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**Elicitation of Preformed Antifungal Compounds by
Non-Pathogenic Fungus Mutants and their Use for the
Prevention of Postharvest Decay in Avocado Fruits**

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**The mechanisms of elicitation of preformed antifungal
compounds by pathogenic and non-pathogenic
mutants and their use for the prevention of
postharvest decay in avocado fruits**

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Index of Content

1. Abstract	2
2. Achievment	
• Significance of the scientific achievments	3
• Potential applications	4
• Estimated impact	5
• Details of Cooperation	5
3. Body of the report	
• Background, scientific and agricultural Relevance of the project	5
• Results	6
• Scientific implications, potential impact On agriculture	7
• List of publications	8
4. Appendices	10

Publication Summary (numbers)

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Refereed (published, in press, accepted)			2	
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Invited review papers			2	
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Ph.D. theses			1	
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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	1	3	5
Sabbaticals				
Postdoctorates			1	

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

Abstract

C. gloeosporioides attacks unripe avocado fruits in the orchard. Germinated spores produce appressoria that germinate and breach the cuticle, but the resultant subcuticular hyphae become quiescent and do not develop further until fruit is harvested and ripens. Resistance of unripe avocado to attack by *C. gloeosporioides* is correlated with the presence of fungitoxic concentrations of the preformed antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene in the pericarp of unripe fruits. The objective of these proposal was to study the signal transduction process by which elicitors induce resistance in avocado. It was found that abiotic elicitors, infection of avocado fruit with *C. gloeosporioides* or treatment of avocado cell suspension with cell-wall elicitor induced a significant production of reactive oxygen species (ROS). Ripe and unripe fruit tissue differ with regard to the ROS production. The unripe, resistant fruits are physiologically able to react and to produce high levels of ROS and increased activity of H⁺ATPase that can enhance the phenylpropanoid pathway and regulate the levels of the antifungal compound-diene, inhibit fungal development, resulting in its quiescence. Interestingly, it was also found that growth regulators like cytokinin could do activation of the mechanism of resistance. Preharvest treatments of cytokinins strongly activated the phenylpropanoid pathway and induce resistance. We have developed non-pathogenic strains of *C. gloeosporioides* by Random Enzyme Mediated Integration and selected a hygromycin resistance, non-pathogenic strain Cg-142 out of 3500 transformants. This non-pathogenic isolate activates H⁺ATPase and induces resistance against *Colletotrichum* attack. As a basis for studying the importance of PL in pathogenicity, we have carried out heterologous expression of *pel* gene from *C. gloeosporioides* in the non-pathogenic *C. magna* and determine the significant increase in pathogenicity of the non-pathogenic strain. Based in this results we can state that pectate lyase is an important pathogenicity factor of *C. gloeosporioides* and found that fungal pathogenicity is affected not by *pel* expression but by PL secretion. Our results suggest that pH regulates the secretion of pectate lyase, and support its importance as a pathogenicity factor during the attack of avocado fruit by *C. gloeosporioides*. This implicates that if these findings are of universal importance in fungi,

control of disease development could be done by regulation of secretion of pathogenicity factors.

Achievements

Significance of the scientific achievements

1. Signal transduction process in resistant fruits

We have found that infection of avocado fruit with *C. gloeosporioides* or treatment of avocado cell suspension with cell-wall elicitor induced ROS production. Ripe and unripe fruit tissue differ with regard to the ROS production in response to pathogen attack. The unripe, resistant fruits are physiologically able to react and to produce high levels of ROS that can enhance the phenylpropanoid pathway and regulate the levels of the antifungal compound-diene, inhibit fungal development, resulting in its quiescence. It was also found that different elicitors can modify the plasma membrane properties and specifically H⁺ ATPase activity and this can lead to the initiation of a cascade leading to physiological processes and induce resistance.

2. Effect of growth regulators for induction of resistance against postharvest pathogens.

As a side project of the culture of avocado cell suspension in vitro we have found that avocado tissue is very responsive to cytokinin treatments. Cytokinin-dependent tissue exposed to TDZ and BAP enhanced the biosynthesis of epicatechin and affect increase the resistance to *C. gloeosporioides* attack. This effect raises a new option for modulation of fruit resistance to postharvest pathogen in avocado fruits by preharvest sprays with growth regulator for the induction of resistance. These results are being further implement in the orchard.

2. Development on non pathogenic mutants using Random Enzyme Mediated Integration

This was done by transformation of *C. gloeosporioides* with a linear fragment of DNA (Hygromycin resistant cassette) in the presence of the restriction

enzyme that was used to generate the linear fragment. Under these conditions the linear DNA integrated into the chromosome at the restriction sites recognized by that particular enzyme. The *C. gloeosporioides* REMI mutants were selected for reduced pathogenicity. These mutants were used as tools for induce resistance against the wild type *C. gloeosporioides*.

4. Heterologous expression of *pel* in non-pathogenic *Colletotrichum* strain as a basis to determine the importance of pectate lyase in pathogenicity.

Our results have clearly shown the contribution of a single *pel* to the pathogenic abilities of *C. magna* and suggest that PL is a pathogenicity factor required for the penetration and colonization of *Colletotrichum* spp.

5. Host pH, a novel mechanism for the regulation of *Colletotrichum gloeosporioides* quiescent infection

We demonstrate the effect of pH in the regulation of PL secretion and its importance during pathogenesis of *C. gloeosporioides*. We suggest that gene expression and protein secretion of PL are not synchronized processes. Even if inducing media can activate gene expression of *pel*, protein PL secretion seems to be affected by post-transcriptional effects directly or indirectly by pH. This environmental effect on protein secretion however affects the conditions for fungal colonization and decay development of *C. gloeosporioides*.

Potential for applications

We believe the potential applications of the findings of this project are based on the development of non-pathogenic transgenic *Colletotrichum* strains that can be used for biological control. Furthermore, the effect of pH on secretion of pathogenicity factors has universal implications if it will found in other pathogens. This could indicate that it might be possible to control diseases by regulation of the secretion. This new idea is the subject of the ms accepted in Environmental and Applied Microbiology what could suggest that a new way for the control of disease was found based in this data.

Intestinally one of the side developments of this project, the use of growth regulators for the control of postharvest disease, was significantly shown and could become a simple way for the control of postharvest diseases.

On the basic part of this proposal we have the first report of signaling events, ROS production, in fruits and specifically in avocado. This could indicate that the use of exogenic reactive oxygen species might be in the future a simple way for inducing resistance in fruits.

Estimated Impact

Presently the intriguing findings where pH regulate the secretion of pathogenicity factor and the finding that oxygen burst occurs in fruits and different in fruits according to their physiological basis, both could represent the most significant findings that might have an impact in the World Postharvest Industry and in Plant Pathology in general.

Details of Cooperation

The Israeli and American groups were constantly in contact by different ways. Visits to the American or Israeli laboratories, discussion in meetings, review of manuscripts. We believe that the personal and scientific interactions developed in this project have been of tremendous importance for the scientific development of all participants

Body of the report

Background, scientific and agricultural relevance of the project

The most important pathological factor limiting fruit life after harvest in subtropical fruits are quiescent infections of anthracnose and stem end rot caused by *C. gloeosporioides* and *Diplodia natalensis*. Our understanding of the processes regulating the resistance of unripe fruits to these fungi comprises a basis for development of methods to prevent fungal attack in ripening fruits.

Prusky and Keen elucidated the mechanism of resistance in avocado fruits to quiescent infections of *C. gloeosporioides* and determined that the major

biocide involved is 1-acetoxy-2-hydroxy-4-oxo-heneicosa-13,15 diene. The mechanism modulating the level of this compound in the peel of ripening fruits is oxidation by the enzyme lipoxygenase. Activity of lipoxygenase is, in turn, regulated by levels of the non-specific inhibitor, epicatechin, present in the peel of unripe but ripe fruits. Two possibilities exist in the prevention of fungal attack in avocado fruit based on these findings: (i), preventing decrease of the antifungal compound and (ii). Inducing greater accumulation of the compound in avocado fruits. Previous work has demonstrated the feasibility of approach (i), primarily through the exogenous application of antioxidant chemicals. In the present project, we examined the possibility of exploiting the approach (ii), through the application of engineered non-pathogenic *C. gloeosporioides* strains. We have found that non pathogenic strains and also pathogenic strains activates a series of early events via activation of H⁺ATPase and NADPH oxidase activities and oxygen burst that down regulate of the mechanism of resistance. Our findings suggest that pathogenicity in fungi might be regulated by secretion of pathogenicity factors

Results

The results are presented in full in the section Appendices, however I would like to refer to two main groups of results that that is the basis of this final report. Inoculation of avocado pericarp tissue with *Colletotrichum gloeosporioides* and treatment of avocado cell cultures with cell-wall elicitor of *C. gloeosporioides* both increased the production of reactive oxygen species (ROS). Protein kinase inhibitors, such as K-252a, and staurosporine and the phosphatase inhibitor microcystin-LR inhibited the release of H₂O₂ from avocado cells suspension. When 1 mM H₂O₂ was exogenously applied to pericarp tissue it enhanced ROS, PAL activity and epicatechin levels. But, when H₂O₂ treatment was applied following staurosporine treatment, PAL activity was no longer induced. The uninduced reactive oxygen production in pericarp tissue of freshly harvested, unripe-resistant fruit was twice as high as in ripe-susceptible fruit. Challenge inoculation of resistant fruit further increased the reactive oxygen level, however this increase did not occur in the susceptible fruits. The present findings are consistent with the hypothesis that

production of ROS is induced by fungal infection of unripe fruits and consequently may modulate resistance, resulting in the inhibition of fungal development and quiescence.

The second group of results that I would like to significantly highlight is the fact that the present project as a result of an in-depth study on the pathogenicity factors of one of the most difficult postharvest pathogen of stored fruits has found that decay development can be modulated by the secretion of pathogenicity factors. When *C. gloeosporioides* was grown in inducing media, *pel* transcript was initially observed at pH 5.0, with an increase up to 5.7 and then declined. Whereas PL secretion was detected starting from pH 5.8 and increased up to 6.5. PL secretion was not detected as long as the pH of the inducing media was kept lower than 5.8. This behavior differed from PG where transcription and protein secretion in inducing media, occurred starting from pH 5.0 and up to 5.8. When pH was tested in the pericarp of unripe, freshly harvested cv. Fuerte fruits, resistant to *C. gloeosporioides* attack, a pH value of 5.5 was detected. However in ripened fruits, pH value of the pericarp increased up to 6.3 and decay symptoms were observed. Two cultivars Ardit and Ettinger that are resistant to *C. gloeosporioides* also after ripening, showed pH values in the pericarp lower than 5.5. Present results suggest that pH regulates the secretion of pectate lyase, and support its importance as a pathogenicity factor during the attack of avocado fruit by *C. gloeosporioides*.

Scientific Implication, potential impact on agriculture

The production of ROS in avocado fruit is reported for the first time to depend on the physiological stage of fruit ripening. It is possible that one of the factors affecting the induction of resistance in unripe fruit is the capability to react and the presence of naturally high reactive oxygen levels, compared to the reduced capability to react in ripe fruits. In the avocado fruit, *C. gloeosporioides* spores that land on the fruit cuticle germinate within few hours and produce appressoria from which penetration hyphae breach the fruit cuticle. We hypothesize that the infection point will subsequently become the local source for reactive oxygen production that will activate the phenylpropanoid pathway, preserve and/or increase the levels of the

antifungal compound-diene, and ultimately inhibit fungal development, resulting in its quiescence.

The regulation of PL and PG secretion in *in vitro* and *in vivo* conditions can emphasize the importance of PL for the transformation of quiescence of *Colletotrichum* to active infection and decay initiation. While PG can be secreted at low pH, when no symptoms are expressed, PL secretion is directly correlated decay initiation. This simple observation could indicate that in the future pathogenicity could be modulated by pH conditions naturally present in the host or exogenously supplied to the host.

List of publications

Publication in meetings

1. Prusky, D., Kobiler, I., Ardi, R., Beno-Moalem, D. and Yakoby, N. 1998. Mechanism of defense of unripe avocado fruits against *Colletotrichum gloeosporioides* attack. *Phytoparasitica* 26:354.
2. Prusky, D., Kobiler, I., Ardi, R., Beno-Moalem N. Yakoby and Madi, L. 1999. Avocado fruit-*Colletotrichum gloeosporioides* Interactions: a model system for the study of host-pathogen interaction in harvested fruits. *Phytoparasitica* 27:146.
3. Yakoby, N., Kobiler, I., Dinoor, A. Prusky, D., 1999. The level of pH at the avocado pericarp modulates the pathogenicity of *Colletotrichum gloeosporioides*. *Phytoparasitica* 27:147.
4. Moualem-Beno, D. and Prusky, D. Signaling processes involved in the activation of the defense mechanism of avocado fruits against *Colletotrichum gloeosporioides*. *Phytoparasitica* 27:147
5. Yakoby, N., Kobiler, I., Dinoor, A., Prusky D. 1999. PH regulates the secretion of pectate lyase from *Colletotrichum gloeosporioides*: possible role in fruits susceptibility. *Molecular Plant Microbe Interactions* 4:78. Amsterdam
6. Yakoby, N., Freeman, S. Keen, N. Dinoor, A. and Prusky, D. 1999. Increase pathogenicity of *Colletotrichum* following the heterologous expression of pectate lyase. *Molecular Plant Microbe Interactions*. 2:56. Amsterdam
7. Vinokur, Y., Beno-Mualem, D. and Prusky, D. 1999. Effect of cytokinins on the induction of resistance of avocado fruits against *Colletotrichum gloeosporioides*. *Molecular Plant Microbe Interactions* 15:167. Amsterdam.
8. Vinokur, Y., Beno-Mualem, D., and Prusky, D., 1999. Development of mesocarp and pericarp avocado-Kinetin regulated tissue culture. *International Society of Tissue Culture*, Jerusalem. 1998

Edited Book Chapters

9. Prusky, D., I. Kobiler, D. Beno-Moalem, and N. Yakoby. 2000.
Mechanisms of defense of unripe avocado fruits to *Colletotrichum gloeosporioides* attack. In Host specificity, pathology and host pathogen interaction of *Colletotrichum*. Ed. D. Prusky, S. Freeman and M. Dickman. APS series In Press.
10. D. Prusky, S. Freeman and M. Dickman, 2000.
Host Specificity, pathology and host pathogen Interactions of *Colletotrichum*. APS Press, Based in BARD Workshop.

International Journals

11. D. Beno- Moualem and D. Prusky. 2000.
Early events during quiescent infection development by *C. gloeosporioides* in unripe avocado fruit.
Phytopathology: In Press
12. Jakoby, N., Kobiler, I., Dinoor, A., and Prusky D. 2000. pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits.
Applied and Environmental Microbiology: In Press
13. Yakoby, N., Freeman, S. Dinoor, A., Keen, N.T. and Prusky D. 2000.
Expression of pectate lyase from *Colletotrichum gloeosporioides* in *C. magna* and its contribution to fungal pathogenicity. Molecular Plant Microbe Interactions. Submitted.
14. Dalila Beno-Moualem, Yakov Vinokur, and Dov Prusky. 2000.
Cytokinins induces epicatechin content and resistance against *Colletotrichum gloeosporioides* in avocado fruits.
Phytochemistry: Will be submitted.

In preparation

15. Dalila Beno-Moualem, and Dov Prusky. 2000.
Early elicitation of plasma membrane H⁺ATPase and oxygen burst as a result of elicitor treatments in avocado fruits.
16. Yakoby, N. Dinoor, A., and Prusky D. 2000
The use of REMI mutants to follow early interactions events in *Colletotrichum*- avocado interactions.
17. Yakoby, N., Dinoor, A., and Prusky D. 2000.
Disruption of pel as a way for demonstration the importance of pectate lyase during pathogenicity of *Colletotrichum gloeosporioides*

1. MECHANISM OF ELICITATION OF AVOCADO FRUITS BY BIOTIC AND ABIOTIC ELICTORS.

Early events during quiescent infection development by *C. gloeosporioides* in unripe avocado fruits

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Abbreviations: Phenylalanine ammonia lyase, PAL; 3,3'-diaminobenzidine, DAB Reactive oxygen species, ROS; Cyt c; Standard error, SE

Abstract

Inoculation of avocado pericarp tissue with *Colletotrichum gloeosporioides* and treatment of avocado cell cultures with cell-wall elicitor of *C. gloeosporioides* both increased the production of reactive oxygen species (ROS). However, whereas the production of ROS could be detected within minutes in avocado cell suspensions, it was detected only after 2 hours following inoculation of pericarp tissue. Protein kinase inhibitors, such as K-252a, and staurosporine, and the phosphatase inhibitor microcystin-LR inhibited the release of H₂O₂ from avocado cells suspension. When 1 mM H₂O₂ was exogenously applied to pericarp tissue it enhanced ROS, PAL activity and epicatechin levels. But, when H₂O₂ treatment was applied following staurosporine treatment, PAL activity was no longer induced. The uninduced reactive oxygen production in pericarp tissue of freshly harvested, unripe-resistant fruit was twice as high as in ripe-susceptible fruit. Challenge inoculation of resistant fruit further increased the reactive oxygen level, however this increase did not occur in the susceptible fruits. The present findings are consistent with the hypothesis that production of ROS is induced by fungal infection of unripe fruits and

consequently may modulate resistance, resulting in the inhibition of fungal development and quiescence.

Beno-Moualem, D. and Prusky, D. 1999. Early events during quiescent infection development of *C. gloeosporioides* in unripe avocado fruits.

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INTRODUCTION

C. gloeosporioides Penz. (anamorph *Glomerella cingulata* (Stonem.) Sapulda, et Schrenk) attacks unripe avocado fruits in the orchard. Germinated spores produce appressoria that germinate and breach the cuticle, but the resultant subcuticular hyphae become quiescent and do not develop further until fruit is harvested and ripens (38). Significant physiological changes occur in the host fruit that enable activation of the quiescent pathogen. Four mechanisms have been hypothesized to explain the resistance of unripe fruit to fungal attack: (1) lack of nutritional requirements for the pathogen; (2) presence of preformed antifungal compounds; (3) presence of inducible antifungal compounds; and (4) lack of activation of fungal pathogenicity factors.

Resistance of unripe avocado to attack by *C. gloeosporioides* is correlated with the presence of fungitoxic concentrations of the preformed antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene (diene) in the pericarp of unripe fruits (40). Decrease of diene concentration and resultant increased susceptibility has been attributed to the activity of the host derived enzyme lipoxygenase, which oxidizes the compound (41). The activity of lipoxygenase is regulated by the levels of the flavan-3-ol, epicatechin (22), a natural antioxidant present in the pericarp that declines during fruit ripening (41). Increased diene concentration was accompanied by an increase in the transcriptional activation of genes involved in the biosynthesis of the flavonoid, epicatechin (2). However, no increase in the epicatechin could be induced in ripe fruit (22). Differences in host gene expression during fruit ripening seem to be the rule in the host-pathogen interactions of harvested produce.

The transient and rapid production of ROS, including superoxide, H_2O_2 , and hydroxyl radical, by various elicitors is well documented (1,3,7,32,34,50,51). It is not clear whether ROS produced in plants in response to a pathogen or

elicitor, have direct antimicrobial effects (5). But the ROS are considered to be involved in various defensive reactions such as lipid peroxidation (23,53), oxidative cross-linking of cell wall proteins (9), peroxidative polymerization of cinnamoyl alcohol and reinforcement of plant cell walls by the formation of lignin polymers (19,24), phytoalexin production (1,13,53) and programmed cell death (28). No reports have been published on the importance of ROS in the initial stages of quiescence development in fruits by pathogens during postharvest.

Several reports indicate that ROS synthesis occurs at the extracellular surface of the plasma membrane via a one-electron reduction of molecular oxygen (3). It has been previously reported that the transfer of electrons from a cytosolic electron donor (NADPH) to electron acceptors at the extracellular surface of the plasma membrane may lead to: i. acidification of the cytosol; ii. alkalinization of the extracellular medium; and iii. decrease in the plasma membrane potential (17,18,31,47). These changes induced by pathogens and biotic elicitor compounds mediate a variety of early cellular events, including membrane depolarization, changes in the permeability of the plasma membrane to calcium ions, protons, potassium and chloride ions (35). All these effects could contribute to transduction and amplification of signals (27). In this report we have found that infection of avocado fruit with *C. gloeosporioides* or treatment of avocado cell suspension with cell-wall elicitor induced ROS production. Ripe and unripe fruit tissue differ with regard to the ROS production in response to pathogen attack. The unripe, resistant fruits are physiologically able to react and to produce high levels of ROS that can enhance the phenylpropanoid pathway and regulate the levels of the antifungal compound-diene, inhibit fungal development, resulting in its quiescence (39).

MATERIALS AND METHODS

Plant material and experimental treatments

Experiments were carried out with freshly harvested avocado fruits [*Persea americana* Miller var. *drymifolia* (Schldl. and Cham.) S.F. Blake] cv. Fuerte, from an orchard at Kibbutz Givat Brenner, Rehovot, Israel. Experiments were carried out during three consecutive harvesting seasons, and repeated at least

three times per season. The average value of 3-5 replications of one single experiment is usually presented. In some cases the average of all the replications from repeated experiments are presented with their pooled standard error (SE). The differences between means were analyzed by ANOVA. Fruits of fairly uniform size and weight (250-270 g) were harvested, washed in water and used 1-2 hours after harvest for experiments. Pericarp tissue strips (about 1 mm thick, almost free of mesocarp tissue) were cut, washed several times with water, and aerated for 20 h in 0.2 mM CaSO_4 . The pericarp strips were then placed with the interior side in contact with the filter paper saturated with 0.2 mM CaSO_4 , and the upper cuticular side was used for fungal inoculation.

Suspension cultures were generated from pericarp callus cultures by transferring 1 cm³ of callus tissue into 40 ml of the medium described by Prusky et al. (38), with the growth regulator picloram 50 µg/liter medium, without agar, in a 250-ml Erlenmeyer flask. Cells for experiments were grown at room temperature with continuous agitation at 96 rpm on a rotary shaker.

Pericarp inoculation and H₂O₂ treatment

A single-spore isolate of *C. gloeosporioides*, isolate Cg-14 (43), was obtained previously from decayed avocado fruits. The fungus was normally maintained on PDA at 20°C. A spore suspension (2×10^6 spores per ml) was brushed over pericarp strips (42). In some cases pericarp strips were also brushed with a freshly prepared solution of 1mM or 5mM H_2O_2 (Merck, Germany). As a control, pericarp strips were brushed with distilled water only. Following inoculation or the H_2O_2 treatment, plasma membranes were isolated.

Extraction of epicatechin

Epicatechin was extracted from 1mm thick avocado pericarp, as previously described (43). Three grams (fresh weight) of pericarp were ground in 95% ethanol (1:10 w/v). The ethanol extract was dried in a rotary evaporator, and the residue was re-dissolved in 10 ml of 1 mM NaCl and extracted twice with 90-ml ethyl acetate. Epicatechin was quantitated by reverse phase-HPLC (RP-18) column eluted with 55% methanol and 1% acetic acid in double

distilled water. Calculation of epicatechin level was based on comparison of the HPLC peak areas quantitated at 275 nm with those of the standard.

Extraction and activity of PAL

Samples for PAL activity were extracted by blending 5 g avocado pericarp in 50 ml of cold acetone (-20°C) and the homogenate filtered out with Whatman no.1. This process was carried out four times. The pericarp powder was dried at room temperature overnight, and stored at -20°C until used. PAL was extracted from 100 mg of pericarp powder by stirring for 2 h at 4°C with 5 ml of 0.1M sodium borate buffer, pH 8.8. The extract was centrifuged at 10,000 g for 20 min at 4°C and the supernatant was used as an enzyme source. The reaction mixture for PAL consisted of 10 mM L-phenylalanine (Aldrich), 0.1 M borate buffer (pH 8.8) and 1.5 ml of enzyme extract in a final volume of 5 ml. The reaction was incubated at 37°C for 60 min and terminated by adding 0.1 ml of 5 N HCl. The product, cinnamic acid was extracted into 7 ml of diethyl ether and quantitated by measuring the amount of trans-cinnamic acid formed at 269-nm (30).

Elicitor preparation from cell walls of *C. gloeosporioides*

Conidia of *C. gloeosporioides* from single-spore cultures were used to inoculate in Erlenmeyer flasks (500ml) containing 200 ml of potato dextrose broth (PDB). The flasks were incubated at 25°C, with continuous agitation at 100 rpm for 7 days. Harvesting and disruption of the hyphae was carried out as described by Prusky et al. (43) with glass beads (0.45-0.5 mm) agitated at maximum speed in a Braun MSK homogenizer for four periods of 45 s each, with the tube being cooled in ice (6). The broken cell walls were sedimented in centrifuge tubes at 1,000 g for 5 min and washed six times with 50 ml of distilled water. The residual walls were lyophilized, re-suspended in distilled water (10 mg/ml), and autoclaved for 30 min. The autoclaved suspension was centrifuged at 5,000 g for 10 min at 4°C. The supernatant was filtered through a Millipore filter (0.2 µM), and the collected fraction was evaporated to 2% of its initial volume. The sample were loaded on a DEAE-Cellulose column washed with 100 ml of 10mM phosphate buffer at pH 8.0. The fraction was

eluted first with 50 ml of 25mM phosphate buffer pH 8.0 and secondly by 50 ml NaCl, 500 mM (4). Both fractions were pooled, concentrated partially by lyophilization and finally dialyzed twice against 2 liter of double distilled water and used as the elicitor source. Soluble sugars were quantified by the anthrone method, as a parameter for elicitor concentrations.

H₂O₂ determination by spectrofluorimeter and diaminobenzidine

H₂O₂ production in cultured avocado cells was measured by monitoring the scopoletin conversion to nonfluorescent product (48). The cells suspension washed twice with a medium containing 2% sucrose, 1 mM NaH₂PO₄, 1 mM (NH₄)₂SO₄, 1 mM MgSO₄, 1 mM CaCl₂, 25 mM KNO₃, and 20 mM MES adjusted to pH 6.6. The cells were concentrated to 10⁶ cells/ml with washing medium. The cells were allowed to stand for 0.5 h at room temperature, 200 µl of cells suspension were placed in ELISA plates and 0.1 mM scopoletin (10 µl) and 30 µM horseradish peroxidase (10 µl) were added. When fluorescence values had stabilized (5-10 min) the stimulus was added. The effect of specific inhibitors was tested by adding the inhibitors 5-min before the addition of the stimulus. Staurosporine, K-252a and microcystin LR were added from stock solutions prepared in dimethylsulfoxide (DMSO). Similar amounts of DMSO were added to control treatment. The optimal inhibitor's concentration determined by testing a series of concentrations. Higher concentration of the inhibitor did not further decrease the H₂O₂ production by avocado cells suspension. Oxidation of scopoletin by H₂O₂, catalyzed by peroxidase, was measured as the decrease in fluorescence recorded by an automatic reading Microplate-Fluorometer-FLUOstar (BMG, Germany). The sample was excited at 350nm and the emission measured at 460nm. The fluorometric units were calculated according to standard curve of H₂O₂ concentrations.

For *in vivo* reactive oxygen production, disks of pericarp tissue (1-cm diameter) were placed in 3,3' diaminobenzidine (DAB, Sigma) solution containing 0.2 mM CaSO₄, according to Thordal-Christensen et al. (52). The reactive oxygen generated in the tissue reacts with DAB resulting in a dark-brown pericarp. At various times after inoculation transferring the disks to 20ml of 96% ethanol stopped the reaction. The tissue was boiled and the metric lightness units were determined with a Minolta Chromameter (CR-200,

Japan). Maximal value of metric lightness (45 units) was obtained in the untreated control tissue. Disks of pericarp placed in DAB solution, treated with increasing concentrations of H_2O_2 (0-50 mM) resulted in darker tissue and a reduction of lightness units (the value with 50mM was 31 units). The regression coefficient between H_2O_2 concentrations (0-50 mM), and the units of lightness was $r=0.934$. Similar regression coefficients were obtained in three independent experiments.

Preparation of microsomes and isolation of plasma membranes.

Plasma membranes were isolated from 40 g of pericarp strips after treatment with 0.2 mM CaSO_4 for 24 h, as described above. Plasma membrane was isolated at several periods after inoculation with *C. gloeosporioides*. Tissue was homogenized in a Waring blender at 4°C and the microsomes were prepared according to Giannini et al. (16). The microsomes were suspended in 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 (26). The final composition of the phase system was: 6.4% (w/w) of each, dextran T500 and polyethylene glycol (PEG 3350) in the suspension medium. The final upper phase was diluted 1:10 with resuspension medium: 330 mM sucrose, 1 mM dithiothreitol (DTT), 5 mM NaF and 5 mM MES-bis-tris propane (MES-BTP), pH 7.8, and the purified plasma membranes were pelleted at 55,000g. The pellet was redissolved and brought to a concentration of 1-3 mg protein/ml, and adjusted to pH 7.0 with 330 mM sucrose, 5 mM MES-BTP and 1 mM DTT. Protein was assayed according to Bradford (8). ATPase activity in these plasma membranes was about 87% vanadate sensitive.

Reactive oxygen production measured by cytochrome c reducing activity.

Reactive oxygen production was determined by cytochrome c reduction dependent on NADPH oxidation according to Owen et al. (36). By this method for each molecule of reactive oxygen production one molecule of cytochrome c reduction is detected Mayo et al. (33). 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 started with 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 superoxide dismutase (SOD) as described by Owen et al. (36,14).

RESULTS

Reactive oxygen production in avocado fruit tissue triggered by fungal infection and fungal cell wall elicitor.

Plasma membranes of isolated pericarp tissue that had been pre-inoculated with *C. gloeosporioides*, showed a rapid increase in superoxide production, measured by cytochrome c reduction. The ROS production by the isolated plasma membrane of inoculated pericarp increased by three fold within 1 h and remained higher up to 4 h after inoculation compared to the uninoculated pericarp tissue (Fig. 1). Addition of fungal cell-wall elicitor to avocado cells suspension enhanced the cytochrome c reduction within 1 h after treatment (Table 1).

H₂O₂ production in avocado fruit tissue, elicited by direct fungal infection and fungal cell-wall elicitor.

Reactive oxygen was initially detected by DAB staining 2 h after avocado pericarp inoculated with *C. gloeosporioides*. Avocado pericarp inoculated with *C. gloeosporioides* at 0 time showed 42.7±2.3 metric lightness units and 2 h later it decreased to 40.1±1.3 units. The lightness further decreased to 37.8±2.8 and 35.6±1.3 units, 10 and 20 h after inoculation, respectively. When cultured avocado cells suspension were treated with cell-wall elicitor (6 µg of glucose equivalents), release of H₂O₂ was detected within a few minutes, as determined by loss of scopoletin fluorescence (Fig 2). Increasing amounts of elicitor (15 and 30 µg of glucose equivalents) further increased the generation of H₂O₂ by the avocado cell culture.

Effect of protein kinase and phosphatase inhibitors on H₂O₂ release by avocado cell suspension system treated with fungal cell wall elicitor

Incubation of cultured avocado cells in the presence of protein kinase inhibitor, such as K-252a (1 μ M) and staurosporine (10 μ M), inhibited H₂O₂ release by 70-94% (Table 2). The phosphatase inhibitor, microcystin-LR (1 μ M), also inhibited H₂O₂ release by 75%. While, the Ca²⁺ ionophore alone, A-23187 stimulated the release of H₂O₂ from cultured avocado cells almost threefold.

Effects of challenge inoculation of *C. gloeosporioides* and H₂O₂ on enhancement of epicatechin, PAL and ROS production.

Early reports showed that challenge inoculation of whole avocado fruits enhanced the level of epicatechin and the activity of PAL in the fruit pericarp within 24 h (2,39). Inoculation of isolated pericarp tissue with *C. gloeosporioides* increased the epicatechin level by twofold within 6 h (Fig. 3). During the same period, PAL activity increased as measured by the increment of cinnamic acid from 3.5 to 6 μ mol/mg protein/h (results not shown). Treatment of pericarp tissue with exogenous H₂O₂ (1mM) doubled reactive oxygen production by the plasma membrane within 30-min (Fig. 4). H₂O₂ treatment induced a 1.5-fold increase in PAL activity within 60 min and a 2.5-fold increase 6 h after the treatment (Fig. 5B). A similar pattern of increase was observed in the epicatechin level, that increased from an initial value of 400 to 1200 μ g/g.f.wt. 6 h after the treatment (Fig. 5A). Treatment of the pericarp tissue with 5 mM H₂O₂ induced faster increases in PAL activity and epicatechin levels than 1 mM H₂O₂ 1 h after the treatment. However, PAL activity and epicatechin levels in the 5 mM H₂O₂ exogenously treated pericarp started to declined 1 h after treatment, while at 1 mM H₂O₂, the activity increased continuously during the 5 hours following the treatment. When avocado pericarp was treated with 1 μ M staurosporine by brushing the pericarp 20-min before its exposure to 1 mM H₂O₂, PAL activity was inhibited and was similar to that found in untreated pericarp tissue (Fig. 6).

Reactive oxygen production in pericarp of unripe-resistant and ripe-susceptible avocado fruits.

The ROS levels in uninoculated plasma membranes from pericarp of unripe-resistant fruits, was 2.3 fold higher than the reactive oxygen production

in ripe-susceptible fruit (Fig. 7). Inoculation of pericarp in resistant fruits showed that ROS levels in the resistant tissue was 4.3 fold higher than the ripe fruit (Fig. 7).

DISCUSSION

The ROS produced in response to pathogen attack or elicitor treatment have been hypothesized to have direct antimicrobial effects and to play a role in defense mechanisms by triggering lignin accumulation, lipid peroxidation, phytoalexin production, and the hypersensitive response (5). One of the probable sources of ROS induced by early interactions with fungal infection is NADPH oxidase located in the plasma membranes (45). In our experiments, *C. gloeosporioides* infection activated the production of ROS by three-folds in plasma membranes of avocado pericarp within 1 h after inoculation. Production of ROS was also detected in cultured avocado cells 8 min after treatment with cell-wall elicitor prepared from *C. gloeosporioides*. Production of ROS is thus one of the most rapid biochemical responses detected in avocado pericarp tissue (1,14).

Programmed cell death induced by H_2O_2 has been reported in several plant systems (20,28). However in the present study no obvious cell death was observed in the avocado pericarp tissue nor in cultured avocado cells where reactive oxygen production was detected. H_2O_2 was easily detected in cultured avocado cells, as reported for other cells suspension systems (29,49). Eight minutes after cultured avocado suspension cells were exposed to cell-wall elicitor of *C. gloeosporioides*, a 10-fold increase in H_2O_2 production was observed. In inoculated pericarp, however, release of reactive oxygen was detected by DAB staining only after several hours. It might be possible that, the delay in H_2O_2 detection in the tissue, is a result of partial destruction of the generated reactive oxygen, since significant activities of catalase and peroxidase are found in the avocado pericarp (Beno-Moualem and Prusky, unpublished).

The ability of the cell-wall elicitor to enhance H_2O_2 release in a cultured avocado cells suspension system is promoted by the rapid binding to/or interaction with the plasma membrane. Binding of several oligo- or polyglucans from fungal origin to soybean microsomal membranes has been

reported (10,11). Avocado fruit pericarp, similar to other plant organ (34), might recognize the pathogen or the cell-wall elicitors at very early stages in the infection process and react with rapid generation of reactive oxygen. These findings imply that the plasma membranes of avocado fruit pericarp acts as a site for the initial binding of elicitor molecules which triggers the defense response (39). In previous work it was found that, influx of Ca^{2+} , exchange of H^+/K^+ across the cell membrane and activation of ion channels occurred within 10 min after elicitor treatment, before ROS are released (35). We demonstrated here that, the avocado cells suspension could be activated to generate H_2O_2 by the addition of the Ca^{2+} ionophore A-23187, which allows the influx of the Ca^{2+} ions. In other systems where specific elicitors were used, increased endogenous Ca^{2+} also was found to stimulate the release of H_2O_2 (21,35,49). Additionally, protein kinase inhibitors such as staurosporine and K-252a significantly reduced the ROS production in cultured avocado cells suspension induced by *Colletotrichum* cell wall. The phosphatase inhibitor, microcystin-LR also inhibited the reactive oxygen production. From the action of kinase and phosphatase inhibitors one can deduce that phosphorylation/de-phosphorylation processes might regulate the induction of ROS in the avocado fruit tissue.

Prusky et al. (43) reported that challenge inoculation of intact fruits with pathogenic or non-pathogenic *Colletotrichum* strains enhanced PAL activity and other phenylpropanoid biosynthetic enzymes within 24 h. Following the early reports of Doke (14), the connection between ROS production and phytoalexin synthesis has been explored in several interactions (1,12,25,46). Apostol and co workers (1) reported that exogenous H_2O_2 (0.5-1mM) added to soybean suspension cells, was sufficient to induce glyceollin. In this work we found that avocado fruit, similar to other plant organs, increases reactive oxygen production, PAL activity and epicatechin levels after fungal inoculation. We found that application of exogenic H_2O_2 to pericarp tissue also induces increase of the epicatechin level, which indicates that the reactive oxygen level produced during the *Colletotrichum*-avocado interaction, leads to the activation of phenylpropanoid pathway. However, inhibition of this process by pre-treatment of pericarp with staurosporine before the H_2O_2 application, inhibited PAL activity, suggesting that protein kinase activity is required for the

activation of phenylpropanoid biosynthesis in the avocado system. It is possible that the application of H_2O_2 may trigger a second oxidative burst as shown by Park et al. (37) in potato slices. In their system also, staurosporine inhibited the induction of subsystemic oxidative burst by H_2O_2 . This might indicate that H_2O_2 stimulates also a novel secondary signaling process in avocado tissue.

The production of ROS in avocado fruit is reported for the first time to depend on the physiological stage of fruit ripening. In the pericarp of freshly harvested unripe -resistant fruits, reactive oxygen production was 0.168 ± 0.026 compared with only 0.072 ± 0.014 $\mu\text{mol cyt c/mg protein/min}$ in the ripe-susceptible tissue. It is possible that one of the factors affecting the induction of resistance in unripe fruit is the capability to react and the presence of naturally high reactive oxygen levels, compared to the reduced capability to react in ripe fruits (42,44). Induction of H_2O_2 production has been described in parsley cell suspensions and cucumber where the response can be further enhanced by 2,6-dichloroisonicotinic acid, salicylic acid, or methyl jasmonate, and also by cuticle abrasion (15). In the avocado fruit, *C. gloeosporioides* spores that land on the fruit cuticle germinate within few hours and produce appressoria from which penetration hyphae breach the fruit cuticle. We hypothesize that the infection point will subsequently become the local source for reactive oxygen production that will activate the phenylpropanoid pathway, preserve and/or increase the levels of the antifungal compound-diene, and ultimately inhibit fungal development, resulting in its quiescence (2).

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LITERATURE CITED

1. Apostol, I., Hienstein, P.F., and Low, P.S. 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiol.* 90:109-116.
2. Ardi, R., Kobiler, I., Keen, N.T., and Prusky, D. 1998. Involvement of epicatechin biosynthesis in the resistance of avocado fruits to postharvest decay. *Physiol. Mol Plant Pathol.* 53: 269-285.
3. Auh, C.K., and Murphy, T.M. 1995. Plasma membrane redox enzyme is involved in the synthesis of O₂ and H₂O₂ by *Phytophthora* elicitor-stimulated in the synthesis rose cells. *Plant Physiol.* 107: 1241-1247.
4. Ayers, A.R., Ebel, J., Valent, B., and Albersheim, P. 1976. Host pathogen Interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. *Plant Physiol.* 57: 760-765.
5. Baker, C.J., and Orlandi, E.W. 1995. Active oxygen in plant pathogenesis. *Annu. Rev. Phytopathol.* 33: 299-322.
6. Bartnicki-Garcia, S., Bracker, C.E., Reyes, E., and Ruiz-Herrera, J. 1978. Isolation of chitosomes from taxonomically diverse fungi and synthesis of chitin microfibrils *in vitro*. *Exp. Mycol.* 2:173-192.
7. Bottini, A., Vernoesi, C., Pontier, D., Esquerre-Tugaye, M.T., Belin, J.P., Rusterucci, C., and Ricci, P. 1994. Differential responses of tobacco cells to elicitors from two *Phytophthora* species. *Plant Physiol. Biochem.* 32: 373-378.
8. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72: 248-254.
9. Bradley, D.J., Kjellbom, P., and Lamb, C.J. 1992. Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein - A novel, rapid defense response. *Cell* 70: 21-30.
10. Cheong, J.J., and Hahn, M. 1991. A specific high-affinity binding site for the hepta-b-glucoside elicitor exists in soybean membranes. *Plant Cell* 3: 137-147.

11. Cosio, E. G., Frey, T., and Ebel, J. 1990. Solubilization and characteristics of the binding sites for fungal β -glucans from soybean cell membranes. *FEBS Lett* 264: 235-238.
12. Devlin, W.S., and Gustine, D.L. 1992. Involvement of the oxidative burst in phytoalexin accumulation and the hypersensitive reaction. *Plant Physiol.* 100: 1189-1195.
13. Doke, N. 1983. Generation of superoxide anion by potato tuber protoplasts upon the hypersensitive response to hyphal wall components of *Phytophthora infestans* and specific inhibition of the reaction by suppressors of hypersensitivity. *Physiol. Plant Pathol.* 23: 359-367.
14. Doke, N. 1985. NADPH-dependent O_2^- generation in membrane fractions isolated from wounded potato tubers inoculated with *Phytophthora infestans* and to the hyphal wall components. *Physiol. Plant Pathol.* 27: 311-357.
15. Fauth, M., Merten, A., Hahn, M.G., Jeblick, W., and Kauss, H. 1996. Competence for elicitation of H_2O_2 in hypocotyls of cucumber is induced by breaching the cuticle and is enhanced by salicylic acid. *Plant Physiol.* 110: 347-354.
16. Giannini, J.L., Gildensoph, L.H., and Briskin, D.P. 1987. Selective production of sealed plasma membrane vesicles from red beet (*Beta vulgaris* L.) storage tissue. *Arch. Biochem. Biophys.* 254: 621-630.
17. Grabov, A., and Bottger, M. 1994. Are redox reactions involved in regulation of K^+ channels in the plasma membrane of *Limnobium stoloniferum* root hairs? *Plant Physiol.* 105: 927-935.
18. Guern, J., Mathieu, G., Ephritikhine, C.I., Luttge, U., Marre, M.T., and Marre, E. 1988. Intracellular pH modification linked to the activity of the ferricyanide driven activity of the plasmalemma redox system in *Elodea densa* leaves, *Acer pseudoplatanus* and *Catharanthus roseus* cells. In FL Crane, DJ Morre, H Low, eds, *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth*, Life Sciences, Vol 157, (Plenum Publishing, New York) p 412.
19. Hammerschmidt, R., and Kuc, J. 1982. Lignification as a mechanism for induced systemic resistance in cucumber. *Physiol. Plant Pathol.* 20: 61-71.

20. Harding, S.A., and Roberts, D.M. 1998. Incompatible pathogen infection results in enhanced reactive oxygen and cell death responses in transgenic expressing a hyperactive mutant calmodulin. *Plant* 206: 253-258.
21. He, S.Y., Huang, H.C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpinPss: a protein that is secreted via the hrp pathway and elicits the hypersensitive response in plants. *Cell* 73: 1-20.
22. Karni, L., Prusky, D., Kobiler, I., Bar-Shira, E., and Kobiler, D. 1989. Involvement of epicatechin in the regulation of lipoxygenase activity during activation of quiescent *Colletotrichum gloeosporioides* infections of ripening avocado fruits. *Physiol Mol. Plant Pathol.* 35: 367-374.
23. Keppler, L.D, and Novacky, A.1987. The initiation of lipid peroxidation during bacteria-induced hypersensitive reaction. *Physiol. Mol. Plant Pathol.* 30: 233-245.
24. Kohle, H., Young, D.H., and Kauss, H. 1984. Physiological changes in suspension cultured soybean cells elicited by treatment with chitosan. *Plant Sci.* 33:221-230.
25. Kondo, Y., Hanawa, F., Miyazawa, T., and Mizutani, J. 1993. Detection of rapid and transient generation of activated oxygen and phospholipid hydroperoxide in soybean after treatment with fungal elicitor by chemiluminescence assay. In, B Fritig, M Legrand, eds, *Mechanisms of Plant Defense Responses.*, Kluwer, Dordrecht, pp. 148-151.
26. Larsson, C., Widell, S., and Kjellbom, P. 1987. Preparation of high purity plasma membranes. *Methods Enzymol.* 148: 558-568.
27. Leon, J., Lawton, M.A., and Raskin, I. 1995. Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. *Plant Physiol.* 108: 1673-1678.
28. Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H₂O₂ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583-593.
29. Linder, W.A., Hoffmann, C., and Grisebach, H. 1988. Rapid elicitor-induced chemiluminescence in soybean cell suspension cultures. *Phytochemistry* 27: 2501-2503.
30. Lisker, N., Cohen, L., Chalutz, E., and Fuchs, Y. 1983. Fungal infections suppress ethylene induced phenylalanine ammonia lyase activity in grapefruit. *Physiol. Plant Pathol.* 22: 331-338.

31. Marre, M.T., Moroni, A., Albergoni, F.G., and Marre, E. 1988. Plasmalemma redox activity and H⁺-extrusion. I. Activation of the H⁺-pump by ferricyanide-induced potential depolarization and cytoplasm-acidification. *Plant Physiol.* 87: 25-29.
32. Mathieu, Y., Sanchez, F.J., Droillard, M.J., Lapous, D., Lauriere, C and Guern, J. 1996. Involvement of protein phosphorylation in the early steps of transduction of the oligogalacturonide in tobacco cell signal cells. *Plant Physiol. Biochem.* 34: 399-408.
33. Mayo, L.A., and Curnutte, J.T. 1990. Kinetic microplate assay for superoxide production by neutrophils and other phagocytic cells. *Methods in Enzymology*, vol., 186:567-575.
34. Mehdy, C.N. 1994. Active oxygen species in plants defense against pathogens. *Plant Physiol.* 105:467-472.
35. Nurnberger, T., Nennstiel, D., Jabs, T., Sacks, W.R., Hahlbrock, K., and Scheel, D. 1994. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell* 78: 449-460.
36. Owen, T.G.J and Hancock, J.T. 1994. Assays of plasma membrane NADPH oxidase. *Methods Enzymol.* 186: 222-229.
37. Park, H., Miura, Y., Kawakita, K., Yoshioka, H. and Doke, N. 1998. Physiological mechanisms of a sub-systemic oxidative burst triggered by elicitor induced local oxidative burst in Potato tuber slices. *Plant Cell Physiol.* 39(11):1218-1225.
38. Prusky, D. 1996. Quiescent infections by postharvest pathogens. *Ann. Rev. Phytopath.* 34: 413-434.
39. Prusky, D., and Keen, N.T. 1993. Involvement of preformed antifungal compounds in the resistance of subtropical fruits to fungal decay. *Plant Dis.* 77: 114-119.
40. Prusky, D., Keen, N.T., Sims, J.J., and Midland, S.L. 1982. Possible involvement of an antifungal diene in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. *Phytopathology* 72: 1578-1582.
41. Prusky, D., Keen, N.T., and Eaks, I. 1983. Further evidence for the involvement of a preformed antifungal compound in the latency of

- Colletotrichum gloeosporioides* on the unripe fruits. *Phytopathology* 72: 1578-1582.
42. Prusky, D., Karni, L., Kobiler, I., and Plumbey, R. 1990. Induction of a preformed antifungal compound in unripe fruits: Effect of challenge inoculation with *Colletotrichum gloeosporioides*. *Physiol. Mol. Plant Pathol.* 37: 425-435.
 43. Prusky, D., Freeman, S., Rodriguez, R.J., and Keen, N.T. 1994. A non-pathogenic mutant strain of *Colletotrichum magna* induced resistance to *C. gloeosporioides* in avocado fruits. *Mol. Plant-Microbe Interact.* 7: 326-333.
 44. Prusky, D., Hamdan, H., Ardi, R., and Keen, N.T. 1996. Induction of biosynthesis of the epicatechin in avocado suspension cells treated with an enriched CO₂ atmosphere. *Physiol. Mol. Plant Pathol.* 48: 171-178.
 45. Pugin, A., Frachiesse, J.M., Tavernier, E., Bligny, R., Gout, E., Douce R., and Guern, J. 1997. Early events induced by the elicitor cryptogein in tobacco cells: Involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. *Plant Cell* 9: 2077-2091.
 46. Rogers, K.M., Albert, F., and Anderson, A.J. 1988. Lipid peroxidation is a consequence of elicitor activity. *Plant Physiol.* 86:547-553.
 47. Rubinstein, B. and Stern, A. 1986. Relationship of transplasmalemma redox activity to proton and solute transport by roots of *Zea mays*. *Plant Physiol.* 80: 805-811.
 48. Root, R.K., Metcalf, J., Oshino, N., and Chance, B. 1975. H₂O₂ release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* 55: 945-955.
 49. Schwacke, R., and Hager, A. 1992. Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca⁺⁺ and protein-kinase activity. *Planta* 187:136-141.
 50. Sutherland, M.W. 1991. The generation of oxygen radicals during host plant responses to infection. *Physiol. Mol. Plant Pathol.* 39: 79-93.
 51. Tavernier, E., Wendehenne, D., Blein, J.P., and Pugin, A. 1995. Involvement of free calcium in action of cryptogein, a proteinaceous elicitor of hypersensitive reaction in tobacco cells. *Plant Physiol.* 109: 1025-1031.

52. Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D.B. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* 11: 1187-1194.
53. Vera-Estrella, R., Blumwald, E., and Higgins, V.J. 1993. Non-specific glycopeptide elicitors of *Cladosporium fulvum*. Evidence for involvement of active oxygen species in elicitor-induced effects on tomato cell suspension. *Physiol. Mol. Plant Pathol.* 42: 9-22.

Table 1.

Effect of cell wall extract of *C. gloeosporioides* on reactive oxygen production in avocado cells suspension

	$\Delta_{OD} (530-570)$ after min		
	30	60	120
Elicitor (15 μ g/equivalent Glu)	0.000	0.016 $\pm$ 0.003	0.043 $\pm$ 0.010
Elicitor (30 μ g/equivalent Glu)	0.000	0.040 $\pm$ 0.012	0.100 $\pm$ 0.023
Ionophore A-23187 (1 μ M)	0.054 $\pm$ 0.019	NT	NT

The difference in cytochrome *c* reduction $\Delta_{OD} (530-570)$ in the absence and presence of SOD is used as a measurement of O_2^- production since each O_2^- reduces ferric cytochrome *c* to its ferrous form (Mayo et al., (33). The ionophore A-23187 was used to test the capability of the avocado cells suspension to release ROS. Means and SE are based on the data from one experiment of four that gave similar results. Each treatment was replicated five times. NT- not tested.

Table.2

Effect of cell wall extract from *C. gloeosporioides* on the release of H₂O₂ from avocado cells suspension

	H₂O₂ (nmol)
Cells only	0.14±0.022
Elicitor (60 µg/equivalent Glu)	1.01±0.26
Staurosporine (10 µM) + Elicitor	0.306±0.092
K-252a (1 µM) + Elicitor	0.15±0.068
Microcystin LR (1 µM) + Elicitor	0.254±0.076
Ionophore- A23187 (5 µM)	2.87±0.402

Cell wall elicitor from *C. gloeosporioides* prepared as described in Materials and Methods. Release of H₂O₂ from 10⁶ avocado cells suspension within 10 min after adding elicitor and various inhibitors. The release of H₂O₂ was measured as oxidation of scopoletin 350/460 nm in the presence of peroxidase. Means and SE are based on the results of one from 4 experiments that showed similar results. Each treatment was replicated three times.

Legends for figures

Fig 1. Time course of reactive oxygen production in plasma membrane isolated from pericarp tissue inoculated with *C. gloeosporioides* strain Cg-14. Bars represent standard error from one representative experiment run 4 times with 5 replications. The reactive oxygen determined as Cytochrome c reduction in the absence and presence of SOD according to the method of Owen et al (36). Each molecule of reactive oxygen produce one molecule of cytochrome c is reduce Mayo et al., (33).

Fig 2. Effect of *C. gloeosporioides* elicitor on the release of H₂O₂ from avocado cells suspension. Different concentrations of elicitor were added to the avocado cells suspension (10⁶ cells) and their effects were compared to the untreated control. Bars represent standard error. The data from 4 experiments with 3 replications per treatment were pooled.

Fig 3. Epicatechin levels in avocado fruits pericarp in response to inoculation with *C. gloeosporioides* strain Cg-14. The pericarp were sampled from freshly harvested fruits, incubated for 24 h in aerated solution of 0.2 mM CaSO₄ and then inoculated as described in Materials and Methods. Bars represent standard error. The data from 3 experiments with 3 replications per treatment were pooled.

Fig 4. Time course of reactive oxygen production in plasma membrane isolated from pericarp tissue treated with 1 mM H₂O₂. Bars represent standard error from one representative experiment run 4 times with 3 replications.

Fig 5. Epicatechin levels and PAL activity in the pericarp of avocado fruits cv. Fuerte treated with H₂O₂. Pericarp was sampled from freshly harvested fruits, incubated in aerated solution of 0.2 mM CaSO₄ for 24 h and then treated with H₂O₂ as described in Materials and Methods. **A.** Epicatechin levels, and **B.** PAL activity. Bars represent standard error. The data from 3 experiments with 5 replications per treatment were pooled.

Fig 6. Effect of staurosporine on the activity of PAL, induced by H_2O_2 , in isolated pericarp tissue of avocado fruits. Pericarp tissue was sampled from freshly harvested fruits, incubated in aerated solution of 0.2 mM $CaSO_4$ for 24 h. The pericarp brushed four times during 20min with 1 μ M staurosporine, followed by treatment with 1 mM and 5 mM H_2O_2 . Bars represent standard error. The data from 3 experiments with 3 replications per treatment were pooled.

Fig 7. Reactive oxygen production in plasma membrane isolated from pericarp of unripe-resistant and ripe-susceptible fruits. The resistant fruits and the susceptible fruits were harvested on the same day. The susceptible fruit were stored for 12 days to ripe completely and become soft. Both pericarp tissues were inoculated with *C. gloeosporioides* strain Cg-14. Activity was tested 4h after inoculation, at the same time as non-inoculated fruit. Bars represent standard error. The data from 4 experiments with 5 replications per treatment were pooled.

Fig. 1

Fig 3

Fig 4

Fig 5

Fig 6

Fig. 7

2. Early elicitation of plasma membrane ATPase and oxygen burst as a result of elicitor treatments in avocado fruits

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Manuscript in Preparation

INTRODUCTION

Defense response of plant to challenge inoculation or to different abiotic elicitors dependent on ability to recognize at early stage. The recognition expressed in various physiological or biochemical activities. For instance, production of reactive oxygen intermediates (Doke and Ohashi, 1988; Baker and Orlandi, 1995), ion fluxes (Jabs et al., 1997), kinase cascade (Levine et al., 1994) and synthesis of antimicrobial compounds (Dixon and Harrison, 1994).

The plant plasma membranes H⁺ATPase play an important role, in the physiology and biochemistry of plant cell. We try to determine whether different biotic or abiotic elicitors operate the same complex cascade of events. We focused on obtaining early or late responses, a quantitative description of several activities of the plasma membranes.

MATERIALS AND METHODS

Plant material and experimental treatments

Experiments were carried out with freshly harvested avocado fruits [*Persea americana* Miller var. *drymifolia* (Schidl. And Cham.) S.F. Blake] cv. *Fuerte*, from an orchard at Kibbutz Givat Brener, Rehovot, Israel. Fruits of fairly size and weight (250-270g) were harvested and used immediately (1-2h) for experiments. Pericarp tissue strips (about 1mm thick, almost free of mesocarp) were cut, washed several times with water and aerated for 20h in 0.2mM CaSO₄.

Elicitor treatment.

The pericarp strips placed with the wound side in contact with the filter paper saturated with 0.2mM CaSO₄, and the upper cuticular side was used for fungal inoculation, or brushing with 1mM H₂O₂. CO₂ or ethylene were injected to glass jar (volume of 600 ml) contained the pericarp strips. 30% CO₂ were obtained from commercial, high-pressure cylinders. Avocado strips was exposed to 50ppm ethylene for 4 hs. At the end of the incubation periods, plasma membranes were isolated for measuring H⁺-ATPase, and NADPH oxidase activities. PAL activity and epicatechin content was extracted and measured as described before.

Rb⁺ uptake

Pericarp tissue discs (5mm diameter and 1mm thick, almost free of mesocarp) were prepared as described earlier. Five discs pre-incubated in vials contained 0.2mM CaSO₄ for 30 min at 25°C. 30% CO₂, 50ppm ethylene, 1mM H₂O₂ or inoculation with *C.gloeosporioides* applied to the avocado discs. The [⁸⁶Rb]K⁺ was added to the vial at the last 20 min of the reaction. Uptake of [⁸⁶Rb]K⁺ into 70 mg of avocado pericarp (5 discs) was assayed in 5 ml of 0.2mM CaSO₄ and 0.1mM[⁸⁶Rb]K⁺ 700Bq/umol. Exchangeable was removed with three washings over a 5 min period in 15 ml of ice cold 10mM CaSO₄. The discs were transformed to vials for scintillation counting and 0.2 ml of 1N NaOH for discoloration and then 4 ml of Opti-Fluor O (Packard). The samples were counted in scintillation counter MR-300 Lumitron (Automatic liquid scintillation system).

RESULTS

Rb⁺ uptake

Treatments with 30% CO₂, exposure to 50 ppm ethylene, H₂O₂ and inoculation with *C. gloeosporioides* enhanced Rb⁺ uptake by pericarp's discs. Treatment with 30% CO₂ or 50ppm ethylene initiated the Rb⁺ uptake without a lag time, while in challenge inoculation delayed the initial response (Fig. 1). Treatment with 30% CO₂ resulted in high Rb⁺ uptake (169%) within 30 min, Ethylene also stimulated the Rb⁺ uptake by 82%. Inoculation with Cg-14 stimulated the Rb⁺ uptake only after 4 hours and the uptake increased (Fig. 2). The Rb⁺ uptake was not effected when 1mM H₂O₂ were added to the pericarp discs.

Addition of 1mM vanadate (VO_4) (H^+ ATPase inhibitor) to the incubation medium of the control inhibited the Rb^+ uptake by 45% (Table 1). When 1mM vanadate were added 10 min before supplement of CO_2 or the ethylene, the Rb^+ uptake were inhibited by 61% or 68%. Inhibition of Rb^+ uptake by 10^{-6}M staurosporine, protein kinase inhibitor, was less efficient.

Reactive oxygen production

Slices of pericarp treated with different elicitors, and the reactive oxygen production was measured in the extracted plasma membranes (Table 2). 1mM H_2O_2 or 50ppm ethylene activated the reactive oxygen production within 0.5h. Two and half-hours later ROS decreased. Treatment with 30% CO_2 or inoculation with Cg-14 stimulated the reactive oxygen production within 1h. CO_2 and challenge inoculation enhanced ROS by 3.5 folds within 2h. But in CO_2 treated tissue ROS decreased 2 h later, while in inoculated tissue ROS was still high at this time.

PAL activity

PAL activity was stimulated rapidly by ethylene and CO_2 (Fig 3 A,B). Activity increased 3 folds within 3h when pericarp slices were exposed to 50ppm ethylene, and 2.5 folds within 1h following exposure to 30% CO_2 . PAL activity increased gradually when avocado tissue was treated with 1mM H_2O_2 ; 6 hs after treatment the increase was 2 folds. Challenge inoculation with *C. gloeosporioides* also enhanced PAL activity almost twice 4h after infection (Fig 4).

Epicatechin level

Epicatechin content present in the pericarp, was affected differently by the biotic or the abiotic elicitors. Epicatechin content increased from 400 to 660ug/g.f.w within 1-2h, follow the 30% CO_2 applied to avocado pericarp (Fig. 5). Exposure of avocado pericarp to ethylene did not affect epicatechin level, despite the enhancement in PAL activity. Treatment with 1mM H_2O_2 gradually increased the antioxidant-epicatechin, the level increased by 2.5 folds within 6h. Epicatechin increased by 50% after 6-10h also by inoculation with *C. gloeosporioides*.

DISCUSSION

It has been hypothesized by Beno-Mualem and Prusky (unpublished) that the infection point of *C. gloeosporioides* is the local source for reactive oxygen production that will activate the phenylpropanoid pathway, preserve and/or increase the levels of the antifungal compound-diene, and ultimately inhibit fungal development, resulting in its quiescence. In the present work it was tested if different elicitors can affect modify the plasma membrane properties and specifically ATPase activity and this can lead to the initiation of a cascade leading to physiological processes.

REFERENCES

- Baker CJ, Orlandi EW (1995).
Active oxygen in plant pathogenesis. *Annu Rev. Phytopathol.* 33:299-321.
- Dixon RA, Harrison MJ, Lamb CJ (1994).
Early events in the activation of plant defense responses. *Annu Rev Phytopathol* 32:479-501.
- Doke N, Ohashi N (1988).
Involvement of an O₂- generating system in the induction of necrotic lesions on tobacco leaves infected with tobacco mosaic virus. *Physiol Mol Plant Pathol* 32:163-175.
- Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D (1997).
Elicitor-stimulated ion fluxes and O₂- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Natl Acad Sci. USA* 94:4800-4805.
- Levine A, Tenhaken R, Dixon RA, Lamb CJ (1994).
H₂O₂ from oxidative burst orchestrates the plant hypersensitive disease resistance responses. *Cell* 79:583-593.

TABLE 1

Effect of different inhibitors on the Rb^+ uptake.

Control	21.20 $\pm$ 4.32
Control + VO_4 1mM	9.58 $\pm$ 0.7
Control + Staurosporine 10^{-6}M	14.26 $\pm$ 2.4
Control + DPI 0.05mM	16.96 $\pm$ 3.3
Ethylene	43.89 $\pm$ 5.9
Ethylene + VO_4 1mM	13.93 $\pm$ 1.58
Ethylene + Staurosporine 10^{-6}M	21.91 $\pm$ 2.41
Ethylene + DPI 0.05mM	38.14 $\pm$ 2.53
CO_2	56.52 $\pm$ 4.76
CO_2 + VO_4 1mM	21.86 $\pm$ 2.17
CO_2 + Staurosporine 10^{-6}M	37.05 $\pm$ 6.2
CO_2 + DPI 0.05mM	40.42 $\pm$ 0.9

Table 2: Effect of different treatments on the generation of O_2^- coupled with oxidation of NADPH.

Control	0h	0.147 $\pm$ 0.022
Control	4h	0.178 $\pm$ 0.06
CO_2	1h	0.514 $\pm$ 0.087
CO_2	2h	0.309 $\pm$ 0.07
CO_2	4h	0.213 $\pm$ 0.082
Ethylene	0.5h	0.231 $\pm$ 0.052
Ethylene	1h	0.294 $\pm$ 0.049
Ethylene	2h	0.182 $\pm$ 0.052

Effect of different treatments (30% CO_2 , 50ppm ethylene) on the generation of O_2^- coupled with oxidation of NADPH. Avocado peels were exposed to different treatments. Ripe avocado, peels from ripe fruit. The activity was measured in extracted plasma membranes, by reduction of Cytochrome *c*, it expressed in $\mu\text{mol}/\text{mg}$ protein/min.

Fig 1.

Fig. 1. Effect of 30% CO₂ treatment on Rb⁺ uptake

Fig. 2

Fig 2. Effect of 50-ppm ethylene treatment on Rb⁺ uptake.

Fig 3.

Fig. 3 Effect of 30% CO_2 or 50 ppm ethylene on PAL activity

Fig 4

Fig. 4 Effect of inoculation with *C. gloesporioides* on PAL activity (A) and epicatechin content in pericarp tissue (B)

Fig 5

Fig. 5 Effect of 30% CO₂ and 50 ppm ethylen on epicatechin contents on pericarp tissue.

3. Cytokinins induces epicatechin content and resistance against *Colletotrichum gloeosporioides* in avocado fruits

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Abstract

Cytokinin treatment of avocado fruit and avocado mesocarp or pericarp callus enhanced epicatechin content. Increase in the cytokinin thidiazuron (TDZ) in the medium from 0.9 to 1.8 μ M tripled the epicatechin concentration of a mesocarp cytokinin-dependent callus clone. However, TDZ treatments to a selected mesocarp cytokinin-independent clone, did not affect epicatechin levels. Preharvest treatments of avocado fruits with TDZ (10 μ M) and benzylaminopurine (40 μ M) increased in fruit pericarp the activities of flavanone-3-hydroxylase and dihydroflavanone-reductase, the amount of epicatechin and resistance to *C. gloeosporioides*. It is suggested that the cytokinins TDZ and BAP induce the pathway affecting the synthesis of the flavonoid-epicatechin and as a consequence affects fruit resistance to postharvest pathogens.

Abbreviation: [TDZ:N-phenyl-N'-(1,2,3-thidiazol-5-yl)ureal] TDZ; benzylaminopurine, BAP; phenylalanine ammonia-lyase, PAL; flavanone-3-hydroxylase, F3H; and dihydroflavanone-reductase, DFR;

Introduction

Resistance of unripe avocado fruit to attack by *C. gloeosporioides* is correlated with the presence of fungitoxic concentrations of the pre-formed antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene in the pericarp of unripe fruit. The decrease of the pre-formed diene level in ripen fruit and the consequent increased susceptibility, has been attributed to the oxidation of the antifungal compound by the enzyme lipoxygenase. Lipoxygenase activity increases during avocado ripening following the decline in LOX-inhibitory activity by the flavan-3-ol, epicatechin, a natural antioxidant present in fruit pericarp. Increase in the levels of the pre-formed antifungal diene by different elicitors occurs by increase in epicatechin level and inhibition of the LOX-mediated catabolism of the antifungal diene. Increase in epicatechin levels is regulated by transcriptional activation of genes i.e. PAL, CHS and F3H involved in the flavonoid biosynthesis.

Cytokinins regulate growth and development in plants, but secondary metabolism is also affected. Early works of Miller [9] described that callus tissues of *Glycine max*, quickly increased the concentration of the deoxyisoflavone, diadzein in response to cytokinins. Cytokinins also were the causal effector by which the wound signal are directed to synthesis JA or SA [9]. However no reports have indicate the effect of cytokinins as an activator of phenylpropanoid pathway that may regulate fungal development. In the present work we have demonstrated that cytokinins induce epicatechin synthesis in avocado fruits is dependent on cytokinin sensitivity of the tissue. Cytokinin-dependent tissue exposed to TDZ and BAP enhanced the biosynthesis of epicatechin and in whole fruit affect increase the resistance to *C. gloeosporioides* attack. This effect raises a new option for modulation of fruit resistance to postharvest pathogen in avocado fruits.

Results and Discussion

Effect of cytokinin sensitivity of callus of avocado fruit tissue on epicatechin content. In order to determine the effect of cytokinins on activation of phenylpropanoid pathway, clones with differential sensitivity to

cytokinins were selected. Sub-cultivation of acquired primary callus was carried out on MS medium with increasing concentrations of picloram (0.01-0.1mg/L), kinetin (0.1-0.4mg/L) or TDZ (0.9-1.8 μ M). After the fifth sub-cultivation two clones from mesocarp (MC-1 and MC-2) and one from pericarp (PC-1) origin were isolated. MC-1 was cytokinin-independent; it grew on medium without kinetin, formed hard round callus with white color. MC-2 was dependent on 0.1 mg/L kinetin for growth. MC-2 formed soft, irregular callus, gray with yellow-brown inclusions (Fig. 1). PC-1 is independent for growth on cytokinins. PC-1 grows hard, with light - pink color and flat, regular round form is independent for cytokinin but sensitive to 1.8 μ M concentrations of TDZ (Fig. 1).

Growth of MC-2 and PC-1 in the presence of 0.9 μ M TDZ stimulated the epicatechin level by 64% and 212% respectively. Further increase of TDZ up to 1.8 μ M increased the epicatechin content in MC-2 by 96%, but suppressed the growth in the pericarp clone. This is contrast to MC-1 that is cytokinin-independent as PC-1 but its' growths is not affected by higher TDZ concentrations. This is not the first report where cytokinins enhance flavonoid biosynthesis in callus tissue. Cytokinins increased the flavonoid formation of scopolotein and scopolon in tobacco tissue culture [6]. Benzyladenine increased phenolic acid content and the activity of O-methyltransferase in tobacco cells culture [7], and the synthesis of the deoxyisoflavone, diadzein [9] in callus tissues of *Glycine max*. However in avocado the cytokinin-epicatechin induction is dependent on the cytokinin sensitivity of the tissue. TDZ treatments to the cytokinin-dependent MC-2 callus, significantly enhanced epicatechin level while growth of the cytokinin-independent MC-1 callus in the presence of similar TDZ concentrations did not affect epicatechin content (Table 1).

Interestingly PAL activity in cytokinin-dependent MC-2 clone is three folds higher than in the cytokinin-independent MC-1 (Table 2). In both mesocarp clones however, PAL activity was not affected by the increase in TDZ concentrations. This could be expected in the cytokinin-independent callus where epicatechin is not induced, but not in the cytokinin-dependent one. This suggests only a partial relationship between PAL activity and the final epicatechin product. Epicatechin increase was the result of more critical

enzymes in epicatechin synthesis. Activity of DFR, the last enzyme before epicatechin product, increased from 20.3 to 80.5 DFR pmolDHQ / mg protein⁻¹hour⁻¹ in the cytokinin dependent callus as a result of the presence of 0.9 μ M TDZ, while DFR activities in the cytokinin-independent MC-1 callus remained around the 20.5 $\pm$ 2.0 pmol DHQ / mg protein⁻¹hour⁻¹.

MC-1 and PC-1 are both cytokinin-independent but differed in the sensitivity of TDZ. While MC-1 did not show any increase in epicatechin concentration as a result of cytokinin increase, PC-1 showed a tremendous increase at 0.9 μ M TDZ but became necrotic at higher cytokinin levels. (Fig. 1) what suggest that cytokinin-epicatechin induction is strongly dependent in the treated tissue.

Effect of pre-harvest cytokinin treatments on PAL, F3H and DFR activities, epicatechin content and *C. gloeosporioides* development in pericarp of avocado fruits. Spraying avocado fruits with 10 μ M TDZ or with 40 μ M BAP 14 days before harvest increases epicatechin content in the pericarp tissue (Table 3). Seven days after treatment epicatechin levels increased by 95% and 170% in 10 μ M TDZ and 40 μ M BAP respectively. Fourteen days after the cytokinins treatment, epicatechin contents were still higher than in untreated fruits. Also in whole fruits cytokinin-induced epicatechin synthesis occurred without a stimulation of PAL activity, but F3H activity was enhanced almost by two folds (Table 4). The behaviour of the activity of PAL was already found in citrus callus where CitCHS2 rather than PAL play an important role on the switching of flavonoid synthesis. The most significant effect of cytokinin treatments was on DFR activity that increased from 24.5 in control to 44.3 and 65.5 pmol DHQ / mg protein⁻¹hour⁻¹ in TDZ and BAP treated fruits, suggesting the specific effect on the last steps of phenylpropanoid biosynthesis.

Treated fruits that were harvested and inoculated with *C. gloeosporioides* showed a 50% inhibition in decay development 10 days after inoculation (Fig. 2). In *in vitro* tests, TDZ or BAP had no direct effect on *C. gloeosporioides* germination and germ tube elongation (results not shown). What is the mechanism(s) by which cytokinins induce resistance in avocado? Our results indicate that cytokinin induced metabolic shifts toward synthesis of antioxidant not only in callus as found before [6,7], but affect the whole fruit. This effect

modulates the whole mechanism of resistance of avocado by preventing the decrease of the antifungal diene. However cytokinins might have other effects. Hare and van Staden [5] showed that TDZ inhibited soybean cytokinin oxides and increase the physiological available cytokinins. Also it was demonstrated that TDZ induced accumulation of purines (adenosine, zeatin and dihydrozeatin), either as cytokinins or as sources of metabolic energy. It seems to us that TDZ increases the available supply of purines for cellular responses and the accumulated concentration of cytokinin. Although the exact mechanism by which cytokinin affect the phenylpropanoid pathway is not known, it is clear that a physiological stress that lead the phenylpropanoid pathways was activated.

Cytokinins however might have a secondary effect of avocado resistance. Chaitanya and Naithani [2] considered that kinetin inhibits and/or reduces membrane damage by suppressing oxidative stress, and enhancing superoxide dismutase that could reduce peroxidation of phospholipids. Also Lesham (1987) suggested that cytokinins might reduce $\cdot O_2$ -scavenging level. This suggest that cytokinins beside their effect on epicatechin synthesis might affect the level of $\cdot O_2$ and indirectly inhibited the lipoxygenase activity resulting in a suppressed decrease in the pre-formed compound and delayed disease expression.

EXPERIMENTAL

Source of material and experimental treatments. Experiments were carried out with avocado fruits [*Persea americana* Miller var. *drymifolia* (Schldl. and Cham.) S.F. Blake] cv. Fuerte, from an orchard at Kibbutz Givat Brenner, Rehovot, Israel. Fruits of fairly uniform size and weight (250-270 g) were harvested, washed in water and used immediately after harvest for experiments. Primary callus of avocado fruits, mesocarp or pericarp from 10 week-old fruit (5 cm length) was initiated on basal MS solid growth medium, with different combination of IAA (0.1-2 mg/L), 2,4-D (0.01-0.4 mg/L) and kinetin (0.1-2 mg/L) at 25°C. For prevention of surface cells oxidation, the explants were incubated with PVP (av. mol wt. 40,000) and vitamin C (250:10 mg/L). Subcultivation of acquired primary callus was carried out on MS

medium with different combination of picloram (0.01-0.1mg/L), kinetin (0.1-0.4mg/L).

Cytokinin treatment. Developing callus of about 0.3g was transferred to plates containing increasing concentrations of TDZ and BAP. Whole avocado fruit were sprayed until fully covered, 7 or 14 day before harvest with 10 μ M TDZ or 40 μ M BAP dissolved in 0.01% dimethyl sulphoxide (DMSO) and 0.02% Triton X-100. Control fruits were sprayed with 0.01% DMSO and 0.02% Triton X-100 only. Fruits were left uncover until they were harvested and transferred to the lab for extraction or inoculation.

Extraction of epicatechin. Epicatechin was extracted from 1mm thick avocado pericarp slices or from callus tissue, as previously described. Three grams (fresh weight) of tissue were ground in 95% ethanol (1:10 w/v). The ethanol extract was dried in a rotary evaporator, and the residue was re-dissolved in 10 ml of 1 mM NaCl and extracted twice with 90-ml ethyl acetate. Epicatechin was quantitated by RP-HPLC. Calculation of epicatechin concentration was based on comparison of the HPLC peak areas with those of authentic standards.

Extraction and activity of enzymes. Activity of PAL, F-3-H and DFR was measured in avocado tissue culture and pericarp. The enzymes extracted from avocado pericarp (5 g) was blended in 50-ml cold acetone (-20°C) and the homogenate filtered out with Whatman no.1 and blended three more times. The pericarp powder was dried at room temperature overnight, and stored at -20°C until used. Enzymes were extracted from 100 mg acetone powder of pericarp by stirring for 2 h at 4°C, in the presence of 4 ml of 0.1 M sodium borate pH 8.8 and polyclar AT. Fresh tissue culture (500 mg) was homogenized in the same described buffer in a mini-beadbeater (Biospec) with 1/3 volume of glass beads (0.5mm). One-ml extract was centrifuged at 10,000 g for 20 min at 4°C and the supernatant was used as an enzyme source. The reaction mixture for PAL consisted of 10 mM L-phenylalanine (Aldrich), 0.1 M borate buffer (pH 8.8) and 1.5 ml of enzyme extract in a final

volume of 5 ml. The enzyme mixture was incubated at 37°C for 60 min and terminated by adding 0.1 ml of 5 N HCl. The product, cinnamic acid was extracted into 7 mL of diethyl ether and quantitated by measuring the amount of trans-cinnamic acid formed at 269-nm.

F3H activity was tested in a reaction mixture that consisted on 0.6 ml of 50mM Tris pH 7.4, 0.015mM naringenin (Roth, HPLC grade), 5mM NADPH and 100µg enzyme extract. The reaction was incubated for period of 1 or 2 hours at 37°C. The reaction was terminated with 50µl of 5N HCl and the enzyme product- naringenin- extracted 3 times with 1ml ethyl acetate. The upper layer were collected and evaporated under N₂. The samples were dissolved with 0.2ml 55% methanol 1% acetic acid in double distilled water. Naringenin was analyzed with reverse-phase HPLC (RP-18, EcoPack, Merck), and quantified by comparison with known standards. The activity is given as disappearance of naringenin in µmol.

DFR activity was assay as a double step reductase sequence starting with dihydroquercetin (DHQ) converted to catechin, according to Stafford and Lester [11]. The 0.6 ml reaction mixture contained 50µg DHQ, 5mM NADPH and 200µg of protein from the crude extract in 50mM tris pH 7.4. The reaction was stopped with 50µl of 5N HCl, and the metabolites extracted as in F3H assay. Enzyme activity is presented as disappearance of DHQ in pmol/mg prot/h DHQ.

Fruit inoculation. A single-spore isolate of *C. gloeosporioides*, isolate Cg-14 obtained from decayed avocado fruits was used for inoculation experiments. The fungus was normally maintained on M₃S media at 20°C. Inoculation was carried out by placing 10µl-spore suspension (2×10^6 spores/ml) on the avocado pericarp. Six inoculation spots on the longitudinal axis of the fruit, each three from both sides were done. Following inoculation fruits were maintained at ca. 90% RH for 24 h and then transferred to normal humidity conditions (70-80% RH) at 20°C until fruit ripening and symptom developed. Symptoms of decay on the pericarp were expressed as a diameter of brown pericarp tissue. Ten fruits were inoculated per treatment (total of 60 inoculated spots/treatment) and experiments were repeated three times.

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REFERENCES

- 1.- Ahl Goy, P., Singer, H., Reist, R., Aichholz, R., Blum, W., Schmidt E. and Kessmann H. *Planta*, 1993, **191**,200.
- 2.- Chaitanya, K.S.K., Naithani, S.C. *J Plant Growth Regul* 1998, **17**, 63.
- 3.- Hahlbrock K and Scheel D (1989). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1989, **40**, 347.
- 4.- Hare, P.D. and van Staden, J. 1994. *Physiol. Plant.* 1994, **91**,128.
- 5.- Hare PD and van Staden J. *Plant Cell Physiol.* 1994, **35**, 1121.
- 6.- Hino F, Okazaki M and Miura Y. 1982. *Agric. Biol. Chem.* 1982, **46**,195.
- 7.- Kuboi, T. and Yamada, Y. *Phytochemistry* 1996,**15**,397.
- 8.- Lesham, Y.Y. *Physiol Plant.*1987, **69**, 551.
- 9.- Miller, C.O. *Planta* 1969, **87**, 26.
- 10.- Sano H, Seo S, Koizumi N, Niki T, Iwamura H and Ohashi Y. *Plant Cell Physiol.* 1996, **37**, 762.
- 11.- Stafford HA and Lester HH. *Plant Physiol.* 1984 **76**, 184.
- 12.-Takaya M, Masayuki K, Yasuhiko T, Tomoko Ei and Mitsuo O. *Plant Cell Physiol.* 1999, **40**, 651.
- 13.- Terao J, Piskula M and Yao Q. *Archives of Biochem. and Biophy.* 1994, **308**, 278.
- 14.- Victor JMR, Murthy BNS, Murch SJ, KrishnaRaj S and Saxena P.K. *Plant Growth Regulation* 1999, **28**, 41.

Table 1.

Levels of epicatechin in selected callus clones from mesocarp and pericarp avocado fruit exposed to increasing concentrations of TDZ

Callus tissue	Epicatechin** ($\mu\text{g/g}$. fresh weight)		
	Control	TDZ 0.9 μM	TDZ 1.8 μM
MC-1	0.237 $\pm$ 0.05	0.398 $\pm$ 0.07	0.338 $\pm$ 0.07
MC-2*	2.918 $\pm$ 0.32	4.783 $\pm$ 0.81	9.283 $\pm$ 1.66
PC-1	4.727 $\pm$ 0.86	14.795 $\pm$ 2.33	Hardly grown

*Control for clone MC-2 includes 0.1 mg/L kinetin.

Table 2

PAL activity in selected callus clones from mesocarp and pericarp avocado fruit exposed to increasing concentrations of TDZ.

Callus tissue	PAL activity ($\mu\text{mol mg protein}^{-1}\text{h}^{-1}$)		
	Control	TDZ 0.9 μM	TDZ 1.8 μM
MC-1	4.2 $\pm$ 2.4	5.8 $\pm$ 1.2	6.2 $\pm$ 0.9
MC-2*	13.1 $\pm$ 3.7	17.5 $\pm$ 1.4	15.1 $\pm$ 2.3
PC-1	12.6 $\pm$ 1.8	5.9 $\pm$ 1.1	(Hardly grown)1.5 $\pm$ 0.2

*Control for clone MC-2 includes 0.1 mg/L kinetin.

Table 3

Effect of TDZ and BAP on epicatechin concentration of pericarp
avocado fruits cv. Fuerte

Time after treatment	Epicatechin ($\mu\text{g/g f. wt}$)		
	Control	TDZ 10 μM	BAP 40 μM
7 days	475 $\pm$ 161	968 $\pm$ 202	1349 $\pm$ 326
14 days	543 $\pm$ 192	1055 $\pm$ 188	1040 $\pm$ 176

Table 4

Effect of TDZ and BAP treatment on PAL, flavanone-3-hydroxylase
and flavanone D reductase activity on pericarp of avocado fruit 14
days after treatment cv. Fuerte fruits.

Enzyme activity (units)	Activity($\mu\text{mol mg protein}^{-1}\text{hour}^{-1}$)		
	Control	TDZ 10 μM	BAP 40 μM
PAL ($\mu\text{mol/ mg prot/h}$)	1.81 $\pm$ 0.5	2.29 $\pm$ 0.19	1.91 $\pm$ 0.38
F-3-H($\mu\text{mol/ mg prot/h}$)	6.50 $\pm$ 1.7	12.00 $\pm$ 1.8	11.50 $\pm$ 0.5
FDR (pmol/ mg prot/h)	24.5 $\pm$ 5.5	44.3 $\pm$ 6.1	65.5 $\pm$ 13.6

Legends

Fig 1: Effect of TDZ on avocado mesocarp or pericarp callus. Mesocarp MC-1 and pericarp PC-1, are cytokinin-independent clones; MC-2 is a mesocarp cytokinin-dependent (0.1mg/L kinetin in the medium).

Fig 2. Effect of preharvest cytokinin treatments on decay development on avocado fruits cv. Fuerte caused by *C. gloeosporioides*. Fruit were sprayed with solution containing 0.01% DMSO, 0.02% Triton X-100 and either 10 μ M TDZ or 40 μ M BAP 14 days before harvest. The control fruits sprayed with DMSO and Triton X-100 only. Inoculation carried out after and incubated at 20° C.

Development of non pathogenic mutant with reduced pathogenic performance for biological control of subtropical fruits.

C. gloeosporioides attacks avocado fruits during growth in the orchard but causes symptoms of decay after harvest when fruits ripen and become ready to eat. Resistance of the fruits is dependent of the levels of epicatechin that regulates all the mechanism of resistance. Epicatechin can affect directly fungal development since it can inhibit two of the enzymes, polygalacturonase and pectate lyase, produce during *Colletotrichum* attack. The objective of the work of this project was to: 1. study and develop non pathogenic mutant that could induce resistance, 2 to study the possible regulation of secretion of the enzymes as a basis for pathogenicity and to 3. heterologously express the *pel* gene of *C. gloeosporioides* in the non pathogenic strain of *C. magna* as a basis to determine the importance of PL in pathogenicity.

4. Development of non-pathogenic strains of *C. gloeosporioides* *Colletotrichum gloeosporioides* tagged mutants obtained by REMI opens a new way to investigate pathogenicity process on avocado fruits.

INTRODUCTION

The phytopathogenic fungus *Colletotrichum gloeosporioides* Penz. (anamorph *Glomerella cingulata* (Stonem). Spauld, et Schrenk), is a very important post-harvest pathogen attacking a wide variety of tropical and sub tropical fruits (Coates et al., 1993. Prusky et al., 1992).

The damage caused by *C. gloeosporioides* led rises to the question as to what genes are involved in the fungi attack.

The development of *C. magna* mutant (Path-1) by UV treatment (Freeman and Rodriguez, 1993) that does not attack avocado fruits made it possible to start investigating the pathogenicity process. The mutant was found to have a delayed secretion of pectolytic enzymes (Wattad et al., 1995). The problem with the mutant would be to locate and to identify the mutation since it is not tagged. The mutant analysis would have to involve complementation with

cosmid library. This process is time consuming since it involves the screening of thousands of transformants for the restoration of pathogenicity. Therefore, tagged mutants will facilitate a fast way to isolate and analyze the mutated genes.

Several approaches have been explored to mutagenize pathogenicity-related genes. Gene disruption is one approach where one looks into the importance of a specific gene. This approach was used to gene disrupt pectin lyase from *C.gloeosporioides* (Bowen et al., 1995).

Restriction enzyme mediated integration (REMI) was a high yield transformation frequency, simple plasmid integration approach. REMI was first reported in yeast (Schiestl and Petes., 1991) for studying the mechanism of illegitimate recombination. Since then, REMI protocols were adopted to other systems facilitating an increased transformation in *Dictyoslelium discoideum* (Kuspa and Loomis., 1992), and fungi like *Cochliobolus heterostrophus* (Yang et al., 1996), *Magnaporthe grisea* (Shi et al., 1995), *Ustilago maydis* (Bolker et al., 1995), *Alternaria alternata* (Akamatsu et al., 1997), *Colletotrichum* sp. (Redman and Rodriguez., 1994), *Penicillium paxilli* (Itoh and Scott., 1997).

This process involves the transformation of an organism with a linear fragment of DNA in the presence of the restriction enzyme that was used to generate the linear fragment. Under these conditions the linear DNA integrates into the chromosome at the restriction sites recognized by that particular enzyme (Schiestl and Petes., 1991. Kuspa and Loomis., 1992). For *U.maydis* it has been reported that transforming linear DNA molecules are integrated into the genome either randomly or in tandem copies (Wang et al., 1988, Kronstad et al., 1989, Fotheringham and Holloman., 1989).

In this paper we show a way for the analysis of *C.gloeosporioides* REMI mutants, that were selected for reduced pathogenicity. We show a phenotypic and genotypic way to analyze the mutant strain.

MATERIAL AND METHODS

Fungal isolates, fruit, Bacterial strains

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. pathogen on avocado fruits (*Persea americana* Miller var. *drymifolia* (Schidl. and Cham.) S. F. Blake 'Fuerte') was kept as spores at -80°C, in 10mM NaP buffer (pH 7.2) and 40% glycerol.

Competent cells from *E.coli* strain, DH-5 α , were used for plasmid amplification and plasmid excision.

Growth conditions

In order to obtain hyphae for protoplasts preparation, the fungi was grown in liquid M3S media (Freeman et al., 1996) for 5 days at 22°C. Hyphae was collected by vacuum filtration and subjected into fungal transformation. For plasmid amplification, bacteria containing the different plasmids (see plasmid generation) were grown on LB media supplemented with 100 μ g/ml of ampicillin, at 37°C for 16h.

Plasmid mini prep was done using MiniPrep wizard (Promega, by manufacturer instructions) to obtain 4 μ g plasmid DNA per 5ml growing bacteria.

Vector construction

The hph gene (Hygromycin phosphotransferase) was used as selectable marker that gives resistance to hygromicine. The gene was subcloned from pHA-1.3 (Redman and Rodriguez, 1994), by restriction with HindIII and Xba-1 (promega) generating 2.13kb, to ligated into pGEM-7Z (promega), restricted with the same enzymes. The product pGH-1 (5.13kb) was use as transformation vector.

REMI- Restriction Enzyme Mediated Integration and mutants screening

Fungal protoplasts were obtained as was describe by Redman and Rodriguez, 1994. The protoplasts were transformed with HindIII or XbaI restricted pGH-1 (4 μ g), in the presence of 20U restriction enzyme, as described by Redman and Rodriguez, 1994. The transformation solution was incubated for 48h at 22°C and was grown on HM media (Redman and Rodriguez, 1994)

supplemented with 100µg/ml hygromycin (HM+Hyg). Transformed colonies were collected 4-8 days after the transformation.

The colonies were transferred to new HM+Hyg plates. In order to get fungi spores, and to check transformation stability, the fungus was transferred and grown on M3S media plates.

Pathogenicity test

The selected colonies were tested for their maceration ability. The mutants were inoculated on the fruit's flesh and the rate of maceration was compared with the maceration performances of the wild type. Fungal maceration occurred within 48h at 22°C. The colonies were checked for peel maceration ability, using spot inoculation, by placing a 3 mm cut cube from the growing plate (with the fungi) on the fruit's peel. Results obtained within 5-7 days. Colonies that showed reduced or non-maceration ability were kept for future analysis.

Southern blot analysis

Fungal DNA was obtained as described by Rodriguez., 1993. One g of dry hyphae was used to extract 50µg DNA. The DNA was double cleaned from protein and restriction enzyme inhibitors.

Fungal DNA (5µg) was restricted for 15h with 20u of EcoRI or HindIII or XbaI+XhoI. The restricted DNA was run on 0.8% agarose gel for 15h. The DNA was blotted to nylon membrane (HiBond+, Amersham, by manufacturer instructions), using the capillary method.

ATPase activity.

Avocado pericarp tissue were exposed to different treatments, plasma membranes were separated. Vanadate sensitive plasma membranes ATPase was determined in the presence of 0.01% polyethylene 20 cetyl ether (Brij 58) and in the presence or absence of 0.2mM Na₃VO₄. ATPase activity was assayed as described previously by (Jacoby and Rudich, 1987), with minor variations: 4ug of plasma membranes protein were incubated for 30 min at 35°C in 0.5ml medium composed of 40mM Mes-tris (pH 6.5), 0.25M sucrose,

100mM KCl, 3mM MgSO₄ and 3mM ATP. NADPH oxidation activity was tested as described in the first chapter.

Rubidium uptake

The pericarp tissue disks (about 5 mm diameter and 1 mm thick, almost free of mesocarp) were prepared as described for pericarp strips in the first chapter of this report. The disks were pre-incubated for 30 min in 0.2 mM CaSO₄ at 25 °C. CO₂, ethylene or other treatments were applied as described in the results. The [⁸⁶Rb]K⁺ was added to the vial at the last 20 min of the reaction. Uptake of [⁸⁶Rb]K⁺ into 70 mg of tissue the avocado disks (5 disks) was assayed in 5 ml of 0.2 mM CaSO₄ and 0.1 mM [⁸⁶Rb]K⁺ 700 Bq/μmol). Exchangeable was removed with 3 washings over a 5 min-period in 15 ml of ice cold 10 mM CaSO₄. The disks were transferred to vials for scintillation counting and added 0.2 ml of 1 N NaOH for discoloration and then 4 ml of Opti-Fluor O (Packard). The sample were counted in scintillation counter MR 300 Lumitron (Automatic liquid scintillation system)

Fruit defense

For biocontrol experiments, 18 fruits cv. Fuerte, were brushed with one month old spores of GH142 (0.75 X 10⁶ spores/ml) and 18 others were brushed with water as control. Twenty four hours later, all fruits were spot inoculated with *C. gloeosporioides* by placing 10 μl of spore suspension (10⁶ spores/ml) at 6 points, three at each side of the longitudinal axes of the fruit. Fruits were then kept at 22°C, in 90% humidity for 10-12 days. For diene level experiment, 14 fruits were brushed with GH142 as written above, and 14 others were brushed with water (this fruits were not inoculated with *C. gloeosporioides*).

Diene extraction

Ten g of avocado pericarp and mesocarp (1-2 mm), were homogenized in 95% ethanol in Omni-mixer (Sorvall) at full speed for 3 min. The ethanol extract was dried in a rotary evaporator at 40°C, re-dissolved in 10-ml distilled water, and the organic phase extracted by fractionation with dichloromethane. Following two extraction processes, the organic phases were pooled, dried with MgSO₄ anhydrous (Riedel-deHaen), and evaporated to dryness. Samples

were re-dissolved in 1 ml of ethanol AR (Bio Lab) and analyzed by HPLC as described by Prusky et al., (1991). The average values of three separate extractions are reported.

Results

Vector construction

One available plasmid for fungi transformation, that contained the *hph* gene was pHA-1.3 (Redman and Rodriguez, 1994). The problem with this plasmid was its ability for self replication (out side of the fungal genome), due to the telomer fragment in the vector. In order to perform REMI experiment and to maximize mutation incidences, we need a vector that his survival will depend on genome integration. The enhanced integration will result in an increased amount of Mutation Mediated Gene Disruption (MMGD). To solve the problem we recently constructed a pGEM-7Z base vector contains the *hph* cassette from pGH-1.3 creating pGH-1 (Fig. 1).

REMI and mutants screening

pGH-1 was transferred into the fungal protoplasts as supercoil DNA and as linear DNA. Transformation of pGH-1 as linear plasmid procedure which 24 U of HindII or XbaI restriction enzymes were added to the transformation reaction, resulted with 10-fold increase in the transformed colonies. This data is less then what was reported by Redman and Rodriguez., 1994 (22 fold). The explanation can be due to the telomer that enables non-integrating plasmids to generate transformats colonies.

The transformation ratio gave 100 transformants per 1 µg plasmid DNA per 10^7 protoplasts. Ten transformations gave 3000 colonies, which were transferred to new HM+Hyg plates. All 3000 colonies were subjected to pathogenicity test (see material and methods). 14 colonies were found to be with different degree of decreased pathogenicity (Table 1). The strains were divided into a few categories: growing on plates; slow (CgM-182, CgM-627, CgM-809), well (GH-142, CgM-6, CgM-25, CgM-104, CgM-114, CgM-154, CgM-392, CgM-807, CgM-1150, CgM-1210, CgM-1230,). Growing on flesh; non (CgM-182, CgM-627, CgM-809, CgM-1150), reduced (GH-142 <figure 2>,

CgM-25, CgM-114, CgM-154), normal (CgM-6, CgM-104, CgM-392, CgM-807, CgM-1210, CgM-1230). Growing on peel; non(CgM-6, CgM-25, CgM-114, CgM-182, CgM-627, CgM-809, CgM-1150, CgM-1210), reduced (GH-142 <figure 3>, DgM-104, CgM-154, CgM-392, CgM-807, CgM-1230). Sporulation ,low (CgM-182, CgM-627, CgM-809, CgM-1210), normal (All others).

Analysis of the mutant GH-142;

Phenotype analysis of the mutants was done. GH-142 produce appressoria at the same time, shape and rate as the wild type (Fig. 4). Differential pathogenicity determined as maceration ability showed that GH-142 had reduced maceration on peeled and re-peeled avocado fruits (Fig. 5). CgM-1150 was found to be lack of appresorium formation and was unable to attack the fruit pericarp with a redundant ability to attack the mesocarp (data not shown).

Molecular analysis

In order to find out the integration location of the plasmid and the reason for the mutation, we received two different colonies mediated plasmid excision.

Restricting GH-142 gDNA with EcoRI generated 8 kb colonie. EcoRI generated a plasmid containing 3 kb pGEM-7Z, 0.7 kb of *hph* gene to the EcoRI site, and 4.3 kb of fungal DNA (Fig. 6).

In order to locate the mutation, Southern blot analysis was done. HindIII+XbaI and XbaI+XhoI resistricted gDNA, of GH-142 compared with Cg-14, was screened with *hph* (2.13 kb) as a probe, generating a 4kb and 3.9 kb bands with GH-142 and not with Cg-14 (Fig 7). Southern blot analysis, using Hyg gene as probe, of CgM-1150 restricted with XbaI or EcoRI, generated ~3.4Kbp band and ~5.0Kbp band respectively (Data not shown).

Sequencing and sequences analysis

Currently we have 5 different clones mediated plasmid excision (not showed). One clone, p142E1 was generated by EcoRI restricted genomic GH-142 DNA. The plasmid was sent to the "Biological Services, Weizman Institute". The sequences were compared with the Gene Bank, using GCG program.

Currently we are analyzing the DNA sequences and we did not define any specific genes.

O₂ generation-dependent NADPH Oxidation and H⁺-ATPase activity.

Plasma membranes of isolated pericarp tissue that had been pre-inoculated with *C. gloeosporioides* showed a rapid increase in the O₂ generation coupled with NADPH oxidation. The activity increased by three folds within 1-2h after inoculation (Fig 8). While the reduced pathogenicity strain, GH-142 enhanced the activity level by six folds within 2h. Inoculation with the non-pathogenic strain CgM-1150 had no effect on the activity level.

The activity of vanadate sensitive plasma membranes H⁺-ATPase was also measured after inoculation. The H⁺-ATPase activity level increased by three folds within 2h after inoculation with *C. gloeosporioides* (Fig. 9). Higher response (four folds) was found after 2h when avocado pericarp were exposed to the reduced pathogenicity strain, GH-142, and 2h latter the activity dropped to the control level. Inoculation with CgM-1150 strain had no response in the H⁺-ATPase activity.

Rb⁺ Uptake.

The Rb⁺ uptake by avocado pericarp discs was measured after inoculation. Exposure of the *C. gloeosporioides* spores to the pericarp tissue enhanced the Rb⁺ efflux (Fig 10). The GH-142, the reduced pathogenicity strain, increased the Rb⁺ uptake by two folds within 2h. Inoculation with the CgM-1150, did not effected the Rb⁺ efflux, as the other activities.

Diene level and fruits protection

Antifungal diene was extracted starting from the second day after GH142 inoculation until symptoms appearance (Fig 11). The level of diene was significantly higher at the protected fruits during the checked 10 days. The level of diene increased immediately after fruit inoculation with GH142, and reached a 2.5 time fold increase over the control 3-4 days.

Decay development started in unprotected fruits 9 days after fruit inoculation (Fig 12). However decay development in GH142 inoculated fruits was

significantly reduced. Pre-inoculation with CgM-1150 before the inoculation with Cg-14 did not effect fruit resistance (Data not shown).

DISCUSSION

Little is known about the pathogenicity process of the fungus *C. gloeosporioides* while it switches from quiescent to an active pathogen. So far, a conservative approach was used in order to investigate the pathogenicity process. Dickman et al. (1985), described that UV mutants of *C. gloeosporioides* lacking in cutinase production could not attack papaya fruits. Also Hwang et al., 1995, reported that a reduced pathogenicity strain of *C. gloeosporioides* after disruption of a gene that is specifically expressed during appressorium formation. Till now the importance of pectolytic enzymes during pathogenicity was questioned by the work of Bowen et al. (1995) that disrupted one of the pectin lyase genes from *C. gloeosporioides* without any effect on pathogenicity on apples. These approaches handle very specific genes, while pathogenicity might be the result of several, and still unknown genes.

REMI on the other hand, gives us a tool that can explore mutation in the fungus transformant, and we can decide on the selected phenotype required. We choose to look for reduced/non pathogens, in order to understand the pathogenicity secret.

On the contrary to the mutants obtained from *Ustilago maydis* where they showed no ability to attack nor induced anthocyanins biosynthesis (Bolker et al., 1995), we obtained several mutants (Table 1), that show a different degree of pathogenicity. This array of mutants can serve as a bank of disrupted properties to investigate different pathogenicity factors involved in fungal attack.

Unlike the vector used by Redman and Rodriguez., 1994, to transform *Colletotrichum* spp. that had a telomer enabling self replication (without integration), we constructed a vector that will maximize the integration efficiency, where it's survival is integration dependent.

The development of a fast efficient screening way was crucial tool in this work. The selection of pathogenicity on peeled avocado fruit, showed symptoms with

48h result enabling us to screen 3000 transformed strains within the three months of the avocado-harvesting season.

Mutation rate was found to be 0.5%, compared to *U. maydis* 1.4% (Bolker et al., 1995) or 0.27% in *D. discoideum* (Kuspa et al., 1992). This result suggests that there might be several genes that play a role in the pathogenicity process. Some mutants have shown growth limitation and this might limit their pathogenicity. However, several other mutants had no growth limitations on in vitro media, but still show significant decay reduction in vivo. This indicates that we have disrupted new genes whose product plays an essential role in fungal attack on the fruit. These genes may be metabolically involved in fungal growth and may not only be required exclusively for fungus attack. The detailed analysis of one REMI mutant strain can exhibit the great potential in tagged mutants, for the studying of such complex processes as pathogenic development.

Development of mutants has results in the capability to analyze the mechanism of elicitation of resistance in avocado fruits. Our results in *C. gloeosporioides* indicate that the pathogen needs the ability to breach through the fruit cuticle to start the initial interaction. Cg-14 and Cg142 did not differ in appressoria formation. Both infected fruits but differ in the rate of decay development. However isolate CgM-1150 did not produce appresoria and could not infect fruits.

Since Cg-142 induce protection against Cg-14 and CgM-1150 did not it was suggested that the protection is induced by the capability of the pathogen to breach the fruit cuticle and start the initial elicitation process. When the effect of Cg 142 and CgM-1150 were compare on the effect on ATPase, NADPH oxidase and rubidium uptake it was found a significant effect of Cg-142 compared to the non-pathogenic mutant. Effect on ATPase, NADPH oxidase and rubidium uptake could implied that inoculation activated the plasma membranes proton pump, the depolarization of the membranes occurred simultaneously or nearby the generation of reactive oxygen species that leads to the elevation of epicatechin levels (Beno and Prusky, 1998). Ardi et al (1998) showed that epicatechin protect the antifungal diene degradation, in the fruits. The signals that lead to the elevation of epicatechin lead to the high

level of antifungal diene that leads to the protection of fruit against the attack of *C. gloeosporioides*.

LITERATURE CITED

- 1) Akamatsu, H., Itoh, Y., Kodama, M., Otani, H., Kohmoto, K. 1997. AAL toxin deficient mutants of *Alternaria alternata* tomato pathotype by restriction enzyme mediated integration. *Phytopatology*. 87:967-972.
- 2) Binyamini, N., Schiffmann-Nadel, M. 1972. Latent infection in avocado fruit due to *Colletotrichum gloeosporioides*. *Phytopatology*. 62:592-594.
- 3) Bolker, M., Bohnert, H. U., Braun, K. H., Gorl, J., Kahmann, R. 1995. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.* 248:547-552.
- 4) Bowen, K. J., Templeton, D. M., Sharrock, R. K., Crowhurst, R. N., Rikkerink, H. A. R. 1995. Gene inactivation in plant pathogen *Glomerella cingulata*: three strategies for the disruption of the pectin lyase *pnl* A. *Mol. And Gen. Genet.* 246:196-205.
- 5) Coates, L. M., Muirhead, I. F., Irwin, J. A. G., Gowanlock, D. 1993. Initial infection processes by *Colletotrichum gloeosporioides* on avocado fruit. *Mycol. Res.* 97:1363-1370.
- 6) Dickman BM. 1985. Cutinase deficient mutant of *Colletotrichum gloeosporioides* are nonpathogenic to papaya fruit. *Physiol. Mol. Plant Pathol.* 28:235-42.
- 7) Fotheringham, S., and Hollman, W. K. 1989. Cloning and disruption of *Ustilago maydis* *Ustilago maydis* genes. *Mol. Cell Biol.* 9:4052-4055.
- 8) Freeman, S., Katan, T., Shabi, E. 1996. Characterization of *Colletotrichum gloeosporioides* isolates from avocado and almond fruits with molecular and pathogenicity tests. *Appl. and Environ. Microbiol.* 62:1014-1020.
- 9) Freeman, S., Rodriguez, J. R. 1993. Genetic conversion of fungal plant pathogen to a nonpathogenic, endophytic mutualist. *Science*. 260:75-78.
- 10) Hwang C-S, Flaishman AM, Kolattukudy EP. 1995. Cloning of gene expressed during appressorium formation by *Colletotrichum gloeosporioides* and marked decrease in virulence by disruption of this gene. *The Plant Cell* 7:183-93.

Figure1: Plasmid construction.

DNA plasmid (pGH-1) was constructed by the ligation of 2.13 Kb, *hygB* gene from pHA-1.3 (Redman and Rodriguez, 1994), restricted with *HindIII* and *XbaI*, into the same restriction sites in pGEM-7Z, to create a two single restriction sites plasmid (*HindIII* and *XbaI*). The pGH-1 was used for the transformation of *C. gloeosporioides* isolate 14 (Cg-14) protoplasts after restriction with *HindIII* and transformants were screened on HM+HygB media (Redman and Rodriguez, 1994).

Figure 2: Patogenicity test on plesh

Pathogenicity of 3000 Hyg resistant mutants were screened and one transformant (GH-142) was found to be less pathogenic compared to the wild type (Cg-14).

Figure 3: Pathogenicity test on peel

Gh-142 was spore inoculated on avocado peel and maceration ability was compared to the wild type (Cg-14)

Figure 4: Appressoria formation

Formation of appressoria (A) of Cg-14 (left) and GH-142 (right) on avocado fruit.

Figure 5: Differential maceration

Peeled (left) and repeeled (right) avocado fruits inoculated with Cg-14 and GH-142 spores

Figure 6: Plasmid excision

Restriction gDNA mediated plasmid excision with EcoRI restriction enzyme, generated a 8kb plasmid. The plasmids were restricted with HindIII and XbaI generating the 3kb pGEM fragment and the 2.13kb hph gene (pGH-1) and the 5kb hph + flanking region.

Figure 7: Southern blot analysis

Genomic DNA was extracted (Rodriguez et al., 1993) from GH-142 and Cg-14 and run on 0.8% agarose gel after restriction with HindIII + XbaI and XbaI + XhoI. DNA was screened hph gene (Hyg) as a prob.

Fig. 8. Time course of NADPH-dependent cytochrome c reducing activity in plasma membrane isolated from pericarp tissue inoculated by different strains of *C. gloeosporioides*. The WT, Cg-14, and the two mutants GH 142 (appressoria forming mutant) and CgM-1150 (non-appressoria forming mutant).

Fig 9. Time course of H^+ ATPase activity in plasma membrane isolated from pericarp tissue inoculated by different strains of *C. gloeosporioides*. The WT, Cg-14, and the two mutants GH 142 (appressoria forming mutant) and CgM-1150 (non appressoria forming mutant).

Fig 10. Uptake of [^{86}Rb] K^+ into pericarp disk following inoculation with different fungal strains of *C. gloeosporioides*. The WT, Cg-14, and the two mutants GH 142 (appressoria forming mutant) and CgM-1150 (non-appressoria forming mutant).

Fig 11. Effect of inoculation of freshly harvested fruit cv. Fuerte with the *C. gloeosporioides* mutant GH 142 on the level of the antifungal diene in avocado pericarp. At different days after inoculation the pericarp of the fruit was peeled and the antifungal diene concentration analyzed.

Fig 12. Effect of pre-inoculation of freshly harvested fruit cv. Fuerte with the *C. gloeosporioides* mutant GH 142 on decay development by the wild type Cg-14. GH 142 was inoculated by dipping the whole fruit in a spore suspension (10^6 spores/ml) and one-day later the same fruits were spot inoculated with the 10 ul of the wild type suspension (10^6 spores/ml).

Table 1:

**Reduced pathogenicity mutants of *C.gloeosporioides*
obtained by REMI**

Number	Strain	Media	Flesh	Peel	Sporulation
1	GH-142	well	reduced	reduced	normal
2	CgM-6	well	normal	non	normal
3	CgM-25	well	reduced	non	normal
4	CgM-104	well	normal	reduced	normal
5	CgM-114	well	reduced	non	normal
6	CgM-154	well	reduced	reduced	normal
7	CgM-182	slow	non	non	low
8	CgM-392	well	normal	reduced	normal
9	CgM-627	slow	non	non	low
10	CgM-807	well	normal	reduced	normal
11	CgM-809	slow	non	non	low
12	CgM-1150	well	non	non	normal
13	CgM-1210	well	normal	non	low
14	CgM-1230	well	normal	reduced	normal

5. Expression of Pectate Lyase from *Colletotrichum gloeosporioides* in *C. magna* and its Contribution to Fungal Pathogenicity

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ABSTRACT

To test the contribution of pectate lyase (PL) to fungal pathogenicity, a pectate lyase gene (*pel*) from the avocado pathogen *Colletotrichum gloeosporioides*, isolate Cg-14, was expressed in *C. magna*, a pathogen of cucurbits which causes minor symptoms in watermelon seedlings and avocado fruits. Wildtype isolate L2.5 of *C. magna* was transformed with pPCPH-1 containing hph-B as a selectable marker and the 4.1-kb genomic *pel* clone. Southern-blotting hybridization, using the 4.1-kb genomic *pel* clone or 2.13-kb hph-B cassette as probes, detected integration of *pel* in transformed *C. magna* isolates Cm-PL-3 and Cm-PL-10. Western blot analysis using antibodies against *C. gloeosporioides* PL detected a single PL band secreted by *C. magna* at a

molecular mass of 41.5 kDa whereas the PL of *C. gloeosporioides* had a molecular mass of 39 kDa. When PL activity was measured 4 days after inoculation in the pectolytic enzyme-inducing media (PEIM), transformed isolates Cm-PL-3 and Cm-PL-10 showed additive PL activity of both *C. gloeosporioides* and *C. magna*. Transformed isolates also showed additive maceration capabilities on avocado pericarp compared to *C. magna* alone, but did not reach the maceration ability of *C. gloeosporioides*. However, more severe maceration and damping off developed in watermelon seedlings inoculated with the transformed isolates versus either of the two wildtype isolates, which showed no symptom development on watermelon seedlings during the same period. Results clearly show the contribution of a single *pel* to the pathogenic abilities of *C. magna* and suggest that PL is a pathogenicity factor required for the penetration and colonization of *Colletotrichum* spp.

Additional keywords: Pathogenicity factor, *pel*, pectolytic enzymes, postharvest decay.

INTRODUCTION

Colletotrichum gloeosporioides, a pathogen of avocado fruits, has been found to produce endopolygalacturonase (endo-PG) (Prusky et al. 1989), pectin lyase (PNLA) (Bowen et al. 1995), pectin methyl esterase (PME) (Ortega 1996) and pectate lyase (PL) (Wattad et al. 1997) during the colonization of infected tissue. Evidence of the importance of PL secretion during *Colletotrichum* colonization on avocado fruits has been found in a number of studies: 1) reduced pathogenicity of a *C. magna* mutant (Path-1) with limited secretion of PL (Wattad et al. 1995); 2) inhibition of decay following co-inoculation of *C. gloeosporioides* spores with PL antibodies; 3) the inhibitory activity of pectolytic enzymes by the host flavonoid epicatechin which correlated with the inhibition of symptom development (Wattad et al. 1994) and 4) a lack of decay development under conditions which do not permit PL secretion (Yakoby and Prusky, unpublished). These results suggest that PL is

a limiting factor during the early stages of pathogenesis (Wattad et al. 1997; Yakoby and Prusky, unpublished).

Cell wall-degrading enzymes (CWDEs) are considered to play a role in the pathogenesis of bacteria and fungi on their hosts (Collmer and Keen 1986; Ried and Collmer 1988; Annis and Goodwin 1997). A significant amount of work has been published on the role of pectolytic enzymes, but only a few reports have shown their clear involvement in fungal pathogenicity (Shieh et al. 1997; ten Have et al. 1998). Of the several approaches which have been taken to demonstrate their involvement in host pathogenicity: 1) gene disruption, 2) gene complementation of a deficient mutant, 3) heterologous gene expression and 4) antisense gene expression, the first has been considered the most direct tool (Bowyer et al. 1995). However, when targeting disrupted mutants in complex systems such as that of CWDEs, where several enzymes are secreted during fungal attack, the disruption of a single enzyme may be complemented by the activity of the other secreted CWDEs (Hamer and Holden 1997). Indeed, most publications on disrupted CWDEs show no reduction in pathogenicity, for example: disruption of PNLA from *C. gloeosporioides* and pectate lyase (PL) from *Nectria haematococca* did not reduce their virulence (Bowen et al. 1995; Gou et al. 1995). Mutations in all known *pel* genes of *Erwinia chrysanthemi* reduced its virulence but the bacteria was still able to macerate the tissue (Ried and Collmer 1988), suggesting that a second set of PLs was activated (Kelemu and Collmer 1993). Gene disruption of the two endo- β -1,4-xylanases from *Magnaporthe grisea* did not have any effect on pathogenicity and revealed the secretion of one more xylanase enzyme (Sheng-Cheng et al. 1997). These reports suggest that the secretion of other CWDEs, that shares a similar or over-lapping enzymatic activity can easily overcome the loss of one enzyme. On the other hand, disruption of *pecA*, encoding the endo-PG P2c in the saprophytic fungus *Aspergillus flavus*, resulted in decreased cotton ball invasion (Shieh et al. 1998) and disruption of *Bcpg1*, one of the genes encoding PG, reduced *Botrytis cinerea* colonization by 22 to 50% (ten Have et al. 1997).

In the present work, we expressed *pel* from *C. gloeosporioides* in *C. magna*, a minor pathogen of cucurbits, and transformed the weak pathogen into an

aggressive pathogenic strain. The heterologous expression of *pel* in *C. magna* increased decay on avocado and maceration on watermelon seedlings, suggesting the involvement of the expressed PL in host penetration and colonization.

RESULTS

Cloning of *pel* from *C. gloeosporioides*.

A 4.1-kb clone encoding *pel* was isolated by screening a specific genomic library created in λ -ZAP (Strategene, La Jolla, CA, USA) at the *EcoRI* restriction site. A partial sequence (1.52 kb) of the clone is published in GenBank (accession number AF052632). Comparison of the genomic clone to the *pel* cDNA clone showed no introns in the latter. The sequence surrounding the ATG start codon, 5' CAAGATGAA 3', closely resemble the Kozak sequence, 5' CAMMATGNC 3', identified in the genes of filamentous fungi and higher eukaryotes genes (Ballance 1986; Kozak 1986). The putative amino acid leader sequence contains 39 amino acids and exhibited 63% identity and 70% positivity to the leader sequence of *pel* 2 from *Glomerella cingulata* f. sp. *malvae* (accession number AF156985) (Fig. 1). The α -amino end of the mature secreted PL was found by comparison to the cDNA gene (Wattad et al. 1997) and is indicated by an asterisk above the first amino acid (Fig. 1).

Vector construction and *C. magna* transformation.

All vectors were pGEM-7Z-based. The 2.13-kb *hph*-B gene (hygromycin phosphotransferase) was subcloned from pHA-1.3 (Redman and Rodriguez 1994) into pGEM-7Z at sites *XbaI* and *HindIII*, generating pGH-1 (Fig. 2A). The 4.1-kb *pel* clone (Fig. 2B), restricted with *EcoRI*, was blunt-ended and subcloned into the plasmid pGH-1 at the blunt-ended *XbaI* site, generating the 9.23-kb pPCPH-1 (Fig. 2C). Protoplasts of *C. magna* hyphae were transformed with 1 μ g of pPCPH-1 and selected as previously described (Redman and Rodriguez, 1994). Two transformed isolates, Cm-PL-3 and Cm-PL-10, from 20 harboring colonies, were randomly selected for further

analysis. One isolate, GH-2, transformed with pGH-1, was selected and further analyzed for the effect of hygromycin on virulence. The isolate was found to be hygromycin-resistant, contained the hph-B cassette, and possessed the same virulence abilities as wildtype *C. magna* (data not shown).

Isolate analysis: Southern and western blotting, PL activity.

Southern blot analysis of the transformed *C. magna* isolates and two wild types, *C. gloeosporioides* and *C. magna*, showed integration of the plasmid at different sites in the genome, when probed with the 4.1-kb *pel* clone (Fig. 3A) or with the 2.13-kb hph-B cassette (Fig. 3B).

Western blot analysis of the proteins secreted from Cm-PL-3 and Cm-PL-10 into PEIM revealed the presence of two bands of 39 kDa and 41.5 kDa, corresponding to the PL bands in *C. gloeosporioides* and *C. magna*, respectively (Fig. 4). When all isolates were grown for 4 days in liquid M₃S media containing sucrose, neither PL was secreted, due to catabolic repression by the added sugar (data not presented).

PL activity of the proteins secreted into PEIM, 4 days after isolate growth, was 3.1×10^{-3} units/min for *C. gloeosporioides*, and 4.1×10^{-3} units/min for *C. magna*. Increased activity reaching 168 % and 231 % of *C. magna* was observed in Cm-PL-3 and Cm-PL-10, respectively (Fig. 5). Activity pace is the slope calculated from the regression line formula ($r^2 = 0.999$, for all 4 isolates). All PL activities were significantly different ($p < 0.01$) between the different isolates.

Virulence of the transformed isolates on avocado fruits and watermelon seedlings.

Germinated conidia of wildtype *C. magna* isolate L2.5 produced appressoria that breached the fruit cuticle but caused only a 2-mm diameter lesion after 5 days (Fig. 6A). Decay caused by the wild type *C. gloeosporioides* isolate Cg-14 was four fold larger in diameter than that caused by wildtype *C. magna* (Fig. 6A). Transformed isolates Cm-PL-3 and Cm-PL-10 showed a 1.8-fold increase in maceration compared to the wildtype *C. magna* isolate (Fig. 6B).

When detached watermelon seedling stems were inoculated, both transformed isolates caused significantly larger lesion development 24 and 48

h post inoculation (Fig. 7 A and B). PL secretion during the colonization of watermelon seedling stems by Cm-PL-3 and Cm-PL-10 was detected 48 h post-inoculation, for Cg-14 and L2.5, PL secretion was detected only after 120 h (Fig. 7C). Inoculation of watermelon with the two wildtype isolates had caused no symptoms six days post-inoculation, whereas Cm-PL-10 and Cm-PL-3 caused damping off of the seedlings and leaf necrosis, respectively (Fig 8).

DISCUSSION

In a complex system like that of CWDEs, where several enzymes are secreted during fungal attack, gene disruption of one enzyme may be compensated by the activity of the other secreted CWDE genes (Hamer and Holden 1997). Most reports on the disruption of a single or combinations of CWDEs failed to prove their role in fungal attack (i.e. Ried and Collmer 1988; Sheng-Cheng et al. 1997; Scott-Craig et al. 1998). Based on these findings, the approach of working on one enzyme in a complex system, versus the disruption of more upstream regulatory genes, has been questioned (Walton 1999).

In the present work, we chose *C. magna*, a weak pathogen of avocado and watermelon that colonize both hosts (Prusky et al. 1994), to evaluate the contribution of pectate lyase to the pathogenic abilities of the fungus. We used the full *pel* clone of *C. gloeosporioides*, with its native control system, to evaluate the contribution of the heterologous expressed gene to the virulence on both hosts.

Heterologous expression was used to test the role of PG (*pg1*) from *Fusarium oxysporum* f. sp. *lycopersici* in a *pg1*-deficient isolate of *F. oxysporum* f. sp. *melonis*, where the PG-expressed isolate showed no change in virulence (Di Pietro and Roncero 1998). It was also used for the heterologous complementation of the melanin biosynthetic pathway from *Alternaria alternata* in a deficient *M. grisea* mutant that restored the latter's pathogenicity (Kawamura et al. 1997).

The expressed *pel* clone was isolated from a genomic library of *C. gloeosporioides* and found to have no introns upon comparison to the cDNA clone. The deduced amino acid sequence was compatible with the size of the

protein (Wattad et al. 1997). The putative leader sequence exhibited high homology to the leader sequence of PL from *G. cingulata* f. sp. *malvae* but contained an additional five amino acids, suggesting different mechanisms of enzyme secretion between the two fungi.

The heterologous expression of *pel* from *C. gloeosporioides* in *C. magna* increased the virulence of the transformed isolates on avocado fruits and watermelon seedlings. To our knowledge, this is the first report of molecular genetic evidence for the involvement of PL, and the third involving specific pectinases, in fungal pathogenesis (Shie et al. 1997; ten Have et al. 1998).

PL activity was additive for the transformed isolate Cm-PL-3 and significantly more than additive for Cm-PL-10, relative to the two-wild type isolates *C. gloeosporioides* and *C. magna*. Although both isolates were transformed with the same gene, pectate lyase activity was significantly greater for transformed isolate Cm-PL-10 than isolate Cm-PL-3. The differences in activities can be explained by the different patterns of gene integration into the fungal genome, observed by Southern blot analysis, which may have affected the expression of the integrated *pel*, as has been described for higher plants and fungi (MacKenzie et al. 1993).

Pathogenicity bioassay can have a marked effect on data interpretation. Transformed isolates Cm-PL-3 and Cm-PL-10 caused lesion development and dumping off, respectively on a whole seedling, unlike the two wild type isolates. When inoculation was carried out by pricking detached watermelon stems, Cm-PL-10 generated significantly faster lesion development compared to Cm-PL-3 and the two wild type isolates *C. gloeosporioides* and *C. magna* were significantly less virulent. These results are in agreement with Cm-PL-10 increased PL activity found *in vitro*.

The transformed isolates, Cm-PL-3 and Cm-PL-10, also showed enhanced virulence on avocado pericarp, relative to wildtype *C. magna* but were significantly less aggressive in comparison to *C. gloeosporioides*. Inoculation of avocado mesocarp with the different isolates showed no difference in maceration development (data not presented). The fact that the transformed isolates were more aggressive on avocado pericarp, but these differences were eliminated when inoculation was carried out in the avocado

mesocarp, further supports the importance of PL for the initial stages of fungal host-colonization.

Our system further elucidates the importance of PL as pathogenicity factor, not only for fungal penetration and colonization of watermelon seedlings, but also for the initial stages of avocado fruits colonization. The addition of PL to wild type *C. magna* caused increased virulence, but virulence expression differed between hosts and on the same host depending on the assay. This supports the heterologous expression as a tool for determine the importance of specific genes in pathogenicity, but at the same demonstrates the complexity of the system that requires more than one factor to express the pronounced contribution of a single enzyme to fungal pathogenicity.

MATERIALS AND METHODS

Fungal isolates, media and growth conditions.

Isolate Cg-14 of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (teleomorph: *Glomerella cingulata*), pathogen of avocado fruits (*Persea americana* Mill var. *drymifolia* [Schidl. And Cham.] S. F. Blake 'Fuerte') and wildtype isolate L2.5 of *Colletotrichum magna* S. F. Jenkins & Winstead (teleomorph: *Glomerella magna*) were maintained in 10 mM Na-phosphate buffer (pH 7.2), 40% glycerol at -80°C. Three-week old conidia were harvested from M₃S plates (Tu 1985) and used for culture and/or fruit inoculation.

For detection of pectolytic enzyme secretion, 1x10⁶ conidia/ml of each of the *Colletotrichum* strains were inoculated into a 250 ml Erlenmeyer flask containing 100 ml of pectolytic enzyme-inducing media (PEIM), at pH 3.8, including Na-polypectate and pectin as the sole carbon source (Prusky et al. 1989).

Bacterial strains were grown on LB or NZY media according to the protocol of the ZAP Express cDNA Synthesis Kit (Stratagene, La Jolla, CA).

Isolation of genomic *pel* from *C. gloeosporioides* and DNA analysis.

Lyophilized *C. gloeosporioides* hyphae harvested from M₃S media were subjected to DNA extraction (Rodriguez 1993). Fungal DNA (10 µg) was

restricted with *EcoRI*. Southern blotting analysis was performed by running half of the restriction mixture on a 0.8% agarose gel followed by blotting onto a BioTrace NT membrane (Pall Gelman Sciences, Ann Arbor, MI). The membrane was screened with a 1.1-kb *pel* cDNA clone (GenBank, accession number U32942), labeled with P^{32} using Prime-a-Gene labeling system (Promega, Madison, WI).

One band at 4.1 kb showed a single copy of the gene (Fig. 3). A genomic library was constructed by running the second half of the restriction mixture under the same conditions. DNA in the range of 3.5 to 4.5 kb was extracted from the gel using Gene Elute Spin Columns (Supelco, Bellefonte). Extracted DNA fragments (50 ng) were subjected to ligation reaction in 1 μ g λ gt-11 arms restricted with *EcoRI* (Boehringer Mannheim, Mannheim, Germany), using ligase (Promega). Ligation products were packed with Gigapack II Gold (Stratagene). Bacterial strain Y1090 (Promega) was grown to an O.D₆₀₀ of 0.5 on LB supplemented with ampicillin. Bacteria (600 μ l) was mixed with 20 μ l of packed products dilution, 5×10^{-4} pfu, and spooled into 3 ml of soft NZY followed by spooling on NZY plates. Plates were incubated at 37°C overnight, and plaque-lift hybridization was performed as described by Sambrook et al. (1989), using nitrocellulose membrane (Pall Gelman Sciences). Plaques were screened three times as described for the Southern blotting analysis. Eleven plaques were extracted using Wizard Lambda Prep (Promega). DNA was restricted with *EcoRI* and blotted onto a NT membrane as described for the Southern blot. One band at 4.1 kb was detected following the hybridization with the 1.1-kb *pel* clone. The 4.1-kb band was extracted from the gel and subcloned into pGEM-7Z (Promega) creating plasmid pPEL1.1.

Sequencing of the genomic *pel*.

pPEL1.1 was restricted with *SphI* creating 3.6 kb (containing 3.0 kb pGEM vector and 0.6 kb of *pel* clone) and 3.5-kb fragments. pGEM-7Z (Promega) restricted with *HindIII*, was blunt-ended using Klenow fragment (Promega) and dephosphorylated using CIAP (Promega). The 3.5-kb fragment was blunt-ended using T4 DNA polymerase (Promega). The vector was subjected to ligation with the 3.5-kb fragment, creating p1.2 $\uparrow$ P (data not shown).

The vector contained two *Xba*I restriction sites and one site was point-mutated by restriction with *Xba*I followed by Klenow fragment reaction and ligation, creating p1.2 $\uparrow$ PX(-), containing *Sph*I next to the *Xba*I restriction sites. The plasmid was subjected to deletion reaction with the Erase-Base System (Promega). Deleted plasmids DNA were sequenced at the Biological Services of Weizmann Institute (Rehovot, Israel). The results were analyzed with the Wisconsin package GCG and compared to sequences in GenBank.

Vector construction and transformation.

The 2.13-kb *hph*-B gene (hygromycin phosphotransferase) was subcloned from pHA-1.3 (Redman and Rodriguez 1994) into *Hind*III and *Xba*I restriction sites of pGEM-7Z (Promega) generating the 5.13-kb pGH-1.

pPEL1.1 was restricted with *Eco*RI, and the 4.1-kb *pel* was extracted and subjected to Klenow fragment reaction (Promega). pGH-1 was restricted with *Hind*III and also subjected to Klenow fragment reaction. The blunt-ended plasmid was dephosphorylated with CIAP (Promega). The reaction mixture was run on an agarose gel and the dephosphorylated pGH-1 was extracted using Gene Elute Spin Columns (Supelco), and ligated to the 4.1-kb *pel*, creating a 9.23-kb plasmid, pPCPH-1.

When hyphae were needed for protoplast preparation, 1×10^6 spores were first shaken for 4 days at 150 rpm, 22°C, in 250 ml of M₃S media and then subjected to transformation procedure (see Transformation).

C. magna protoplasts obtained as described by Redman and Rodriguez (1994) were transformed with 1 μ g pPCPH-1 and with 1 μ g of pGH-1, as a control for hygromycin resistance. The transformation solution was incubated for 48 h at 22°C followed by plating on HM media (Redman and Rodriguez 1994) supplemented with 100 μ g/ml hygromycin (HM+Hyg).

Transformed colonies were collected 4 to 8 days after the transformation. The colonies were transferred to new HM+Hyg plates. In order to get fungal spores, and to check transformation stability, the fungi were transferred to M₃S media plates and a single spore was then plated on HM+Hyg. Spores from the single spore isolation were collected and kept at -80°C as described earlier.

Southern analysis, western analysis and PL activity.

Fungal DNA was extracted as described by Rodriguez (1993) from 1 g of dry mycelium yielding 50 µg DNA.

Fungal DNA (5 µg) was restricted for 15 h with 10 U of *EcoRI* or *HindIII* restriction enzyme (Promega). The restricted DNA was run on a 0.8% agarose gel for 4 h at 30 V. The DNA was blotted onto a HyBond+ nylon membrane (Amersham, Buckinghamshire, UK), by capillary method, with 20 X SSC. All hybridizations were carried out at 65°C and washes were with 0.1 X SSC.

Protein extraction for western blot analysis and PL activity was performed by inoculating 1×10^6 of fungal conidia into PEIM and growing for 4 days. The fungus was harvested by centrifugation at 12,000 X g for 10 min. The clear supernatant was concentrated by rotavapor (Buchii, Flawil, Switzerland) at 42°C to 15 ml. The concentrated media was subjected to overnight dialysis against 5 L 50 mM Tris-HCl, pH 8.5 and then reconcentrated to 5 ml as described. Protein concentration was determined by BioRad protein assay using standard dilution of BSA (Sigma, St. Louis, MO).

Each sample (10 µg) was run on a 12.5% SDS-polyacrylamid gel (Mini-Protean II, BioRad), for 1 h at 150 V constant voltage, and western blot analysis was performed with PL antibodies as described by Wattad et al. (1995).

PL activity was measured as described by Collmer et al. (1988). All activities were conducted with 5 µg protein, which was found to be on the linear portion of the curve for 5 min in a spectrophotometer (Uvikon 820, Kontron, Basel, Switzerland) at 232 nm. Activity was expressed as the accumulated absorption change of 1 µmol of the 4,5-unsaturated galacturonide product of Na-polygalacturonate per minute, at 25°C.

Five inoculated detached watermelon seedlings were ground under liquid nitrogen using a pestle and mortar and then vortexed for 10 seconds with 5 ml of 50 mM tris HCl (pH 8.5). Plant debris were separated from the supernatant by centrifugation at 12,000 X g for 10 min. The supernatant was treated as described above. Protein samples (15 µg of each) were subjected to western blotting analysis.

Pathogenicity assay on avocado fruits and watermelon seedlings.

Twenty freshly harvested avocado fruits (cv. Fuerte) were inoculated with 10 μ l conidia (1×10^6 conidia/ml) from each of the three different isolates per fruit (*C. gloeosporioides*, *C. magna* and the tested transformed isolates, Cm-PL-3 or Cm-PL-10) with two repetitions for each isolate. The data shown is the mean of 20 fruits per experiment with standard error. Decay development was monitored during fruit storage at 20°C, by measuring lesion diameter. The results show one representative experiment, out of three independent ones.

Ten-day old watermelon seedlings (cv. Odem), susceptible to *C. magna*, were spot inoculated on the apex with 20 μ l of conidia suspension at concentration of 4×10^6 conidia/ml of *C. gloeosporioides*, *C. magna*, Cm-PL-3 and Cm-PL-10. Plants were maintained in a greenhouse at 25°C in 90% humidity for 48 h. Water inoculation was used as a control. Disease symptoms were observed 6 to 7 days after inoculation, resulting in necrosis or plant mortality. Pathogenicity to watermelons was also tested on 10-day old detached watermelon (cv. Odem) seedling stems which were surface-sterilized by immersion for 30 s in 3% sodium hypochlorite. Thereafter, the stems were wounded by pin pricking and inoculated with 5 μ l per stem of 4×10^6 conidia/ml of each of the isolates. The stems were placed on sterile filter paper in Petri dishes and maintained at 25°C. Tissue colonization was measured 24 and 48 h after inoculation. Eight stems were inoculated per isolate and water as a control. All experiments were conducted three times.

LITERATURE CITED

- Annis, L. S., and Goodwin, H. P. 1997. Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *Eur. J. Plant Pathol.* 103:1-14.
- Ballance, D. J. 1986. Sequences important in gene expression in filamentous fungi. *Yeast* 2:224-236.
- Bowen, K. J., Templeton, D. M., Sharrock, R. K., Crowhurst, N. R., and Rikkerink, A. H. E. 1995. Gene inactivation in the plant pathogen *Glomerella cingulata*: three strategies for the disruption of the pectin lyase gene *pnIA*. *Mol. Gen. Genet.* 246:196-205.

- Bowyer, P., Clarke, B. R., Lunness, P., Daniels, M. J., and Osbourne, A. E. 1995. Host range of a plant pathogenic fungus determined by a saponin detoxification enzyme. *Science* 267:371-374.
- Collmer, A., and Keen, T. N. 1986. The role of pectic enzymes in plant pathogenesis. *Ann. Rev. Phytopathol.* 24:383-409.
- Collmer, A., Ried, L. J., and Mount, S. M. 1988. Assay methods for pectic enzymes. *Meth. Enzymol.* 161:329-335.
- Di Pietro, A., and Roncero, G. I. 1998. Cloning, expression, and role in pathogenicity of *pg1* encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. *Mol. Plant-Microbe Interact.* 11:91-98.
- Gou, W., Gonzalez-Candelas, L., and Kolattukudy, E. P. 1995. Cloning of a novel pectate lyase gene *pelB* from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of the gene product expressed in *Pichia pastoris*. *J. Bacteriol.* 177:7070-7077.
- Hamer, J. E., and Holden, D. W. 1997. Linking approaches in the study of fungal pathogenesis: a commentary. *Fungal Genet. Biol.* 21:11-16.
- Kawamura, C., Moriwaki, J., Kimura, N., Fujita, Y., Fuji, S., Hirano, T., Koizumi, S., and Tsuge, T. 1997. The melanin biosynthesis genes of *Alternaria alternata* can restore pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 10:446-453.
- Kelemu, S., and Collmer, A. 1993. *Erwinia chrysanthemi* EC16 produces a second set of plant inducible pectate lyase isozymes. *Appl. Environ. Microbiol.* 59:1756-1761.
- Kozak, M. 1986. Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. *Nature* 308:241-247.
- MacKenzie, D. A., Jeenes, J. D., Belshaw, N. J., and Archer, D. B. 1993. Regulation of secreted protein production by filamentous fungi: recent developments and perspectives. *J. Gen. Microbiol.* 139:2295-2307.
- Ortega, J. 1996. Pectolytic enzymes produced by the phytopathogenic fungus *Colletotrichum gloeosporioides*. *Tex. J. Sci.* 48:123-128.
- Prusky, D., Freeman, S., Rodriguez, R. J., and Keen, N. T. 1994. A nonpathogenic mutant strain of *Colletotrichum magna* induces resistance to

- C. gloeosporioides* in avocado fruits. *Mol. Plant-Microbe Interact.* 7:326-333.
- Prusky, D., Gold, S., and Keen, N. T. 1989. Purification and characterization of an endopolygalacturonase produced by *Colletotrichum gloeosporioides*. *Physiol. Mol. Plant Pathol.* 35:121-133.
- Redman, S. R., and Rodriguez, J. R. 1994. Factors affecting the efficient transformation of *Colletotrichum* species. *Exper. Mycol.* 18:230-246.
- Ried, L. J., and Collmer, A. 1988. Construction and characterization of an *Erwinia chrysanthemi* mutant with directed deletions in all of the pectate lyase structural genes. *Mol. Plant-Microbe Interact.* 1:32-38.
- Rodriguez, J. R. 1993. Polyphosphate present in DNA preparations from filamentous fungal species of *Colletotrichum* inhibits restriction endonucleases and other enzymes. *Anal. Biochem.* 209:291-297.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Scott-Craig, J. S., Cheng, Y., Cervone, F., De Lorenzo, G., Pitkin, J. W., and Walton, J. D. 1998. Targeted mutants of *Cochliobolus carbonum* lacking the two major extracellular polygalacturonases. *Appl. Environ. Microbiol.* 64:1497-1503.
- Sheng-Cheng, W., Kyung-Sik, H., Darvill, G. A., and Albersheim, P. 1997. Deletion of the two endo- β -1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. *Mol. Plant-Microbe Interact.* 10:700-708.
- Shieh, M., Brown, R. L., Whitehead, M. P., Carey, J. W., Cotty, P. J., Cleveland, T. E., and Dean, R. A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion of *Aspergillus flavus* in cotton balls. *Appl. Environ. Microbiol.* 63:3548-3552.
- ten Have, A., Mulder, W., Visser, J., and van Kan, L. A. J. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact.* 11:1009-1016.
- Tu, J. C. 1985. An improved mathur's medium for growth, sporulation and germination of spores of *Colletotrichum lindemuthianum*. *Microbiosis* 44:87-93.

- Walton, J. D. 1999. Fungal virulence factors: some things are and some things aren't. sp35. p. 20. *In*: Abstracts of the 9th International Congress on Molecular Plant-Microbe Interactions.
- Wattad, C., Dinoor, A., and Prusky, D. 1994. Purification of pectate lyase produced by *Colletotrichum gloeosporioides* and its inhibition by epicatechin: A possible factor involved in the resistance of unripe avocado fruits to anthracnose. *Mol. Plant-Microbe Interact.* 7:293-297.
- Wattad, C., Freeman, S., Dinoor, A., and Prusky, D. 1995. A nonpathogenic mutant of *Colletotrichum magna* is deficient in extracellular secretion of pectate lyase. *Mol. Plant-Microbe Interact.* 8:621-626.
- Wattad, C., Kobilier, D., Dinoor, A., and Prusky, D. 1997. Pectate lyase of *Colletotrichum gloeosporioides* attacking avocado fruits: cDNA cloning and involvement in pathogenicity. *Physiol. Mol. Path.* 50:197-212.

Figures:

Fig 1. Alignment of the deduced amino acid sequences of the *pel* clone leader sequences from *C. gloeosporioides* (Cg-14) and *G. cingulata* f. sp. *malvae* (Gc-malvae). The identical sequence lies along the centerline, + indicates positive amino acids. * is above the start of the N-terminal of the secreted PL. Alignment was done by NCBI FASTA program.

Fig 2. The transformation vector was constructed by subcloning the 2.13-kb *hph*-B gene (hygromycin phosphotransferase) from pHA-1.3 (Redman and Rodriguez 1994) into *Hind*III and *Xba*I restriction sites of pGEM-7Z (Promega) generating the 5.13-kb pGH-1 (A). The 4.1-kb clone of *pel* (see arrow in Fig. 3A) was subcloned from λ -ZAP genomic library into pGEM-7Z, at the *Eco*RI restriction site, generating plasmid pPEL1.1 (B). pPEL1.1 was restricted with *Eco*RI, and the 4.1-kb *pel* was extracted and subjected to Klenow fragment reaction. The plasmid pGH-1 was restricted with *Hind*III and also subjected to Klenow fragment reaction. The blunt-ended plasmid was dephosphorylated with CIAP. The reaction mixture was run on an agarose gel and the dephosphorylated pGH-1 was extracted from the gel and subjected to ligation with the 4.1-kb *pel*, creating a 9.23-kb plasmid pPCPH-1 (C).

Fig 3. Southern blot analysis of the transformed isolates, Cm-PL-3 and Cm-PL-10, compared to wildtype isolate Cg-14 of *C. gloeosporioides* and wildtype isolate L2.5 of *C. magna*. Fungal DNA (5 μ g) was restricted with *Eco*RI or *Hind*III. Restricted DNA was run on a 0.8% agarose gel, blotted and probed with 4.1-kb *pel* (A) or 2.13-kb *hph*-B (B).

Fig 4. PL secretion from the wildtype isolate Cg-14 of *C. gloeosporioides*, wildtype isolate L2.5 of *C. magna*, and the transformed isolates, Cm-PL-3 and Cm-PL-10, grown and harvested 4 days after inoculation to PEIM with 1×10^6 conidia. Clear supernatant (100 ml) was concentrated by rotavapor at 42°C to 15 ml, dialyzed, and further concentrated to 5 ml. 10 μ g of protein was per

lane onto a 12.5% SDS-polyacrylamide gel. A western blot was probed with PL antibodies.

Fig 5. PL activity was measured for Cm-PL-3 (Δ) and Cm-PL-10 (X) relative to wildtype *C. gloeosporioides* isolate Cg-14 ($\diamond$) and wild type *C. magna* isolate L2.5 ($\square$). 4-day old PEIM culture was harvested and treated as described in Fig. 4. The concentrated supernatant (5 μ g) was subjected to PL activity assay (units/ml). Units were calculated as a function of absorbance and the extinction coefficient 4600 M⁻¹cm⁻¹. The results describe the accumulated absorption of the 4,5-unsaturated product of Na-polygalacturonate. The experiment was conducted 3 times and the results describe the average of three repetitions of one representative experiment. All isolates' activities are significantly different from each other ($p < 0.01$).

Fig 6. Decay development caused by wildtype *C. gloeosporioides* isolate Cg-14 ($\diamond$), wildtype *C. magna* isolate L2.5 ($\square$) (A). (B) Isolate L2.5 ($\square$) and transformed isolates Cm-PL-3 (Δ) and Cm-PL-10 (X), on avocado fruits. Freshly harvested avocado fruits were inoculated with 10 μ l conidia (1×10^6 conidia/ml) of three different isolates per fruit (*C. gloeosporioides*, *C. magna* and the tested transformed isolate), with two repeats of each isolate. Inoculation was performed on 20 fruits. The data shown is the mean of these 20 fruits for each experiment and the standard error. Decay diameter was monitored during fruit storage at 20°C. The results show one representative experiment out of three repetitions.

Fig 7. Lesion development on detached watermelon seedling stems (cv. Odem) formed by wildtype *C. gloeosporioides* isolate Cg-14, wildtype *C. magna* isolate L2.5, Cm-PL-3 and Cm-PL-10 and water. Detached 10-day old watermelon (cv. Odem) seedling stems were wounded by pin-prick and inoculated with 5 μ l per stem of each of the different isolates at 4×10^6 cinidia/ml. The extent of tissue colonization was measured 24 and 48 h after inoculation (A). (B) Detached stems 48 h post-inoculation. (C) PL detection from inoculated stem tissue 48 h and 120 h post-inoculation. Eight stems were

inoculated with each isolate and water as a control. Stems were maintained under fluorescent light at 20°C in 90% humidity for 48 h. All experiments were conducted three times. Letters above the bars in (A) represent significant differences between the isolates ($p < 0.01$).

Fig 8. Symptoms of necrotic development and damping off on watermelon seedlings (cv. Odem) caused by wildtype *C. gloeosporioides* isolate Cg-14, wildtype *C. magna* isolate L2.5, Cm-PL-3 and Cm-PL-10. Ten-day old watermelon seedlings, susceptible to *C. magna*, were spot-inoculated on the apex with 20 µl of conidial suspension at a concentration of 4×10^6 conidia/ml of the different isolates. Plants were maintained in a greenhouse at 25°C in 90% humidity for 48 h. Water inoculation was used as a control. Pathogenicity was observed 7 days after inoculation as developing necrosis and damping off.

Fig. 1 N. Yakoby:

Cg-14	1	MKFTGIAS	TLALLAS	VASATPT	PTVRDGA	AVDPRT	VGKRAA	41
		MKFTG+AS	ALLA	ASA+PT	PTV DG	R +	KRAA	
Gc-malvae	1	MKFTGLASW	-ALLAIA	ASASPT	PTVEDG	-----	RVLSKRAA	35

*

N. Yakoby

Fig. 2

Fig 4

Fig 3B

Fig 3A

Fig 8

N. Yakoby

Fig. 5

N. Yakoby

Fig. 6A

Fig. 6B

Fig. 7A

Fig 7C

Fig 7B

6. Secretion regulation of pectolytic enzymes as a basis for pathogenicity

Host pH, a novel mechanism for the regulation of *Colletotrichum gloeosporioides* quiescent infection

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ABSTRACT

When *C. gloeosporioides* was grown in inducing media, *pel* transcript was initially observed at pH 5.0, with an increase up to 5.7 and then declined. Whereas PL secretion was detected starting from pH 5.8 and increased up to 6.5. PL secretion was not detected as long as the pH of the inducing media was kept lower than 5.8. *Colletotrichum* hyphae transferred from PL secreting conditions at pH 6.5 to 3.8 did not secrete PL. However immediate secretion was detected when hyphae was transferred to pH 6.0. This behavior differed from PG where transcription and protein secretion in inducing media, occurred starting from pH 5.0 and up to 5.8. When pH was tested in the pericarp of unripe, freshly harvested cv. Fuerte fruits, resistant to *C. gloeosporioides* attack, a pH value of 5.5 was detected. However in ripened fruits, pH value of the pericarp increased up to 6.3 and decay symptoms were observed. Two cultivars Ardit and Ettinger that are resistant to *C. gloeosporioides* also after ripening, showed pH values in the pericarp lower than 5.5. Present results suggest that pH regulates the secretion of pectate lyase, and support its importance as a pathogenicity factor during the attack of avocado fruit by *C. gloeosporioides*.

INTRODUCTION

Inhibition of *C. gloeosporioides* (anamorph *Glomerella cingulata* (Stonem). Spauld, et Schrenk) colonization and fungal quiescence occurs in avocado fruits following appressoria germination. The pathogen attacks early developing fruits and remains quiescent during fruit growth. Only after harvest and fruit ripening the pathogen renews its development causing fast developing brown-black spots on the pericarp and soft rot in the mesocarp symptoms of anthracnose (Prusky, 1996). Early reports by Verhoeff (1974), Swinburne (1993) and Prusky (1996) suggested that significant physiological changes must occur in the host and in the pathogen during fruit ripening to activate quiescent infections (Jeffries et al, 1990. Simmonds, 1941). Prusky (1996) suggested several hypothesis to explain the resistance mechanism of unripe fruits: (1) the unripe host affects the nutritional requirements of the pathogen; (2) the presence of preformed antifungal compounds in unripe fruit that declines during ripening; (3) the presence of inducible antifungal compounds

in unripe fruit that decline during ripening; and (4) activation of fungal pathogenicity factors. Considerable work has shown the involvement of the antifungal compounds in resistance of avocado fruits to postharvest pathogens. Few reports, however have described capability of secretion of enzymes as a factor for fungal attack. *Colletotrichum* produces an array of pectolytic enzyme, polygalacturonase (PG) (Prusky et al., 1989), pectin lyase (PNLA) (Bowen, et al, 1995), and pectinesterase (PME) (Ortega, 1996). The importance of PL secretion during *Colletotrichum* attack in avocado fruits was suggested by the inhibition of decay development in the presence of anti-PL and the reduced secretion of PL by a non pathogenic mutant of *C. magna* (Wattad et al., 1994,1995). This suggests that PL is a limiting factor for pathogenesis (Wattad et al., 1995).

Specific gene disruption can provides usually, the best tool to demonstrate the involvement of pectolytic enzyme in pathogenesis (Bowyer et al., 1995). However, in some cases gene disruption experiments suggested that several genes that were considered to be important for pathogenicity did not affect symptom development. Disruption of PNLA from *C. gloeosporioides* and PL from *Nectria haematococca* did not reduce there virulence (Bowen et al., 1995, Guo et al., 1995a). Mutation of all the *pel* genes of *Erwinia chrysanthemi* reduced its virulence but still was able to macerate the tissue (Ried and Collmer., 1988). They suggested that as a result of specific gene disruption, a second set of PLs might be activated.

What is the relation between gene expression of pectolytic enzymes encoding genes, protein secretion and symptom development by the pathogens.? Environmental conditions can affect protein production and secretion in different organisms (Annis and Goodween., 1997, MacKenzie et al., 1993, Condamine and Robert-Baudouy., 1995).

Dean and Timberlake (1989) were the first to describe the possible effect of pH on pectolytic enzyme secretion of *A. nidulance* in culture media but they did not show this effect in *planta*, because of the limited pathogenic ability of this saprophytic fungus. While PG of *A. nidulance* was found to be produced at low pH values, PL accumulated when media pH increased and became conducive to PL activity (Dean and Timberlake., 1989). Also in *Metarhizium anisopliae*, ambient pH affected the expression of cuticle-degrading enzymes

and hydrophobin that were suggested to have a role in the pathogenicity (St.Leger et al., 1998).

An in depth study was done in yeast systems. pH affected the secretion of extracellular proteases in the yeast *Yarrowia lipolytica* and growth of *Candida albicans* (Otero and Gaillardin., 1996). Mutation in *PHR1* and *PHR2* from *Candida albicans* created a pH-growth dependent mutant (Muhlschlegel and Fonzi., 1997). pH also regulates the biosynthesis of secondary metabolites like sterigmatocystin and aflatoxin in *Aspergillus* spp. and also penicillin in *Aspergillus nidulans* (Espeso et al., 1993., Otero and Gaillardin., 1996, Shah et al., 1991. Keller et al., 1997). In *A. nidulans*, the activation of *pa/F*, responsible for activating the product of the *pacC* gene, a transcriptional activator of genes, required for the growth under alkaline conditions (Maccheroni et al., 1997. Orejas et al., 1995).

In this report, we demonstrate the effect of pH in the regulation of PL secretion and its importance during pathogenesis of *C. gloeosporioides*. We suggest that gene expression and protein secretion of PL are not synchronized processes. Even if inducing media can activate gene expression, protein secretion seems to be affected by post-transcriptional effects directly or indirectly by pH. This environmental effect on protein secretion, however affects the conditions for fungal colonization and decay development of *C. gloeosporioides*.

MATERIAL AND METHODS

Fungus, media and growth condition

C. gloeosporioides (Penz.) Penz. & Sacc. Isolate Cg-14 was obtained from decayed avocado fruits and used in all experiments. Culture spores were stored in 10mM Na phosphate buffer (pH 7.2) and 40% glycerol at -80°C. Cultures were initiated with 0.5 to 1 X 10⁶ spores per ml.

Ten to twenty days old spores were harvested from M₃S plates (Tu et al. 1995). Five hundred ml Erlenmeyer flask containing 250 ml media with Na-polypectate and pectin as sole carbon source (Pectolytic Enzymes Inducing Media, PEIM) (Prusky et al., 1989) were inoculated with *Colletotrichum* spores at a concentration of 5x10⁵ /flask, and grown at 24°C,

on an orbital shaker at 150 rpm. The initial pH of the PEIM media was 3.8. In some cases the media was adjusted to pH 6.0 using 5 N NaOH. In other experiments hyphae mass was transferred after 4-5 days of growing in liquid M_3S , to fresh PEIM.

Fruits, inoculation conditions

Avocado fruit, *Persea americana* Miller var. *drymifolia* (Schidl. and Cham.) S. F. Blake 'Fuerte' cvs. Fuerte, Ardit and Ettinger were harvested from an orchard at Kvutzat Shiller, Israel. Inoculation was carried out on 10 freshly harvested fruits, by placing 10 μ l of spore suspension (10^6 spores/ml) at 6 points, three at each side of the longitudinal axes of the fruit. Fruits were then kept at 22°C, in 90% humidity for 10-12 days

pH measurements

pH was measured using a flat pH electrode (Sensorex, USA). Media measurement was done in 2-3 ml aliquots samples at different periods after fungal inoculation. Pericarp pH was determined by gently scarping the wax, using scalpel and placing the electrode against the exposed area. Mesocarp pH was determined following peeling the pericarp with a peeler to the depth of 1-2 mm. Electrode was placed directly against the exposed tissue. All measures were repeated in 3 fruits at 3 different places at longer transversal axis of each fruit, at any given time. Comparison between direct pH measurements and pH measurements of the homogenate of 5 g of pericarp tissue in the presence of 10 ml of DDW at different stages during fruit ripening, resulted in similar pH values.

Detection of PL and PG in liquid media and fungal hyphae

Colletotrichum hyphae was harvested and separated from the culture media by centrifugation at 10,000 rpm for 10 minutes at 4°C. The hyphae was washed twice with sterile water (250 ml) by re-suspending the precipitated. The hyphae was then frozen under liquid nitrogen, lyophilized and stored at -80°C until use. Supernatant of the culture media was concentrated by rotor-evaporator (Buchii) at 42°C to 15 ml. The concentrated culture media

was dialyzed over night (45Kd molecular weight cut, Sigma) against 5 L Tris-HCl 50mM, pH 8.5 and then concentrated to 5 ml as above and stored at -20°C .

Proteins were sampled directly from the media and/or extracted from fungal hyphae using Tri-Reagent (Sigma, by manufacturer instructions). Protein sample was quantified by BioRad protein assay using BSA as standard dilutions (Sigma). Five μg (unless written differently) from each sample were loaded in 12.5% SDS-PAGE (Mini-Protean II, Bio-Rad), for 1 h at 150 V constant voltage and Western blot analysis was performed with PL antibodies as described by Wattad et al, 1995 and PG (Prusky et al., 1997).

To determine the presence of high degree of glycosylation of PL before secretion, protein were extracted from hyphae grown at PEIM as described before at the proper pH values and then 10 μg of protein sample, was subjected to endoglycosidase F according to manufacturer's instructions (Boehringer Mannheim).

RNA extraction, Northern blot

Total RNA was extracted using Tri-Reagent (Sigma) by grounding 0.2 g lyophilized hyphae 3 times with mortar and pestle under liquid nitrogen. RNA was quantified by GeneQuant (Pharmacia Biotech, USA).

Northern blot analysis was conducted by running 10 μg of total RNA in 1.1% formaldehyde denaturing agarose gel (Shambrook et al., 1989). The RNA was blotted to Hybond+ (Amersham) nylon membrane by capillary method (Shambrook et al., 1989), using 20 X SSC. The RNA was fixed by baking for 2 hours at 80°C and subjected to hybridization using 1.1 Kbp *pel* full clone as probe, as described by Wattad et al, (1995) or 487 bp PG clone obtained by PCR cloning (unpublished). A 487 bp, putative *pg* (accession number AF116507, Gene Bank), *cgpg1*, was obtained by PCR from 3 μg gDNA of *C. gloeosporioides*, using two oligonucleotides (5' CCTCAACGGCATCAAGGTACC 3' as a forward primer and 5' CAGGCATGCGTCCTGGTTGTA 3' as a reverse primer). The primers were constructed based on the alignment of *clpg1* and *clpg2* from *C. lindemuthianum* (Centis et al, 1997). The reaction used Thermal Reaction

(Hybaid), Taq polymerase (Promega). The following cycles: 95°C 5 min 1 cycle, 95°C 1 min, 60°C 1.5 min, 72°C 1 min 10 cycles, 94°C 1 min, 60°C 1.5 min, 72°C 1 min 20 cycles and 72°C for 10 min 1 cycle. The fragment was subcloned into pGEM-T Easy (Promega) and transferred to DH5- α using Gene Pulser II (BioRad). Colonies were selected on LB media supplemented with 100 μ g/ml of ampicillin with blue/white selection. DNA was sequenced at the "Biological Services" (Weizman Institute, Rehovot, Israel). The results were analyzed in the Wisconsin package GCG version compared to the Gene Bank. Ethidium bromid stained gel was used for RNA quantification (Fig 8 and 8.1).

Diene extraction

Ten g of avocado pericarp and mesocarp (1-2 mm), were homogenized in 95% ethanol in Omni-mixer (Sorvall) at full speed for 3 min. The ethanol extract was dried in a rotary evaporator at 40°C, re-dissolved in 10-ml distilled water, and the organic phase extracted by fractionation with dichloromethane. Following two extraction processes, the organic phases were pooled, dried with MgSO₄ anhydrous (Riedel-deHaen), and evaporated to dryness. Samples were redissolved in 1 ml of ethanol AR (Bio Lab) and analyzed by HPLC as described by Prusky et al., (1991). The average values of three separate extractions are reported.

RESULTS

Media pH changes, PL secretion in PEIM by *C. gloeosporioides*

Three days after *C. gloeosporioides* spores inoculation of PEIM, the pH values was 3.8 and increased in the next 3 following days to 6.5 (Figure 1A). Western blot analysis of culture filtrate, detected PL secretion when the media pH exceeded 6.0 (Fig 1B) and an the amount further increased with the pH increase.

The relation between pH and PL secretion

PL was detected in PEIM 5 days after *C. gloeosporioides* was grown and the pH increased from 3.8 to pH 6.0. However if the hyphae was transferred to fresh PEIM at pH values 3.8 and 6.0, no PL was detected at the range values

of 3.8-5.2 while an immediate secretion occurred at pH 6.0-6.5 (Fig 3). If fungal mass grown in non-inducing conditions, and transferred to inducing conditions in PEIM and continuously titrated up to pH values not higher than 3.8, 4.9, 5.4 and 5.8, PL was only detected when the pH increased over values of 5.8 (Fig 4).

The level of PL and PG regulation

When *C. gloeosporioides* was grown in PEIM starting from 3.8 and up to 6.5 Northern blot analysis of RNA extracted from harvested hyphae of *C. gloeosporioides* at pH levels from 3.8 and up to 6.5 detected *pel* transcripts at pH 5.0 with an increased strength of the transcript up to pH 5.7. Further increase in pH reduced the transcription level (Fig 8B). PL secretion, however, was detected only when the media pH increased over 5.8 (Fig 8A).

The same protein samples were run and screened with PG antibodies that.

When the expression of polygalacturonase was tested at increasing pH levels, high transcription was detected at pH 5.0. Transcript intensity increased to peak at 5.7 followed by decrease at pH 5.8 (Fig 8.1 B) while PG secretion was detected PG secretion at all pH levels, in the same intensity (Fig 8.1 A).

Western blot analysis of proteins extracted from the fungus hyphae, screened with PL antibodies, showed PL protein in different stages of processing, at different pH levels (Fig 9A). Treatments with endoglycosidase F to 10 μ g protein extracted from the dried hyphae resulted in a migration of high MW bands to lower MW bands (Fig 9B). This may suggest that PL is in the hyphae at different stages of glycosilation.

pH levels in pericarp and mesocarp during avocado development and ripening

pH values in the pericarp from 50 days after fruit set (un-mature fruits) up to 200 days later (over mature fruits) increased from 4.9 to 5.5. (Fig 5). No symptoms of decay were observed during this period and the infections remained quiescent. When avocado cv. Fuerte fruits were harvested at the regular harvesting period and stayed to ripen an increase in pH value from 4.5 to 5.8 occurred. pH further increased during ripening with enhanced symptom

development (Fig 6). pH values in the mesocarp were 6.4 ± 0.2 during all the ripening period. Interestingly cvs. Ardit and Ettinger, that are resistant to *Colletotrichum* attack, showed a pH level in the pericarp of 5.5 ± 0.2 and these values did not increase during ripening. In both cultivars the pH in the mesocarp was 6.5 ± 0.2 (Fig 7A and B).

Antifungal diene concentration in the pericarp of freshly harvested resistant cv. Ardit and cv. Ettinger were about 350-ug.g.f wt. This amounts is significantly lower than levels present in the susceptible Fuerte fruits where the concentrations in freshly harvested fruits range on the level of 1000-1200 ug.g.fr wt. But decrease to subfugitoxic concentrations of about 300 ug.g.fr wt. when they ripen.

DISCUSSION

In this paper studied the effect of environmental pH, on pathogen-host interaction, by controlling the secretion of pectolytic enzymes.

Quiescent infection of *C. gloeosporioides* on avocado fruit was suggested to be affected by various conditions like the levels of preformed antifungal diene and epicatechin that had inhibition effect on PL and PG activity (Prusky 1996). Fungus quiescence was kept as long as the level of the Diene and epicatechin enabled the inhibition of fungal growth. Unlike the inhibition effect of epicatechin on PL and PG, we suggest a prior mechanism, at the transcription and post transcription levels that affects the production and secretion of pathogenicity factors. The role of cell wall degrading enzymes as pathogenicity factor is not well established, but it consider to play a role during fungal attack (Di Pietro and Roncero, 1998. Wattad et al, 1995. ten Have et al, 1998). The effect of ambient pH on the secretion of enzymes and secondary metabolites was vastly discussed in the *Aspergilluse* spp. for *pacC* *palF* and penicillin (Orejas et al, 1995. Espeso et al, 1993. Maccheroni et al, 1997). In *Candida albicans* pH played a role in regulating the *PHR1* and *PHR2* genes which are being strongly expressed under alkaline and acidic environmental pH respectively. Dean and Timberlake (1989) showed the effect of pH on PL and PG secretion by *A. nidulans*, where PG accumulation under low pH conditions, when environmental conditions are better for PG activity, and PL accumulate just when the media pH increase to be conducive for PL activity. Unlike Dean

and Timberlake (1989) who worked with a non-pathogenic fungus, St. Leger et al (1998) suggested that the insect pathogen *M. anisopliae* respond to the cuticle pH that represents a physiological signal that triggers the production of cuticle degrading enzymes mediated insect attack. Also in *C. gloeosporioides* when hyphae mass was transferred from PL secretion conditions at pH 6.5 to a low pH at 3.5 and high pH PEIM at 6.0, an immediate PL secretion was detected at pH 6.0. But no secretion was detected at low pH media (Fig 3). This can suggest that PL secretion is dependent on the levels of the media pH. PL was not secreted to the medium as long as the media pH was lower than 5.8 unlike PG that was secreted at the same intensity at all checked pHs. Dean and Timberlake in *Aspergillus* (1989) showed that the level of mRNA and enzyme secretion do not correlate. In *Colletotrichum* we showed that PL mRNA and protein levels are not correlated (Fig 8 A and B). mRNA is initially detected at 3.5 and increased up to 5.7 and then decrease. Protein secretion was detected at pH 5.8, what might indicate that post-transcriptional mechanisms control the PL processing and secretion. When endoglycosidase F was activated on the protein samples extracted from fungal hyphae, the deglycosilation affected the migration of high MW proteins to lower MW values. This migration may suggest that deglycosilation is a possible process occurring during PL and might be affected by the pH conditions. Although we did not get any band that is compatible to the mature secreted protein, we expect that more than one glycosilation type of enzymes are involved in that processing.

PG differing from PL was found to be secreted first at low pH as was described by Dean and Timberlake (1989) and Prusky et al (1989). PG secretion continued at the same level during all pH levels, whereas the transcription level that started at high level in pH 5.0 followed by a decrease in the transcription level at 5.4. From pH 5.4, an increase in *pg* transcription accompanied by pH elevation (Fig 8.1 A and B). Centis et al (1997) found two different *pgs* in *C. lindemuthianum* that had different transcription and roles during saprophytic and parasitic stages of the fungus development. The two genes have 61% identity in the protein level, that can explain cross detection of *pgs* transcription. This *pgs* can explain the fluctuating levels in the *pg* transcription, suggesting a different pH effect on each of the two different *pgs*.

So far we did not isolate any additional *pg*. The clear decrease in transcript level both for *pel* and *pg*, at pH 5.8, can indicate a common mechanism for transcription control. The transcription repression can be controlled by PL product on the level of both genes.

Garibaldi and Bateman (1971) suggested the connection between the pH of host pectin and the enzymes isoelectric point, of the pathogen. They claimed the possibility for a charge repelling between the enzyme and the pectin, which protect the tissue from degradation. The pH difference between pericarp and mesocarp, of almost a whole pH unit, in all freshly harvested fruit, can be attributed to a defense mechanism of the fruit against microorganisms. The germ tube of *Colletotrichum* becomes quiescence at the first layer(s) of the pericarp, where the pH is relatively low. But when pericarp pH increases over 5.8 the pathogen can release the enzyme and also renew its development (Fig 5). Moreover, cvs. like Ettinger and Ardit, with have pH values in the pericarp during ripening of only 5.5 behave as resistant (Fig 7 A and B). However in all cvs. mesocarp have a pH value of approximately 6.4 (Fig 5, 7A and B) and this tissue showed susceptibility during all physiological stages of fruit development and growth (Kobiler *et al.*, 1993).

The regulation of PL and PG in *in vitro* and *in vivo* conditions can emphasize the importance of PL for the transformation of quiescence of *Colletotrichum* to active infection and decay initiation. While PG can be secreted at low pH, when no symptoms are expressed, PL secretion is directly correlated decay initiation.

LITERATURE

- 1) Annis, L. S., Goodwin, H. G. 1997. Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. *Europ. J. of Plant. Pathol.* 103:1-14.
- 2) Bowyer, p., Clarke, B. R., Lunness, P., Daniels, M. J., Osbourne, E. A. 1995. Host range of a plant pathogenic fungus determined by a saponin detoxification enzyme. *Science*. 267:371-374.
- 3) Bowen, K. J., Templeton, DM., Sharrock, R. K., Crowhurst, N. R., Rikkerink, A. H. E. 1995. Gene inactivation in the plant pathogen *Glomerella*

cingulata: three strategies for the pectin lyase gene *pnlA*. *Mol. Gen. Genet.* 246:196-205

- 4) Buhr, T. L., Dickman, B. M. 1994. Isolation, characterization, and expression of a second β -Tubulin-encoding gene from *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. *Appl. Environ. Microbiol.* 60:4155-4159.
- 5) Centis, S. Guillas, I. Sejalón, N. Esquerre-Tugaye, M.-T. Dumas, B. 1997. Endopolygalacturonase genes from *Colletotrichum lindemuthianum*: cloning of *CLPG2* and comparison of its expression to that of *CLPG1* during saprophytic and parasitic growth of the fungus. 1997. *Mol. Plant-Microbe Interact.* 10:769-775.
- 6) Coates, L. M., Muirhead, I. F., Irwing, J. A. G., Gowanlock, D. H. 1993. Initial infection processes by *Colletotrichum gloeosporioides* on avocado fruit. *Mycol. Res.* 97:1363-1370.
- 7) Collmer, A., Keen, T. N. 1986. The role of pectic enzymes in plant pathogenesis. *Ann. Rev. Phytopathol.* 24:383-409
- 8) Condemine, G., Robert-Baudouy. 1995. Synthesis and secretion of *Erwinia chrysanthemi* virulence factors are coregulated. *Mol. Plant-Microbe Interact.* 8:632-636.
- 9) Dean, A. R., Timberlake, E. W. 1989. Regulation of the *Aspergillus nidulans* pectate lyase gene (*peIA*). *The plant cell.* 1:275-284.
- 10) Di Pietro, A., Roncero, G. I. 1998. Cloning, expression, and role in pathogenicity of *pg1* encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. *Mol. Plant-Microbe Interact.* 11:91-98.
- 11) Espeso, A. E., Tilburn, J., Arst, N. H., Penalva, A. M. 1993. pH regulation is a major determinant in the expression of a fungal penicillin biosynthetic gene. *The EMBO Journal.* 12:3947-3956.
- 12) Freeman, S., Rodriguez, J. R. 1993. Genetic conversion of fungal plant pathogen to a nonpathogenic, endophytic mutualist. *Science.* 260:75-78.
- 13) Guo, W. Gonzalez-Candelas, L. Kolattukudy, E.P. 1995. Cloning of a novel constitutively expressed pectate lyase gene *pelB* from *Fusarium solani* f. sp. *Pisi* (*Nectria haematococca*, mating type VI) and characterization of the gene product expressed in *Pichia pastoris*. *J. of Bacteriol.* 177:7070-7077.

- 14) Jeffries, P., Dodd, J. C., Jeger, M., Plumbley, R. A. 1990. The biology and control of *Colletotrichum* species on tropical fruits crops. *Plant Pathol.* 39:343-466.
- 15) Kang, Y., Huang, J., Mao, G., He L-Y., Schell, A. M. 1994. Dramatically reduced virulence of mutants of *Pseudomonas solanacearum* defective in export of extracellular proteins across the outer membrane. *Mol. Plant Microbe Interact.* 7:370-77.
- 16) Keller, P. N., Nesbitt, C., Sarr, B., Phillips, D. T., Burow, B. G. 1997. pH regulation of sterigmatocystin and aflatoxin biosynthesis in *Aspergillus spp.* *Phytopathology.* 87:643-648.
- 17) Maccheroni, W. Jr., May, S. G., Martinez-Rossi, M. N., Rossi, A. 1997. The sequence of *palF*, an environmental pH response gene in *Aspergillus nidulans*. *Gene.* 194:163-167.
- 18) MacKenzie, A. D., Jeenes, J. N., Belshaw, J. N., Archer, B. D. 1993. Regulation of secreted protein production by filamentous fungi: recent developments and perspectives. *J. Gen. Microb.* 139:2295-2307.
- 19) Muhlschlegel, A. F., Fonzi, A. W. 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. *Mol. Cell. Biol.* 17:5960-5967.
- 20) Orejas, M., Espeso, A. E., Tilburn, J., Sarkar, S., Arst Jr, N. H., Penalva, A. A. M. 1995. Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of carboxy-terminal moiety. *Genes Development.* 9:1622-1632.
- 21) Ortega, J., 1996. Pectolytic enzymes produced by the phytopathogenic fungus *Colletotrichum gloeosporioides*. *Texas J. Sci.* 48:123-28
- 22) Otero, C. R., Gaillardin, C. 1996. Dominant mutation affecting expression of pH-regulated genes in *Yarrowia lipolytica*. *Gen. Genet.* 252:311-319.
- 23) Prusky, D., 1996. Pathogen quiescence in postharvest diseases. *Annu. Rev. Phytopathol.* 34:413-34
- 24) Prusky, D., Gold, S., Keen, N. T. 1989. Purification and characterization of an endopolygalacturonase produced by *Colletotrichum gloeosporioides*. *Physiol. Mol. Plant Pathol.* 35:121-33

- 25) Prusky, D., Freeman, S., Rodriguez, J. R., Keen, T. N. 1994. A nonpathogenic mutant strain of *Colletotrichum magna* induces resistance to *C. gloeosporioides* in avocado fruits. 7:326-333.
- 26) Prusky, D., Plumbley, R. A. 1992. Quiescent infections of *Colletotrichum* in tropical and subtropical fruits. In *Colletotrichum: Biology, Pathology and Control*, ed. Baily, J. A., Jeger, M. J. pp. 289-307. Waillingford, UK: CAB Int.
- 27) Prusky, D., Plumbley, R. A., Kobilier, I. 1991. The relationship between antifungal diene levels and fungal inhibition during quiescent infection of unripe avocado fruits by *Colletotrichum gloeosporioides*. Plant Pathology. 40:45-52.
- 28) Punt, J. P., Veldhuisen, G., Van der Hondel, J. J. M. A. C. 1994. Protein targeting and secretion in filamentous fungi. Antonie van Leeuwenhoek. 65:211-216.
- 29) Ried, L. J., Collmer, A. 1988. Construction and characterization of an *Erwinia chrysanthemi* mutant with directed deletions in all of the pectate lyase structural genes. *Mol. Plant Microbe Interact.* 1:32-38
- 30) Rikkerink, A. H. E., Solon, N. R., Templeton, D. M. 1994. Integration of vectors by homologous recombination in the plant pathogen *Glomerella cingulata*. *Curt. Genet.* 25:202-208.
- 31) Sambrook, J., Fritsch, F. E., Maniatis, T. 1989. Molecular cloning: a laboratory manual. 2nd. edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- 32) Simmonds, J. H. 1941. Latent infection in tropical fruits discussed in relation to part played by species of *Gloeosporium* and *Colletotrichum*. *Proc. R. Soc. Queensl.* 52:92-120.
- 33) Steward, E., Hawser, S., Gow, R. A. 1989. Changes in internal and external pH accompanying growth of *Candida albicans*: studies of non-dimorphic variants. *Arch. Microbiol.* 151:149-153.
- 34) ten Have, A., Mulder, W., Visser, J., van Kan, L. A. J. 1998. The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. *Mol. Plant Microbe Interact.* 11:1009-1016.

- 35) St. Leger, J. R., Joshi, L., Roberts, D. 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. *Applied and Environmental Microbiology*. 64:709-713.
- 36) Swinburne, T. R. 1983. Quiescent infections in post-harvest diseases. In: Post- Harvest pathology of fruits and vegetables. Ed. Dennis, C. pp. 1-21. London Academic
- 37) Templeton, D. M., Sharrock, R. K., Bowen, K. J., Crowhurst, N. R., Rikkerink, A. H. E. 1994. The pectin lyase-encoding gene (*pnl*) family from *Glomerella cingulata*: characterization of *pnlA* and its expression in yeast. *Gene*. 142:141-146.
- 38) Verhoeff, K. 1974. Latent infections by fungi. *Annu. Rev. Phytopathol.* 12:99-110
- 39) Walton, D. J. 1994. Deconstructing the cell wall. *Plant Physiol.* 104:1113-1118.
- 40) Wattad, C., Dinoor, A., Prusky, D. 1994. Purification of pectate lyase produced by *Colletotrichum gloeosporioides* and its inhibition by epicatechin: possible factor involved in the resistance of unripe avocado fruits to anthracnose. *Mol. Plant Microbe Interact.* 7:293-297.
- 41) Wattad, C., Freeman, S., Dinoor, A., Prusky, D. 1995. A nonpathogenic mutant of *Colletotrichum magna* is deficient in extracellular secretion of pectate lyase. *Mol. Plant Microbe Interact.* 8:621-26

Figures

Fig 1. Changes in pH values (A) and secretion of PL protein (B) in pectolytic-enzyme-inducing-media (PEIM) by *C. gloeosporioides*. Spores of *C. gloeosporioides* (5×10^5) were inoculated into 250 ml PEIM and shook at 150 rpm and 22 °C. The culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods.

Fig 2. Effect of PEIM pH on PL secretion by *C. gloeosporioides* following transfer from PL secreting conditions. Spores of *Colletotrichum* (1.5×10^6) were inoculated to PEIM and let to grow for 5 days up to pH 6.5. Mycelia mat was then washed, and transferred to PEIM at pH 3.8 and 6.0. The culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods.

Fig 3. Effect of PEIM titration to specific pH values on the secretion of PL by *C. gloeosporioides*. *Colletotrichum* spores (1.5×10^6) were inoculated into M₃S and let to grow for 4 days. The mycelia mat was then washed thoroughly and transferred to PEIM that was titrated to pH 3.8, 4.9, 5.4 and 5.8 with 32% HCl. The culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods.

Fig 4. Effect of PEIM pH values on mRNA expression of *pel* from *C. gloeosporioides* and on the secretion of PL by the fungus. *C. gloeosporioides* was grown in PEIM and at different pH levels, hyphae was harvested and then subjected to RNA extraction. At the same pH the culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods. Hybridizations were carried out at 63°C. The radioactive probe for *pel* was the full-length cDNA *pel* clone described in Materials and Methods. (A) Western blots of analysis, (B) RNA analysis and (C) ethidium bromide RNA loading

Fig 5.. Effect of PEIM pH values on *pg* expression from *C. gloeosporioides* and on the secretion of PG by the fungus. *C. gloeosporioides* was grown in PEIM and at different pH levels, hyphae was harvested and then subjected to RNA extraction. At the same pH the culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PG by Western blot as described in Materials and Methods. Hybridization were carried out at 63°C. The radioactive probe for *pg* was the 487 bp length probe obtained by PCR as described in Materials and Methods. The radioactive probe for *pel* was the full-length cDNA *pel* clone described in Materials and Methods. (A) Western blots of analysis, (B) RNA analysis and (C) ethidium bromide RNA loading

Changes in media pH and pectate lyase secretion
during fungus development

Fig 1. Changes in pH values (A) and secretion of PL protein (B) in pectolytic-enzyme-inducing-media (PEIM) by *C. gloeosporioides*. Spores of *C. gloeosporioides* (5×10^5) were inoculated into 250 ml PEIM and shook at 150 rpm and 22 C. The culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods.

Effect of pH on the secretion of pectate lyase following the transfer from secretion inducing condition

Fig 2. Effect of PEIM pH on PL secretion by *C. gloeosporioides* following transfer from PL secreting conditions. Spores of *Colletotrichum* (1.5×10^6) were inoculated to PEIM and let to grow for 5 days up to pH 6.5. Mycelia mat was then washed, and transferred to PEIM at pH 3.8 and 6.0. The culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods.

Effect of media titration on pectate lyase secretion

Initial pH	Days of growth		Final pH
	1	4	
3.8			4.9
3.8			5.4
3.8		—	5.8
6.0	—	—	7.0

Fig 3. Effect of PEIM titration to specific pH values on the secretion of PL by *C. gloeosporioides*. *Colletotrichum* spores (1.5×10^6) were inoculated into M₃S and let to grow for 4 days. The mycelia mat was then washed thoroughly and transferred to PEIM that was titrated to pH 3.8, 4.9, 5.4 and 5.8 with 32% HCl. The culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods.

Changes in pericarp and mesocarp pH during
cv. Fuerte development

Fig. 4. Changes in the pericarp (♦) and mesocarp (■) pH values of avocado fruits cv. Fuerte at different days after fruit set.

Fig 5. Changes in pericarp pH value (◆) of avocado fruits cv. Fuerte during ripening after harvest. Firmness (▲) is presented as a parameter of ripening. Arrow indicated the time of decay initiation (DI) and of decay symptoms (DS) by *C. gloeosporioides* following inoculation of freshly harvested fruits.

Changes in pericarp and mesocarp pH during
cvs. Ardit and Ettinger ripening

A

B

Fig 6. Changes in the pericarp (□) and mesocarp (◇) pH values of avocado and firmness (Δ) fruits cv. Ardit (A) and Ettinger (B) during ripening after harvest.

Effect of media pH on *pel* expression

Fig 7. Effect of PEIM pH values on mRNA expression of *pel* from *C. gloeosporioides* and on the secretion of PL by the fungus. *C. gloeosporioides* was grown in PEIM and at different pH levels, hyphae was harvested and then subjected to RNA extraction. At the same pH the culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PL by Western blot as described in Materials and Methods. Hybridizations were carried out at 63°C. The radioactive probe for *pel* was the full-length cDNA *pel* clone described in Materials and Methods. (A) Western blots of analysis, (B) RNA analysis and (C) ethidium bromide RNA loading

Effect of media pH on *pg* expression

Fig 8. Effect of PEIM pH values on *pg* expression from *C. gloeosporioides* and on the secretion of PG by the fungus. *C. gloeosporioides* was grown in PEIM and at different pH levels, hyphae was harvested and then subjected to RNA extraction. At the same pH the culture media was concentrated, dialyzed and further concentrated before analyzed for the presence of PG by Western blot as described in Materials and Methods. Hybridizations were carried out at 63°C. The radioactive probe for *pg* was the 487 bp length probe obtained by PCR as described in Materials and Methods. The radioactive probe for *pel* was the full-length cDNA *pel* clone described in Materials and Methods. (A) Western blots of analysis, (B) RNA analysis and (C) ethidium bromide RNA loading

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