Analysis of The Morphology of *Citrus limon* Seedlings Inoculated whith *Trichoderma harzianum* and *Alternaria alternata*

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ABSTRACT

ANALYSIS OF THE MORPHOLOGY OF *Citrus limon* SEEDLINGS INOCULATED WITH *Trichoderma harzianum* AND *Alternaria alternata*. Luz M. Pérez, Daniela Seelenfreund, Jorge Garrido and Angela Roco. Universidad de Chile. Casilla 233. Santiago-Chile.

Lemon seedlings were inoculated with conidia from *Trichoderma harzianum* or *Alternaria alternata*, and were observed by optical and electron microscopy. Observations were made at time periods corresponding to maximal induction of the phenylpropanoid pathways, measured as the activity of phenylalanine ammonia-lyase (PAL). The induction of this pathway leads to the synthesis of defense metabolites, umbelliferone and scoparone, which are part of the hypersensitive response of lemon seedlings against inoculation by these fungi.

Seedlings inoculated with *T. harzianum* showed degradation of cell walls, an observation that can be explained due to the cellulolytic activity of this fungus. On the other hand, *A. alternata* produces loss of cell structure without obvious disruption of cell walls, which correlates with its pectinolytic activity.

Results showing the kind of damage observed in lemon seedlings, is related to the major enzyme secreted by each fungus. Therefore, the complete destruction of the plant cell wall is not necessary to induce the hypersensitive reaction, since both *T. harzianum* and *A. alternata* are able to elicit this response.FITOPATOLOGIA 29 (3) 1994: 202-206.

INTRODUCTION

The hypersensitive reaction is characterized by the appearance of localized necrotic zones in the plant tissue, when an incompatible interaction between a microorganism and a plant is produced. Through this response, the plant effectively limits the spread of the pathogen thus preventing the development of the disease. At the molecular level, changes such as an increase in respiration, accumulation and oxidation of phenolic compunds, loss of permeability of cell membranes and production of phytoalexins are observed (3,12). A number of evidences indicate that one of the major subcellular structures involved in the response is the plant cell wall. Cell walls not only maintain the structural integrity of the plant cell, but in addition their components play an active role as ion exchangers (4) and in the defense against inducing antifungal proteins (6) and releasing oligosaccharides that may act as elicitors and putative second messengers (20, 21).

Citrus species are infected by a number of fungal pathogens (13). Most studies have been done on fruits in a research effort directed to improve post-harvest conditions (11). Also, ultraestructure of *Citrus* species has been examined mainly in fruits and focused towards specialized tissues, such as secretory cavities (7, 8), flavedo (15) and gland structure (10).

Citrus limon seedlings develop a hypersensitive reaction when inoculated with *Trichoderma harzianum* (19) and *Alternaria alternata* (17). This hypersensitive reaction is characterized by the induction of the synthesis of umbelliferone and scoparone (18). Synthesis of scoparone has been previously reported in some *Citrus* species, although these studies did not include a morphological analysis (1,2,11).

In this work we examine structural changes of *C. limon* seedlings after inoculation with *T. harzianum* and *A. alternata*, two fungi usually found associated

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with sooty molds (16), at time periods where maximal PAL induction has been observed (17,19).

MATERIALS AND METHODS

Chemicals. All chemicals were reagent grade.

Biologicals. *C. limon* seeds were directly obtained from ripe fruits and washed extensively with tap water. Seeds were then heated at 50°C for 10 min., quickly cooled with sterile water at room temperature, surface sterilized with 10% NaCl for 30 min., rinsed with sterile water and placed on sterile pleated filter paper imbibed with 1% Captan. For germination, seeds were incubated at 28°C at 100% humidity in complete darkness. Once hypocotyls reached 2-3 cm length, the seedlings were transferred to light (3000 lux) in a 16/8 L/D period and grown for an additional 1-2 weeks. These seedlings were used for all experiments.

T. harzianum and *A. alternata* were isolated from *Citrus* trees infected with sooty molds (16), and were grown on solid Mandels medium (14) containing 1 g *Citrus* pectin per liter. Conidia were harvested using a 0.85% NaCl solution , as described (5). They were used to inoculate lemon seedlings as previously described (18,19). Petri dishes, containing six seedlings were incubated at 28°C in complete darkness: 24 hours for *A. alternata* and 32 hours for *T. harzianum*. These time periods were selected because they corresponded to the time of maximal phenylalanine ammonia-lyase (PAL) activation observed after inoculation with *A. alternata* (20) or with *T. harzianum* (19). Controls were mock inoculated with the same volume of the saline solution, and were incubated at 28°C in complete darkness for 32 hours.

Light Microscopy (LM).

Seedlings treated as above were cut into 6 sections spanning the tip of the hypocotil through the radicle. Slices of 1 mm were cut from each section and fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer pH 7.4 during 3 hours. The tissue was embedded in Epon, stained with uranyl acetate and toluidine blue.

Transmission Electron Microscopy (TEM)

Slices from seedling sections prepared as above were fixed during 3 hours in 2% glutaraldehyde in 0.2 M cacodylate buffer pH 7.4 and postfixed in 1% OsO_4 for 1 hour. Tissues were embedded in Epon after dehydration in acetone series and thin sections were stained with lead citrate and uranyl acetate.

RESULTS AND DISCUSSION

Tissues from seedlings obtained 32 hours after inoculation with *T. harzianum* and 24 hours after inoculation with *A. alternata* were first examined by LM. At these time periods,the maximal PAL induction has been reported for lemon seedlings inoculated with *T. harzianum* (19) or with *A. alternata* (17), respectively. The organization of inoculated (Fig 1a, 1b, 1e and 1f) and control (Fig 1c and 1d) hypocotyl tissues differed markedly. *T. harzianum* produced extensive disruption of cell walls (Fig. 1a and 1b), while *A. alternata* (Fig. 1e and 1f), induced a general loss of tissue structure. Similar results were obtained when root tissues were analyzed (results not shown). Observation of the same materials under TEM confirmed that cell walls and plasma membranes from seedlings inoculated with *T. harzianum* showed a notorious breakdown (Fig 2a) that was absent in mock inoculated healthy control seedlings (Fig. 2b). On the other hand, cell walls of specimens inoculated with *A.alternata*, showed areas where the cell wall had lost its original shape (see arrows), although destruction or breakdown was not apparent (Fig. 2c).

It has been reported that *T. harzianum* mainly secretes cellulases (2.6 µ/mg protein for total cellulolytic and 46.5 µ/mg protein for endoglucanase activities), although a low level of endopolygalacturonase has been detected (0.4 µ/mg protein) (16). The destruction of cell walls observed in lemon seedlings after 32 hours of inoculation with *T. harzianum*, correlates well with the major enzymes produced by this fungus.

On the other hand, *A. alternata* mainly secretes endopolygalacturonase (105.8 µ/mg), while it only produces 0.9 µ/mg protein of endoglucanase. Total cellulolytic activity was not detected in this species (16). The loss of tissue organization and integrity of cell wall structure observed both by LM and TEM in lemon seedlings after 24 hours of inoculation with *A. alternata* agrees with the enzymes secreted by the fungus. In the absence of total cellulolytic activity, and only low levels of endoglucanase, complete destruction of cell walls cannot occur.

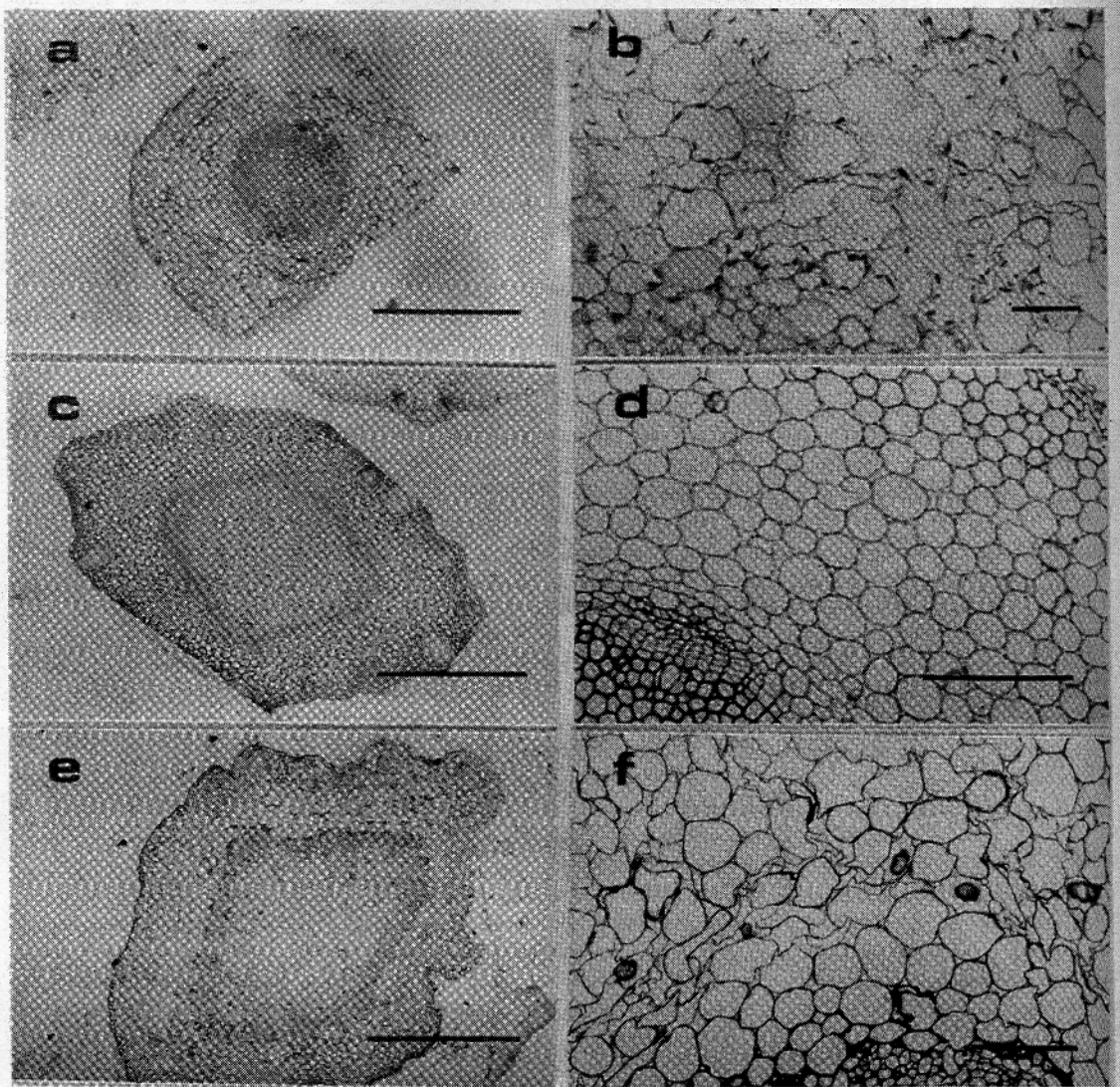

Figure 1. Light microscopy at low and high magnification of hypocotyls from lemon seedlings inoculated with *Trichoderma harzianum* or *Alternaria alternata*. Seedlings inoculated with *T. harzianum* were fixed after 32 hours of treatment (Figs. 1a and 1b). Control seedlings mock inoculated with saline solution and fixed after an incubation of 32 hours (Figs. 1c and 1d). Seedlings inoculated with *A. alternata* analized after 24 hours of treatment (Figs. 1e and 1f). Bar: 1000 µm (Figs. a, c, e,); 200 µm (Fig. b); 400 µm (Fig. d); 300 µm (Fig. f).

From this work we conclude that the morphology of the attacked cells can be correlated with the major enzymes secreted by each fungus species. Nevertheless, it is noteworthy to point out that although each fungus attacks the cell wall by different mechanisms, both are able to induce the hypersensitive response. Therefore, it is not necessary to produce the complete destruction of the cell wall, to induce this reaction. Previous work has shown that endopolygalacturonase from *A. alternata*, catalyzes the formation of pectic fragments from lemon seedlings (9), which in turn act as elicitors of the plant defense response (20).

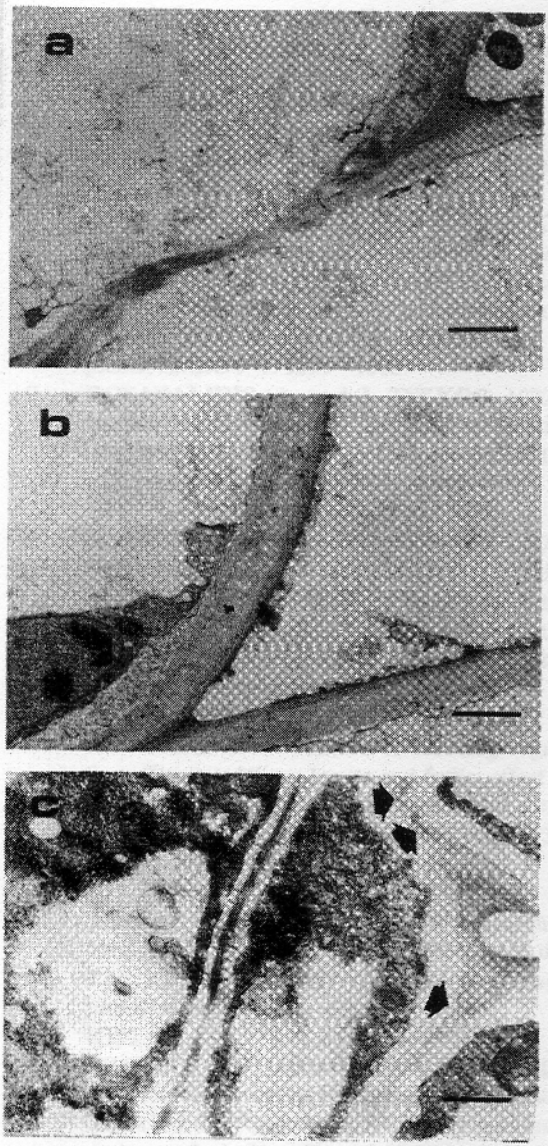

Figure 2. Transmission electron microscopy of hypocotyls from lemon seedlings inoculated with *Trichoderma harzianum* or *Alternaria alternata*. Seedlings inoculated with *T. harzianum* fixed after 32 hours of treatment (Fig. 2a). Control seedlings mock inoculated with saline solution and fixed after an incubation of 32 hours (Fig.2b). Seedlings inoculated with *A. alternata* analyzed after 24 hours of treatment (Fig. 2c). Bar: 120 nm (Figs. a, b); 100 nm (Fig. c). Arrows show distorted areas in the plant cell wall and surrounding structures.

Hence, it is posible that the low pectinase activity excreted by *T. harzianum* (16) could account for the release of enough oligosaccharides to enable the elicitation of the hypersensitive response (19).

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RESUMEN

Plántulas de limonero fueron inoculadas con conidias de *Trichoderma harzianum* o *Alternaria alternata*, y fueron observadas por microscopía óptica y electrónica de transmisión a los tiempos en que las plántulas presentan la máxima inducción de la vía fenilpropanoide, cuantificada a través de la reacción catalizada por la fenilalanina amonio liasa (PAL). La inducción de esta vía conduce a la síntesis de los metabolitos de defensa umbeliferona y escoparona, y corresponde a uno de los eventos de la reacción de hipersensibilidad que desarrollan las plántulas de limonero en respuesta a la inoculación por estos hongos.

Las plántulas inoculadas con conidias de *T. harzianun* muestran una degradación de la pared celular, hecho que coincide con su capacidad celulolítica. A su vez, *A. alternata* produce una desorganización de la estructura celular, sin degradación evidente de la pared, lo cual se correlaciona con su actividad pectinolítica.

En consecuencia , el daño producido en las plántulas de limonero se correlaciona con las enzimas mayoritarias secretadas por cada hongo. Sin embargo, la destrucción total de la pared celular vegetal no es necesaria para que se desencadene la reacción de hipersensibilidad, ya que tanto *T. harzianum* como *A. alternata* son capaces de inducir esta respuesta defensiva.

REFERENCES

1. **AFEK, U., A. STEJNBERG and S. CARMELY.** 1986. 6, 7 dimethoxy coumarin, a Citrus phytoalexin confering resistance against *Phytophthora* gummosis. Phytochemistry 25: 1855 -1856.
2. **AFEK, U., and A. STEJNBERG.** 1988. Accumulation of scoparone, a phytoalexin associated with resistance of *Citrus* to *Phytophthora citrophthora*. Phytopathology 78: 1678 - 1682.
3. **AGRIOS, G.N.** 1988. Plant Pathology. Third Edition. Academic Press Inc., New York.

4. **ALBERSHEIM, P.** 1976. The primary cell wall in "Plant Biochemistry". Third Edition (J. Bonner and J. E. Varner, eds.) pp. 225 - 274. Academic Press Inc., New York.
5. **A.O.A.C.** 1980. Official Methods of Analysis of the association of Official Analytical Chemists. 13 Ed. (W. Horwitz, ed.) p. 61.
6. **BOHLMAN, H., S. CLAUSER, S. BEHUKE, H. GIESE, C. HILLER et al.** 1988. Leaf-specific thionins of barley-a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defense mechanism of plants. EMBO J. 7: 1559 - 1565.
7. **BOSABALIDIS, A. and I. TSEKOS.** 1982a. Ultrastructural studies on the secretory cavities of *Citrus deliciosa* Ten. I. Early stages of the gland cells differentiation. Protoplasma 112: 55 - 62.
8. **BOSABALIDIS, A. and I. TSEKOS.** 1982b. Ultrastructural studies on the secretory cavities of *Citrus deliciosa* Ten. II. Development of the essential oil-accumulating central space of the gland and process of active secretion. Protoplasma 112: 63 - 70.
9. **FANTA, N., A. QUAAS, P. ZULUETA and L. M. PEREZ.** 1992. Release of reducing sugars from *Citrus* seedlings leaves and fruits; effect of treatment with pectinase and cellulose from *Alternaria* and *Trichoderma*. Phytochemistry 31: 3359 - 3364.
10. **HEINRICH, G., W. SCHULTZE and R. WEGWNER.** 1980. Zur Kompatimentierung der Synthese Von Mono- end Sesquiterpenen des atherischen Ols bei *Poncirus trifoliata*. Protoplasma 103: 115 - 129.
11. **KIM, J. J., S. BEN-YEHOSHUA, B. SHAPIRO, Y. HARRIS and S. CARMELI.** 1991 Accumulation of scoparone in heat-treated lemon fruit inoculated with *Penicillium digitatum* Sacc. Plant Physiol 97: 880 - 885.
12. **KUC, J. and J. S. RUSH.** 1985. Phytoalexins. Arch. Biochem. Biophys. 236: 455 - 472.
13. **LATORRE, B.** 1992. Enfermedades de las plantas cultivadas. Tercera edición. Ediciones Universidad Católica de Chile.
14. **MANDELS, M., L. HONTZ and J. NYSTYROM.** 1974. Enzymatic hydrolysis of waste cellulose. Biotech. Bioeng. 14: 1471 - 1473.
15. **PEREZ, L. M. and GARRIDO, J.** 1985. Ultrastructure of cells from *Citrus sinensis* flavedo. Arch. Biol. Med. Exp. 18: 41 - 55.
16. **PEREZ, L. M., S. METTIFOGO, M. ZALDIVAR and M. MUSALEM.** 1991. Sooty molds infecting citrus species in Chile: identification of the associated fungi and their ability to excrete plant cell wall hydrolyzing enzymes. Fitopatologia 26: 28 - 38.
17. **PEREZ, L. M., M. CHIONG, R. BRAVO, A. ROCO and P. CASTAÑEDA.** 1993. The action of conidia, endopolygalacturonase and fragments from cell walls of *Alternaria alternata* on *Citrus limon* seedlings is suppresed by antielicitor antibodies. Fitopatología 28: 86 - 92.
18. **PEREZ, L. M., M. PAVANI, A. QUAAS and A. ROCO.** 1994. Umbelliferone and acoparone are synthetized by *Lemon* seedlings in the hypersensitive response against *Alternaria alternata, Trichoderma harzianum* and other elicitors. Fitopatologia 29 : (in press).
19. **QUAAS, A., M. CHIONG, A. ROCO, R. BRAVO, D. SEELENFREUND and L. M. PEREZ.** 1993. Antibodies against fungal conidia and antibiotics inhibit phenylalanine ammonia-lyase activation in *Citrus*. J. Plant Physiol. 141: 393 - 397.
20. **ROCO, A., P. CASTAÑEDA and L. M. PEREZ.** 1993. Oligosaccharides released by pectinase treatment of *Citrus limon* seedlings are elicitors of the plant response. Phytochemistry 33: 1301 - 1306.
21. **VARNER, J. E. and L. S. LIN.** 1989. Plant cell wall architecture. Cell 56: 231 - 239.