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Title - Biocontrol of Insect Pests Using Aschersonia Species

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Principal Investigator: Prof. Ephraim Cohen
Grantee Institution: Faculty of Agricultural, Food and Environmental Quality Sciences,
The Hebrew university of Jerusalem, Rehovot, Israel
Tel. 972-8-9489718
Fax 972-8-9466768
e-mail address: ecohen@agri.huji.ac.il

Collaborator: Prof. Valentina Yasnosh
Institution: Institute of Plant Protection, 82 Chavchavadze Ave.
380062 Tbilisi, Republic of Georgia
Tel. 955-32996634
Fax 955-32001153
e-mail address: cisia@mymail.ge
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C. Executive Summary

The purpose of the project is to investigate the possible use of entomopathogenic fungi to biologically control important agricultural pests such as diaspidids and whiteflies. Entomopathogens belonging to the genus Aschersonia was isolated from diseased insects, bioassayed in the laboratory and mass-cultured on autoclaved millet. The sensitivity of conidia to solar-simulated radiation was investigated and photostabilization experiments were conducted using clays and clay-adsorbed photoprotectants. The range of insect pests sensitive to the entomopathogen was studied, primarily in Georgia, in order to identify the ones towards which control measures will be directed in the future.

It has been shown that the entomopathogenic fungus Aschersonia f. Georgia is effective against species of armored scales (Diaspididae) like Crysomphalus dictyospermi and the soft scale (Coccidae) Coccus hesperidum. The entomopathogen was also very effective against 1st and 2nd instar larvae of the cottony scale Neopulvinaria innumerabilis. The fungus was not effective against softscales (Coccidae) like Ceroplastes japonicus and C. sinensis (all stages) and against coccids of the family Pseudococcidae (Mealybugs). The favorable developmental stages for infection of the sensitive coccids were the 1st and 2nd instar larvae.

Five different potent photostabilizers were examined for their effects on the viability of Aschersonia f. Georgia. It was found that the positively charged photoprotectants such as acriflavin, berberine and palmatine are toxic to the fungal spores. The three compounds caused severe reduction in spore viability at extremely low concentrations. Two anionic dyes (Naphthol Yellow S (NYS) and Fast green (FG)) did not affect spore viability at very high concentrations. In a further study we found that the three cationic photostabilizers were cytotoxic for a mammalian cell line whereas the two anionic dyes were nontoxic at very high concentrations. The research proceeded using the photostabilizing capacity of matrices based on four types of clay (attapulgite, montmorillonite, kaolinite and bentonite) onto which the polycationic chitosan was adsorbed. These complexes served as a platform for the adsorption of the two toxicologically-safe anionic photostabilizers, NYS and FG. The composite matrices were highly effective in providing excellent photoprotection for Aschersonia spores.

It is believed that the joint project has a profound impact on labs and colleagues in the Kanchaveli Institute. The equipment and supplies as well as knowledge and exposure to skills gained and transferred were imperative for conducting experiments in the Georgian Institute. It also provided a solid basis for a sustainable capacity to independently perform same or similar research activities.

D. Research Objectives

Chemical control has been plagued by myriad problems associated with short and long term toxic effects, negative impact on the environment and the development of resistance to large number of pests. The current trend in both developed and developing countries is to gradually reduce dependence, and ultimately, ban broad spectrum, mostly neuroactive synthetic pesticides from being applied to control pests
in agriculture and public health. The quest is for cost-effective alternative measures, that are selective, safe to the environment and with minimal impact on non-targets. Biocontrol agents including entomopathogenic fungi obviously have to potential to become such safe alternatives that are friendly to the environment and are compatible with integrated pest management (IPM) programs (Butt and Goettel, 2000; Federici, 1999; Menn and Hall, 1999; Wraight and Carruthers, 1999). The main objectives of the project were to find the range of insect pests sensitive to the entomopathogen, as well as to provide measures of protection to photosensitive spores. As the fungus was isolated from homopteran species, insect pests taxonomically related to this group were selected for bioassay. Biocontrol agents, including entomopathogenic fungi, are sensitive to solar irradiation (Ignoffo et al., 1972, 1977; Morris, 1983). Consequently, any successful performance in the field should address the above problem. To overcome environmental constraints like UV radiation, the photolabile spores were protected using novel composite matrices composed of clay, a positively charged biopolymer like the aminosugar chitosan and toxicologically-safe anionic photostabilizers like FG and NYS.

E. Methods and Results

Isolates –
Two Aschersonia isolates, which were obtained from infected insects (Japanese scale) in Georgia, were purified from contaminating microorganisms by successive transfers on PDA Agar plates. The pure isolates are being maintained on slants and on PDA Petri dishes. To obtain large quantities of spores the entomopathogens were cultured in 250 ml Erlenmeyer flasks on autoclaved millet or rice at 24°C. The spores were collected by washing cultures with sterile distilled water containing 0.05% Tween-80. The concentration of spore in the suspension was determined using a hemocytometer.

Sensitivity of a range of insect pests to Aschersonia –
The isolate (from infected Japanese scale) was purified at the Rehovot laboratory and tested on a variety of insect pests from the Eastern part of Georgia. The insect species are the following: Aspidiotus nerii Bouche', Coccus hesperidum L., Neopulvinaria innumerabilis (Rathan), Diaspis echinocacti Bouche', Eriococcus buxi Fonsc., Unaspis euonymi Comst., Carulaspis visci Schr. Most of the treated insects were in the adult (female) stage, only in the case of N. innumerabilis the larval stage was used. Plant leaves or branches infested with insects were sprayed with a suspension containing 6x10^8 spores per ml. The sprayed plants were covered with a plastic bag for 3 days and then was removed. Insect mortality was recorded on the 10th day.

There are great differences in sensitivity of coccid and diaspidid insect pests to the Aschersonia isolate. Mortality of A. nerii and C. hesperidum is much higher as compared to the other pests. The entomopathogen is less aggressive against E. buxi, U. euonymi and C. visci.
The previous report (September 1999 - December 2001) mostly covered research accomplished by the Israeli partner. Extension of the grant period was asked as the Georgian partner did not get any money for conducting their part of the research, for reasons discussed later in the report. Nevertheless, the Georgian team has accomplished to carry out some experiments and the results are summarized below.

The investigations were conducted in the Guria region. In this region, which is neighboring Adjara (Batumi), heavy infestation of the Japanese scale was observed in citrus groves of Ozurgeti, Shroma, Thvermagala, Natanebi and other places.

The results (Table 1) establish that the entomopathogenic fungus *Aschersonia* is pathogenic to a different degree to the five species of armored scale: *Lopholeucopsis japonica* Ckll., *Chrysomphalus dictyospermi* Morg., *Aonidiella citrina* Coq., *Lepidosaphes gloveri* Pack., and *L. becki* Newn. It is noteworthy that the last species should be recorded as a new host for the entomopathogen.

Although abundant among other scales, *C. dictyospermi* is the least infected by *Aschersonia* (8.4%). In contrast, the Japanese scale, *Lopholeucopsis japonica*, is a less abundant and, comparatively, can be regarded as a minor pest, is 100% infected by the fungus. It was also observed that *A. citrina* was appreciable infested (by 57.2%) by the entomopathogen.
Table 1. Distribution of armored scale and infestation by *Aschersonia* in the Guria region.

<table>
<thead>
<tr>
<th>Species of insects</th>
<th>Distribution (numbers of trees with scales)</th>
<th>Scales (No. per leaf)</th>
<th>Scales infested with <em>Aschersonia</em> No. per leaf</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysomphalus dictyosperma</em></td>
<td>50</td>
<td>16.7</td>
<td>1.4</td>
<td>8.4</td>
</tr>
<tr>
<td><em>Aonidiella citrina</em></td>
<td>65</td>
<td>20.1</td>
<td>11.5</td>
<td>57.2</td>
</tr>
<tr>
<td><em>Lepidosaphes beckii</em></td>
<td>45</td>
<td>20.2</td>
<td>3.8</td>
<td>19.0</td>
</tr>
<tr>
<td><em>Lepidosaphes gloveri</em></td>
<td>25</td>
<td>16.9</td>
<td>3.2</td>
<td>19.0</td>
</tr>
<tr>
<td><em>Lopholeucapsis japonica</em></td>
<td>25</td>
<td>11.5*</td>
<td>11.5*</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*Number per cm² of tree trunk

In 2002 results obtained from Guria and Adjara regions are summarized in Table 2. The Japanese scale *Lopholeucapsis japonica* has become a major pest of many crops along the Black sea coast of Georgia including citrus, tea and ornamental and wild plants. Since the heavy infestations that were observed in the 1980's, an increase in biodiversity and efficacy of natural enemies occurred that resulted in reducing the pest populations below the economic threshold of 25 scales per cm² of tree trunk. In particular, the importance of the entomopathogenic fungus *Aschersonia* as the leading biocontrol factor has emerged.

Table 2. Distribution of the Japanese scale *Lopholeucapsis japonica* and the entomopathogenic fungus *Aschersonia* spp. in citrus groves

<table>
<thead>
<tr>
<th>Location</th>
<th>Date*</th>
<th>Number of scales/cm² of tree trunk</th>
<th>Number of scales infected by fungus</th>
<th>% mortality of scales</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guria, Shorma farm</td>
<td>19.07</td>
<td>35.2</td>
<td>13.3</td>
<td>37.6</td>
</tr>
<tr>
<td>Guria, Shroma farm</td>
<td>21.10</td>
<td>9.2</td>
<td>6.2</td>
<td>67.3</td>
</tr>
<tr>
<td>Adjara, Akhalsheni farm</td>
<td>25.02</td>
<td>22.5</td>
<td>7.0</td>
<td>31.1</td>
</tr>
<tr>
<td>Adjara, Akhalsheni farm</td>
<td>05.08</td>
<td>19.4</td>
<td>7.3</td>
<td>17.0</td>
</tr>
</tbody>
</table>

*2002

The density of the Japanese scale in the summer of 2002 in the Guria farm was high (35.2 insects per cm²). This number, which dropped to 9.2 per cm², was parallel with
the high mortality rate of 67.3% due to the entomopathogen. In the Adjara farm *Aschersonia* was ineffective.

<table>
<thead>
<tr>
<th>Guria region</th>
<th>Insect species</th>
<th>Scales per leaf or trunk** area</th>
<th>Infected scales number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shorma* (9.06.03)</td>
<td><em>Chrysothemalus dictyospermi</em></td>
<td>1.1</td>
<td>0.1</td>
<td>9.0</td>
</tr>
<tr>
<td>** (9.06.03)</td>
<td><em>Lopholeucapsis japonica</em></td>
<td>30.4**</td>
<td>11.5</td>
<td>37.8</td>
</tr>
<tr>
<td>** (19.07.02)</td>
<td>&quot;</td>
<td>35.3</td>
<td>13.3</td>
<td>37.6</td>
</tr>
<tr>
<td>** (21.07.03)</td>
<td>&quot;</td>
<td>9.2</td>
<td>6.2</td>
<td>67.3</td>
</tr>
<tr>
<td>Thsvermagala* (23.07.03)</td>
<td><em>Lopholeucapsis japonica</em></td>
<td>3.2**</td>
<td>0.04</td>
<td>1.3</td>
</tr>
<tr>
<td>** (01)</td>
<td>&quot;</td>
<td>25.0**</td>
<td>11.5</td>
<td>46.0</td>
</tr>
<tr>
<td>** (01)</td>
<td><em>Chrysothemalus dictyospermi</em></td>
<td>16.7**</td>
<td>1.4</td>
<td>8.2</td>
</tr>
<tr>
<td>** (01)</td>
<td><em>Aonidiella citrina</em></td>
<td>20.1**</td>
<td>11.5</td>
<td>57.2</td>
</tr>
<tr>
<td>** (01)</td>
<td><em>Lepidosaphes becki</em></td>
<td>20.2**</td>
<td>3.8</td>
<td>19.0</td>
</tr>
<tr>
<td>** (01)</td>
<td><em>Lepidosaphes gloveri</em></td>
<td>16.9**</td>
<td>3.2</td>
<td>19.0</td>
</tr>
<tr>
<td>Adjara region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Akhalsheni (14.10.003)*</td>
<td><em>Aonidiella citrina</em></td>
<td>2.4</td>
<td>0.5</td>
<td>20.8</td>
</tr>
<tr>
<td>** (14.10.03)</td>
<td><em>Lepidosaphes gloveri</em></td>
<td>1.6</td>
<td>0.1</td>
<td>6.3</td>
</tr>
<tr>
<td>** (14.10.03)</td>
<td><em>Lopholeucapsis japonica</em></td>
<td>24.6**</td>
<td>11.6</td>
<td>47.2</td>
</tr>
<tr>
<td>** (25.02.02)</td>
<td>&quot;</td>
<td>22.5**</td>
<td>7.0</td>
<td>31.1</td>
</tr>
<tr>
<td>** (05.08.02)</td>
<td>&quot;</td>
<td>19.4**</td>
<td>3.3</td>
<td>17.0</td>
</tr>
</tbody>
</table>

*Various farms at the Goria and Adjara regions.*

It has been established that the entomopathogenic fungus *Aschersonia* is distributed and infected four armored scale insects: *Lopholeucapsis japonica* Ckl., *Chrysothemalus dictyospermi* Morg., *Aonidiella citrina* Coq., and *Lepidosaphes gloveri* Pack. A fifth species (*L. becki* Newn.) that is infested with the fungus has been recently observed. As in the previous observations the Japanese scale, *L. japonica*, is very sensitive to infection by *Aschersonia*. The other armored scale insects are only moderately infected by the entomopathogen.

The natural infection by *Aschersonia* and sensitivity of different diaspidid insects is summarized in Table 3. The Japanese scale *Lopholeucapsis japonica* is very sensitive to the entomopathogen as infection rates of 37.6-67.3% and 17.0-47.2%, were observed in Goria and Adjara regions, respectively. Diaspidids like *Aonidiella citrina* (20.8-57.2%) and *Lepidosaphes becki* (20.2%) were also infected by *Aschersonia* at appreciable rates. It appears that *Chrysothemalus dictyospermi* was the least sensitive among the examined diaspidids to the fungus. Applying conidia of the entomopathogenic fungus revealed that also *C. dictyospermi* can be appreciably infected by *Aschersonia* as up to 64.6% of this insect were infected 15 days post treatment with an inoculum load of $8 \times 10^6$ conidia per ml (Table 4).
Table 4. Mortality of insects following infection with conidia of *Aschersonia*

<table>
<thead>
<tr>
<th>Insect pest</th>
<th>Conc. of conidia (per ml)</th>
<th>% mortality (after days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysomphalus dictyospermi</em></td>
<td>8x10^6</td>
<td>0.1  5.9  29.2  64.6</td>
</tr>
<tr>
<td><em>Chrysomphalus dictyospermi</em>*</td>
<td>3x10^7</td>
<td>1.6  5.6  32.8  60.0</td>
</tr>
<tr>
<td><em>Coccus hesperidum</em>**</td>
<td>8x10^6</td>
<td>1.4  8.9  33.7  55.9</td>
</tr>
</tbody>
</table>

1Experiments were conducted in 1999 (September 21-October 18)
*At Batumi park on *Quercus glauca* (larvae, females)
**At Baumi botanical garden on citrus trees (larvae, females)
***Tbilisi laboratory on citrus potted plants (larvae, females)

Table 5. Efficacy of *Aschersonia* in controlling various coccids and diaspids—laboratory experiments

<table>
<thead>
<tr>
<th>Insect b</th>
<th>Plant</th>
<th>Conc. of codidia (per ml)</th>
<th>Infected insects, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspidiotus neri</em></td>
<td>Oleander sp.</td>
<td>5.6x10^6**</td>
<td>55.2</td>
</tr>
<tr>
<td><em>Carulaspis caruell</em></td>
<td>Thuya orientalis</td>
<td>&quot;</td>
<td>13.3</td>
</tr>
<tr>
<td><em>Diaspis ecinicaeti</em></td>
<td>Opuntia sp.</td>
<td>&quot;</td>
<td>35.3</td>
</tr>
<tr>
<td><em>Unaspis evonymi</em></td>
<td>Evonymus sp.</td>
<td>&quot;</td>
<td>17.3</td>
</tr>
<tr>
<td><em>Eriococcus buxi</em></td>
<td><em>Buxus sempervirens</em></td>
<td>&quot;</td>
<td>12.8</td>
</tr>
<tr>
<td><em>Coccus hesperidum</em></td>
<td>Citrus sp.</td>
<td>8.0x10^6***</td>
<td>54.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Citrus (potted plant)</td>
<td>6.0x10^6***</td>
<td>61.4</td>
</tr>
<tr>
<td>&quot;</td>
<td>Ornamental tree</td>
<td>&quot;</td>
<td>61.4</td>
</tr>
<tr>
<td><em>Neopulvinaria innumerabilis</em></td>
<td>Vine, leaves</td>
<td>6.0x10^6****</td>
<td>77.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>Vine, leaves</td>
<td>7.2x10^7******</td>
<td>50.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Potato, green shoot</td>
<td>8.3x10^7******</td>
<td>8.8</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>Malus sp.</em>, leaves</td>
<td>5.6x10^6*</td>
<td>31.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>Potato, green shoot</td>
<td>3.5x10^9*****</td>
<td>2.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Vine, leaves</td>
<td>3.5x10^9*****</td>
<td>80.5</td>
</tr>
<tr>
<td><em>Chrysomphalus dictyospermi</em></td>
<td><em>Magnolia grandiflora</em></td>
<td>1.5x10^5**</td>
<td>7.3</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>Quercus glauca</em></td>
<td>1.5x10^5**</td>
<td>10.5</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>Magnolia grandiflora</em></td>
<td>2.4x10^9***</td>
<td>51.5</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>Quercus glauca</em></td>
<td>2.4x10^9***</td>
<td>66.8</td>
</tr>
</tbody>
</table>

aThe experiments were conducted during 1998-2000.
bInfected insects were females and 1st and 2nd instar larvae.
Days post infection - *10; **15; ***7; ****3; *****5.

Table 5 summarizes results of laboratory experiments in which various coccids and diaspids on a variety of plants were treated with different concentrations of *Aschersonia*. As in the previous table it was observed that *C. dictyospermi* is sensitive to the entomopathogen. A higher load of spores yielded increased rates of infection with scales on both *Magnolia grandiflora* and *Quercus glauca*. Similarly, the coccid *Coccus hesperidum* was also sensitive to the fungus (42.3-61.4% infection) on both citrus and ornamental tree at a conidium load of about 7x10^7 per ml. In contrast, it is interesting to note that apparently the plant species had an indirect effect of the rate of
infection. High infection rates were observed with *Neopulvinaria innumerabilis* on vine and apple tree (31.1-80.5%), whereas very low infection (2.0-8.8%) was found when the insect was grown on green shoots of potato.

Table 6. Efficacy of *Aschersonia* in controlling diaspidids on different plant - field experiments

<table>
<thead>
<tr>
<th>Diaspidid</th>
<th>Plant</th>
<th>Conc. of conidia (per ml)</th>
<th>Infected insects, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Neopulvinaria innumerabilis</em></td>
<td>Vine</td>
<td>$3.5 \times 10^6$</td>
<td>7.2</td>
</tr>
<tr>
<td><em>Chrysomphalus dictyospermi</em></td>
<td>Citrus</td>
<td>**</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Citrus</td>
<td>**</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>Ficus sp.</em></td>
<td>**</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Citrus</td>
<td>$3 \times 10^7$</td>
<td>22.4</td>
</tr>
<tr>
<td>&quot;</td>
<td><em>Quercus glauca</em></td>
<td>$8 \times 10^6$</td>
<td>42.1</td>
</tr>
</tbody>
</table>

*Experiments conducted during 1998-2000.*

*Tbilisi private garden; *Batumi region - field.

Days post infection *15; **10.*

Figure 2. The effect of Tween-80 on germination of *Aschersonia* spores

Five different photostabilizers were examined as to their effect on spore viability of *Aschersonia*. Viability of spores exposed to very high concentrations of the toxicologically-safe anionic dyes such as NYS and FG were not affected. The positively charged photoprotectants such as acriflavin, berberine and palmatine
drastically reduced spore germination and acriflavin was highly inhibitory as a IC\textsubscript{50} value is about 7\mu. These results were corroborated with the cytotoxicity bioassay using osteosarcoma (human) 143B.TK\textsuperscript{−} cultured cells (Figure 1). The cationic dyes palmatine and berberine display a 50% reduction in the viability of the cultured cells at a level of 3-5 mM. Compared to the above, acriflavin is extremely toxic with IC\textsubscript{50} value three orders of magnitude lower (2\mu M). In comparison, the anionic photostabilizers were completely non-toxic at a very high concentration of 1M.

![Figure 1. Cytotoxic effects by several photostabilizing compounds in 143B.TK\textsuperscript{−} osteosarcoma cultured cells. NYS (Naphthol Yellow S) and FG (Fast Green) were non cytotoxic at a 1M level. Probit 5 = 50% inhibition.](attachment:image)

Clay surface, which is negatively-charged was converted to positive by adsorption of the polycation polymer chitosan. Illustration of such conversion is evident by measuring the Zeta potential values using various concentrations of chitosan adsorbed to montmorillonite (Figure 2). At chitosan concentration of 0.03% the initial negative charge is close to zero, while a level of 0.05% a dramatic conversion occurred and the measured Zeta potential exceeded +20 mV.
Figure 2. Zeta potentials of montmorillonite-chitosan complexes. Chitosan was dissolved in lactic acid and adsorbed onto the clay, and the Z.P. (Zeta potential) values of the complex were determined.

The four positively-charged clay-chitosan complexes were used as a platform for adsorbing the anionic dyes FG and NYS. The dyes were easily adsorbed onto the clay-chitosan matrix and the values in Table 7 show the amount of stabilizers adsorbed to the various clay complexes.

Table 7. Adsorption of anionic dyes to various clay-chitosan complexes.

<table>
<thead>
<tr>
<th>Clay-chitosan</th>
<th>Adsorption (mmole/gr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FG</td>
</tr>
<tr>
<td>attapulgite</td>
<td>0.45</td>
</tr>
<tr>
<td>montmorillonite</td>
<td>0.77</td>
</tr>
<tr>
<td>bentonite</td>
<td>0.43</td>
</tr>
<tr>
<td>kaolinite</td>
<td>0.95</td>
</tr>
</tbody>
</table>

A solution of the dye dissolved in distilled water (0.2M) was added to 50 mg of clay-chitosan complex suspended in distilled water. The mixture was stirred overnight and spun for 25 min at 11,000 rpm. The pellet was washed three times by centrifugation and dried by lyophilization. The amount of dye adsorbed to a clay-chitosan matrix was calculated from concentration changes in solution before and after the centrifugation steps, using the extinction coefficients of the relevant dye.
The cation exchange capacity rates of the clays (in meq/100gr) as reported in the literature are: 5-30 for attapulgite, 75-100 for montmorillonite, 70-90 for bentonite and 5-17 for kaolinite.

Data presented in Table 1 clearly show a significant adsorption of the anionic dyes onto the clays. Depending on the clay examined, NYS was adsorbed in the range of 1.45-2.15 mmoles per gr clay. Adsorption of FG, with a molecular weight more than twice higher compared to NYS, was by 2-3 folds lower (0.43-0.95 mmoles per gr). FG is adsorbed at a greater rate to montmorillonite and kaolinite compared to the other two clays. NYS is adsorbed more or less at the same amount to montmorillonite, bentonite and kaolinite, but less to attapulgite.

![Figure 3](image1.png)

Figure 3. The effect of various clays on the germination rate of *Aschersonia* spores. The spores were exposed to 5% suspension of clays. Bent.- bentonite; mont. - montmorillonite; attap. - attapulgite; kaol. - kaolinite.

Clays *per se* like bentonite and montmorillonite up to 5% in aqueous suspension have no inhibiting effect on spore germination (Figure 3). Slight inhibition effects were observed when the fungus spores were exposed to attapulgite and kaolinite.

![Figure 4](image2.png)

Figure 4. Germination rates of *Aschersonia* spores exposed to artificial near-UV irradiation.

The *Aschersonia* species sporulates large multicellular spores (70-80 microns in length). Spores and germinating spores could be easily visualized even when mixed
with the opaque suspension of clay matrices used for photoprotection. To facilitate the photostabilization experiments, short periods of about 3 hrs of irradiation is advantageous. It was found that under the setup of the irradiation chamber where the irradiation source is 35 cm away from the unprotected spores, germination was blocked after 3 hrs of irradiation (Figure 4). A 50% reduction in germination is evident after about 100 min of irradiation.

Figure 5. Micrographs of germinating and non-germinating Aschersonia sp. spores.

The advantage of working with large spores (Figure 5) resides in the directness of spore counting, thus avoiding the time consuming colony plaque units (CFUs) procedure.

Figure 6. The photostabilizing effect of clays on Aschersonia spores. Spores were mixed with various levels of bentonite and kaolinite and exposed to irradiation for 3 hrs.
Figure 7. The photostabilizing effect of clays on *Aschersonia* spores. Spores were mixed with various levels of montmorillonite and attapulgite and exposed to irradiation for 3 hrs.

Clays to a considerable degree are capable of photoprotection (Figures 6 and 7). Up to 60% of *Aschersonia* spores survived a 3 hrs irradiation period when mixed with bentonite and attapulgite at the highest levels (5 mg/ml). Compared to the above, under similar conditions about 30% and 10% of the spores germinated when mixed with kaolinite and montmorillonite, respectively. The photostabilizing capacity of clays may be attributed to their light dispersing property.

Figure 8. The photostabilizing effect of kaolinite-chitosan-dye matrices on *Aschersonia* spores exposed to 3 hrs of irradiation. Irradiated samples covered with aluminum foil served as control.
Figure 9. The photostabilizing effect of attapulgite-chitosan-dye matrices on *Aschersonia* spores exposed to 3 hrs of irradiation. Irradiated samples covered with aluminum foil served as control.

Figure 10. The photostabilizing effect of montmorillonite-chitosan-dye matrices on *Aschersonia* spores exposed to 3 hrs of irradiation. Irradiated samples covered with aluminum foil served as control.
Figure 11. The photostabilizing effect of bentonite-chitosan-dye matrices on \textit{Aschersonia} spores exposed to 3 hrs of irradiation. Irradiated samples covered with aluminum foil served as control.

Invariably, clay-chitosan-FG matrices were powerful photostabilizers (Figures 8-11). After 3 hrs of irradiation, which blocks germination of unprotected spores, 100\% of spores germinated at a level of 5\% clay matrices based on montmorillonite, attapulgite and kaolinite. Also with the bentonite matrix (Figure 11), only a mere 10\% of the spores were non-viable. The effect was dose-dependent and related to the amount of clay matrix in the suspension. Even the lowest level of clay complexes examined (0.05\%) was sufficient to substantially photoprotect spores, and the level of germination was 50\%-60\% with bentonite, kaolinite and attapulgite. (about 20\% with montmorillonite). In contrast, NYS (Figures 8-10) was not effective in matrices based on attapulgite and bentonite, with a little difference in photostabilization as compared to the relevant clays \textit{per se}. It is evident, that when photoprotection is not a result of the clay the photostabilizing potency of NYS complexes is apparent. With clays like montmorillonite and kaolinite, which as such, contributed less photoprotection, the corresponding NYS-containing matrices provided significant photostabilization.

\textbf{F. Impact, relevance, and Technology Transfer}

The intensive study involving clay-based photostabilizing matrices has been essential for improved field performance of photolabile biocontrol agents such as \textit{Aschersonia}. Already we have ingredients for viable formulations that contain photoprotecting clay-chitosan-dye matrices. Repeated laboratory assays using UV source that simulates solar radiation have clearly demonstrated their extensive capacity for photostabilization of the biocontrol agent. The above studies are essential for devising appropriate formulations and for carrying out large field experiments. Field observations in different regions in Georgia revealed that insect pests like coccids and diaspidids are infected by \textit{Aschersonia}. The findings generated by the project are important for implementing biocontrol measures and to avoid relying on chemical pesticides. Aside from the practical aspects mentioned above, the project
benefited the scientists and labs involved in the project. Although studies focused on *Aschersonia*, various species of entomopathogenic fungi (*Verticillium, Beauveria, Fusarium*) from Georgia orchards were isolated, identified and are ready for further research in the future in both countries. Technologies developed in Rehovot that involve propagation (solid-substrate fermentation) of *Aschersonia*, preparing photostabilizing matrices, irradiation and bioassay techniques were practiced by scientists from the Kanchaveli Institute, Tbilisi. The scientists spend two training periods (first and last years of the project) in Israel to ensure technology transfer. Some small equipment and chemicals purchased for the Georgian team helped in the research conducted there. Obviously, the acquired experience will have a profound impact on the scientific capabilities and on ways research is carried in the relevant labs and departments, and as a whole, at the Georgian Institute. As the project is completed, skills gained and equipment will help for sustaining research on biocontrol agents such a fungal entomopathogens as alternatives for chemical control measures.

G. Project Activities/Outputs

**Meetings:** The Israeli PI (E. Cohen) traveled to Tbilisi, Georgia on July 1998. He met there the collaborating team at the Kanchaveli Institute to start and coordinate the research. On October 1998 the Georgian collaborator (V. Yasnosh) travel to Rehovot. Another meeting took place in Jerusalem (July 25-30, 1999) where the Georgian PI and Dr. Tabatadze from of her research team attended the International Congress of Plant Protection (IPPC). Presenting abstracts and being exposed to the international community of scientists who study various aspects of plant protection were of tremendous importance. Attending such an international conference created an excellent opportunity to meet scientists from the international community and be exposed to current ideas, novel approaches and techniques associated with pest control. During the above meetings progress, problems and accomplishments related to the project were discussed.

**Training:** The first cycle of training for one month was carried out at Rehovot on October 1988. Dr. L. Chkhaidze and Dr. E. Tabatadze from the Georgian team of Prof. Yasnosh participated in the training program that involved isolation and culturing of *Aschersonia* as well as conducting bioassays. On the way back to Georgia they carried with them equipment, supplies and materials purchased in Israel to facilitate the planned experimental research.

Dr. E. Tabatadze spent about 7 weeks for a second training period (December, 2003 - January, 2004) in Rehovot. During this visit she conducted various experiments related to photostabilization of *Aschersonia* using novel clay-polymer-dye matrices devised in the PI lab.

**Publications:**


H. Project Productivity

The project accomplished and met most of its original objectives. We isolated a virulent *Aschersonia* species from infected insects collected in Georgia, and succeeded in obtaining large quantities of conidia by solid-state fermentation using substrates such as millet or rice. As consistent field performance of biocontrol agents such as fungal entomopathogens hinges on photostabilization of photosensitive spores, a significant part of the studies were directed to achieve this goal. Obviously, during a normal course of a research emphasis might be modulated and placed on new approaches that nevertheless are in line with overall goals. For example we have conducted extensive studies with novel matrices (not included and detailed in the original objectives) of clay-chitosan that readily adsorb negatively-charged photostabilizing dyes like the toxicologically safe Fast Green and Naphthol Yellow S. The notable photostabilizing capacity of such matrices on *Aschersonia* conidia has been demonstrated, and consequently, should serve the purpose of improved formulation and field performance of biocontrol agents. Planned field experiments in Georgia with stabilized formulations of *Aschersonia* were not carried out. The reason, as mentioned in previous reports, is due to chronic problems of money transfer to the Georgian partner.

I. Future Work

The project has the evident potential for future work that includes a variety of important aspects. 1) Improved mass production of spores and formulations. 2) Field experiments to generate data concerning efficacy of the entomopathogen. 3) In addition to *Aschersonia*, photostabilized formulations with other fungal entomopathogens, which are known to exist in nature in Georgia, can be examined. 4) Approaching commercialization of the biocontrol agent as a mycoinsecticide.

J. Literature cited


