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Biological Control of Greenhouse and Sweetpotato Whiteflies by Entomopathogenic Fungi

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3. EXECUTIVE SUMMARY

The aims of the present project were to develop effective screening procedures for mycoinsecticides against whiteflies and employ them for selecting such strains from *Verticillium lecanii* and *Beauveria bassiana*. An additional objective was to assess the genetic and phenotypic diversity of these fungi in relation to virulence. Most of the above mentioned objectives were achieved during this project. One hundred strains of the following fungi obtained from various hosts and geographic locations were screened and evaluated for virulence on *Bemisia tabaci* and *Trialeurodes vaporariorum*: *Verticillium lecanii* (35), *Beauveria bassiana* (15), *Paecilomyces fumosoroseus* (28), *Paecilomyces farinosus* (2), *Conidiobolus coronatus* (2), *Conidiobolus* spp. (8), *Hirsutella* sp. (7) and *Verticillium* sp. (3). Highly efficient virulent strains of either *V. lecanii* or *B. bassiana* and an additional, still unidentified, *Verticillium*-like strain were selected by the procedures developed. All these strains were effective against all stages of the whiteflies with the exception of eggs which were immune. Only very few strains of *B. bassiana* and the *Verticillium*-like strain (but not *V. lecanii*), were effective under high temperatures and low humidity which are common to greenhouses in either, Kazakhstan or Israel . The study on genetic diversity of *V. lecanii* in relation to virulence was summarized in an enclosed paper published in *Phytoparasitica*.

The main contribution of this project to Kazakhstan and Israel is the obtained effective mycoinsecticides strains which can now be employed for biocontrol of whiteflies. Additional general contributions are: a) Development of screening procedures under *in vivo* conditions b) The lack of relationship between genetic diversity or geographic distribution of *V. lecanii* and virulence and c) The emphasis on the need to obtain highly virulent fungal strains which will be adapted to climatic conditions (ecotypes) where they will be employed. The cooperation achieved between the 2 countries was very good and resulted in a common publication.

4. RESEARCH OBJECTIVES

The management of insect pests, particularly whiteflies, is becoming most difficult because whiteflies are developing high resistance to most, if not all, agricultural pesticides. Due to the high costs of development fewer insecticides are available. The situation has been aggravated by the greater public concern for safe methods of management and the public demand for the use of management strategies with minimal impact on the environment. Therefore, most countries' government regulations of chemical pesticides are becoming more stringent. In developing countries such as Kazakhstan, additional concern such as shortage of foreign exchange reserves to purchase pesticides, which are all imported, and food security may be key issues. Therefore there is a strong tendency to shift from high reliance on chemical pesticides to an Integrated Pest Management approach (IPM). Biological control is a fundamental component of IPM. One of the most underutilized types of biological control is entomopathogenic fungi.

In spite of increasing use of these fungi commercially and otherwise in various countries, research is still needed for selecting effective mycoinsecticides against whiteflies which can perform under conditions available in Kazakhstan and Israel. The goal of this project was to develop effective strains of *Verticillium lecanii, Beauveria bassiana* as well as other fungi for biocontrol of the whiteflies *Trialeurodes vaporariorum* (Greenhouse whitefly, GW) and *Bemisia tabaci* (Sweetpotato whitefly, SPW) for use in IPM systems under greenhouse conditions in Kazakhstan and Israel. In order to achieve this goal we have pursued the following objectives:

1. **Development of an efficient procedure for evaluation of *V. lecanii* and *B. bassiana* strains for enhanced virulence against GW and SPW and epizootic efficacy.**
2. **Selection of effective fungal strains for utilization as mycoinsecticides against GW and SPW in IPM programs.**
3. **Evaluation of the genetic and phenotypic diversity exhibited by *V. lecanii* and *B. bassiana* in relation to virulence.**
4. **Development of a DNA-fingerprint procedure for strains identification.**

In addition to the financial support of AID, this project was supported by the Israeli Department of Agriculture and Kazakh Academy of Sciences which provided laboratories and greenhouse facilities and services as well as additional technical help.

The results of the project are presented in 3 parts. The first one describes the development of evaluating procedures of the fungal strains (objective 1) as well as selection of strains for virulence (objective 2). The second part provides data on the survival ability of selected strains under suboptimal conditions and epizootic potential (objective 2). The third part describes the evaluation of genetic and phenotypic diversity (objective 3) and its potential development for DNA-fingerprint procedure for strains identification (objective 4).

5. METHODS AND RESULTS

PART I

I. Development of efficient procedures for evaluating *V. lecanii* and *B. bassiana* strains for biocontrol of GW and SPW.

The following procedures were developed and optimized for initial screening of entomopathogenic fungi for virulence against GW and SPW under *in vitro* and *in vivo* conditions.

Materials and Method

Isolation and cultivation of fungi

The majority of the fungal isolates used in this study were isolated in Israel and in Kazakhstan whereas the rest were obtained from other different countries. The location and the insect from which they were isolated are listed in Table 3. Cultures were routinely maintained on Sabouraud Dextrose Agar (SDA) or malt extract agar.

Preparation of conidia suspension

Fungal strains were cultivated on malt extract agar for 10 days at 25°C. Spores were harvested using a solution containing 0.005% triton X100. The spore suspension was filtered through several layers of cheesecloth to remove mycelial mates. The concentration of spores in the final suspension was determined by haemocytometer. Spore preparation used for bioassay was obtained by diluting concentrated spores with 0.005% triton X100 to a final concentration of 10^7 spores/ml. The number of colony forming units (CFU) was determined by plating the suspension on agar medium prior to inoculation.

Insect rearing

A laboratory population of *Bemisia tabaci* was maintained on cotton plants in a greenhouse. In order to obtain a uniform age of *B. tabaci*, 50-100 adults were placed on

small cotton plants for 24-36 hours. Then all the adults were removed and plants with eggs were transferred into environmental growth chambers for 16-18 days. Insects were used for bioassay when the larvae were in early stage of transfer to pupae. Ten to twelve replications were used for each strain.

Bioassay procedure for larvae and eggs of *B. tabaci*

Individual cotton leaves with about equal density of insects were sorted before inoculation. Leaves (or leaf sectors) with insects were immersed into conidial suspension or into 0.005% triton X100 as a control for 8-10 seconds. The leaves were then placed on a filter paper for 20-30 min. until dried. This step was necessary for preventing the development of saprophytic fungi. Dry leaves were transferred into assay containers with a moist filter paper and placed in an incubator with temperatures alternating between 25°C (14 hr) during the day and 20°C (10 hr)during the night. The filter papers were kept moist throughout the bioassay but care was taken to prevent excessive moisture which may stimulate development of saprophytes on the leaves.

Mortality of larvae was determined daily by counting the number of infected versus noninfected individuals per each leaf. Mortality of eggs was determined for 1-day-old and 4-day-old eggs respectively. The leaves were infected by the fungus as described earlier. Infection of eggs was determined by the following criteria: a) direct development of mycelium on eggs, b) number of hatching larvae and c) infection of hatching larvae.

Bioassay procedure for pupae and adults of *B. tabaci*

Spraying of adults with spore suspension resulted in high mortality. The high sensitivity of this procedure caused some difficulties in determining the degree of virulence among the various fungal strains. Therefore a procedure was developed which was based on inoculating ready-to-emerge pupae and recording the infection of adults after emergence.

Small plants with 2-4 leaves were inoculated with adults of *B. tabaci* for 24-30 hr. The adults were then removed and the plants were transferred into environmental chambers for 20-22 days. A leaf with 50-100 ready-to-emerge pupae was retained on each plant whereas the others were removed. The leaf was sprayed with conidial suspension (10^7 spores/ml) and covered by a plastic frame with an outlet. The opening was closed with several layers of cheesecloth. For maintaining the moisture, the cheesecloth was

irrigated with water each day. Virulence was determined by the following criteria: 1) direct infection of pupae, 2) the number of adults emerged and 3) infection of emerging adults.

Bioassay procedure for larvae of *T. vaporariorum*

Selection of entomopathgenic fungi against *T. vaporariorum* (GW) were conducted on growing cucumber plants in the laboratory. Small cucumber plants (4-5 leaves) were inoculated with 30-50 adults per leaf of GW for 48 hrs. Then, all adults were removed and plants with eggs were transferred into environmental growth chambers for 10 days. Insects were used for bioassay when larvae were in 2-3 stage. Four leaves of each plant were used for each treatment.

Larvae on leaves were sprayed with 1 ml conidial suspension per leaf ($5x10^7$ spores/ml) made with 0.01% triton X100. Plants were placed in plastic chambers at 24-26°C and RH- 96-100% with 16-hour day-light. Mortality of larvae was determined by counting the number of infected versus non-infected individuals per each leaf during a period of 7 days.

Results and Discussion

Optimization of an *in vitro* bioassay procedure for screening pathogenic fungi.

The present research was aimed at developing a more precise and simple bioassay procedure for the rapid evaluation of virulence of fungal strains. It is well known that many parameters such as temperature, humidity, age of insect, physiological condition of the host and the pathogen (which may be influenced by their prior cultivation procedure) etc. may affect the bioassay. Furthermore, whiteflies may have some unique features which may require further considerations. The bioassay procedures for *in vitro* evaluation of virulence described in 'Materials and Methods' were found optimal, in comparisons with other various procedures.

One crucial factor which may influence the degree of conformity of the bioassay is the the population density of the insect. The density of the populationon leaves is generally not uniform yet it may affect the microconditions of the leaf niche. Thus higher

density may greatly conduce the development of the pathogen by increasing secretion of sugars and other nutrients and facilitating the transmission of the pathogen.

In order to examine the effect of larvae density on the uniformity of the results, leaf section of 8 cm^2 were selected with densities ranging from 20 to 200 insects (2.5-25 insects/cm^2). The leaf sectors were inoculated with *V. lecanii* strain Is-5. Results in Fig. 1 indicate that a density of 5-10 insects/cm^2 secured the most uniform data. Therefore further experiments for *in vitro* selection were conducted with leaf sectors of this density.

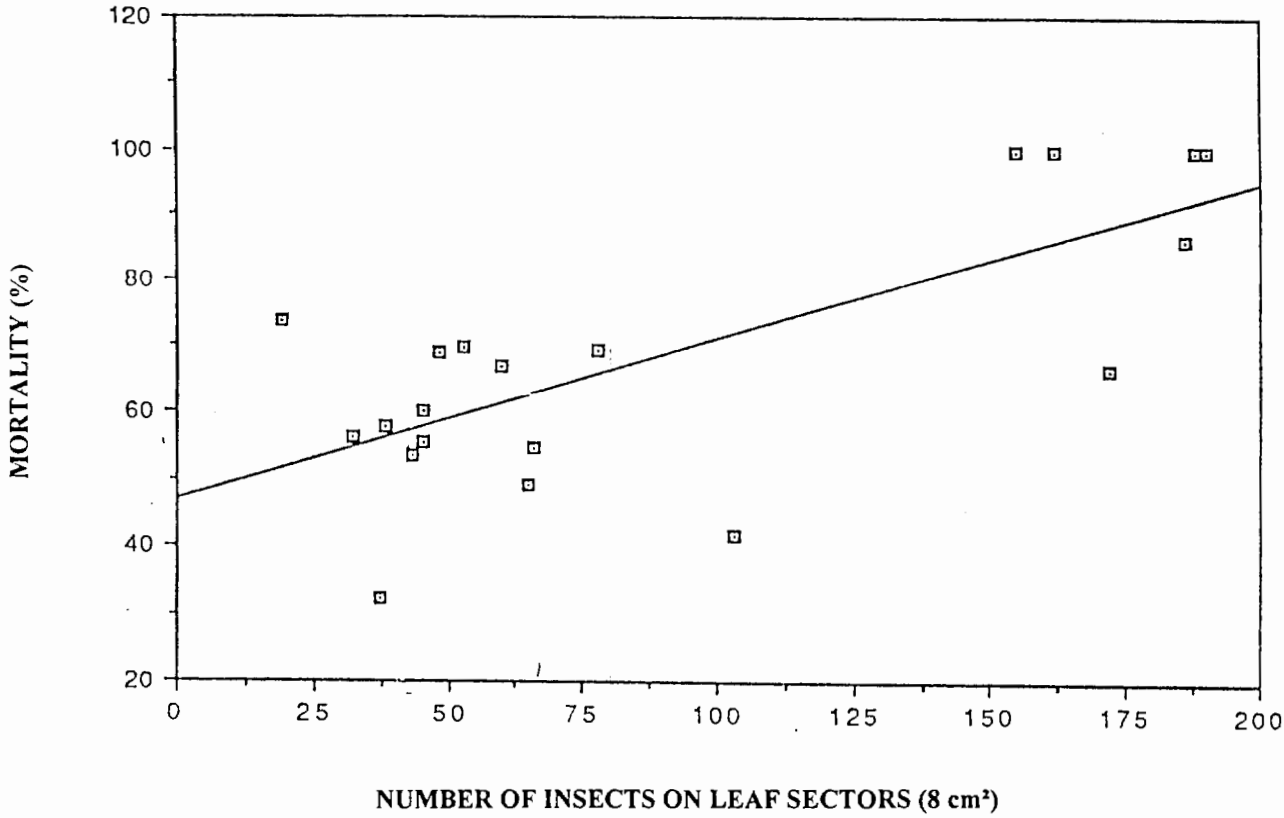
Development of *in vivo* bioassay procedure for screening

A major problem in screening of virulent fungal strains is the uneven distribution of the pathogen on the leaf. Generally, after incubation of 4-5 days the fungus appears to cover groups of insects which demands a gentle removal of the mycelial mates to expose individual insects for counting and calculating LC_{50}. This process terminates the experiment and prevents the follow-up on infection dynamics. Such a limitation is particularly severe under *in vivo* conditions where it is difficult to design sufficiently uniform replications to compensate for the damage of the treatment after recording the results. In order to cope with this problem we have examined the possibility of using a qualitative bioassay procedure for estimating the degree of infection. Infection was scored as follows:

Score	Degree of infection (%)
1	20
2	40
3	60
4	80
5	100

The degree of infection within a group of insects was determined visually. Results given in Table 1 indicate a significant correlation between larvae mortality obtained by direct counting of individual insects and the newly developed qualitative procedure in which estimation of infection percentage was made on a group of insects. Thus the estimated percent of mortality by direct counting of infected larvae with strain Is-5 gave an average of 68±19.5% whereas the average value for mortality obtained by the new procedure was calculated according to the score to be 72±17.2%.

Fig 1. Mortality of *B. tabaci* larvae as a function of insect density on leaf sectors.



Experimental details are described in the text.

Table 1. Comparison of *in vitro* and *in vivo* procedures for estimating larvae mortality of *B. tabaci* by *V. lecanii*

Leaf No.	No. of larvae inoculated	No. of larvae infected	Mortality (%)[a]	Score for degree of infection[b]
1	186	160	86	4
2	172	114	66.2	3
3	60	40	66.6	3
4	32	18	56.3	3
5	118	87	73.7	4
6	78	54	69.2	4
7	45	25	55.6	3
8	66	36	54.5	3
9	48	33	68.8	4
10	43	23	53.5	3
11	53	37	69.8	4
12	188	188	100.0	5
13	37	12	32.4	3
14	155	155	100.0	5
15	190	190	100.0	5
16	103	43	41.7	2
17	45	27	60.0	3
18	38	22	57.9	3
19	65	32	49.2	3
20	162	162	100.0	5
			X = 68±19.5	X = 3.6±0.86

[a] - Moratlity percentage was determined by testing individual insects

[b] - Degree of infection was estimated by the score described for the qualitative *in vivo* procedure. Score of infection of 3.6 is equivalent to 72% mortality (see text).

The above mentioned results were carried out under *in vitro* conditions in which the distribution of the larvae on the leaf could be controlled by selecting leaf sectors with reasonably even distribution. However, under *in vivo* conditions such an ideal situation does not exist. In order to overcome the uneven development of the fungus on the leaf, was divided into sectors according to insect distribution. The area of each sector from the total area of the leaf was also estimated. For example: a leaf was divided into 3 sectors (S) as follows: $S_1=1/2$, $S_2=1/4$ and $S_3=1/4$. The score of mortality (M) was first calculated per each sector e.g. : $M_1=4$, $M_2=3$, $M_3=2$, and the values of the sectors were combined to give the mortality percentage for the whole leaf (M_X) according to the following formula:

$$M_X = (M_1 \times S_1 + M_2 \times S_2 + M_n \times S_n) \times 20$$

M_1, M_2, M_n - mortality; S_1, S_2, S_n - relative area of leaf sectors

The calculations for the above mentioned example will be as follows:

$$M_X = [M_1\ (4) \times S_1\ (1/4) + M_2\ (3) \times S_2\ (1/4) + M_3\ (2) \times S_3\ (1/2)] \times 20 =$$

$$[4/4 + 3/4 + 2/2] \times 20 = 55\%$$

On the basis of the variabillity of the experimental data it was calculated that 12-15 replications will be required to obtain a standard deviation (SD) of 20-25%.

The above mentioned procedure allowed estimation of mortality on whole plants. In order to further test the performance of the qualitative estimating procedure for mortality, in comparison to the *in vitro* method, it was performed with detached leaves , however the leaves were not selected for even distribution of insects. Cotton plants with different numbers of infested leaves were inoculated by spore suspension of *V. lecanii* strain Is-5 (10^7 spores/ml) in 0.005% triton X-100. The leaves were then removed and transferred to a moist chamber, as previously described. Mortality was first estimated by the qualitative estimation procedure and then the mycelial mates which covered groups of larvae were removed and mortality was reestimated by direct counting of individual larvae. Results obtained (not shown) clearly indicated high correlation between the 2 methods and both practically gave the same results. It should also be emphasized that the qualitative estimation method takes a considerable shorter time and is much easier to perform.

Table 2. Determination of the degree of infection by *V. lecanii* strain Is-5 on intact cotton plants using the qualitative *in vivo* estimation method [a]

Plant No.	Leaf No.	Leaf sector with identical infection	Degree of infection
1	1	1	1
	2	1	1
2	3	1	1
	4	1	1
	5	1/2	5
		1/2	4
	6	1/2	5
		1/2	3
3	7	1/5	5
		4/5	1
	8	1	1
4	9	1	1
5	10	1/10	5
		9/10	1
6	11	1/2	2
		1/2	1
7	12	1	1
	13	1	1
	14	1	1
	15	1	1
8	16	1/2	4
		1/2	2
	17	1	1
	18	1/4	4
		3/4	2
	19	1	2
9	20	4/5	5
		1/5	2
	21	1/5	5
		4/5	1
	22	1/5	4
		4/5	1
	23	1	1

[a] - Description of the procedure is given in the text

In order to test the qualitative estimation method, 9 cotton plants with different numbers of infested leaves were inoculated by spore suspension of *V. lecanii* strain Is-5 (10^7 spores/ml) in triton X-100. The plants were covered by plastic containers for keeping moist atmosphere. Results presented in Table 2 demonstrate how the latter procedure is being performed on whole plants. High degree of variability is observed in the results. Statistical analysis of our initial results indicated that because of the high variability, a large number of leaves (up to 100) will have to be evaluated for each strain in order to obtain a significant value for comparing the performance with other fungal strains (results not shown). It appears, therefore, that the qualitative estimation method should be limitted for comparing only the best strains which were selected by the *in vitro* procedure. However, the major advantage of this procedure is that it allows estimation of strain performance under actual conditions present in the greenhouse and throughout the growing season.

II. Selection of *V. lecanii* strains for virulence against GW and SPW

V. lecanii strains were isolated from different insects and one from a rust fungus and were obtained from various geographic locations representing 8 countries. Pathogenicity of these strains on larvae of *B. tabaci* is shown in Table 3. Results indicate that the most virulent strains of *V. lecanii* which caused above 70% mortality of larvae were isolated from *Myzus persicae* and *Acyrthosiphon pisum* in Israel. These strains were superior to "mycotal" a commercial product of *V. lecanii* originally developed in 1982 by Microbial Resources Ltd. against whiteflies on cucumber and tomato and caused 54% mortality (Table 3). It is noteworthy that these highly virulent strains were isolated from aphids rather than whiteflies. Although only one fungal isolate was obtained from *B.* tabaci which showed 43.5% mortality, 6 isolates were obtained from the greenhouse whitefly *T. vaporariorum*. Interestingly, the virulence among these isolates varied from 56.3% to 16.3. These results could suggest that no correlation exists between the insect from which *V. lecanii* was isolated and the degree of virulence on whiteflies. The lack of such a correlation is in accordance with the broad specifity of *V. lecanii* as an insect pathogen.

Table 3. The origin of fungal isolates used in the study, and their virulence on larvae of *Bemisia tabaci*.

Verticillium spp isolates	SOURCE Insect / Location[a]	Larvae mortality (%) X (SE)[d]
Verticillium lecanii		
974	*Pulvinaria floccifera* (T)	0 (0)
3460	*Protopulvinaria pyriformis* (Is)[e]	9 (2.4)
973	*Cossus cossus* (T)	10.5 (2.4)
313	*"Vertalec"*[b]	11.5 (2.4)
Myc	*"Mycotal"*[c]	54 (4.7)
3456	*Plant leaf* (Is)[e]	17.5 (3.6)
M9	*Puccinia graminis* (R)	8 (2.6)
M2	*Pseudococcus citri* (G)	1.8 (0.2)
R6	*Trialeurodes vaporariorum* (R)	7.3 (1.4)
R4	*Aleyrodidea* sp. (R)	4.5 (1.7)
R3	*Trialeurodes vaporariorum* (R)	18.5 (1.9)
R1	*Trialeurodes vaporariorum* (R)	56.3 (4.1)
Is11	*Bemisia tabaci* (Is)	43.5 (3.4)
M4	*Hemileia vasatrix* (R)	8.75 (1.8)
Is1	*Oxycarenus hyalinipennis* (Is)	47.3 (4.8)
Is2	*Myzus persicae* (Is)	75 (4.9)
Is5	*Myzus persicae* (Is)	83 (2.4)
Is6	*Acyrthosiphon pisum* (Is)	73 (3.0)
3457	Unidentified coccid *(Coccidae)* (Is)[e]	0.5 (0.3)
2638	*Coccus hesperid* (C)[e]	39.8 (4.3)
2557	*Aphis gossipii* (Is)[e]	4.5 (1.5)
3113	*Lecanium cornii*[e]	29.5 (4.7)
2614	*Coccus hesperidum* (Is)[e]	24.5 (3.4)
3112	*Pulvinaria floccifera* (T)[e]	0 (0)
2585	Unidentified coccid *(Coccidae)* (Is)[e]	6 (1.3)
2842	*Saissetia oleae* (Is)[e]	8.3 (0.9)
2584	Unknown (Is)[e]	16 (2.3)

Table 3 (continued)

Verticillium spp isolates	SOURCE Insect / Location[a]	Larvae mortality (%) X (SE)[d]
Verticillium lecanii		
Kaz1	*Trialeurodes vaporariorum* (K)	16.3 (1.6)
Kaz13	*Trialeurodes vaporariorum* (K)	33.3 (5.5)
Kaz14	*Trialeurodes vaporariorum* (K)	46.3 (4.5)

[a]- Is - Israel, T - Turkey, C - Cyprus, G - Georgia, K - Kazakhstan, I - Italy, R - Russia.

[b]- *V. lecanii* strains used for production of commercial preparations against aphids (Vertalec).

[c]- *V. lecanii* strains isolated from commercial preparations against whiteflies (Mycotal).

[d]- Mortality of larvae in 4 days.

[e]- Israeli isolates obtained from the collection of Prof. R. Kenneth, Dept. of Plant Pathology, the Faculty of Agriculture, Hebrew University.

Table 4. Pathogenicity of *Verticillium lecanii* strains on *B. tabaci* larvae as expressed by LT_{50}.

LT_{50} 3.2-4.0	LT_{50} 4.0-7.0	$LT_{50} > 7$
Is1 (Israel)	*Is11 (Israel)	*IsH2 (Israel)
Is2 (Israel)	*IsH1 (Israel)	2557 (Israel)
Is5 (Israel)	2842 (Israel)	2574 (Israel)
Is6 (Israel)	3456 (Israel)	2585 (Israel)
Is1 (Israel)	3460 (Israel)	2668 (Israel)
*R1 (Russia)	M3 (Russia)	2614 (Israel)
Mycotal	*M5 (Russia)	3457 (Israel)
	M9 (Russia)	R2 (Russia)
	*Kaz1 (Kazakhstan)	R5 (Russia)
	*Kaz13 (Kazakhstan)	*R6 (Russia)
	*Kaz14 (Kazakhstan)	M4 (Russia)
	A973 (Italy)	*R3 (Georgia)
	2638 (Cyprus)	M2 (Georgia)
	3113 (Turkey)	A974 (Turkey)
	A313 (Vertalec)	3112 (Turkey)

Infection was performed by 10^7 conidia / ml

* Strains originally isolated from whiteflies; The other strains have been isolated from other insects.

The variability in virulence of *V. lecanii* strains as expressed by LT_{50} (lethal time) is given in Table 4. The pathogenicity tests of *V. lecanii* strains on GW were conducted on cucumber plants growing under laboratory conditions in Kazakhstan. Results presented in Table 5 indicate that the virulence of *V. lecanii* on GW varied from 10.4% to 86.9% after 7 days. Strains isolated from either GW or SPW showed different levels of virulence and no correlation could be established between the insect from which the strains were isolated and virulence towards the insect-host. Generally, strains which were highly virulent towards SPW showed also high virulence towards GW (e.g. Is-5, Is-6, "Mycotal").

Tests conducted with several strains of *V. lecanii* with a mixed population (several developmental stages) of GW showed differential virulence with high mortality of larvae and low mortality of adults. These preliminary results lead to comparative studies in which each developmental stage of GW was tested individually (Table 6). Results indicated that eggs were not infected with any of the strains, however hatched larvae (6.7%-23.1%) were infected within 7 days. The larvae was the most susceptible stage whereas the pupae showed lowest susceptibility. Additional observations indicate that strains which exhibited the highest virulence towards adults showed the lowest virulence towards larvae and pupae. In contrast, four highly virulent strains towards larvae showed different levels of pathogenicity towards adults. It is possible that some strains have differential specificity to different developmental stages of the whitefly (as found in Kazakhstan). However, this claim should be substantiated by testing a far larger number of fungal strains and cannot be based on the limited number of strains tested.

III. Selection of *B. bassiana* strains for virulence against GW and SPW

Screening of *B. bassiana* strains to SPW were conducted on the basis of the *in vivo* qualitative estimation method in Israel. Results showed that strains of *B. bassiana* were less virulent to SPW. Mortality of SPW caused with different *B. bassiana* isolates have already varied from 6.1 to 26% after 4 days (Table 7).

Table 5. Pathogenicity of *V. lecanii* isolates on GW and SPW

Isolates	Source	Larvae mortality (%)	Level of pathogenicity to	
	Insect / Location	GW in 7 days (X±SE)	GW	SPW
2574	*Ceroplastes pforidensis* (Is)	36.5±4.1	L	L
R6	*Trialeurodes vaporariorum* (R)	22.9±5.4	L	L
Kaz-7	*Trialeurodes vaporariorum* (K)	24.4±5.1	L	-
Kaz-10	*Trialeurodes vaporariorum* (K)	10.4±3.3	L	-
R-2	*Aleyrodidea* sp. (R)	17.9±3.2	L	L
X-1	*Bemisia tabaci* (Is)	29.6±4.3	L	M
Kaz-1	*Trialeurodes vaporariorum* (K)	55.5±4.9	M	L
V-c-3	*Trialeurodes vaporariorum* (K)	63.5±6.2	M	-
X-2	*Bemisia tabaci* (Is)	51.7±4.8	M	L
2842	*Saissetia oleae* (Is)	58.8±5.8	M	L
M-2	*Pseudococcus citri* (G)	58.3±4.9	M	L
Kaz-14	*Trialeurodes vaporariorum* (K)	53.0±6.0	M	M
Is-1	*Oxycarenus Hyalinipennis* (Is)	58.5±6.1	M	M
Kaz-12-5	*Trialeurodes vaporariorum* (K)	72.4±6.3	M	-
Is-6	*Acyrthosiphon pisum* (Is)	85.9±4.6	H	H
Is-4	*Myzus persicae* (Is)	86.9±3.7	H	H
Kaz-13	*Trialeurodes vaporariorum* (K)	80.7±4.0	H	M
"Mycotal"	*"Mycotal"*	86.9±5.8	H	H

Is - Israel, R - Russia, K - Kazakhstan, G - Georgia.

L - low pathogenicity (< 50%); M - middle pathogenicity (50%-75%);

H - high pathogenicity (> 75%)

Table 6. Pathogenicity of *V. lecanii* strains to different developmental stages of GW .

Strains of	Mortality (%) of GW (X±SE)				
V. lecanii	eggs	larvae stage II	larvae stage III	pupae	adults
V-c-3	19.4±3.5	63.1±3.5	42.1±9.5	27.1±3.1	86.7±5.9
Kaz-12-5	9.4±1.7	84.9±5.1	73.5±5.5	15.2±5.4	32.1±4.5
B-1	6.7±3.1	63.3±7.6	51.4±5.1	21.3±4.5	43.1±7.7
K-16	23.1±5.2	72.7±4.8	67.3±3.7	18.1±3.9	39.3±8.1
Tvi-p-82	12.4±2.7	32.3±2.5	34.7±5.8	10.1±2.1	89.4±8.2
Control	3.5±1.5	7.1±0.9	5.3±1.2	7.5±1.9	4.2±1.3

Table 7. Pathogenicity of *B. bassiana* strains to *B. tabaci* larvae.

Isolates	Source	Mortality (%) of (X±SE)	
	Insect / Location	4 days	7 days
OX	*Oxycarenus hyalinipennis* (Is)	6.1±0.4	12.0±0.5
BZ	*Bemisia tabaci* (Is)	16±0.5	38.5±2.0
K-2	*Zeuzera pyrina* (R)	26±2.1	27±1.6
25	Unknown (R)	22±0.9	41.0±2.2
28	Locusta sp. (R)	13±0.5	46±1.9
Kaz-6	*Trialeurodes vaporariorum* (K)	18±1.2	35±1.6
Kaz-15	*Trialeurodes vaporariorum* (K)	7±0.4	42±1.2
Kaz-21	*Trialeurodes vaporariorum* (K)	12±0.4	57±3.0

Is - Israel, R - Russia, K - Kazakhstan.

Table 8. Pathogenicity of *B. bassiana* strains on GW and SPW

Isolates	Source Insect / Location	Larvae mortality (%) in 7 days (X±SE)	Level of pathogenicity to GW	Level of pathogenicity to SPW
Kaz-2	*Trialeurodes vaporariorum* (K)	50.0±4.4	M	-
Kaz-5	*Trialeurodes vaporariorum* (K)	52.1±5.9	M	-
Kaz-6	*Deroceras cancasucum* (K)	42.6±4.7	L	L
Kaz-10	*Trialeurodes vaporariorum* (K)	24.1±3.9	L	-
Kaz-15	Unidentified insect (K)	61.4±3.7	M	M
Kaz-11	*Trialeurodes vaporariorum* (K)	15.7±3.3	L	-
Kaz-21	*Trialeurodes vaporariorum* (K)	82.3±5.5	H	M
By	*Trialeurodes vaporariorum* (U)	55.4±4.2	M	-

K - Kazakhstan, U - Ukraina.

L - low pathogenicity (< 50%); M - middle pathogenicity (50%-75%);
H - high pathogenicity (> 75%)

* Level of pathogenicity to BT on basis of date in Table 7.

Selection of *B. bassiana* to GW were conducted on growing cucumber plants (Kazakhstan). The majority of the tested strains were isolated from different insects in Kazakhstan and only strain By was isolated in Ukraina. Results indicated that the majority of strains showed medium virulence towards GW. Only a single strain (Kaz-21) showed high virulemce (Table 8). It may by concluded that the occurrence of virulent strains of *B. bassiana* on SPW and GW in our selections was quite rare.

PART II

IV. The potential use of *V. lecanii* and *B. bassiana* strains for biocontrol of whiteflies.

The use of fungi for biocontrol of insects depends not only on the degree of virulence but also on additional traits which may determine their efficacy under field conditions. These traits include: a) survival ability (epiphytic fitness) of the entomopathogenic fungus on leaf surface in the absence of insects hosts, b) transmission of infection from one developmental stage of the host insect to the next one, c) epizootic potential, e.g. the spread of fungal infection to untreated plots and d) survival capacity under suboptimal greenhouse conditions and resumption of growth, sporulation and virulence after conditions have optimized. The foregoing parameters were investigated during the screening of fungal strains against whiteflies.

Materials and Methods

Bioassay procedure

Bioassay tests were conducted using *B. tabaci* populations on cotton plants. Treatment of insects and determination of mortality were carried out as described earlier.

Determination of survival under extreme conditions

Fungal isolates where grown on malt agar plates at 25°C for 7 days. Agar discs (0.8 cm) were removed from the culture by a cork borer and transferred to the center of each of the new malt agar plates and the latter were then incubated under various temperatures. Colony growth was measured after 3 and 6 days whereas sporulation rate was evaluated after 10 days. Six replications were used for each strain.

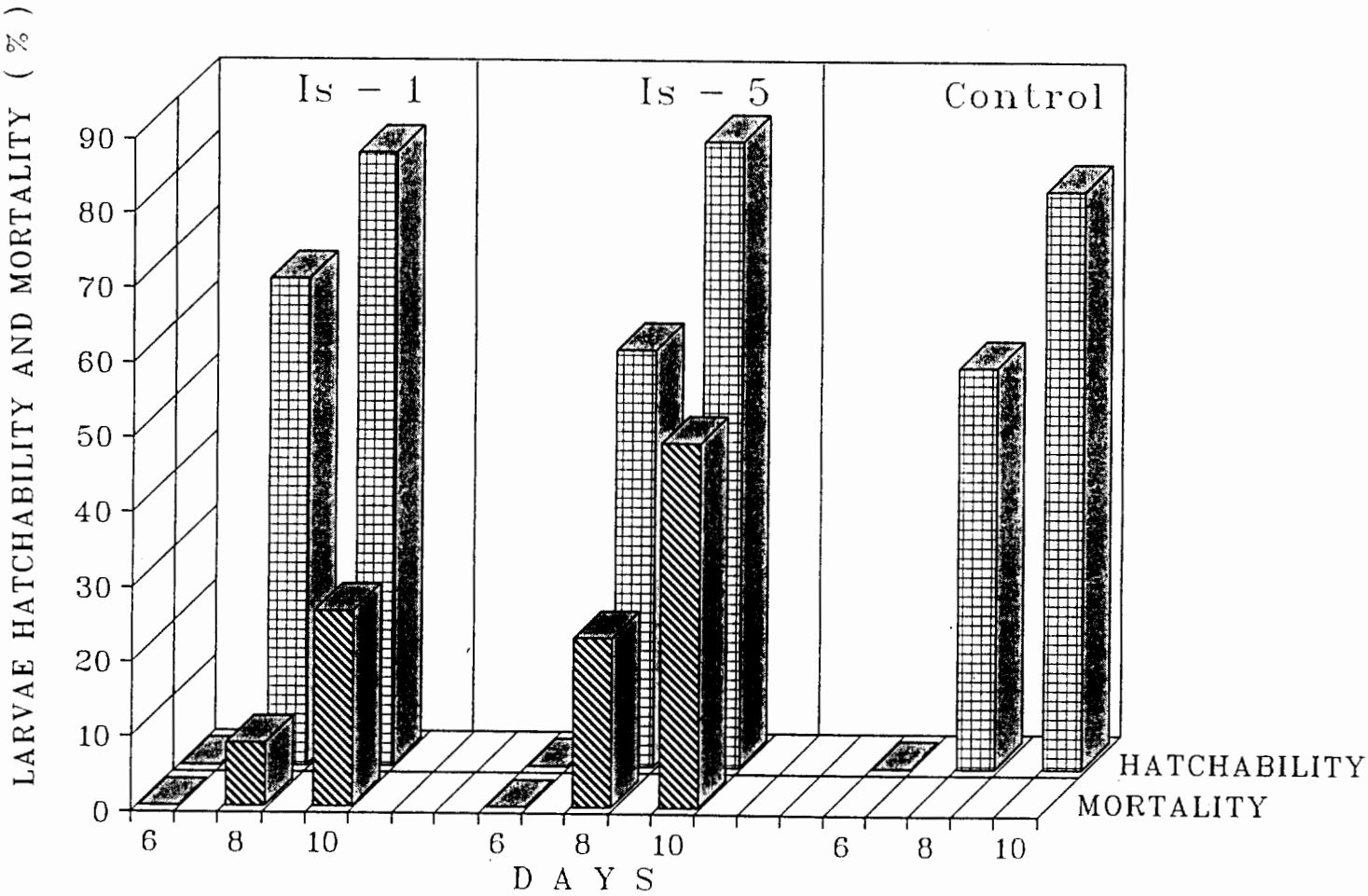
For investigating the temperature tolerance of the fungal strains, the inoculated malt agar plates were incubated at various high temperatures for 1-6 days and each day 3 plates (for any strain tested) were transferred to 25°C for growth resumption and for determination of the period required for resuming sporulation. The agar disc containing the culture was removed from the plate and the center of the agar plate was tested daily by microscopic observation for 3 weeks.

Results and Discussion

a) Survival ability and transmissability of infection

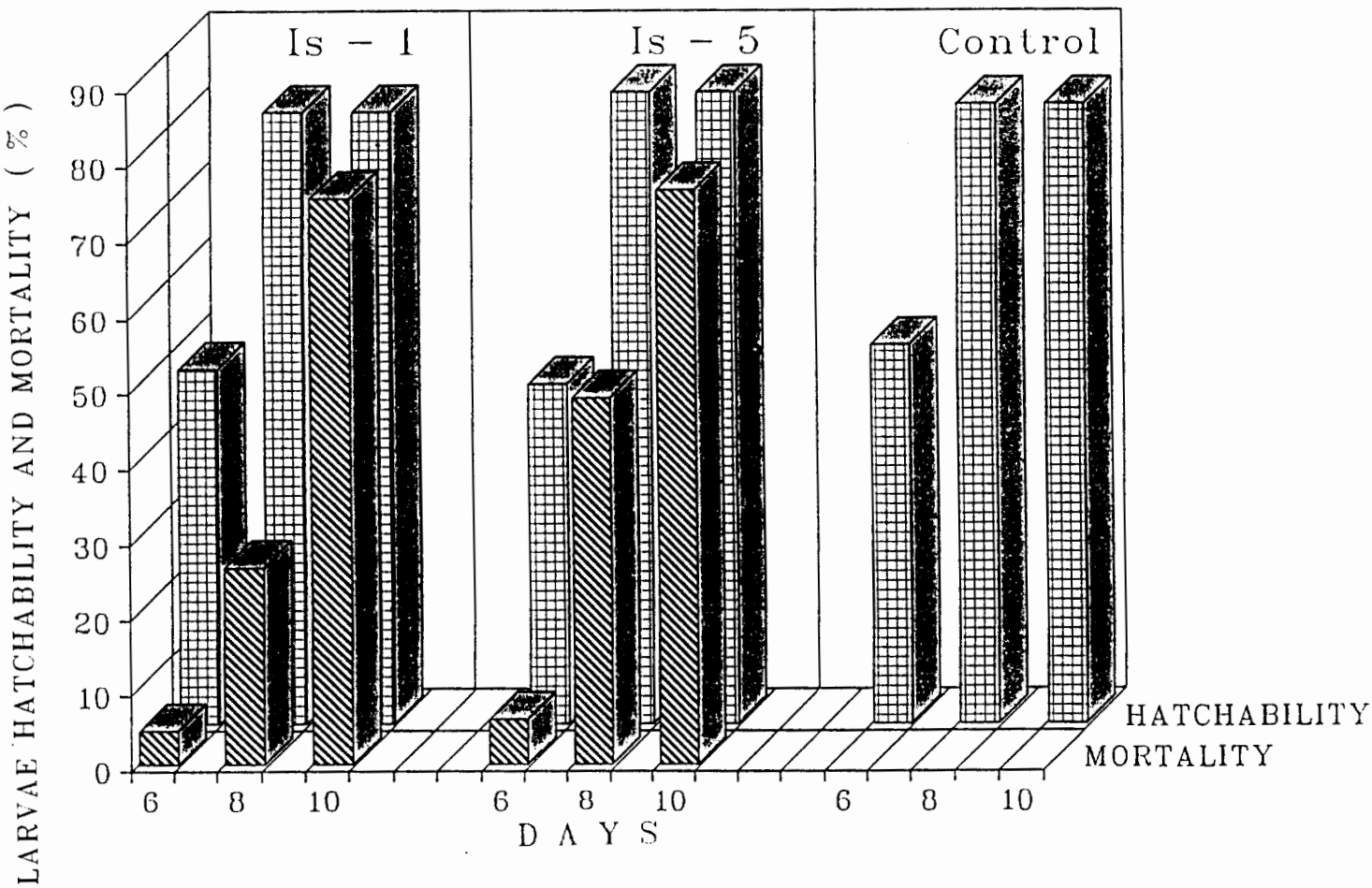
It is generally known from numerous previous studies that various developmental stages of insects exhibit differential sensitivity towards fungal infection. Furthermore, artificial inoculation experiments revealed that eggs of GW and SPW are practically immune to infection by *V. lecanii* and *B. bassiana*. Nevertheless, our experiments indicated that inoculation of mixed whitefly populations (e.g. different stages) by fungal spores caused eggs to be in contact with other infected stages and resulted in eggs covered with fungal hyphae. Hatching larvae emerging from such eggs became infected within 2-3 days. Furthermore, fungal spores which have remained on the leaf or eggs surface, were also able to infect the hatching larvae. The rate of hatching larvae infection was dependent on eggs age. Thus hatchability of larvae from one-day-old eggs which were treated with spores of *V. lecanii* strains was started after 6-7 days and reached approximately 60% and 80% at 8 and 10 days respectively (Fig. 2). The mortality of hatched larvae from one-day-old eggs was 20% or lower after 8 days and 45% or lower after 10 days as compared to non-treated control. In contrast, hatchability as well as mortality were considerabley higher in four-day-old eggs inoculated with spores of *V. lecanii* (Fig. 3). In the latter experiment hatchability was equivalent to the untreated control and reached about 50% and 80% after 6 and 8 days respectively. The mortality of the larvae was correlated with hatchability and started after 6 days, reaching approximately 70% after 10 days. These results indicate that eggs inoculation has no effect on the rate of hatchability but does affect the mortality of hatching larvae. The rate of mortality depends on the length of the exposure period of larvae to an affective fungal inoculum. The fact that inoculation of one-day-old eggs caused lower mortality than

Fig 2. Pathogenicity of *Verticillium lecanii* to *Bemisia tabaci* larvae after inoculation of 1-day-old eggs.



1-day-old eggs in cotton leaves were inoculated by 2 strains (Is-1 and Is-2) of *V. lecanii* as described in Materials and Methods. For further details see text.

Fig 3. Pathogenicity of *Verticillium lecanii* to *Bemisia tabaci* larvae after treatment of 4-day-old eggs.



Experimental details are as in Fig 5., except that 4-day-old eggs were inoculated.

four-days-old eggs (Figs. 2,3) reflects on one hand a decline in the effectivity of the inoculation within time but on the other hand indicates its survival on leaf surfaces for at least 8 days during which infectivity has been preserved.

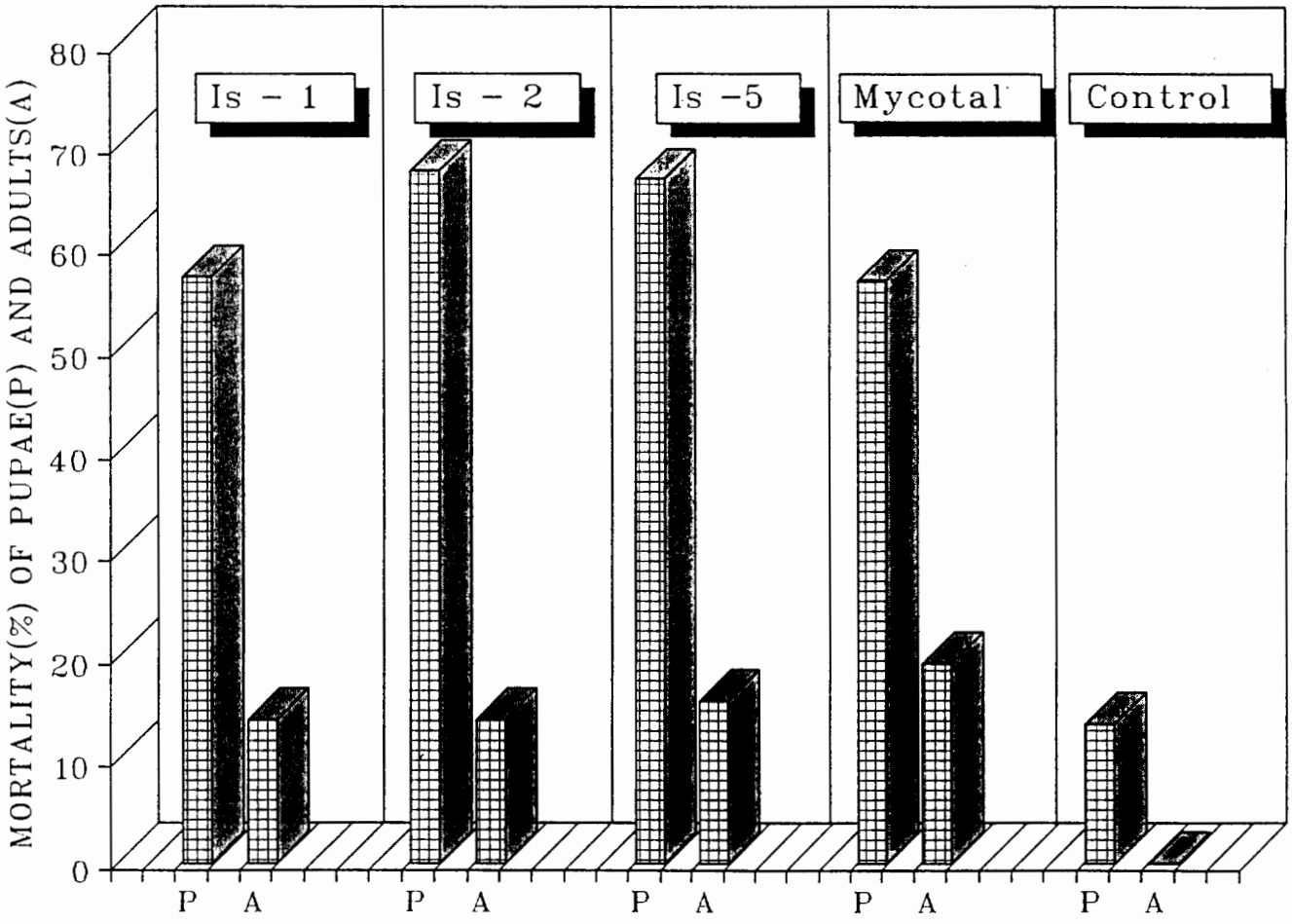
The effect of virulence of *V. lecanii* strains Is-1, Is-2 and "Mycotal" on pupae and adults following inoculation during the pupae stage of *B. tabaci* is illustrated in Fig. 4. Both pupae and subsequently emerging adults were infected. The mortality of pupae reached 52-68% and that of emerging adults reached 20-25%. No significant differences could be observed among the three isolates tested. The total percentage survival of the whitefly population was 15-25% after fungal infection as compared to 90% in the control. These results demonstrate again the transmissability of the selected *V. lecanii* strains from one developmental stage to the other.

b) Evaluation of epizootic potential

Vertical spread (along the stem and leaves of individual plants) versus horizontal spread (from one plant to another) was found to be insignificant for strains of both *V. lecanii* and *B. bassiana*. Following fungal treatments mycosed adults were only rarely found after 2-3 weeks on new growing parts of the plant or adjacent untreated plants. The horizontal transmission of infection to plants infested with larvae of SPW was slightly more successful and reached 3-5% infection with *B. bassiana* larvae and 5-10% for larvae of *V. lecanii*. The latter results were also recorded 2-3 weeks after contact with plants containing infected adults.

The ability of the selected fungal strains to survive on infected host-insect cadavers and maintain infectivity after inoculation for a long period under suboptimal conditions (e.g. low humidity which exists in the greenhouse) was examined in the following experiment: Cotton plants, which were heavily infested with *B. tabaci*, were sprayed with fungal spores. In the first phase of this experiment the plants were transferred into conditions for disease development (25°C, RH 100%). The following fungal strains were used: a) *V. lecanii* (Is-2) b) *B. bassiana* (Kaz-6) and c) An unidentified *Verticillium*-like strain which was isolated from *B. tabaci* during natural epizootic occuring in a greenhouse in Israel. Results of this part of the experiment revealed that disease development following the artificial inoculation was approximately equal for all three fungi. Death of larvae was observed 3-4 days after inoculation and

Fig 4. Virulence of *Verticillium lecanii* strains to *Bemisia tabaci* pupae and adults.



Experimental details are described in Materials and Methods. Mycotal means a *Verticillium lecanii* strain isolated from the commercial preparatum "Mycotal".

mortality reached maximal values of 50-70% after 6-7 days. In the second phase of this experiment the heavily SPW - infested and fungal - treated plants were transferred into low humidity conditions (RH 30-40%) and placed near cotton plants which were infested with *B. tabaci* but untreated with fungi. The plants were left for 10 days in order to allow adults to fly between the plants as well as transfer of fungal spores from treated to untreated plants. During this period no epizootic developemnt could be observed in any of the untreated plants.

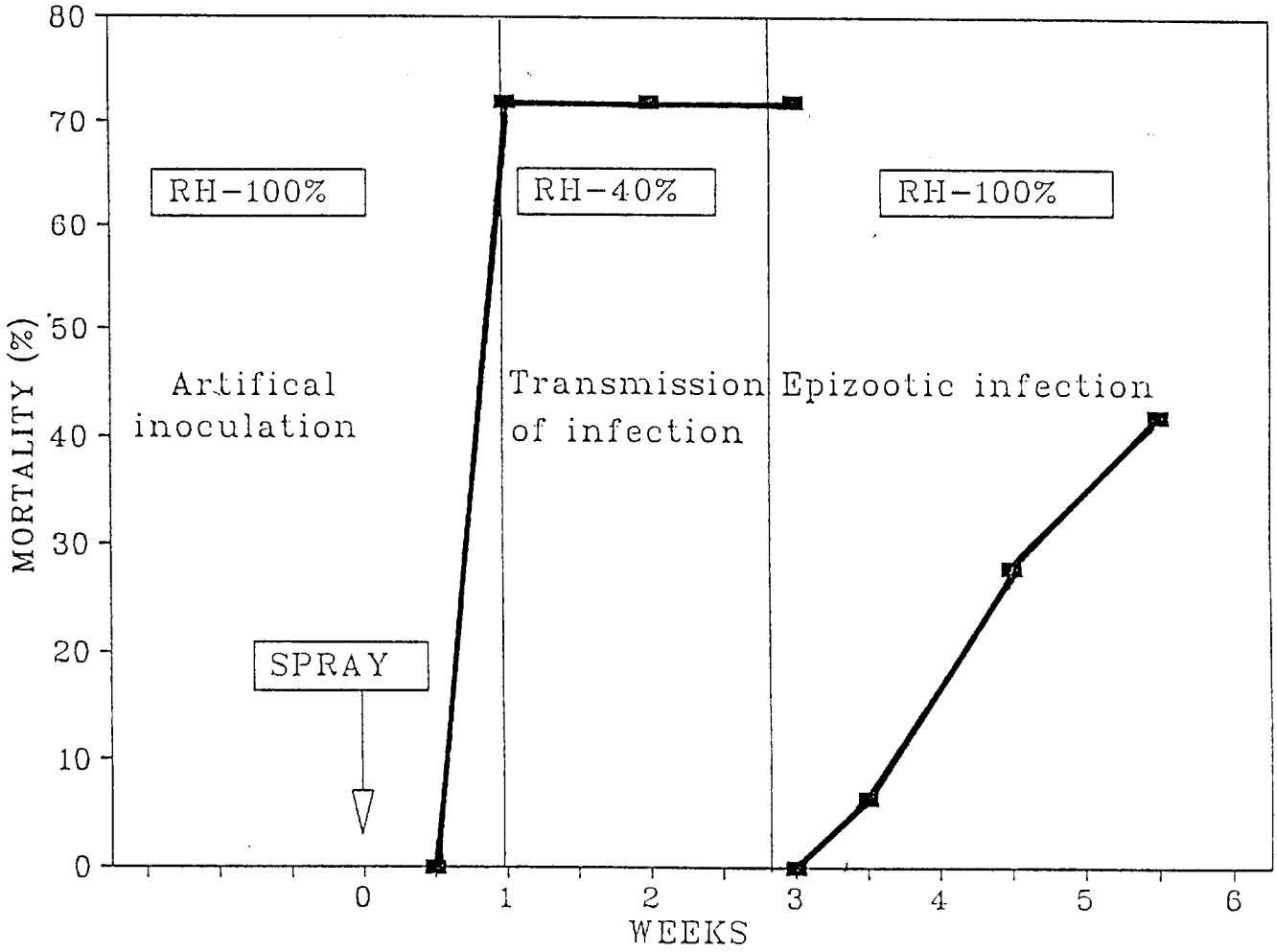
After 10 days the plants which were transferred into optimal conditions for epizootic development (25°C, RH 100%). Although dead adults, due to fungal infection were observed after 4-5 days with all the 3 fungal isolates, epizootic infection was developed only with the *Verticillium*-like isolate and not with the *V. lecanii* and *B. bassiana* isolates. The infection due to the latter isolate gradually increased and reached 45% after 6 days (Fig. 5). Results obtained emphasize the significance of screening for epizootic potential in addition to virulence under optimal conditions. It appears that fungal strains of the same species could vary in their epizootic potential. The latter character should be crucial for performance under field condtions.

c) Effect of extreme temperatures on survival ability

The fluctuation in greenhouse and field temperatures during day and night or during the growing season may often become suboptimal to fungal development. Our observations indicate that the greenhouse temperatures in Israel and Kazakhstan may reach 45-48°C which can be maintained for a relatively long period. Therefore, the need to use isolates which can develop at higher temperatures and can tolerate extreme temperatures without loosing virulence and capacity to resume optimal growth and sporulation once temperatures become normal, is of utmost significance. Three virulent strains representing 3 different species were selected for studies on their performance in various temperatures: a) *V. lecanii* (Is-6) b) *B. bassiana* (Bz) and c) *Verticillium*-like sp.

All the above mentioned isolates did not differ significantly in their sensitivity to low temperatures and resumed growth at 8-10°C (Table. 9). However, the 3 isolates exhibited differential sensitivity for sporulation at low temperatures. Thus sporulation could be initiated at 12°C for *V. lecanii* (Is-6), 16°C for *B. bassiana* (Bz) and 20°C for *Verticillium*-like sp. The optimal temperatures range for growth and sporulation differed considerabley between Is-6 and the *Verticillium*-like sp. Thus growth and sporulation was

Fig 5. Epizootic potential of the *Verticillium*-like isolate



Experimental details are described in the text.

at the range of 20-25°C for *V. lecanii* and 25-32°C for the *Verticillium*-like sp., whereas *B. bassiana* kept a broader range of 20-30°C . Furthermore, the sporulation of the *Verticillium*-like strains was higher at 30°C than 25°C. Of particular interest were the maximal temperatures which may be relevant to greenhouse conditions during Summer in Kazakhstan and Israel. Results indicate (Table 9) that the *Verticillium*-like sp. could grow and sporulate at 36°C as compared to 32°C and 30°C for sporulation of the Bz and Is-6 strains respectively. These results illustrate the differential adaptation of various species to growth and sporulation at existing higher temperatures in Kazakhstan and Israel.

Additional experiments were aimed at assessing the effect of exposure to extreme temperatuers on the ability of the fungal strain to resume growth and sporulation under optimal conditions. The latter effect is a function of both the temperature and the duration in which the fungus survived at a given high temperature. The product of these 2 factors will be referred to as heat tolerance.

Experiments indicate that *V. lecanii* isolates could resume normal growth and sporulation after incubation of 6 days at 35°C (e.g. heat tolerance = 6x35 = 210). Whereas all strains of *B. bassiana* and the *Verticillium*-like strains could resume sporulation after incubation for 6 days at 37.5°C (heat tolerance of 225). However, the heat tolerance of all fungi was radically reduced at higher temperatures. For example, the *V. lecanii* strain (Is-6) could sporulate after 1 day at 42°C whereas the 2 other strains did not.
Relatively higher temperature resistance was exhibited by the *B. bassiana* strains with the highest resistance in strain Kaz-6. The latter was isolated in Kazakhstan and could sporulate after 2 days at 45°C. One of the *B. bassiana* strains (Bz) lost the ability for growth after 1 day heat at 42°C or 2 days heat at 38-40°C, while the ability for sporulation was completely lost after 1 day incubation at 38°C as compared to 6 days incubation at 36°C. It is noteworthy that the ability for sporulation after the heat treatment could not be resumed in the following generation. In general the temperature tolerance of the *Verticillium* sp. was similar to the level of the *B. bassiana* strains inspite of the higher optimal temperature range of the former species (Table 9). The duration of heat to which the fungus was exposed exerted a significant influence on the speed at which sporulation could be resumed. The resumption of sporulation by strains of either *B. bassiana* or the *Verticillium* sp. was generally faster than *V. lecanii* strains. For example, after heat treatment of 36°C for 3 days or 40°C for 1 day *V. lecanii* could sporulate only after 15 days as opposed to 5 days in case of *B. bassiana*. The above mentioned property might be

Table 9. Effect of temperature range on growth and sporulation of various entomopathogenic fungal strains.

Strain designation	Temperatures (°C)					
	Minimum		Optimum		Maximum	
	G	S	G	S	G	S
V. lecanii; Is-6	8	12	20-25	20-25	30	30
B. bassiana; BZ	8	16	20-30	20-30	34	32
Verticillium-like sp.	10	20	25-32	25-32	36	36

crucial for induction of epizootic under arid or semiarid climates since temperatures above 36°C may occur quite frequently. Therefore during selection special care should be given not only to optimal temperature for growth rate and sporulation but also the ability of the fungal strain to regenerate sporulation following heat treatment.

V. Phenotypic diversity of fungal isolates in relation to virulence (the studies were performed in Kazakhstan)

Identification of traits that might be responsible for virulence of fungal pathogens on insects would facilitate the selection of isolates for biological control. Previous workers determined that *V. lecanii* virulence for the aphid *Macrosiponiella sanborni* is associated with the following traits: fast germination, high sporulation rate, an absence of extracellular amylase activity and high extracellular chitinase activity (Jackson *et al*, 1985). In this study, we have examined the diversity of some phenotypic, physiological and biochemical traits in relation to virulence towards the whiteflies GW and SPW.

Materials and Methods

Size determination of conidiospores and blastospores

Conidiospores were harvested from 7-day-old culture plates on SDA by washing with water. Blastospores were obtained from Sabouraud liquid medium (SLM) after 7 days. The size of spores and blastospores was recorded with a micrometer in the ocular of a microscope. Results represent an average of 100 spores or blastospores.

Growth rate determination

Discs (4 mm) were removed from 7-day-old culture plates and transferred to the center of fresh SDA plates. Diameters of the growing colonies were recorded daily during a week using 5 plates for each strain.

Dry mycelium was determined after 7 day growth in Erlenmyers containing 50 ml SLM on a shaker at 25°C. Four replications were used for each strain.

Determination of sporulation rate

Conidiospores were harvested from 12-day-old cultures of SLM with 10 ml of 0.005% Triton X100 per plate and their number was determined with an haemocytometer. The number of blastospores on SLM were determined after 7-day-growth in SLM.

Conidial and blastospore germination

Conidiospores were harvested from 7-day-old plates by washing with sterile water. Blastospores were harvested from 7-day liquid culture by filteration and washing with sterile water. Spores were used to inoculate SDA plates in a microchamber, which ensured the observation of spore germination by a microspore.

Extracellular enzyme production

Production of protease and chitinase were determined in agar plates according to Pavluschin, 1979. The following substrates were used: fatless mick agar for protease;

0.3% purified colloidal chitin prepared from crab spell for chitinase. Enzyme production was estimated according to the diameter cleared area in the agar plate. Lipase production was determined by a titration method of fatty acids (Nagaoka & Yamada, 1969). Catalase production was determined according to Bonnichsen, 1955, whereas peroxidase and polyphenoloxydase production was determined by a colorimetic method (Karjgina, 1986).

Results and discussion*

Phenotypic and biochemical traits associated with virulence

Phenotypic and biochemical variability was evaluated by a number of criteria:
a) size of conidia b) rate of mycelial growth and sporulation on solid media,
c) production of blastospores on liquid media, d) rate of spore germination.
Spore size of either *V. lecanii* or *B. bassiana* isolates was not related to virulence. On the other hand, radial growth rate and blastospore production of *V. lecanii* isolates were correlated with virulence. Growth rate of *V. lecanii* isolates with high virulence on solid media was 1.3-1.5 time lower, than growth rate of isolates with low virulence. In contrast, the number of blastospores after 5 days growth in liquid media was 1.5-2.0 time higher for isolates of *V. lecanii* which exhibit high virulence. However no correlation of the above mentioned traits could be detected with virulence of *B. bassiana*.
Spore germination on the insect cuticle is considered the first stage of fungal pathogenesis process. Fast germination is especially significant under suboptimal conditions since the survival time is reduced. In addition the rate of blastospores germination in the haemolymph of infected insects can have an influence on fungal spread throughout the insect and final sporulation and dissemination.
Our observation indicated that conidia and blastospores of strains with low virulence started to germinate during 18-24 hrs and reached maximal rate of 60-95% during 36 hrs. In contrast, conidia and blastospores of high virulence strains started to germinate during 10-12 hrs and reached maximal germination of 90-100% during 24-48 hrs. Thus the ability for fast germination was charachteristic of high virulence strains of both, *V. lecanii* and *B. bassiana*.

* Due to the limitted number of pages requested for the final report the tables containing the data of this section are not included in the report.

6. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

At the end of this project we have reached the point in which efficient mycoherbicides have been selected in Kazakhstan and Israel and are now available for application. We have learned as in preceding similar studies that it is not sufficient to have potent virulent strains which can perform well in the laboratory but rather emphasis should also be exerted on adaptation of the strains to the ecological niche where they are expected to perform.

Although the project has formally ended, it continues in both Kazakhstan and Israel. We assume that because of the perceived smaller potential dollar return compared with agrochemicals, most large companies are hesitant to become involved in fungal production technology. Furthermore, expertise and continuous assessment of quality is requested to make the mycoinsecticides control fulfill their potential. Therefore, to promote the use of fungal pathogens, especially in developing nations, suitable in-country mass-production techniques are needed to be advised. Such techniques should deliver viable and stable inoculum, should be simple to implement and maintain & utilize local media ingredients. Production must be cost-effective. Consequently, studies in Kazakhstan and Israel are continuing to design and test mass-production techniques for the selected fungal strains which have already proven to be effective under limited greenhouse trials. These techniques can then be transferred to local companies and/or cooperative farmers.

As a result of this project, the laboratory in Kazakhstan is now better equipped and able to perform future goals with modern techniques such as the use of DNA for studying genetic diversity in fungi and identify fungal strains by DNA fingerprinting. In this particular aspect the capability of the laboratory in Kazakhstan has dramatically improved.

7. PROJECT ACTIVITIES / OUTPUTS

The following papers were either published as manuscripts or presented in meetings:

Mor, H., Gindin, G., Ben-Zeev, I. S., Raccah, B., Geschtovt, N. U., Ajtkhozina, N. and Barash I. (1996) Diversity among isolates of *Verticillium lecanii* as expressed by DNA polymorphism and virulence towards *Bemisia tabaci*. *Phytoparasitica*. 24:111-118

Gindin, G., Barash I., Ben-Zeev, I. S. and Raccah, B. (1994) Biological control experiments with fungal pathogens of the sweetpotato whitefly *Bemisia tabaci*. The 7th Conference of the Entomological Society of Israel. p. 84.

Barash I., Mor, H., Gindin, G., Raccah, B., and Ben-Zeev, I. S. (1994) Selection and possible genetic manipulation of entomopathogenic fungi for biocontrol. International *Bemisia* Workshop, Shoresh, Israel. p. 35-36.

Ben-Zeev, I. S., Gindin, G., Barash I. and Raccah, B. (1994) Identification of entomopathogenic fungi attacking *Bemisia tabaci* in Israel. International *Bemisia* Workshop, Shoresh, Israel. p. 36.

8. PROJECT PRODUCTIVITY

The project has accomplished all the proposed goals. Furthermore, during the third year techniques for mass-production of selected strains and their application in larger scale have been initiated in Kazakhstan.

9. FUTURE WORK

The development of cost-effective mass-production techniques, quality control and methods of application (simple formulations) during IPM of various crops. The sensitivity of the fungal strains to various chemicals used during IPM should be determined. It is anticipated that local companies supported by farmers may be established for mass-production. The transfer of knowledge to the farmer is also considered an important component of future work.

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7. Pavluschin, V. A. (1979) Factores of *Beaeveria bassiana* virulence and pathogenesis of insects diseases. PhD Thesis. 205 p.VIZR, Leningrad.