% Tween 80 and 40µM crystal violet and sonicated for several minutes. The spore concentration in the resulting suspension was determined with a hemocytometer (Table 5).

Table 5. Fungal sporulation at different temperatures

Genera	No. of spores / mm^2 after 20 d		
	20°C	25°C	30°C
Beauveria	4 – 6 x 10^6	3 – 6 x 10^6	5 – 6 x 10^6
Metarhizium	5 – 6 x 10^6	5 – 6 x 10^6	4 – 6 x 10^6

The full results generated from this research appear in the Activity Report prepared by Mr. Aysheshim (Appendix A).

Single dose screening assays against sorghum chafer. During the course of this project, the sorghum chafer, *Pachnoda interrupta*, surfaced as a significant insect pest in Ethiopia. Fungi have great potential as a component of IPM for this pest. Because of the serious damage caused by this insect, the Ethiopian cooperators felt this would be an excellent target pest to include in this project. They had a collection of 19 indigenous isolates, most of them recovered from coleopteran insects, with which to initiate this work.

Single dose assays were conducted in Ethiopia on all 19 isolates against larvae and sexually mature adult beetles to select the most virulent isolates warranting further evaluation. For each of

the 19 test isolates, 50 ml of a $1x10^8$ conidia/ml suspension in sterile 0.1% Tween (wetting agent) was prepared. Four batches of 10 larvae (similar age) and sexually mature beetles (5 male and 5 female) were immersed in each suspension (total of 40 beetles per concentration) for 10 sec. Controls were dipped in 0.1% Tween. The insects were transferred into plastic Petri dishes (120 mm diam) containing moist soil (16.67% water by volume) and held at 28°C. Every day following treatment, a slice of ripe banana was added to each dish, and insect mortality was assessed.

All of the test isolates caused mortality both to the larvae and adults, though the degree of virulence varied. Over 26% mortality among the larvae occurred 5 d after treatment. Two *M. anisopliae* isolates (PPRC14 and PPRC19) produced higher mortality rates than the other isolates. They induced 100% mortality among the larvae and adults at days 8 and 10, respectively. Likewise, among the tested isolates, the earlier median lethal time recorded both for the larvae and adults were by isolates PPRC14 (5.51 and 6 d, respectively) and PPRC19 (4.3 and 4.5 d, respectively). This study demonstrated that the larvae were more susceptible to fungal infection than the adults.

Objective 2. Assess the suitability of this technique in farmer cooperatives in Israel and Ethiopia and evaluate the quality and quantity of fungal material produced.

The original project included testing of the mass production method in farmer cooperatives in Israel and Ethiopia. Owing to continued political unrest and concern for the personal safety of the US and Ethiopian scientists who were scheduled to visit Israel, this second phase of the project, i.e., the planned testing of the mass production techniques in collaboration with researchers and farmers in Israel was cancelled. In its place, a second training program was organized at UVM. Mr. Seneshaw Aysheshim, the trainee who participated in the first training program (see below) returned to UVM from 16 January through 15 March 2002, during which time he tested and validated the mass production process developed in earlier stages of this project. Several fungi were tested in the system to ensure that the methods developed were robust enough to be applied to a broad range of fungal isolates. Training was also provided in harvesting and preservation techniques. In addition, quality control issues were addressed, e.g., how to ensure the product is free from contamination; the importance of regular testing to assure that products are of high viability and that insecticidal activity is maintained (i.e., ways of preventing attenuation of virulence). This ensures that the product reaching farmers is safe and of high and consistent quality.

From 33 fungal isolates collected in Ethiopia, two were selected for further assessment in the mass-production system, based on bioassays carried out against WFT at the ERL, and spore production studies in Israel. The spore production of these two indigenous isolates were compared with two exotic isolates. Untreated sorghum and millet were used as inexpensive substrates on which to produce the fungi. Full results are included in the Activity Report by S. Aysheshim (Appendix II) and will be briefly summarized herein. The viability of spores produced from agar slants was compared with those from the solid substrates (sorghum and millet), and in general no significant differences were detected. The rate of mortality for WFT ranged from around 40–70% among the test isolates. There was no evidence that the potency of the spores against the insect host was reduced as a result of the mass-production system we had devised.

Spore yield was also assessed for the two solid substrates. Millet produced more spores than sorghum for all test isolates (Table 6). Both grains maintained good structural integrity following autoclaving, but the millet was easier to mix than the sorghum. Though millet was clearly a better

solid substrate for mass production, it is significantly more expensive in Ethiopia, which may preclude its common use for fungal production.

Table 6. Spore productivity on sorghum and millet

Fungal Isolates	No. of spores / 10 ml grain		Relative productivity of millet (M/S)
	Sorghum (S)	Millet (M)	
PPRC 56	1.15×10^{10}	1.53×10^{10}	1.33
PPRC 29	9.50×10^{8}	4.85×10^{9}	5.10
IMI 330189	3.05×10^{9}	5.85×10^{9}	1.92
726	3.25×10^{9}	3.50×10^{9}	1.08

A full description of the mass-production procedure is contained in the Activity Report (Appendix II) and will not be repeated here. This procedure was utilized by the Ethiopian cooperator and resulted in large amounts of viable fungal spores.

In June 2003, Dr. M. Brownbridge, insect pathologist at the ERL, spent 4 days at the Plant Protection Research Centre (PPRC), Ambo, to review progress in the fungal research activities and construction of the mass-production facility. A laboratory, equipped for applied research in insect pathology through the current project, had been established and technical personnel hired to staff the facility. A designated room within the center was identified for remodelling into a mass production facility and plans were drawn up for construction, which began soon after the visit. Two days were spent providing basic training in insect mycology and basic microbiological procedures to a new technician, in anticipation of his assuming responsibility for maintenance and lab-scale production of fungi for experimental purposes.

Dr. S. Gouli led a 10-day intensive training course to four research technicians at PPRC, Ambo in October 2004. The technicians leamed basic techniques by instruction and doing, covering a range of topics from preparation of mycological media to mass-production of fungi on cereal grains. The laboratory established for insect mycology was well equipped for productive research. The training program provided the technical personnel with all of the skills necessary to carry out their respective duties in such a program, where they were to serve as support staff for the primary scientists. Special emphasis was placed on identifying fungal entomopathogens, and learning to distinguish them from common saprophytes and potentially hazardous contaminants. Training was also provided in the use of safety equipment, e.g., respirators, gloves, required when harvesting conidia from grains in the mass production facility.

Construction of the mass production facility was completed during this training session, with additional materials, e.g., electric heaters, shelving, purchased in Addis Ababa and local markets. Locally-available grains were tested for their suitability as growth substrates for the mass production process. The process originally entailed production of fungi in autoclavable plastic bags. These cost approx. US$1.00 each, and are difficult to obtain in Ethiopia. We thus sought to identify a local alternative. Various plastic shopping bags were tested for their ability to withstand autoclaving, and suitability for mass production (Fig. 4). Several types were found that satisfied

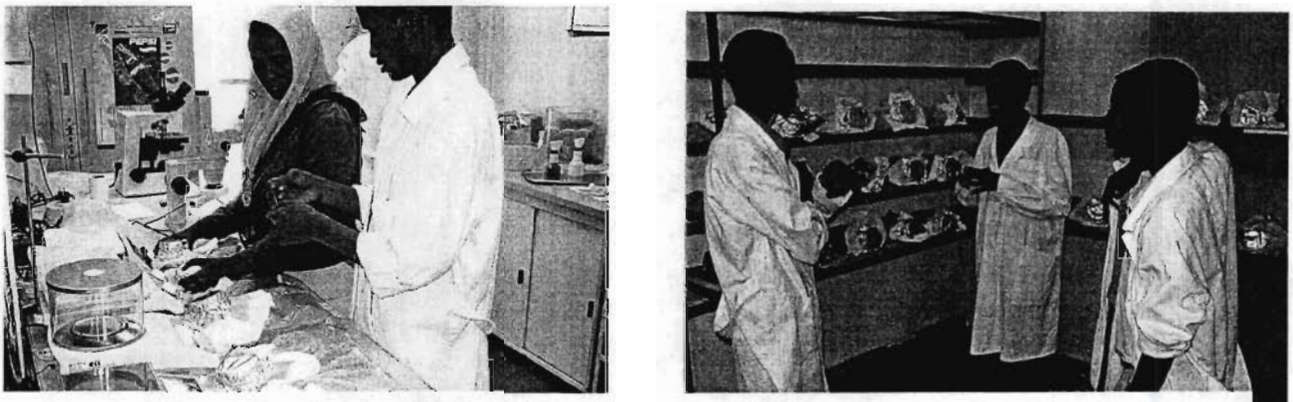

Figure. 4. Preparation of grains for mass-production of fungi; inside the mass production room, technicians discuss the procedure. Note colored autoclavable plastic shopping bags used in the production process.

these criteria; these are manufactured and sold in Addis Ababa and cost <$2 for 80 bags, or approx. $0.02 each, a considerable cost savings.

Objective 3. Present a practical comprehensive educational program on the basics of entomopathogenic fungi to Ethiopian technical personnel.

Educational programs at many levels and for several different audiences were presented throughout this project, and will be discussed separately. Beginning and advanced technical training programs in insect pathology, with a focus on insect-killing fungi, were held at the UVM ERL. In addition, UVM scientists travelled to Ethiopia to provide hands-on training at the research facility to ensure that the techniques were being implemented correctly. Two events served to introduce other scientists and pest managers in Ethiopia to the value of fungi for pest management.

Technical Training Programs. The goal of these training programs was to introduce Ethiopian trainees to the basic principles of entomopathogenic fungi as they relate to the development of these microbes for IPM. Emphasis was placed on the characterization of fungal isolates to determine their suitability for their use in IPM of a specific target pest, and for mass production. An 8-week beginning level training course at the UVM ERL was developed to be presented in a practical manner with an appropriate mix of direct one-on-one instruction and hands-on exercises. The following curriculum was established:

A. Insect rearing to support bioassay for fungal pathogenicity determinations
   a. Western flower thrips (WFT), *Frankliniella occidentalis*
   b. Tarnished plant bug, *Lygus lineolaris*
   c. Green peach aphid, *Myzus persicae*

B. Basic Microbiological Techniques
   a. Fungal isolation and recovery
   b. Preparation of different agar mediums
   c. Preparation of serial dilutions
   d. Growing and harvesting fungi on agar medium

C. Standard Bioassay Techniques
   a. Fungal treatment methods
      Immersion, Direct application, Indirect application, Precision spray using a Potter's tower
   b. Munger cell assays, On-plant and in-soil assays
   c. Specialized assays with range of test organisms
      Western flower thrips, Tarnished plant bug, Green peach aphid

D. Entomopathogenic fungal identification
   a. Collection and preparation of microbials for identification
   b. Use of keys to identify insect-killing fungi
   c. Critical characters of entomopathogenic fungi
   d. Basic procedures for storage of fungal isolates

E. Trials for development of Ethiopian entomopathogenic fungal isolates
   a. Pathogenicity using western flower thrips
   b. Rate of growth, sporulation and germination

F. Methods of mass production of entomopathogenic fungi
   a. Laboratory techniques and on-farm techniques
   b. The importance of temperature, moisture and sanitation

This training program was presented to Mr. Seneshaw Aysheshim from 21 September – 30 November 2000 (see Appendix I). All of the laboratory exercises were done using entomopathogenic fungal isolates collected in Ethiopia by the trainee. Following is a list of the Principal Instructors that contributed to this training program:

Dr. Bruce L. Parker, Entomologist
Dr. Michael Brownbridge, Insect Pathologist
Dr. Vladimir Gouli, Insect Pathologist
Dr. Robert Jones, Insect Rearing Specialist
Mr. Alek Adamowicz, Pathology Technician
Dr. Svetlana Gouli, Microbiologist
Dr. Margaret Skinner, Entomologist
Dr. Richard A. Humber, Mycologist, USDA, ARS
Mr. William Reid, Insect Pathologist
Mr. Steven LaRosa, Insect Rearing Specialist

As indicated in Objective 2 above, testing of the mass production techniques in Israel was cancelled and instead Mr. Aysheshim took part in an advanced training program at the UVM ERL from January 16 through March 15, 2002, during which time he tested and validated the mass production process developed in earlier stages of this project. A report of this training program is found in Appendix II.

Unfortunately, Mr. Aysheshim left EARO unexpectedly in June 2002, which necessitated the selection of individuals to take over the work responsibilities for the project. Working with Dr. Bekele (EARO, Holetta), two scientists at the EARO facility in Ambo were identified, Dr. Mohammed Dawd and Mr. Wondirad Mandefro. While both are trained entomologists, neither had experience in insect pathology. An intensive training course was thus organized at UVM from 26 January through 2 March 2003 (see reports in Appendix III & IV). The course curriculum was

revised slightly to address issues of particular interest to these trainees and included the following topics:

1. Basic Microbiological Techniques
   - Preparation of different agar media
   - Growing and harvesting fungi on agar medium
   - Enumeration of fungal concentrations using a hemocytometer
   - Preparation of serial dilutions for bioassay
2. Identification of Fungi
   - Preparation of fungi for identification
   - Use of keys to identify insect-killing fungi
   - Critical characteristics of entomopathogenic fungi
3. Comparison of methods for isolation of fungi from soil
   - Baiting, Soil dilution, Direct inoculation, Print method, Electrostatic method
4. Bioassay of insects
   - Western flower thrips and soil insects (using *Spodoptera exigua*)
5. Characterization of entomopathogenic fungi – identification of desirable traits for mass production and field efficacy
   - Fungal growth rate at different temperatures
   - Conidial germination rate at different temperatures
   - Spore production by different fungal isolates
6. Mass production of fungi
   - Preparation of inoculum for mass production
   - Evaluation of 5 different substrates for fungal growth
   - Selection of optimal moisture levels for spore production
   - Mass production in glass jars, plastic bags and liquid substrates
   - Quality control issues
7. Long-term storage of fungi
   - Preparation of slant cultures and preservation under oil
   - Refrigerated storage
   - Storage in ultra-low temperature freezer

Following is a list of the Principal Instructors at the Entomology Research Laboratory, Univ. of Vermont, who contributed to the organization and initiation of the training program:

Dr. Bruce L. Parker, Entomologist
Dr. Michael Brownbridge, Insect Pathologist
Dr. Vladimir Gouli, Insect Pathologist
Dr. Scott Costa, Entomologist and Biostatistician
Mr. Tom Doubleday, Greenhouse Manager
Dr. Svetlana Gouli, Microbiologist
Dr. Margaret Skinner, Entomologist
Dr. Robert Jones, Insect Rearing Specialist
Mr. Don Tobi, Entomologist

As mentioned in Objective 2, ERL scientists visited Ethiopia twice during the project to provide in-country support and training to the cooperators and their technical personnel. Dr. M. Brownbridge spent several days in June 2003, and Dr. S. Gouli spent 10 days there in October, 2003. This follow-up training expanded the scope of the training to a broader audience, and provided guidance and encouragement to the Ethiopian cooperators to continue the research.

Seminars and Workshops. Dr. Brownbridge participated in the Eleventh Annual Conference of the Crop Protection Society of Ethiopia (CPSE) in Addis Ababa (June 5-6, 2003), where he gave an invited paper titled 'Biological control using insect pathogenic microorganisms and their potential for the management of agricultural pests in Ethiopia'. Participation in this conference provided an opportunity to interact with other Ethiopian researchers and extension specialists with interests in biological control, and to learn more about on-going research. Some of these specialists were recruited to take part in the biological control workshop later that year.

The Biological Control Workshop at the Upper Awash Agro-Industry Enterprise was coordinated jointly by the UVM ERL and collaborators from Ethiopia. The program was designed to stimulate interest among the broader EARO personnel in this project (see Appendix V for agenda and list of participants). A greater goal was to promote greater awareness of the benefits of IPM and biological control, to discuss the role of natural enemies, including insect-killing fungi, in pest suppression and the importance of their conservation, and to examine alternative pest management strategies with farmers and extension personnel.

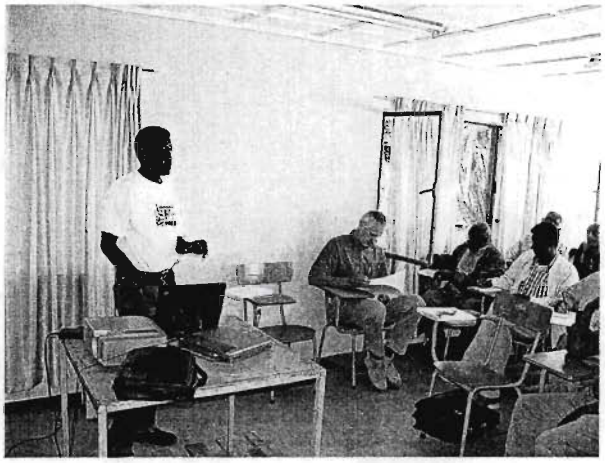

Fig. 5. Dr. Feredu Azerefegne presenting on insect pest problems at the Upper Awash Enterprise.

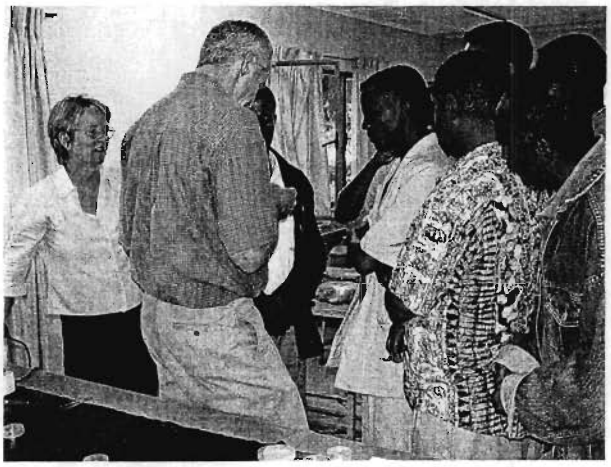

Fig. 6. Louise Labuschagne & Dr Michael Brownbridge with a hands-on demonstration of natural enemies found at Mertijeju.

Thirty people attended the day-long workshop, including entomologists, pathologists and agronomists, and featured presentations on citrus pests and diseases, biological and cultural control tactics, and conservation methods that could be utilized in their management (Fig. 5). The morning sessions were followed by hands-on sessions to familiarize attendees with natural enemies of pests and diseases (Fig. 6). The specimens presented were collected the day before the workshop from citrus orchards and field crops located on the farm, so that all of the natural enemies on display were indigenous. For the demonstration sessions, three display stations were established and participants divided into 3 groups to maximize the hands-on experience, and interactions with presenters. This is an excellent way of disseminating information and maintaining a high level of participation and interest. All attendees were provided with a hand lens, and instruction in its correct use for viewing and recognizing pests and natural enemies. The workshop was very well received by all attendees, many of whom had not previously understood the impact of natural enemies on pest populations, and were unaware of their presence in citrus and other crops on the farm. In addition to the formal training program, the workshop allowed presenters to interact, and enabled collaborative linkages to be established that will be beneficial in future projects.

F. Impact, Relevance and Technology Transfer

The main goal of the project was to develop the capacity to study and produce insect-killing fungi for crop protection in Ethiopia. Through the training programs given to EARO scientists and technical personnel and the equipment and supplies provided to establish a mass-production facility at the Ambo Plant Protection Research Center, this capacity has been achieved. Procedures for mass-producing insect-killing fungi were developed and tested that are appropriate for Ethiopia, and have been compiled into manual form. As a result of this project, research in Ethiopia is underway to investigate the use of insect-killing fungi for management of pests such as sorghum chafer and desert locusts in staple food crops, and thrips in citrus, a valuable cash crop for export. Establishment of a mass production facility and training in mass production techniques has allowed researchers to produce inoculum for field and laboratory study. This technology may also be applied to the production of antagonistic fungi used to suppress soil-borne plant diseases, e.g., *Trichoderma harzianum*, which is also being evaluated within EARO. The short- and long-term benefits of the project to agricultural research are thus being realized.

A significant portion of the budget was used to purchase equipment to establish a research laboratory at the PPRC, Ambo. The laboratory is now fully equipped to conduct basic research, as well as provide fungal inoculum for research and management purposes. Combined with improvements to the infrastructure of the Research Centre, the scientific training ensured that technical personnel are able to use the equipment correctly. This allowed objectives within the current project to be achieved, and has also strengthened the overall capacity of the EARO to do research on insect pathogens for many years in the future.

Technology transfer was a fundamental component of this project, and the multiple ways that this was addressed is specifically described in the summary of Objective 3 above. Briefly, educational programs at many levels and for several different audiences were presented. Beginning and advanced technical training programs in insect pathology, with a focus on insect-killing fungi, were held in the US, led by scientists at the UVM ERL. In addition, UVM scientists travelled to Ethiopia to provide hands-on training at the Ambo research facility to ensure that the techniques were being implemented correctly. Finally two events served to introduce other scientists and pest managers in Ethiopia to the value of fungi for pest management and biological control in general.

G. Project Activities/Outputs

1. Meetings Held:

Meeting Title/Type	**Dates of Program**	Attendance	**Location**	**Purpose**
Coordina-tion Meeting	25 – 31 July 2000	B.L. Parker, M. Skinner, M. Brownbridge, S. Gouli, V. Gouli, I. Barash	Burling-ton, VT	To coordinate activities relating to the project and develop a work plan
Coordina-tion Meeting	25 – 31 July 2000	B.L. Parker & M. Skinner	Several locations Ethiopia	To coordinate activities relating to the project, develop a work plan, identify cooperators, assess equipment needs for establishment of the insect pathology lab

Meeting Title/Type	Dates of Program	Attendance	Location	Purpose
Coordination Meeting	25 July 2000	T. Gebremedhin, M. Skinner, E. Bekele, S. Aysheshim, B.L. Parker	Addis Ababa, Ethiopia	To discuss the project, develop a work plan, identify cooperators and determine primary pest problems and role of fungi in existing agricultural systems
Coordination Meeting	26 July 2000	S. Aysheshim, M. Skinner, B.L. Parker, E. Bekele A. Kidane & K. Ali	Holetta Agric. Research Center	To identify cooperators and assess equipment needs for establishment of the insect pathology facility
Coordination Meeting	27 July 2000	S. Aysheshim, M. Skinner & B.L. Parker	Ambo Agric. Res Center	To identify cooperators and assess equipment needs for establishment of the insect pathology facility
Coordination Meeting	30 July 2000	E. Bekele, D. Dawit, S. Aysheshim, M. Skinner, B.L. Parker	Addis Ababa	To discuss the project in general, develop a work plan, identify cooperators and assess equipment needs for establishment of the insect pathology facility
Coordination Meeting	November 2000	B.L. Parker, V. Gouli, M. Skinner, G. Gindin, M. Brownbridge, S. Gouli, S. Aysheshim	UVM, Burlington, VT, USA	To discuss the project, develop a work plan and establish standardized bioassay methods for use by all cooperators
Workshop Planning	February 2003	M. Brownbridge, S. Gouli, V. Gouli,D. Mandefro, M. Dawd	UVM, Burlington, VT, USA	To discuss workshop program, logistics and audience
Workshop Planning	3-4 June 2003	D. Mandefro, E. Bekele, M. Dawd ,M. Brownbridge	Addis Ababa	To discuss workshop program, logistics and audience
Coordination Meeting	October 2003	D. Mandefro, E. Bekele, M. Dawd ,M. Brownbridge	Addis Ababa	To evaluate the workshop program, discuss the status of the project and future opportunities for collaboration

2. Training Programs Provided:

Purpose of Training	Program Dates	Trainee	Site
Basic microbiological training	21 Sept. – 30 Nov. 2000	S. Aysheshim	UVM, VT, USA
Advanced microbiological training on specialized bioassay methods	October 2000	G. Gindin	UVM, VT, USA
Advanced microbiological training	16 Jan. – 15 Mar. 2002	S. Aysheshim	UVM, VT, USA
Basic microbiological training	26 Jan. – 2 Mar. 2003	M. Dawd & W. Mandefro	UVM, VT, USA
In-country training in advanced fungal mass production techniques	June 2003	M. Dawd, W. Mandefro, and technical personnel	Ambo Research Center, Ethiopia
Eleventh Annual Conference of the Crop Protection Society of Ethiopia	5-6 June 2003	Conference participants from Ethiopia	Addis Ababa

Purpose of Training	Program Dates	Trainee	Site
In-country training in advanced fungal mass production techniques	October 2003	M. Dawd, W. Mandefro, and technical personnel	Ambo Research Center, Ethiopia
IPM and Biological Control	6 October 2003	M. Dawd, W. Mandefro, E. Bekele, Ethiopian pest managers and specialists, see list of participants, App. V	Mertijeju, Upper Awash Agro-Industry Enterprise, Ethiopia

3. Publications.

Gouli, V., S. Gouli, B.L. Parker, M. Brownbridge & M. Skinner. 2006. Manual for Mass Production of Insect-Killing Fungi for Ethiopia. The University of Vermont, Entomology Research Laboratory, Burlington, VT.

H. Project Productivity

As evidenced by this report of the activities and research completed, the proposed goals were definitely accomplished. In fact more was achieved than originally projected to be done for this project despite minimal financial remuneration. However, significant challenges were faced throughout the project which made accomplishing the objectives difficult. The first challenge occurred at the initiation of the project. There was a lapse of several years from the time the proposal was submitted to the time it was funded. During that time, our projected cooperator, who had completed a Ph.D. degree in insect pathology at UVM, had been deported from Ethiopia as a result of the conflict with Eritrea. This required identification of new collaborators before the project could proceed. We were able to find an appropriate replacement, but that took time and delayed project progress. The project as originally proposed included a significant role for the Israeli cooperators, in particular testing of the mass-production methods at farmer cooperatives. This aspect of the project had to be replaced with testing at UVM because of political unrest in Israel. This reduced the direct involvement of the Israeli cooperators that had been projected. The program was again challenged with the unexpected departure of the scientist originally assigned to the program by the Ethiopian project leader. To ensure that the benefits of the project were sustained at its completion, we had to revise the original plan again by offering training to two additional Ethiopian researchers. Finally, communication among the cooperators has been difficult throughout the project. This is in large part a result of the limited internet infrastructure in Ethiopia. Despite these numerous difficulties, we were pleased with the overall accomplishments, which we believe have benefited the farmers of Ethiopia, and will continue to do so for the future.

I. Future Work

There are no specific plans at this time to continue collaboration on insect-killing fungi among the US and Ethiopian scientists. However, should funds become available we will pursue further investigation of this promising biological control option.

J. Literature Cited None

K. Appendices

I. Activity Report, S. Aysheshim (November 2000)

II. Activity Report, S. Aysheshim (March 2002)

III. Training Report, M. Dawd (March 2003)

IV. Training Report, W. Mandefro (March 2003)

V. Agenda, Biological Control Training Program (Oct. 2003)

VI. Trip Report: Biological Control Opportunities at the Upper Awash Agro-Industry Citrus Enterprise
by Michael Brownbridge

APPENDIX I

Activity Report, S. Aysheshim (November 2000)

INSECT PEST MANAGEMENT WITH FUNGI: A MASS PRODUCTION TECHNIQUE FOR FARMERS

Cooperative Development Research Project C-16-125

Activity Report

by

Seneshaw Aysheshim

Ethiopian Agricultural Research Organization
Plant Protection Research Center, Ambo
Ethiopia

November 30, 2000

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Preface

Citrus thrips and sorghum chaffer are among the high priority insect pests in Ethiopia requiring critical attention. Control of these pests using chemical insecticides couldn't bring satisfactory solution on top of the fact that they are very expensive and environmentally unsafe.

Entomopathogenic fungi have strong potential as viable control agents for both insects, as evidenced with other closely related insect pests. The Entomology Research Laboratory (ERL) at the University of Vermont, USA, together with scientists from Israel and Ethiopia have launched a project on the development of indigenous biological control agents which can be produced locally to provide an inexpensive, non-toxic and self sustaining means of pest management.

One component of the project is to give short-courses on the use of entomopathogens in IPM for researchers in order to facilitate promotion of the technology transfer to Ethiopian farmers. Based on this, a three month on-job training was given at the ERL focusing on techniques of insect bioassay, rearing of insects, fungal mass production and growth optimization studies. This paper reports activities carried out at the ERL and the major outcomes of the research.

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Acknowledgments

I wish to express my appreciation to Prof. B. L. Parker, Drs. M. Skinner and M. Brownbridge for initiating this research project and organize the training. I am in particular grateful to Dr. S. Gouli and W. Reid for the outstanding assistance and for making my stay more enjoyable. I thank Dr. Richard Humber for identifying the specimens and sharing his identification skills. The technical assistance of Drs. B. Jones and S. Costa, S. LaRosa, A. Adamowicz and Panagiotis Lekkas is highly appreciated. I am thankful to all the staffs of ERL for making my visit pleasant and enjoyable. I also thank my employer, Ethiopian Agricultural Research Organization, for allowing me to spend three months at the University of Vermont. The research was funded by the US Agency for International Development.

MAJOR OUTPUTS

- Training on rearing of thrips, tarnished plant bug and green peach aphid; mass production of fungi and assaying techniques.
- Over 40 indigenous fungal isolates, collected from Ethiopia, were identified and their pathogenicity examined on western flower thrips under laboratory condition.
- Training on the essentials of insect pathology and identification of entomopathogenic fungi and common saprophytes.
- Characterization of Ethiopian fungal isolates for growth rate and spore production.
- All the Ethiopian fungal isolates have been purified and sent to Ethiopia for further studies and use.

Insect rearing

1. Western Flower Thrips:

The western flower thrips (WFT), *Frankliniella occidentalis* (Thysanoptera: Thripidae) is an economically important pest of a range of crops in the United States and Europe (Bryan and Smith, 1956; Yudin et al. 1986). Current control measures involve use of non-selective pesticides. Since recent time, however, considerable attention has been given to the use of entomopathogenic fungi as a potential biological control agent.

The Entomology Research Laboratory (ERL) at the University of Vermont has been doing research on the biological control of this pest using laboratory-reared thrips. Rearing of thrips under laboratory condition provides a healthy colony and even-aged insects at all stages of development. This minimizes variability in bioassay results associated with age differences of the test insects. The method described below has been used for the past eight years at ERL and supplied up to 5000 thrips per week (Doane, et al 1998).

Materials:

- Rearing chamber with outside dimension of 62.2 x 54.6 x 81.3 cm
- An Archer 80 mm fan
- A 61 cm florescent light fixture with a 20 watt cool white bulb
- Clear acrylic boxes (15.8 x 10.0 x4.2 cm)
- Cotton
- Distilled water
- Bean leaves

Methods:

Clear acrylic boxes (15.8 x 10.0 x 4.2 cm) were used for rearing of the thrips. A double layer of cotton (6 x 29 cm) was cut in half, and one half split to make two thinner cotton layers. These two thinner layers were wrapped around the bottom and sides of the other half. Then, about 250 ml of distilled water was added to the box while pressing the cotton with the middle finger to evenly distribute the water. Excess water was then pressed out to avoid standing water in the box. A slit was made in the cotton using a spatula and the petiole of a bean leaf (2 per box) was inserted into the water-saturated cotton that supplies moisture throughout the development of the insect. Care was taken to ensure that the edges of the leaf overlap slightly. This allows movement of thrips from leaf to leaf without them being trapped in the moist cotton. The bean leaves were used both as a source of food and egg laying. Apple pollen was also put at the base of each leaf to stimulate fecundity. The beans were grown in small plastic six-pack planters in standard potting medium that become ready 12-14 days after sowing.

Mated females were then transferred to each of the plastic boxes using aspirators. Female thrips are noticeably larger than males when viewed at 10x magnification, and are assumed mated if males are present in the same box. The thrips boxes were wrapped with black electrical tape to prevent thrips escape and kept in the rearing chamber, which

is made of 6.35 mm Luan Plywood, with outside dimensions of 62.2 x 54.6 x 81.3 cm (Fig. 1). The chamber holds 25 thrips boxes (Fig. 2). The door is 64.8 x 40.0 cm and is hinged at the base, opening outward with a toggle at the top to hold it closed. A ventilator is also positioned in the intake hole, which is covered on the inside with a fine nylon screening. A 20-watt florescent lamp is mounted inside the top of the chamber that provided an inside temperature of 24-25°C and photoperiod of 16:8 (L:D). The thrips rearing chambers were kept in an enclosed room with thrips proof screening.

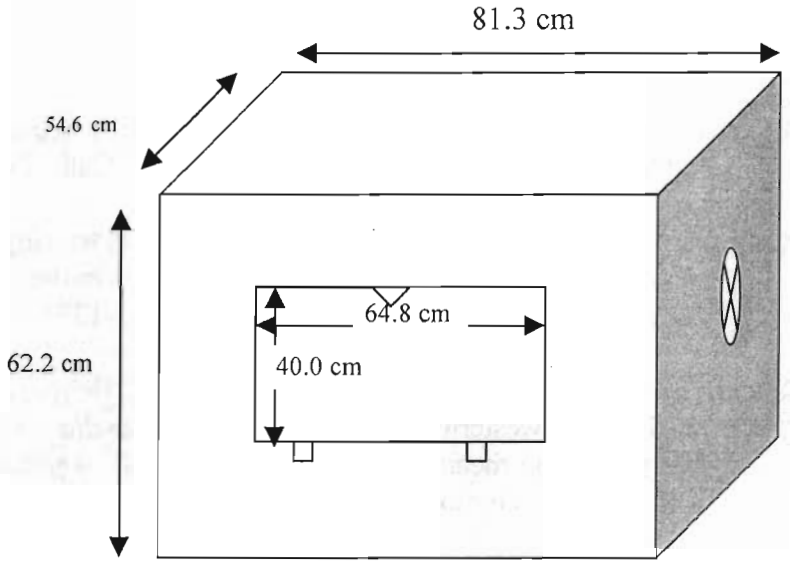


Fig. 1. Chamber used for rearing of western flower thrips.

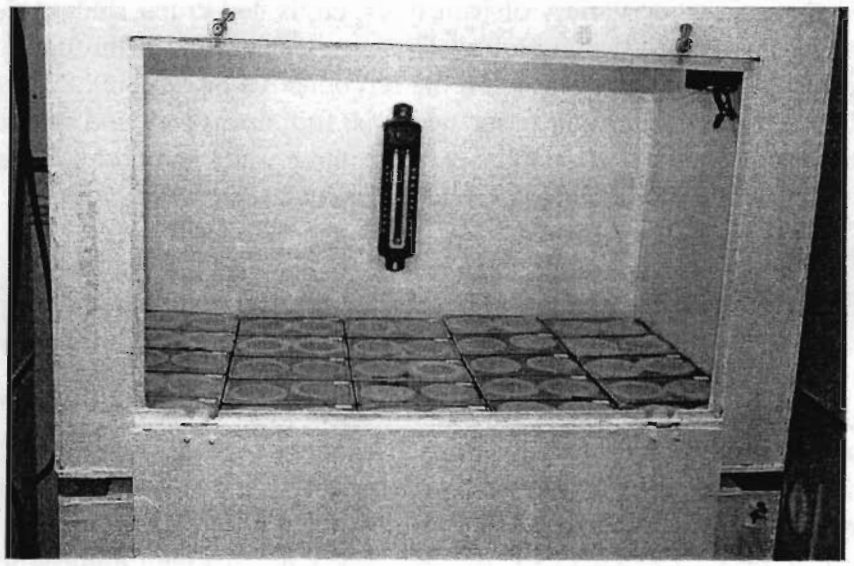


Fig. 2. Thrips boxes inside the rearing chamber.

First instar thrips were usually noted four days after the adult females are caged. Hence, all adults were removed with an aspirator 48 h after introduction. When the newly emerged thrips become second instars (eight days old), they were transferred into small vials and used in bioassays.

Old boxes were put in a freezer after thrips have been removed in order to minimize contamination. After the boxes have been frozen for at least 24 h, the contents were removed and disinfected by soaking in 10% bleach for 20 minutes and then washed with detergent before reused.

References:

Bryan, D. E. and R. F. Smith. 1956. The *Frankiniella occidentalis* (Pergande) complex in California (Thysanoptera: Thipidae). Univ. Calif. Pub. Entomol. 10: 359-410.

Yudin, L. S., J. J. Cho and W. C. Mitchel. 1986. Host range of western flower thrips, *Frankiniella occidentalis* (Thysanoptera: Thripidae), with special reference to *Leucaena glauca. Environ. Entomol.* 15: 1292-1295.

Doane, E. N., Parker, B. L., LaRosa, S., Skinner, M., Boone, J. and Pivot, Y. 1998. Mass rearing of western flower thrips, *Frankinella occidentalis* (Thysanopetera: Thripidae) on beans. Technical Note #4. Agricultural Experiment Station, University of Vermont.

2. Tarnished Plant Bug

Tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) is a serious pest of a wide variety of fruit trees, cultivated crops, and garden flowers with a host range of over 300 plant species. The insect is distributed throughout North America. Both the nymphs and adults feed on the reproductive organs (buds, flowers, young fruits and plant tissues) resulting in tissue necrosis, fruit abscission and seed deformation. The ERL has a colony of TPB established some three years ago and has a size of 1500-2000 adults with an egg production of 4000-5000 per week.

Materials and Methods:
Chemically untreated green bean (*Phaseolus vulgaris* L. - 'blue lake') pods and broccoli (*Brasica oleracea* var.*itlicaa* Plenck - "packman" or "premium") flowers were used to feed the adults and nymphs, respectively.

The rearing cage was made from four pieces of PVC sewer pipe. The long and the three short sections were used to make the main body and the bottom and the removable top for the cage, respectively. Two types of cages were used for the rearing. Matured adults were put in circular cages (15 x 10 cm diam. x height) with eight holes drilled at equal distance in the upper wall to allow the insertion of the bean pods (Fig.3). The nymphal rearing cages had a dimension of 10 by 7.5 cm with an opening on the top. Bottom and

top openings for the adult and nymphal rearing cages were covered with 20 and 50 mesh nylon, respectively.

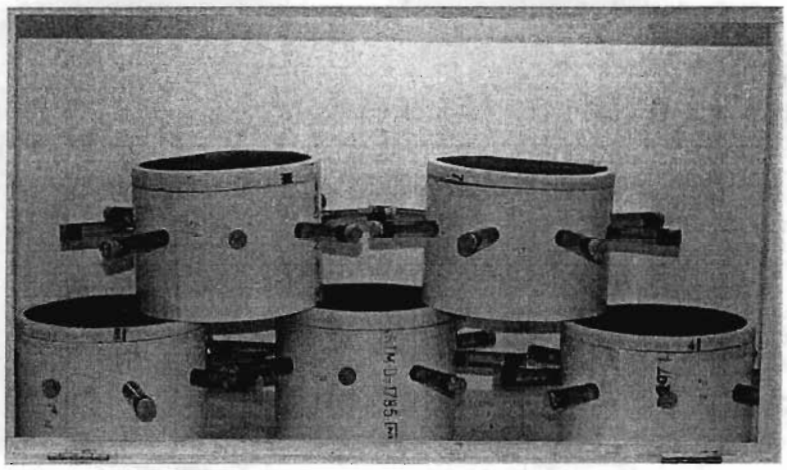


Fig 3. Rearing cage of TPB used for oviposition.

Adult rearing and egg deposition:
Females were allowed to deposit their eggs on a facial tissue paper (Angel soft brand). The bean pods were wrapped with double layers of tissue paper (2.5 cm wide) and inserted into the scintillation vial (7 cm) filled with water. About 200-250 adult TPB were put in each cage containing 6-8 bean pods. The adults fed on bean pods and laid most of the eggs on the tissue paper. The pods together with the tissue paper were replaced every 3-4 days.

Egg extraction and preparation:
All the tissue papers from one adult cage were unwrapped from the bean pods and put in a separatory funnel for egg extraction. About 300 ml water was added and the tissue beaten with a pair of forceps until pulped. An additional 600 ml of water was added before the mixture is stirred by an electronic stirrer with a metal drill bit (1.2 cm in diameter). The stirrer was operated in alternate speed of high (500 rpm) to low (200 rpm) until the circulation is just sufficient to prevent the fibers from settling. Under this condition eggs fell out of the gently circulating pulp and settled to the bottom of the funnel, where they were drained into a plastic container. Most eggs were collected within 5 minutes of stirring. Eggs obtained from the extraction process were washed several times to get rid of small tissue fibers before water was drained through a filter paper with a glass powder funnel. Eggs on the filter paper were then spread out, counted and transferred to a petri dish with a tissue paper on the bottom. About 10 drops of water were put on the tissue paper to keep the overlaid filter paper moist.

Egg hatching:
Eggs hatch in about 7 days at 25 °C. Each petri dish with eggs were inspected daily for the moisture and signs of hatching. Often, the red eye-spots and green abdomen in the developing nymphs become visible five days after extraction. The newly emerged nymphs were fed a small piece of broccoli floret. After 24 hours, nymphs and hatching

eggs on the filter paper were transferred to a plastic container with two layers of tissue paper (7 x 14 cm). The container was tightly sealed with a lid to maintain humidity inside.

Nymph rearing:
Newly hatched nymphs were transferred daily from the plastic containers to nymphal rearing cages and supplied with a broccoli floret. Each nymph rearing cage could hold 200-300 nymphs. Fresh broccoli florets were replaced daily except for the first 3-4 days to avoid disturbance of the mall nymphs.

References:

Parrot, W. L., Jenkins, J. N., Maxwell, F.G. and Bostick, M. L. 1975. Improved techniques for rearing the tarnished plant bug, Lygus lineolaris (Palisot de Beauvois). Miss. Agric. Exp. Stn. Tech. Bull. 72.

Slaymaker, P. H. and Tugwell, N. P. 1982. Low-labor method for rearing the tarnished plant bug (Hemiptera: Miridae). *J. Econ. Entomol.* 75: 487-488.

Snodgrass, G. L. and McWilliams, J. M. 1992. Rearing tarnished plant bug (Heteroptera: Miridae) using a tissue paper oviposition site. *J. Econ.. Entomol.* 85: 1162-1166.

Wilson, R. L. 1973. Rearing Lygus bug on green beans: a comparison of two oviposition cages. *J. Econ. Entomol.* 66: 810-811.

3. The Green Peach Aphid

The green peach aphid (GPA), *Myzus persicae* (Sulzer), is a cosmopolitan insect (Radcliffe 1982), which feeds on over 400 different plants and vectors more than 100 different plant viruses (Mackauer & Way 1976). Resistance to most pesticides also has made control of this pest very difficult (Devonshire 1989).

Even aged rearing
Green pepper plants, *Capiscum annuum* cv: Lantern, were grown in Metromix 20 with a constant feed 60 ppm 20-20-20. Third, fourth or fifth true leaves were excised and the petiole was immersed in a 50-ppm solution of 20-20-20 all purpose fertilizer held in a creamer cup (~30ml) glued to the bottom of a greenhouse flat (30cm x 50cm). A total of 30 leaves were inoculated with four adult GPAs for a period of 48 hours at which time, the adults were removed individually with a camel's-hair brush. The remaining progeny averaged between ten to twenty GPA per leaf.

Bioassay preparation
Creamer cups were prepared by pouring 5 ml of 0.7% water-agar media into the bottom of a 3-cm tight fitting Falcon® brand petri dish (Fig. 3). A pepper leaf disc (cv: Lantern)

was cut using a scalpel blade to the exact dimensions of the dish. These discs were then innoculated with eight 10 day old GPA from the even aged colony and capped with an inverted creamer cup to which the bottom had been vented with a circle of thrips proof mesh (2.54 cm diameter). The rearing was done at room temperature (~23°C). After twelve to sixteen hours, adults were removed. The resulting progeny varied between twelve and twenty five per cup and were held for an additional twenty-four hours so that all became 2nd instars.

Bioassay treatment

Bioassay dishes were treated individually using a Potter's precision spray tower. Three ml of test suspension were used per dish at 15 psi using a fan nozzle. After application, the dishes were covered with the vented creamer cup and the rim was sealed with parafilm. A second creamer cup was used to cap the vented cup for the first 24 hours to maintain free water for germination to occur. Dishes were held for six days post treatment between 22°C and 25°C and mortality assessed using a dissecting stereoscope (10x).

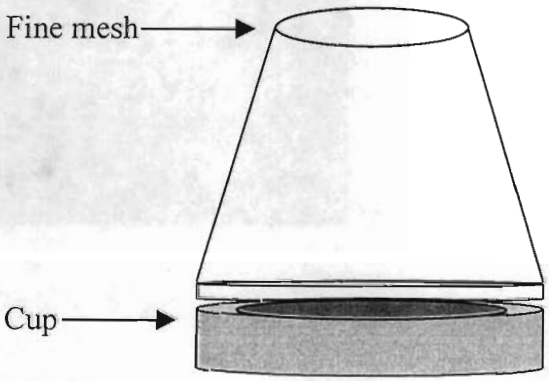


Fig. 4. Creamer cups used for maintaining and bioassaying of aphids.

References:

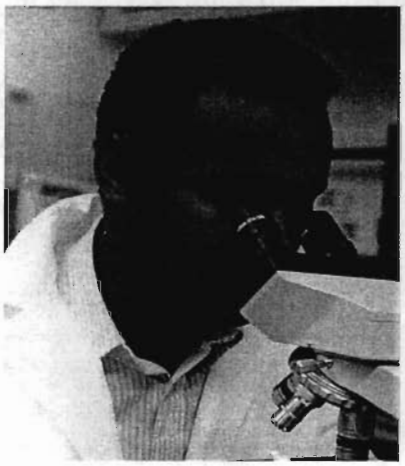
Devonshire, A. L. 1989. Resistance of aphids to insecticides, pp. 123-139. *In* A. k Minks & P. Harewiijin (eds), aphids, their biology, natural enemies and control. Elsevier, Amsterdam.

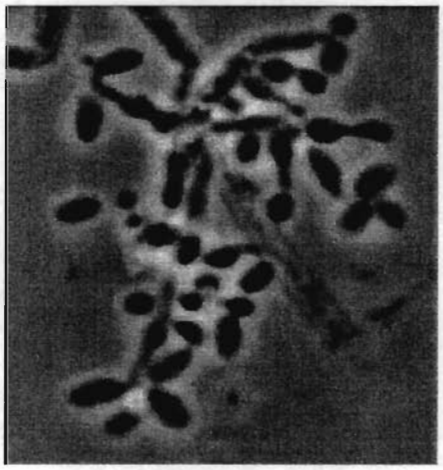
Mackauer, M. & Way, M. J. 1976. *Myzus persicae* Sulz., an aphid or world importance pp. 51-120. *In* V. L. Delucchi (ed), Students in biological control. Cambridge University press, Cambridge.

Radcliffe, E. b. 1982. Insect Pests of Potatoes. *Annu. Rev. entomol.* 27: 173-204.

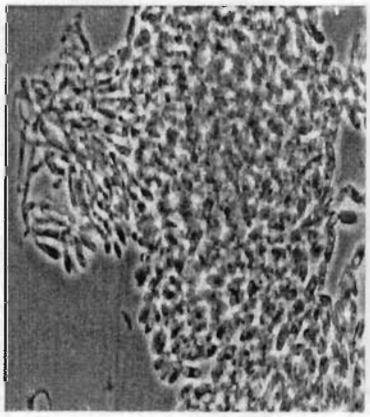
Identification of fungi

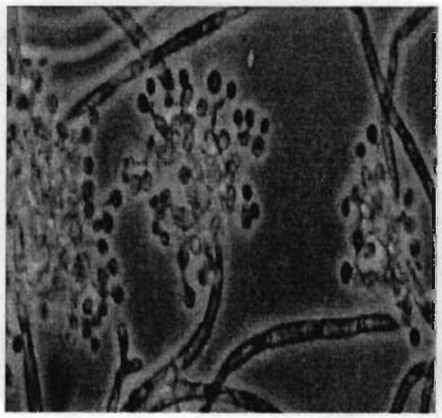
Identification of the fungal isolates was made through examination of the growth morphology and microscopic features of conidiogenous cells using cover-slip and scotch tape techniques. Dr. Richard Humber, from Cornell University confirmed that 17 of the isolates were *Metarhizium anispoliae*, 14 *Beauveria* sp, 1 *Verticillium lecanii*, 2 *Trichoderma* sp., 4 *Fusarium* sp, 1 *Exerohilum* sp. One of the *Beauveria* isolates (BB) has also been identified as a new species. Below are some of the microscopic features of the isolates examined.

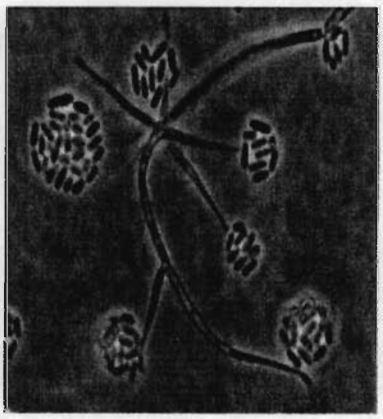




a)







b) c) d)

Fig. 5. Conidial structures of some Ethiopian fungal isolates. a) A new *Beauveria* sp. b) *Metarhizium anisopliae*, c) *Beauveria bassiana* d) *Verticillium lecanii.*

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Pathogenicity test

It is well known that fungal isolates, even of the same species, usually vary considerably with regards to pathogenicity, growth and spore production. Hence, in order to find a pathogen that could be useful under Ethiopian conditions, careful selection has to be done both in terms of virulence, spore production and applicability.

Since the virulence of fungal isolates could vary depending on the assaying temperature, we first examined the possibility of maintaining the test insect, western flower thrips (WFT), at high temperatures before running the assay.

The effect of high temperature on thrips:
A preliminary experiment was done to determine the response of WFT exposure to high temperatures. Two age groups, namely 2nd instars and adults, were used in the study. One group of the insects were kept in Munger cells containing filter paper and a fresh bean leaf.

The second group of insects were put in small plastic petri plates (60 x 15 mm) containing water-agar media, moist filter paper and bean leaf. Thrips survival was assessed after five days of exposure to 22, 25 and 30°C, dividing the insects into dark and light condition.

Results

It was found that 30°C was lethal to all the thrips. Moreover, it made the leaves wilt and dried the media quickly. At 25°C, rapid growth of saprophytic fungi, mainly *Fusarium* sp. occurred resulting in high mortality. All the test insects survived well when incubated at 22°C and hence this temperature was selected to carry out the pathogenicity trials.

Evaluation of pathogenicity:
A total of 33 fungal isolates obtained from Ethiopia were maintained on quarter-strength Sabouraud dextrose agar medium containing 0.25% w/v yeast extract (SDAY) and used in the assay (Annex 1). Suspensions of each isolate were prepared in 0.01% tween 80 adjusting the concentration to 5 x 10^6 conidia/ml. The pathogenicity of the isolates was tested against western flower thrips by applying 0.7 ml of each suspension onto two 7cm-diameter filter papers placed in the Munger cells (Fig. 6).

About twenty thrips were put on a filter paper and then a bean leaf disk (~2 cm diameter) was placed underside down in contact with the filter paper. The second spore-treated filter paper was placed over the top of the leaf disc and thrips and clamped together within a Munger cell. Four replicates were used per treatment. The cells were placed in plastic bags to prevent drying and held at 22 °C. Control insects were treated with 0.01% sterile Tween 80, and mortality recorded seven days after treatment.

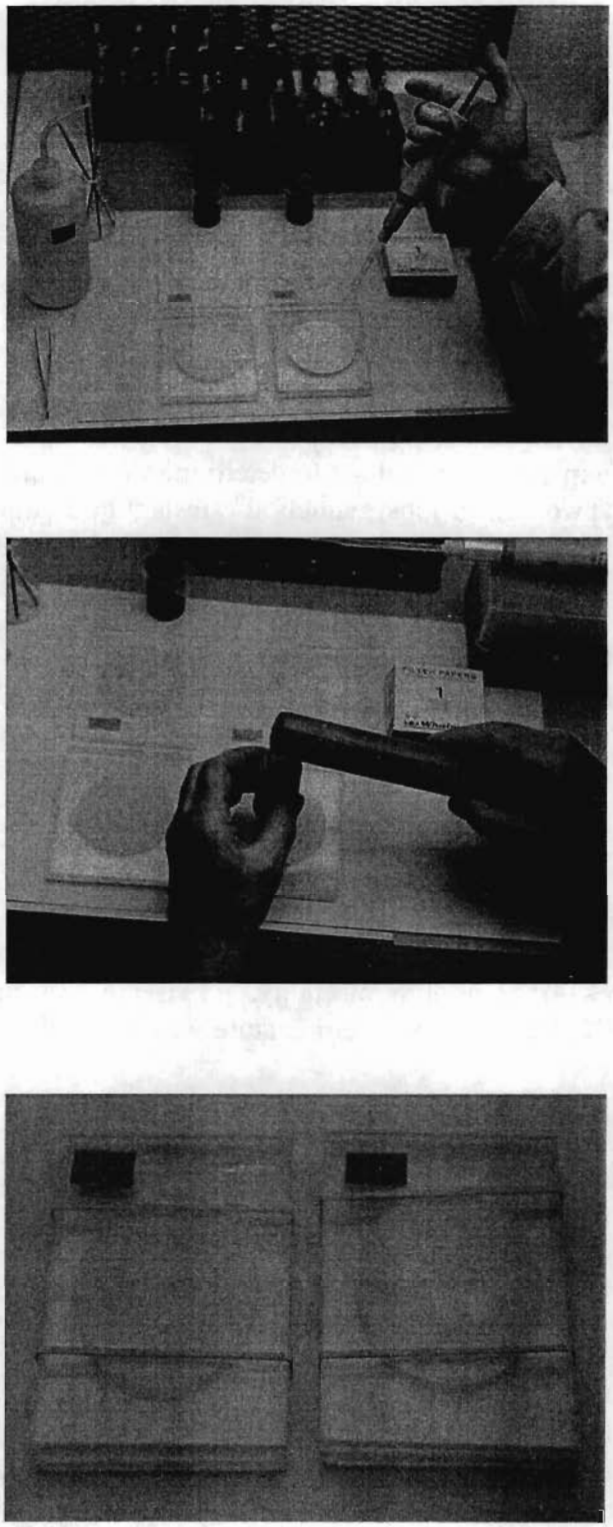


Fig 6. Application of fungal suspension using Munger cells.

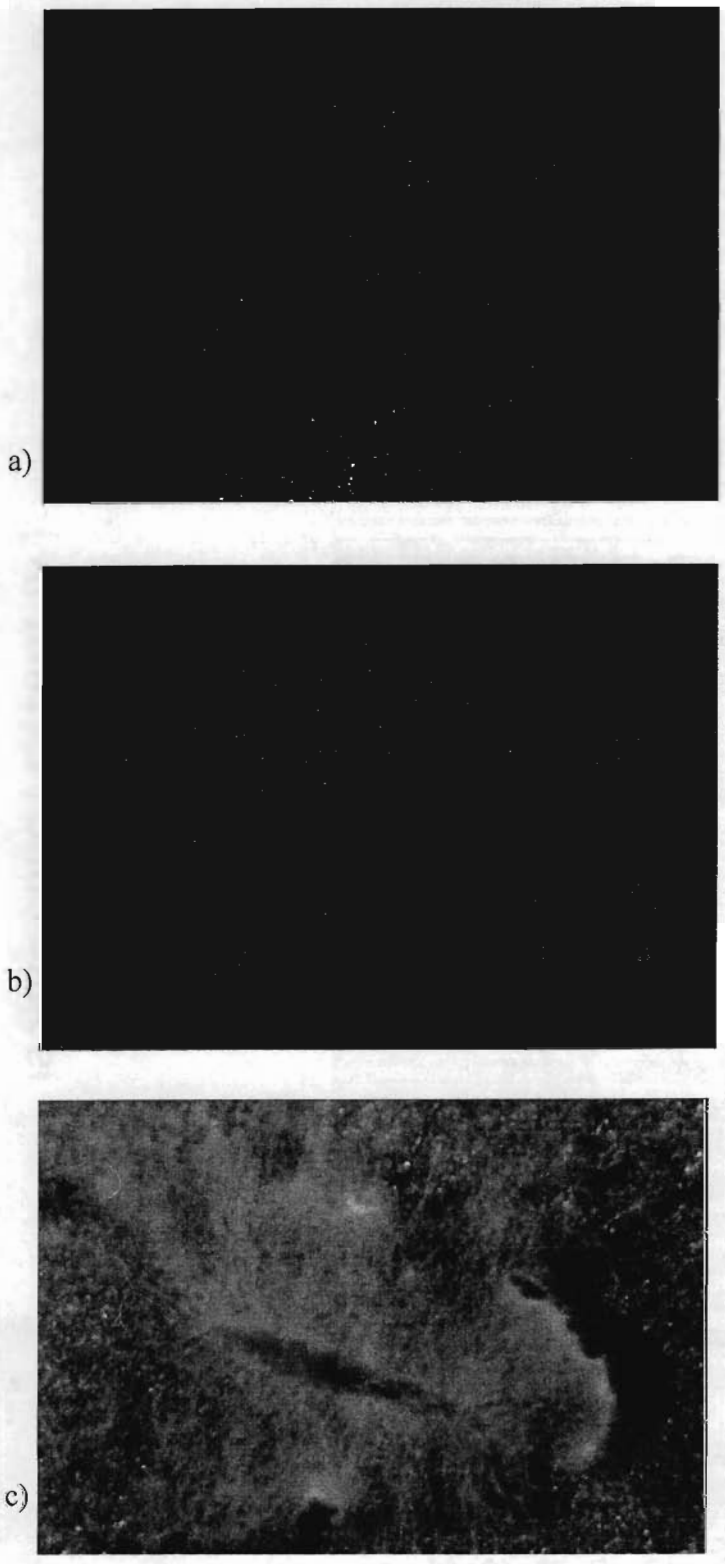


Fig. 7. Cadaver of thrips with propagules of a)*Verticillium lecanii*, b)*Beauveria bassiana* and c) *Metarhizium anisopliae.*

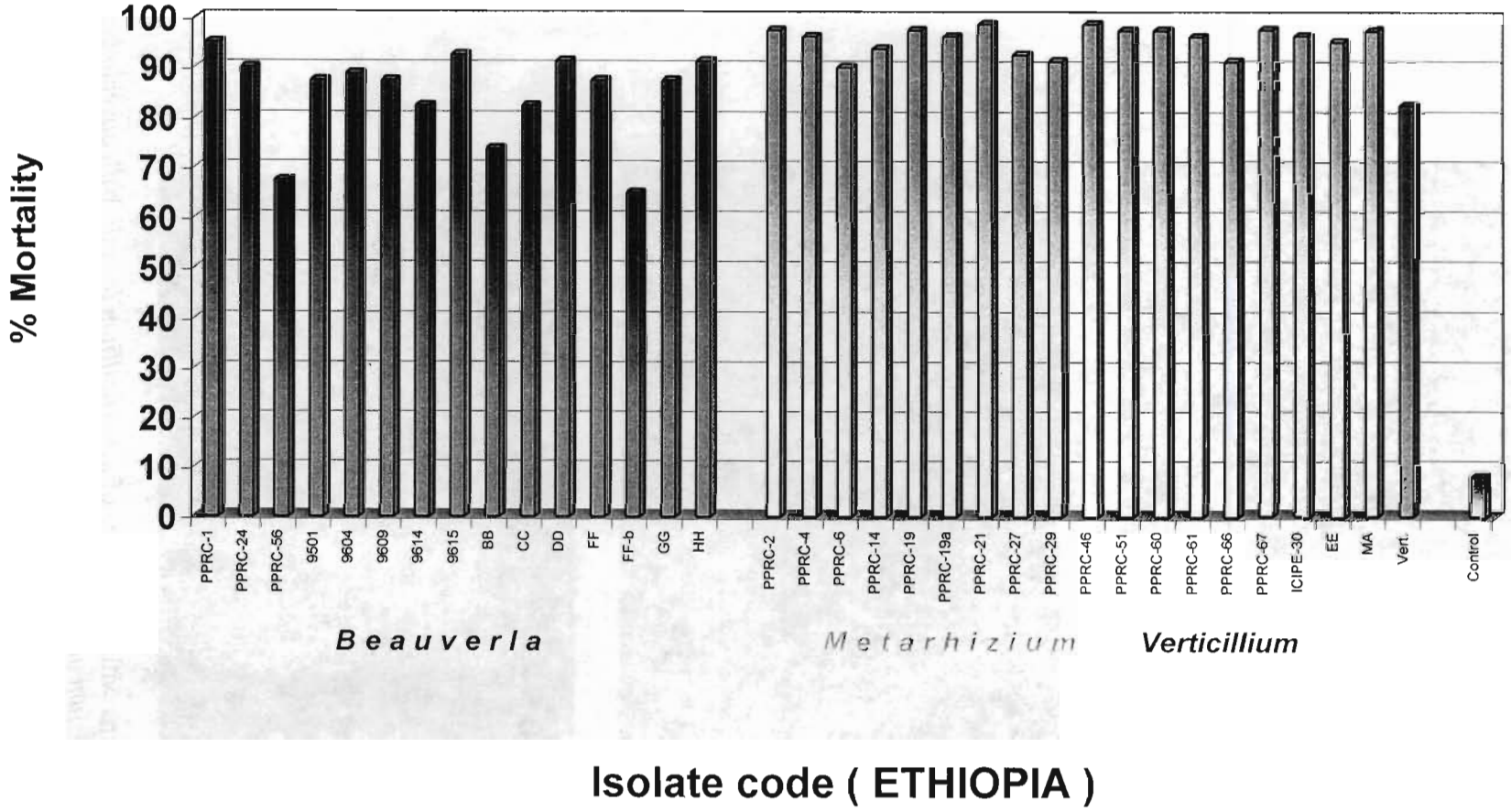
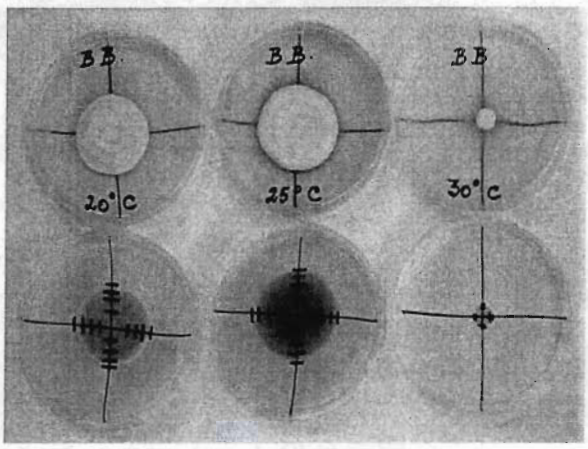


Fig. 8. Pathenogenicity of the different fungal isolates against WFT.

As indicated in Table 1.preliminary screening trial showed that all of the tested fungal isolates had pathogencity towards WFT. Further assays will be conducted to determine the dose-mortality relationship of each isolate.

Growth rate studies

Evaluation of the effect of temperature on growth rate:
The growth rates of 33 fungal isolates from Ethiopia was measured at various temperatures (20, 25, 30 and 35°C) on SDAY ($1/4^{th}$) media. A suspension of each of the isolates was prepared adjusting the spore concentration to 1 x 10^6 conidia/ml. A circular disc of filter paper (1/4" diameter) was placed in the center of each petri dish and inoculated with 10µl of each suspension. Four replicates were used for each isolate and temperature tested. Each plate was wrapped with parafilm and put in plastic sheets to avoid desiccation. The rate of radial growth was recorded every five days for twenty days (Fig. 9a).

a) 

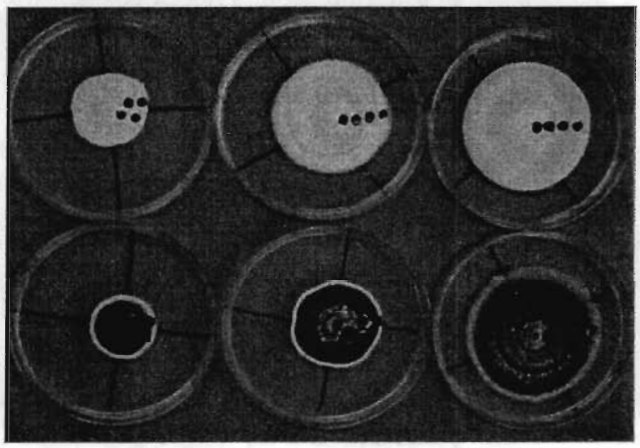
b) 

Fig. 9. a) Growth rate of *Beauveria* isolate at different temperatures b) Circular agar blocks removed for measurement of spore production.

Effect of temperature on spore production:
Four circular agar discs were taken from each plate using a cork borer along the radial growth of 5, 10, 15 and 20 days after inoculation (Fig. 9b). The discs were then put in test tubes containing 10 ml of 0.6% green shield, 0.1% Tween 80 and 40µM crystal violet, sonicated and the spore concentration in the resulting suspension determined using a hemacytometer. The spore count was used to estimate the rate of sporulation at the respective temperatures.

Statistical analysis for the data of growth rate measurement and spore production is being undertaken using SAS.

Mass production of fungi

Phase 1: *Determination of the optimum amount of water for moisturizing of grains*

One of the attractive features of entomopathogenic fungi as a biocontrol agent is the ease of production. Cheap substrates such as grains could be used for mass production, and easily adapted by small-scale farmers in Ethiopia.

Grains of wheat and millet together with wheat bran were selected for the preliminary mass production experiment. The first step we determined was the optimum amount of water required to moisten the grains. Accordingly, water: grain mixtures (v/v) in the ration of 0.5 : 1, 1 : 1, 2 : 1 and 3 : 1 were examined.

About 1800 cc of grains (wheat or millet) was measured and put in autoclavable bags. Water was added to each of the bags, as in the ratio indicated above together with citric acid (0.4ml /l). Citric acid was added to make the pH acidic and minimize microbial contamination. Then, the bags were kept in a water bath (70°C) for an hour. After which time, the same volume of wheat bran was added to each of the bags and autoclaved at 121°C for 55 minutes.

Three days later, visual observation was made on the wetness or stickiness of the steriled grains in autoclavable bags. Below are some of the physical features noted.

Table 1. Physical appearance of grains at different water-grain mixture.

Grain	Water : grain ratio (v/v)	Physical appearance
Wheat + water	0.5:1	Dry, not enough moisture for fungal growth
Wheat + wheat bran + water	0.5:1	Inadequate moisture
Millet + water	0.5:1	Enough moisture for fungal growth
Millet + wheat bran + water	0.5:1	Enough moisture for fungal growth
Wheat + water	1:1	Not sticky, may be suitable for fungal production
Wheat + bran + water	1:1	Not sticky
Millet + water	1:1	Not sticky
Millet + wheat bran + water	1:1	Not sticky, suitable for fungal growth
Millet + wheat bran + water	2:1	Sticky
Wheat + wheat bran + water	2:1	Not sticky NB: it is recommended to reduce the vol. of water for good aeration and management
Wheat + water	2:1	Sticky
Millet + water	2:1	Sticky
Wheat + water	3:1	Very sticky, not suitable for fungal production
Wheat + wheat bran + water	3:1	Very sticky, not suitable for fungal production
Millet + water	3:1	very sticky, not suitable for fungal production
Millet + wheat bran + water	3:1	Very sticky, not suitable for fungal production

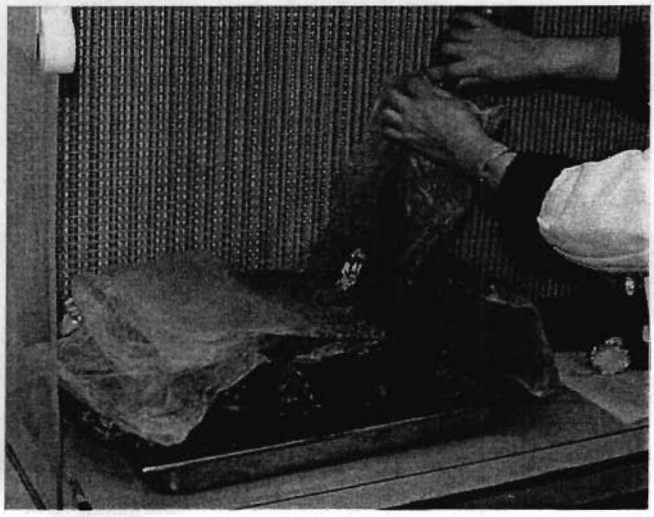
From the physical features, mixture of grain and water (at the ratio of 1:1) was found less sticky and furnished adequate moisture. The addition of wheat bran facilitated good aeration and easy mixing that is suitable for fungal growth.

Phase 2: *Mass production of fungi using grains as a substrate*

Isolates of *Beauveria* sp. (BB and DD), *M. anispoliae* (PPRC 2) and *V. lecanii* grown on SDAY media ($1/4^{th}$) were used for inoculation. The spores were harvested and suspended in 10 ml of 0.01% Tween 80. From this, ten more disposable plastic petri plates were prepared for each of the isolates to get more number of spores. Fifteen days after inoculation, spores of each isolate were harvested and pooled to form a large suspension.

The spores obtained from this culture were used to inoculate about 150 g of moistened sterile millet grains placed in wide mouthed jars (500 ml). The jars were hand-shaken daily to avoid clamping of grains and provide aeration. Following incubation at 25°C for fifteen days, the spores were harvested using 100 ml of sterile 0.01% Tween 80. The crude fungal suspension (25 ml per bag) was used to inoculate grains in large autoclavable bags (24 x 36 in). After 10 days of incubation, the bags were opened and contents dried in a de-humidified room.

Further development of mass production techniques shall be done at the Volcani center and Tel Aviv University, Israel by the year 2001.



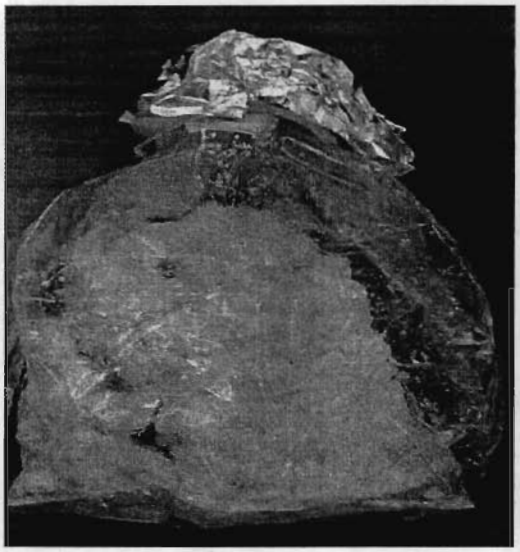


Fig. 10. Steps for the mass production of fungi.

Annex 1. Fungal isolates from Ethiopia

Isolate Code	Species	Location collected	Host insect	Collected by	Isolated by
1. PPRC 21	*M a. var. anisopliae*	Berber (North Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
2. PPRC 14	*M a. var. anisopliae*	Dedeaa (North Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
3. PPRC 60	*M a. var. anisopliae*	Rufe Kure (N shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
4. pprc 60 (a)	*Exserohilum sp.*	Rufe Kure (N Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	S. Gouli
5. PPRC 29	*M . anisopliae*	Gobenayetu (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
6. PPRC 46	*M a. var. anisopliae*	Sefi Beret (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
7. PPRC 1	*B. bassiana*	Harae (N. shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
8. pprc (1B)	*Fusarium sp.*	Harae (N. shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	S. Gouli
9. PPRC 2	*M a. var. anisopliae*	Ashan (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
10. PPRC 66	*M a. var. anisopliae*	Awaketu (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Esayas Samuel
11. PPRC 4	*M a. var. ?anisopliae*	Shewa Robit (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
12. PPRC 6	*M a. var. anisopliae*	Kewot (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
13. PPRC 19	*M a. var. ?anisopliae*	Rufe Kure (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
14. PPRC 51	*M a. var. anisopliae*	Shewa Robit (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
15. PPRC 61	*M a. var. ?anisopliae*	Awaketu (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Esayas Samuel
16. PPRC 27	*M a. var. anisopliae*	Dedeaa (North Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
17. PPRC 67	*M a. var. anisopliae*	Kewot (N. Shoa)	*Pachnoda interrupta*	Mulugeta Negeri	Esayas Samuel
18. PPRC 56	*B. bassiana*	Berber (N.Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
19. PPRC 39	*Fusarium* sp.	Sefi Beret (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
20. PPRC 37	*Fusarium* sp.	Sefi Beret (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
21. PPRC 54	*Fusarium* sp.	Shewa Robit (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Esayas Samuel
22. PPRC 24	*B. bassiana*	Dedeaa (North Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
23. PPRC 3	*Trichoderma* sp.	Harae (N. shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
24. PPRC 11	*Trichoderma* sp.	Rufe Kure (N Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
25. PPRC 40	*Asperigilus* sp.	Sefi Beret (N. Shoa)	*Pachnoda interrupta*	Seneshaw Aysheshim	Seneshaw Aysheshim
26. 9615	*Beauveria* sp. ?	Awassa	Spider (Arachinida)	Adane Kassa	Adane Kassa
27. 9614	*Beauveria* sp. ?	Awassa	Ground beetle	Adane Kassa	Adane Kassa
28. 9604	*B. bassiana*	Bugae (Arbaminch road)	*Aceraea acerata*	Adane Kassa	Adane Kassa
29. EE	*M a. var. ?anisopliae*	Alamata (Tigray)	Crustacean (sow pill)	Seneshaw Aysheshim	Seneshaw Aysheshim
30. FF	*B. bassiana*	Ashengae (Tigray)	Coleoptera (adult)	Seneshaw Aysheshim	Seneshaw Aysheshim
31. ff (B)	*B. bassiana*	Ashengae (Tigray)	Coleoptera (adult)	Seneshaw Aysheshim	S. Gouli
32. DD	*B. bassiana*	Debremarkos (Gojam)	Coleoptera (adult)	Seneshaw Aysheshim	Seneshaw Aysheshim
33. HH	*B. bassiana*	Ashengae (Tigray)	Coleoptera (adult)	Seneshaw Aysheshim	Seneshaw Aysheshim
34. CC	*B. bassiana*	Woldiya (Welo)	Coleoptera (adult)	Seneshaw Aysheshim	Seneshaw Aysheshim
35. *BB	***Beauveria* sp. ?**	Gusquam	Spider (Arachinida)	Seneshaw Aysheshim	Seneshaw Aysheshim
36. GG	*B. bassiana* ?	Ashengae (Tigray)	Coleoptera (adult)	Seneshaw Aysheshim	Seneshaw Aysheshim
37. ICIPE 30	*M a. var. anisopliae*	Kenya	? Tsetse fly	?	?
38. 9501	*B. bassiana*	Tikur Inchini (West Shoa)	Chafer grub	Bayeh Mulatu	Adane kassa
39. 9609	*B. bassiana* ?	Mugundo (Dila road)	*Blosyrus rugulosus*	Adane kassa	Adane kassa
40. MA	*M a. var. anisopliae*	Arba Minch	Soil	Mamuye Hadis	Mamuye Hadis
41. Verticilium	*V. lecanii*	Kefa	From coffee rust	Tefesetewold Biratu	Tefesetewold Biratu

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Annex 2. Activities

Week 1.

***Thursday* (21-09-00)**

0900: Tour of entomology laboratory facilities
1245: Reception in Bio-Research conference room. Getting acquainted with the staffs.
1315: Tour of Burlington

***Friday* (22-09-00)**

0900: Meeting with Kathleen Ford, Office of International Affairs
0930: Overview of thrips, whitefly and aphid rearing by Dr. Bob. J., Steve L. and Bill. Reid
1200: Lunch
1300: Review of basic microbiological techniques (Dr. S. Gouli)
1500: Media preparation

Week 2.

***Monday* (25-09-00)**

0800: Thrips rearing; bean planting, prepare thrips boxes and oasis cubes (Dr. Bob)
1200: Lunch
1300: Introduction to standard bioassay techniques (Dr. S. Gouli)
1500: Techniques of microscope slide preparation

***Tuesday* (26-09-00)**

0800: Thrips rearing (Dr. Bob)
0830: Monitoring of insects in the bean growing room using yellow sticky traps.
0930: Prepare thrips rearing boxes
1000: Transfer of adult thrips using aspirator
1200: Lunch
1300: Prepare spore suspension (Dr. S. Gouli)
1500: Thrips bioassay using Ethiopian fungal isolates

Wednesday (27-09-00)

0800: Thrips rearing (Dr. Bob)
0900: Appointment with Dr. Alan Gotlieb, Chairman of the Department of Plant and Soil Science
1000: Transfer of adult female thrips for egg laying
1200: Lunch

1300: Introduction to symptoms of major fungal, protozoal and viral diseases (Dr. S. Gouli)
1500: Microscopic examination of caterpillar haemolymph for microsporida

Thursday **(28-09-00)**

0800: Thrips rearing (Dr. Bob)
0900: ID card was issued
1000: Introduction of TBP rearing by Steve
1200: Lunch
1300: Field collection of symptomatic insects (Dr. S. Gouli)
1600: Microscopic examination of sporulated cadavers of insects;

Friday **(29-09-00)**

0800: Thrips rearing; thinning of bean plants, prepare thrips boxes and transfer of adult females for egg oviposition (Dr. Bob)
1200: Lunch
1300: Isolation of fungal pathogens (*Entomophtorales* sp.) from field collected aphids.

Week 3.

Monday **(02-10-00)**

0800: Thrips rearing (Dr. Bob)
0945: Discuss the weekly activities with Prof. Parker and Dr. Scott
1000: Transfer of adult female thrips for egg oviposition
1200: Lunch
1300: Preparation and observation of microscope slides (Dr. S. Gouli)
Isolation of fungi under a dissecting microscope.
Cleaning up a contaminated culture

Tuesday **(03-10-00)**

0800: Thrips rearing (Dr. Bob)
1200: Lunch
1300: Prepare spore suspension (Drs. Svetlana and Galina)
1500: Data collection of thrips mortality

Wednesday **(04-10-00)**

0800: Thrips rearing (Dr. Bob)
1200: Lunch
1300: Mass production using grains (Drs. Svetlana and Galina)
Prepare spore suspension, inoculation

***Thursday* (05-10-00)**

0800: Thrips rearing
1000: Prepare new thrips boxes and transferring of matured female thrips for egg laying
1200: Lunch
1300: Determine the appropriate grain and water mixture suitable for mass production of fungi

***Friday* (06-10-00)**

0800: Thrips rearing
1200: Lunch
1300: Introduction to fungal identification (Drs. Svetlana and Galina)
1500: Prepare SDAY (one quarter) media for growth and spore production study

Week 4.

***Monday* (09-10-00)**

0800: Thrips rearing
1200: Lunch
1300: Microscopic slide preparation using scotch tape and cover slip technique for identification (Drs. Svetlana and Galina)
1600: Observation on the wetness and stickiness of grains at various levels of water for spore production

***Tuesday* (10-10-00)**

0800: Slide preparation for fungal identification (using scotch tape and cover slip methods)
1200: Lunch
1300: Bioassay of thrips using two fungal isolates

***Wednesday* (11-10-00)**

Lecture by Dr. Richard Humber
0800: Essentials of insect pathology
1200: Lunch
1300: Essentials of insect pathology

***Thursday* (12-10-00)**

0800: Essentials of insect pathology
1200: Lunch
1300: Identification of Ethiopian fungal isolates with Dr. Richard Humber

***Friday* (13-10-00)**

0800: Identification of Ethiopian fungal isolates (Dr. Richard Humber)
1200: Lunch
1300: Meeting on the ongoing research activity to be undertaken in Israel
1600: Study the microscopic structures of fungi

Week 5.

Monday **(16-10-00)**

0800: Aphid rearing (with Bill Reid)
1200: Lunch
1300: Study the response of thrips at higher temperatures (25 and 30°C)
1400: Discussion was made on how to measure the rate of growth and spore production (Drs. Svetlana and Galina)
1500: Prepare SDAY (one quarter) media (14 l)

Tuesday **(17-10-00)**

0800: Autoclaving the media
0900: Pouring of media into 700 petri dishes
1200: Lunch
1300: Pouring of media (continued)

Wednesday **(18-10-00)**

0800: Aphid rearing (with Bill)
Transfer of aphids to initiate new colonies
1200: Lunch
1300: Prepare materials for growth optimization study; ¼" circular paper disc placed onto 700 petri plates

Thursday **(19-10-00)**

0800: Prepare a suspension of 40 fungal isolates
0900: Adjust the spore concentration to 1 x 10^6 conidia/ml
1200: Lunch
1300: 10µl of each of the fungal suspension inoculated onto the circular paper discs (4 replicates per each isolate and T°)

Friday **(20-10-00)**

0800: Inoculation (continued)
1200: Lunch
1300: Meeting on the weekly activities

Week 6.

Monday **(23-10-00)**

0800: Aphid rearing (with Bill)
1200: Lunch
1300: Determination of percentage of germination of the fungal isolates

Tuesday **(24-10-00)**

0800: Prepare a suspension of four fungal isolates
0900: Adjust the concentration to 5 x 10^6 conidia/ml

1000: Assay on thrips using Munger cells
1200: Lunch
1300: Prepare a fresh culture of fungal isolates (replicating each of the eight isolates 10 times)
1500: Growth rate of the fungal isolates measured
1600: A solution of about 20 liters of 0.6% green shield, 0.01% tween 80 and 40 μM crystal violet prepared

***Wednesday* (25-10-00)**

0800: Aphid rearing (with Bill); prepare aphids for bioassay
1200: lunch
1300: Measuring of the growth rate of 40 fungal isolates
1500: Viability test

***Thursday* (26-10-00)**

0800: Aphid rearing (with Bill); prepare aphids for bioassay
1200: lunch
1300: Test tube slants of SDAY (one quarter) media prepared

***Friday* (27-10-00)**

0800: Aphid rearing (with Bill); prepare aphids for bioassay
1200: lunch
1300: Viability test

Week 7.

***Monday* (30-10-00)**

0800: Aphid rearing
1200: Lunch
1300: Data recording on the growth rate measurement study
1500: Mass production of fungi using grains

***Tuesday* (31-10-00)**

0800: Discussion with Dr. Scott Costa regarding with the new research proposal "Conservation of biodiversity in Ethiopia: emphasizing on entomopathogenic fungi".
1100: Report writing
1200: Lunch
1300: Data on the mortality of thrips recorded
1500: Assay on thrips using 5×10^5 and 5×10^6 conidia/ml at 25°C.

***Wednesday* (1-11-00)**

0800: Aphid rearing
1200: Lunch
1300: Mass production using millet grains

Thursday **(2-11-00)**

0800: Aphid rearing
1200: Lunch
1300: Microscopic examination of different fungal species

Friday **(3-11-00)**

0800: Media preparation
1200: Lunch
1300: Meeting on the weekly research activity with Prof. Parker and Dr. Scott
1330: Prepare cultures of the 33 fungal isolates
1500: Growth rate measurement
1600: Data recording on the thrips mortality

Saturday **(4-11-00)**

0900: Inoculation of fungal isolates onto SDAY ($1/4^{th}$) media
Growth rate measurement

Sunday **(5-11-00)**

1400: Data recording on the thrips mortality

Week 8.

Monday **(6-11-00)**

0800: Aphid rearing; transfer aphids for assaying
1200: Lunch
1300: Report writing

Tuesday **(7-11-00)**

0800: Aphid rearing; transfer aphids for fungal spraying
1200: Lunch
1300: Prepare grains for mass production:

Wednesday **(8-11-00)**

0800: Aphid rearing
1200: Lunch
1300: Growth rate data recording of fungal isolates
1430: Circular agar blocks were transferred from the cultures to quantify the amount of spore production at various temperatures

Thursday **(09-11-00)**

0800: Prepare a fungal suspension of 14 fungal isolates, adjusting to 5×10^6 conidia/ml
1200: Lunch
1300: Bioassay done on thrips using 14 fungal isolates
1700: Data recording on the growth rate of fungi;

Circular agar blocks transferred from the cultures to quantify the amount of spore production

Friday **(10-11-00)**

0800: Report writing
1200: Lunch
1300: Attend weekly meeting with Prof. Parker and Dr. Scott
1330: Spore counting using hemacytometer

Week 9.

Monday **(13-11-00)**

Trip to Boston to get an airport transit visa of Germany

Tuesday **(14-11-00)**

0800: Spore counting
1200: Lunch
1300: Spore counting
Bioassay on thrips using eight isolates

Wednesday **(15-11-00)**

0800: Report writing
1200: Lunch
1300: Spore counting

Thursday **(16-11-00)**

0800: Spore counting
1200: Lunch
1300: Spore counting
1400: Data recording on the mortality of thrips
1600: Prepare grains for mass production of fungi

Friday **(17-11-00)**

0800: Quantify spore production
1200: Lunch
1300: Weekly meeting with Prof. Parker and Dr. Scott
1330: Quantify spore production
1600: Mass production of fungi using millet grains (Inoculation of fungal suspension)

Week 10.

Monday (20-11-00) Report writing

Tuesday (21-11-00) Report writing

Wednesday (22-11-00)
Report writing
Bioassay on thrips

Thursday (23-11-00) HOLIDAY (THANKSGIVING DAY)

Friday (24-11-00) Report writing

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APPENDIX II

Activity Report, S. Aysheshim (March 2002)

Insect Pest Management with Fungi: A Mass Production Technique for Farmers

Cooperative Development Research Project C-16-125

Activity Report

for the training that was held between 16 January and 15 March 2002

By

Seneshaw Aysheshim

15th March 2002

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Acknowledgments

I thank Prof. Bruce L. Parker and Drs. Margaret Skinner and Michael Brownbridge for arranging the training. I am in particular indebted to Dr. Svetlana Gouli for her guidance, kindness and friendship that made my stay more enjoyable. My special thanks also go to Dr. Vladmir Gouli for facilitating my transportation and his technical assistance throughout my stay. I thank very much Dr. Scott Costa for his highly valued consultation and dedication in ordering scientific equipment purchased for Ethiopia. Thanks are due Mr. William Reid for introducing me to techniques in entomopathogenic nematodes. I appreciate the assistance of Dr. Bob Jones for supplying test insects. I appreciate the support and encouragement I received from graduate students: Panagiotis Lekkas and Mohammed El Damir that made my stay more interesting. The US Agency for International Development supported the research and training.

Major outputs of the training

- Methods of maintaining cultures for long-term storage
- Aseptic procedures to be followed for mass production
- Details of mass production of fungi using grains as a cheap substrate
- Production of clean dry spore powder
- Quality control
  - Estimation of yield per gram of substrate
  - Number of conidia per gram of dry powder
  - Viability of dry spore powder and test of virulence

Introduction

Sorghum chafer (*Pachnoda interrupta*) and Citrus thrips are major production constraints of sorghum and citrus in Ethiopia, respectively. *P. interrupta* has become a threat for production of sorghum, maize and several other cereal crops and fruits in Northern Ethiopia spreading alarmingly every year. The damage on 1300 hectares in 1993 extended to over 112,000 hectares by September, 2001 (MoA, 2001). Damage estimates showed that it could result in up to 80% grain loss to Sorghum and 25% to maize. Current control methods entirely depend on direct spraying and baiting with insecticides though without much success. It is apparent that biological control agents could play an important role. Entomopathogenic fungi could contribute a lot in the control of these pests assuming their pathogenecity, ease of production and contact penetration through the cuticle. Several entomopathogenic fungi have been isolated from dead cadavers of *P. interrupta* and other beetles in Ethiopia. During the initial stage of this training, which took place between September and December 2000, the focus was on the identification, screening and characterization of 40 of the entomopathogenic fungal isolates collected from Ethiopia under laboratory conditions.

The project has now further developed with two Ethiopian isolates selected for further development and production. These fungi demonstrate the feasibility of large-scale production. There is a wide range of solid substrates available for use in the production of fungi for biological control. Previously reported as suitable for production of conidia include rice, wheat, wheat bran, maize, millet, oats, barley and sorghum. The choice of substrate depends on a number of factors, including local availability, cost and isolate preference.

Sorghum, *Sorghum bicolor*, is the second largest (after maize) crop produced in Ethiopia (Statistical Abstract, 1998). It is mainly used for human consumption.

Price of sorghum varies with location and season within Ethiopia ranging from birr 54/qt to 200/qt (Ethiopian Food Security Network, 2001) with an average annual price of 84/qt (1 USD ≅ 8.5 Eth Birr). Compared to other crops, it is considered inexpensive and easily available making it a good candidate for fungal production. Accordingly, the second phase of the training was designed to adopt simple and cost-effective mass production techniques of fungi using sorghum as a cheap substrate.

Objectives of the training:

- To develop a simple and cost effective mass production technique of entomopathogenic fungi for Ethiopian farmers

Methodologies

Two fungal isolates were selected from 33 *Metarhizium* and *Beauveria* isolates collected from Ethiopia based on bioassays carried out on western flower thrips, *Frankliniella occidentalis*, (Thysanoptera: Thripidae) at the University of Vermont and conidial production studies in Israel. The spore production of these two native isolates was compared with two exotic isolates. Chemical untreated sorghum and millet Pioneer hybrids were used as cheap substrates for mass production; the latter crop was used for comparison purpose. Fresh cultures of fungi were prepared from long storage and used to inoculate sterile grains put in glass jars. Spores produced on the grains were used to inoculate autoclave bags filled with ~ 450 g of sterile sorghum or millet grains. Mass production of clean *Metarhizium* cultures in less aseptic condition was also successfully attempted. A brief account of each of the detailed methodologies are summarized as follows:

1. Preparation of fungal isolates for mass production

The four isolates examined in this study were: *Metarhizium anisopliae* strain PPRC 29; isolated from *P. interrupta* adult in Ethiopia, *Beauveria bassiana* strain PPRC 56; isolated from *P. interrupta* adult in Ethiopia, *Beauveria bassiana* strain

726, isolated from chrysomelid beetle in the US and IMI 330189; isolated from grasshopper in Niger.

Test tubes from long storage are not used for direct inoculation of the jars. Therefore, it was important to prepare fresh cultures from each long-term stored slant. About 10 ml of sterile 0.01% tween 80 was added to each test tube and vortexed for 5-10 minutes. Then, 0.1 ml of each suspension was transferred onto petri dishes containing quarter strengthened SDYA media and incubated at 24°C for 15 days. From this, slants containing fresh cultures of each isolate were prepared to be used for inoculation of the jars.

To produce enough clean spores for use to inoculate grains, conidia from four 15 day old cultures were harvested by flooding with 10 ml of sterile 0.01% tween 80 into each test tube. The tubes were vortexed and the resultant spore suspension was pooled together into a sterile Erlenmeyer flask which was ready for jar inoculation.

2. Selection of substrate

Solid medium was chosen for spore production over liquid medium, because it requires less expensive substrates, less sophisticated equipment and more control over contamination.

For maximum sporulation, a good surface area to volume ratio is essential. Individual substrate particles should remain separate after hydration and sterilization. Substrate particles which clump together when water is added reduce the surface area to volume ratio, limiting the space on which sporulation can occur. An ideal substrate should not only contain particles of the correct dimensions, but also maintain its structural integrity during preparation for the production process (Bradley *et al.*, 1992).

Although rice is the most commonly substrate used for production of fungal conidia, sorghum was chosen considering its availability, cost and physical characteristics such as grain size and hydration properties.

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However, since cereal grains vary considerably in nutrient status even among varieties of the same species, it may be necessary to further select a variety of sorghum yielding more consistent results of spore quantity.

3. Selection of moisture levels of grains

The optimum moisture content for fungal growth and conidiation needs to be identified for each substrate/isolate combination. Moisture content plays a significant role in the final yield of conidia.

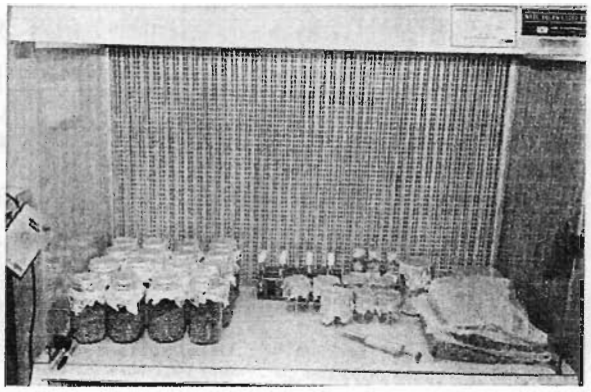
Following a series of experiments, an optimum moisture content for sorghum with *B. bassiana* and *M. anisopliae* cultures was determined as 1:0.75 (ratio of volume of grain to the amount of water added). Likewise, an optimum moisture content for millet was determined as 1 : 0.5 (volume of millet to the volume of water). Grains were best moistened with hot tap water. To minimize possible bacterial contamination, the pH of the grain to water mixture was adjusted to approximately 4 by adding 0.4 ml of 2.6 M citric acid per liter.

4. Inoculation of jars

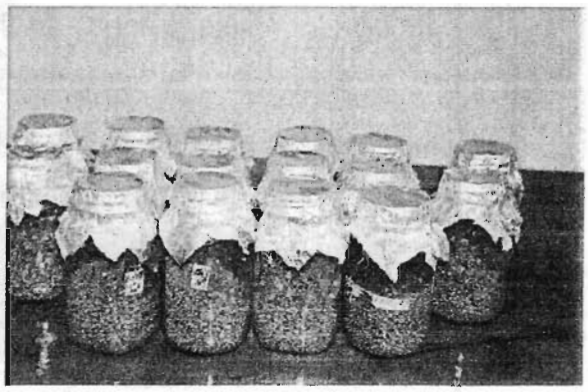
One third of the volume of jars was filled with dry grains of sorghum or millet. This facilitated easy mixing and even distribution of spore production. Hot water was added to the jars with the ratio indicated in section 3 and maintained in a water bath (90-100°C) for one hour. The jars were then autoclaved for one hour at 121°C and 15 psi. There was an increase in volume of the substrate (by approximately 2.5 to 3 times) following autoclaving.

Jars were inoculated with 2 ml of $\geq 10^7$ conidia per ml of fungal suspension following an overnight cooling of the grains. Inoculation using a sterile syringe was advantageous to discharge a known volume of the suspension and reduce the chance of contamination. It was possible to directly insert the needle of the syringe and inoculate the fungal material through layers of paper towel and cheesecloth. Inoculated jars were incubated at an ambient temperature of 22 -

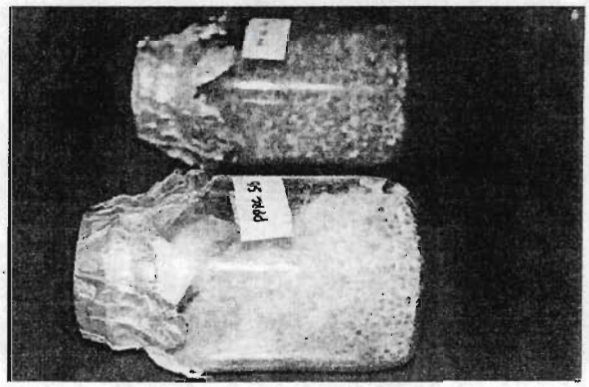
25°C for 10 - 15 days while mixing every 3 – 5 days. In order to avoid excess humidity and condensation, the aluminum covers were removed from the jars after about 5-7 days. Large amount of spores were obtained fifteen days after inoculation, which were used to inoculate bags and biotest. Below are some pictures of inoculation process.



a) Autoclaved jars ready for inoculation in a laminar flow hood



b) Volume of grains should be close to one third the volume of the jar



C) Volume of grain above half the volume of jars encourages accumulation of vapor and disables effective mixing

d) Inoculated jars were placed in horizontal position to maximize aeration.

NB: -Do not inoculate jars directly from slants stored in mineral oil

-Recommended amount of grain: 30 - 50% of volume of the jar.

-Prepare enough sterile water and sterile 1 ml pipette tips in advance.

-Use septic technique thoughout this procedure

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5. Bioassay on WFT using spores obtained from grains

In order to compare the virulence of spores produced on sorghum grains in autoclave bags, bioassays were carried out using western flower thrips as a test insect. These results were compared with the results of fresh spores grown in slants. For each isolate a standard suspension of 5 x 10^6 spores/ml was prepared using sterile 0.01% tween 80 and applied onto two 7 cm diameter Whatman No. 1 filter papers (at the rate of 0.7 ml per each filter paper) placed inside Munger cell. Twenty unsexed second instar thrips were used for each Munger cell, replicating each experiment four times. Insects were supplied freshly cut bean leaf (2 cm diameter) and kept at a constant 22 °C and light regime of 18/6 hours. Mortality was recorded seven days after inoculation. Viability of spores grown on each of the medium was determined one day prior to bioassay.

Results:

Preliminary biotest results showed that spores obtained from grains had no inferior virulence compared with slant cultures (Figs. 1 and 2). Test strains PPRC 56, PPRC 29, IMI 330189 and 726, grown in grains, resulted in mean mortalities of 70%, 65%, 42.5% and 52.5% respectively. Where as spores obtained from slant cultures of PPRC 56, PPRC 29, IMI 330189 and 726 resulted in mean mortalities of 38.9%, 52.5%, 63.8% and 45.5%, respectively. Compared with the previous results, mortality was low for all isolates in both cases. Although assay conditions and dose levels were the same throughout the experiment, the age group of test insects was variable possibly influencing insect mortality.

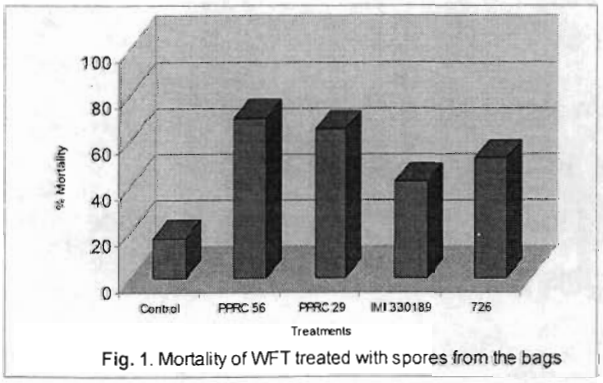

Fig. 1. Mortality of WFT treated with spores from the bags

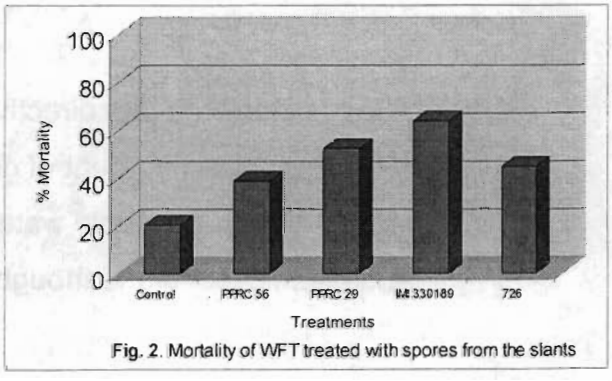

Fig. 2. Mortality of WFT treated with spores from the slants

6. Determination of spore productivity and spore viability

The amount of spores produced inside jars using sorghum and millet as a substrate was determined by taking 12 day old culture of the four isolates: IMI 330189, PPRC 56, PPRC 29 and 726. To avoid release of spores in the isolation room, jars were opened in the mass production room and a volume of 10 ml of each grain culture was transferred into sterile Erlenmeyer flasks containing 100 ml of sterile 0.01% Tween 80. Spores were harvested by gently shaking the resultant suspension for 30 minutes using an orbital shaker. 10 ml of the suspension was taken out and filtered. Haemocytometer counts of the recovered spore suspension gave estimates of the total conidial yield. Serial dilutions plated on ¼ SDYA media were used as estimates of the viable conidial yield. Viability was determined by counting the percentage germination of conidia after 24 hours incubation at 22°C.

Results

Millet gave higher spore yield than sorghum for all isolates tested. In particular, PPRC 29 yielded five times the number of spores when grown on millet, but produced less when cultivated on sorghum than the remaining isolates (Table 1). PPRC 56 produced the highest number of spores both on sorghum and millet, the later yielding 1.3 times more spores. It was also interesting to note that from a small volume (10 ml) of sorghum or millet grains, a large number of spores (1.15×10^{10}, 1.53×10^{10} on sorghum and millet, respectively) could be obtained with high yielder strains such as PPRC 56. From this, it may be possible to roughly estimate that grains in a ten-liter container could yield about 10^{13} spores, which is sufficient to spray a hectare of land for some strains.

Table 1. Spore productivity of sorghum and millet substrates for different isolates

Isolates	No. of spores produced in 10 ml grain volumes of		Relative productivity of millet (**M/S**)
	Sorghum (S)	**Millet (M)**	
PPRC 56	1.15×10^{10}	1.53×10^{10}	1.33
PPRC 29	9.5×10^{8}	4.85×10^{9}	5.1
IMI 330189	3.05×10^{9}	5.85×10^{9}	1.92
726	3.25×10^{9}	3.5×10^{9}	1.08

Both millet and sorghum grains maintained good structural integrity following autoclaving. Mixing of millet was easier than sorghum, and the presence of its shell didn't affect growth of fungi. On the other hand, microscopic examination showed that both Beauveria and Metarhizium strains had little preference of the sorghum shell and conidiated mainly on grinned grains without the shell, suggesting that broken sorghum is most suitable than whole grain. The study showed that millet is the choice for mass production depending on its availability. However, considering the comparable spore production and the cost and availability in Ethiopia, sorghum appears to be better and is recommended for use in mass production.

Results on spore viability

All except PPRC 29 had high germination rate within 24 hours at 22°C (Table 2). Germination speed could bias the viability of a spore. Therefore, it is important to maintain an optimum temperature of each strain when incubate. *Metarhzium* strain PPRC 29 was originally isolated in a warm area about 230 km North East of Addis Ababa. Low viability could be due to low (22 °C) room temperature that influenced the germination speed (Table 2), and possibly relatively high moisture of grains.

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Table 2. Viability of spores obtained from slants and grains in bags and jars.

Percentage viability of spores from			
	Slants	**Jars**	**Bags**
PPRC 56	100	100	99
PPRC 29	45	16.3	58.5
IMI 330189	99	99	88
726	100	99	100

The amount of spores found per gram of fine powder, powder of spores obtained by sieving the grinned grain and crude grain was also compared. The dry grains were carefully poured into a fine polyester monofilament mesh (~300 μm), and were contained in a large plastic bag tied with a knot to seal it. The sieve was shaken inside the bag for several minutes and allowed to stand before opening. Spore powder that settled to the bottom of the bag was collected and placed in small plastic bags. 0.1 g of the spores was then weighed and suspended in 10 ml of sterile 0.01% Tween 80. The resultant spore suspension was diluted to 1:100 and ultrasonicated to release clumped spores. Haemocytometer counts of the spore suspension gave estimates of the total conidial yield.

Result

Conidia powder collected by sieving had the highest amount of spores per gram (Table 3). Blending the old sorghum grains, which has been sieved once, also provided additional fine powders of conidia. Number of conidia per gram, however, is lower than the first clean powders. Large amounts of conidia were recovered from whole grain even after sieving, which showed that all materials could be useful. It is important to maintain harvested conidia powders under low moisture content as low as 5%.

Table 3. Number of conidia per gram of dry spore powder and grain

Isolates	Number of spores produced per 1 gram		
	Clean powder	Powder obtained after grinned	Grain
726	1.42×10^{10}	Not examined	4.05×10^{9}
IMI 330189	5.02×10^{10}	7.02×10^{9}	8.55×10^{8}
PPRC 29	2.68×10^{10}	8.55×10^{9}	6.12×10^{8}

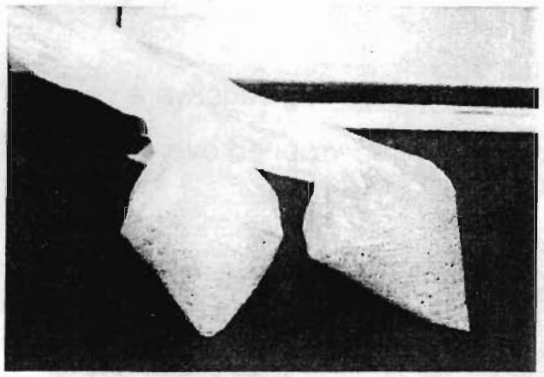
Although spores were sieved inside a large plastic bag to avoid dusting of spores in the room, it was essential to wear special mask equipped with fine filters. Care must be taken while sieving, as inhaling too many spores of any fungi could cause allergies to some people.

7. Mass production using autoclaveable bags

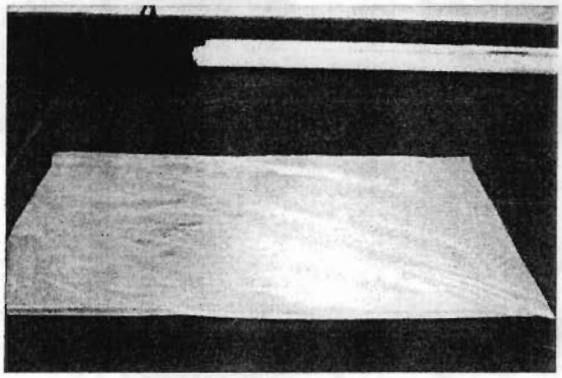
The development and optimization of simple production procedure is invaluable for the supply of reliable material. We have used autoclaveable bags and clean polyethylene bags to demonstrate the possibility of mass production of fungi.

A cup of (~500 ml volume) sorghum grains was put in an autoclaveable bag (300 x 450 mm), and filled with 375 ml of hot water (i.e. grain to water ratio, 1:0.75) to moisten the grains. To minimize bacterial contamination citric acid was added as indicated in section 3. After an hour in a water bath (100°C), bags were autoclaved at 121°C and 15 psi for an hour. Any clumps of sorghum were separated manually while cooling. Bags were inoculated the next day with 10 ml of $\geq 10^{7}$ conidia/ml and mixed thoroughly. In most of the cases a high density of spores was used to ensure enough supply of clean inoculum. The opening of bags was covered with layers of sterile paper towel, fine cheese cloth and aluminum sheet. Procedures used in packing of grains for autoclaving and inoculation are shown in the pictures below.

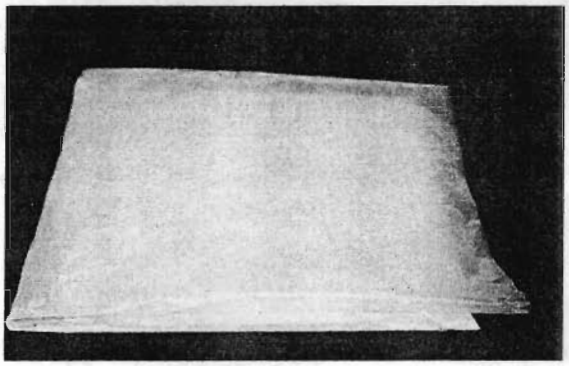
Each bag was filled with 500 ml (~450 grams) of sorghum and cooked in a water bath (1 hr)



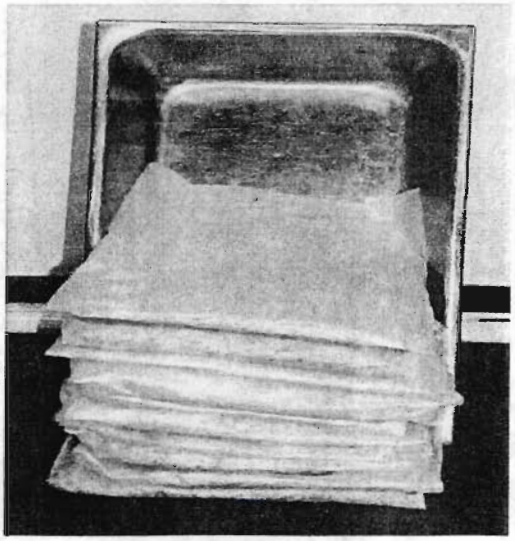
Cooked grains were distributed flat; this helped efficient use of space.



Grains were spread to one half and wrapped with the remaining half. This minimized contamination and facilitated inoculation process



Bags ready to be autoclaved. Large number of leveled bags could be autoclaved at a time



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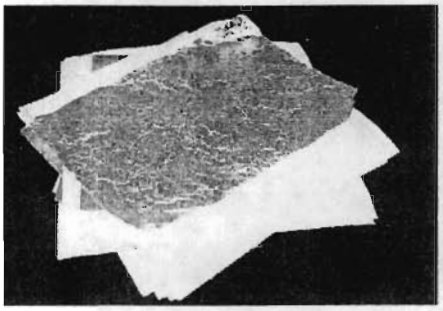
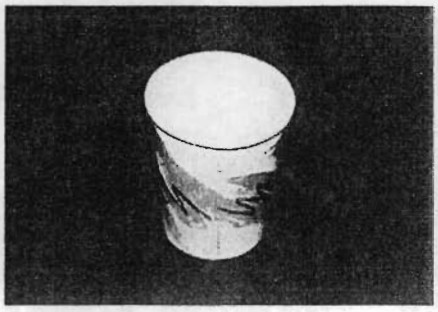
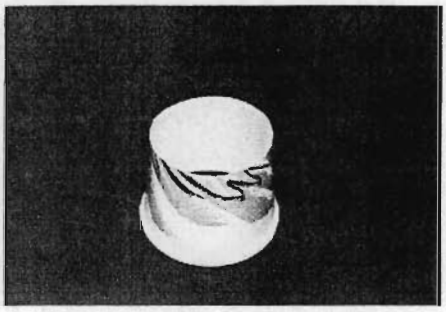
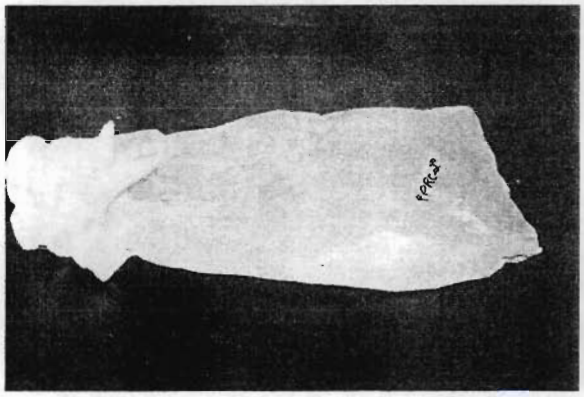
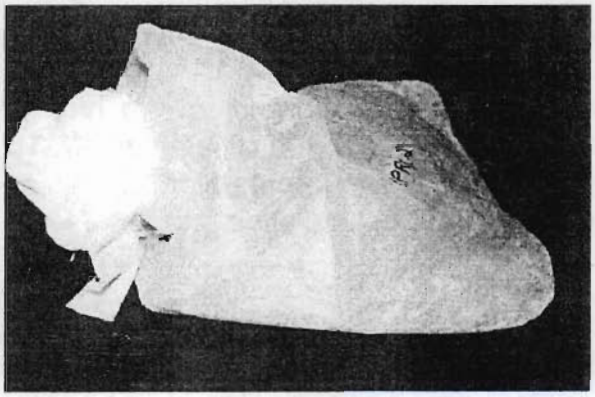
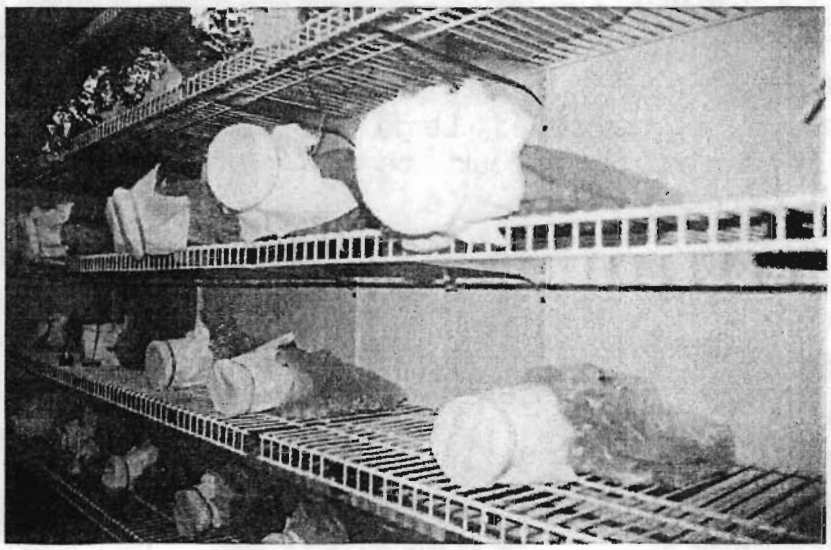
Opening of autoclave bags were covered with paper towel, fine cheese cloth and aluminum paper wrapped over the caps shown below.

Aeration was enhanced by placing inoculated bags as indicated in the right picture.



The standard growth condition was 22 ± 2°C under natural photoperiod for 3 weeks.

Aluminum covers were removed from the bags after 5-7 days to minimize vapor formation. Grains were mixed every 3-5 days to maintain an even distribution of spore production. Bags were also inspected everyday for the presence of any unusual growth of fungi or bacterial contaminants until five days after inoculation. After 12 days all covers of the bag openings were removed to further dehydrate the culture, as spore viability is reduced under moist conditions.

The inoculation room should be isolated and better equipped with a bactericidal lamp. For large scale production the job is better handled with two individuals, one holding and opening the autoclave bags while the other pouring the fungi suspension.

Caution! -Check for any contamination in the first five days. Discard if any!
-Never inoculate bags from contaminated cultures!
-Inoculate autoclave bags not later than one day following autoclaving.
-Make sure that the grains are not over cooked
-Make sure there is no condensation in the autoclave bags after enough spores are produced

Common contaminant encountered in culture bags was *Scopulariopsis* sp., which has globose with truncate base spores.

8. Room for mass production

The Mass production room should have good ventilation and an ambient temperature of 20-25°C. It is essential to separate the mass production room from the isolation and insect rearing rooms. A big ventilator could be placed inside and an opening hole made on one of the walls to allow fresh circulation of

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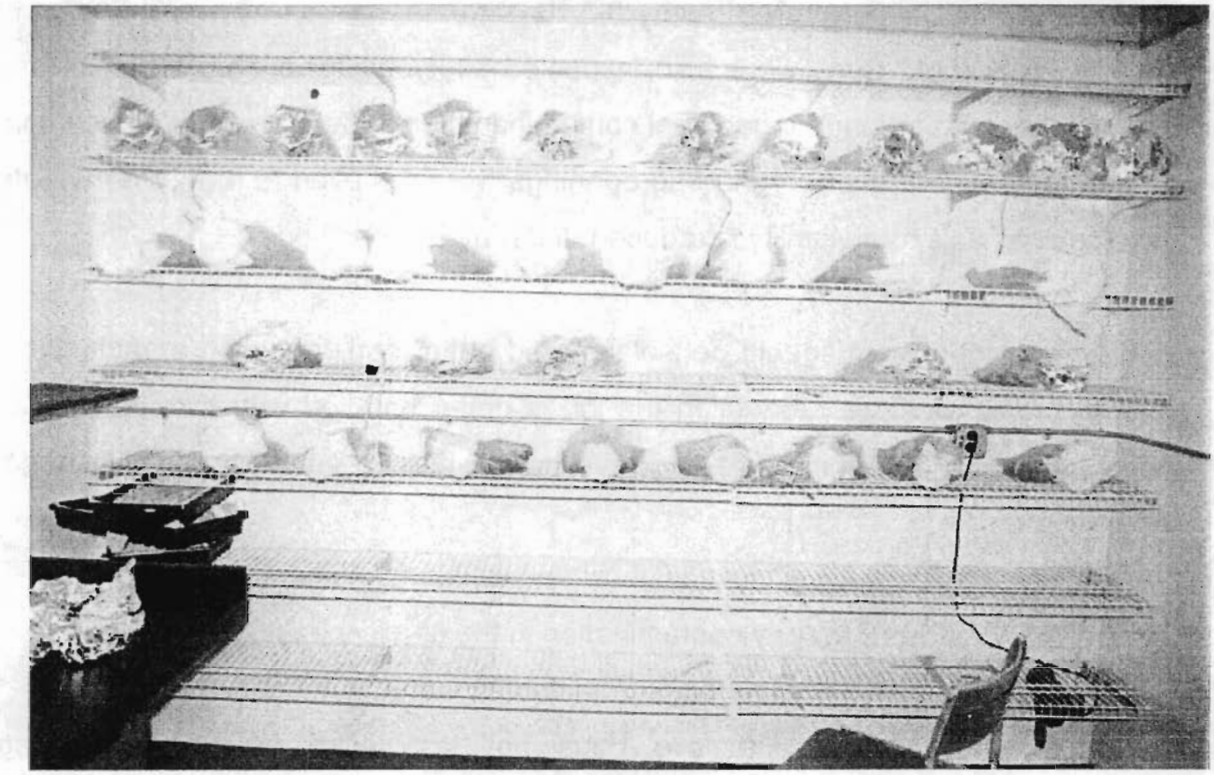
the room. *Metarhizium* and *Beauveria* sp. appeared to be less affected with the light intensity of the room.



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Bradley, C. A., Black, W. E., Kearns, R. and Wood, P. 1992. Role of production technology in mycoinsecticide development. *In*: Leatham, G. F (*ed*) *Frontiers in industrial mycology*. London: Chapman and Hall, pp. 160-173.

Annex:

Quarter strengthened SDYA Media

2.5 g Neopeptone
10 g Dextrose
2.5 g Yeast extract
15 g Agar
1 l Distilled water

Full strength SDYA media

10 g Neopeptone
40 g Dextrose
10 g Yeast extract
15 g Agar
1 l Distilled water

APPENDIX III

Training Report, M. Dawd (March 2003)

Insect pest management with fungi: Amass production technique for farmers

Cooperative development research project C-16-125

Activity report

For the training held January 26-March 2, 2003

By

Mohammed Dawd

Ethiopian Agricultural Research Organization

Entomology research laboratory

University of Vermont

March 2, 2003

Acknowledgement

I express my deepest gratitude and respect to professor Bruce L. Parker, Dr. Margaret Skinner and Dr. Michael Brownbridge, for their effort for making the training possible. And the effort made to convince the USA and British embassies is unforgettable.

I thank Dr. Svetlana Gouli and Dr. Vladmir Gouli for the training course, which was very educative and attractive.

Last but not least, I appreciate Dr. Scott Costa, for analyzing some of the data and his lecture on statistical analysis, Tom for his help to get our food items, Mohammed E Damir, Don and Amanda for their friendship and necessary help through out the coarse of study.

Table of Contents

Introduction

Pachnoda interrupta (sorghum chafer) is a major pest of sorghum. Sorghum is largely grown in draught affected areas and serves as a staple food. Damage to this crop by this pest aggravates serious food shortages caused by drought conditions which repeatedly affect Ethiopia. It has been estimated that up to 80% of the potential crop yield is lost these pests.

Citrus thrips cause a lot of damage to citrus crops throughout the year in Ethiopia. The only citrus exporting enterprise and a major earner of foreign exchange, the Upper Awash Agro Industry may have to cease its citrus production activities due to thrips. Other thrips species are primary pests of vegetable crops where they significantly reduce yields. Resource poor farmers who rely on citrus for their wellbeing are now abandoning production of these fruits and the land area in citrus production is shrinking. Cotton is another important crop threatened by thrips where farmers are using mixtures of obsolete insecticides to control insects which may lead to cross-resistance, in addition to health and environmental risks they pose. Their non-discriminate activity also kills natural enemies which are present in a natural ecosystem. Not only do these beneficials help suppress many pest species, but some may also be utilized as biological control agents. The wide spread use of broad-spectrum use of insecticide can lead to a decline in biodiversity and change in the ecosystem.

Just over two decades ago, it was estimated that about 40 % Ethiopia was forested. Today, it is estimated that this has declined to approximately 4 %. The deteriorating forest resource is further threatened by insect pests, particularly termites. Termites are the major pests attacking live trees and wood products in Ethiopia in general, Wellega, Bale and Borena areas in particular. The devastation and shrinking of forestland will worsen food security problems through environmental degradation (erosion, pollution) and shortages of wood will lead to shortage of raw materials for construction of houses.

A multi dimensional approach must be taken to provide a solution to all of these pest problems - IPM (Integrated Pest Management). Biological control is a fundamental component of IPM. Use of entomopathogenic fungi within an IPM program will help secure food and protect the environment while avoiding resistance, resurgence and residue problems, which result from overuse of synthetic pesticides.

The experience and the knowledge of the University of Vermont (UVM) entomologists can help Ethiopia. The existing USAID-funded project needs to be strengthened with the support of additional organizations such as, FAO and other international agencies which, by supporting agricultural research in Ethiopia, will greatly assist the resource poor farmers in this country.

Introduction of equipment and safety measures

Equipment

The following equipment used for fungal production and preservation was demonstrated.

Micro biological hood (different types), incubators, ice chamber, sonicator, centrifuge, vortex, shaker, stirrer, autoclave, oven, water bath, phase-contrast and stereomicroscopes, munger cell, Hemocytometer, macro and micropipettes, different syringes and other small scientific equipments, fridges and cold rooms, sensitive balance and different glassware

Chemicals used in fungal research

Alcohol (75 and 95 %), Tween 80, glycerol, lactophenol cotton blue

Materials for safety measures

Eyewash and shower stations, fire blanket

Basic principles in antiseptic and aseptic techniques in the laboratory.

Antiseptic (sterilizing technique)

The microbiological hood is the most important pieces of laboratory equipment, providing an opportunity to work under sterile conditions. It should be inspected each year and the filter changed if found to be defective. Before working in the hood, it is important to clean the hands and working surface using 75 % alcohol. The hood should be turned on 15 minutes before starting any activity and all unnecessary equipment and materials should be removed. If the hood is equipped with ultra-violet light (ULV), this must be turned off before work begins., since it has a carcinogenic effect on the skin. Plastic materials are not protective can be broken by ULV and it is not necessary to depend on gloves. Bleach (5 %) can be used to sterilize/ dispose off biological objects that cannot be autoclaved, such as, plastics, plant materials and insects. But care should be taken when using the material and hands and eyes should be protected. A Bunsen burner and alcohol are used inside the hood to sterilize metal equipment, such as, needles and spatula. Thermo-resistant materials (pipette, glassware) can be sterilized by autoclave. The

unwanted biological material, such as, fungi should be autoclaved and dispose off. Labeling is important before autoclaving any thing to avoid confusion.

Safety measures and operation of equipment

Laboratory equipment needs to be used with care and requires regular maintenance. Microscopes should always be carried with two hands supporting the bottom, during any movement.
The autoclave should not be opened until the sterilization cycle has been completed and the internal pressure is zero. Autoclave should not be open until it reads zero. Before starting work with the autoclave, it is important to check water level and ensure there is sufficient for operation. During the operation of centrifuge, speed should be increased slowly to avoid any hazard. Materials should be placed correctly in the rotors to ensure that they are balanced. Sonicators are also hazardous and care must be taken when placing materials in the water because it will damage tissues. Ultra low temperature deep freezers are used for long-term storage of fungi and protective gloves must be worn during operation. Incubators shakers, stirrers, etc. need to be adjusted and checked regularly during operation to avoid accidents and ensure that they are working efficiently.

Isolation of fungi from soil

Objective

To compare different methods for isolation of entomopathogenic fungi

Materials

Hood, shaker, vortex, incubator, oven, sensitive balance, selective media, test tubes, pipettes, pipette tips, distilled water, 0.1%Tween 80, Petri dishes, slide cover slips, spatula, alcohol and Bunsen burner, soil and entomopathogenic fungi spore at the rate 100 spores per gram of soil.

Methods

In this experiment 5 methods were administered, bait method, soil dilution method, direct inoculation method, print method and electrostatic method. Each method had 4 replications samples brought from the field and 3 for forest samples. The experiment was repeated 3 times and 1000 spores of entomopathogenic fungi added per gram of soil.

Bait method

Twenty-four small plastic cups with lids were prepared and 2/3 filled with forest soil. Three insect larvae (*Galleria mellonella*, wax moth) were added to each plastic, which then was closed. The cups were then inverted and held at 22^0c to favor fungi growth. Observations were made every 3 days. The cups were inverted every 3-4 days to force the larvae to move through the soil and trap the available infective fungal propagules. Mortality was recoded after 15 days and the data computed using SAS soft ware.

Soil dilution method

Twenty to thirty gm of soil was added to each of 3 beakers and later divided into 2 parts, one part was used to calculate the moisture content of the soil and the second was used to prepare a soil dilution. Approximately 10 gm of soil was added to a 250ml erlenmeyer flask containing 50 ml 0.1% Tween 80. Flasks were transferred to an orbital shaker , where they were held at---rpm. After shaking, the flasks were removed and 10 ml of soil solution taken from each flask and filtered through a sterile cheese cloth. The stock solution was diluted 10x and 100x for plating on selective media. Both B.bassiana and M.anisopliae selective media were used (see appendix), and 0.5 ml of the stock solution, 10x and 100x dilutions used to prepare a spread plates. To do this, each of the respective suspensions was spread over the entire surface of the medium using a bent glass rod. A total of 72 Petri dishes were inoculated (3 solutions x 2 fungi x 4 replications x3 people) and incubated at 22^0c. Plates were checked every 3 days for the sign of fungal growth. It was regularly checked every 3 days for fungal growth.

Direct inoculation method

In this method 0.01 gm of soil was placed on the glass cover slip(11x11mm). One to two drop of distilled water with 0.1% Tween 80 was then pipetted on to the cover slip and the soil was thoroughly mixed with a Tween using spatula. Mixing was done inside a hood to minimize the external contamination. Using a forceps, the cover slip was then pulled over the surface of the selective media to distribute the resulting soil suspension over the entire surface of the medium. The resulting plates were held in an incubator at 22^0c. Plates were examined every 3-4 days for 15, days and the fungi growing on the media were identified through a stereo microscope.

Print method

Five to ten g of soil was placed on a Petri dish. A clean test tube lid (diam. of 20 mm) was moistened by touching the surface a selective growth media and then pressed to the soil

surface. Small soil particles adhered to the moist lid, 10 consecutive prints were produced on the surface of the selective media. Using this process, 24 Petri dishes were inoculated and were incubated at 22°c. plates were observed every 3-4 days for 15 days. Fungi growing on the medium were identified using stereo and phase contrast microscopy

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Electrostatic method

Five to ten g of soil was placed on sterile Petri dish. The plastic lid of a test tube that was rubbed against a sterile paper towel to establish electroststatic charge on the lid . The lid was held 1-2 cm. above the surface of the soil and the fine particles were attracted to the charged plastic. Then, the lid was used to produce 10 consecutive prints on the selective media evenly inoculating the entire surface. The process was repeated on 24 Petri dishes with all the work being done under the hood in sterilized condition. The Petri dishes were incubated at 22°c and were observed every 3-4 days for 15 days. Fungi growing on the *B. bassiana* and *M. anisopliae* selective media were identified under stereo and phase contrast microscopes.

Result

Two types of soil (field and forest soil) were utilized in this study. A greater number of entomopathogenic fungus were obtained from both types soils using the soil dilution method (Table1 and 2). The other methods (direct, print and electrostatic) resulted in the isolation of saprophytic fungi (*Fusarium* spp.and *Penicillium* spp.), which made it difficult to isolate entomopathogenic fungi. *Fusarium* was a particular problem on plates using print and direct inoculation methods. Approximately 1000 *Fusarium* colonies were obtained on the *B. bassiana* selective medium using direct inoculation method (Table 1). A major difference observed between the two types of soils was the availability of *Trichoderma* spp. (13.3 CFU from the forest soil (Table 2). Over all, the Electrostatic method was the poorest technique used and no *B. bassiana* and *M. anisopliae* were isolated.

Table 1.Types of fungi isolated from field soil using different methods and mean number of CFU/plate

Isolation technique	Selective media	Bb	Ma	Fus.	Pen.
Dilution method	Bb				
Stock solution		13.0	-	-	-
10x dilution		4.0	1.0	1.0	-
100x dilution		0.5	-	-	-
Total		17.5	1.0	1.0	
	Ma				
Stock solution		1.0	3.0	0.3	
10x dilution		-	-	5.0	0.5
100x dilution		0.3			
Total		1.3	3.0	5.3	0.5
Direct inoculation	Bb	0.5		1000.0	2.5
	Ma	1.5	0.3	62.0	22.5
Total		2.0	0.3	1062.0	25.0
Print method	Bb	0.5	0.3	1.3	2.3
	Ma			1.8	5.3
Total		0.5	0.3	3.1	7.6
Electro static method	Bb	-	-	-	-
	Ma	0.3	-	9.3	21.0
Bait method*	Bb				
	Ma				

- * = No count was done, Bb= *B. bassiana* , Ma= *M. anisopliae*
- - = 0

Table 2. Types of fungi isolated from forest soil through different methods and CFU/number of colonies

Isolation technique	Selecti ve media	Bb	Ma	Fus.	Pen.	Tricho.
Dilution method	Bb					
Stock solution		12.7	-	-	-	-
10x dilution		8.3	-	-	-	-
100x dilution		2.0	-	1.0	-	-
Total		23.0		1.0		
	Ma					
Stock solution		8.0	4.3	-	-	3.3
10x dilution		14.7	26.0	-	-	3.0
100x dilution		1.0	7.0	-	-	
Total		23.7	37.3			6.3
Direct inoculation	Bb	0.3				
	Ma	-	-	6.0	-	7.0
Total				6.0		7.0
Print method	Bb	1.0	-	-	1.0	1.0
	Ma	-	-	1.0	-	12.3
Total				1.0		13.3
Electro static method	Bb	1.0	-	-	2.0	1.0
	Ma	-	-	-	-	7.0
Total		1.0			2.0	7.0
Bait method*	Bb					
	Ma					

- * = No count was done, - = 0, Bb=*B. bassiana*, Ma= *M. anisopliae*, Pen =*penicillium* Fus =*Fusarium*, Tricho= *Trichoderma*

Bioassay of thrips

Material

Culture (*B. bassiana*, and *M.anisopliae* 14 days old), incubator, autoclave, vortex, sonicator, munger cell, thrips (2^{nd} instar), vials, spatula, test tubes, pipettes pipette tips, cheese cloth, filter paper, forceps, distilled water, Tween 80

Methods

0.7 ml of each fungus ($5x10^6$ conidia /ml 0.01% Tween 80) was pipetted to 7 cm diam. white man # 1 filter papers that were placed on Munger cells. Twenty second instar Western flower thrips (*Frankliniella occidentalis*) were placed on the surface of the wet filter paper. The two filter papers were placed together with the thrips inside sandwiched between the two cells, the complete Munger cell was held together with two rubber bands. Total of There 7 treatments were tested against thrips, each replicated 4 times. The cells with the thrips were incubated at 22^0c with the light: dark regime of 16: 8.

Mortality data was taken 6 days after treatment and the data was analyzed using SAS stat soft ware at 5% significant level.

Result

Five entomopathogenic fungi and 2 controls were tested against the western flower thrips. The percentage mortality achieved from *B. bassiana* treatment (49.4) was significantly higher all other treatments at 5% level (Table 3). *V. Lecani* (29.7%)and *M. anisopliae* (24.7%) treatments were Higher than the controls. Additional replicated trials are necessary to confirm these findings.

Table 3. Percentage mortality of western flower thrips treated with different fungi*

Treatment	Alive	Dead	Mortality
Beauveria bassiana	10	9.75	49.4%
Metarhizium anisopliae	15.25	5	24.7%
Paecilomyces fumoseroceus	17	4	19%
Verticillium lecanii	13.75	5.8	29.7%
Fusarium sp.	17	3	15%
Control + Tween 80	17	3	15%
Control (Distilled water)	17.75	2.25	11.3%

* Average of 4 replications

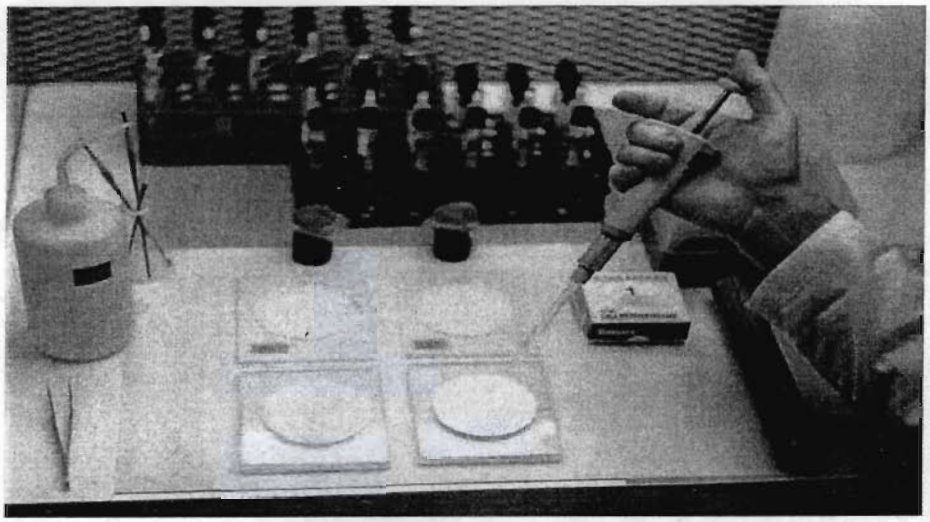


Fig1.innoculating filter papers on Munger cells.

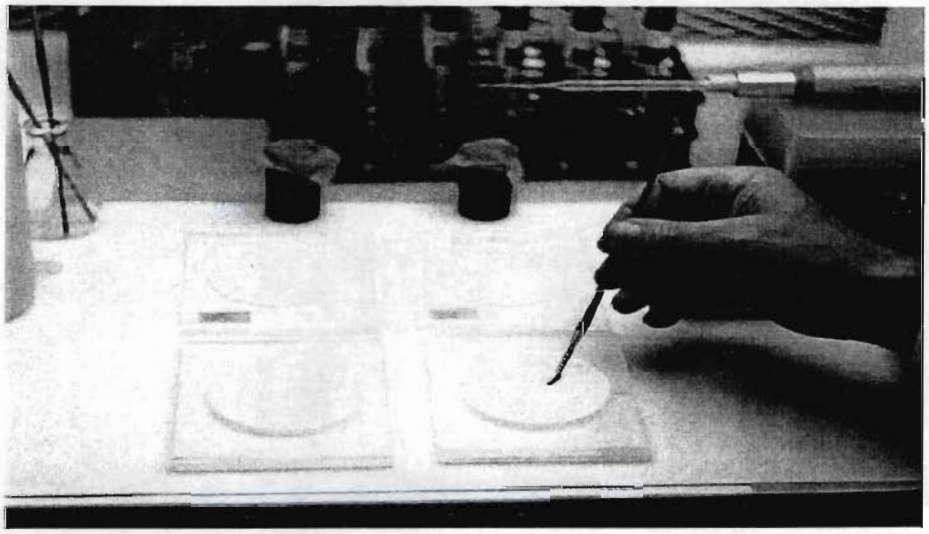


Fig.2 Spreading suspension over the lower disk using a paint brush

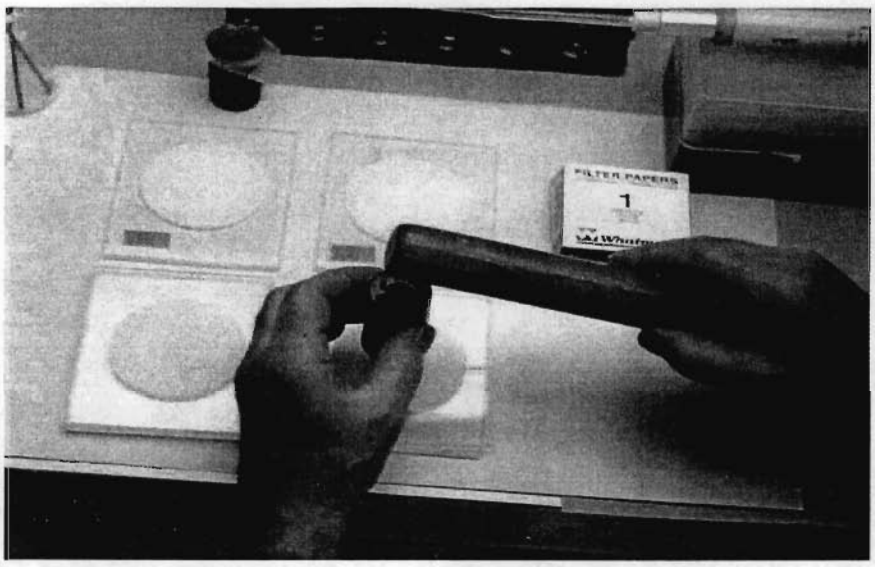


Fig.3. Transferring the second instar thrips on the surface of the lower filter paper

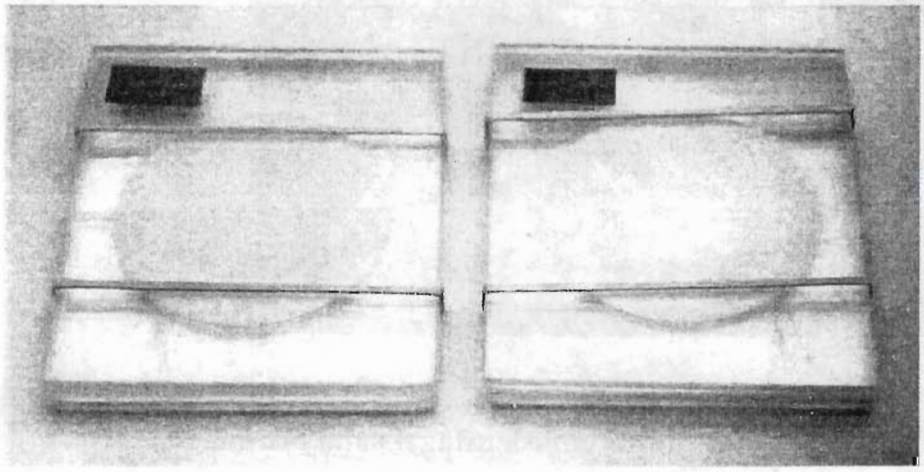


Fig. 4. closure of the assembled cells

Speed of growth and spore production

Materials

Standard growth medium (quarter-strength SDYA), Petri dishes, entomopathogenic fungi (*Beauveria bassiana* and *Metarhizium anisopliae),* pipettes, pipette tips (Plaster and plastic), incubators, filter paper, hemocytometer, ruler, distilled water, Tween 80, sonicator, vortex, phase contrast microscope and marker

Methods

A suspension containing 10^6 conidia / ml of B. bassiana or M. anisopliae were prepared. Ten micro liter of each was pipetted on to a small filter paper disk (6.35mm diameter) placed in the center of a media plate. Four replicates were produced (total of 16 Petri dishes) for each fungus. Plates were incubated at 15, 20 25 and 30 ^{0}c. The diameter of the developing colonies was measured every 5 days for 15 consecutive days along the same two axes. Aftern15 days 3 samples removed from each plate using cork—was and were transferred to test tubes containing 0.01% Tween 80 and small quantity of 75% alcohol the alcohol was added to kill the fungal spores to facilitate counting by haemocytometer. Test tubes were sonicated to homogenize spore suspension prior to counting on a haemocytometer under phase contrast microscopy. Samples were taken from fungi grown at 15 ^{0}c as virtually no growth occurred at this temperature. Spore concentration was achieved using the following formula.

X = Ax400/80x dilutionx50000, where A is the number of spores counted from 5 cells.

Results

Radial growth of *B. bassiana*, 603 was the highest at 20 ^{0}c after 15 days (colony diameter = 19.75 mm), but spore production at this temperature was low (1.9 x 10 ^{6}). The highest level of spore production was obtained at 25 ^{0}c (2.7 x10^7) although the radial growth is 18.75mm(Table 4 and 5). In this case, there was no positive correlation between radial growth and spore production.

Radial growth (42.75 mm) and spore production (9.2x $10^0$6/ml) by *M. anisopliae* was at 30^0c (Table 4 and 5). Spore production of spore (4.5x10^6/ml) was recorded at 20^0c and the radial growth was lowest. This suggests that there is a positive correlation between radial growth and spore production. Based on this result, *M. anisopliae* IMI strain grows better at higher than *B. bassiana*, 603.

Table 4. Radial growth of *B.bassiana* and *M.anisopliae* to the different temperatures diameter measured in mm temperature.

Temperature	Day	Fungal isolates	
		M.anisopliae (IMI)	*B. assiana* (603)
20°C	5	11.13	7.5
	10	24.75	13.5
	15	35	19.75
25°C	5	17	9.75
	10	37.25	14.75
	15	53.75	18.75
30°C	5	21.5	6.5
	10	42.75	11
	15	61	15.5

Table 5. Effect of temperature on spore productivity of *B. bassiana* 603 and *M. anisopliae* IMI

Species	Temperature	Mean no. Of spore/ml
M.anisopliae IMI	20°C	1.9x10^6
	25°C	5.6x10^6
	30°C	9.2x10^6
B.bassiana 603	20°C	4.5x10^6
	25°C	2.7x10^7
	30°C	8.7x10^6

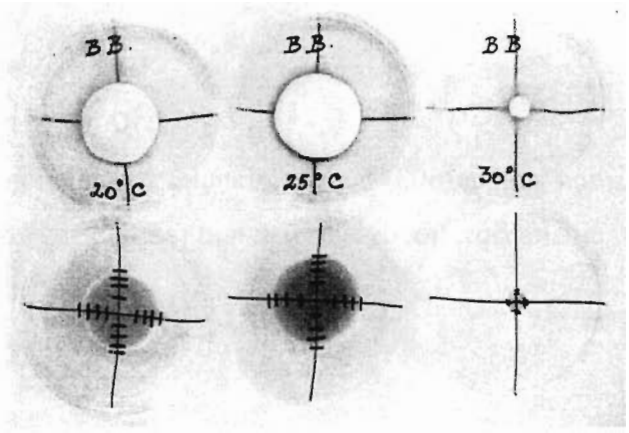


Fig. 5. Measuring the radial growth

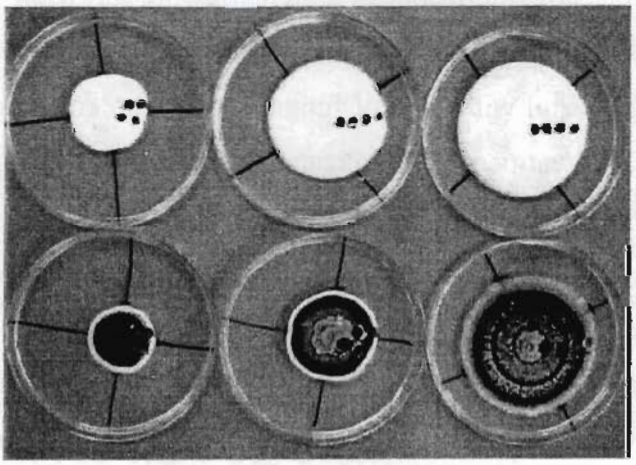


Fig. 6. Sampling the colony

Methods of treatment of soil pests

Materials

Cups, top pan digital balance, soil, *B. bassiana* isolate (603) both in granular formulation an spore suspension, spatula, beaker, pipettes, pipette tips, hood, leaf disk and insect armyworm (*Spodoptera exigua*), 6th instar

Methods

Thirty-two plastic cups were prepared and 20 g sterile soil added to 16 of them. The remaining 16 were filled with 20 g non-sterile soil. Four treatments were applied to the soil control 1, drenching the soil with 2ml.0.01% Tween 80; control 2,drenching the soil with 2 ml. Tween 80 and mixing in 2 g untreated millet grains; 3, grains inoculated 7d earlier with B.bassiana, isolate, 603; and 4, drenching the soil with 2 ml of fungal suspension containing 2.5x 10^6 conidia /ml. 0.01% Tween 80. Each treatment was replicated 4 times. In all 32 cups, 2 ml of 0.01% Tween 80 was added to moisten the soil. In the 2^{nd} un treated control, 2 g of un treated millet grains were added to serve as a granule or control all treatments were thoroughly mixed with the seed to have homogeneous distribution of spores or granules. Experiments were set up at two time intervals; 4d prior to addition of the insects were added to the cups and the day on which insects were added to the cups. Eight insects were added to each cup and bean leaf disks were also placed inside to feed the larvae and avoid starvation, so that mortality would be due to the entomopathogenic fungi.

Result

Day zero application.

The granular soil treatment out performed other methods applied on a day zero; 100 % mortality was recorded for both types of soil (sterilized and non-sterilized). The drench method slightly caused lower mortality against *S. exigua* 87.5 % mortality in sterile and 68.8 % in non-sterile soil (Table 6). Mortality in both control treatments was 9.4 %.

Treatment applied 4 days earlier

Both granular and drench methods caused 100 % mortality in the sterile soil. However, control mortality was also high so these results are inconclusive. In the non-sterile soil, the drench treatment caused lower mortality (59.46) than granular treatment (96.91) insects (Table 6) indicating that the granular treatment looks superior to soil drench.

Table 6. Percentage mortality of armyworm treated by *B .bassiana* using different methods

soil type	Treatments	Days of application	
		4 day before(%)	0 day(%)
Sterile	Bb, Granular	100	100
	Bb, drench	100	87.5
	Control, granular	53.1	9.4
	Control, drench	87.5	9.4
Non-sterile	Bb, Granular	96.9	100
	Bb, drench	59.4	68.8
	Control, granular	12.3	31.3
	Control, drench	18.8	3.1

Mass Production Technique of Entomopathogenic Fungi

1.To determine moisture level for the growth of fungi

2.To evaluate different crop grains for mass production of entomopathogenic fungi

Materials

Different crop grains (sorghum, millet, corn, wheat), fungal culture (*Beaveria bassiana* and *Metatrhizium anisopliae*), erlenmeyer flask distilled water, citric acid (800microlitrer), water bath, autoclave, hood, vortex, paster pipettes, filter, syringe, filter paper, cheese cloth, paper towel and Bunsen burner

Methods

Different moisture ratios (1:0.25, 1: 0.5, 1:0.75, 1: 1.00, 1: 1.25, 1: 1.50, 1:1.75 and 1:2.00 volume of grain: volume of water) were prepared using 100 ml of sorghum grain, millet grain, ground wheat, wheat bran or cracked corn in autoclavable glass jars. Four hundred micro liters of citric acid was added to each jar to stabilize the PH.

Comparison of cooked and soaked grains for production of fungi

1. Preparation of cooked grain preparation

   All the grain types and all grain: water ratios were held in a water bath at 100^0c for 30 minutes. The glass jars then removed and autoclaved for 60 minutes. The grains were allowed to cool to ambient temperature. Jars were shaken to break apart grains, which were stuck together prior to inoculation. Suspension of fungal spores were prepared (10 ml.), and 2 ml. suspension was used to inculcate each jar , using a syringe to apply the inoculums to the grains.

2. Preparation of soaked grains (uncooked)

The different types of grain (100g) were soaked in 1.5 to the volume of water to volume of grain. Grains were left for over- night to absorb the water. The next morning, excess water was removed and the grains were auto clave sterilized for 30 minutes. The sterilized grain was removed from the autoclave and cooled to the ambient temperature prior to inoculation as described above.

The cooked and soaked grains were maintained at room temperature (22 ^{0}c) and the observed every 3 days. Jars were shaken at this time to prevent caking of the grains. Spore yield was determined after 15 days using the following formula.

X =Ax4000/80xdilutonx1000

Preparation of suspension

To prepare a spore suspension for inculcating the grains, conidia were harvested from 14d old culture using a spatula to dislodge the conidia into 0.01% Tween 80 which was pipetted on to the plate. The resulting suspension was transferred to a test tube to serve as the stock suspension. The spore suspension was used to prepare the inoculums, which contained $1x10^8$ conidia/ml. Two ml. of this suspension used to inoculate the grains in the glass jars.

Inoculum was similarly prepared for the *M. anisopliae* isolate, and 2 ml of a 10^8/ml suspension was used to inoculate each of the jars.

Result

1. **Evaluation of different moisture level for the mass production of fungi**

This experiment was studied on *M.anisopliae* the conidia concentration was increasing as the moisture increases up 1:1.25(v/v) moisture level (Table 7). However due to its stickiness, it will be difficult to harvest the powder and is not preferable to work with this moisture level. The other moisture levels(!:1.75and 1:2.00) were rejected due to its coagulation character and high level of stickiness. The best moisture level preferred was 1:0.75 grain to water level combination. It was not sticky and the grain enlargement was also good as to the observation.

The best grain crops evaluated for the mass production of entomopathgenic fungi (*B.bassiana*) were millet and sorghum (table 8).

Table 7. evaluation of different moisture level for mass production of *M. anisopliae*

Moisture level v/v	grain enlargement	stickiness/ caked	fungal growth	conidial conc./ml	conidial conc./200ml
1:0.25	low	not sticky	poor	$1.6x10^6$	$3.1x10^7$
1:0.50	good	not sticky	good	$8.3x10^6$	$1.7x10^9$
1:0.75	very good	not sticky	good	$3.3x10^7$	$6.6x10^9$
1:1.00	good	slightly sticky	good	$4.5x10^7$	$9.1x10^9$
1:1.25	good	sticky	good	$7.3x10^7$	$1.5x10^{10}$
1:1.50	good	very sticky	good	-	-
1:1.75	good	very sticky	good	-	-
1:2.00	good	very sticky	good	-	-

Table 8. evaluation of different crop grain for the mass production of *B. bassiana* at 1:0.5 v/v grain to water

grain type	grain enlargement	stickiness/ caked	fungal growth	conidial conc./ml	conidial conc./200ml
soaked wheat bran	poor	sticky	poor	-	-
cooked wheat bran	poor	sticky	poor	-	-
soaked corn	good	not sticky	good	$1.2x10^7$	$2.4x10^9$
cooked corn	good	not sticky	good	$1.2x10^7$	$2.4x10^9$
soaked broken wheat	good	not sticky	good	-	-
cooked broken wheat	good	not sticky	good	-	-
soaked millet	very good	not sticky	good	$2.0x10^7$	$4.1x10^9$
cooked millet	very good	not sticky	good	$1.8x10^7$	$3.6x10^9$
soaked sorghum	very good	not sticky	good	$3.2x10^7$	$6.4x10^9$
cooked sorghum	very good	not sticky	good	$2.8x10^7$	$5.6x10^9$

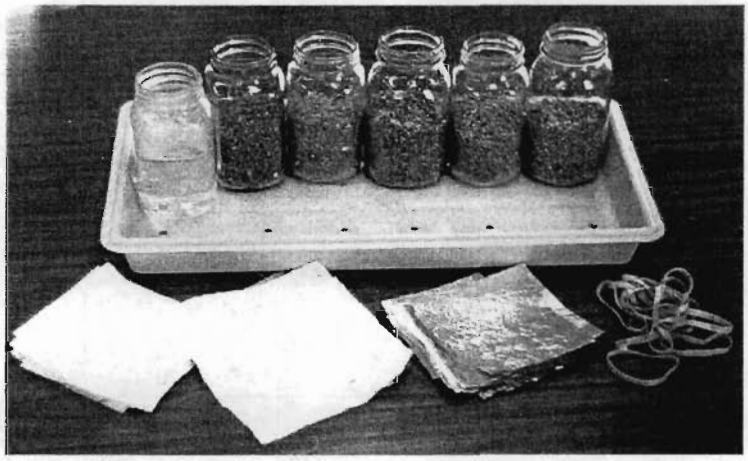


Fig. 7. Materials for mass production

Fig. 8. Jars prepared for inoculation

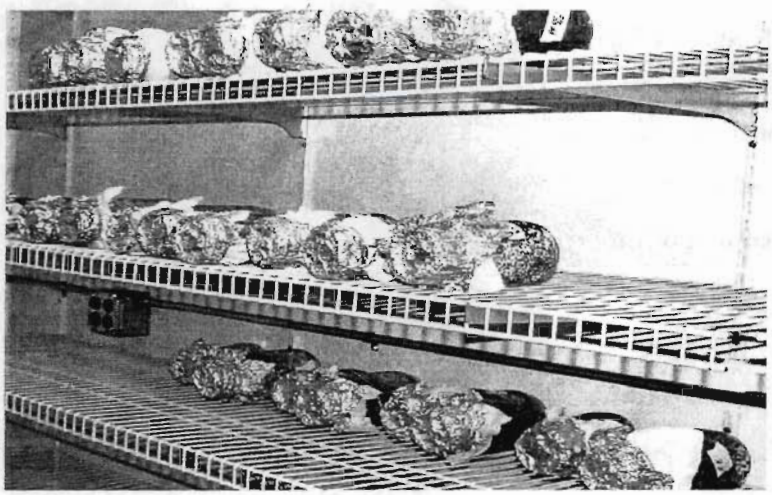


Fig.9. Glass jars with the entomopathogenic fungi in the growth room

I .Appendix

1. Recipe standard and selective e media used in these studies

1. Quarter strength sabroud dextrose agar supplemented with 0.25% (w/v)yeast extract.

- Neopeptone 2.5g
- Dextrose 10g
- Yeast extract 2.5g
- Agar 15g
- Citric acid 0.4 ml (50g in 1000ml distilled water)
- Add water to the total volume of 1 liter

After sterilization of the medium, antibiotics can be added.

- Penicillium G 5ml. (0.01g/100ml of dist. Water
- Streptomycin 12.5 ml (0.05g/100ml dist. Water

Selective media for *M. anisopliae*

- Chloramphenicol 0.25g
- Dodine 0.3g
- Agar 15g
- Rose Bengal 0.05g
- Wheat gram extract 30g
- Benlate 2ml stock (0.01g in 100 ml dist. Water)
- Crystal violate 2ml stock (0.5g in 100 ml dist. Water)
- Add water to make 1liter

In case of germ extract, malt extract agar can be substituted either with agar or without

2. Selective media for *B. bassiana*

- Neopeptone 10g
- Dextrose 10g
- Yeast extract 10g
- Agar 15g
- Dodine 0.45g
- Rose Bengal 0.05g

Add distilled water to make the volume 1 liter

After out clave

- Penicillium G 2ml (0.2g in 100 ml of dist. Water)
- Sterptomycin5ml stock solution (0.8g in 20 ml dist. Water)

Appendix II

Daily training activities

Jan.27/03

Arrival in UVM

Jan.28/03

Introduction with entomology laboratory staff

Tour of facilities and demonstration of equipment

Jan.29/03

Introduction with laboratory instruments

Safety measures and handling of equipments and different fungi

Jan.30/03

Isolation of fungi from soil-Five methods

Bait method

Soil dilution method

Print method

Direct inoculation

Electrostatic method

Jan.31/03

Isolation of fungi continued

Feb.03/03

Basic antiseptic and aseptic techniques used in mycology

Insect bioassay techniques

Feb.04/03

Enumeration of fungi using hemocytometer

Bioassay technique continued

Feb.05/03

Speed of growth and productivity of fungi

Mass production technique of entomopathogenic fungi

Feb.06/03

Preservation/storage of media and main ingredients

Meeting with Dr. Brown bridge to evaluate what has been done so far

Feb.7/03

Inoculation technique of saprophytic fungi and purifying technique

Feb.10/03

Preparation of selective and common media

Feb. 11/03

Isolation of fungi from soil

Inoculation of fungi isolated from soil

Feb.12/03

Mass production technique

Slide preparation technique

Scotch tape method

Sandwich method

Feb.13/03

Mass production technique continued

Feb.14/03

Preparation of liquid media

Method of treatment of soil pests

Feb. 18/03

Checking mass production (experiment II)

Transferring liquid media to a plastic box

Experimental design and analyses of data (SAS)

Rearing of thrips

Feb.19/03

Checking of mass production experiment I

Data recording on isolation technique of fungi in the soil.

Isolation and inoculation of *Trichoderma* and *Paecilomyces* for identification purposes (aseptic)

Feb.20/03

Checking speed of growth experiment (marking petri dishes)

Checking isolation of fungi from soil experiment

Feb.21/03

Checking mass production technique experiment II

Meeting with Michael Brown bridge to discuss general issues.

Feb.24/03

Checking and recording observation on method of soil treatment experiment

Observation on mass production technique experiment conidial and I count through phase contrast microscope for all treatments.

Feb.25/03

Checking and recording of isolation of fungi from soil experiment II (recording types of fungi and CFU for all methods)

Feb.26/03

Conidial count of *B. bassiana* liquid media

Discussion on long storage methods

General discussion with Dr. Michael Brown bridge, general issues.

APPENDIX IV

Training Report, W. Mandefro (March 2003)

Insect pest management with fungi: A mass production technique for farmers

Cooperative Development Research Project C-16-125

Activity report
For the training held between January 26 and March 2 3003

By
WONDIRAD MANDEFRO

Ethiopian Agricultural Research Organization
Entomology Research Laboratory
University of Vermont

March 2, 2003

Acknowledgments

I would like to thank Prof. Bruce Parker, Dr Michel Brownbridge and Dr Margaret Skinner for arranging the training. In particular, Dr Brownbridge is thanked for organizing the training, facilitating accommodations and assisting all our needs on a daily basis. The training would have not been successful without Drs Vladimir Gouli and Svetlana Gouli unreserved assistance, guidance and friendship. They have also provided us with transportation to and from the laboratory. I would also like to thank Dr Scot Costa for an excellent lecture on statistics.

I want to express my gratitude to Tomas Doubleday for taking care of our groceries and Don Tobi for handling our paper works. Mohammed El Damir, Amanda and Jane, thanks for your lively hospitality and friendship. I could not have survived the coldest winter of Vermont without your smiles. EARO is duly acknowledged for allowing me to participate in this training. The cost of the training is covered by USAID.

Table of contents

Major output of the training

The training course has provided me with the basic principles and techniques of insect pathology, with a particular focus on fungal entomopathogens. The major techniques and methods learned during the course of the training were:

- Culture, quantification and preservation of fungal isolates
- Methods of mass production of fungi on grains
- Use of aseptic techniques for microbiological studies
- Different methods of conducting insect/ fungus bioassays

I. Introduction

The report presents a compilation of various experiments and activities carried out at the Entomology Research Laboratory (ERL), University of Vermont during four weeks of training from January 26 to February 28. The experiments are presented individually. However, methodologies related to general mycology and insect pathology are all described together. More emphasis is given to detailed protocols and procedures hoping that it may serve as a reference tool.

II. Comparison of methods for isolation of fungi from soil

Five different methods of isolation of were compared for their effectiveness in recovering fungus from soil. Soil samples were collected from a nearby farm and forest and air-dried at room temperature. Spores of *Beauveria bassiana* and *Metarhizium anisopliae* were thoroughly mixed into the soils to obtain a concentration of $1x10^3$ conidia g^{-1} dry soil before doing the experiment. The experiment was done on both soils. The methods of isolation were:

1. Bait method
2. Soil dilution method
3. Direct inoculation method
4. Print method
5. Electrostatic method

1. Bait method

The bait method is based on the use of susceptible larvae of the greater wax moth, *Galleria mellonela*. Three third instar *G. mellonela* were put into small plastic containers of about 200ml. Soil was placed on top of the larvae until approximately 2/3 of the container was filled. The containers were incubated at 22°C. Every two days, the containers were inverted, so that the larvae continually had to move through the soil, and were thus repeatedly exposed to infective conidia. Insect mortality was checked after 15 days. Dead insects were transferred to a clean Petri dish lined with a moist to promote fungal outgrowth and sporulation on the cadaver. Fungi were cultured on artificial media and pure cultures obtained through successive transfer for identification.

2. Soil dilution method

The method is useful for estimation of the number of fungi colony forming units (CFU) in soil, i.e. the number of fungal propagules per g of soil, as well as in isolation of new strains. Soil sample was divided into two 10-15g sub-samples. The first sub-sample was used to make a soil suspension while the second was used to determine the moisture content of soil. To determine moisture content, the sample was placed in a beaker and held in an oven at 150°C for 3 days, after which the soil was re-weighed and the moisture content determined.

The first sub-sample was transferred to a 500ml Erlenmeyer flask of 200ml 0.1% Tween 80 was added to it. The flask was [laced on a shaker at 300 rpm for 30 minutes. About

10ml of the resulting soil suspension was filtered through cheesecloth into a 20ml test tube. The suspension was then vortexed and diluted 10 and 100 times; 0.5 ml suspensions was spread over the surface of both *M. anisopliae* and *B. bassiana* selective media (Annex 1). Twelve replicate plates were prepared for each for each dilution and incubated at 22°C. After 7-d, plates were examined and colonies of *B. bassiana* and *M. anisopliae* counted under dissecting microscope. The experiment was then repeated again using a different soil sample.

3. Direct inoculation method

Four replicate 10 mg soil samples were weighed on separate cover slips. Two to three drops of sterile distilled water were added to each soil sample and mixed well with a spatula. The resulting soil suspension was then smeared uniformly over the surface of *B. bassiana* and *M. anisopliae* selective media. The plates were incubated at 22°C. After 7-d, the number of *M. anisopliae, B. bassiana* and total fungal colonies were counted.

4. Print method

A dry, sieved soil sample was put into a clean Perti dish. Flat-bottomed and circular plastic caps were touched onto a surface of fresh nutrient media and then to the soil surface. Small soil particles, adhered to the moist object, was then used to print at least 5 consecutive times onto surface of a plate containing selective media. Twelve replicate plates were produced. The plates were incubator at 22°C, and examined after 7-d for fungal colonies.

5. Electrostatic method

This method is based on the attraction of small particles to electrostatically-charged materials. A dry flat-bottomed plastic cap was charged by rubbing on a paper towel. The charged plastic was held over the surface of the dried and sieved soil sample at a distance of about 2-3 cm for 30 seconds. Small particle were attracted to the plastic material and was printed a minimum of 5 times onto a surface of the selective media. Four replicate plates were produced. The plates were incubated at 22°C, and examined after 7-d.

Result

Mean number of colonies of *M. anisopliae*, *B. bassiana*, *Fusarium* sp., *Paecilomyces* sp., *Penicillium* sp. and *Trichoderma* sp. recovered through four different methods in field and forest soils were summarized in tables 1 and 2. Dilution method recovered more number colonies of *M. anisopliae* and *B. bassiana* than other methods. 10x dilution has the highest number of colonies from both soils. Other methods are less sensitive in isolation of the two fungal species from soil.

The bait method was fund to be less sensitive to isolate *B. bassiana* and *M. anisopliae*. No insect was found to be infected with two species in both soils. In general, both selective media were effective to suppress most saprophytic fungi. However, *Fusarium* sp. and *Paecilomyces* sp. from field and forest soils, respectively, had prolific growth on *B. bassiana* selective media. While *Trichoderma* sp. dominated *M. anisopliae* selective media.

Table 1. A summary of mean number of colonies isolated from farm soil.

Method	Selective media	Mean number of colonies					Total colonies
		Ma	Bb	Fus	Paec	Penic	
Soil dilution, Stock	Bb	0.2	4.3	0.4	0	0.08	7.9
	Ma	1	2.6	1.8	0.2	0.17	7.7
Soil dilution, 10x	Bb	0.2	0.8	0	0	0	1.8
	Ma	0.08	0.3	0	0	0	0.5
Soil dilution, 100x	Bb	0	0.08	0	0	0	0.08
	Ma	0	0	0	0	0	0
Direct inoculation	Bb	0	0.2	1333	0	0.8	1333
	Ma	0.08	0.5	20.5	0.25	7.6	28.9
Print	Bb	0.08	0.2	0.4	0	0.8	1.7
	Ma	0	0	0.7	0	2.2	3.8
Electrostatic	Bb	0	0	0	0	0	0.08
	Ma	0	0.08	2.3	0	6.2	12.5

NB: Ma: *M. anisopliae*, Bb: *B. bassiana*, Fus: *Fusarium* spp., Paec: *Paecilomyces* sp., Penic: *Penicilium* sp.

Table 2. Summary of mean number of colonies isolated from forest soil.

Method	Selective media	Mean number of colonies						Total colonies
		Ma	Bb	Fusar	Paec	Penic	Trich	
Soil dilution, Stock	Ba	0	4	0	0	0	0	322.2
	Ma	0.9	2	0	0	0	1.9	49.7
Soil dilution, 10x	Bb	0	2.4	0	0	0	0	49.2
	Ma	8.1	3.2	0.4	0	0	0.6	53.3
Soil dilution, 100x	Ba	0.1	0.8	0	0	0	0	8.9
	Ma	1.8	0.2	0	0	0	0	9.8
Direct inoculation	Ba	0	0.2	0	0	0	0	8.7
	Ma	0	0	18	0	0	28	65
Print	Bb	0	0.3	0	0	0.6	0.3	6.1
	Ma	0	0.2	0	0	0	3.8	5.6
Electrostatic	Ba	0	0.2	0	0	0.7	0.4	6.1
	Ma	0	0	0	0	0	2.3	2.7

NB: Ma: *M. anisopliae*, Bb: *B. bassiana*, Fus: *Fusarium* spp., Paec: *Paecilomyces* sp., Penic: *Penicilium* sp., Trich: *Trichoderma* sp.

III. Bioassay of insects /Pathogenecity test

There is considerable genetic variation among different isolates of the same species of fungi in relation to their pathogenecity, speed of growth, spore productivity and temperature requirements. Knowledge of these factors is critical in the selection of potent strain for use in biological control.

1. Bioassay of western flower thrips

Fungal suspensions of *M. anisopliae* IMI 330189, *B. bassiana* 603, *Verticillium lecanii* and *Paecilomyces fumosoroceus* were prepared from 2 week-old cultures grown on ¼ strength SDAY (Annex 1). Suspensions of the isolates were prepared in 0.01% (w/v) Tween 80 and adjusted to $5x10^6$ conidia ml^{-2}. 0.7ml of each suspension was applied to two 7cm-diameter Whatman # 1 filter papers on Munger cells (Fig. 1 - 4). About 20 adult western flower thrips (*Frankliniella occidentalis*) were put onto a filter paper and were provided with 2 cm diameter fresh bean leaf disc for food. The Munger cells were carefully and immediately closed and held together wit a rubber band. Two controls, 0.01% Tween 80 and distilled water were set up for comparison against the fungal treatments. Four replicate cells were set up for each treatment. The cells were held in plastic bags to prevent desiccation and kept at 22°C in an incubator with 16 / 8 light / dark regime. Mortality data was collected seven days later opening each Munger cell. Dead insects were observed under dissecting microscope for evidence of fungal infection.

Results

Summary on percentage mortality of thrips were presented in table 3. Data were analyzed using ANOVA, which showed that significantly higher levels of mortality were obtained with the *B. bassiana* treatment only in comparison with the Tween control. All other treatments were no significantly different at $P \leq 0.05$.

Table 3. Summary of average percentage mortality of WFT 7 days after treatment.

Treatment	Mortality
Beauveria bassiana	49.4%
Metarhizium anisopliae	24.7%
Paecilomyces fumosoroseus	19%
Verticillium lecanii	29.7%
Fusarium sp.	15%
Control + Tween 80	15%
Control (Distilled water)	11.3%

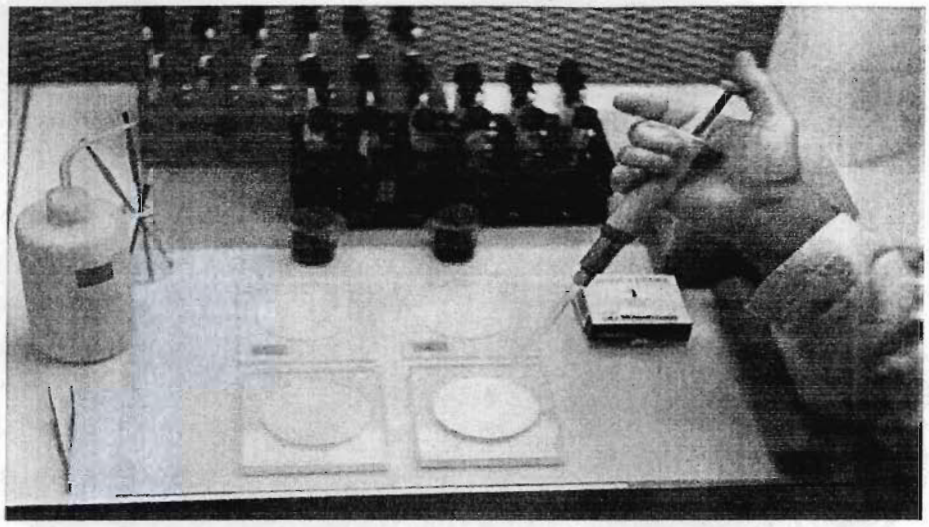


Fig. 1. Inoculating filter papers on Munger cells.

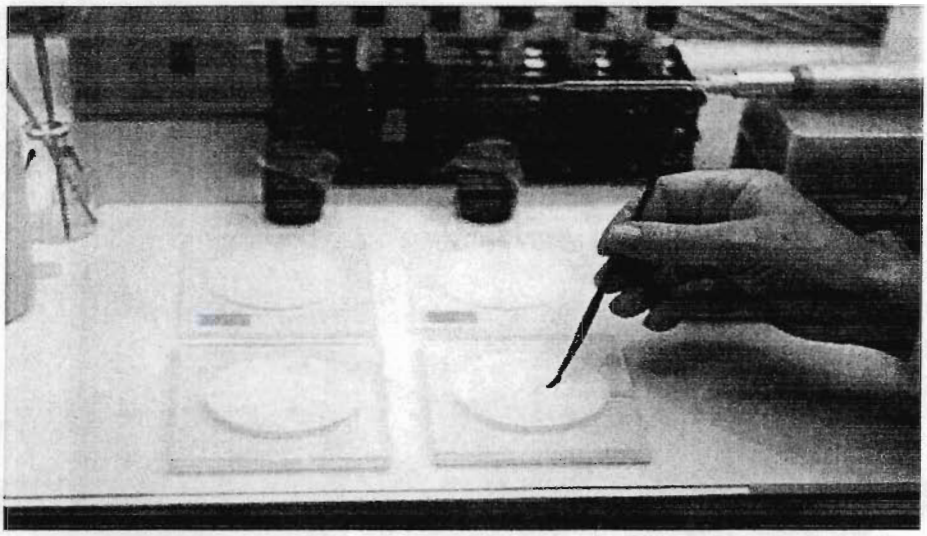


Fig. 2. Spreading suspension over the 'lower' disc using a paint brush.

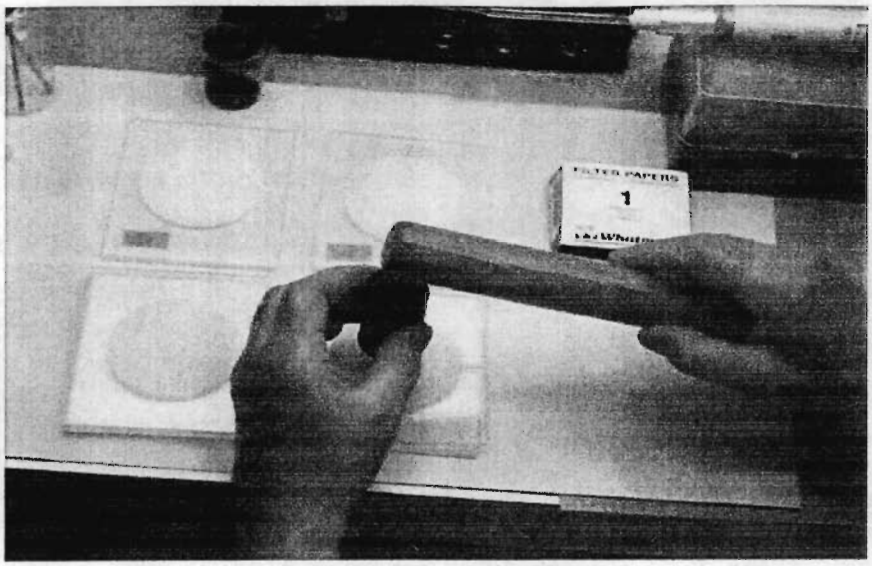


Fig. 3. Transfer of second instar thrips onto the surface of the 'lower' filter paper.

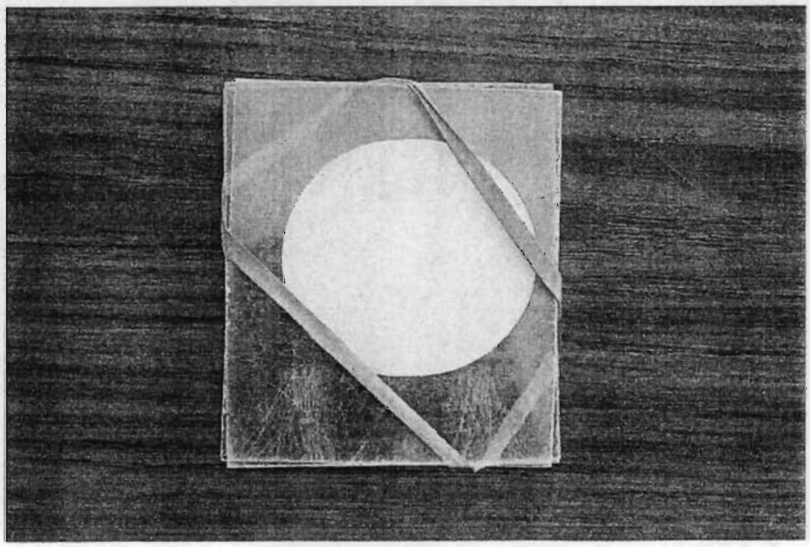


Fig. 4. Secure closure of the assembled cells using rubber bands.

2. Bioassay of soil insects

The effect of several variables on the efficacy of *B. bassiana* in soil were tested. The experiment was set up to determine the impact of sterile and non-sterile soil, two methods of application (drench and granular) and two times of treatment application; (4 days before and day of insect release into the soil) on the efficacy of *B. bassiana* 603 against the beet army worm (*Spodoptera exigua*).

Twenty grams of sterile and non-sterile soil were weighed into plastic containers. A drench treatment was prepared as spore suspension in 0.01% Tween 80 at a concentration of $2.5x10^7$, while one-week-old *B. bassiana* cultures grown on sorghum was used as a granular treatment. In the drench application, 2ml of fungal suspension were added to the soil and mixed well to obtain a homogenous distribution and a final spore concentration of around $2.5 x10^5$ conidia gm^{-1}. Two ml of 0.01% Tween 80 were added to soils prior to addition and incorporation of 2g granules. The controls were treated with 0.01% Tween only (drench) or Tween plus sterile sorghum grains (2g/ 20g soil). After thorough mixing of the soils, eight fourth instars were added into each container, which were then closed and held a 22°C. Fresh bean leaves were added to each container to provide the larvae with food (Fig. 5).

After one week the number of dead and living insects were counted by removing the entire contents.

Results

Due to time constraint, only summarized data is presented without statistical analysis Table 4). Therefore effects of individual treatments and their interactions could not be observed at present. However, in all combinations granular application of *B. bassiana* had more than 95% mortality followed drench application. Similarly, sterile mortality is higher in sterile soil that non-sterile soil.

Table 4. Effect of different soils, treatment formulations and time of application on efficacy of *B. bassiana* against *S. exigua.*

Soil type	Treatments	Percent mortality	
		Days of application	
		4 day before	0 day
Sterile	Bb, Granular	100	100
	Bb, drench	100	87.5
	Control, granular	53.1	9.4
	Control, drench	87.5	9.4
Non-sterile	Bb, Granular	96.9	100
	Bb, drench	59.4	68.8
	Control, granular	12.3	31.3
	Control, drench	18.8	3.1

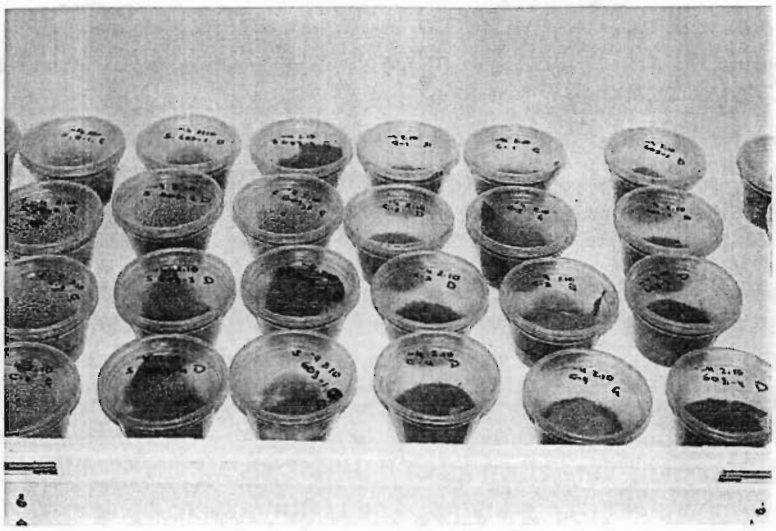


Fig. 5. Experiment set up for bioassay of soil insects.

IV. Growth rate and spore production

Definition of the optimal temperatures for growth rate and spore production is essential to the selection of fungal isolates well suited to the environment in which they will be used. Growth ands spore production was assessed at four different temperatures, 15, 20, 25 and 30°C. Spore suspensions of two isolates, *M. anisopliae* IMI 330189 and *B. bassiana* 603, were prepared at a concentration of $1x10^6$ spore ml^{-2}. A circular disc of filter paper (6 mm diameter) was placed in the center of ¼ strength SDAY media and inoculated with 10µl of suspension. Four replicate plates were setup for each isolate. All Petri dishes were sealed with Parafilm and incubated at the respective temperatures. Every 5 days after inoculation, the diameter of the colony was measured along two axes until day 15, to provide an indication of the radial growth rate at the respective test temperatures.

To determine spore productivity, four small circular agar blocks were sampled along a radial axis on day 15 using cork borer (Fig. 6, 7). The disks were placed in test tubes containing 10ml 0.01% Tween 80. The tubes were stored at 4°C overnight. Spores were stored in refrigerator overnight and sonicated for 5 minutes to disrupt spore clumps and then vortexed to homogenize the suspension. Spore concentration was determined using a hemocytometer. Neither isolates grew at 15°C and hence no data were collected from this temperature.

Data on radial growth and spore productivity were summarized to determine the optimum temperature of isolates.

Result

Summary of radial growth and spore productivity is presented in tables 5 and 6. Statistical analysis, however, has not been done.

Table 5. Diameter of *M. anisopliae* and *B. bassiana* colonies overtime when incubated at different temperatures.

Temperature	Day	Fungal isolates	
		M. anisopliae	***B. bassiana***
20°C	5	11.13	7.5
	10	24.75	13.5
	15	35	19.75
25°C	5	17	9.75
	10	37.25	14.75
	15	53.75	18.75
30°C	5	21.5	6.5
	10	42.75	11
	15	61	15.5

Table 6. Spore production by *M. anisopliae* and *B. bassiana* at four different temperatures.

Species	Temperature	Average spore yield/ml
M. anisopliae IMI 330189	20°C	$1.9x10^6$
	25°C	$5.6x10^6$
	30°C	$9.2x10^6$
B. bassiana 603	20°C	$4.5x10^6$
	25°C	$2.7x10^7$
	30°C	$8.7x10^6$

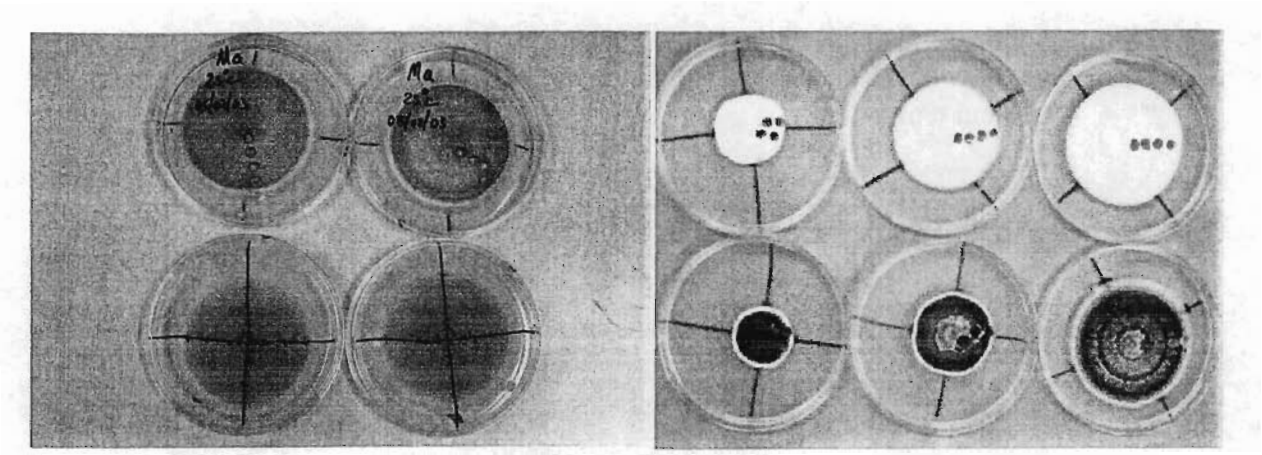


Fig. 6. a. Measuring the radial growth (colony diameter) of *B. bassiana* and *M. anisopliae*. Note: measurements were taken along the same axes 5, 10 and 15 d after inoculation.
b. Colony sampling to determine spore production. Samples taken 15d after inoculation.

V. Mass production of fungi

Studies were done to select the best moisture content, grain substrate and preparation methods for mass production of the fungi. Comparison of solid and liquid culture was also done to determine both the quality and quantity of spores produced in each system. The objectives of the experiment were:

- To identify the best grain substrate
- To observe effects of impact of different moisture level on the substrate.
- To observe effect of cooking vs. soaking on the texture of the grain, substrate.
- To compare spore yield and quality from solid and liquid substrates.

1. Evaluate the suitability of different substrates for fungal growth
100 ml of different grains (millet, sorghum, broken wheat, broken maize and wheat bran) were measured and put into a 500 ml glass jar in two replicates. 50ml of tap water containing 0.4 ml liter^{-1} of citric acid was added to one set of jars and cooked in a boiling water bath for 2 hours and then autoclaved immediately for 1 hour. The other set of jars were soaked with an excess of water and left overnight. The following day, the excess water was drained off and the jars were autoclaved for 1 hour. Spore suspensions of *B. bassiana* 603 were prepared at concentration of 1x10^{8}. Jars were inoculated with 1ml of spore suspension and held at 24°C. (see figs. 7, 8 and 9 for set-up procedures).

2. Selection of optimal moisture levels for spore production

Eight different moisture levels were tested for their effects on fungal growth on sorghum grains. The experiment was set up as described above, but different volumes of water were added to the jars to achieve different moisture levels in the substrate. Eight different ratios of grain: water were selected, ranging from 1: 0.25 to 1: 2 (V:V) with an incremental difference of 0.25 between treatments. After adding water to the jars, the grains were all cooked in a boiling water bath for 2 hours and autoclaved for 1 hour. After cooling to room temperature, the jars were inoculated with 1 ml of *M. anisopliae* suspension containing $1x10^8$ spore ml^{-1}. After inoculation the jars were held at room temperature (24°C) and the contents of the jars were mixed every 3 days.

3. Mass production in plastic bags

Using jars only a limited amount of spores can be produced. Larger containers must be used to produce large quantities of conidia fir use in use in bio-control programs. Plastic bags are good alternatives as they are inexpensive and easy to handle.

500ml of sorghum was placed in an autoclavable plastic bags and water was added at different ratios to select the optimum moisture content for spore production. Five ratios of water: sorghum were tested 0.5, 0.75, 1, 1.25 and 1.5: 1 (V:V). After adding the water plus 0.4 ml $liter^{-1}$ of citric acid, bags were cooked for two hours and then autoclaved for 1 hour. After cooling to room temperature, the bags were inoculated with 5ml spore suspension containing $1x10^8$ spores ml^{-1}. The grains were mixed thoroughly to enhance homogenous distribution of the spores throughout the growth medium at 24°C. Every 3 days, the contents were thoroughly mixed to promote growth throughput the medium. To provide air exchange the opening of each bag was covered with a layer of paper towel, cloth, and aluminum foil that were held in place with a rubber band (Fig. 11).

4. Mass production in liquid substrate

Mass production of fungi in liquid culture could significantly decrease the costs associated with their production. However, there are disadvantages to using this system, and the propagules produced are frequently less stable than those produced by solid substrates. Industrial mass production of microorganisms is largely done in liquid media in fermenters. This experiment was done to compare spore yield on a solid substrate (sorghum) and in a liquid medium (¼ strength SDY). 100ml SDY was poured into a 250ml shake flasks and autoclaved. Both media were inoculated with 1ml of $1x10^8$ spores ml^{-1} of *M. anisopliae* IMI and *B. bassiana* 603. Flasks were incubated on a horizontal flask shaker at 24°C, 200 rpm for. After 5 days, the whole contents were poured into a pan covered with nylon mesh and left to dry at room temperature. Spore productivity of solid substrate on sorghum and liquid culture will be made with experiment V.3 and 4 data.

Both substrates were not ready for harvest at the time of compiling this report.

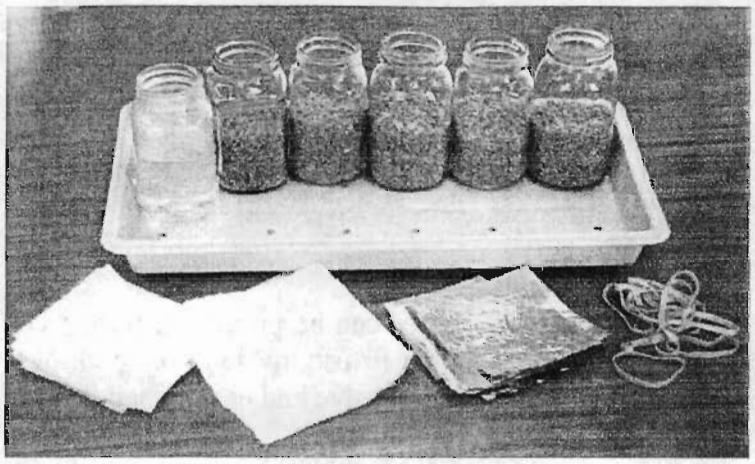


Fig. 7. Material used for mass production of fungi in glass jars.

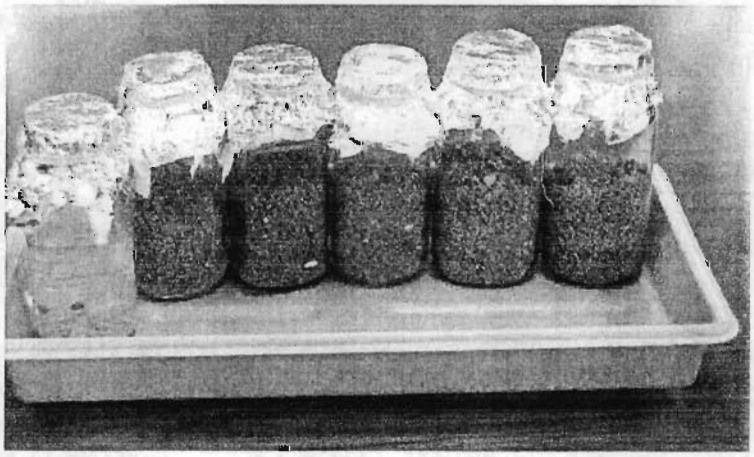


Fig 8. Jars ready for inoculation.

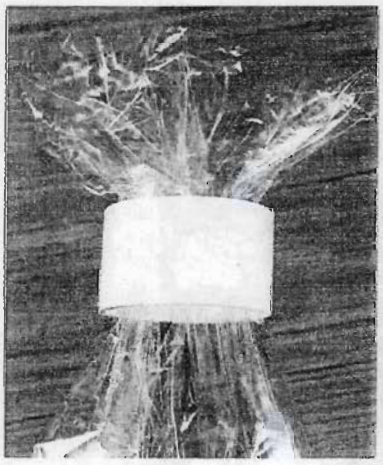


Fig. 9. Pictures demonstrating how to close plastic bags after inoculation.

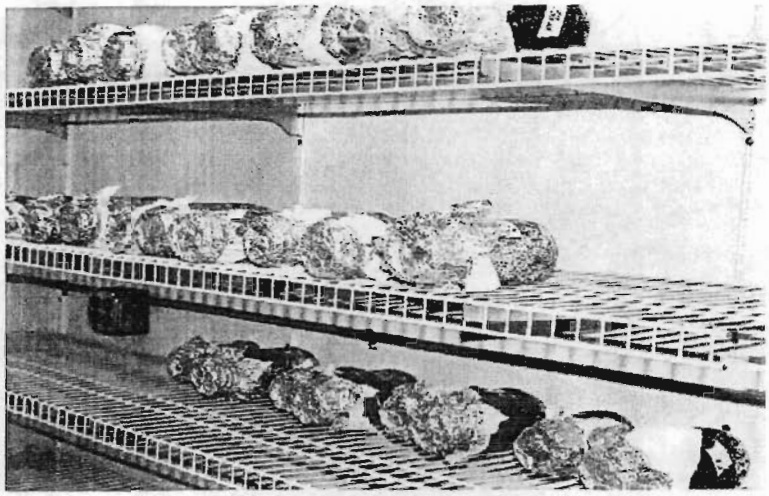


Fig. 10. Incubating inoculated jars in growth room.

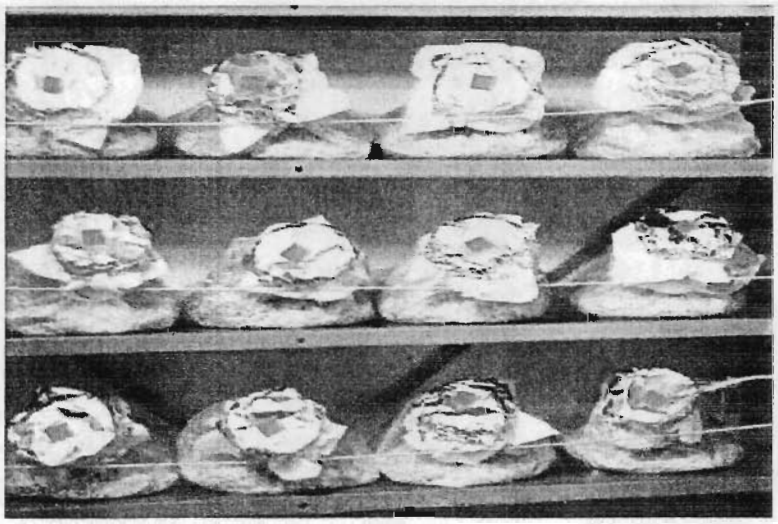


Fig. 11. Incubation of plastic bags after inoculation.

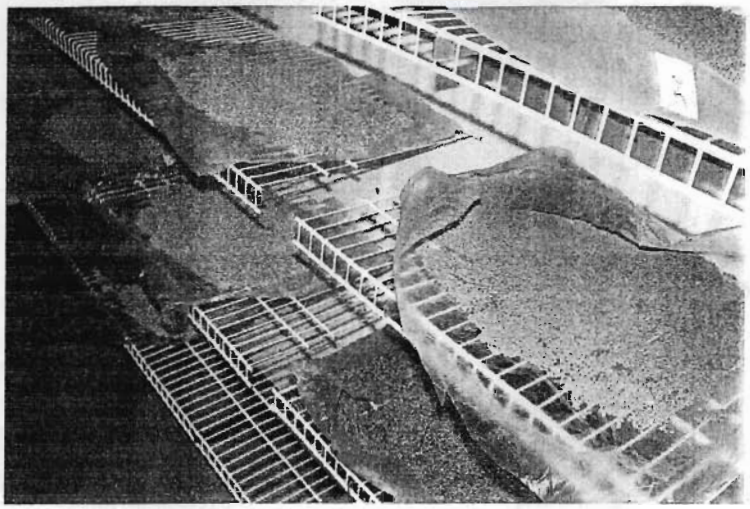


Fig. 12. Drying grains before harvesting spores opening plastic bags.

Results

Observations on the texture, moisture content and stickiness of the different grains in both sets of experiments are summarized in tables 1 and 2. According to our observation, cooking in a water bath is the preferred method for preparation of the grain substrate rather than soaking. Millet and sorghum were better than the other substrates in terms of their suitability for mass production and ease of use.

Table 7. Comparison of different grains substrates for their suitability for mass production of *B. bassiana* 603 at a ratio of 1: 0.5 grain, water (V/V).

Grain type	Type of cooking	Observation on quality of grains after moisture	Remark
Wheat	Cooked	Not sticky, good moisture, good in nutrient but not workable	Not good
	Soaked	Very sticky and stony	Not good
Corn	Cooked	Highly sticky, not good as growth media	Not good
	Soaked	Not very sticky, did not absorb enough moisture, fairly good for growth though	Not good
Millet	Cooked	Uniform grain size, slightly sticky	Good
	Soaked	Good in volume, fungal growth	Very good
Sorghum	Cooked	Good texture and volume	Very good
	Soaked	Good texture and volume, no difference from the cooked	Very good
Wheat bran	Cooked	Very sticky, not good as growth medium if used alone	Not good
	Soaked	Very sticky, not good and poor as a growth medium	Not good

Table 8. Effect of different grain: water (V:V) ratios on the texture of sorghum grains and their suitability as substrates for mass production of *M anisopliae* IMI 330189.

Grain type	Grain: water ratio	Observation	Remark
Sorghum	1: 0.25	Very dry, grain not opened	Not good
	1: 0.5	Good, not sticky but slightly dry	Fair
	1: 0.75	Ample moisture, grains opened	Very good
	1: 1	Good moisture, grain opened, slightly sticky	Fair
	1: 1.25	High moisture, grain sticky & coagulate	Not good
	1: 1.5	Very high moisture, very sticky	Not good
	1: 1.75	Very high moisture, not possible to mix	Not good
	1: 2	Very high moisture, not possible to mix	Not good

Table 9. Spore yield of *M. anisoplae* and *B. bassiana* from sorghum in jars.

Solid substrate			
		***M. anisoplae* IMI**	***B. bassiana* 603**
Substrate	**Grain: moisture ratio**	**Spores ml^{-1} of grain**	**Spores ml^{-1} of grain**
Sorghum	1: 0.25	$3.15x10^{9}$	$3.55x10^{10}$
	1: 0.5	$1.66x10^{10}$	$4.1\ x10^{10}$
	1; 0.75	$6.6\ x10^{10}$	$2.3\ x10^{10}$
	1: 1	$9.05\ x10^{10}$	
	1: 1.25	$1.46\ x10^{11}$	

Table 10. Comparison of spore yield of *B. bassiana* on different grains using two methods of soaking in jars

Grain type	**Method of moisture**	**Spore ml^{-1}**	**Total spore in 200ml of grain**
Millet	Cook	$1.8x10^{7}$	$3.6x10^{10}$
	Soak	$2.1x10^{7}$	$4.1x10^{10}$
Corn	Cook	$1.2x10^{7}$	$2.3x10^{10}$
	Soak	$1.2x10^{7}$	$2.4x10^{10}$
Sorghum	Cook	$2.8x10^{7}$	$5.6x10^{10}$
	Soak	$3.3x10^{7}$	$6.5x10^{10}$

VI. Long term storage of fungi

If kept in appropriate conditions, fungal cultures can be kept in storage for quite indefinitely. Long term storage is essential element of research in microorganisms. Cultures in those storage remains unchanged without change in their behaviors. Demonstration was made on techniques of long term storage in deep freezes at –80°C and cold room storage.

VIII. Appendix

I. Recipes general and selective media used in these studies.

1. Quarter strength Sabroud dextrose agar supplemented with 0.25% (w/v) yeast extract.

- Neopeptone 2.5 g
- Dextrose 10 g
- Yeeast extract 2.5 g
- Agar 15 g
- Citric acid 0.4 ml (50 g in 100 ml dH_2O)
- Add water to a total volume of 1 liter.

After sterilization antibiotics can be added after filter sterilization (stock solution kept in refrigerator)

- Pencillin G 5 ml (0.01 g/100 ml dH_2O)
- Streptomycin 12.5 ml (0.05 g / 100 ml dH_2O)

2. Selective media for *Metarhizium anisopliae*

- Chloramphenical 0.25 g
- Dodine 0.3 g
- Agar 15 g
- Rose Bengal 0.05 g
- Wheat germ extract 30 g
- Benlate 2 ml stock (0.1 g in 100 ml dH_2O)
- Crystal violate 2 ml stock (0.5 g in 100 ml dH_2O)
- Add water to make it 1 liter

In case of wheat germ extract, malt extract agar can be substituted either with agar or without.

3. Selective media for *Beaveria bassiana*

- Neopeptone 10 g
- Dextrose 40 g
- Yeast extract 10 g
- Agar 15 g
- Dodine 0.45 g
- Rose Bengal 0.05 g
- Add distilled water to make volume 1 lt

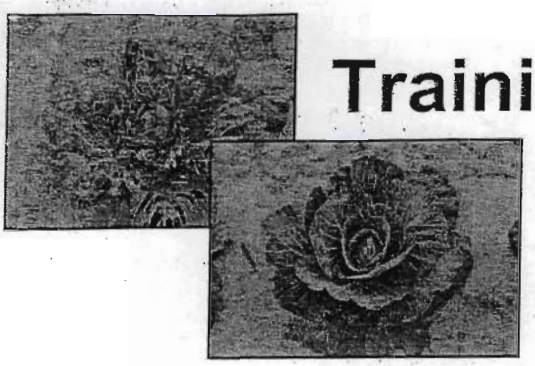
After autoclave

- Penicillin G 2 ml of stock solution (0.2 g in 100 ml dH_2O)
- Streptomycin 5 ml stock solution (0.8 g in 20 ml dH_2O)

APPENDIX V

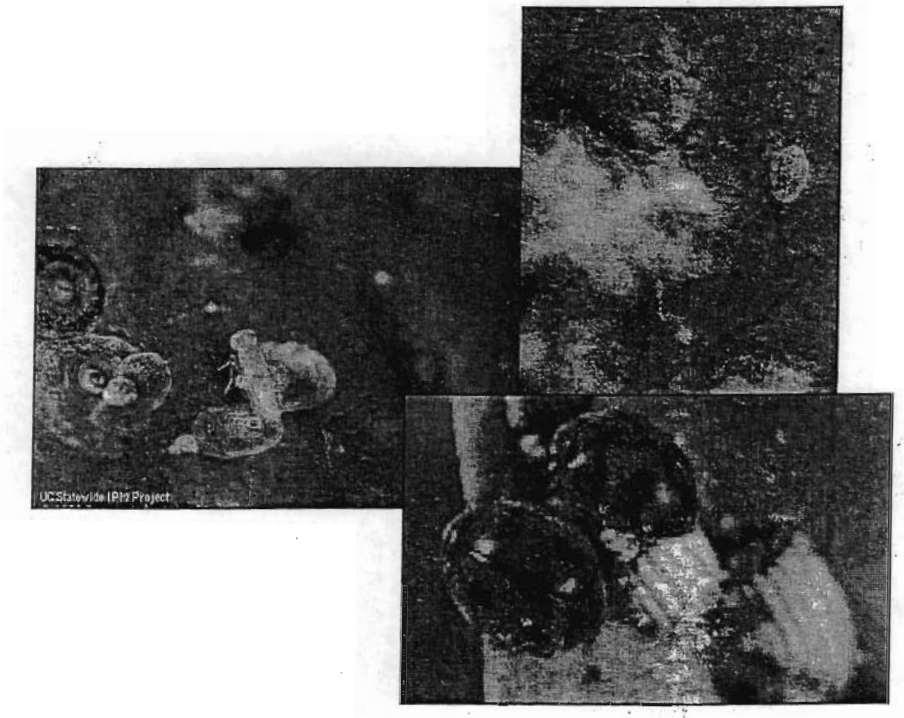
Agenda and List of Participants

Biological Control Training Program (Oct. 2003)



Training Workshop

"Biological Control Opportunities at the Upper Awash Agro-Industry Enterprise"

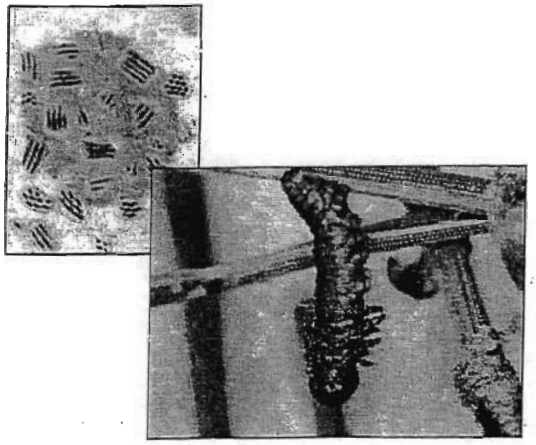


Venue: Mertijeju, Upper Awash Agro-Industry Enterprise

Date: October 6, 2003

Sponsored by:

- Upper Awash Agro-Industry
- USAID
- EARO



29

TRAINING PROGRAM ON BIOLOGICAL CONTROL OPPORTUNITIES IN CITRUS PRODUCTION AT UPPER AWASH AGRO INDUSTRY ENTERPRISE (UAAIE)

6 OCTOBER, 2003 (Monday)

8:30 - 9:00AM Registration

Session I: Plenary Session

9:00 – 9:10AM	Welcoming Address ***Eshetu Bekele (Dr.), HARC, Holetta***
9:10 – 9:20AM	Opening Address ***Haile Mariam Kidanu, Manager UAAIE, Ambo***
9:20 – 9:45AM	Insect pest problems of citrus at UAAIE ***Feredu Azerefegne (Dr.) Debub University*** ***Mohammed Dawd (Dr.), PPRC, Ambo***
9:45 – 10:00AM	Natural enemies of insect pests (predators, parasites, pathogens) ***Michael Brownbridge (Dr.), University of Vermont, USA***
10:00 – 10:20AM	**Coffee break**
10:20 – 10:50AM	Disease problems of citrus and the natural enemies ***Temesgen Belayneh, PPRC, Ambo***
10:50 – 11:20AM	Environmental manipulation through the use of mulches and cover crops: Effects on natural enemy populations and other benefits ***Michael Brownbridge (Dr.), University of Vermont, USA***
11:20 – 11:35AM	Tools used in crop monitoring (sampling, pest identification) and the value of action thresholds ***Mohammed Dawd (Dr.)/ Michel Brownbridge (Dr.)***
11:35 – 11:55AM	Fundamental difference between chemical and biological control strategies ***Mohammed Dawd (Dr.), PPRC, Ambo***

11:55 – 12:15AM	Critical considerations in the formulation and implementation of a biocontrol program ***Louise Labuschagne (Dr.), The Real IPM Company, Kenya***
12:15 – 12:40AM	Proposed Research activities, their purpose and goals, to be done at UAAIE ***Wondirad Mandefro, PPRC, Ambo***

12:40 – 13:40PM **Lunch**

Session II: Practical (Afternoon Session)

13:40 – 15:00PM	Practical demonstration of specimens ***Organizers***
15:00 – 15:20AM	**Coffee break**
15:20 – 17:40PM	Practical demonstration of specimens ***Organizers***
17:40 – 18:00PM	Closing remarks ***Tesfahun Fenta (ESTC)***

Training workshop
"Biological control Opportunities at Upper Awash Agro-industry Enterprise"

October 6, 2003
Mertijegu, Upper Awash

Registration form

No.	Name	Profession	Institution	Signature
1	Andualem Engida	Horticulturist	UAAIE	
2	Fassil T/Belay	Res. Branch	UAAIE	
3	Bezawork Mekonnen	Entomologist	UAAIE	
4	Enyew Tarekegn	Veg. Expert	UAAIE	
5	Abdella Shafi	Crop Protection	UAAIE - TIBILA.	
6	Tesfahun Fenta	Crop Protection	ESTC	
7	Seife Bankuwalu	"	Upper - Aw.	
8	Eshetu Bekele	Pl. Pathology	EARO	
9	Louise Labuschagne	IPM agronomist	the Real IPM Company	
10	Temesgen Belayneh	pathologist	EARO/PPRC	
11	Ferdu Azerefegne (Ph.D.)	Entomologist.	Debub University	
12	Wondirad Mandefro	Nematologist	EARO/PPRC	
13	Berhanemeskel Mehari	Farm Manager	UAAIE	
14	Tesfaye Tessema	" "	"	
[illegible]	[illegible]	Operation [illegible]	UAAIE	

Training workshop
"Biological control Opportunities at Upper Awash Agro-industry Enterprise"

October 6, 2003
Mertijegu, Upper Awash

Registration form

No.	Name	Profession	Institution	Signature
1	Tsebe Gefaneh	Agriculturist	U.A.A.I	
2.	Tebest Gf/Kidanu	Plant protection	U.A.A.I	
3	Yimam Mamo	Agriculturist	U.A.A.I	
4	Michael Brownbridge	Insect pathologist	Univ. of Vermont	
5	Yonas Peresse	plant protection	H.D.F	
6	Kassahun Yeman	Unit manager	H.D.E	
7	MOHAMMED DAWD	Entomologist PPRC, EARO	EARO	
8	Amsalu Bekele	Asr. Dep head	UAALE	
9.	Fitsum-Berhan Kidanu	Agro-Economist-Horticulture D-E	H.D.E	
10	Hailemariam Kidanu	General manager Plant Science	UAA-E	
11	Tesfaye Woldemeskel	Fruit Expert	U.A.A.I	
12	Girma Kelo	Research Agrometeorologist	PPRC/EARO	

Training workshop
"Biological control Opportunities at Upper Awash Agro-industry Enterprise"

October 6, 2003
Mertijegu, Upper Awash

Registration form

No.	Name	Profession	Institution	Signature
1	Fesseha - Ayalew	Farm Manager	UAAIE	
2	Solomon Aimaw	Crop protection Head	H.D.E	
3				

APPENDIX VI

Trip Report:

Biological Control Opportunities at the Upper Awash Agro-Industry Citrus Enterprise

By

Michael Brownbridge

Biological Control Opportunities at the Upper Awash Agro-Industry Citrus Enterprise

Michael Brownbridge, Ph. D.,
University of Vermont, Entomology Research Laboratory
Burlington, VT 05405, USA

Citrus fruits comprise the bulk of the fruit production (>60%) on lands held by the Upper Awash Agro-Industry Enterprise. High-quality fruits are produced for export and domestic consumption, and fruit products (juices, pastes) for both local and export markets. The industry has the potential to increase its access to export markets, thereby becoming a major driving force in the economic and agricultural development of Ethiopia. Export crops generate hard currency, and expanded production further serves the local market, alleviating the need for food imports and conserving foreign exchange. The farms can serve as leaders in innovative agricultural production, with multiple benefits to other farm holdings within the enterprise (vegetables and other food crops), and the farming community in general. Furthermore, the existing infrastructure provides a level of expertise and organization not found in other agricultural sectors of Ethiopia, enhancing the likelihood of successful implementation of new pest management and production technologies, and sustainability. However, citrus production, particularly oranges, mandarins, and grapefruit has been adversely affected in recent years by a combination of insect, disease, weed and soil problems, leading to a net decline (~ 15%/year) in production capacity. Several key pests have been identified as major impediments to improved fruit production:

- Citrus thrips
- Citrus leaf miner
- Red scale
- Mediterranean fruit fly
- False codling moth
- Phytophthera
- *Fusarium*

In addition, the presence of high levels of calcium carbonate in the soil, high salinity levels, and aging fruit stock adversely affect the uptake of nutrients and productivity. Soil problems are probably compounded by the flood irrigation system used.

These pests and diseases can be controlled using conventional pesticides, but such an approach is not sustainable. Nor is it desirable to the long-term health and well-being of farm workers, who are exposed to high levels of toxins in the citrus orchards (many of the products currently used are based on 'old' chemistries). Repeated use of pesticides leads to resistance, degradation of the environment, and has a negative impact on biodiversity. Furthermore, chemical control agents must be imported, putting a drain on foreign exchange reserves. Also, there are now strict guidelines imposed by the European Union (the main export market for Ethiopian fruits) on pesticide residue levels that are permitted on food crops. This situation has brought about major changes in the way export food crops are produced in other East African countries, most notably Kenya. Crop protection has changed from one that was wholly reliant on synthetic pesticides, to one that is now largely based on biological control. Although surveys of

natural enemies have been carried out in Ethiopia, presently none are utilized in crop protection programs. An opportunity exists to target several key pests of citrus with biological control agents, and to develop a coordinated IPM strategy for their regulation. It is impractical to attempt to control all citrus pests with biologicals, but some approaches offer the potential to eliminate several pests concurrently.

Ongoing research in the US has shown that placement of composted organic mulches around the base of avocado trees can suppress avocado thrips survival by >50%. In addition to creating a physical barrier over the soil which impedes successful thrips pupation, the mulches appear to create an environment that is conducive to the incidence and activity of a range of natural enemies, including predatory mites, insect-parasitic nematodes, and entomopathogenic fungi. The role of these natural enemies in thrips suppression is currently under investigation, with the long-term view to their possible development as bio-amendments which are 'seeded' into the mulch prior to laying in the orchards. Use of mulches has many other benefits, including suppression of root rots through the increased incidence of antagonistic microbes such as *Trichoderma* which flourish in the modified environment. Furthermore, orchard health is improved because of increased water conservation, improved soil fertility and weed control. Could this approach be applied in citrus orchards in Ethiopia? There is potential to test this approach, as long as a source of mulch (e.g., trash from sugar cane processing facilities) can be identified. Such approaches could provide means of controlling thrips, root rots and, potentially, other insects pupating in the soil, such as leaf miners. The feasibility of utilizing such a system in conjunction with the current flood irrigation system needs to be assessed.

Several microbial control agents may be actively utilized in citrus. Entomopathogenic fungi have been shown to be effective against thrips and other insect pests in soil. Application of granular or other formulations to the soil around the base of trees could provide a means of augmenting fungal inoculum levels, controlling thrips and leaf miners, both of which fall to the ground to pupate. Antagonistic fungi such as *Trichoderma* may also be incorporated into the soil for control of root rots. The role that insect-parasitic nematodes may play in the regulation of these pests also warrants investigation.

Various arthropod natural enemies have been recovered in surveys conducted in citrus orchards. These include leaf miner parasites, various unidentified hymenopteran parasitoids, and generalist predators such as lacewings, *Orius* spp., ladybugs and staphylinid beetles. Their impact could be enhanced with reductions in pesticide use, and manipulation of the natural habitat. Growing a diverse variety of plant species, and flowering plants in particular, can enhance biological control by providing natural enemies with shelter, nectar, pollen and alternate prey. Natural enemies will multiply in such areas and migrate out over a period of time to provide a steady supply of beneficials that will help control pests in adjacent crops. Cover crops are widely used in orchards and vineyards in the US, providing many benefits and enhancing production of the primary crops. Cover crops could be planted in between the rows of citrus trees in Ethiopia. Careful selection of crops would ensure the presence of flowering plants at key times during citrus development and pest population build-ups, and heat and drought-tolerant varieties would ensure consistent ground cover. Additional benefits may be derived from the use of flowering plants such as African marigolds, clovers and vetch, whose roots produce chemicals that can kill, repel or otherwise suppress population development of plant parasitic nematodes,

which have also tentatively been implicated in the decline of Upper Awash citrus orchards. Cover crops can also suppress weed growth (reducing the need for use of herbicides) and improve soil quality.

Undoubtedly, changes in agronomic practices are also necessary to improve citrus production. This includes renewal of aging production stock; improved spacing of trees to prevent overcrowding and competition for resources; improved irrigation techniques to conserve water and decrease the build-up of salts in the soil; and increased soil fertility. These factors must be investigated to ensure the long-term health and productivity of the citrus enterprise; pest control is only one component of what must, ultimately, be a comprehensive crop improvement program that will require an investment of financial and technological resources.

Formal collaborative links have already been established between researchers at the EARO and the University of Vermont, Entomology Research Laboratory (ERL), and representatives of the Upper Awash Agro. Industry Enterprise. Entomopathogenic fungi and their development for insect pest management form the primary focus of the initiative between EARO and the ERL. A mass-production facility has been established at the PPRC, Ambo. One of the goals of the project is to test and assess the role of fungi in the management of thrips and leaf miners in citrus, and this will also be a primary focus of future collaborative proposals. There is also opportunity to expand this collaboration to include other aspects of pest and citrus management. Institutional support for this initiative to develop a bio-based IPM strategy for citrus will be critical to its long-term success and implementation.

Education is critical to the successful implementation of any biological control program. Thus, we propose to conduct a day-long workshop at facilities near the Merti Jeju farm, alongside the Merti fruit and vegetable processing plant, in early October 2003. The goal of the workshop is to provide technical training in concepts and tools used in biological control and IPM; a greater awareness of the role natural enemies can play in crop protection; and to present specimens of common predators, parasites, parasitoids and pathogens. The workshop will also enable us to engage the stakeholders and pest management professionals in discussions relevant to the implementation of biological control, and to provide an overview of proposed research activities that will be done in the orchards to evaluate biological control strategies. This will serve several purposes: it will serve as a primer in biological control to promote greater awareness, understanding, and acceptance of this pest management approach, and a realistic appreciation of what can be achieved; it will allow us to outline major differences between a biologically-based IPM program and a conventional, pesticide-based crop protection strategy; and lastly, it will allow us to solicit input from participants, and ensure their cooperation in research and implementation phases of biological control strategies. Participants must "buy-into" and be a part of this program if we are to succeed in promoting biological control as a viable alternative to conventional pest management techniques. An additional benefit will be that the workshop will bring together different researchers and educators working in biological control, enabling greater interaction and formulation of a coordinated, collaborative approach to the development of bio-based IPM strategies for citrus production.