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

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### 3. Executive Summary:

During the 6 years of the project we studied various approaches for the control of postharvest diseases in subtropical fruits. These approaches included the natural selection of reduce pathogenicity strains and at the same time the production of transgenic non-pathogenic strain of *Colletotrichum* by REMI: (Restriction enzyme mediated integration) technique. Another approach that was tested was the expression of peptides with toxic properties to fungi in yeast strains. Each of the approach showed positive results. The REMI mutant isolates allowed us to demonstrate the importance of appressorium formation dynamics in triggering avocado fruit resistance. This observation indicates that early signaling by REMI mutants succeeded in elevating resistance in fruit naturally infected with *C. gloeosporioides*. We suggest that the induction of fruit resistance is a result of greater epicatechin accumulation in the fruit, derived from early signals generated by delayed appressorium formation and invading hyphae, in contrast to the fast penetration by the wild-type isolate.

The second approach we have used is the transformation of yeast for the expression to of various peptides. The peptide that was chosen is resistant to plant proteases and represents just one of many possible small peptide candidates that could be used for yeast expression. The engineering of yeast expressing an antifungal peptide is a new approach for the biocontrol of postharvest pathogens. The lack of activity towards nontarget organisms by the peptide and the fact that we have used *S. cerevisiae* as a delivery system, suggests that this method should provide a safe alternative to other method for controlling postharvest diseases.

The third approach that was tested was the selection of natural reduced pathogenic strain of *Colletotrichum capsici*. The ability of *Colletotrichum capsici* isolater to effectively control mango anthracnose caused by *C. gloeosporioides* in the field at the different stages of mango development represents a simple approach for disease control. The capability of this isolates for disease control was tested at different stages of growth by weekly and fortnightly treatments and promising result have bee obtained.

## 4. Research Objectives

The intention of this research is to develop new technology for the control of quiescent infections of subtropical fruits by enhancing mechanism of resistance that have been already elucidated by utilizing non or reduced pathogenesis mutants or yeast with direct effect on the fungus. 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.

Molecular biology transformation by REMI: Restriction enzyme mediated integration technique (Redman and Rodriguez, 1994) was used for obtaining mutant of *C. gloeosporioides*. The efficiency of this mutant for induction of resistance was be tested in Israel and found that it induces resistance.

### 2. Expression of toxic peptides on yeast mutants.

The possibility that specific peptides that affect fungal attack were tested as well.

## 5. Methods and Results

### 5.1 Delopment of *Colletotrichum gloeosporioides* REMI Mutants as Biocontrol Agents against Anthracnose Disease in Avocado Fruits

## INTRODUCTION

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 fruit, fungal isolate, and growth media.** Experiments were carried out with freshly harvested fruit of the avocado cultivar Fuerte (*Persea americana* Mill. var. *drymifolia* (Schltdl. & Cham.) S. F. Blake) from an orchard at Kibbutz Givat Brenner, Israel. A single-conidium isolate of *C. gloeosporioides*, Cg-14, was obtained from a decayed fruit of the avocado cultivar Fuerte, and conidia were maintained in 10 mM Na-phosphate buffer (Ph 7.2), 40% glycerol, at  $-80^{\circ}\text{C}$ . Three-week-old conidia were harvested from Mathur's medium (M3S) plates (3), counted with the aid of a hemacytometer (Brand, Wertheim, Germany), and used for culture and fruit inoculation. Tissue treatment and inoculation were performed as previously described (4). Experiments were conducted in two consecutive harvesting seasons and repeated at least three times per season. The data presented are usually the average values of three to five replications of a single experiment. In some cases the average of all the replications of repeated experiments are presented with their pooled standard error.

### **Fruit protection assays and phenotypic characterization of**

**transformed isolates.** Twenty freshly harvested avocado fruits (cv. Fuerte) were dipped for 30 s in suspensions of conidia ( $5 \times 10^5$  conidia/ml) of the two *C. gloeosporioides* mutants (Cg-M-142 and Cg-M-1150) and then stored at 90% relative humidity and  $20^{\circ}\text{C}$  for 24 h. Thereafter, the treated fruit was spot-inoculated with a Cg-14 conidial suspension ( $1 \times 10^6$  conidia/ml) (36). Appressoria were observed under a light microscope (BH-2 Olympus, Tokyo, Japan) in a 0.5-mm-deep slice of

inoculated exocarp cut out with a scalpel 24 and 48 h after inoculation. Isolate growth rates were measured in five replicates on exocarp and on glass slides by inoculation with 10 µl of the conidial suspension described above. All experiments were repeated at least three times over two consecutive avocado seasons. Exocarp inoculation was carried out by brushing exocarp strips with a conidial suspension ( $2 \times 10^6$  conidia/ml) (22). As a control, exocarp strips were brushed with distilled water only.

**Extraction of epicatechin.** Epicatechin was extracted from 3 g of 1-mm-thick avocado exocarp strips, as previously described (21). Epicatechin was quantified in a reverse-phase high-performance liquid chromatography (HPLC) (RP-18) column eluted with 55% methanol and 1% acetic acid in double-distilled water. Calculation of the epicatechin level was based on comparison of the HPLC peak areas quantified at 275 nm with those of the standard, (2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol (Sigma, Jerusalem, Israel).

**Extraction of PAL and analysis of PAL activity.** Samples for analysis of PAL activity were extracted by blending 5 g of avocado exocarp in 50 ml of cold acetone ( $-20^\circ\text{C}$ ) and collecting the homogenate on Whatman no. 1 filter paper. This process was carried out four times. The exocarp powder was dried at room temperature overnight and stored at  $-20^\circ\text{C}$  until used. PAL was extracted from 100 mg of exocarp powder stirred for 2 h at  $4^\circ\text{C}$  with 5 rnl of 0.1 M sodium borate buffer, pH 8.8. The extract was centrifuged at  $10,000 \times g$  for 20 min at  $4^\circ\text{C}$ , and the supernatant was used as an enzyme source. The reaction mixture for PAL consisted of 10 mM L-phenylalanine (Aldrich, Jerusalem, Israel), 0.1 M borate buffer (pH 8.8), and 0.75 ml of enzyme extract in a final volume of 2.5 ml. The reaction was incubated at  $37^\circ\text{C}$  for 60 min and terminated by the addition of 0.1 rnl of 5 N HCl. The product, cinnamic acid, was extracted in 3.5 ml of diethyl ether and quantified by measuring the amount of *trans*-cinnamic acid formed at 269 nm (15).

**Preparation of microsomes and isolation of plasma membranes.**

Plasma membranes were isolated from 40 g of exocarp strips after treatment with 0.2 mM  $\text{CaSO}_4$  for 24 h. Plasma membranes were isolated at several periods after inoculation with *C.gloeosporioides*. Tissue was homogenized in a Waring blender at  $4^\circ\text{C}$ , and microsomes were prepared as previously described (10).

The microsomes were suspended in a suspension medium (330 mM sucrose, 5 mM KCl, and 5 mM potassium phosphate buffer, pH 7.8), and the plasma membranes were separated by two-phase partitioning (13). The final composition of the phase system was 6.4% (wt/wt) dextran T500 and 6.4% (wt/wt) polyethylene glycol (PEG 3350) in the suspension medium. The final upper phase was diluted 1:10 with a resuspension medium (330 mM sucrose, 1 mM dithiothreitol, 5 mM NaF, and 5 mM MES-bis-tris propane, pH 7.8), and the purified plasma membranes were pelleted by centrifugation at 55,000 × *g*. The pellet was redissolved in 330 mM sucrose, 5 mM MES-bis-tris propane, and 1 mM dithiothreitol adjusted to pH 7.0 and brought to a concentration of 1 to 3 mg protein/ ml. Protein was assayed according to the Bradford method (6).

**Reactive oxygen production measured by cytochrome *c* reducing activity.** The production of ROS was determined by cytochrome *c* reduction dependent NADPH oxidation, as previously described (18). Reduction of the cytochrome *c* molecule produces one molecule of reactive oxygen (16). The assay consisted of 20 μM cytochrome *c* (Sigma type VI), 100 μM NADPH, 25 mM potassium phosphate buffer (pH 7.5), and 10 μg of plasma membrane protein in a total volume of 1 ml. The reaction was initiated with the addition of NADPH, and the rate of cytochrome *c* reduction was measured on a dual-wavelength spectrophotometer at 550 nm, by the difference in the absence and presence of 100 μg of superoxide dismutase, as described by Owen and Hancock (18).

**H<sup>+</sup>-ATPase activity and rubidium uptake.** Vanadate-sensitive plasma membrane was determined in the presence of 0.01% polyethylene 20 cetyl ether (Brij 58) and in the presence or absence of 0.2 mM Na<sub>3</sub>VO<sub>4</sub>. H<sup>+</sup>-ATPase activity was assayed as previously described (11), with variations: 4 μg of plasma membrane protein was incubated for 30 min at 35°C in 0.5 ml of a medium composed of 40 mM MES-tris (pH 6.5), 0.25 M sucrose, 100 mM KCl, 3 mM MgSO<sub>4</sub>, and 3 mM ATP. H<sup>+</sup>-ATPase activity in the sample plasma membrane was found to be 87% vanadate sensitive. For rubidium uptake, exocarp tissue disks (about 5 mm in diameter and 1 mm thick, almost free of mesocarp) were prepared as described for microsome preparation. The disks were preincubated for 30 min in 0.2 mM CaSO<sub>4</sub> at 25°C. [<sup>86</sup>Rb]K<sup>+</sup> was added

to the vial for the last 20 min of the reaction. The uptake of [<sup>86</sup>Rb]K<sup>+</sup> into 70 mg of avocado disk tissue (from five disks) was assayed in 5 ml of 0.2 mM CaSO<sub>4</sub> and 0.1 mM [<sup>86</sup>Rb]K<sup>+</sup> (700 Bq/μmol). Exchangeable was removed with three washings over a 5-min period in 15 ml of ice-cold 10 mM CaSO<sub>4</sub>. The disks were transferred to vials for scintillation counting; 0.2 ml of 1 N NaOH was added for discoloration, and then 4 ml of Opti-Fluor O (Packard, Groningen, The Netherlands) was added. Samples were counted in an MR 300 Lumitron Automatic liquid scintillation system (Packard).

**Extraction and quantification of the antifungal diene.** A 10-g sample of avocado exocarp (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 and then redissolved in 10 ml of distilled water, and the organic phase was extracted by fractionation with dichloromethane. After two extractions, the organic phases were pooled, dried with anhydrous MgSO<sub>4</sub> (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 (26). The average value of three separate extractions is presented. The experiment was repeated twice in each of two consecutive avocado seasons.

## RESULTS

**Phenotypic analysis of REMI mutants.** Two *C. gloeosporioides* REMI mutant isolates, Cg-M-142 and Cg-M-1150, were compared to the wild-type (WT) isolate Cg-14 for conidial germination, germ tube elongation, and appressorium formation on glass slides (Fig. 1A) and on avocado exocarp (Fig. 1B) 24 and 48 h after inoculation. Cg-M-142 and Cg-M-1150 were similar to the wild type in germination and germ tube elongation on the exocarp, but the two mutants were retarded on glass slides. Appressorium formation was delayed in Cg-M-142 and absent in Cg-M-1150 on both glass slides and exocarp. Appressorium formation by Cg-M-142 was similar to that of the WT within 48 h after inoculation, while Cg-M-1150 did not produce appressoria at all (Fig. 1A and B), even after 96 h (data not presented). Analysis of germ tube elongation showed that Cg-M-142 and Cg-M-1150 formed longer germ tubes on inoculated fruit than on glass slides (Fig. 1A and B).

**Effect of preinoculation with REMI mutants on the development of decay.** Inoculation of avocado fruit with Cg-M-142 increased the level of the antifungal diene approximately threefold within 4 days (Fig. 2). The diene level remained higher in treated fruit even 11 days after inoculation with Cg-M-142. Decay symptoms were delayed by 2 days in fruit preinoculated with Cg-M-142, compared to the control fruit and fruit preinoculated with Cg-M-1150. Diene levels were similar in fruit inoculated with Cg-M-1150 and the control fruit, during the monitored period. In addition, the development of symptoms in fruit preinoculated with Cg-M-1150 was similar to that in control fruit (Fig. 2).

**H<sup>+</sup>-ATPase activity, rubidium uptake, and O<sub>2</sub> generation dependent NADPH oxidation.** Exocarp slices inoculated with the mutant strains were tested for early signaling effects. H<sup>+</sup>-ATPase activity in the plasma membrane extracted from exocarp inoculated with Cg-M-142 increased fourfold within 2 h after inoculation (Fig. 3A) and 2 h later dropped to the same level as the control. Inoculation with Cg-M-1150, however, had no effect on H<sup>+</sup>-ATPase activity 2 h after treatment, with a small increase 4 h after treatment. This observation was further confirmed by monitoring Rb<sup>+</sup> uptake, which showed a pattern similar to that of H<sup>+</sup>-ATPase activity, while Cg-M-1150 followed the same pattern as the control (Fig. 3B). A transient sixfold increase in NADPH oxidase activity of exocarp inoculated with Cg-M-142 occurred 1 to 2 h after inoculation (Fig. 4). In exocarp inoculated with Cg-M-1150, cytochrome *c* reduction was unaffected 2 h after inoculation and increased slightly 4 h after inoculation (Fig. 4).

**Induction of PAL and epicatechin level.** Inoculation of exocarp slices with Cg-M-142 increased PAL activity more than twofold within 10 h (Fig. 5A), whereas PAL activity was reduced in exocarp inoculated with Cg-M-1150, compared to the control. Epicatechin also increased twofold in exocarp inoculated with Cg-M-142, whereas no change in epicatechin levels was found after inoculation with Cg-M-1150 (Fig. 5B). Epicatechin levels increased when whole avocado fruit was preinoculated with Cg-M-142 or Cg-M-1150. However, only the epicatechin increase in fruit inoculated with Cg-

M-142 was significant (a twofold increase within 120 h after inoculation) (Fig. 6), while no significant increase was found in fruit inoculated with Cg-M-1150.

## DISCUSSION

Development of disease caused by pathogens in the genus *Colletotrichum* requires adhesion of conidia to the surface of host tissue, followed by conidial germination and the formation of appressoria to penetrate the host tissue (3,19). Two reduced-pathogenicity REMI mutants, Cg-M-142 and Cg-M-1150 (36), did not differ from the wild-type *C. gloeosporioides* in germination rate or germ tube elongation on fruit exocarp. The most striking differences between the two mutants and the wild type were (i) the lack of appressorium formation by Cg-M-1150 and the delayed appressorium formation by Cg-M-142 and (ii) the capability of Cg-M-142 to induce resistance to the wild-type *C. gloeosporioides* isolate. The importance of appressoria in fungal attack has been extensively studied (30,37); however, it has not been examined for its importance in triggering signals leading to increased resistance.

Avocado fruit exocarp, like other plant organs (7,9), recognizes penetrating hyphae of wild-type *C. gloeosporioides* appressoria at the level of the host plasma membrane (4). This recognition occurs by the release of soluble cell wall sugars, probably sensed by a heterotrimeric G-protein receptor that activates a G-protein (32,33) that transduces the signal by activating one or more membrane-bound phosphates, resulting in the dephosphorylation of the host plasma membrane H<sup>+</sup>-ATPase and activation of NADPH oxidase (4) (Fig. 7). We cannot exclude the presence of other receptors in the plasma membrane that may sense the penetrating fungus as well. However, activation of H<sup>+</sup>-ATPase and H<sub>2</sub>O<sub>2</sub> production was stimulated by the appressorium-forming isolate Cg- M-142 and not by the non-appressorium-forming isolate Cg-M- 1150. The latter caused a marginal delayed increase in both signals, had no effect on rubidium uptake, and failed to alter fruit resistance. The lack of induction of resistance indicates the importance of appressorium formation in initiating defense signals, such as the proton electrochemical gradient across the plasma membrane (28). In avocado fruit, H<sup>+</sup>-ATPase was induced by the penetrating appressoria, along with Rb<sup>+</sup> uptake, confirming the opening of K<sup>+</sup> channels as an early reaction to fungal invasion. ROS

production has been shown to be an early response in plant pathogen interactions in general (1,17) and in avocado fruit (4). Inoculation of avocado exocarp tissue with the wild-type Cg-14 increased ROS production threefold within 2 h, while inoculation with Cg-M-142 increased ROS production six fold, which can explain its increased resistance effect. Cg-M-1150 did not significantly affect ROS levels. In addition, the application of Ca<sup>2+</sup> ionophore (A-23187), which opens Ca<sup>2+</sup> channels, stimulated the production of H<sub>2</sub>O<sub>2</sub> (4) (Fig. 7). Thus the level of H<sub>2</sub>O<sub>2</sub> in the fruit may predict the level of fruit resistance against fungal attack. In addition to these responses, Cg-M-142 activated the resistance related phenylpropanoid pathway, where PAL activity was higher and epicatechin accumulation was 30% higher than that induced by the wild-type Cg-14 (4). Moreover, in vivo defense experiments on whole fruit showed a threefold increase in epicatechin production in fruit inoculated with Cg-M-142, compared to the control.

Activation of the phenylpropanoid pathway increases the production of epicatechin, an antioxidant, which protects the reduction of the antifungal diene by inhibiting lipoxygenase (2). Epicatechin has also been found to inhibit *C. gloeosporioides* polygalacturonases and pectate lyase (34,35) (Fig. 7), and the latter was recently found to be an important virulence factor in fruit-fungus interactions (35). The induction of PAL activity by H<sub>2</sub>O<sub>2</sub> in avocado fruit is in agreement with the results of studies of *Arabidopsis* and tobacco cell cultures, in which the addition of exogenous H<sub>2</sub>O<sub>2</sub> triggered PAL expression (14,17). This suggests that PAL activity is stimulated after direct penetration by appressoria, as was observed in barley leaves after inoculation with *Erysiphe graminis* or *E. pisi* (29), via the production of H<sub>2</sub>O<sub>2</sub>. Although Cg-M-1150 showed a marginal increase in early signals as well as in PAL activity, it did not have any effect on fruit resistance. It is possible that compounds secreted by the fungus during its growth on the exocarp generate this effect. Since PAL is the first enzyme in the phenylpropanoid pathway that branches into various routes, the increase in PAL may affect other downstream products unrelated to resistance. The genetic differences between the wild-type Cg-14 and its derived isolate Cg-M-142 in their ability to induce resistance has not yet been elucidated. Several genes have been reported to affect appressorium formation (37). A nonpathogenic *C. lagenarium*

mutant in mitogen-activated protein (MAP) kinase, CMK1 gene, showed reduced conidiation and failed to form appressoria (30).

The disruption of *Botrytis cinerea* *BMP1*, a gene encoding MAPkinase, prevented appressorium formation and pathogenicity (38). Yang and Dickman (37) showed that strains of *C. trifolii* deficient in protein-kinase A were unable to infect intact alfalfa plants, likely because of a failure to penetrate. Cg-M-142 has been shown to harbor a single REMI integration site, which suggests a single gene disruption, whereas two independent integration sites have been found in Cg-M-1150, possibly disrupting one or two genes to cause its unique phenotype (36). The genes disrupted by REMI technology in both mutants are currently under investigation. Although the flanking regions have been cloned, we did not find any homology in the known published genetic data. The two REMI mutant isolates allowed us to demonstrate the importance of appressorium formation dynamics in triggering fruit resistance. In general appearance, the fruit inoculated with Cg-M-142 was less decayed than fruit inoculated with Cg-M-1150 or with the control (data not shown). This observation indicates that early signaling by Cg-M-142 had succeeded in elevating resistance in fruit naturally infected with *C. gloeosporioides* as well.

We suggest that the induction of fruit resistance is a result of greater epicatechin accumulation, derived from early signals generated by delayed appressorium formation and invading Cg-M-142 hyphae, in contrast to the fast penetration by the wild-type isolate (35,36). This fast penetration by the wild-type isolate has generated a fast and transient activation of resistance mechanism, but this effect did not delay symptom development in ripening fruit (20). Lack of appressorium formation by the Cg-M-1150 mutant is assumed to be the reason for the lack of induction of resistance and failure to colonize the fruit.

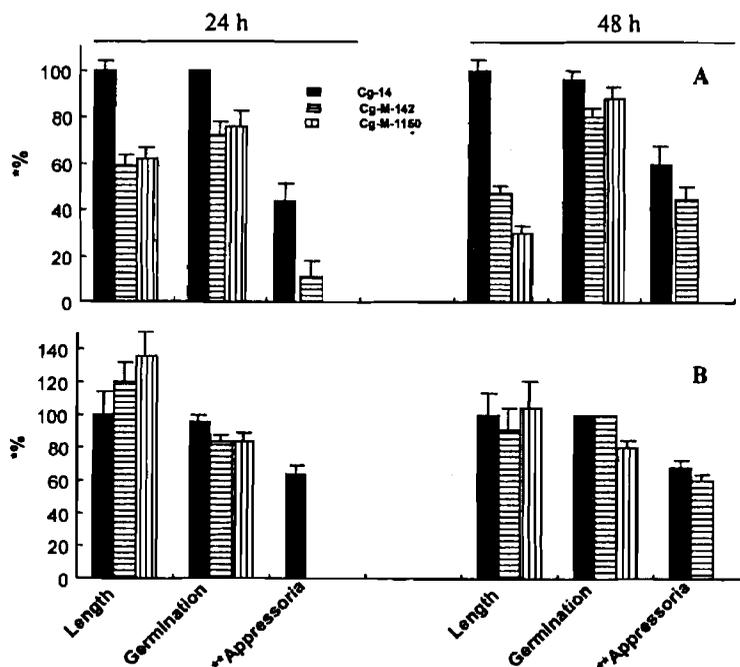
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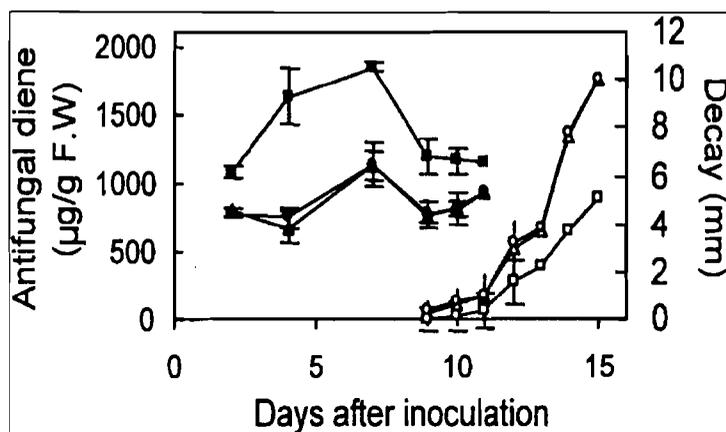
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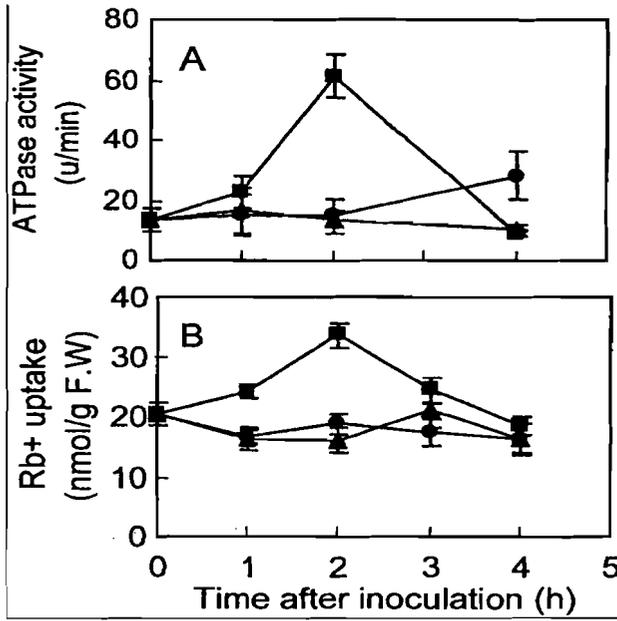


**Fig. 1.** Conidial germination, germ tube elongation, and appressorium formation by *Colletotrichum gloeosporioides* wild-type isolate Cg-14 and the reduced-virulence mutants Cg-M-142 and Cg-M-1150 on glass slides **A**, and on exocarp of avocado fruit of the cultivar Fuerte **B**, 24 and 48 h after inoculation. The data and standard errors are from three replications. \*Percentages relative to the wild type. \*\*Percentage of germlings that formed appressoria.

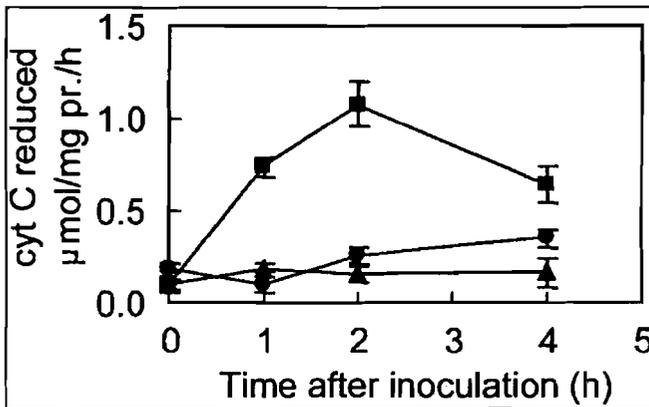


**Fig. 2.** Development of decay caused by *Colletotrichum gloeosporioides* isolate Cg-14 in avocado fruit of the cultivar Fuerte following preinoculation with the reduced-virulence mutants Cg-M-142 (■) and Cg-M-1150 (●) or with water as a control (▲). Ten freshly harvested fruits were dipped for 30 s in a conidial

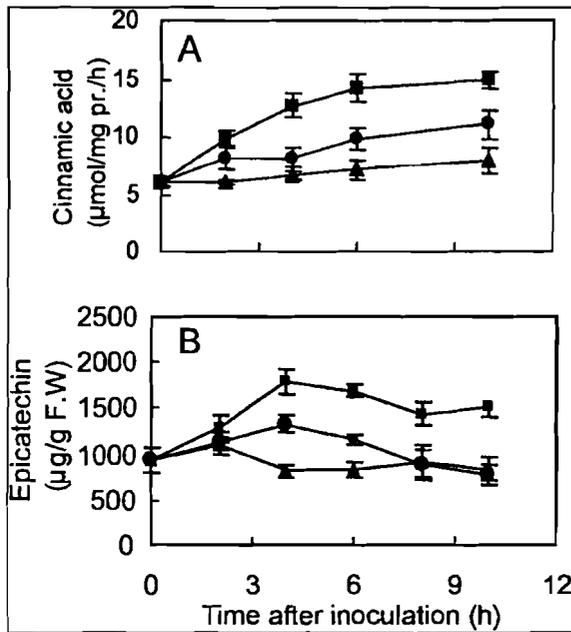
suspension of Cg-M-142 or Cg-M-1150 or in water, inoculated 24 h later at six points with the wild-type Cg-14, and incubated for 15 days. The effect of the reduced-virulence mutants on the antifungal diene was done in parallel by inoculation of the whole fruit with spores of the mutant isolates or with water treatment only. Levels of diene (filled symbols) and decay development (open symbols) were recorded. The experiments were repeated three times in two consecutive harvesting seasons. The data and standard errors are from one representative experiment.



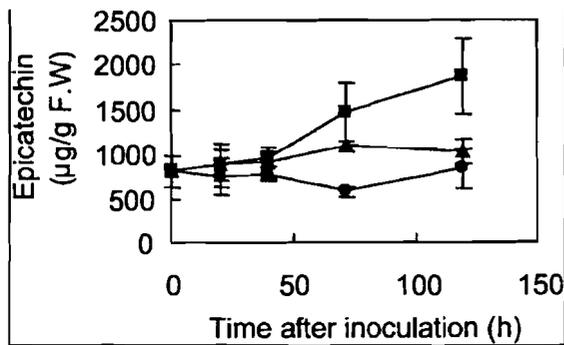
**Fig. 3.** Effect of preinoculation of avocado fruit exocarp with *Colletotrichum gloeosporioides* mutant isolates Cg-M-142 (■) and Cg-M-1150 (●) or with water as a control (▲) on the induction of plasma membrane H<sup>+</sup>-ATPase A, and rubidium uptake B, on extracted plasma membranes. The bars represent standard error. The data from three experiments with five replications per treatment were pooled. The experiments were repeated in two consecutive avocado seasons.



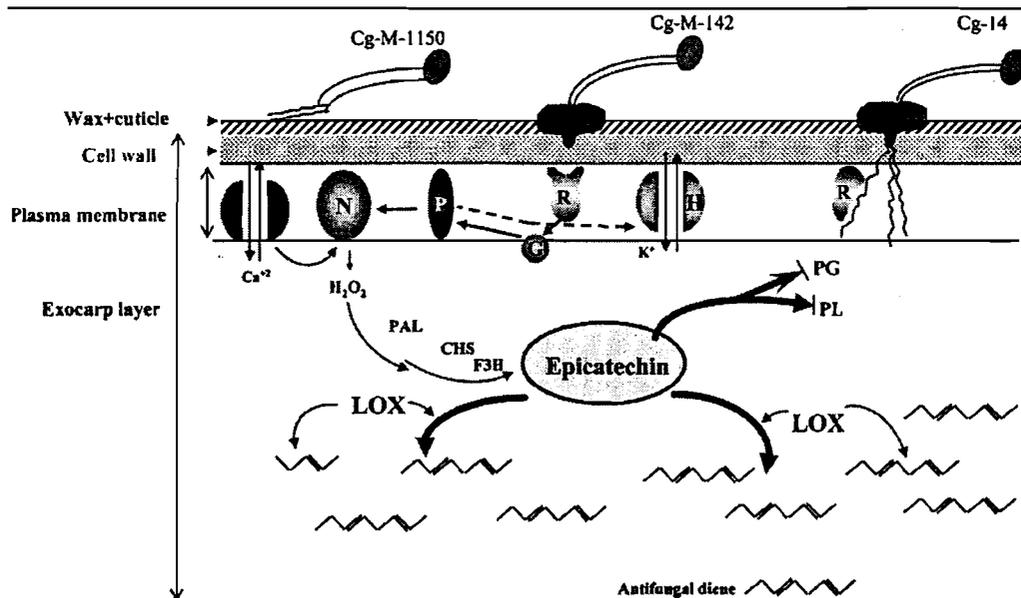
**Fig. 4.** Time course of the production of reactive oxygen species in plasma membranes isolated from avocado fruit exocarp inoculated with *Colletotrichum gloeosporioides* mutant isolates Cg-M-142 (■) and Cg-M-1150 (●) or with water as a control (▲). The bars represent standard error from one representative experiment run four times with three replications. The experiment was repeated in two consecutive avocado seasons.



**Fig. 5.** Phenylalanine ammonia lyase activity **A**, and epicatechin **B**, in slices of avocado fruit exocarp inoculated with *Colletotrichum gloeosporioides* mutant isolates Cg-M-142 (■) and Cg-M-1150 (●) or with water as a control (▲). The bars represent standard error. The data from three experiments with five replications per treatment were pooled. The experiments were repeated in two consecutive avocado seasons.



**Fig. 6.** Epicatechin in avocado fruit exocarp inoculated with *Colletotrichum gloeosporioides* mutant isolates Cg-M-142 (■) and Cg-M-1150 (●) or with water as a control (▲). The bars represent standard error in one representative experiment run four times with three replications. The experiment was repeated in two consecutive avocado seasons.



**Fig. 7.** General scheme of proposed interaction between fruit of the avocado cultivar Fuerte and *Colletotrichum gloeosporioides* isolates Cg-14, Cg-M-142, and Cg-M-1150, during the attack/protection actions of the different strains. The scheme was generated from our data and data published in other fungal systems as cited in the text. N = NADPH-oxidase. P = phosphates. G = heterotrimeric G-protein. H = H<sup>+</sup>-ATPase. R = receptor. Vertical arrows = ion channels. PAL = phenylalanine ammonia lyase. CHS = chalcon synthase. F3H = flavonone-3-hydroxylase. LOX = lipoxygenase. PG = polygalacturonase. PL = pectate lyase.

## **5.2 Expression of an antifungal peptide in *Saccharomyces*: a new approach for biocontrol of the postharvest disease caused by *Colletotrichum coccodes*.**

### **INTRODUCTION**

Losses from postharvest pathogens on fruits and vegetables have been principally managed by the use of synthetic fungicides (10). Consumer concerns about possible risks associated with the use of fungicides, along with development of pathogen resistance to certain fungicides, have resulted in an intensive search for safer, more effective control options that pose minimal risk to human health and the environment. Significant progress has been made in developing potential biological alternatives to synthetic fungicides for the control of postharvest diseases of fruits and vegetables (27). The use of microorganisms, particularly yeasts occurring naturally on the surface of fruits or vegetables usually has been preferred for the control of postharvest disease (6, 8, 15,16,18,25). Yeasts are suitable as biocontrol agents of postharvest diseases since: i) they rapidly colonize and survive on fruit surfaces for long periods of time under different conditions; ii) they use available nutrients to proliferate rapidly, limiting nutrient availability to the pathogen; and iii) they are generally unaffected by fungicides used commercially.

Several yeast antagonists have been reported to effectively inhibit the development of postharvest pathogens on various fruits (6,7,8,23). Among these yeasts, *Pichia guilliermondii* Wicherham, and *Candida oleophila* Montrocher were developed into commercial products (9,14). However one of the major problems with the use of those products is the insufficient and inconsistent performance under commercial conditions. Consequently they are used in combination with low concentrations of postharvest fungicides (9) or by using preharvest treatments with an antagonist (1). Another significant problem is that the reported antagonists are mainly used to control wound pathogens but not for pathogens invading directly through the cuticle and causing quiescent infection (28).

The current study was undertaken to investigate the possibility of expressing a DNA sequence in yeast to allow for the production of an antifungal peptide to produce an improved biological control organism (12). Small antibacterial peptides with lytic activity have been found in a broad variety of species (3). Two main structural patterns

were identified; one characterized by a  $\beta$ -sheet structure with two or three intramolecular disulfide bonds and a second group possessing an  $\alpha$ -helical motif typical of cecropin families (2) that were used in the present work. Most of the work on the antimicrobial properties of cecropin A and B peptides has concentrated on their activities against plant-pathogenic bacteria. Consequently, these peptides have been studied with a view to engineering bacterial disease resistance in plants. However, antifungal activity was also reported for these peptides (5,21). In an effort to improve the control of postharvest decay by biological means, we have developed a new approach to control postharvest pathogens by expressing a lytic peptide in *Saccharomyces cerevisiae* Hansen. We chose the tomato fruit fungal pathogen *Colletotrichum coccodes* (Wallr.) S.J. Hughes as a target to demonstrate the potential of bioengineered yeast in disease control.

## **MATERIALS AND METHODS**

Tomato fruits, fungal isolates and growth media. Tomato fruits (*Lycopersicon esculentum* cv. Roma) were obtained from a local grocery store. A single-spore isolate of *C. coccodes* was obtained from a decayed tomato (cv. Roma). Three-week-old conidia were harvested from Mathur's medium (M<sub>3</sub>S) plates (26) and used for culture and/or fruit inoculation. Spore production was estimated on 3-week-old colonies on M<sub>3</sub>S plates at 20°C by counting spores with a haemocytometer (Brand, Wertheim, Germany).

Antifungal activity of the synthesized peptide. The antifungal peptide represents an internal portion of cecropin A. The peptide (WKLFKKILKVL) was synthesized by Genosys (Genosys, The Woodlands, TX). Antifungal activity against *C. coccodes* was tested in water solutions at 0.5  $\mu$ M and up to 5 mM of the pure peptide. Peptide stock solutions were prepared at 2-fold concentrations of the final concentrations tested and mixed at a 1:1 ratio (5 $\mu$ l of each solution) with a *C. coccodes* suspension of  $2.5 \times 10^5$  spores/ml. Spore germination was carried out on a glass slide at 25°C and high humidity over 12 h. Germinated spores were those where the germ tube was twice the length of the spore.

Fruit protection assays were carried out in the presence of the synthesized peptide. Fruits were inoculated by wounding to a depth of 2-3 mm and placing 10  $\mu$ l of *C. coccodes* spore suspension ( $10^6$  spores/ml) at two points, one on each side of the longitudinal axis of 15 fruits. The fruits were then incubated at 20°C, in 90% relative humidity for 6 days.

Construction of invertase-antimicrobial peptide fusion, yeast transformation and isolate selection. The invertase signal sequence from *S. cerevisiae* Hansen (4) was cloned by two rounds of PCR. In the first round a 626 bp fragment was obtained using primers IF1 (5'-GGGTTTTTCCATGGAG-3') and IR1 (5'-GAAGTAGCATGGCCCC-3'). The fragment was gel purified (Zymo Research, Orange, CA ) and used for the second round of amplification with primers IF2 (5'CATACTCTAGAGAAACAAGC3') and IR2 (5'P-CGTTTGTTCATTGATTGGC-3'). The second round yielded a 124 bp fragment that was gel purified and used for ligation to the antimicrobial peptide sequence.

The antimicrobial peptide fragment was amplified from a prior construct (19) by using primers PEPF (5'P-TGGAACTCTTTAAG-3') and PEPR (5'-CTCGAGCTACTAGAGCACTTTGAGAATCTTCTTAAAGAGTTTCC-3'). The prior construct was developed through sequential PCR to provide the complete antimicrobial sequence for an internal eleven amino acid sequence of cercropin A, modified for protease resistance (5). Each of the prior PCR reactions used Thermopol polymerase (New England Biolabs, MA) and the cycling parameters of 94°C, 5 min. followed by thirty cycles of 94°C, 1 min 53°C, 1 min, 72°C, 1 min, and a final cycle of 72°C for 10 min. The PCR product was gel purified and used in a ligation reaction with the invertase signal sequence (Fast-Link, Epicentre, Madison, WI). One tenth of the reaction volume was used to amplify the ligated product using primers IF2 and PEPR, ExTaq polymerase (Fisher Scientific, Chicago, IL), and the previously described cycle parameters. The PCR product was cloned using pCR2.1-TOPO (Invitrogen, Carlsbad, CA).

The invertase signal:antimicrobial peptide insert was released from subsequent plasmid preparations by double digestion with *Xho*1 and *Xba*1, sites contained within primers PEPR and IF2 respectively. The end-digested fragment was gel purified

before use in ligation. The shuttle vector pRS413 (Stratagene, La Jolla, CA) was digested with the same restriction enzymes and gel purified. The insert and pRS413 were ligated (Fast-Link ligase) and used for bacterial transformation. Insert-containing colonies were identified by PCR screening and the plasmid propagated. The shuttle vector pRS413 contains the autonomous replication sequence (ARS) and centromeric sequence (CEN6) which allows for high copy replication in a non-integrative manner. The purified plasmid containing the invertase signal:antimicrobial peptide sequence and the plasmid alone, were used for transformation of YPH500 yeast cells (Stratagene) using the lithium acetate-polyethylene glycol-mediated transformation at 30°C (11) and selected on synthetic dextrose (SD) minimal media with amino acids, lacking histidine, at the same temperature. Histidine-complemented colonies were collected 8 days later and transferred to new yeast-peptone-adenine-dextrose (YPAD) (Difco Laboratories, Detroit, MI) agar plates. Colonies were transferred back to L-histidine-lacking media to test complementation stability and then back to YPAD agar.

**Selection of biologically active *S. cerevisiae* transformants and fruit protection assays.** Histidine -complemented colonies were grown in 1 ml YPAD broth for 24 h at 30 C and 25 µl of the yeast suspension was combined with 5 µl of *C. coccodes* spores ( $1.2 \times 10^6$  spores per ml). Treated spores in the presence of the yeast cells were germinated as described before. For fruit protections assays the suspension containing the growing yeast cells and the *Colletotrichum* spores were incubated at room temperature for 12 h and then transferred to freshly wounded tomato fruits. Control inoculations included spores of the wild type *C. coccodes* in a mixture with 25 µl of the YPAD media inoculated 24 hs earlier with either untransformed *S. cerevisiae* (WT) and *S. cerevisiae* containing the vector pRS413 only (YWT). Fruits were incubated for 6 days as described before. All experiments were repeated at least three times

**FPLC analysis of yeast extracts.** Fast protein liquid chromatography was carried out with a Liquid Chromatography Controller LCC-500 Plus (Pharmacia Biotech, AB, Uppsala, Sweden) using two Superose<sup>R</sup>-12 filtration columns (NR1013) connected in series (Pharmacia, Uppsala, Sweden). The columns were equilibrated

with 50 mM phosphate, 150mM NaCl (PBS), pH 7.0 that was filtered through a 0.45 µm Millipore membrane (Millipore Corp., Bedford, MA) prior to use. Sample volumes of 50 µl were applied to the columns and eluted with PBS at a flow rate of 0.6 ml/min. Peptides were detected by continuous absorbance readings at 280 nm and chart speed of 2mm/min.

To determine the active fraction in FPLC runs, aliquots of 40 µl out of 2 ml FPLC fractions were mixed with 5 µl of *C. coccodes* spores ( $1.2 \times 10^6$  spores per ml) and incubated as described before.

**Plasmid rescue and sequencing.** One of the colonies of the transformant Y-20, exhibiting inhibitory activity, was transferred to YPAD broth and cultured overnight. Cells were pelleted, resuspended in 10 mM Tris pH 7.5 and incubated for two hours at 37° C with 0.1 mg/ml of lyticase (Sigma, St. Louis, MO). Protoplasts were lysed and extracted with phenol:chloroform and the DNA precipitated with 1/20 vol. 2M sodium acetate and 3 vol. ethanol from the supernatant. Precipitated DNA was pelleted after overnight storage at -20° C, re-suspended in water and used to transform DH5α competent cells (Gibco BRL, Gaithersburg, MD). A plasmid from a transformed colony was sequenced to verify the presence of the construct (Center for Agricultural Biotechnology, University of Maryland, College Park, MD).

## RESULTS

**Antifungal activity of the synthesized peptide.** The synthesized peptide, WKLFKKILKVL, at 50 µM, inhibited germination of *C. coccodes* by 100% (Fig. 1) and showed an ED<sub>50</sub> of 13.45. At lower concentrations germination was not inhibited but hyphal growth was reduced. When spores of *C. coccodes* were germinated at 5 µM a percentage could germinate but the mycelium subsequently lyses and no further growth was observed (Fig. 2).

Decay development in tomato fruits following direct inoculation with *C. coccodes* incubated in the presence of the synthesized peptide was completely inhibited at 50 µmol and partially inhibited at 5 and 0.5 µmol (Fig. 3).

**Construction of invertase-antimicrobial peptide fusion.** We relied on phosphorylated primers to ligate the invertase signal sequence to the antimicrobial peptide sequence in the proper reading frame (Fig. 4). Use of one terminal primer from each fragment for selection of the ligated product avoided the need for additional cloning steps. Colonies containing the insert were identified at each step by PCR screening. Two final yeast transformants, Y-20 and Y-47, were further analyzed for their capability to secrete the peptide and inhibit *C. coccodes*.

**FPLC analysis of yeast peptide production.** FPLC runs of the YPAD extracts of Y-20, Y-47 containing the sequences encoding for the invertase signal:antimicrobial peptide and YWT-41 containing only the plasmid, were compared to the synthesized peptide dissolved in water. A peak was observed at a retention time of 59 min in the synthesized peptide sample and in samples from Y-20 and Y-47, but not in the YWT-41 sample. This peak contained the biological activity and inhibited germination of *C. coccodes* by ca. 70% (data not shown).

**Germination assays and fruit protection assays of transformed isolates.** Germination of *C. coccodes* spores in the presence of YPAD extract of the yeast transformants Y-20 and Y-47 did not inhibit germination but completely prevented growth of hyphae after spore germination. Inoculation of tomato fruits with spores of *C. coccodes* treated for 24 h with extracts of Y-20 and Y-47 resulted in no symptom development after 6 days of incubation at 20 C, compared to the control transformed yeast YWT-41 and untransformed WT strains (Fig. 5 A,B).

## DISCUSSION

Small peptides related to the host defense system of a variety of organisms have generated increasing agricultural interest based on the prospect of genetically engineering disease resistance in plants (17,22). The activity of antimicrobial peptides has been mainly tested against bacterial pathogens and a few fungal pathogens (5). Thus, these peptides are useful models for expression in transgenic plants to obtain resistance to fungal pathogens. However the directed expression of such peptides in yeast gives a new perspective on their use for biological control of pathogens and on the direct interaction of the peptide with the target pathogen. At 50  $\mu$ M the synthetic

peptide inhibited germination of *C. coccodes* with a significant reduction of inhibition at 5  $\mu$ M, suggesting a similar range of effect on the activity reported against *Phytophthora infestans* and *Fusarium* sp. (5). We found *C. coccodes* germination to be more sensitive than *C. accutatum* since in this species inhibition of germination was only partially inhibited by 50  $\mu$ M of the synthetic peptide (result not shown). When assessing the impact of antimicrobial peptides on fungal targets it is important to observe the fungus through out the exposure period. While germinating spores formed fungal biomass, it lost viability through disruption of membrane integrity. Thus, although the spores germinated, the hyphae may be unable to initiate an infection. The fact that germinated spores of *C. coccodes*, in the presence of extracts containing the transformed yeast, were unable to cause disease suggests that the peptide is biologically active against germinated spores in fruit tissue. The affected hyphal integrity possibly resulted in the lack of actively growing fungi since no symptom development was observed following inoculation on the fruit. The mechanism of action of the peptide, is believed to be interaction of the amphipathic cationic peptide with the phospholipids on the target cell membrane, followed by either channel formation or simple membrane disruption (24). The ability to adopt an amphipathic  $\alpha$ -helical structure in a membrane environment is thus the major requirement for activity in this type of peptide (13). By incubating the pathogen in the presence of *Saccharomyces cerevisiae* transformants Y-20 and Y-47, we have been able to completely inhibit fungal growth of *C. coccodes* and the subsequent decay development from germinated spores in tomato fruits. The mechanism of action of this peptide enables a direct interaction between the antifungal peptide and the target pathogen membrane, resulting in a rapid inhibition of the pathogen. This simple mechanism of action is very important in rapidly localizing the germinated spores of the attacking pathogen in the wounded tissue. Since biological control agents are applied as a postharvest treatment, the dynamics of secretion of the active peptide should be fast enough to overcome the rapid development of germinated spores at the infection site so that it will fit to the stringent conditions where the biological control agent should act. In spite of the fact that the expressed yeast is able to produce

biologically active peptides, further work will be needed to confirm the widespread applicability of this method for enhancing biological control.

The transformed yeast has the advantage over transgenic plant strategies in being available for application to a wide range of vegetable and fruit crops. While some crops are readily transformed, many are not. The yeast is readily transformed and various peptides may be rapidly introduced and expressed. The direct expression of peptides in yeasts could also prevent degradation problems encountered when peptides are expressed in plants. Recent studies indicate that cecropin B peptides are degraded by limited proteolysis and the extent of this degradation varies from one plant species to another (20). The direct interaction of Y-20 and Y-47 with the target pathogen does not preclude the possible effect of proteases from fruits affecting the peptide, but our results suggest that biological control by the peptide is feasible. The peptide we chose is resistant to plant proteases and represents just one of many possible small peptide candidates that could be used for yeast expression. The engineering of yeast expressing an antifungal peptide is a new approach for the biocontrol of postharvest pathogens. We envision further improvements and modifications by introducing other peptides, as well as engineering other yeast genera. The lack of activity towards nontarget organisms by the peptide and the fact that we have used *S. cerevisiae* as a delivery system, suggests that this method should provide a safe alternative to other methods for controlling postharvest diseases.

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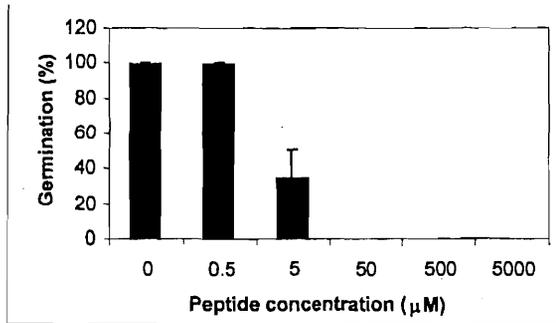
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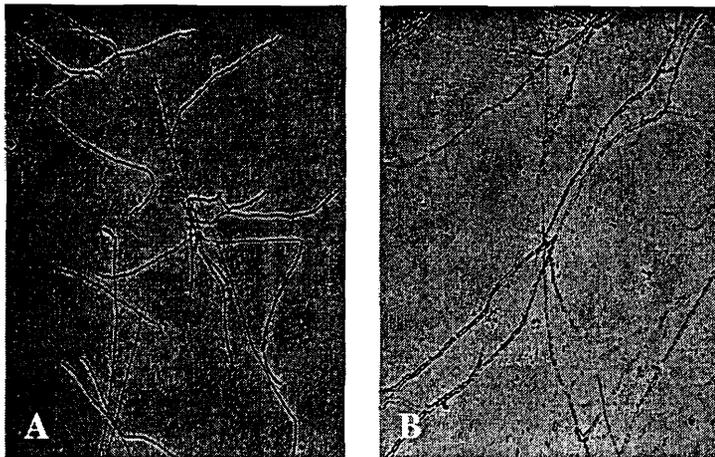
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## Figures

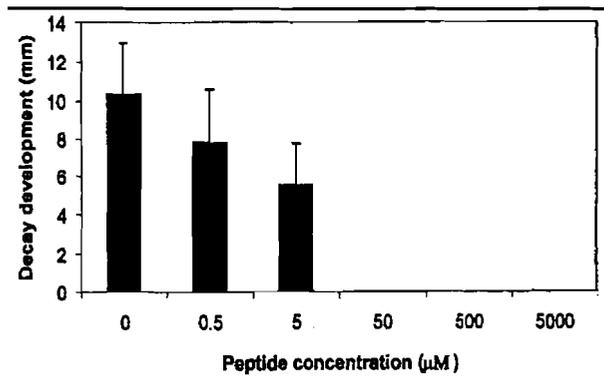


**Fig. 1.** *In vitro* antifungal activities of synthetic antifungal cecropin A-based peptide on *C. coccodes* germination. Fungal germination is expressed as a percentage of the germination in control spores (100% germination in water without the peptide is about 87%). Three or more replicates of each bioassay were performed, each time with a different spore suspension.



**Fig. 2.** Effect of synthetic antifungal peptide on *C. coccodes* germinated hyphae. Spores were exposed to 10  $\mu\text{M}$  of the pure cecropin A-based peptide. Three or more replicates of each bioassay were performed, each time with a different spore suspension. **A**, Hyphae in water, **B**, Hyphae in water plus peptide. Notice the loss of hyphal integrity in the presence of the peptide.

Observations were done with a phase contrast light microscope (BH-2 Olympus).



**Fig. 3.** *In vivo* antifungal activities of synthetic antifungal cecropin A-based peptide on the inhibition of anthracnose caused by *C. coccodes*. Decay development is determined as diameter of decay compared to the untreated spores. Three replicates of each inoculation were performed, each time with a different spore suspension.

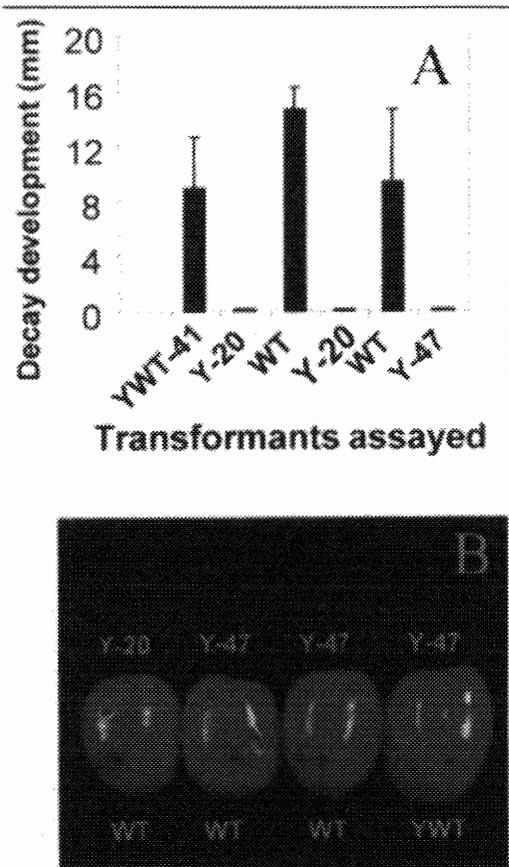
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TCTAGAGAAACAAGCAAAACAAAAGCTTTTCTTTTCACT
XbaI
AACGTATATGATGCTTTTGCAGCTTTCCTTTTCCTTTTG
M L L Q A F L F L L
GCTGGTTTTGCAGCCAAAATATCTGCATCAATGACAAACTG
A G F A A K I S A S M T N W
GAAACTCTTTAAGAAGATTCTCAAAGTGCTCTAGTAGCTC
K L F K K I L K V L
GAG
XhoI

```

restriction sites needed for ligation into pRS413.

**Fig. 4.** Construction of invertase leader sequence and antimicrobial peptide sequence. Two primers IR2 (in bold) and AMPF (in italics) were phosphorylated to allow for ligation in the proper reading frame. Terminal primers contained the



**Fig 5.** Decay development caused by *C. coccodes* in tomato fruits co-inoculated in the presence of transformed yeast isolates expressing the peptide. **A**, Decay development measurements, **B**, Symptom development of anthracnose. Isolates Y-20 and Y47 are transformed yeast expressing the antifungal peptide. Isolate YWT is a transformed yeast containing only the vector pRS413 and WT is the wild-type yeast strain.

### **5.3. Effect of Weekly and Fortnightly Sprayings of *Colletotrichum capsici* Mutant at Different Stages of Mango Development on Fruit Anthracnose Infection**

The approach used in Philippines was the selection of natural isolated of *Colletotrichum capsici* developing on peppers planted within the mango fruit trees.

#### **Methodology**

The ability of *Colletotrichum capsici* mutant (Isolate 013M<sub>4</sub>) to effectively control mango anthracnose in the field at the different stages of mango development was determined. The effect of the frequency of 013M<sub>4</sub> applications on the development of fruit anthracnose was also tested. Application of 013M<sub>4</sub> at the different stages of growth was done weekly and fortnightly with the following treatments: a) weekly spraying starting at pre-bloom; b) weekly spraying at mango flowering; c) weekly spraying at fruit set; d) fortnightly (once in 2 weeks) spraying starting at pre-bloom; e) fortnightly spraying starting at mango flowering and f) fortnightly spraying starting at fruit set.

A standardized inoculum suspension of 10<sup>6</sup> spores/ml from 7-day old cultures of 013M<sub>4</sub> was used and 48 hours later, the test organism was challenge inoculated with the pathogen, *Colletotrichum gloeosporioides*, using the same inoculum concentration (10<sup>6</sup> spores/ml).

Periodic spraying of the experimental units was then done following the given spray schedule until the fruits were at the green nature stage when they were ready for harvest. Three control set-ups for each growth stage of mango were used: a) untreated; b) inoculated with the pathogen only; and c) application with 013M<sub>4</sub> only.

#### **Results**

The *Colletotrichum capsici* mutant (Isolate 013M<sub>4</sub>) was highly effective in reducing the incidence of fruit anthracnose infection irrespective of the stage of mango development when treatment with the mutant was initiated. When spraying with the mutant was started at the pre-bloom stage, weekly or fortnightly (once in two weeks) treatments were equally effective in reducing fruit anthracnose (Table 1). The same

trend as above was observed when treatment with the mutant was started at the flowering stage (Table 2) as well as at fruit set (Table 3).

These results indicate that treatment with the mutant once in two weeks (fortnightly) is just as effective as weekly treatment. Thus, to save time, money and effort, fortnightly spraying is preferred.

Table 1. Effect of weekly and fortnightly application of *Colletotrichum capsici* mutant (013M<sub>4</sub>) on anthracnose infection at the pre-bloom stage of mango development

<i>Treatment</i>	Average Percentage Infection (%)	
	Schedule of Application	
	Weekly	Fortnightly
Pathogen only	25.30a	23.66a
013M <sub>4</sub> +Pathogen	8.53b	7.65b
013M <sub>4</sub> only	8.20b	9.76b
Control	8.02b	8.02b

<sup>1</sup> Figures with the same letter (s) are not significantly different from each other at LSD<sub>.05</sub>.

<sup>2</sup> Average % infection based on the total surface area of the fruit infected.

<sup>3</sup> Inoculations were done weekly and fortnightly at the pre-bloom stage of mango development.

<sup>4</sup> Fruits were harvested at 120 days after flower induction and data were gathered 10 days after harvest when the fruits turned yellow.

Table 2. Effect of weekly and fortnightly application of *Colletotrichum capsici* mutant (013M<sub>4</sub>) on anthracnose infection at the flowering stage of mango development

<i>Treatment</i>	Average Percentage Infection (%)	
	Schedule of application	
	Weekly	Fortnightly
Pathogen only	19.02a	18.65a
013M <sub>4</sub> +Pathogen	6.36bc	8.50c
013M <sub>4</sub> only	3.95b	4.58bc
Control	3.23b	3.23b

<sup>1</sup> Figures with the same letter (s) are not significantly different from each other at LSD<sub>.05</sub>.

<sup>2</sup> Average % infection based on the total surface area of the fruit infected.

<sup>3</sup> Inoculations were done weekly and fortnightly at the flowering stage of mango development.

<sup>4</sup> Fruits were harvested at 120 days after flower induction and data were gathered 10 days after harvest when the fruits turned yellow.

Table 3. Effect of weekly and fortnightly application of *Colletotrichum capsici* mutant (013M<sub>4</sub>) on anthracnose infection at the fruit set stage of mango development

<i>Treatment</i>	Average Percentage Infection (%)	
	Schedule of application	
	Weekly	Fortnightly
Pathogen only	19.57a	14.92b
013M <sub>4</sub> +Pathogen	2.63c	2.27c
013M <sub>4</sub> only	1.51c	1.38c
Control	1.75c	1.75c

<sup>1</sup> Figures with the same letter (s) are not significantly different from each other at LSD<sub>.05</sub>.

<sup>2</sup> Average % infection based on the total surface area of the fruit infected.

<sup>3</sup> Inoculations were done weekly and fortnightly at the fruit set stage of mango development.

<sup>4</sup> Fruits were harvested at 120 days after flower induction and data were gathered 10 days after harvest when the fruits turned yellow.

#### **5.4. Effect of Inoculation with the Pathogen, *Colletotrichum gloeosporioides* on Anthracnose Development in Fungicide Treated Trees**

##### **Methodology**

The effect of inoculation with the pathogen, in fungicide treated plants was determined. The pathogen, *Colletotrichum gloeosporioides*, was inoculated weekly and fortnightly at the pre-bloom, flowering, and fruit set stages of mango development. An inoculum concentration of  $10^6$  spores/ml from 7-day old cultures of *C. gloeosporioides* was prepared and inoculated weekly and fortnightly at the different growth stages of mango mentioned earlier. Fungicide was applied twice during the entire mango season along with the pesticides and fertilizers as well as other routine farm practices.

A control set-up with untreated or uninoculated experimental units was provided for each growth stage of mango. Mango fruits were harvested at the green mature stage (120 days after flower induction) and incubated at the laboratory for 10 days. Symptoms of anthracnose infection were observed and noted.

##### **Results**

Inoculation of mango with the pathogen, *Colletotrichum gloeosporioides* at the pre-bloom stage of mango development appeared to be the most devastating among the other growth stages. Application of the pathogen at this stage, whether inoculation was done weekly or fortnightly, resulted to zero harvest as the experimental units had already fallen off before the green mature stage has been reached. This goes to show that the pre-bloom stage is the most susceptible stage to mango anthracnose infection (Table 4).

Between the flowering and the fruit set stages, the fruit set stage seemed to be less susceptible to mango anthracnose as 14.36 and 16.36 average % infections which were not significantly different from one another were noted when inoculated weekly and fortnightly, respectively, at the fruit set stage. These were significantly different from the weekly and fortnightly average % infections at the fruit set stage at 45.0 and 28.82% infections, respectively.

These results show that the usual farm practice of fungicide application was not able to control anthracnose infection with the inoculation of *C. gloeosporioides* at the different growth stages of mango. High average % infection based on disease severity was observed which were significantly different from that of the control which was not inoculated with the pathogen.

Table 4. Effect of Inoculation with the Pathogen, *Colletotrichum gloeosporioides* on Anthracnose Development in Fungicide Treated Trees

Treatment	Average Percentage Infection (%)		
	Schedule of application		
	Weekly	Fortnightly	Control
Pre-bloom	No fruits harvested	No fruits harvested	5.07a
Flowering	45.0b	28.82c	4.62a
Fruit Set	14.36d	16.36d	3.61a

<sup>1</sup> Figures with the same letter (s) from a to c are not significantly different at LSD<sub>.05</sub> in comparing treatments in a row and w to y in comparing treatments in a column.

<sup>2</sup> Average % infection based on the total surface area of the fruit infected.

<sup>3</sup> Inoculations were done weekly and fortnightly at the fruit set stage of mango development.

<sup>4</sup> Fruits were harvested at 120 days after flower induction and data were gathered 10 days after harvest when the fruits turned yellow.

### 5.5 Stability and Efficacy of *Colletotrichum capsici* Mutant (013M<sub>4</sub>) and the Pathogen, *Colletotrichum gloeosporioides* After Storage for Various Lengths of Time at 10°C

#### Methodology

The test isolate from *Colletotrichum capsici* which had been altered through UV radiation showing reduced pathogenicity (013M<sub>4</sub>) and the pathogen, *Colletotrichum gloeosporioides* were grown in prune agar medium and incubated for 10 days in the

case of *C. capsici* and 7 days in the case of *C. gloeosporioides* to allow sufficient sporulation of the organisms.

Cultures of 013M<sub>4</sub> and *C. gloeosporioides* were stored for different lengths of time (0, 1, 2, 3, 4, 5, 6, 8, 10, and 12 months) at 10°C. The stability of both organisms after storage for each length of time at 10°C was tested. The effectiveness of 013 M<sub>4</sub> in inducing resistance to anthracnose in mango and the virulence of *C. gloeosporioides* were tested after storage at 10°C for various storage periods mentioned earlier. Pathogenicity tests were conducted and the efficacy of both 013M<sub>4</sub> and *C. gloeosporioides* was evaluated.

## Results

The effectiveness of *Colletotrichum capsici* mutant, 013M<sub>4</sub> reduce the incidence of anthracnose in mango and the virulence of the pathogen, *Colletotrichum gloeosporioides* after storage of the organisms for different periods were determined. Prolonged storage of the organisms at 10°C did not seem to affect the efficiency of either 013M<sub>4</sub> or *C. gloeosporioides* (Table 5).

Results showed that the average percentage infection of 013M<sub>4</sub> on the first month was not significantly different from that on the 2<sup>nd</sup>, 3<sup>rd</sup>, 4<sup>th</sup>, 5<sup>th</sup>, 6<sup>th</sup>, 8<sup>th</sup>, 10<sup>th</sup>, and 12<sup>th</sup> months. This showed that the reduced pathogenicity characteristic of 013M<sub>4</sub> was maintained. The same was true for *C. gloeosporioides* as the average percentage infections were not significantly different from one another when the pathogen was stored for 1 month at 10°C until 12 months, suggesting the stability of the organism in the growth medium. In the same manner, challenge inoculation of the pathogen, *C. gloeosporioides*, with 013M<sub>4</sub> did not show any variability as the average percentage infections upon storage for 1, 2, 3, 4, 5, 6, 8, 10, and 12 months at 10°C were not significantly different from one another. This only implied that the effectiveness of 013M<sub>4</sub> to reduce anthracnose infection was not affected by the length of time the organism was stored in prune agar medium.

Table 5. Efficacy of *Colletotrichum capsici* Mutant (013M<sub>4</sub>) and the Pathogen, *Colletotrichum gloeosporioides* After Storage for Various Lengths of Time at 10°C.

Length of Storage	Average Percentage Infection (%)		
	<i>C. gloeosporioides</i> only	013M <sub>4</sub> + <i>C. gloeosporioides</i>	013M <sub>4</sub> only
1 month	88.25a	35.00b	3.75c
2 months	78.75a	26.25bd	6.75ce
3 months	75.00a	22.50be	4.50cf
4 months	72.50a	36.25b	13.75cde
5 months	62.25a	26.50bd	5.50ce
6 months	77.75a	35.00b	8.00ce
8 months	74.00a	33.75b	5.5ce
10 months	85.25a	27.5bd	8.00ce
12 months	68.75a	21.25bdef	3.75c

<sup>1</sup> Data based on the average of four replications.

<sup>2</sup> Average % infection based on the total leaf surface area infected.

<sup>3</sup> Figures with the same letter(s) are not significantly different at LSD<sub>0.05</sub>.

## 5.6 Test on the Stability and Efficacy of *Colletotrichum capsici* Mutant (013M<sub>4</sub>) and the Pathogen, *Colletotrichum gloeosporioides* in Filter Paper Disks After Storage for Various Lengths of Time

### Methodology

Cultures of *Colletotrichum capsici* mutant, 013M<sub>4</sub> and the pathogen, *Colletotrichum gloeosporioides* were maintained in sterilized filter paper disks placed in a desiccator with silica gel at 0°C. For *C. gloeosporioides*, sterile paper disks were placed on the surface of prune agar plates containing the 4-day-old growth of the organism while for 013M<sub>4</sub>, disks were placed in potato agar plates. The organisms were incubated for 10 days at room temperature and allowed to grow until the disks were covered with the fungal growth. The disks were then kept in sterile coin envelopes which were stored in a desiccator containing silica gel at 0°C.

The efficacy of 013M<sub>4</sub> and *C. gloeosporioides* were tested through periodic inoculation and challenge inoculation. Filter paper disks stored for a particular length of time were transferred to a fresh suitable medium and allowed to grow and sporulate in the said medium. A spore load of 10<sup>6</sup> spores/ml was used in the succeeding inoculations in testing the efficiency of the organisms. The effectiveness of 013M<sub>4</sub> to reduce anthracnose infection and the virulence of the pathogen after storage for various lengths of time was evaluated.

### Results

The effect of storage of *Colletotrichum capsici* mutant, 013M<sub>4</sub> and the pathogen, *Colletotrichum gloeosporioides* grown and maintained in filter paper disks for various lengths of time was determined. The efficiency of 013M<sub>4</sub> in reducing anthracnose infection and the virulence of the pathogen appeared not to be affected after 5 months of being maintained in filter paper disks (Table 6). However, slower growth rate and sporulation of both 013M<sub>4</sub> and *C. gloeosporioides* were observed as storage in filter paper disks of the organisms was prolonged. More slants which contained the fungal growth with spores were needed to produce the required inoculum concentration of 10<sup>6</sup> spores/ml.

Both 013M<sub>4</sub> and *C. gloeosporioides* failed to grow after 6 months of incubation, when the filter paper disks containing the fungal growth with spores were transferred

to potato agar slants and prune agar slants which would serve as the growth media of the said organisms. Maintenance of 013M<sub>4</sub> and *C. gloeosporioides* in filter paper disks as stock cultures appeared not to be an effective preservation technique since loss of viability of the organisms was observed when subjected to such storage condition.

Table 6. Efficacy of *Colletotrichum capsici* Mutant (013M<sub>4</sub>) and the Pathogen, *Colletotrichum gloeosporioides* in Filter Paper Disks After Storage for Various Lengths of Time

Length of Storage	Average Percentage Infection (%)		
	<i>C. gloeosporioides</i> only	013M <sub>4</sub> + <i>C. gloeosporioides</i>	013M <sub>4</sub> only
0 month storage	96.50a	30.00b	5.00c
1 month storage	90.75ad	37.50b	3.25c
2 months storage	87.25 ad	35.00b	4.25c
3 months storage	83.25 ad	43.75b	6.50c
4 months storage	80.00ad	41.25b	8.5c
5 months storage	75.75d	35.00b	6.5c

Data based on the average of four replications.

<sup>2</sup> Average % infection based on the total leaf surface area infected.

<sup>3</sup> Figures with the same letter(s) are not significantly different at LSD<sub>.05</sub>.

## 5.7. Mass Production of *Colletotrichum capsici* Mutant and Pathogen, *Colletotrichum gloeosporioides* Inoculum

### Methodology

A suitable growth medium which would allow proliferation of the spores of *Colletotrichum capsici* mutant, 013M<sub>4</sub> and the pathogen, *Colletotrichum gloeosporioides*, was determined. The organisms were grown in various media: Potato Dextrose Agar, Potato Agar, Prune Agar, Mango Decoction Agar, Pepper Decoction Agar. After sufficient growth of the organisms covered the agar or after 7 days of incubation, a thin cellophane and disinfected leaves of mango and pepper were placed over the surface of the agar containing the fungal growth. The cellophane and the mango and pepper leaves were disinfected in 95% ethanol for 10 minutes. The organisms were allowed to grow over the surface of the cellophane and the mango and pepper leaves. After 14 days of incubation, the cellophane and the leaves were removed from the medium and were suspended in 10 ml water. Spores were dislodged from the plastic film and the leaves using a sterile wire loop.

Several broth media which would allow profuse sporulation of 013M<sub>4</sub> and *C. gloeosporioides* were prepared in 250 ml Erlenmeyer flasks. Using a cork borer, disks from 7-day old cultures of 013 M<sub>4</sub> and *C. gloeosporioides* were inoculated in different broth media: Sweet Potato Broth, Mango Decoction Broth, Pepper Decoction Broth, Potato Broth and, Prune Broth. The flasks containing the different broth media were agitated using a rotary shaker to facilitate aeration. The spore load in each of the broth media was counted after 14 days of incubation at room temperature with constant shaking of the culture. The number of spores observed in broth media, leaves, and plastic film were compared.

### Results

In mass production of *Colletotrichum capsici* mutant, 013M<sub>4</sub>, Potato Dextrose Agar and Potato Agar appeared to be the most suitable medium as highest number of spores was counted when grown in these media (Table 7). Sporulation was further enhanced when pepper leaves which were previously disinfected in 95% ethanol were placed over the surface of the medium with the fungal growth. An average spore

concentration of  $3.86 \times 10^6$  in 10 ml water was counted when Potato Agar medium was amended by adding pepper leaves over the surface of the medium after 7 days of incubation when the organism has already sufficiently grown in the medium. Using Potato Dextrose Agar medium,  $2.68 \times 10^6$  spores were counted when the fungal spores were dislodged from the pepper leaves in 10 ml water. The average number of spores counted using the two media were not significantly different from one another.

Less sporulation, however, was observed when a cellophane was placed over the surface of the agar and when the organism was grown in liquid shake culture medium. The average spore concentrations when plastic film and when liquid shake culture medium were used, were significantly different from that of the average spore concentration when leaves were placed over the surface of the media.

Table 7. Mass Production of *Colletotrichum capsici* mutant, 013M<sub>4</sub> in Different Growth Culture Media

Growth Culture Media	Average Spore Concentration
Mango Leaves	
Potato Dextrose Agar	$2.68 \times 10^6$ ab
Potato Agar	$3.86 \times 10^6$ a
Prune Agar	$2.00 \times 10^5$ c
Cellophane	
Potato Dextrose Agar	$1.05 \times 10^6$ bc
Mango Decoction Agar	0 c
Pepper Decoction Agar	$3.75 \times 10^4$ c
Prune Agar	$8.75 \times 10^4$ c
Shake Culture	
Camote Broth	$2.50 \times 10^4$ c
Mango Decoction Broth	$1.25 \times 10^4$ c
Potato Dextrose Broth	0 c
Pepper Decoction Broth	$1.25 \times 10^4$ c
Potato Broth	$1.25 \times 10^4$ c

<sup>1</sup> Data based on the average of four replications.

<sup>2</sup> Average spore concentration based on the total number of spores counted.

<sup>3</sup> Figures with the same letter(s) are not significantly different at LSD<sub>0.05</sub>.

Sporulation of the pathogen, *Colletotrichum gloeosporioides* appeared to be favored when grown in Prune Agar and Potato Dextrose Agar which was amended with mango leaves disinfected in 95% ethanol, showing the highest number of spores with  $5.09 \times 10^6$  spores and  $5.64 \times 10^6$  spores, respectively (Table 8). Less number of spores were counted when cellophane was placed over the surface of the media and

when liquid shake culture media was used. The average number of spores from broth media and from the media amended with cellophane were significantly different from the average number of spores from that of the media amended with the mango leaves.

Table 8. Mass Production of the Pathogen, *Colletotrichum gloeosporioides* in Different Growth Culture Media

Growth Culture Media	Average Spore Concentration
Mango Leaves	
Potato Dextrose Agar	5.64 x 10 <sup>6</sup> a
Potato Agar	8.00 x 10 <sup>5</sup> bc
Prune Agar	5.09 x 10 <sup>6</sup> a
Cellophane	
Potato Dextrose Agar	3.75 x 10 <sup>4</sup> b
Mango Decoction Agar	1.25 x 10 <sup>5</sup> b
Pepper Decoction Agar	2.00 x 10 <sup>5</sup> b
Prune Agar	3.88 x 10 <sup>5</sup> b
Shake Culture	
Sweet Potato Broth	1.58 x 10 <sup>6</sup> cd
Mango Decoction Broth	5.00 x 10 <sup>5</sup> b
Potato Dextrose Broth	1.94 x 10 <sup>6</sup> d
Pepper Decoction Broth	5.25 x 10 <sup>5</sup> b
Prune Broth	1.63 x 10 <sup>5</sup> b

<sup>1</sup> Data based on the average of four replications.

<sup>2</sup> Average spore concentration based on the total number of spores counted.

<sup>3</sup> Figures with the same letter(s) are not significantly different at LSD<sub>0.05</sub>.

## 5.8 Determination of the Mechanism of Action of *Colletotrichum capsici* Mutant (013M<sub>4</sub>) in Reducing Anthracnose Infection

### 5.8.1 Test for volatile compounds

#### Methodology

From the stock culture, a loopful of the agar containing *Colletotrichum capsici* mutant (013M<sub>4</sub>) was transferred to potato dextrose agar plates. After four days of incubation, when 013M<sub>4</sub> had sufficiently grown and was already established in the agar media, the petri dish cover was removed and was then replaced by another dish containing a newly transferred *C. gloeosporioides*, also grown in potato dextrose agar. The two organisms contained in separate plates were incubated on top of the other. The

plates were secured using a parafilm and were then observed for any inhibitory effect to *C. gloeosporioides* due to possible release of volatile compounds by 013M<sub>4</sub>.

## Results

The mode of action of *Colletotrichum capsici* mutant (013M<sub>4</sub>) in reducing anthracnose infection caused by *Colletotrichum gloeosporioides* appeared to be due to factors other than production of volatile compounds. This was based on the observation that uninhibited growth of the pathogen, *C. gloeosporioides* still occurred.

### 5.8.2 Effect of *Colletotrichum capsici* Mutant (013M<sub>4</sub>) culture filtrate

#### Methodology

The effect of the culture filtrate from *Colletotrichum capsici* mutant (013M<sub>4</sub>) on growth, sporulation and spore germination of the pathogen, *Colletotrichum gloeosporioides*, was determined.

A spore suspension was made from 7-day old cultures of 013M<sub>4</sub> and was passed through sterilized Seitz filter with several layers of filter papers to extract the filtrate excluding the spores. After the filtrate had been collected, a loopful was obtained and checked if spores are still present. The filtrate should be free of any spores or mycelia.

To test the effect of the filtrate on growth of the pathogen, the growth media was amended with the incorporation of the filtrate. Ten milliliters of the filtrate was added to a flask containing 250 ml of melted potato dextrose agar when the media had already cooled just before pouring into petri plates. A loopful of the agar from the stock culture containing growth of *C. gloeosporioides* was then inoculated to the amended petri plates. The plates were incubated for 7 days and were observed for any inhibition of growth when grown in media amended with the culture filtrate of 013M<sub>4</sub>. The number of spores was also noted to determine if the culture filtrate would affect sporulation of *C. gloeosporioides*.

The effect of the filtrate on germination of the spores of *C. gloeosporioides* was also studied. Different proportions of the culture filtrate were added to the spore

suspension of *C. gloeosporioides* in sterile glass slides to make 1:1, 1:2, 1:3 and 1:10 dilutions. The slides were incubated in a moist chamber and the percentage germination after 24, 48, 64, and 72 hours were noted. Data were based on the first 200 spores that were counted. Culture filtrate was also applied to mango leaves to determine its ability to reduce anthracnose infection. The filtrate was spray inoculated at different time intervals: simultaneous inoculation to *C. gloeosporioides* and 24, 48, and 72 hours prior to *C. gloeosporioides* inoculation. Drop inoculation of the filtrate was also done by placing 5µl of the filtrate to the same spot in the leaf where 5µl of *C. gloeosporioides* spore suspension had already been inoculated. Inoculated leaves were incubated and were observed for symptom development.

## Results

The filtrate used was extracted from 7-day old cultures of *Colletotrichum capsici* mutant (013M<sub>4</sub>). The acidity of the filtrate was determined and was noted to be at pH 6.

Amending the growth media of 013M<sub>4</sub> by the addition of 10 ml of the culture filtrate to 250 ml melted potato dextrose agar did not affect the growth of *Colletotrichum gloeosporioides*. Growth of the pathogen was not in any way inhibited and after 7 days of incubation, was not observed to be different from the control. Sporulation was also not affected when culture filtrate was incorporated to the growth medium.

Germination was likewise not affected when the spores were incubated with the culture filtrate from 013M<sub>4</sub>. Spores treated with the culture filtrate had about the same percentage germination as the control, if not higher. Normal elongation of germ tube was also observed even with the application of the filtrate. Neither spray application of the culture filtrate to the mango leaves prior or simultaneous to inoculation of the pathogen, *C. gloeosporioides*, inhibited or reduced anthracnose infection in mango leaves. Infection was still prevalent even upon treatment with the 013M<sub>4</sub> culture filtrate regardless of the stage the filtrate was applied. Drop inoculation of the filtrate at the same spot the pathogen was inoculated, likewise developed anthracnose infection at the inoculated area.

### **5.8.3. Test for antagonistic activity using dual culture**

#### **Methodology**

This test was done by growing *Colletotrichum capsici* mutant (013M<sub>4</sub>) and *Colletotrichum gloeosporioides* in the same petri plates containing the agar medium. Both organisms were seeded on the agar medium on the same day and this was done by placing small agar disks containing growth of the organism at the opposite ends of the plates. The plates were incubated at room temperature for 7 days and were observed for any inhibition of growth.

#### **Results**

Dual culture tests showed that growth of the pathogen, *C. gloeosporioides* and *Colletotrichum capsici* mutant (013M<sub>4</sub>) was somewhat inhibited. The hyphae of 013M<sub>4</sub> and *C. gloeosporioides* could not be easily differentiated when viewed under a microscope at the junction where the two cultures met. Further tests are still needed to determine any direct or specific action of 013M<sub>4</sub> against *C. gloeosporioides* to see if competition for space and/or nutrients or parasitism may be factors that can be considered in determining the effect of 013M<sub>4</sub> on *C. gloeosporioides*.

## **6. Impact Relevance and Technology Transfer**

In this project we studied and developed several innovative technologies for disease control of postharvest pathogens in Philippines and Israel. All the knowledge that was accumulated from this project in the Volcani Center was transferred to the University of Philippines, Department of Plant Pathology, at Los Banos through the trainee Mrs. Jeniffer Nim, which was involved in developing and testing the new technologies.

In Israel the approach used was to develop mutants of the wild type *Colletotrichum gloeosporioides* with 1. Reduced pathogenicity that induces the natural resistance and 2. Mutants secreting specific toxic peptides that inhibit fungal attack. While in Philippines the approach was to select natural isolates of *Colletotrichum* with reduced pathogenicity. Both approaches represent new developments for disease control.

Presently in the Philippines the use of the natural-reduced pathogenicity isolates is being tested in large scale.

## **7. Project Activities/Outputs:**

**List of meeting and trainees held for the entire project.**

On 2003, Dr. Prusky visited Philippines and discussed the development of the project as well visited several mango orchards.

On August 2005, the trainee Mr. Jeniffer Nim came to the Department of Postharvest Science and stay in Israel for the period of 18 months until December 2006. This was an extraordinary visit since Jennifer become involved in all the different technologies used in the Department of Postharvest. She was involved as part of her MsC thesis in the understanding of the etiology and pathogenicity of aspects and a manuscript is being written in present days.

Reports of the findings of the project were reported in the International Mango Symposium in 2002 that took place in Brazil in the year 2002 and in the International Society of Plant Pathology that took place in New Zealand in 2003.

## **8. Project productivity:**

### **Lectures in International Symposium**

1. Prusky, E. Ilag, L. Ackerman, M., Kobilier . I. Fuchs, I. 2002. New Approaches for the control of postharvest diseases of mango fruits. International Mango Symposium, Brazil.
2. Prusky, D., Shalom, Y., Kobilier, I. Akerman, M. and Fuchs, Y. 2003. International Mango Symposium, Brazil.
3. Niem,J., I. Miyara, Y., Reuveni, M., Etedgui, Y., Fleishaman, M., and D. Prusky. 2006. The mechanism of differential resistance of apple fruits to *Alternaria alternata* causing moldy-core disease in apple fruit. Israel Society of Phytopathology, Bet Dagan, Israel.

## **Publications**

1. Yakoby, N., Zhou, R., Kobilier, I., Dinoor, A., and Prusky, D. 2001. Development of *Colletotrichum gloeosporioides* REMI mutants as biocontrol agents against anthracnose disease in avocado fruits. 2001 *Phytopathology* 91:143-148.
2. Jones, R.W. and Prusky D. 2002. Expression of an antifungal peptide in *Saccharomyces*: a new approach for biocontrol of the postharvest disease caused by *Colletotrichum coccodes*. *Phytopathology* 92:33-37.
3. Prusky, E. Ilag, L. Ackerman, M., Kobilier . I. Fuchs, I. 2003. New Approaches for the control of postharvest diseases of mango fruits. *Acta Horticulture* 645: 129-137.
4. Prusky, D., Shalom, Y., Kobilier, I. Akerman, M. and Fuchs, Y. 2003. Postharvest fungicide treatments in mango fruits according to the level of quiescent infection of pathogens at harvest. *Acta Horticulturae* 645: 605-617.

## **9. Future Work:**

The use of M13 will be further tested in Philippines in a broader scale for further testing.