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INDUCE RESISTANCE IN SUBTROPICAL FRUITS FOR THE PREVENTION OF DECAY INITIATED FROM QUIESCENT INFECTING PATHOGENS

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Table of Contents

Summary.....3

Section I

One. Research objectives.....3

Two. Research accomplishments.....4

c. Future Work.....15

Section II

Collaboration, Travel, Training 15

Executive Summary-

The work that was done in the first year included the search for new non-pathogenic mutants that can be used for the control of postharvest diseases in subtropical fruits. Our approach has been to look for natural mutants in Philippines and molecular engineered mutants in Israel. Both mutants were tested against the disease in the respective countries and initially good responses were found.

Section I

a. Research objectives

The intention of this research is to develop new technology for control quiescent infections of subtropical fruits by enhancing mechanism of resistance that have been already elucidated by utilizing the non or reduced pathogenesis mutants or direct elicitor of the resistance mechanism. The kind of non pathogenic *Colletotrichum* strain are similar to the Path-1 that infects avocado fruit and does not cause significant disease symptoms but appears to stimulate host defense responses. Host organisms to be studied include avocado and mango fruits. The pathogens will be *Colletotrichum gloeosporioides* in avocado and mango fruit, and selected Colletotrichum strains.

1. **Selection and development of non-pathogenic *Colletotrichum* mutant.** It is imperative for the fungus in order to induce resistance to penetrate the fruit cuticle. Based in our early results *Colletotrichum* is the best organism that can penetrate the fruit cuticle and enhance the host defense response. Several steps for the development of non-pathogenic strains will be done parallel.

Natural selection: non-pathogenic strain will be isolated from small limited lesion in decaying fruit. We will test whether several *Colletotrichum* sp. from host other than avocado and mango, can enhance resistance to *Colletotrichum*. We expect to detect other non-pathogenic strains that

breached the peel, remain as endophytes, but are not between those known pathogen attacking mango or avocado.

UV treatments: UV treatment will be used for the development of new mutants and selection similarly as described previously (Freeman and Rodriguez, 1992). This method has been very efficient for obtaining the Path-1 mutant.

REMI- Restriction enzyme mediated integration technique (Redman and Rodriguez, 1994) will be used for obtaining mutant of *C. gloeosporioides*. This method was used for tagging and cloning of genes required for pathogenicity by Nir Yakoby in Prusky's lab. An efficiency of 80 transformants per microgram of DNA was obtained by this method in the Isreali Lab. The efficiency of this mutant for induction of resistance was tested in Israel and found that it induces resistance. We suggest that this same mutant should be tested in Philippines. For this purpose the mutant will be sent to Philippines and there the efficiency of the mutant tested.

Following the development of specific isolates that are non-pathogenic will be selected by any of those methods they will be tested for induce resistance.

b. Research accomplishments

Phillipines

Selection of natural mutants with reduced pathogenicity that induces resistance against *Colletotrichum* attacking mango fruits.

About 30 strains of *Colletotrichum* originated from different plants species were tested and found that 3 of them are non pathogenic on mango fruits and one isolate has shown reduced pathogenicity. This first year was mainly directed to the selection of reduced pathogenicity strains that induce resistance. A report of the whole work is described below.

Israel

This first year was used to select and test the pathogenicity of mutant obtained by the method of REMI- Restriction enzyme mediated integration technique according to Redman and Rodriguez, 1994.

Nir Yakoby in Prusky's lab has used this method for obtaining mutants with reduce pathogenicity. We have tested about 2000 transformants from where 14 isolates were obtained with reduced pathogenicity. We are presently testing their capability to induce resistance against the wild type *C. gloeosporioides* attacking avocado fruits. A report of this work is presented below.

PHILLIPINES

I. SUSCEPTIBILITY OF MANGO FRUITS TO *Colletotrichum gloeosporioides* AT DIFFERENT STAGES OF FRUIT DEVELOPMENT

Methodology

The susceptibility of mango fruits to the anthracnose pathogen, *Colletotrichum gloeosporioides* (*C. g.*), at various stages of fruit development was tested. Mango fruits at different stages of development were inoculated both in the field and in the laboratory.

For the laboratory experiment, mango fruits were picked (harvested) and inoculated at 57-71 days after flower induction (DAFI); 85-106 DAFI; 115 DAFI (green mature stage); colorbreak stage, and ripe stage. Another trial was made on mango fruits of another tree using the same procedure but at slightly different developmental stages. Fruits were inoculated at 65-100 DAFI; 110 DAFI; 120 DAFI (green mature), colorbreak and ripe stages. At each stage of fruit development, mango fruits were inoculated with 10^6 and 10^5 spore concentration of the pathogen (*C. g.*) and enclosed with plastic to enhance germination of the spores. The plastic cover was removed after 48 hours and symptoms were observed after 7 days of incubation.

In the field experiment, mango fruits were inoculated while on the tree with 10^5 concentration of *C. g.* at 57 DAFI; 71 DAFI; 85 DAFI; and at 106 DAFI, on the first trial. Fruits were also inoculated with *C. gloeosporioides* and with the challenge fungus, *C. capsici,* at all stages of fruit development. This means to say that the same fruits were inoculated repeatedly at each fruit stage. Mango fruits were harvested at 115 DAFI. In the second trial, mango fruits were inoculated at 65 DAFI; 86 DAFI; 100 DAFI; and at 110 DAFI. Inoculation at all stages of fruit development was likewise done. Fruits were harvested at 120 days after flower induction. Infection was observed and rated after symptoms were apparent.

Results

Results of the laboratory tests on the susceptibility of mango fruits showed that mango fruits are most susceptible to *Colletotrichum gloeosporioides* (Table 1.1a) at the early stages of fruit development and when they are ripe.

The highest infection rate was observed in fruits harvested 57-71 days after flower induction (DAFI) at 90.5% with the 10^6 spore concentration while 86.2% with 10^5. The least infection was observed at the green mature and the colorbreak stages of fruit development. An infection rate of 59.0% at 10^6 spores/ml and 45.5% at 10^5 was observed at the green mature stage while 53.2 and 46.2% infection was observed at the colorbreak stage with 10^6 and 10^5 spores/ml inoculum, respectively. At ripening,

however, the fruits became more susceptible again to anthracnose infection. An infection rate of 88.8% at 10^6 spores/ml and 81.8% at 10^5 was observed at the ripe stage of mango fruit development.

Table 1.1a. Susceptibility of Harvested Mango Fruits at Different Stages of Development to *Colletotrichum gloeosporioides* (Trial I)[1]

Stages (days after flower induction or **DAFI**)	Average % Infection Trial I	
	At 10^6 spores/ml	At 10^5 spores/ml
57-71	90.5 a	86.2 a
85-106	48.4 b	85.2 a
115 (green mature)	59.0 b	45.5 b
Colorbreak	53.2 b	46.2 b
Ripe	88.8 a	81.8 a

[1] Data based on the average of four replications.
[2] Figures with the same letter (s) in a column are not significantly different at LSD_{05} 14.3397 in 10^6 spore concentration and LSD_{05} 17.1167 in 10^5 spore concentration.
[3] Average % infection based on the total surface area of the fruit infected.
[4] Inoculations were done at different fruit stages: 57-71 days after flower induction (DAFI); 85-106 DAFI; 115 DAFI (green mature stage); colorbreak stage, and ripe stage.

Similar results were obtained in the second trial of the experiment conducted in the laboratory (Table 1.1b). Again, the most susceptible stage to anthracnose infection was the earlier stage of fruit development. This was observed at 65-100 days after flower induction (DAFI) with 85.6% infection with a spore load of 10^6 spores/ml and 82.7% infection with 10^5 spores/ml. As the mango fruit approached maturity, it appeared to develop some kind of resistance to the pathogen, *Colletotrichum gloeosporioides.* This was noted in less infection observed at 110 DAFI, green mature, and colorbreak stages with 68.5, 67.0 and 72.2% infection with 10^6 spore concentration and 54.5, 55.0, and 51.2% with 10^5 spores/ml, respectively. As the fruit ripened, resistance was reduced as observed in the ripe stage with 82.8% infection at 10^6 spores/ml and 73.8% at 10^5.

This confirms the results of the first trial that young fruits (up to 110 days after flower induction) are more susceptible than the green mature fruits and those at the colorbreak stage. However, ripe fruits are as susceptible as the young fruits.

The high infection rates may be explained by the high levels of inoculum applied.

Table 1.1b. Susceptibility of Harvested Mango Fruits at Different Stages of Development to *Colletotrichum gloeosporioides* (Trial II)[1]

Stages (days after flower induction or **DAFI**)	Average % Infection Trial II	
	At 10^6 spores/ml	At 10^5 spores/ml
65-100	85.6 a	82.7 a
110	68.5 b	54.5 b
120 (green mature)	67.0 b	55.0 b
Colorbreak	72.2 b	51.2 b
Ripe	82.8 a	73.8 a

[1] Data based on the average of four replications.
[2] Figures with the same letter (s) in a column are not significantly different $LSD_{.05}$ 7.3756 in 10^6 spore concentration and LSD_{05} 17.1888 in 10^5 spore concentration.
[3] Average % infection based on the total surface area of the fruit infected.
[4] Inoculations were done at different fruit stages: 65-100 DAFI; 110 DAFI; 120 DAFI (green mature), colorbreak and ripe stages.

The field experiment on the susceptibility of mango fruits to *Colletotrichum gloeosporioides* when inoculated while fruits were intact on the tree showed that as the fruit approached maturity, it became more resistant to anthracnose infection (Table 1.2a). This was noted at 106 DAFI with 3.1% infection where the least infection was observed. This was not significantly different at 85 DAFI with 8.6% infection. The highest infection rate was observed when fruits were inoculated with the pathogen at all stages of fruit development with 55.3% infection followed by inoculation at 57 DAFI with 48.0% infection which were not significantly different from each other.

Table 1.2a. Susceptibility of Intact Mango Fruits to *Colletotrichum gloeosporioides* at Different Stages of Development (Trial I)[1]

Stages (days after flower induction or **DAFI**)	Average % Infection Trial I
57	48.0 ac
71	35.1 a
85	8.6 b
106	3.1 b
At all stages	55.3 c

[1] Data based on the average of seven replications.
[2] Figures with the same letter (s) are not significantly different at LSD_{05} 18.2016 in comparing treatments in a column.
[3] Average % infection based on the total surface area of the fruit infected.
[4] Inoculations at 10^5 concentration of *C. gloeosporioides* were done at different fruit stages: 57 DAFI; 71 DAFI; 85 DAFI; 106 DAFI and at all fruit stages. Fruits were harvested at 115 DAFI at the green mature stage.

The second trial of the field experiment showed that infection rate was also highest at the earlier stage of fruit development (Table 1.2b). An infection rate of 35.0% was observed at 65 DAFI which was not significantly different at 86 DAFI with 30.8% nfection. Less infection was noted at 100 and 110 DAFI with 16.0 and 23.1% infection, respectively. Fruits that were inoculated at all stages, showed the highest infection rate (at 64.6%) as expected.

Table 1.2b. Susceptibility of Intact Mango Fruits to *Colletotrichum gloeosporioides* at Different Stages of Development (Trial II)[1]

Stages (days after flower induction or **DAFI**)	Average % Infection Trial II
65	35.0 a
86	30.8 ab
100	16.0 b
110	23.1 b
At all stages	64.6 c

[1] Data based on the average of seven replications.
[2] Figures with the same letter (s) are not significantly different at $LSD_{.05}$ 18.7538 in comparing treatments in a column.
[3] Average % infection based on the total surface area of the fruit infected.
[4] Inoculations at 10^5 concentration of *C. gloeosporioides* were done at different fruit stages: 65 DAFI; 86 DAFI; 100 DAFI; and at 110 DAFI and at all fruit stages.

II. EFFECT OF *Colletotrichum capsici* (013) ON MANGO ANTHRACNOSE

Methodology

The potential of *Colletotrichum capsici* (013) obtained from pepper fruit, to induce resistance to anthracnose in mango fruits, was tested both in the laboratory and in the field.

For the laboratory part, newly harvested mango fruits were inoculated at various stages of mango fruit development: at 57-71 days after flower induction (DAFI); 85-106 DAFI; 115 DAFI (green mature stage); colorbreak stage, and ripe stage in trial I. Fruits were inoculated with different concentrations of the pathogen and of *C. capsici* at each growth stage of the fruit.: $dH_2O \longrightarrow 10^5$ *C. g.*; $dH_2O \longrightarrow 10^6$ *C. g.*; 10^5 013 $\longrightarrow$ 10^5 *C. g.*; 10^6 013 $\longrightarrow$ 10^6 *C. g.*; 10^5 013 $\longrightarrow 10^6$ *C. g.*; 10^6 013 $\longrightarrow$ 10^5 *C.g.* *C. capsici* was inoculated 48 hours prior to the inoculation with *C. gloeosporioides*. Fruits were enclosed with a plastic cover after each inoculation to facilitate germination which was removed after 2 days. Symptoms were observed after 7 days of incubation.

Mango fruits that were intact on the tree were inoculated in the field at various stages of maturity: 57 days after flower induction (DAFI); 71 DAFI; 85 DAFI; and 106 DAFI. Inoculation at all stages of fruit development on the same fruits was also done meaning that the same fruits were inoculated repeatedly at each fruit stage. Fruits were inoculated with 10^6 spore concentration from a 7-day old culture of *C. capsici* at each fruit stage and then enclosed with plastic. The control set-up was sprayed with distilled water. After 48 hours, the plastic cover was removed and the fruits were then inoculated with 10^5 concentration of the 7-day old culture of the pathogen, *C. g.* Control set-up was inoculated with the pathogen only. Fruits were also enclosed with plastic which was removed after 48 hours. Mango fruits were harvested at 115 days after flower induction. A second trial was made using the same procedure as the above. Fruits were inoculated at 65 DAFI; 86 DAFI; 100 DAFI; and 110 DAFI. Inoculation at all stages was also done. Fruits were harvested at the green mature stage, 120 days after flower induction. Infection was observed and rated after symptoms occurred or after the fruits turned yellow.

Results

Comparing treatments in a column using letters a to c, the least infection was generally noted at the green mature and colorbreak stages at each of the different concentrations of the pathogen and of *C. capsici* (Table2.1). The highest infection was observed at the earliest stage of fruit development (57-71 days) and at the ripe stage. In comparing treatments in a row using letters w to y to determine the effect of *C. capsici* application prior to *C. gloeosporioides* inoculation, it appeared that *C. capsici* at 10^6 spores/ml significantly reduced anthracnose infection when applied to green mature (115 DAFI), colorbreak and ripe fruits when the inoculum level for the pathogen was 10^5 spores/ml. No significant differences were found at the susceptible fruit stages (57-106 DAFI) nor when the pathogen spore load was higher (10^6 spores/ml).

Table 2.1. Effect of *Colletotrichum capsici* (013) Application on Anthracnose Development in Harvested Mango Fruits

Fruit Stages	Average % Infection					
	Pathogen at 10^6 spores/ml			Pathogen at 10^5 spores/ml		
	Pathogen only	Pathogen and 013 (10^6 spores/ml)	Pathogen and 013 (10^5spores/ml)	Pathogen only	Pathogen and 013 (10^6 spores/ml)	Pathogen and 013 (10^5 spores/ml)
57-71 DAFI	86.2 a w	81.9 a w	84.9 a w	88.8 a w	81.5 a w	79.5 a w
85-106 DAFI	85.2 a w	75.6 a w	79.0 a w	75.4 ab w	74.8 a w	75.4 a w
115 DAFI	43.0 b x	39.5 b x	47.0 b wx	63.2 b wy	36.8 b x	77.0 a y
Colorbreak	46.2 b xy	40.0 b x	60.8 bc wy	64.5 b w	47.0 b xy	65.0 a w
Ripe	73.8 a wx	66.2 a x	70.8 ac x	88.8 a w	70.8 a x	74.0 a wx

[1] Data based on the average of four replications.
[2] Figures with the same letter (s) from a to c are not significantly different at LSD_{05} 16.6279 in comparing treatments in a column and w to y in comparing treatments in a row.
[3] Average % infection based on the total surface area of the fruit infected.
[4] Inoculations were done at different fruit stages: 57-71 days after flower induction (DAFI); 85-106 DAFI; 30.8% 115 DAFI (green mature stage); colorbreak stage, and ripe stage.

The field experiment, where intact fruits on the tree were treated with *C. capsici* (99-013) and the pathogen, showed that *C. capsici* had no statistically significant effect ($LSD_{.05}$) on anthracnose development. However, the absolute values indicated some reduction in percentage infection when *C. capsici* was applied, compared to when only the pathogen was present (Table 2.2a and Table 2.2b). Further experiments are needed to determine the effect of *C. capsici* under field conditions, particularly the effect of increasing the frequency of its application.

Table 2.2a. Effect of *Colletotrichum capsici* (Isolate 99-013) Application on Anthracnose Infection in Intact Mango Fruits in the Field (Trial I)[1]

Growth Stages (days after flower induction or **DAFI**)	Average % Infection (Trial I)	
	Pathogen only (*C. g.*)	99-013 + *C. g.*
57	48.0 a	38.3 a
71	35.1 a	20.6 a
85	8.6 a	7.3 a
106	3.1 a	1.1 a
All Stages	55.3 a	40.7 a

[1] Data based on the average of 7 replications.
[2] Figures with the same letter (s) in a row are not significantly different at LSD_{05} 18.2016.
[3] Average % infection based on the total surface area of the fruit infected.
[4] Inoculations were done at different fruit stages: at 57 days after flower induction (DAFI), 71 DAFI, 85 DAFI, 106 DAFI and at all fruit stages.

Table 2.2b. Effect of *Colletotrichum capsici* (Isolate 99-013) Application on Anthracnose Infection in Intact Mango Fruits in the Field (Trial II)[1]

Growth Stages (days after flower induction or **DAFI**)	Average % Infection (Trial II)	
	Pathogen only (*C. g.*)	99-013 + *C. g.*
65	35.0 a	19.6 a
86	30.8 a	15.4 a
100	16.0 a	13.3 a
110	23.1 a	13.2 a
All Stages	64.6 a	53.3 a

[1] Data based on the average of 7 replications.
[2] Figures with the same letter (s) in a row are not significantly different at LSD_{05} 18.7538.
[3] Average % infection based on the total surface area of the fruit infected.
[4] Inoculations were done at different fruit stages: at 65 days after flower induction (DAFI); 86 DAFI; 100 DAFI; 110 DAFI and at all fruit stages.

III. EFFECT OF POSTHARVEST TREATMENT WITH THE CHALLENGE ORGANISM, *Colletotrichum capsici* ON MANGO ANTHRACNOSE USING DIFFERENT APPLICATION TIMES

Methodology

The effect of postharvest treatment with *Colletotrichum capsici* on mango anthracnose using different frequency and application times was determined. Seven days prior to harvesting, the mango fruits were inoculated with 10^5 spore concentration of the pathogen, *Colletotrichum gloeosporioides*. The mango fruits were harvested at the green mature stage (120 days after flower induction).

Different treatments with *C. capsici* were applied to the harvested mango fruits that were previously inoculated with the pathogen. Inoculation with *C. capsici* at 10^6 spore concentration was done: (a) within 24 hours after harvest; (b) 24 and 72 hours after harvest; (c) within 48 hours after harvest; (d) 48 and 96 hours after harvest; (e) 72 hours after harvest; and (f) 96 hours after harvest. The control set-up, which was also inoculated 7 days before harvest with the pathogen, *C. g.*, was not inoculated with *C. capsici*.

Results

The postharvest treatment of mango fruits with the challenge organism, *C. capsici*, appears to have some potential in reducing anthracnose infection due to *C. gloeosporioides*. However, the time and frequency of application of *C. capsici* affected the effectiveness of the postharvest treatment. Results (Table 3) showed that the least infection was observed when *C. capsici* was applied twice, at 24 and at 72 hours after harvest, as indicated by only 10.1% infection compared to the control inoculated with the pathogen only with 59.7% infection. One application, whether at 24, 48, 72 or 96 hours after harvest, resulted in higher infection than the dual application (24 and 72 hours).

Table 3. Postharvest Treatment of Mango Fruits with *Colletotrichum capsici* at Different Application Times[1]

Application Time of *C. capsici*[1]	Average % Infection
24 hours after harvest	49.1 ad
24 and 72 hours after harvest	10.1 b
48 hours after harvest	35.1 ac
48 and 96 hours after harvest	32.1 ac
72 hours after harvest	24.6 bc
96 hours after harvest	30.4 ac
Pathogen only (Control)	59.7 d

[1] Fruits were previously inoculated with the pathogen 7 days before harvest.
[2] Data based on the average of four replications.
[3] Figures in a column with the same letter (s) are not significantly different at LSD_{05} 19.3663.
[4] Average % infection based on the total surface area of the fruit infected.

RESEARCH PLANS

1. Conduct studies to determine the optimum frequency of *C. capsici* application in the field.
2. Determine the need for postharvest application of *C. capsici*, its frequency and time of application.
3. Continue to search for more effective indigenous Colletotrichum isolates against anthracnose development.
4. Develop a Colletotrichum mutant that is effective against anthracnose.

ISRAEL

Delopment of *Colletotrichum gloeosporioides* REMI Mutants as Biocontrol Agents Against Anthracnose Disease in Avocado Fruits

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The phytopathogenic fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph: *Glomerella cingulata*) is an important postharvest pathogen attacking a wide variety of tropical and subtropical fruits (7,22). *C. gloeosporioides* spores germinate on avocado fruits in the orchard by growing a germ tube that develops an appressorium (3). The appressorium germinates infection hyphae that remain quiescent in the cells of the fruit's epidermal layer until the fruit ripens and softens during storage (20). The importance of *Colletotrichum* spp. as a worldwide economic problem and the search for biological means to control quiescent infection have led to the development of tools to control decay.

The search for biological control strategies against fruit and vegetables diseases after harvest has intensified in recent years, particularly with the banning of several pesticides (32). Most biocontrol strategies are directed towards wound pathogens and involve the use of antagonist bacterial strains that produce antibiotics, compete successfully for nutrients or produce lytic enzymes that affect germinating fungal hyphae by possible direct degradation of cell wall-degrading enzymes or direct action against the pathogen's hyphae (6,8,9,32,33). The search for biocontrol agents against pathogens causing quiescent infections has been more difficult since the infecting hyphae are

protected from microorganisms once the pathogen has penetrated the plant cuticle. Thus, few reports describe the biological control of quiescent infections in general and in avocado in particular (12,14,16). In the absence of a potentially direct interaction between the antagonist and quiescent infecting pathogens, we developed *C. gloeosporioides* reduced-pathogenicity mutants, which induce avocado fruit resistance to anthracnose, as an approach to controlling quiescent *Colletotrichum* infections.

In *Colletotrichum* spp., transformation-mediated genetic manipulation was established to enable heterologous gene expression (34), targeted gene disruption (5) and random gene disruption by restriction enzyme-mediated integration (REMI) (25). REMI involves the transformation of an organism with a linear fragment of DNA in the presence of the restriction enzyme used to linearize the fragment (19). Under these conditions, the linear DNA integrates into the chromosome at the restriction sites recognized by that particular enzyme (13,18,28). REMI was first used in yeast (28) to study the mechanism of illegitimate recombination. REMI protocols were adjusted to other systems, facilitating increased transformation-mediated gene disruption in *Dictyostelium discoideum* (18), *Cochliobolus heterostrophus* (35), *Magnaporthe grisea* (29), *Ustilago maydis* (4), *Alternaria alternata* (1), *Colletotrichum* spp. (25) and *Penicillium paxilli* (11).

Here we describe the development of reduced-virulence mutants, generated by REMI, and their use to elevate the natural resistance mechanism of avocado fruit, as a way of reducing the severity of anthracnose during storage.

MATERIALS AND METHODS

Avocado fruits, fungal isolates and growth media. Avocado fruits (*Persea americana* Miller var. *drymifolia* (Schidl. And Cham.) S. F. Blake 'Fuerte') were obtained from Kibbutz Givat Brenner, Israel. A single-spore isolate of *C. gloeosporioides* (Penz.) Penz. & Sacc. (Teleomorph: *Glomerella cingulata*), Cg-14, was obtained from a decayed cv. Fuerte avocado fruit and spores were maintained in 10 mM Na-phosphate buffer (pH 7.2), 40%

glycerol at -80°C. Three-week-old conidia were harvested from Mathur's medium (M_3S) plates (30) and used for culture and/or fruit inoculation. Spore production was estimated on 3-week-old colonies on M_3S plates at 20°C by spore-counting with hemacytometer (Brand, Wertheim, Germany).

Vector construction, fungal transformation and isolate selection. The *hph-B* cassette (hygromycin phosphotransferase), conferring resistance to hygromycin, was subcloned from pHA-1.3 (25) by restriction with *Hin*dIII and *Xba*I (Promega, Madison, WI), generating a 2.13-kb fragment that was ligated into pGEM-7Z (Promega), restricted with the same enzymes. The product, pGH-1 (5.13 kb), was use as a transformation vector. For transformation, 4-day-old growing mycelia were harvested and 3 g digested with Novozyme 234 (Calbiochem, La Jolla, CA) and -glucuronidase (Sigma, St. Louis, MO) to obtain fungal protoplasts, which were transformed with pGH-1 (1 μg) in the presence of 24 units of *Xba*I restriction enzyme, as initially described by Redman and Rodriguez (25). The transformation suspension was incubated for 48 h at 22°C and selected on media containing 100 μg/ml hygromycin (HM) (25). Hygromycin-resistant colonies were collected 4 to 8 days later and transferred to new HM plates. Colonies were transferred to M_3S plates to enhance sporulation and a single-spore culture was obtained. The mutants were sporulated by transferring the colonies to M_3S media plates and in order to test transformation stability, spores were transferred back to HM plates.

Hygromycin-resistant colonies were tested for their ability to cause symptoms on the pericarp and maceration of the mesocarp of peeled fruits. Freshly harvested fruits were inoculated on both sides of their longitudinal axis; three points of *C. gloeosporioides* isolate Cg-14 and three of the transformed isolate. Inoculation was carried out on the pericarp and mesocarp (after peeling a 1 to 2-mm thickness of pericarp) by placing a 3-mm^2 disk of media taken from the edge of a 4-day-old colony on HM plates and from HM without supplemented-hygromycin for Cg-14. Mesocarp maceration (2-3 mm deep, dark spots) occurred within 48 h and pericarp symptoms (darkening) within 5 to 7 days at 22°C. The diameters (mm) of the decay and maceration caused by the transformed isolates were compared

with that caused by Cg-14. Colonies that showed a lack of, or reduced macerating abilities were kept for further analysis.

Fruit protection assays and phenotypic characterization of transformed isolates. Fruit protection assays were carried out by dipping 20 freshly harvested avocado fruits (cv. Fuerte) for 30 s in a spore suspension (5 X 10^5 spores/ml) of Cg-M-142 or Cg-M-1150 and then storing for 24 h at 90% relative humidity (RH), 20°C. Fruits were then inoculated with 10 μl of Cg-14 spore suspension (1 X 10^6 spores/ml) at six points, three on each side of the longitudinal axis of the fruit, and incubated at 22°C, in 90% RH for 10 to 12 days. Isolates were characterized for appressorium formation by spot inoculation of the different isolate spores (1 X 10^6 spores/ml) on the fruit's pericarp and observation 48 h later under a light microscope (BH-2 Olympus, Tokyo, Japan), after cutting a 0.5-mm deep slice of the inoculated pericarp with a scalpel. Appressorium formation was photographed by video camera (APPLITEC MSV-800, Azor, Israel). Isolate growth rates were measured on three replicates of M_3S plates or five replicates of the pericarp, by inoculation with 10 μl of spore suspension as already described. Growth rate on the mesocarp was measured by inoculating at six points with a 3-mm^2 square of media with growing isolate (as described). All experiments were repeated at least three times over two consecutive avocado seasons.

Southern blot analysis and plasmid rescue. Fungal DNA was obtained from 0.2 g of lyophilized hyphae as described by Rodriguez (26). Fungal DNA (5 μg) was restricted for 15 h at 37C by 20 units of *Hin*dIII or *Xba*I. Restricted DNA was run on a 0.8% agarose gel for 15 h and blotted on to HyBond+ nylon membrane (Amersham, Buckinghamshire, UK), using the capillary method (27).

All hybridizations were carried out at 65°C and washes were with 0.1 X sodium chloride/ sodium citrate (SSC). The membrane was probed with either a 4.1-kb *pel* gene (accession number AF052632, GenBank) or pGH-1. The blotted membrane was exposed to both X-ray film and Fuji BAS sample screen and the image was captured using the Fuji BAS reader (Fujifilm,

Tokyo, Japan). Bands were quantified using the MacBAS software v2.3 (Fujifilm).

For plasmid rescue, 1 μg of fungal DNA was used. DNA was restricted with 10 units of *Xba*I or *Hin*dIII for 15 h. Restriction enzymes were separated from the DNA using phenol:chlorofom (50:50 v/v) followed by ethanol precipitation. The DNA was eluted and subjected to ligation using ligase (Promega) for 12 h at 4°C. Ligation mixture from each isolate (1 μl) was electroporated, using Gene Pulser II (Bio-Rad Laboratories, Hercules, CA), into DH-5α-competent cells (prepared according to Bio-Rad Laboratories recommendations). Bacteria were grown on Luria-Bertani (LB) media (27) supplemented with 100 μg ampicillin/ml at 37°C for 15 h. Randomly selected colonies were grown for plasmid mini-preparation using Wizard Plus SV Minipreps (Promega). Plasmids were restricted with the same restriction enzyme used for their rescue and run on a 1% agar gel followed by ethidium bromide staining.

Antifungal diene extraction. A 10-g sample of avocado pericarp (1-2 mm thick) was homogenized in 95% ethanol in an Omni-Mixer (Sorvall, DuPont Company, Newtown, CT) at full speed for 3 min. The ethanol extract was dried in a rotary evaporator at 40°C, redissolved in 10 ml distilled water, and the organic phase was extracted by fractionation with dichloromethane. Following two extractions, the organic phases were pooled, dried with anhydrous $MgSO_4$ (Riedel-deHaen, Seelze, Germany), and evaporated to dryness. Samples were redissolved in 1 ml of ethanol AR (Bio Lab, Jerusalem, Israel) and analyzed by HPLC (23). The average values of three separate extractions are presented. The experiment was repeated twice during each year of two consecutive avocado seasons.

SUMMARY OF THE RESULTS

Reduced-pathogenicity mutants of the avocado fruit pathogen *Colletotrichum gloeosporioides* isolate Cg-14 (teleomorph: *Glomerella cingulata*) were generated by insertional mutagenesis using restriction enzyme-mediated integration (REMI) transformation. Following seven transformations, 3500 hygromycin-resistant isolates were subjected to virulence assay by inoculation on mesocarp and pericarp of cv. Fuerte avocado fruits. Fourteen isolates showed a reduced degree of virulence relative compared to the wild type, Cg-14. Two isolates, Cg-M-142 and Cg-M-1150, were further characterized. Cg-M-142 produced appressoria on avocado pericarp similar to Cg-14, but caused reduced symptom development on the fruit's pericarp and mesocarp. Isolate Cg-M-1150 did not produce appressoria, it caused much reduced maceration on the mesocarp and no symptoms on the pericarp. Southern blot analysis of Cg-M-142 and Cg-M-1150 showed REMI at different *Xba*I sites of the fungal genome. Preinoculation of avocado fruit with Cg-M-142 delayed symptom development by the wild-type isolate. Induced resistance was accompanied by an increase in the levels of preformed antifungal diene, from 760 to 1200 g/g FW, 9 days after inoculation, whereas preinoculation with Cg-M-1150 did not affect the level of antifungal diene, nor did it delay the appearance of decay symptoms. The results presented here show that reduced-pathogenicity isolates can be used for the biological control of anthracnose caused by *C. gloeosporioides* attack.

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c. **Future Work**

The identification of natural mutants obtained in Philippines and the REMI mutants obtained in Israel will be further followed. This will include genetic definition of the mutants and pathogenicity tests where decay reduction will be tested.

Section II

Collaboration, Travel, Training

During the period of this annual report one visit toke place not on this project money. The visit will take place by Dov Prusky visiting the Philippines on the 1st of August 1999. Dov Prusky also visited Los Banos for a very short visti during extra project activities. The visits was intented to develop the following year experiments that will be done in both laboratories.