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

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Project Objectives:

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.

Major Accomplishments:

1. Research Related Activities Directly Involving the US Collaborators

A. Cooperative Research with Ethiopian Scientist to Test Fungal Mass Production Methods Developed at the University of Vermont

Owing to the continued tensions in Israel, the 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 cooperative research/testing program was organized at the University of Vermont (UVM). The co-operator from Year 1 of the project, Mr. Seneshaw Aysheshim, was at UVM 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. Several fungi were tested in the system to ensure that the basic methods and approaches developed were robust enough to be applied to a broad range of fungal isolates. A step-by-step approach to the process was taken, whereby Mr.

Ayeshim evaluated the process from the preparation of isolates, to the selection and preparation of substrates, through to the evaluation of relative spore yields and virulence. Quality control issues were also 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 was done to not only provide in-country expertise in the production process which will be adapted to different isolates and production conditions, and to ultimately ensure that the product reaching farmers is safe and of high and consistent quality. A summary report of the activities and data generated on this important testing phase of the project are included below.

Refine and Test Mass Production Techniques for Entomopathogenic Fungi

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 to 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 cooperative research:

- To test a simple and cost effective mass production technique for entomopathogenic fungi which can be adopted in Ethiopia

Methodology

Two fungal isolates were selected from 33 *Metarhizium* and *Beauveria* strains collected in Ethiopia. Selections were made on the basis of their virulence towards western flower thrips, *Frankliniella occidentalis*, (Thysanoptera: Thripidae) in assays carried out at the University of Vermont, and conidial production characteristics which were determined in Israel. The spore production of these two 'native' isolates was compared with two 'standard' exotic isolates. Sorghum and millet (Pioneer hybrids) grains were used as the test substrates for the mass production system; sorghum is readily available in Ethiopia and millet is known to be a good growth substrate and thus allows the comparative value of sorghum s a substrate to be determined. Fresh cultures of fungi were prepared from slants in long-term storage and were used to inoculate sterile grains put in glass jars. Spores produced on the grains were then used to inoculate autoclave bags filled with ~ 450 g of sterile sorghum or millet grains. Mass production of clean *Metarhizium* cultures under conditions that were less sterile was also successfully attempted. A brief account of each of the detailed methodologies are summarized as follows:

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 a chrysomelid beetle in the US; and IMI 330189, isolated from a grasshopper in Niger. Cultures held in long-term storage are not used for direct inoculation of the jars. Fresh cultures must first be prepared. To do this, 10 ml of sterile 0.01% tween 80 was added to each culture tube and vortexed for 5-10 minutes. Then, 0.1 ml of each suspension was transferred onto Petri dishes containing quarter strength SDYA media and incubated at 24°C for 15 days. New slant cultures were prepared from the plates to provide the initial inoculum for the grains.

To produce enough clean spores for use as inoculum, conidia from four 15 day old slant cultures were harvested by adding 10 ml of sterile 0.01% Tween 80 into each test tube. The tubes were vortexed and the resulting spore suspensions pooled in a sterile Erlenmeyer flask. This suspension was used to inoculate the grains in glass jars.

Selection of substrate

Solid substrates were chosen for the mass production system rather than liquid media as they tend to be less expensive, require less sophisticated equipment for production of conidia, provide spores which are more stable, and are less likely to become contaminated.

For optimal sporulation to occur, a good surface area to volume ratio is essential so that cultures receive maximum aeration. 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-used substrate for production of fungal conidia, sorghum was chosen as the primary substrate for these studies considering its availability, cost and physical characteristics, such as grain size and hydration properties. However, since cereal grains vary considerably in nutrient status, even among varieties of the same species, it may be necessary to further select a specific sorghum variety yielding more consistent results of spore quantity.

Selection of moisture levels of grains

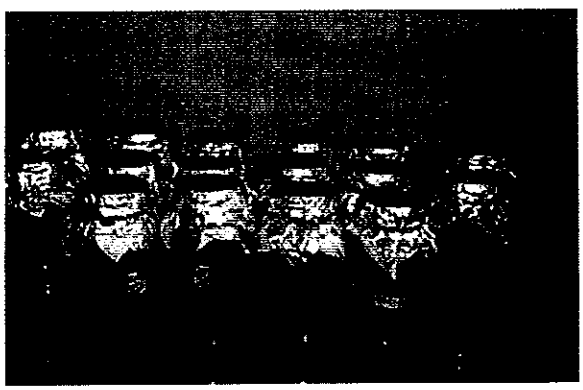
The optimum moisture content for fungal growth and conidiation needs to be identified for each substrate/isolate combination. Moisture content plays a significant role in the final yield of conidia.

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 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 to a liter of water.

Inoculation of jars

One third of the volume of jars was filled with dry grains of sorghum or millet. This facilitated easy mixing and optimized the surface area of substrate for spore production. Hot water was added to the jars with the ratio indicated above and maintained in a water bath at 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 allowed to cool overnight, and were then inoculated with 2 ml of $\geq 10^7$ conidia per ml of fungal suspension. Grains were inoculated using a sterile syringe, allowing a known volume of inoculum to be discharged into each jar, and reducing the chance of contamination. It was



1. Volume of grains in jars should initially be no more than one-third of the volume of the jar; this facilitates easier mixing and greater aeration of the substrate while reducing the build-up of condensation.

2. Inoculated jars in a horizontal position to maximize aeration. Note: aluminum foil covers removed after 5-7 d to increase aeration and reduce humidity inside the jars.

possible to directly insert the needle of the syringe through the lid of the jar (layers of paper towel and cheesecloth) and inoculate the grains. Inoculated jars were incubated at an ambient temperature of 22 - 25°C for 10 - 15 days, gently mixing the substrate every 3 – 5 days by rotating the jars. In order to avoid excess humidity and condensation building up inside the jars and on the substrate, the aluminum covers were removed from the lids of the jars after 5-7 days (after the grains had been colonized by the fungi) to allow greater aeration of the medium. Large quantities of spores were obtained 15 days after inoculation, and were used to inoculate bags for mass production and in tests of viability and virulence.

Summary Notes:

- Jars should not be inoculated directly from slants stored under mineral oil
- Recommended amount of grain: maximum 30% by volume of the jar
- Prepare enough sterile water and sterile 1 ml pipette tips in advance
- Use aseptic techniques throughout to prevent introduction of contaminants onto substrate

Bioassay on WFT using spores obtained from grains

In order to compare the virulence of spores produced on sorghum grains in autoclave bags with that of spores produced on standard mycological medium (SDYA), bioassays were carried out using western flower thrips as a test insect. For each isolate, a standard suspension containing 5 x 10^6 spores/ml was prepared in 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 a Munger cell. Twenty unsexed second instar thrips were added to each Munger cell, replicating each experiment four times. Insects were supplied with a bean leaf disc (2 cm diameter) as food and kept at a constant 22 °C, 18:6 L:D. Mortality was recorded seven days after inoculation. The viability of spores grown on each of the media was determined one day prior to bioassay.

Results:

Preliminary biotest results showed that spores obtained from grains were as virulent as those produced on 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; whereas 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 previous results, mortality was lower for all isolates in both cases. Although assay conditions and dose levels were the same throughout the experiment, the age of the test insects was variable, possibly influencing insect mortality.

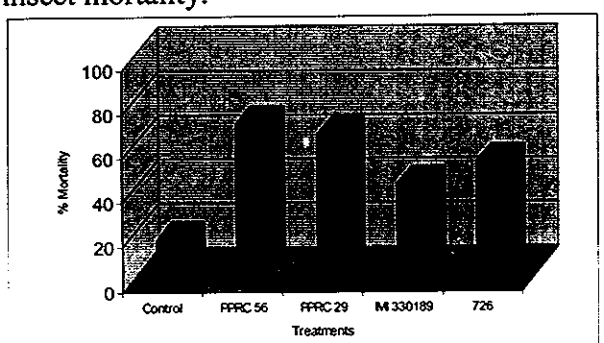


Fig. 1. Mortality of WFT treated with spores from the bags

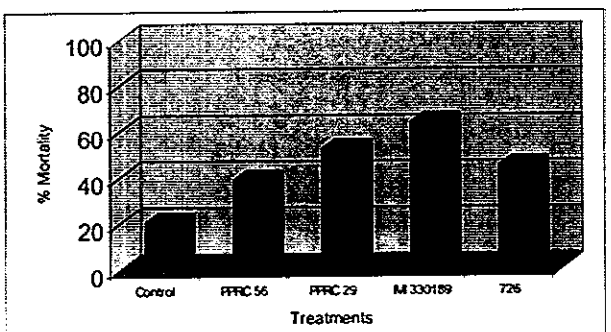


Fig. 2. Mortality of WFT treated with spores from the slants

Determination of spore production and spore viability

The quantity of spores produced inside jars using sorghum and millet as substrates was determined by sampling from 12 day old cultures of the four isolates: IMI 330189, PPRC 56, PPRC 29 and 726. A 10 ml sample of each grain culture was taken and transferred into sterile Erlenmeyer flasks containing 100 ml of sterile 0.01% Tween 80. Spores were dislodged from the grains by gently shaking the flasks for 30 minutes using an orbital shaker. Ten ml of the resulting suspension was then removed and filtered through sterile cheesecloth. Haemocytometer counts were made on the spore suspension to provide an estimate of the total conidial yield per g of substrate for each isolate. Serial dilutions of the suspensions were plated on quarter-strength SDYA to provide an estimate of conidial viability. Viability was determined by counting the percentage germination of conidia after 24 hours of incubation at 22°C.

Results

Spore yield was higher on millet 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 overall on both sorghum and millet, the latter 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 yielding 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 treat a hectare of land.

Table 1. Spore production on sorghum and millet substrates for 4 different isolates

Isolates	No. of spores produced on 10 ml grain substrate		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 after autoclaving. Mixing of millet was easier than sorghum, and the presence of its husk did not affect growth of fungi. On the other hand, microscopic examination showed that both *Beauveria* and *Metarhizium* strains primarily colonized and conidiated on grains without the husk, suggesting that cracked sorghum is more suitable as a substrate than whole grain. The study showed that, from the perspective of spore production, millet is a better choice for mass production. However, considering its comparable cost and availability in Ethiopia, sorghum appears to be better choice for use as a growth substrate and is recommended for use in mass production.

Spore viability. All isolates, except PPRC 29, had a high germination rate within 24 hours at 22°C (Table 2). As only one reading was taken after 24 h, the speed at which germination occurred could obviously bias the results obtained. A major factor influencing the speed of germination is temperature, and its effects vary from isolate to isolate. Therefore, it is important to maintain an optimum temperature for each strain when incubating cultures. For example, *Metarhzium* strain PPRC 29 was originally isolated in a warm area about 230 km North East of Addis Ababa. The relatively low viability of the conidia may have been due to the relatively low (22 °C) room temperature at which the cultures were incubated, which would have an obvious effect on germination speed (Table 2). Incubating the plates at a temperature closer to its optimum for growth and sporulation (ca. 30°C) would probably have produced a different result.

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

Percentage viability of spores after 24h			
	Slants	**Jars**	**Bags**
PPRC 56	100	100	99
PPRC 29	45	16.3	58.5
IMI 330189	99	99	88
726	100	99	100

Spore concentration per gram of spore powder was determined by first removing the conidia from the grains by sieving. The dry grains were carefully poured into a fine polyester monofilament mesh (~300 μm) bag held inside a larger 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 to prevent spore drift out of the bag. The spore powder was collected from the bottom of the bag and packaged in small Ziploc bags. 0.1 g of the spore powder was weighed and suspended in 10 ml of sterile 0.01% Tween 80. The resulting spore suspension was diluted 100 times and the tubes placed inside a sonicator to disrupt clumped spores. Haemocytometer counts on the spore suspension provided an estimate of the conidial concentration and total conidial yield/g of substrate. Samples of substrate remaining after sieving were either ground, or simply suspended in 0.01% Tween, to provide an estimate of the residual number of conidia remaining attached to the grains. The resulting powder or suspension was treated as described above to obtain an estimate of conidial concentration by haemocytometer count.

Results

The conidial powder collected by sieving was essentially free from any substrate and contained the highest number of spores per gram (Table 3). The remaining sorghum grains, once ground, provided additional fine powders of conidia plus substrate. The number of conidia per gram, however, was lower than that for the sieved powders. Large amounts of conidia were also recovered from whole grain, indicating that all materials used in the mass production process could be utilized as a source of inoculum. It is important to maintain harvested conidial powders at a moisture content of <5% to maintain conidial viability.

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

Isolates	Number of spores produced per 1 gram		
	Clean powder	Powder obtained after grinding	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 prevent spore dust from contaminating the room, it was essential to wear a special mask equipped with fine filters. Care must be taken while sieving, as inhaling too many spores of any fungi may cause an allergic reaction in some people.

Mass production using autoclaveable and polythene bags

The development and optimization of a simple production procedure is invaluable for the supply of a consistent, viable product. In this 'scale-up' phase of the mass production system, both autoclaveable and clean polyethylene bags were used to demonstrate the feasibility of mass producing fungi under conditions of optimal sterility, or under conditions where equipment or materials may be lacking.

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 previously. 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. A high density of spores was used to inoculate the bags to ensure rapid colonization of the substrate by the entomopathogen, further reducing the chance of contaminants growing on the medium. The mouths of the bags were covered with layers of sterile paper towel, fine cheese cloth and aluminum foil. Some of the procedures used to prepare and inoculate the autoclavable bags are presented in the pictures below.

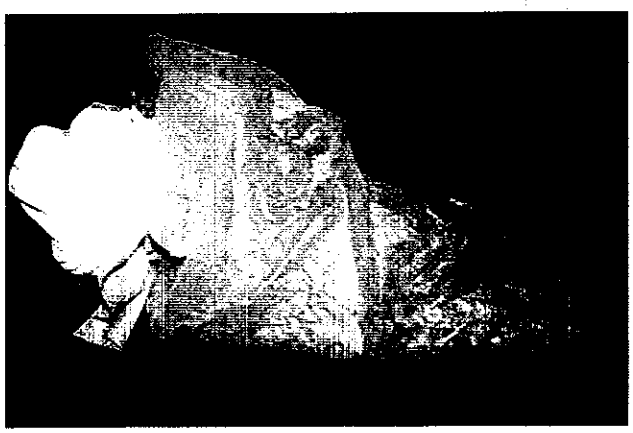
The polythene bags, used in the study were purchased specifically for the work, and had not been previously used. Grains were prepared as described above, but were cooked in the water bath inside a plastic, heat-proof container (100°C, 1 hour). Inside a laminar flow hood, the hot grains were dispensed to the plastic bags, which were closed in the same way as the autoclavable bags. Grains were allowed to cool prior to inoculation with 10 ml of $\geq 10^{8}$ conidia/ml, a higher concentration than that used in the autoclavable bags to further reduce the risk of contaminants growing on the grain.

Cultures were incubated on open shelves inside a mass-production room at 22 ± 2°C under natural light for 3 weeks. Aluminum covers were removed from the bags after 5-7 days to minimize the build-up of condensation inside the bags. Grains were mixed every 3-5 days to ensure

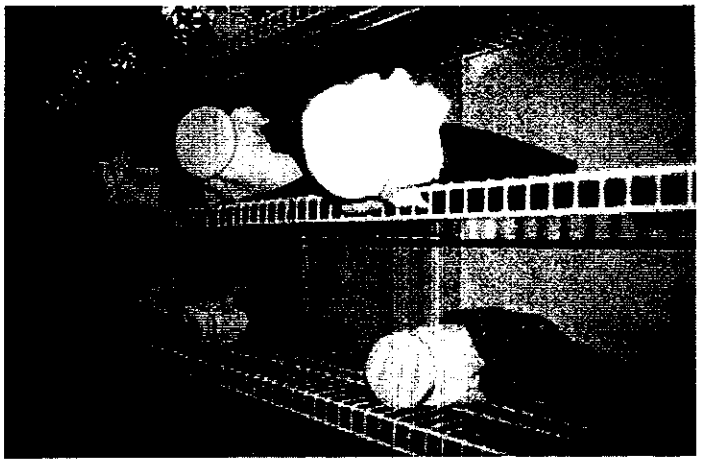
even distribution and growth of the fungi on the substrate and to maximize spore production on the surface of the grains. Bags were also inspected daily for 5 days for the presence of fungal or bacterial contaminants. After 12 days, the bags were opened to dry the conidia and the substrate; spore viability rapidly declines if held under moist conditions.

1. Bags filled with sorghum grains prior to cooking in a water bath for ~ 1 h.

2. Cooked grains 'flattened' to facilitate autoclaving of several bags at the same time.



3. Aeration of inoculated grains enhanced by closing the mouth of the bag around a plastic or paper 'tube' (ca. 10 cm diam.), which was covered with a paper towel and fine cheese cloth. This was covered with aluminum foil for the first 5-7 d which was then removed to prevent build-up of condensation inside the bag.



4. Placement of the bags on shelves inside a mass production room. The room should be isolated and equipped with a bactericidal lamp to reduce the risk of contamination of cultures. Bags can be inoculated inside the room following simple aseptic procedures. For large scale production the job is better handled with two individuals, one holding and opening the autoclave bags while the other inoculates grains with a fungal suspension.

Summary notes:

- Never inoculate bags from contaminated cultures!
- Inoculate autoclave/polythene bags not later than one day after autoclaving or cooking of grains.
- Make sure that the grains are not over cooked – this renders the substrate useless for production.
- Check for any contamination in the first five days. Discard if any observed!
- Make sure there is no condensation in the autoclave bags after sporulation is complete – open bags to dry.

A common contaminant encountered in the culture bags was *Scopulariopsis* sp., which has globose with truncate base spores. Familiarity with common fungal contaminants is essential to ensure the quality and purity of the fungi produced for pest management.

Conidia can be separated from the substrate easily by sieving. Residual conidia may be removed from the remaining substrate, or the grains may be, for example, incorporated into the soil for treatment of soil pests. Additional drying of the conidia may be required for longer term storage/preservation. This can be achieved over silica gel or other anhydrous materials. Spore powders will remain viable for several months if held under cool, dry conditions.

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 facilities to prevent cross-contamination. Ideally, the production room should be located in a separate building A big ventilator could be placed inside and an opening hole made on one of the walls to allow fresh circulation of the room. *Metarhizium* and *Beauveria* sp. appeared to be less affected with the light intensity of the room.

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Major Outputs of this Cooperative Research and Testing of the Mass Production System

- Methods of maintaining cultures for long-term storage were reviewed.
- Protocols for preparation of fungal cultures for inoculation of substrates were tested.
- Aseptic procedures needed to ensure production of a contaminant-free fungal product were detailed.
- A fungal mass production system based on the use of grains as an inexpensive growth substrate was tested and evaluated.
- Spore yield, viability and virulence on different grain substrates were determined.
- Key factors influencing yield were identified; these include: the type of grain substrate, the method of substrate hydration used, the specific fungal isolate; such parameters will need to be determined for each fungal isolate that will be produced using the basic protocols tested.
- Methods of spore preservation were demonstrated.
- Quality control issues were addressed, and means of preventing contamination determined.
- The suitability of the basic system developed for mass production of entomopathogenic fungi was demostrated.

B. Special Training Course in Insect Pathology, with Particular Reference to Fungal Entomopathogens, Their Identification, Characterization, Virulence, and Mass-Production for IPM.

Unfortunately, the primary scientific collaborator, Mr. Aysheshim, left EARO in June 2002. This necessitated the identification of individuals to take over the work responsibilities for the remainder of the project. This was essential to ensure continued progress and to promote implementation of the new technologies developed in Ethiopia. Working with Dr. Bekele (EARO, Holetta), two individuals were duly assigned to the project and to the insect pathology laboratory established at the EARO facility in Ambo. The scientists are: Dr. Mohammed Dawd and Mr. Wondirad Mandefro.

While both scientists are trained entomologists, neither has experience in insect pathology. To ensure progress towards achieving the objectives of the project, and to develop in-country expertise in insect mycology, Dr. Dawd and Mr. Mandefro had to receive formal training in this subject area. An intensive training course was thus organized at the Entomology Research Laboratory, which Dr. Dawd and Mr. Mandefro attended from January 26 through March 2, 2003. The course 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
   - Bait method
   - Soil dilution method
   - Direct inoculation method
   - Print method
   - Electrostatic method

4. Bioassay of insects
   - Bioassay against western flower thrips
   - Bioassay against 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
   - Mass production in plastic bags
   - Mass production in liquid substrates
   - Quality control issues

7. Long-term storage of fungi
   - Preparation of slant cultures and preservation under oil
   - Refrigerated storage
   - Storage in an ultra-low temperature freezer

The goal of the training program was to introduce the 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. This course was presented in a practical

manner with an appropriate mix of direct one-on-one instruction and hands-on activities for Messrs. Dawd and Mandefro in the laboratory and our fungal mass-production facility.

Following is a list of the Principal Instructors at the Entomology Research Laboratory, Univ. of Vermont, that 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

Planning of future research/training activities in Ethiopia

During the visit of Dr. Dawd and Mr. Mandefro, extensive discussions were held on future phases of this project, specifically the form and content of the training workshop that will take place in Ethiopia in 2003, and target insect pests for this new technology which will be the subject of future collaborative efforts. Two researchers from the Univ. of Vermont will participate in the workshop in 2003. It is anticipated that the researchers will remain in Ethiopia for approximately 3 weeks to provide additional one-on-one training and instruction in the insect pathology lab located in Ambo. This will consolidate the training received at UVM earlier this year, and stimulate progress and activities of the EARO personnel on this project. It will also facilitate visits to field sites to discuss IPM, biological control, the role of natural enemies in pest suppression, the importance of their conservation, and alternative pest management strategies with farmers and extension personnel.

In the planned workshop, biological control and IPM form the core subjects that will be covered by presenters. The outline was developed in cooperation with Messrs. Dawd and Mandefro, to address needs and knowledge gaps identified by them that exist within Ethiopian agricultural research organizations, non-governmental organizations, and extension programs:

Biological Control Workshop, Ethiopia 2003

What?

- Workshop on biological control as a component of IPM

Why?

- To create an awareness on the potential use of biological control in IPM in Ethiopia
- To provide a review of current biocontrol research in Ethiopia
- To facilitate an exchange of experience and information on the use of biological control agents
- To identify gaps and needs in biological control research and outreach activities in Ethiopia

How?

Financing (projected in-country expenses; not including costs of participation of UVM collaborators)

- From USAID: $1,500-$2,000
- Matching from local sources, e.g., EARO, Ethiopian Entomological Society, Chemical Companies

Resource persons

- University of Vermont 2
- EARO PPRC 2
- EARO Holetta 1
- EARO Melkassa 1
- Addis University 3
- Regional Extension Specialists 3
- Chemical companies 2
- NGO's 2

Number of papers to be presented: 7-10

Organization of the workshop

The Organizing Committee will be comprised of five members – four from Ethiopia, with representatives from EARO and the University of Addis Ababa (Dawd, Mandefro + 2), and one from the University of Vermont (Brownbridge). They will be responsible for handling all matters pertaining to the organization, fund-raising, and other planning associated with the workshop, and in the solicitation and editing of contributed abstracts and papers for inclusion in a Proceedings. Prior to the workshop, attendees (limited to 50) will be presented with a Program and Abstracts. The Proceedings will be made available after the workshop so that it can include a summary of discussions following each presentation, together with a listing of recommendations for future research directions and needs. Papers will be solicited from key persons to cover topics such as: opportunities and needs for biological control in Ethiopia; integrated pest management and the role of biological control in IPM; reviews of key pests where biological control has a high likelihood of success, e.g., sorghum chafer, *Pachnoda* sp., citrus thrips, African armyworm, *Spodoptera exempta*, locusts; methods of biological control, including conservation, augmentation and innundative releases; organisms used in biological control. We envision a 1-2 day workshop comprising oral presentations and a 'hands-on' session to familiarize participants with natural enemies used in biological control, and those which play a role in the natural suppression of insect pests.

Duration, When and Where?

- The workshop will be for 1½ days
- It will be held in September/October 2003
- The workshop will be held at the PPRC, Ambo

Who Will be Invited to Attend?

- Researchers from the US and EARO, Ethiopia
- University researchers (Ethiopia)
- Private enterprises (agricultural producers and chemical companies)
- Ministry of Agriculture extension specialists and agronomists
- Representatives of various NGO's

In addition to this formal workshop, two additional 'on-site', day-long informal workshops will be presented to extension personnel and researchers. These will consist of site visits to citrus production areas, to review damage by citrus thrips, discuss pest identification, methods of suppression, cultural controls, methods of enhancing the incidence and impact of natural enemies (conservation techniques), recognizing natural enemies, and discussing opportunities to rear and release different beneficial organisms for citrus pest management. IPM techniques and tenets will be presented to attendees. This system is currently heavily and solely reliant on failing pesticides for thrips control. In many instances, pesticides are failing and citrus production is seriously threatened. Citrus, and products from the citrus industry, are important export crops. The second workshop will be held in sorghum producing area to review damage and control options for the sorghum chafer. As a staple food crop, sorghum is extremely valuable to food security in Ethiopia. These workshops will specifically target agronomists, pest managers and extension personnel at these sites.

Budget Report:

USA

Total Budget	**Budgeted**		**Expended**		**USAID**
Expense Item	USAID	Matching	USAID	Matching	Remaining
Salaries	27,897	37,459	12,251.58	24,480	15,645.42
Fringe Benefits	9,680	13,072	4,410.33	8,348	5,269.67
Supplies	3,174	0	145.04	0	3,028.96
Travel	23,009	0	0	0	23,009.00
Consultant	1,000	0	0	0	1,000.00
Equipment	0	0	0	0	0
Total Direct Costs	64,760	50,531	20,186	32,828	47,953.05
Indirect Costs	0	23,981	0	16,447	0
Total	64,760	74,512	20,186	49,275	47,953.05

Ethiopia

Total Budget Period	**Budgeted**		**Expended**		**USAID**
Expense Item	USAID	Matching	USAID	Matching	Remaining
Salaries		21,000.00		14,000.00	
Fringe Benefits					
Equipment	46,642		38,235.44		8,406.56
Supplies	11,800		2,104.29		9,695.71
Travel [a,b,c]	24,740		24,740.00		0.00
Other Direct [a]	12,000		12,000.00		0.00
Total Direct Costs	95,182	21,000.00	77,079.73	14,000	18,102.27
Indirect Costs					
Total	95,182	21,000.00	77,079.73	14,000	18,102.27

[a] A payment of $10,871.39 was received by the UVM ERL to cover costs associated, with the training of Mr. Ayesheshim from January 16-March 15, 2002. The breakdown of expenses is as follows:

Bench fees:	$ 6,000.00
Airfare	$ 2,153.29
Housing:	$ 1,000.00
Food (Meal Ticket):	$ 932.83
Other misc. expenses:	$ 785.27
Total:	$10,871.39

[b] An invoice for $15,566.90 was submitted on April 1, 2003 by the UVM ERL to cover costs associated, with the training of Dr. Dawd and Mr. Wondirad for the period of January 26-March 2, 2003. As of the submission of this report, these funds have not yet been reimbursed to UVM. The breakdown of expenses is as follows:

Bench fees:	$ 7,500.00
Airfare	$ 3,548.54
Housing:	$ 1,300.00
Meals:	$ 1,337.91
Other misc. expenses:	$ 1,880.45
Total:	$15,566.90

[c] In the above report, because insufficient funds were available to cover the full costs of the 2003 training from the travel category, $2,104.29 of these costs were subtracted from the supply category.

2. *Research Related Activities in Israel (2001-2003).*

Prepared by Dr. Galina Gindin (The Volcani Center) and
Professor Isaac Barash (Tel Aviv University)

Objectives:

1. Morphological variability of promising strains and selection of clones with higher sporulation.
2. Evaluation of stability and productivity of promising clones and parental isolates after passages on medium.
3. Evaluation of stability and productivity of promising clones and parental isolates after long-term storage on different medium.
4. Evaluation of virulence of promising clones and parental isolates toward several pests: western flower thrips, *Frankliniella occidentalis*; onion thrips, *Thrips tabaci* and silverleaf whitefly, *Bemisia argentifolii*.

Materials and methods.

Fungi.

Two strains of *M. anisopliae* (M.a.EE and M.a.PPRC-29) and two strains of *B. bassiana* (B.b.FF and B.b. PPRC-56) isolated from an insect in Ethiopia were examined for morphological characters and sporulation. All isolates were maintained on SDYA at 4°C.

Cloning.

Four media, SDA (Difco), SDYA, PDA (potato dextrose agar, Difco), Czapek Dox agar (Sigma), were used for cloning of each isolate. To obtain conidia for cloning, the parental strains were cultivated on SDYA in 7 d at 25°C and young spores were harvested using sterile solution of 0.01% Triton X-100. The spore suspension was vortexed with glass beads in 10 sec. For the *M. anisopliae* strains, 0.05-0.1 ml diethyl ether per 5 ml suspension was added to remove fat and restore the spore chain. After mixing, the suspension with ether was held for 2 hr in tubs with a plastic cork for ether evaporation and then were revortexed. The spore concentration of the suspension was determined by haemocytometry and adjusted by diluting concentrated spores with sterile solution of 0.01% Triton X-100 to a final concentration of 100-200 spores/ml. To obtain clones (i.e., progeny derived from a single cell), small Petri dishes were inoculated 0.1 ml of the final suspension (10-20 spores per dish) and cultivated at 25°C in 7 d. The clones were examined for morphological type of colonies and sporulation. Over 100 clones were obtained and analyzed for each strain on each medium.

Analysis of clones for morphology and sporulation.

Clones obtained on different medium were analyzed for morphological characters (size of colony, density and abundance of mycelium, pigments) and sporulation. For examination of spore productivity of clones, 6-mm blocks from colony centre of clones were cut out after 7 d and transferred in tubs with 2 ml of a solution of 0.01% Triton X-100. To obtain the spore suspension, the block was vortexed with glass beads in 10 sec., and the concentration of spore in suspension was determined by haemocytometry.

Sporulation of selected clones was also investigated by standard disk method.

Passages of promising clones and parental isolates.

Promising clones and parental isolates were subcultured weekly by 15 repeated passages on SDYA. Before passages and after 5, 10 and 15 passages, the parental isolates and clones were examined for morphological type of colonies and sporulation. Sporulation was investigated by standard disk method on SDYA.

Storage of promising clones and parental isolates.

Promising clones and parental isolates were maintained on two media: SDA and SDYA for 1, 3, 6, 9 and 12 mo at 4°C. To investigate the influence of storage duration on productivity, the fungi were subcultured on SDYA and sporulation was investigated by standard disk method.

Bioassay of promising clones and parental isolates on thrips.

The fungi were grown on SDYA in 10 d. Suspensions of each isolate were prepared in 0.01% Triton X-100, adjusting the concentration to $5x10^6$ conidia/ml. The comparative virulence of promising clones and parental isolates to western flower thrips and onion thrips was estimated by standard Munger cell methods at 25°C (4 repetitions with 20 thrips per isolate).

Bioassay of promising clones and parental isolates on *B. argentifolii.*

Fungi were grown on SDYA for 10 d. The spore preparation used for bioassay was adjusted by diluting concentrated spores with 0.01% Triton X-100 to a final concentration of 10^7 spores/ml. Third instar *B. argentifolii* nymphs were used for the bioassay procedures. Individual cotton leaves with approximately uniform distributed insects (5 to 10 insects/cm^2) were selected prior to fungal inoculation. Leaf sectors with about 50 to 100 insects were used. The leaf pieces with nymphs were immersed in a spore suspension for 10 sec, whereas control leaves were immersed in 0.01% Triton X100 for the same length of time. To prevent development of saprophytic fungi, treated leaves were placed for 20-30 min on filter paper to remove excessive moisture. The leaves were then placed in Petri dishes and incubated in a growth chamber at temperatures alternating between 25°C (14 h under light) and 20°C (10 h under dark). A relative humidity (RH) close to 100% was reached by applying a moist filter paper into each of the Petri dishes. To allow aeration, all Petri dishes were opened daily for 25-30 min. The number of insects per leaf sector was counted prior to inoculation. The percentage of dead insects following inoculation and infection was recorded daily. All clones and parental strains were tested using five to six replications.

Results

I. Evaluation of morphological variability of promising strains and selection of clones with higher sporulation

In order to achieve this goal we have pursued the following specific objectives:

1. Cloning of four promising strains, which were selected from natural parental isolates, on different medium.

2. Analysis of morphology variability on each medium and choice medium suitable for further cloning.
3. Evaluation of sporulation for clones with different morphology types.
4. Choice and cultivation of promising clones of the 1st generation and estimation of growth and sporulation by standard disk methods.
5. Second cloning of promising clones and analysis of morphology variability and sporulation of clones of 2nd generation.
6. Repetition of item 3 for clones of the 2nd generation.

Strain *Metarhizium anisopliae* EE.

Effects of different media on the variability in morphology and sporulation of clones.

SDA medium.

After cloning of parental strain on SDA, 105 clones with two morphology types colonies were obtained (Table 1).

Table 1. Clone analysis of parental *M. anisopliae* EE strain on SDA medium for morphological types.

Colony type	Colony diameter, mm (7 d)	Number of clones	% clones
Type I	5-10	58	55.2
Type II, a	3-5	18	17.1
b	6-8	24	22.9
c	9-11	5	4.8
			100.0

1. Clones of type I (55.2%) had abundant cottony mycelium and very weak sporulation (on day 7 <10% colony surface had a green sporulating layer); colony diameter was 5-10 mm.
2. Clones of type II (44.8%) had flat compact colonies with weak aerial mycelium and abundant sporulation (on day 7 >50% colony surface had a green sporulating layer); colony diameter was 3-11 mm.
3. Evaluation of sporulation of colonies with different morphological types revealed that clones of type I had very low sporulation ($<10^5$ spore/cm^2). Sporulation of clones of type II varied from $(20.3 + 6.4) \times 10^6$ to $(64.5 + 16.4) \times 10^6$ sporés/cm^2, depending on colony size.

Table 2. Sporulation of *M. anisopliae* EE clones (spore number x $10^6/cm^2$ on day 7), obtained from parental strain on SDA medium.

No.	Type I clones with diameter 5-10 mm	Type II clones with diameter		
		3-5 mm	6-8 mm	9-11 mm
1	< 0.1	18.6	59.6	43.2
2	< 0.1	17.1	40.0	85.0
3	< 0.1	19.3	37.9	53.6
4	< 0.1	10.7	37.1	67.9
5	< 0.1	15.0	47.1	72.9
6	< 0.1	31.1	44.6	-
7	< 0.1	26.8	37.1	-
8	< 0.1	15.0	46.1	-
9	< 0.1	22.5	40.7	-
10	< 0.1	27.1	34.6	-
Average	< 0.1	20.3	42.5	64.5
SD		6.4	7.3	16.4

SDYA medium

After cloning of the parental strain on SDYA, 114 clones with two morphology types colonies were obtained (Table 3).

1. Clones of type I (20.2%) had abundant cottony mycelium and very weak sporulation (on day 7 <10% colony surface had a green sporulating layer); colony diameter was 4-6 mm.
2. Clones of type II (79.8%) had flat compact colonies with weak aerial mycelium and abundant sporulation (on day 7 more 50% colony surface had a green sporulating layer); Colony diameter was 2-8 mm.

Table 3. Clone analysis of parental *M. anisopliae* EE strain on SDYA for morphological types.

Colony type		Colony diameter, mm (at 7 d)	Number of clones	% clones
Type I		4-6	23	20.2
Type II,	a	2-3	26	22.8
	b	4-5	57	50.0
	c	6-8	8	7.0
				100.0

Evaluation of sporulation of colonies with different morphological types revealed that clones of type I had a very low sporulation (<10^5 spores/cm^2). Sporulation of clones of type II varied from (37.2 +8.0) x 10^6 to (115.2 + 18.4) x10^6 spores/cm^2, depending on colony size (Table 4).

Table 4. Sporulation of *M. anisopliae* EE clones (spore number x 10^6 per cm^2 on day 7) from the parental strain on SDYA.

Clone No.	Type I clones with diameter	Type II clones with diameter		
	5-10 mm	2-3 mm	4-5 mm	6-8 mm
1	< 0.1	24.3	71.4	135.4
2	< 0.1	42.9	75.4	108.2
3	< 0.1	34.6	92.9	133.9
4	< 0.1	28.6	77.1	119.6
5	< 0.1	42.9	96.1	136.1
6	< 0.1	26.4	72.5	92.1
7	< 0.1	41.1	39.3	99.3
8	< 0.1	46.4	70.7	97.1
9	< 0.1	43.2	58.2	-
10	< 0.1	41.4	74.3	-
Average	< 0.1	37.2	72.8	115.2
SD		8.0	16.0	18.4

PDA medium.

After cloning of parental strain on PDA, 107 clones with two morphology types colonies were obtained (Table 5).

Clones of type I (18.7%) had abundant cottony mycelium and very weak sporulation (on day 7 <10% colony surface had a green sporulating layer), colony diameter was 4-8 mm.

Clones of type II (81.3%) had flat compact colonies with weak aerial mycelium and abundant sporulation (on day 7 >50% colony surface had a green sporulating layer). Colony diameter was 2-10 mm.

Evaluation of sporulation of the colonies with different morphological types revealed that clones of type I had a very low sporulation (<10^5 spores/cm^2). Sporulation of clones of type II varied from $16.5 \pm 3.5 \times 10^6$ to $57.9 \pm 9.5 \times 10^6$ spore/cm^2 depending on colony size (Table 6).

Table 5. Clone analysis of parental *M. anisopliae* EE strain on PDA for the two colony morphological types.

Colony type		Colony diameter, mm (7 d)	Number of clones	% clones
Type I		4-8	20	18.7
Type II,	a	2-4	34	31.8
	b	5-7	47	43.9
	c	8-10	6	5.6
				100.0

Table 6. Sporulation of *M. anisopliae* EE clones (spores x $10^6/cm^2$ on day 7) from parental strain on PDA.

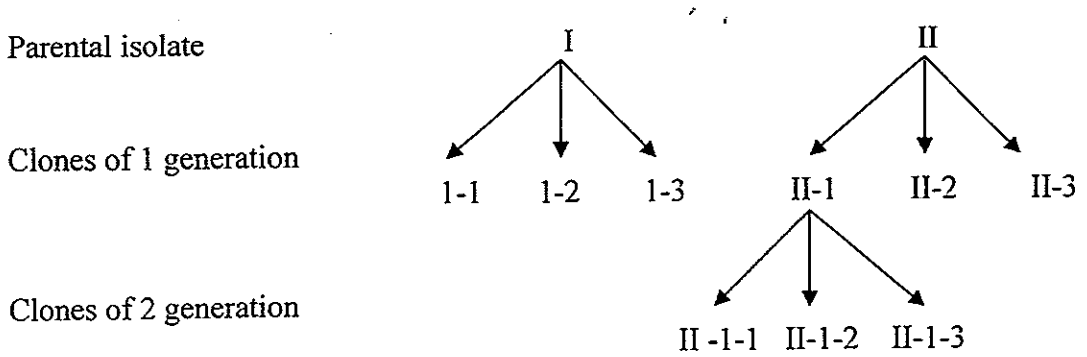
No.	Type I clones, diameter 5-10 mm	Type II clones with diameter		
		2-4 mm	5-7 mm	8-10 mm
1	< 0.1	19.3	55.0	58.9
2	< 0.1	13.9	49.3	61.1
3	< 0.1	18.9	45.4	58.2
4	< 0.1	11.1	45.4	47.1
5	< 0.1	22.5	42.5	73.2
6	< 0.1	15.4	32.1	48.6
7	< 0.1	19.6	46.8	-
8	< 0.1	16.4	37.9	-
9	< 0.1	15.4	46.1	-
10	< 0.1	12.9	38.6	-
Average	< 0.1	16.5	43.9	57.9
SD		3.5	6.5	9.5

Czapek Dox agar

After cloning of the parental strain, 110 clones were obtained. Morphological variation was absent; growth and sporulation were very weakly. Sporulation lever was (0.1-0.2) x10^6 spores/cm^2. The above mentioned results allowed us to conclude, that parental strain of *M. anisopliae* EE contain clones of two types, which significantly differ in sporulation (>50-300 times). Three tested media (SDA, SDYA and PDA) were suitable for cloning, promoting the dissociation of parental isolate into two morphology types with different sporulation level. SDYA and PDA media showed the higher potential to promote the development of clones with high sporulation. The clones with the highest sporulation were obtained on SDYA and this medium was selected for further experiments.

I. Evaluation of variability of clones in morphology and sporulation during firth and second generations.

Analysis of morphology variability and sporulation of clones was carried out by scheme 1.



Repeated cloning of the parental strain on SDYA confirmed the dissociation of the natural isolate on clones of two types (Table 7). From 123 obtained clones, 37 clones had abundant cottony mycelium and very weak sporulation.

Table 7. Repeated clone analysis of parental *M. anisopliae* EE strain on SDYA for morphological types.

Colony type	Colony diameter mm (7 days)	Number of clones	% clones
Type I	4-6	37	30.0
Type II, a	2-3	45	36.6
b	4-5	29	23.6
c	6-8	12	9.8
			100.0

Clones with fast growth and high levels of sporulation from two clonings of parental strain were reinoculated on SDYA in 7 d and then were again cloned to receive clones of the 2nd generation and evaluation of morphology variability and sporulation between the 1st and 2nd generations (see scheme). Results (Table 8) indicated that the number of clones with low sporulution (type I) were reduced 2-2.5 times after the first cloning and more that 3 times after second cloning (II and II-1-1) (Table 8). However, the number of clones with fast growth and higher sporulation (type II-c) remained stable.

Sporulation and growth of the parental strain and selected clones of the 1st and 2nd generations were compared using the standard disk method (Table 8). Sporulation of clones obtained after first and second cloning on SDYA, did not differ from sporulation of parental strain.

Table 8. Growth, sporulation and clone analysis of patental isolates and clone of the 1st and 2nd generation.

Isolate	Growth on day 14 (diameter, mm)	Sporulation on day 14(spores x $10^8/cm^2$)	No. of clones with high sporulation (%)	No. of clones with low sporulation (%)
Parental				
I	40.1 ± 0.3	0.9 ± 0.3	7.0	20.2
II	35.4 ± 1.5	1.3 ± 0.6	9.8	30.1
1st generation				
I-1	36.9 ± 0.9	1.3 ± 0.4	9.8	11.7
I-2	37.6 ± 0.8	1.4 ± 0.3	-	-
I-3	37.9 ± 0.6	1.0 ± 0.2	-	-
II-1	36.4 ± 0.5	1.6 ± 0.3	12.8	13.6
II-2	37.8 ± 1.7	1.4 ± 0.1	-	-
II-3	36.0 ± 0.7	1.8 ± 0.4	-	-
2nd generation				
II-1-1	38.0 ± 0.4	1.5 ± 0.3	8.8	8.0
II-1-2	37.9 ± 0.9	1.4 ± 0.2	-	-
II-1-3	38.5 ± 2.1	1.2 ± 0.2	-	-

Thus, cloning of the parental *M. anisopliae* EE strain reduced the number of clones with low sporulution (type I) by 2-3 times. However, the sporulation level of selected clones on

SDYA did not exceed the sporulation level of the parental strain. It is possible that SDYA promotes the development of clones with higher sporulation (type II). We will compare the sporulation of clones and parental strain on sorghum medium for mass production.

Strain *Metarhizium anisopliae* PPRC – 29.

I. Effects of different media on the variability in morphology and sporulation of clones.

SDA medium

After cloning of the parental strain on SDA, 122 clones were obtained. Clones had flat compact colonies with weak aerial mycelium and an abundant green sporulating layer. Morphology variation was absent, but clones differed in colony size and sporulation zone (Table 9). Colony diameter was 3-10 mm. Evaluation of sporulation of colonies revealed that sporulation varied from $20.8 \pm 4.7 \times 10^6$ to $58.4 \pm 7.3 \times 10^6$ spore/cm^2 depending on colony size (Table 10).

Table 9. Clone analysis of parental *M. anisopliae* PPRC- 29 strain on SDA medium for morphological types.

Colony diam., mm (7 d)	Sporulating zone, mm (7 d)	No. of clones	% clones
3-4	1-3	8	6.6
5-6	2-4	44	36.1
7-8	4-5	64	52.5
9-10	5-7	6	4.9
			100.0

Table 10. Sporulation of *M. anisopliae* PPRC- 29 clones (spores x 10^6/cm^2 on day 7) from the parental strain on SDA.

No.	Colony diameter			
	3-4 mm	5-6 mm	7-8 mm	9-10 mm
1	19.6	38.6	39.6	65.7
2	16.4	30.7	40.4	63.2
3	25.0	30.0	48.2	53.9
4	13.2	33.6	49.6	55.7
5	20.7	38.9	31.1	64.6
6	22.1	30.7	44.6	47.1
7	21.4	39.3	49.3	-
8	28.2	36.4	52.9	-
9	-	31.8	60.0	-
10	-	38.2	52.9	-
Average	20.8	34.8	46.9	58.4
SD	4.7	3.8	8.2	7.3

SDYA medium

After cloning of parental strain on SDYA, 129 clones were obtained. Clones had flat compact colonies with weak aerial mycelium and abundant green sporulating layer. Morphology variation was absent, but clones were differed by colony size and sporulation zone (Table 11). Colony diameter was 2-9 mm. Evaluation of sporulation of colonies revealed, that sporulation varied from 25.8±4.9 x 10^6 to 71.9 ± 14.4 x10^6 spore/cm^2 depending on colony size (Table 12).

Table 11. Clone analysis of parental *M. anisopliae* PPRC- 29 strain on SDYA medium for morphological types.

Colony diameter, mm (7 d)	Sporulating zone, mm (7 d)	Number of clones	% clones
2-3	1-1.5	6	4,7
4-5	2-3	41	31.8
6-7	4-5	72	55.81
8-9	5-6	10	7.8
			100.0

Table 12. Sporulation of *M.anisopliae* PPRC-29 clones (spores x 10^6/cm^2 on day 7) from parental strain on SDYA.

No.	Colony diameter			
	2-3 mm	4-5 mm	6-7 mm	8-9 mm
1	30.4	56.1	46.1	100.0
2	32.5	46.8	45.0	52.1
3	23.2	48.9	51.1	66.4
4	26.8	36.4	55.7	54.6
5	20.0	45.7	42.5	75.0
6	22.1	44.6	66.4	61.4
7	-	39.6	53.2	77.9
8	-	33.9	54.3	85.7
9	-	42.1	65.7	72.9
10	-	40.0	67.9	73.2
Average	25.8	43.4	54.8	71.9
SD	4.9	6.5	9.2	14.4

PDA medium

After cloning of parental strain on PDA, 103 clones were obtained. Clones had flat compact colonies with weak aerial mycelium and abundant green sporulating layer. Morphology variation was absent, but clones were differed by colony size and sporulation zone (Table 13). Colony diameter was 3-6 mm. Evaluation of sporulation of colonies revealed, that sporulation varied from 16.6 ± 7.2 x10^6 to 33.6 ± 7.1 x10^6 spores/cm^2 depending on colony size (Table 14).

Table 13. Clone analysis of parental *M. anisopliae* PPRC- 29 strain on PDA medium for morphological types.

Colony diameter, mm (7 d)	Sporulating zone, mm (7 d)	Number of clones	% clones
3-4	1-2	29	28.2
5-6	3-4	74	71.8
			100.0

Table 14. Sporulation of *M. anisopliae* PPRC- 29 clones (spores x $10^6/cm^2$ on day 7) from parental strain on PDA.

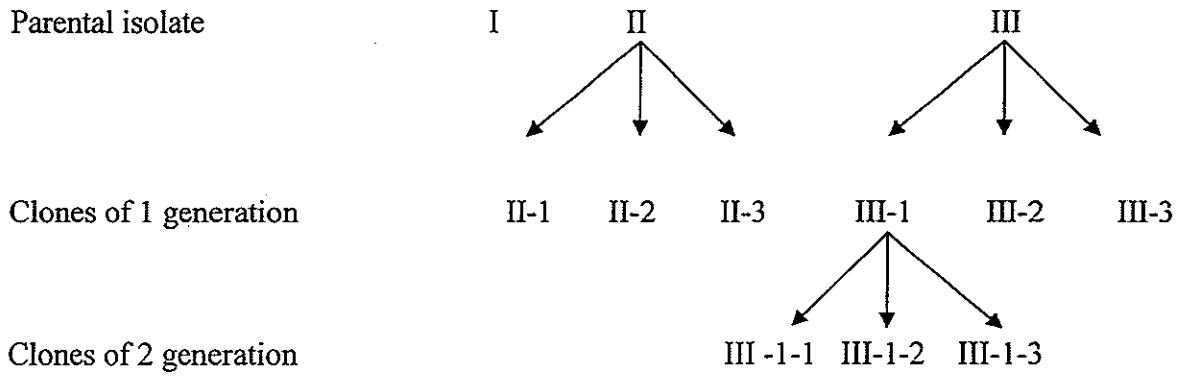
No.	Colony diameter	
	3-4 mm	5-6 mm
1	6.8	41.1
2	32.1	26.4
3	13.6	42.9
4	15.7	35.0
5	20.0	39.6
6	12.9	25.4
7	11.8	29.6
8	23.6	40.0
9	12.1	32.1
10	17.1	23.6
Average	16.6	33.6
SD		7.1

Czapek Dox agar

After cloning of parental strain, 100 clones were obtained. Morphological variation was absent. Clones had very sparse aerial mycelium and sparse green sporulating layer. Colony diameter was 6-8 mm, sporulation lever - 2.9-5.3 x10^6 spore/cm^2. The above mentioned results allowed us to conclude, that parental strain of *M. anisopliae* PPRC- 29 contain clones of owe type, which differ in growth speed and sporulation lever. The clones with most high sporulation were obtained on SDYA and this medium was selected for further experiments.

II. Evaluation of variability of clones in morphology and sporulation during firth and second generations.

In order to select the strain with faster growth and higher sporulation, the promising clones were cloning again on SDYA medium and best clones were evaluated for sporulation level in comparison with parental strain. Cloning was carried out by scheme II.



Clones with fast growth and high sporulation level, obtained from parental strain, were reinoculated on SDYA in 7 d and then were again cloned in order to receive clones of second generation and evaluation sporulation (see scheme). Analysis of results shown, that growth and sporulation of clones, obtained after firth and second cloning on SDYA, did not differ from sporulation of parental strain (Table 15). Further details are described in attached supplements.

Table 15. Growth, sporulation and clone analysis of parental isolates and clone of 1st and 2nd generation.

Isolate	Growth on day 14 (diam., mm)	Sporulation on day 14 (spores x $10^8/cm^2$)
Parental		
I	32.9 + 1.0	1.5 + 0.5
II	31.6 + 1.3	1.9 + 0.3
III	29.8 + 1.5	1.7 + 0.2
1st generation		
II-1	31.9 + 0.6	1.3 + 0.4
II-2	30.0 + 0.8	1.2 + 0.3
II-3	30.6 + 1.1	1.8 + 0.8
III-1	30.8 + 0.6	1.8 + 0.2
III-2	30.9 + 1.8	1.4 + 0.2
III-3	31.8 + 1.3	1.3 + 0.3
2nd generation		
III-1-1	31.1 + 2.2	1.8 + 0.4
III-1-2	31.3 + 1.5	1.5 + 0.7
III-1-3	30.6 + 0.5	1.9 + 0.2

Thus, the sporulation level of selected clones on SDYA did not exceed the sporulation level of the parental strain. It is possible that SDYA promotes the development of clones with higher sporulation. We plan to compare the sporulation of clones and parental strain on sorghum medium for mass production.

Strain *Beauveria bassiana* FF.

I. Effects of different media on the variability in morphology and sporulation of clones.

SDA medium

After cloning of parental strain on SDA, 124 clones with two morphology types colonies were obtained (Table 16).

1. Clones of type I (16.1%) had compact dense white colonies with abundant cottony mycelium, colony diameter was 6-10 mm.
2. Clones of type II (83.9%) had flat large white colonies with eminence in the center; colony diameter was 10-12 mm. The eminence in center was often cream-colored in 12-14 d.

Evaluation of sporulation of colonies with different morphological types revealed that clones of type I had lower sporulation than clones of type II (Table 17).

Table 16. Clone analysis of parental *B. bassiana* FF strain on SDA medium for morphological types.

Colony type	Colony diameter, mm (7days)	Number of clones	% clones
Type I	6-10	20	16.1
Type II	10-12	104	83.9
			100.0

Table 17. Sporulation *B. bassiana* FF clones (spores x $10^6/cm^2$ on day 7), from parental strain on SDA.

No.	Colony type	
	I	II
1	38.6	238.2
2	24.6	275.4
3	3.6	272.9
4	11.4	243.2
5	5.7	204.6
6	5.7	166.1
7	20.4	160.4
8	29.3	120.4
9	9.6	197.9
10	18.6	211.1
11	28.6	214.3
12	7.5	182.5
13	7.1	178.6
14	28.6	189.6
15	37.5	202.1
16	38.9	238.9
17	32.1	253.6
18	9.3	245.7
19	11.4	238.6
20	22.5	257.9
Average	19.6	214.6
SD	12.1	40.8

SDYA medium

After cloning of the parental strain on SDYA, 124 clones with two morphology types colonies were obtained (Table 18).

1. Clones of type I (7.7%) had compact dense white colonies with abundant cottony mycelium, colony diameter was 8-12 mm.
2. Clones of type II (92.3%) had flat large white colonies with eminence in the center, colony diameter was 10-12 mm. The eminence in the center was often cream-colored after 12-14 d.

Evaluation of sporulation of colonies with different morphological types revealed, that clones of type I had lower sporulation, than clones of type II (Table 19).

Table 18. Clone analysis of parental *B. bassiana* FF strain on SDYA medium for morphological types.

Colony type	Colony diameter, mm (7 days)	Number of clones	% clones
Type I	8-12	8	7.7
Type II	10-12	96	92.3
			100.0

Table 19. Sporulation *B. bassiana* FF clones (spores x $10^6/cm^2$ on day 7), from parental strain on SDYA.

No.	Colony type	
	I	II
1	57.5	243.2
2	31.8	271.8
3	21.4	233.9
4	27.5	198.9
5	53.9	236.8
6	66.1	203.6
7	42.9	246.4
8	54.3	186.1
9	-	187.5
10	-	165.7
11	-	195.0
12	-	234.6
13	-	153.6
14	-	203.9
15	-	205.4
16	-	158.2
17	-	150.0
18	-	205.4
19	-	224.3
20	-	206.4
Average	44.4	205.5
SD	16.1	33.2

PDA and Czapek Dox agar

After cloning of parental strain on SDA, 121 clones were obtained. Morphological variation was absent. Clones had compact dense white colonies with abundant cottony mycelium. Colony diameter was 6-10 mm, sporulation level - 146.2 ± 27.8 x10^6 spores/cm^2 (Table 20). After cloning of parental strain on Czapek Dox agar, 100 clones were obtained. Morphological variation was absent. Clones had colorless or white colonies with poor mycelium. Colony diameter was 6-8 mm; sporulation level was 60.8 ± 19.0 x10^6 spores/cm^2 (Table 20).

Table 20. Sporulation of *B. bassiana* FF clones (spores x 10^6 /cm^2 on day 7) from parental strain on PDA and Czapek Dox.

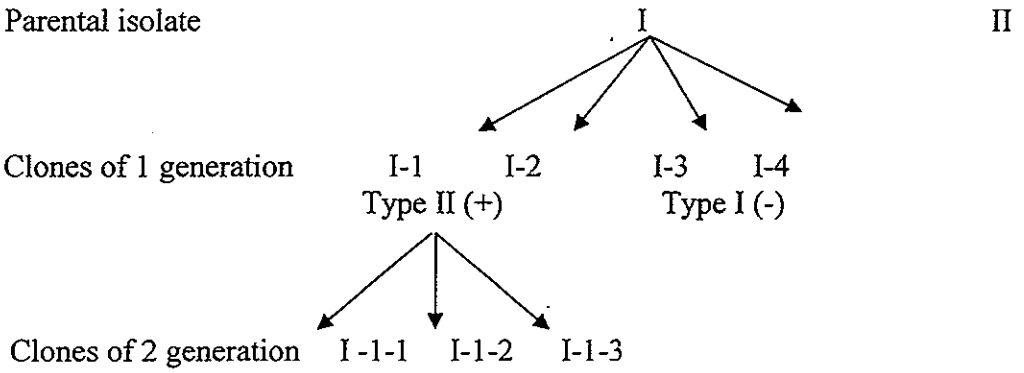
No.	PDA	Czapek Dox agar
1	130.0	99.6
2	115.4	106.4
3	154.3	55.7
4	142.9	30.7
5	100.7	53.2
6	128.2	52.5
7	143.6	72.5
8	198.6	68.9
9	136.8	62.9
10	149.3	42.5
11	133.2	51.4
12	154.6	67.1
13	123.9	71.8
14	134.6	38.6
15	129.3	56.4
16	136.4	61.8
17	134.6	66.4
18	179.6	69.3
19	191.8	33.6
20	205.7	53.6
Average	146.2	60.8
SD	27.8	19.0

The above mentioned results allowed us to conclude, that parental strain of *B. bassiana* FF contain clones of two types, which significantly differ in sporulation (>5-10 times). Two tested media (SDA and SDYA) were suitable for cloning, promoting the dissociation of parental isolate into two morphology types with different sporulation level.

II. Evaluation of variability of clones in morphology and sporulation in the first and second generations.

Analysis of morphology variability and sporulation of clones of two types was carried out by scheme III.

Scheme III.



Repeated cloning of the parental strain on SDYA confirmed the dissociation of natural isolate on clones of two types (Table 21). From 106 obtained clones, 13 clones had compact dense white colonies with abundant cottony mycelium.

Table 21. Repeated clone analysis of parental *B .bassiana* FF strain on SDYA for morphological types.

Colony type	Colony diameter, mm (7 days)	Number of clones	% clones
Type I	6-10	13	12.3
Type II	10-12	93	87.7
			100.0

Clones with high sporulation levels (type II) from first cloning of parental strain were reinoculated on SDYA in 7 d and then were again cloned to receive clones of the 2nd generation and evaluation of morphology variability and sporulation between the1st and 2nd generations (see scheme). Results indicated that the number of clones with high sporulution (type II) remained stable between the first and second cloning (Table 21).

Sporulation and growth of parental strain and clones with low and high sporulation were compared using the standard disc method (Table 22). Sporulation of type II clones, obtained after the first and second cloning on SDYA, did not differ from sporulation of parental strain. However, sporulation of type I clones, was 1.5 times lower than sporulation of parental strain.

Thus, cloning of the parental *B. bassiana* FF strain reduced the number of clones with low sporulution (type I). The sporulation level of selected clones on SDYA did not exceed the sporulation level of the parental strain. It is possible that the SDYA medium promotes the development of clones with higher sporulation (type II). We plan to compare the sporulation of clones and parental strain on sorghum medium for mass production.

Table 22. Growth, sporulation and clone analysis of patental isolates and clones of the 1st and 2nd generation.

Isolate	Growth on day 14 (diameter, mm)	Sporulation on day 14 (number spore x $10^8/cm^2$)	No. of clones with high sporulation (%)	No. of clones with low sporulation (%)
Parental				
I	46.1 + 2.6	4.1 + 0.5	92.3	7.7
II	42.3 + 2.5	3.7 + 1.3	87.7	12.3
1 generation				
I-1 (type II)	42.4 + 1.1	4.1 + 0.4	95.1	4.9
I-2 (type II)	37.0 + 2.0	5.7 + 1.2	97.9	2.1
I-3 (type I)	43.9 + 1.3	2.6 + 0.3	-	-
I-4 (type I)	29.6 + 0.8	2.5 + 0.1	-	-
2 generation				
I-1-1(type II)	40.9 + 1.2	4.3 + 0.4	98.1	1.9
I-1-2 (type II)	40.3 + 1.8	4.7 + 0.8	97.2	2.8
I-1-3 (type II)	39.0 + 0.9	5.0 + 0.5	98.9	1.1

Strain *Beauveria. bassiana* PPRC-56

I. Effects of different media on the variability in morphology and sporulation of clones.

SDA medium

After cloning of the parental strain on SDA, 110 clones with two morphology types colonies were obtained (Table 23).

1. Clones of type I (83.6%) had compact dense white colonies with abundant cottony mycelium, colony diameter was 6-8 mm.
2. Clones of type II (16.4%) had flat crumbly white colonies, colony diameter was 8-10 mm.

Evaluation of sporulation of colonies with different morphological types revealed that clones of type I had lower sporulation than clones of type II (Table 24).

Table 23. Clone analysis of parental *B. bassiana* PPRC-56 strain on SDA for morphological types.

Colony type	Colony diameter, mm (7 d)	Number of clones	% clones
Type I	6-8	92	83.6
Type II	8-10	18	16.4
			100.0

Table 24. Sporulation of *B. bassiana* PPRC-56 clones (spores x $10^6/cm^2$ on day 7) from parental strain on SDA.

No.	Colony type	
	I	II
1	1.1	125.0
2	0.7	176.4
3	1.8	74.4
4	0	83.2
5	1.1	127.9
6	1.1	82.1
7	0.7	123.6
8	1.8	186.1
9	0	93.2
10	0	126.1
11	0.4	126.8
12	0.7	125.0
13	0	114.3
14	1.1	145.7
15	1.1	147.1
16	0.7	118.9
17	0	111.4
18	1.4	138.6
19	1.4	-
20	0.7	-
Average	0.8	123.7
SD	0.6	29.6

SDYA medium

After cloning of the parental strain on SDYA, 126 clones with two morphology type colonies were obtained (Table 25).

1. Clones of type I (88.9%) had compact dense white colonies with abundant cottony mycelium; colony diameter was 6-8 mm.
2. Clones of type II (11.1%) had flat crumbly white colonies; colony diameter was 8-10 mm.

Evaluation of sporulation of colonies with different morphological types revealed that clones of type I had lower sporulation than clones of type II (Table 26).

Table 25. Clone analysis of the parental *B. bassiana* PPRC-56 strain on SDYA for morphological types.

Colony type	Colony diameter, mm (7 days)	Number of clones	% clones
Type I	6-8	112	88.9
Type II	8-10	14	11.1
			100.0

Table 26. Sporulation of *B. bassiana* PPRC-56 clones (spores x $10^6/cm^2$ on day 7) from the parental strain on SDYA.

No.	Colony type	
	I	II
1	8.6	88.2
2	9.6	60.4
3	6.8	133.9
4	4.3	70.7
5	13.2	49.3
6	8.9	52.9
7	7.5	40.7
8	6.1	50.7
9	8.2	46.8
10	7.9	70.7
11	4.6	48.2
12	5.4	64.3
13	7.5	57.5
14	4.6	72.1
15	8.9	-
16	9.6	-
17	11.8	-
18	5.0	-
19	6.1	-
20	5.0	-
Average	7.5	64.0
SD	2.5	24.4

PDA medium

After cloning of parental strain on PDA, 95 clones with two morphology types colonies were obtained (Table 27). Clones of type I (93.7%) had compact dense white colonies with abundant cottony mycelium; colony diameter was 7-8 mm. Clones of type II (6.3%) had flat crumbly white colonies; colony diameter was 9-11 mm. Evaluation of sporulation of colonies with different morphological types revealed that clones of type I had lower sporulation than clones of type II (Table 28).

Table 27. Clone analysis of parental B.bassiana PPRC-56 strain on PDA for morphological types.

Colony type	Colony diameter, mm (7 days)	Number of clones	% clones
Type I	7-8	89	93.7
Type II	9-11	6	6.3
			100.0

Table 28. Sporulation of *B. bassiana* PPRC-56 clones (spores x $10^6/cm^2$ on day 7) from parental strain on PDA.

No.	Colony type	
	I	II
1	12.9	126.1
2	6.8	164.6
3	5.0	124.6
4	6.8	171.8
5	6.8	155.7
6	8.2	136.1
7	8.9	-
8	7.5	-
9	10.7	-
10	10.4	-
11	5.4	-
12	6.1	-
13	8.2	-
14	11.1	-
15	5.4	-
16	5.4	-
17	6.1	-
18	7.9	-
19	8.6	-
20	4.3	-
Average	7.6	146.5
SD	2.3	20.3

Czapek Dox agar

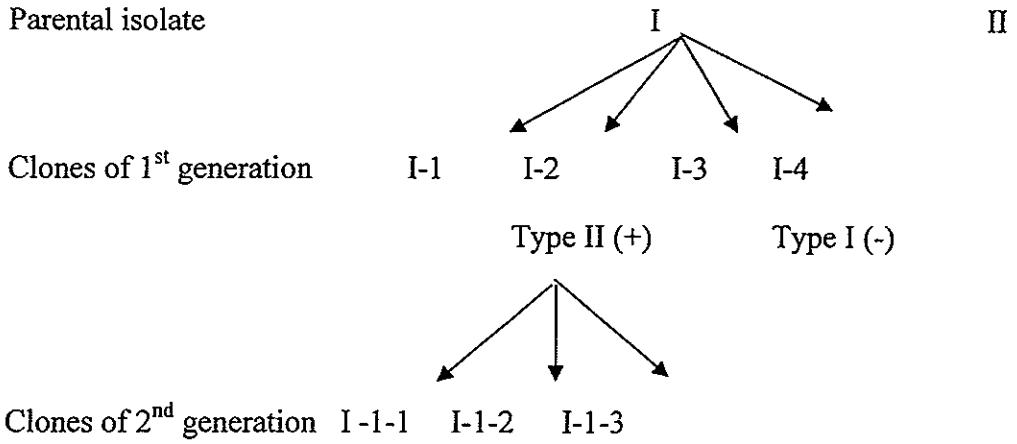
After cloning of the parental strain on Czapek Dox agar, 101 clones were obtained. Morphological variation was absent. Clones had colorless, white or cream colonies with poor mycelium. Colony diameter was 4-10 mm, sporulation lever - 4.3 – 79.3 x10^6 spores/cm^2.

The above mentioned results allowed us to conclude, that parental strain of *B. bassiana* PPRC-56 contain clones of two types, which significantly differ in sporulation (>5-10 times). Three tested media (SDA, PDA and SDYA) were suitable for cloning, promoting the dissociation of parental isolate into two morphology types with different sporulation level.

II Evaluation of variability of clones in morphology and sporulation during firth and second generations.

Analysis of morphology variability and sporulation of clones of two types was carried out by scheme IV.

Scheme IV.



Repeated cloning of the parental strain on SDYA confirmed the dissociation of natural the isolate on clones of two types (Table 29). From 133 obtained clones, 112 clones had compact dense white colonies with abundant cottony mycelium and low sporulation.

Table 29. Repeated clone analysis of parental *B. bassiana* PPRG-56 strain on SDYA for morphological types.

Colony type	Colony diameter, mm (7 days)	Number of clones	% clones
Type I	7-8	112	91.7
Type II	10-12	14	8.27
			100.0

Clones from the parental strain with high sporulation (type II) were reinoculated on SDYA in 7 d and then were again cloned to receive 2nd generation cones and evaluation of morphology variability and sporulation between the 1st and 2nd generations (see scheme). Results indicated that the number of clones with high sporulution (type II) were increased after the first cloning by 7-8 times and remained stable after the second (Table 30). Sporulation and growth of the parental strain and clones with low and high sporulation were compared using the standard disc method (Table 30).

Table 30. Comparison of growth, sporulation and clone analysis of patental isolates and clone of 1st and 2nd generation.

Isolate	Growth, day 14 (diameter, mm)	Sporulation, day 14 (spores x 10^8 /cm^2)	Number of clones with high sporulation (%)	Number of clones with low sporulation (%)
Parental				
I	29.5 + 1.2	1.6 + 1.1	11.1	88.9
II	31.1 + 0.3	1.4 + 0.5	8.3	91.7
1st generation				
I-1 (type II)	29.8 + 0.3	4.5 + 1.2	85.4	14.6
I-2 (type II)	29.5 + 1.0	4.8 + 0.4	74.3	25.7
I-3 (type I)	31.3 + 1.5	0.7 + 0.3	-	-
I-4 (type I)	31.5 + 0.4	1.0 + 0.4	-	-
2nd generation				
I-1-1(type II)	29.6 + 0.9	4.3 + 0.5	71.3	28.7
I-1-2 (type II)	29.0 + 1.7	3.8 + 0.3	87.9	12.1
I-1-3 (type II)	29.4 + 0.6	4.2 + 0.7	83.0	17.0

Sporulation of type II clones, obtained after the first and second cloning on SDYA, was increased by 3 times compared in parental strain. Sporulation of type I clones was 1.5-2 times lower than sporulation of parental strain and 4-5 time lower than sporulation of II type clones.

II. The influence of passages on stability and productivity of promising clones and parental isolates.

Parental strains of *M. anisopliae* (EE and PPRC-29) and their "+clones" did not reduce the spore productivity and did not change the morphological type of colonies after 15 passages on SDYA (Tables 31 and 32).

Table 31. Sporulation of *M. anisopliae* EE (parental strain and "+ clone) after passages on SDYA.

Number of passages	Sporulation of parental strain (spores x 10^8/cm^2)	Sporulation of "+clone" (spores x 10^8/cm^2)
0	1.5 ± 0.4	1.9 ± 0.5
5	1.6 ± 0.3	2.2 ± 0.6
10	1.4 ± 0.3	2.1 ± 0.3
15	1.6 ± 0.3	2.0 ± 0.4

Table 32. Sporulation of *M. anisopliae* PPRC-29 (parental strain and "+"clone) after passages on SDYA.

Number of passages	Sporulation of parental strain (spores x 10^8/cm^2)	Sporulation of "+clone" (spores x 10^8/cm^2)
0	1.8 ± 0.4	2.0 ± 0.3
5	1.7 ± 0.4	2.4 ± 0.6
10	1.5 ± 0.3	1.8 ± 0.3
15	1.5 ± 0.6	2.2 ± 0.4

Productivity of parental strains of *B. bassiana* (FF and PPRC-56) after 5 passages did not reduce significantly, but the sporulation rate decreased 4-10 times (Tables 33 and 34). In

contrast, the spore productivity of their "+clones" did not decrease after 15 passages. The passages caused the morphological modifications of the parental strains colonies, but not in colonies of "+clones".

Table 33. Sporulation of *B. bassiana* FF (parental strain and "+ clone) after passages on SDYA.

Number of passages	Sporulation of parental strain (spores x 10^8/cm^2)	Sporulation of "+clone" (spores x 10^8 /cm^2)
0	3.7 ± 0.5	3.9 ± 0.6
5	2.6 ± 0.5	3.8 ± 0.7
10	0.4 ± 0.2	3.3 ± 0.6
15	0.6 ± 0.4	3.7 ± 0.8

Table 34. Sporulation of *B. bassiana* PPRC-56 (parental strain and "+ clone) after passages on SDYA.

Number of passages on SDYA	Sporulation of parental strain (spores x 10^8/cm^2)	Sporulation of "+clone" (spores x 10^8/cm^2)
0	1.9 + 0.4	4.2 + 0.8
5	1.3 + 0.6	4.0 + 0.3
10	0.4 + 0.1	3.8 + 0.6
15	0.5 + 0.2	4.0 + 0.7

Results indicate that because of repeated passages of *B. bassiana* parental strains, their productivity decreased 4-10 times. In contrast, their preliminary selected "+clones" were stable and retained the sporulation rate after repeated passages. Both *M. anisopliae* parental strains and their "+clones" retained their productivity after repeated passages on SDYA.

II. The influence of storage duration on stability and productivity of promising clones and parental isolates.

The results of storage only during 1-12 mo are summarized in this report, because the storage over a longer period is still continuing. Parental strains of *M. anisopliae* (EE and PPRC-29) and their "+clones" after 12-mo storage on both SDYA and SDA and following cultivation on SDYA did not reduce the spore productivity (Tables 35 and 36).

Table 35. Sporulation of *M. anisopliae* EE (parental strain and "+clone") after storage.

Months of storage	Sporulation (number spore x 10^8 per cm^2) after storage on SDYA		Sporulation (number spore x 10^8 per cm^2) After storage on SDA	
	Parental	"+" Clone	Parental	"+" Clone
0	1.5 ± 0.4	1.9 ± 0.5	1.5 ± 0.4	1.9 ± 0.5
1	1.7 ± 0.3	1.6 ± 0.3	1.7 ± 0.5	1.7 ± 0.6
3	2.2 ± 0.4	2.0 ± 0.5	1.7 ± 0.6	2.3 ± 0.6
6	1.8 ± 0.4	1.6 ± 0.7	1.6 ± 0.7	1.6 ± 0.5
9	1.6 ± 0.3	1.4 ± 0.2	1.5 ± 0.4	1.7 ± 0.6
12	1.4 ± 0.6	1.5 ± 0.3	1.7 ± 0.3	1.8 ± 0.5

Table 36. Sporulation of *M. anisopliae* PPRC-29 (parental strain and "+clone") after storage.

Months of storage	Sporulation (number spore x 10^8 per cm^2) after storage on SDYA		Sporulation (number spore x 10^8 per cm^2) after storage on SDA	
	Parental	"+" Clone	Parental	"+" Clone
0	1.8 ± 0.4	2.0 ± 0.3	1.8 ± 0.5	2.0 ± 0.4
1	1.7 ± 0.4	1.9 ± 0.6	1.6 ± 0.6	2.3 ± 0.5
3	2.3 ± 0.7	1.7 ± 0.8	2.1 ± 0.7	1.8 ± 0.5
6	2.1 ± 0.4	2.0 ± 0.2	1.9 ± 0.6	2.3 ± 0.3
9	2.0 ± 0.2	1.9 ± 0.2	1.9 ± 0.6	1.7 ± 0.6
12	1.8 ± 0.6	2.0 ± 0.3	1.6 ± 0.5	2.1 ± 0.4

Sporulation of the parental strain of *B. bassiana* FF stored on SDA and SDYA decreased 1.5-2 times after 6-12 mo storage, but the spore productivity of *B. bassiana* FF "+clones" did not decrease significantly after 1-12 mo storage on both mediums (Table 37).

Table 37. Sporulation of *Beauveria bassiana* FF (parental strain and "+clone") after storage.

Months of storage	Sporulation (number spore x 10^8 per cm^2) after storage on SDYA		Sporulation (number spore x 10^8 per cm^2) after storage on SDA	
	Parental	"+" Clone	Parental	"+" Clone
0	3.7 ± 0.5	3.9 ± 0.6	3.7 ± 0.6	3.9 ± 0.7
1	3.3 ± 0.9	3.1 ± 1.2	3.0 ± 1.3	4.0 ± 0.5
3	2.9 ± 0.7	3.0 ± 0.9	2.7 ± 1.1	3.1 ± 1.6
6	2.2 ± 0.7	3.6 ± 1.4	1.6 ± 0.5	3.3 ± 1.0
9	2.8 ± 0.4	4.3 ± 0.4	2.1 ± 0.3	4.1 ± 0.5
12	2.5 ± 0.8	3.8 ± 0.3	2.2 ± 0.6	4.2 ± 0.4

Productivity of parental strains of *B. bassiana* PPRC-56 decreased after 6-12 mo storage on SDYA 1.5-2 times and 3-10 times after 3-12 mo storage on SDA (Table 38). Sporulation of *B. bassiana* PPRC-56 "+clones" decreased after 3-6 mo storage on both medium by 1.5-2 times.

Table 38. Sporulation of *B. bassiana* PPRC-56 (parental strain and "+clone") after storage.

Months of storage	Sporulation (spores x $10^8/cm^2$) after storage on SDYA		Sporulation (spores x $10^8/cm^2$) after storage on SDA	
	Parental	"+" Clone	Parental	"+" Clone
0	1.9 ± 0.4	3.8 ± 0.8	1.9 ± 0.5	3.8 ± 0.9
1	1.8 ± 0.6	3.6 ± 0.9	1.7 ± 0.3	3.2 ± 1.2
3	1.9 ± 0.6	2.8 ± 0.8	0.5 ± 0.4	3.2 ± 0.9
6	1.0 ± 0.5	2.5 ± 0.7	0.2 ± 0.1	1.5 ± 0.1
9	1.2 ± 0.7	2.3 ± 0.5	0.6 ± 0.2	1.8 ± 0.4
12	1.0 ± 0.8	2.6 ± 0.4	0.5 ± 0.3	2.0 ± 0.5

Results indicate that both parental *M. anisopliae* strains and their "+clones" can be stored not <12 mo without losing its ability to sporulate on both SDYA and SDA. Productivity of parental strains of *B. bassiana* decreased after 3-6 mo storage, especially on SDA. In contrast, prolonged storage of *B. bassiana* FF "+clones" on both media maintained a high sporulation rate.

The productivity of *B. bassiana* PPRC-56 "+clones" decreased during storage, but less that the productivity of parental strain.

IV. Comparison of virulence between promising clones and parental isolates.

Mortality of thrips, treated with *M. anisopliae* isolates varied from 90-96.3%, whereas for the *B. bassiana* isolates – from 82.5-90.5% (Table 39). Mortality among the control insects was <10%. Virulence of selected clones did not significantly differ from sporulation of parental strain.

Table 39. Virulence of parental strains and "+ clones toward WFT (Munger cells, $5 \cdot 10^6$ spore/ml, 22°C)

Strain	Parental	"+ clone"
M. anisopliae EE	91.3 ± 6.3	90.0 ± 4.1
M. anisopliae PPRC-29	93.8 ± 7.5	96.3 ± 4.8
B. bassiana FF	88.8 ± 7.5	90.5 ± 8.7
B. bassiana PPRC-56	82.5 ± 5.0	90.0 ± 5.8
Control	8.5 ± 4.0	8.5 ± 4.0

Mortality of *B. argentifolii* nymphs recorded on day 4 varied from 5.2-32.2%, as compared to uninoculated whiteflies that was 4.1%. A sharp increase in mortality was recorded for *B. bassiana* strains 6-7 d after inoculation reaching 46.5-62.4%. *M. anisopliae* strains were low virulent caused only 12.3-17.1% mortality after 7 d (Table 40).

Table 40. Virulence of *M. anisopliae* and *B. bassiana* strains toward *B. argentifolii* nymphs.

Strain	Mortality (%)	
	4 days	7 days
M. anisopliae EE	5.2 ± 2.3	12.3 ± 6.7
M. anisopliae PPRC-29	3.9 ± 2.0	17.1 ± 8.2
B. bassiana FF	32.3 ± 10.3	46.5 ± 12.2
B. bassiana PPRC-56	24.5 ± 15.0	62.4 ± 14.2
Control	4.1 ± 2.0	6.5 ± 4.4