Centro de Investigación en Alimentación y Desarrollo A. C.

ADNc's DIFERENCIALMENTE EXPRESADOS EN *Alternaria alternata* TOLERANTE A 2 PROPENIL-ISOTIOCIANATO

POR

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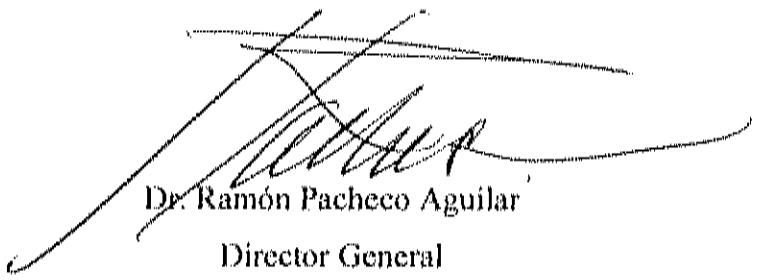

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CONTENIDO

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RESUMEN

El desarrollo de cepas de hongos resistentes a fungicidas sintéticos es un problema en agricultura que destaca la necesidad de desarrollar alternativas para controlar plagas. Una alternativa promisoria son los isotiocianatos, cuya potente actividad fungicida ha sido comprobada en diversos hongos incluyendo *Alternaria alternata*. Sin embargo, después de una exposición crónica, este hongo tolera concentraciones subletales de 2-propenil isotiocianato (2-pITC) mediante un mecanismo no elucidado.

Con el objetivo de identificar los genes inducidos en *A. alternata* en respuesta al tratamiento con 2p-ITC, se construyó una genoteca sustractiva supresiva utilizando los cDNAs de la cepa tratada como "tester" y los cDNAs de la cepa testigo como "driver". Se aislaron y secuenciaron un total de 102 ESTs (Expressed Sequence Tags, por sus siglas en inglés), los cuales generaron 50 unigenes al realizar el ensamblaje (17 contigs and 33 singlets).

El análisis mediante el algoritmo Blastn, reveló que el 20% de los clones fueron similares a secuencias de *Alternaria brassicicola* inducidas por condiciones limitantes de nitrógeno. Asimismo, el análisis con el algoritmo Blastx demostró que el 38% de los unigenes fueron similares a proteínas fúngicas conocidas, el 40% a proteínas hipotéticas y el 18% no presentaron similitud con secuencias conocidas.

La comparación a nivel nucleotídico de los unigenes con el genoma de *A. brassicicola*, reveló que el 90% fueron similares a secuencias de este hongo,

mientras que el 10% correspondió a secuencias no reportadas. A nivel de aminoácidos, el 92% fue significativamente similar a proteínas de *A. brassicicola* mientras que el 8% correspondió a proteínas no reportadas.

El análisis por ontología de genes (GO) asignó los unigenes a procesos biológicos, funciones moleculares y componentes celulares, siendo los más importantes los procesos celulares, actividad de unión y componentes citoplásmicos, respectivamente.

Mediante el análisis por RT-PCR en tiempo real se encontraron incrementos significativos para los genes de opsina a los 30 minutos después del tratamiento; los genes de calmodulina y del transportador se incrementaron significativamente a las 0.5, 1 y 2 horas de tratamiento. Asimismo, se demostró que el gen de la ATPasa de calcio se incrementó a los 0.5, 1, 2 y 12 horas después del tratamiento.

La inducción de la ATPasa y calmodulina sugiere la acción del calcio como segundo mensajero en la transducción de señales en *A. alternata* tolerante al 2p-ITC, mientras que la marcada expresión del transportador ABC sugiere el eflujo como mecanismo de detoxificación.

Se concluye que la adaptación de *A. alternata* al 2p-ITC se debe a la activación de genes codificando proteínas que participan en un mecanismo "no degradativo" de remoción del ITC de la célula; proteínas de respuesta a estrés oxidativo y proteínas para la reparación y mantenimiento de la estructura celular fúngica.

INTRODUCCIÓN Y PROPÓSITO DE LA TESIS

Uno de los principales problemas en el manejo postcosecha de frutas y hortalizas son las enfermedades ocasionadas por hongos y su control se ha realizado principalmente mediante el uso de productos químicos. Sin embargo, actualmente existe una preocupación a nivel mundial por reducir el uso de dichos compuestos en el control de plagas agrícolas. Esto se debe principalmente a los problemas relacionados con el deterioro del medio ambiente y a los efectos adversos para la salud humana, de la residualidad de compuestos tóxicos en productos alimenticios. Asimismo, otro factor importante que debe considerarse para reducir el uso de sustancias sintéticas en campo y postcosecha, es el desarrollo de hongos resistentes a diversas sustancias químicas, que al principio fueron eficaces, pero el uso indiscriminado ha ocasionado la pérdida de su efectividad. Uno de los hongos mas importantes en el cultivo de frutas y hortalizas es *A. alternata*, microorganismo que infecta a una amplia variedad de productos de importancia alimentaria y que ha presentado fenotipos de resistencia frente a distintos compuestos.

Por todo lo anterior, actualmente se estudian una gran variedad de productos de origen natural para su uso en el control de enfermedades fúngicas en agricultura. Dichos productos han probado ser muy efectivos, con el beneficio adicional de que no dañan el ambiente ni la salud humana o animal. Entre los compuestos más prometedores se encuentran los isotiocianatos (ITCs), productos que ya se aplican en campo a nivel comercial, sobre todo en países de Europa y Asia. Sin embargo, existe evidencia experimental que sugiere que los microorganismos podrían adaptarse y tolerar los ITCs, generando el fenómeno de resistencia, como ha ocurrido con los compuestos químicos sintéticos. Este hecho hace indispensable estudiar los posibles mecanismos por los cuales los hongos se defienden y resisten el efecto tóxico de estos compuestos naturales. La información disponible relativa a los mecanismos de resistencia fúngica frente a compuestos sintéticos seguramente será de gran utilidad

para entender mejor los mecanismos involucrados en la resistencia a compuestos naturales. Debido a las ventajas del uso de los compuestos naturales es indispensable generar información que permita el diseño de estrategias que eviten la aparición de la resistencia frente a estos compuestos para poder aprovechar mejor su potencial antimicótico.

El objetivo de este trabajo fue estudiar los genes diferencialmente expresados en *A. alternata* tolerante a isotiocianatos. Conociendo los genes que se activan será posible determinar qué proteínas podrían estar involucradas en el mecanismo de defensa del hongo frente a estos compuestos, contribuyendo a la identificación futura de rutas metabólicas y reacciones clave que podrían ser inhibidas para evitar el fenómeno de resistencia. La información que se ha generado durante el desarrollo de esta investigación, incluyendo una revisión exhaustiva, resultados y conclusiones generales, se han dispuesto de manera condensada en una sinopsis, haciendo referencia según el desarrollo del trabajo, a los artículos generados; mismos que se incluyeron como capítulos después de la sinopsis. Asimismo, en la sinopsis se contrasta la hipótesis y se resalta la contribución científica del trabajo al conocimiento del efecto de isotiocianatos en la expresión genética en hongos.

En el capítulo 1 se aborda la problemática de la resistencia fúngica a compuestos naturales y sintéticos, con una revisión extensa de los eventos bioquímicos y genéticos subyacentes al fenotipo de resistencia en hongos.

En el capítulo 2 se aborda de manera más específica el efecto de isotiocianatos en hongos y se discute la evidencia experimental de que los ITCs ocasionan polimorfismo en microsatélites de *A. alternata*. Asimismo, se plantea la hipótesis de que dicho polimorfismo podría tener efecto en la regulación genética del hongo orientando la respuesta trascriptómica hacia la síntesis de proteínas de defensa como Glutatión S-transferasas (GSTs) y transportadores ABC.

En el capítulo 3 se describe el trabajo realizado para obtener información sobre la cinética de crecimiento de *A. alternata* tratada con 2p-isotiocianato y del

hongo testigo con el objetivo de determinar la ocurrencia de la fase exponencial en ambos casos, asegurando así que ambos hongos se encontraran en estado fisiológico comparable al realizar los análisis genéticos.

En el capítulo 4 se describe el trabajo experimental llevado a cabo para la obtención, clonación, secuenciación, análisis bioinformático y anotación de las secuencias diferencialmente expresadas, así como también la evaluación de la expresión mediante PCR en tiempo real de algunos genes involucrados en la respuesta del hongo a ITCs.

Posteriormente se incluyen los apéndices. El Apéndice I, contiene la publicación de las ESTs (Expressed Sequence Tags) en la base de datos del Banco Mundial de Genes (Gen Bank), con los números de acceso y el archivo de publicación de algunas ESTs. El Apéndice II contiene la publicación de los contigs en el Transcriptome Shotgun Assembly del Banco de Datos de DNA de Japón (DDBJ, DNA Data Bank of Japan), incluyendo también los números de acceso y algunos archivos de publicación de dichas secuencias. En el Apéndice III se describe detalladamente la metodología utilizada para la construcción de la genoteca substractiva y la interpretación de resultados en las etapas más críticas del protocolo.

En el apéndice IV se deja constancia de la presentación de este trabajo en congresos internacionales, así como también de los reconocimientos obtenidos con motivo de la participación en este trabajo.

SINOPSIS

Las frutas y hortalizas son altamente susceptibles a daños mecánicos, desórdenes fisiológicos e infecciones microbiológicas. A nivel mundial, estos factores pueden ocasionar pérdidas postcosecha que van de un 20 a un 50% de la producción total (Barbosa Canovas et al 2003). Entre las infecciones microbiológicas, las enfermedades ocasionadas por hongos fitopatógenos representan una de las principales causas de pérdidas postcosecha. Hasta ahora, la estrategia para controlar las enfermedades en los cultivos, se ha basado casi por completo en la aplicación de productos químicos sintéticos (Rosa y Rodrigues 1999). Tan sólo en los años 2000 y 2001, el uso mundial de plaguicidas superó los 5 billones de libras, siendo los herbicidas, los más usados, seguidos por los insecticidas y los fungicidas. La cantidad de plaguicidas usados en los EU, representó más del 20% del total utilizado mundialmente, más del 25% en el caso de los herbicidas, menos del 10% de los insecticidas y entre el 15 y el 30% de los fungicidas (USEPA 2007).

Desafortunadamente, los plaguicidas son contaminantes que resisten en grado variable la degradación fotoquímica, química y bioquímica, por lo que su vida media es elevada y se acumulan en las cadenas tróficas acuáticas y terrestres (Van der Werf 1996). Otra consecuencia del uso indiscriminado de plaguicidas, es el desarrollo de plagas resistentes (Adachi et al 1996), como ha ocurrido con hongos (Georgopoulos 1987), insectos (Georghiou 1990) y malezas (Lebaron 1991). Esto ha provocado que se suspenda el uso de algunos compuestos químicos, así como el incremento en las cantidades usadas de otros, para controlar las enfermedades (Staub 1991).

Con el objetivo de llevar a cabo un control de plagas más compatible con los principios de la agricultura sostenible, en los últimos años se han estudiado los efectos biológicos de extractos y residuos de plantas (Rosa y Rodrigues 1999, Tiznado-Hernández y Troncoso-Rojas 2006) sobre hongos fitopatógenos. Entre los compuestos que más se han estudiado por su actividad antimicótica, se encuentran los isotiocianatos (Mayton et al 1996), que son productos derivados de la hidrólisis de los glucosinolatos, compuestos producidos por algunas especies de la familia *Brassicaceae*: mostaza, brócoli, repollo, coliflor, rábano, nabo y canola (Fahey et al 2001). Entre los productos de hidrólisis de los glucosinolatos, los isotiocianatos

(ITCs), se han reportado como los antibióticos más potentes en la inhibición del crecimiento de hongos (Mayton et al 1996), bacterias (Tajima et al 1998), nemátodos (Kermanshai et al 2001), insectos (Ratzka et al 2002) y malezas (Brown y Morra 1995).

En el caso de hongos, los ITCs han mostrado una potente actividad fungicida en diferentes especies bajo condiciones *in vitro*, *in vivo* e *in solum* (Tiznado-Hernández y Troncoso-Rojas 2006). Por ello, en los últimos años se está promoviendo la biofumigación, que es el uso de sustancias volátiles provenientes de la biodegradación de materia orgánica, como fumigantes para controlar patógenos de plantas. Entre las especies de plantas que han mostrado su alta efectividad para la biofumigación en la familia *Brassicaceae* se encuentran: *Raphanus sativus* (Rábano), *Sinapis alba* (Mostaza blanca) y *Brassica juncea* (Mostaza de la india).

Entre los principales agentes causantes de enfermedades en productos agrícolas se encuentra miembros del género *Alternaria*, el cual comprende casi 100 especies de hifomicetes dematiáceos que existen en todo el mundo en una gran variedad de hábitats (Simmons 1992). *Alternaria* spp. es capaz de infectar gran variedad de especies de frutas, hortalizas, semillas, granos y ornamentales (Otani et al 1998). En general, los miembros de este género son patógenos foliares que causan una destrucción relativamente lenta de los tejidos del huésped mediante la reducción de la capacidad fotosintética de la planta (Agarwal et al 1997), pudiendo ocasionar en algunos cultivos hasta un 80% de defoliación. Como patógenos poscosecha, estos hongos pueden ocasionar infecciones latentes en frutos, en las que el hongo penetra en el tejido y permanece en estado de reposo hasta que las condiciones favorecen la infección (Rotem 1994).

Experimentos realizados en nuestro laboratorio demostraron que los ITCs son capaces de controlar las infecciones por *Alternaria* en frutos, más efectivamente que algunos fungicidas comerciales sin efectos negativos en la calidad o en el desarrollo fisiológico del fruto (Troncoso-Rojas et al 2005, Troncoso et al 2005). Sin embargo, estudios preliminares han demostrado que aunque *Alternaria alternata* es inhibida al 100% por 2-propenil isotiocianato (2p- ITC), este hongo es capaz de desarrollarse en presencia del compuesto después de una exposición crónica a concentraciones

subletales, observándose un fenotipo diferente en relación al hongo testigo (**Capítulos 1 y 2**).

Existe evidencia sugiriendo que la resistencia a compuestos tóxicos involucra cambios en los genes (Adachi et al 1996) que conducen a cambios fenotípicos (Li et al 2004). Con las técnicas modernas de Biología Molecular se dispone de marcadores de mayor sensibilidad para detectar cambios que ocurren en el ADN de los individuos (Becerra y Paredes 2000). Entre las secuencias más variables del genoma se encuentran los microsatélites (Weber 1990) y se ha demostrado que la mutación en éstos, puede ser dirigida por exposición a señales externas específicas (Schmidt y Mitter 2004) como estrés por químicos genotóxicos derivados de la contaminación ambiental o incluso, por cambio climático (Hoffman et al 2004). Investigaciones realizadas con mutantes de *Arabidopsis thaliana* deficientes en el sistema de reparación de errores en la replicación, han demostrado que los cambios en microsatélites producen cambios fenotípicos como aberraciones morfológicas en hojas, palidez en el color de las hojas, enanismo, flores estériles así como tamaño, forma y color anormal en semillas (Hoffman et al 2004). (**Ver Capítulo 2**).

Otro aspecto muy importante es que todos los organismos han desarrollado mecanismos de transporte activo para la secreción de tóxicos endógenos y exógenos. En los hongos, como en otros organismos, existen varias familias de proteínas integrales de membrana que pueden mediar el transporte de compuestos naturales tóxicos a través de las membranas biológicas. Las dos clases principales de proteínas transportadoras son las proteínas ABC (ATP-binding cassette) y MFS (Major Facilitator Superfamily). En patógenos de plantas estos transportadores tienen una función esencial en la secreción de toxinas específicas y no específicas del huésped, en la protección contra los compuestos de defensa de las plantas y en la resistencia a fungicidas (Del Sorbo et al 2000). (**Ver Capítulo 2**).

Análisis preliminares de las regiones intermicrosatelitales del genoma de *A. alternata* tolerante a 2p-ITC encontraron polimorfismo en las cepas expuestas al compuesto, observándose diferencias en el tamaño y número de alelos en diferentes *loci* de microsatélites, entre las cepas tolerantes y las sensibles (R. Troncoso-Rojas, sometido a Physiological and Molecular Plant Pathology). Por otra parte, se ha sugerido que los cambios en tamaño de regiones intermicrosatelitales pueden deberse

a alteraciones en las regiones simples repetidas que las flanquean (Bussel et al 2005). De acuerdo a lo anterior, los resultados obtenidos con *A. alternata* tratada con 2p-ITC implican potenciales alteraciones en la expresión de algunos genes, lo cual puede deberse a cambios en regiones promotoras de genes, en las señales para la eliminación de los intrones, alteraciones en las regiones 5´ o 3´no traducidas, en los codones para detener la traducción, o en las señales para iniciar la cola de poliadeninas, etc. Esto significa que el tratamiento con 2p-ITC podría inducir cambios en la expresión de genes en *A. alternata* codificantes para proteínas, cuya función es evitar el efecto tóxico del compuesto, como sería el caso de cambios en la expresión de tansportadores y otras proteínas de defensa.

En experimentos realizados con *A. brassicicola* expuesta a 2p-ITC y bencil-ITC se encontró que el tratamiento con estos compuestos activa diferencialmente la transcripción de genes codificantes de glutation-S-transferasas (Sellam et al 2006), enzimas evolutivamente conservadas implicadas en la resistencia a pesticidas en plantas e insectos (Sheehan et al 2001), tolerancia a xenobióticos y respuesta a estrés oxidativo en hongos (Burns et al 2005, Veal et al 2002). Estas enzimas catalizan la conjugación del glutatión a sustratos electrofílicos produciendo compuestos generalmente menos reactivos y más solubles, lo cual facilita su remoción de la célula vía proteínas de membrana transportadoras de conjugados de glutatión (Salínas y Wong 1999).

En otro estudio con *A. brassicicola* tratada con 2p-ITC se reportó la inducción de genes que codifican para glutatión peroxidasas, glutamil-cisteína sintetasas, tiorredoxinas, tiorredoxin-reductasas, oxidoreductasas, citocromo P450, y proteínas de membrana ABC y MFS (Sellam et al 2007). Sin embargo, a diferencia de *A. brassicicola* patógeno especializado en brassicas, *A. alternata* es un patógeno generalista y no está naturalmente adaptado a los isotiocianatos (Giamoustaris y Mithen 1997, Pua y Douglas 2004) (**Capítulo 2**).

De acuerdo a lo anteriormente expuesto, y dada la inexistencia de información relativa al efecto de isotiocianatos en *A. alternata*, hongo de suma importancia como patógeno en pre y postcosecha, y con base en la evidencia experimental de su capacidad para desarrollar tolerancia a compuestos naturales que ya están en uso comercial, se planteó la presente investigación. El objetivo fue aislar

e identificar los genes inducidos en *A. alternata* en respuesta al tratamiento con 2p-ITC bajo la hipótesis de que dicho tratamiento podría inducir proteínas de defensa como GSTs y transportadores ABC. En primer término, se llevó a cabo una cinética de crecimiento de *A. alternata* para determinar la ocurrencia de la fase exponencial del hongo tratado y el hongo testigo. Esto para asegurar que los dos organismos se encontraran en la misma etapa fisiológica al momento de tomar la muestra para la construcción de la genoteca substractiva, garantizando así que los genes diferencialmente expresados que se encontraran, se debieran exclusivamente al tratamiento y no a diferencias en el desarrollo de ambos hongos (**Capítulo 3**).

La extracción del RNA total de las cepas de *Alternaria alternata* tolerante al 2-propenil-isotiocianato y testigo se realizó mediante un protocolo de extracción de RNA para hongos dematiáceos (Islas-Flores et al 2006). La construcción de la genoteca sustractiva se realizó mediante los protocolos SMART PCR cDNA synthesis kit y PCR-Select cDNA substraction kit (Clontech, Palo Alto, CA). Los fragmentos diferencialmente expresados se clonaron en el vector pGEM-T Easy (Promega Corporation) y se transformaron células de *E. coli* JM109 que posteriormente se sembraron en LB-Ampicilina suplementado con IPTG y X-Gal para diferenciar clones recombinantes. Éstos se resembraron en caldo LB-Amp para amplificar los fragmentos insertados. Se extrajo el DNA plasmídico por lisis alcalina (Sambrook et al 1989) y digestión con la enzima de restricción *Rsa*1 para verificar la presencia y el tamaño del fragmento.

Los fragmentos de interés se enviaron a secuenciar al Laboratorio "Genomic Analysis and Technology Core Facility" de la Universidad de Arizona, utilizando el oligonucleótido M13 Forward. El ensamblaje de secuencias se realizó utilizando el programa CAP3 Sequence Assembler (Huang y Maddan 1999) de la Universidad de Lyon. Los contigs y singlets resultantes se analizaron mediante el programa BLAST (Altschul et al 1990) utilizando los algoritmos Blastn y Blastx (GenBank). La anotación de los unigenes y singlets se realizó con el programa Blast2GO, v.2 (Götz et al 2008) disponible en Internet (Bioinformatics Department, Centro de Investigación Príncipe Felipe, Valencia Spain).

De los genes diferencialmente expresados encontrados, se seleccionaron seis, que de acuerdo a lo revisado en la literatura, pudieran estar relacionados con la

tolerancia y/o detoxificación celular fúngica. Se diseñaron oligonucleótidos para dichos genes utilizando el software Primer 3 (Rozen y Skaletsky 2000) y se realizaron experimentos de RT-PCR en tiempo real para evaluar su nivel de expresión a diferentes tiempos después de la aplicación del tratamiento. Se usó el método comparativo C_T ($2^{-\Delta\Delta CT}$) normalizando con el gen de la β-tubulina y como referencia o tiempo cero, el RNA del hongo testigo (**Capítulo 4**).

De la genoteca sustractiva se aislaron, secuenciaron e identificaron 102 ESTs (Etiquetas de Secuencias Expresadas, por sus siglas en inglés) diferencialmente expresadas en respuesta al tratamiento con 2p-ITC, las cuales generaron 50 unigenes (17 contigs y 33 singlets) como resultado del ensamblaje. Posteriormente, como resultado del análisis de los unigenes con el algoritmo Blastn, 20% de éstos fueron similares (83 a 98%) a clones de *A. brassicicola* cultivada en deficiencia de nitrógeno (Cramer et al 2006).

El análisis de las secuencias con el algoritmo Blastx reveló que el 38% de los unigenes fueron similares (60 a 85%) a proteínas fúngicas conocidas (dominios de unión a RNA, permeasas de aminoacidos, transportador ABC, subunidad alfa coatomera, proteínas ribosomales, factor de elongación 1 alfa, opsina, proteina de resistencia a benomil-metotrexato, aldehído deshidrogenasa, aminotransferasa de aminoácidos aromáticos, ATPasas de Ca++, histona acetiltransferasa MYST2, 5-metil-tetrahidropteroil-triglutamato-homocisteina metiltransferasa, calmodulina, RNA helicasa, fumarato reductasa); mientras que un 40% fue similar a proteínas hipotéticas principalmente de *Phyrenophora tritici repentis* (PTRG) y *Phaeosphaeria nodorum* (SNOGs, S-nitroso-glutation). El 18% de las secuencias no fueron similares a secuencias de la base de datos del Banco de Genes. Sin embargo, la comparación a nivel nucleotídico de los unigenes en la base de datos del genoma de *A. brassicicola*, reveló que el 90% fueron similares a secuencias de este hongo, mientras que el 10% correspondió a secuencias no reportadas. A nivel de aminoácidos, el 92% fue significativamente similar a proteínas de *A. brassicicola* mientras que el 8% correspondió a proteínas no reportadas.

En la anotación de secuencias, realizada mediante el programa Blast2GO, el 46% de éstas recibió una o más anotaciones (términos GO), de las cuales el 45% fue

asignado a procesos biológicos, el 43% a funciones moleculares y el 18% a componentes celulares. En procesos biológicos (nivel 2), las categorías mejor representadas fueron procesos celulares (GO: 009987), procesos metabólicos (GO: 0008152) y procesos de desarrollo (GO: 0032502), mientras que en funciones moleculares las actividades mejor representadas fueron unión (GO: 0005488), catálisis (GO: 0003824) y molécula estructural (GO: 0005198).

En la evaluación del efecto del 2 p-ITC en el nivel de expresión, por RT-PCR en tiempo real, se observaron incrementos significativos ($p < 0.05$; pair wise fixed reallocation randomisation test), representados como el número de veces que se incrementó la expresión en comparación con el testigo, en cuatro de los seis genes seleccionados: opsina, ATPasa de calcio, calmodulina y el transportador ABC. Se observó un efecto significativo en el tratamiento de media hora en el caso de la opsina (1.39 veces); en los cuatro tratamientos (0.5, 1, 2 y 12 horas) en el caso de la ATPasa, y en tres tratamientos (0.5, 1 y 2 horas) en el caso de la calmodulina y el transportador ABC. El máximo nivel de expresión de la ATPasa de calcio se observó en el tratamiento de 1 hora (6.24 veces), al igual que para la calmodulina (2.29 veces). Para el transportador ABC, su máximo incremento en expresión (95.8 veces) se observó en el tratamiento de media hora. La expresión relativa para este gen en las muestras de 1 y 2 horas de tratamiento, fue de 25.6 y 3.85 veces, respectivamente. A las doce horas de tratamiento, el nivel de expresión ya no fue significativo (1.38). No se observaron incrementos significativos en la expresión de los cDNAs correspondientes al gen de la proteína SNOG y la 5-methyl-tetra-hidropteroil-triglutamato-homocisteine-metiltransferasa.

Mediante el análisis bioinformático de los clones obtenidos en la genoteca sustractiva, se identificaron secuencias que participan en la regulación postranscripcional de la regulación genética (dominios de unión a RNA) y cDNAs representando proteínas integrales de membrana (permeasas de aminoácidos, transportador ABC CDR4, proteína de resistencia a metotrexato, opsina, ATPasas y fumarato reductasa) involucradas en: transporte de aminoácidos (Hosie et al 2002), secreción de compuestos tóxicos naturales y sintéticos (Del Sorbo et al 2000), resistencia a fungicidas sintéticos (Brôco et al 1999), bombeo de protones (Bieszke et al 1999), mantenimiento de la homeostasis en caso de fluctuaciones en

concentraciones de calcio (Vanoevelen et al 2005) y respiración anaeróbica (Iverson et al 1999). Además, se encontraron secuencias con similitud significativa a proteínas involucradas en respuesta a estrés oxidativo (SNOGs).

El tratamiento con 2 p-ITC podría tener un impacto directo en la utilización del nitrógeno, como lo sugiere el parecido a nivel nucleotídico del 20% de los clones obtenidos con clones de *A. brassicicola* inducidos bajo condiciones limitantes de nitrógeno. Otro hallazgo que sugiere un impacto en la utilización del nitrógeno, es la identificación de permeadas de aminoácidos en nuestra genoteca, ya que en *Saccharomyces cerevisiae* se han reportado permeasas sobreexpresándose bajo limitación de nitrógeno (Magasanik y Kaiser 2002). La presencia de proteínas integrales de membrana como transportadores ABC y MFS sugiere la secreción activa del compuesto tóxico como mecanismo de detoxificación, mientras que las ATPasas sugieren que el tratamiento con 2p-ITC ocasiona cambios drásticos en los niveles de calcio, el cual tiene un papel muy importante como segundo mensajero y es indispensable para la adecuada transcripción, traducción, empaquetamiento y procesamiento de proteínas secretadas (Corbett y Michalak 2000). Estas proteínas son parte de la ruta de secreción y funcionan tanto introduciendo calcio como retirándolo del citosol una vez que el estímulo ha cesado.

Por otra parte, el hallazgo de proteínas SNOGs sugiere la ocurrencia de estrés oxidativo ya que estas proteínas son donadoras de óxido nitroso (NO), compuesto que es un regulador fisiológico en diversos organismos y está involucrado en la protección contra estrés oxidativo y apoptosis (Hoga et al 1997). En otros hongos, genes ortólogos de SNOGs se han relacionado con la síntesis de compuestos de transporte y también con protección frente a estrés oxidativo (Hane et al 2007).

En parte, nuestros resultados sugieren que el estrés ocasionado por el 2p-ITC podría modular la inducción de SNOGs como mecanismo de protección. En mamíferos la óxido nitroso-sintasa endotelial y neuronal son enzimas constitutivamente expresadas que bajo estímulo, sintetizan NO por un mecanismo controlado por pulsos de Ca++/calmodulina. Esto explicaría la inducción simultánea de ATPasas, calmodulina y proteínas SNOGs en *A. alternata* tratada con 2p-ITC. Sin embargo, en el análisis de expresión de los cDNAs correspondientes a estas proteínas, no se comprobó su expresión diferencial. Aún cuando se observó un

incremento en su expresión a las 12 horas de tratamiento, éste no alcanzó a ser significativo. En cambio, el cDNA correspondiente al transportador ABC mostró el más notorio incremento en el nivel de expresión. Es importante mencionar que a la media hora de tratamiento, la expresión del transportador se incrementó 95.8 veces con respecto al testigo, decayendo rápidamente este valor a 25.6 y 3.8 después del tratamientos durante 1 y 2 horas, respectivamente. Aparentemente, este patrón de inducción puede explicarse en relación a la vida media de estos transportadores, ya que se ha reportado que en algunos casos tienen una vida media de 5 días (Wakabayashi et al 2006), por lo que una vez expresados y sintetizados la célula no necesitaría expresarlos nuevamente, al menos por el tiempo que permanezcan funcionales.

Consistentemente con los resultados descritos, el análisis por ontología genética de los genes únicos (anáIsis GO), reveló que procesos celulares, metabólicos y de desarrollo fueron los procesos mejor representados en la categoría de procesos biológicos, mientras que las actividades de unión, catalisis y de molécula estructural, fueron las mejor representadas en la categoría de funciones moleculares. Estas funciones reflejan las actividades de las ATPasas y otras proteínas integrales de membrana, así como de la calmodulina, transferasas y dominios de unión a RNA.

Este trabajo generó información novedosa en cuanto a genes diferencialmente expresados en respuesta a ITCs en *A. alternata*. El hallazgo de transportadores ABC y MFS confirma parcialmente nuestra hipótesis y concuerda con resultados recientemente publicados. Sin embargo no se encontraron enzimas glutatión- S-transferasas. La respuesta fúngica al 2p-ITC está enfocada a la inducción de mecanismos de transporte y secreción para la remoción del ITC de la célula; en respuesta a estrés oxidativo y reparación de DNA dañado, así como en el mantenimiento estructural y crecimiento de hifas. En base a esto podemos afirmar, que es la acción concertada de diferentes procesos y estrategias, lo que hace posible el crecimiento de *A. alternata* en presencia del compuesto tóxico 2p-ITC.

Se recomienda continuar con el estudio de los genes involucrados en la respuesta del transcriptoma de este hongo al 2p-ITC. La regulación de estos genes es un tópico de investigación importante, ya que su estudio podría conducir al diseño de estrategias que permitan desarrollar fungicidas más efectivos y acordes al concepto

de agricultura sostenible. Además, es importante el estudio de los fragmentos de genes para cuyos productos no se encontró similitud significativa con secuencias depositadas en el GenBank, ya que podrían estar participando en mecanismos moleculares de resistencia a fungicidas no estudiados a la fecha.

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CAPÍTULO 1

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Biochemical and Genetic Responses of Fungi to the Toxic Effect of Synthetic and Natural Fungicides

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Abstract: The objective of the present review is to analyze the molecular basis of fungicide resistance mechanism to both synthetic fungicides and the natural fungicides isothiocyanates. The review is focused mainly on *Alternaria* sp., but whenever available, similar studies in other fungi have been included. Fungal resistant strains to dicarboximide and phenylpyrrole fungicides have been found to contain mutations in one of the proteins involved in the signal transduction pathway that regulates the fungal response to osmotic stress. By the other hand, it was found that isothiocyanates induce enzymes like glutathione S-transferase, cyanide hydratase, heat shock proteins, membrane transporters and proteins associated with the oxidative stress response. Also, inter simple sequence repeats polymorphism was recorded as a response to the isothiocyanates treatment. The knowledge about the genetic basis of the response mechanism of *Alternaria* sp. to the isothiocyanates is scarce. Therefore, studies by DNA recombinant technology to analyze the fungi responses to fungicides, will allow knowing the metabolic pathways involved in the phenomena to permit the design of strategies to inhibit key reactions involved in the fungal resistance, reaching a better and sustainable fungal infections control.

Keywords: iprodione, dicarboximide, fungicide résistance, mutations, gene regulation

INTRODUCTION

Produce postharvest losses are due to pre-harvest factors[1], poor harvesting techniques[2], bad practices of handling[3] as well as bacterial[4] and fungal infections[5]. The amount of fruits and vegetables losses have been estimated to range from 5 to 50% in developing countries, being still substantial even in areas with the most advanced technologist available[6]. In some raw products, fungi are the primary spoilage organisms[5]. Out of these, *Alternaria* spp. is one of the most important postharvest pathogens, as it causes destructive leaf spots, blights (foliar and blossom), blemishes and damage to stored products[7,8]. This gender had been reported in apple, broccoli, cauliflower, carrot, potato, cabbage, tomato, pepper, melon, citrics, ornamentals and in a great number of seeds[9].

The main strategy used up to now to control agricultural products infections are mainly based in the use of synthetic chemicals[10]. By the years 2000 and 2001, the world use of chemicals in agricultural production was over 5 billions of pounds of which, more than 20% were used in U.S.A. Out of these, the fungicides represented between 15 and 30%[11].

A serious consequence of the synthetic chemicals use, is the development of resistant populations[12]. This had been reported in fungi[13], insects[14] and weeds[15] and it is an important problem in agriculture, leading to damage on vegetable crops and the chemical's discontinuation or modification of its use[16]. The development of a resistant phenotype to a specific chemical is a sophisticated phenomenon that can result from several mechanisms, including: mutations at specific genes[17-21] causing alterations of the drug target, or changes at expression level generating

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overproduction of the target enzyme, prevention of the entry of the drug at cell membrane level, inhibition of enzymes that convert an inactive drug to its active form and the functioning of a bypass pathway compensating the loss-of-function due to the drug activity,[22]. Furthermore, a resistant phenotype can also be due to degradation of toxic compounds to a less toxic chemicals[23-29] and elimination from the cell by efflux pumps[30-32].

In later years, with the objective to carry out a more environmentally friendly fungi control, efforts had been directed towards the study of the effect of natural compounds present in plants, which are thought to be an alternative compatible with the sustainable agriculture concept[10]. However, the use of natural compounds is limited and still the use of chemicals is a widespread practice.

Based on above, it is important to elucidate the genetic changes due to the fungicides use in fungal strains that became resistant, in order to develop effective and environmentally friendly strategies to control fungi infections[12]. In this review, it was analyzed the recent literature studying the molecular basis of synthetic and natural fungicides resistance in *Alternaria* gender mainly, although other fungi were included when similar studies were found in the literature.

Resistance phenotype to synthetic fungicides: The development of resistance fungi is an important area of study in population genetics of phytopathogenic fungi because it offers a broad view about how the pathogen population responds to a particular selective pressure. The comparison of the genetic structure between resistant and sensitive subpopulations within the same species, can generate data about the processes of fast evolution involved in the resistance phenomena to the fungicide[33]. In general, the development of resistance to a synthetic chemical by a pathogen is a response to the repeated use of a particular fungicide or another one chemically related[34].

Field isolates of different species of *Alternaria* highly resistant to dicarboximides and phenylpyrroles were recently identified[18]. Particularly, the dicarboximide iprodione is widely used as an active ingredient for seed dressing and spray programs to limit outbreaks of diseases on crucifers[19]. The appearance of iprodione resistant *A. alternata* strains was recorded since 1988, less than five years after the release of the fungicide to be used in passion fruit[35]. Strains of *Alternaria alternata* showing resistance to iprodione had also been reported for other crops. Furthermore, field resistance to this compound had been observed in several fungi[18].

The basis of fungal resistance to iprodione is not well understood, although studies with osmotic-sensitive mutants of *Neurospora crassa* suggest that may be a link between dicarboximide resistance and the regulation of osmotic stress. It had been suggested that this fungicide induces intracellular glycerol accumulation that results in abnormally high internal turgor pressure, leading to the rupture of fungal cells[36]. Also, dicarboximides and phenylpyrrole fungicides have been shown to stimulate glycerol synthesis in the wild type *N. crassa* strain, but glycerol synthesis is not induced by these fungicides in the *N. crassa* osmotic-sensitive (*os* mutants)[37], which shows a resistant phenotype to these fungicides. This in turn, provides experimental evidences toward the mode of action of dicarboximides and phenylpyrrole fungicides mentioned above.

The *os-1* gen from *N. crassa* was cloned and found to encode a two component histidine kinase homologous to the Sln1 protein from *Sacharomyces cerevisiae*[38]. A number of *os-1* mutants alleles from *N. crassa* have been cloned and shown to contain various mutations in the DNA region encoding one of the six 90 amino acid tandem repeat regions, at the N-terminus of the histidine kinase protein[17]. This region is not present in the Sln1 homolog from *S. cerevisiae*[39]. It had been proposed that these alterations in Os-1 protein function were responsible for the increased resistance to the carboximide and phenylpyrrol fungicides[39]. A search of genome databases indicated that most filamentous fungi have the *os-1* family histidine kinase genes in their genomes, while dicarboximide-insensitive *S. cerevisiae* and *Schizosaccharomyces pombe* do not[40].

In the fungus *Cochliobolus heterostrophus*, it had been shown that at least three locus were involved in osmosensitivity and resistance to dicarboximide and phenylpyrrole, namely: *Dic1*, *Dic2* and *Dic3*[20]. It was found that the locus *Dic1* confers both, resistance to dicarboximides and phenylpyrrole fungicides and sensitivity to osmotic stress. Further, it was suggested that fungicide resistance in *dic1* mutants is caused by alterations in proteins regulating the cellular response to osmotic stress. In another work, a gene encoding a two component histidine kinase protein from *C. heterostrophus* was cloned and sequenced. It was

demonstrated that this histidine kinase is encoded by *Dic1* by complementation of the *C. heterostrophus dic1* mutant[21]. Moreover, it was found that the predicted protein contained the conserved histidine-kinase domain, the response regulator domain and six repeats of a novel 92-amino acid unit at the N-terminus. Furthermore, the authors found that the osmosensor of *C. heterostrophus* does not have a membrane-spanning domain, whereas Sln1p of *Sacharomyces cerevisisae* contains two. These last findings further suggested that the proteins encoded by *Dic1* (Dic1p) and *Sln1* (Sln1p) regulate response to osmotic stress by different mechanisms of signal transduction.

With the goal to elucidate the molecular basis of the phenotype in each *C. heterostrophus* mutant strain, the mutant *dic1* alleles were cloned and compared in sequences with the wild-type strain[21]. A fragment isolated from *C. heterostrophus* by PCR and genomic library screening showed significant homology to the histidine kinase gene encoding *Os-1* from *N. crassa*; it was denominated *Bmhk1*. In order to determine whether the phenotypes of any of the *dic1* mutants were conferred by mutations in the *Bmhk1* gene, mutants strains were transformed with the wild type *Bmhk1* gene. Representatives of each mutant strain, *Dic1* (E4504), *Dic2* (E4503) and *Dic3* (N9005), were used. In the *dic1* mutant strain E4504, introduction of the wild type *Bmhk1* reversed the osmosensitivity and fungicide resistant phenotypes, while the phenotypes of *Dic2* and *Dic3* mutants were not affected. These results suggested that phenotypic alterations observed in *dic1* mutant strains are due to mutations in the *Bmhk1* gene. In order to identify the mutations present in *dic1* gene, genes isolated from 10 mutant strains were sequenced and compared with those from the wild type. Strains E4504, N9006, N9010 and E4509 appear to be null mutant. These strains are highly sensitive to osmotic stress and highly resistant to dicarboximide and phenylpyrrole fungicides suggesting that Dic1p is essential for osmotic regulation and resistance to both classes of fungicides.

In the case of the *dic1* mutant strain E9002, the DNA sequence of *Dic1* gene revealed that the protein encoded lacked two of the six N-terminal repeats (2 and 4). In *dic1* mutant strains E9001, N9001 and N9013 the mutations were located in the N-terminal amino acid repeats of Dic1p resulting in a different single amino acid substitution. The repeats altered were 1, 5 and 5 respectively. These mutant strains were highly sensitive to osmotic stress and highly resistant to both dicarboximide and phenylpyrrole fungicides. Therefore, the aminoacid repeat domain of Dic1p may be playing an important function in dicarboximide resistance[21].

It was proposed that the amino acid repeats of the Dic1p protein are the site of action of dicarboximides, while phenylpyrroles may target the kinase region of the protein[41]. However, the strains E9003 and N9017 with an amino acid substitution within the kinase domain and the response regulator respectively, were both moderately resistant to both classes of fungicides. Furthermore, strain E9003 was highly sensitive to osmotic stress, while strain N9017 was only slightly affected. The authors suggested that mutations within the kinase and regulator domains affect the signal transduction of the osmosensing pathway in a different way, resulting in variable response to the stress compounds.

The fungal resistance to the carboximide iprodione and the phenylpyrrole fludioxonil, as well as salt tolerance (in order to test their osmotic tolerance) were tested in field strains of *Alternaria alternata*[18]. There were found two isolates Aa 8508 and Aa 8740, that were completely inhibited by 4 μg mL^{-1} of iprodione, while isolates Aa 8501 and Aa 8495 were highly resistant to this fungicide. Similar results were obtained with the phenylpyrrole fungicide fludioxonil. The resistant strains analyzed in this study were isolated from sites which were previously sprayed with the dicarboximide fungicides. Phenotypic testing confirmed that these two isolates showed high level of resistance to iprodione and fludioxonil[18] , although with base in their growth characteristics, others authors have suggested that these strains are only moderately resistant[19].

In the test for sensitivity to osmotic stress the mycelial growth behavior of all *A. alternata* isolates was significantly reduced at 4% sodium chloride, while the fungicide resistant isolates were only slightly more sensitive to osmotic stress than the sensitive isolates[18]. The authors proposed that *A. alternata* resistance to iprodione was mediated by a mutation in a two-component histidine kinase (HK) gene. They amplified a genomic fragment encoding a two component HK protein from an iprodione sensitive field isolates of *A. alternata* showing high homology to previously identified genes from other fungal species, like *N. crassa*[42] and *Botrytis cinerea*[43]. *A. alternata* HK gene, designated *AaHK1*, was more similar to the HK from *B. cinerea* (Bos1), showing 80% amino acid identity on

the sequenced region. The partial amino acid sequence of AaHK1 protein also showed the characteristic protein structure of fungal HK with 6 repeated amino acid domains of approximately 90 amino acids in length followed by a kinase domain[18].

Sequence comparison of fungicide sensitive isolates with fungicide resistant isolates, revealed the presence of a mutation in each one of the resistant isolates. In one fungicide-resistant isolate (Aa 8495), a duplication of an 11 base pairs (bp) sequence was found within the first amino acid repeat domain at bp position 108, causing a frame shift and resulting in the presence of a premature stop codon at amino acid position 95. In another fungicide resistant isolate (Aa 8501) a 4 bp deletion was detected within the fifth amino acid repeat between bp 1349 and 1352 which also produces a frame shift leading to a termination of the open reading frame at amino acid position 434. In each case, a null mutation was produced. This was the first report of a dicarboximide resistant fungal isolate with a mutation within the first amino acid repeat region[18].

In a recent work, the *AbNIK1* gene, encoding a two component histidine kinase protein, was isolated from a wild type dicarboximide and phenylpyrrole sensitive *Alternaria brassicicola* strain and compared with the corresponding histidine kinase genes from fungicide resistant strains (field and laboratory mutant strains)[19]. Mutations were found in the *AbNIK1* gene in four of five field resistant strains (Abra3, Abra41, Abra7407 and Abra40) and the two laboratory mutants (Abra20M and Abra43M). Furthermore, one of the fungicide resistant field strain (AbraCP), showed no mutation in the *AbNIK1* gene. Based in this evidences, the fungicide resistant isolates were separated in two groups based on their sensitivity to osmotic stress. The first group showed a moderately osmosensitivity and high fungicide-resistant phenotype (3 field strains (Abra3, Abra41, Abra7407) and 1 laboratory strain (Abra20M); while the second group showed a low osmosensitivity and high fungicide resistant phenotype (one field strains: Abra40 and 1 laboratory strain: Abra43M).

All the strains of the first group had null mutations, resulting from premature translation termination because of a frameshift within repeat 4, or the presence of stop codons within repeat 5 or near the G-2 box. A mutation within repeat 4, which consisted of an identical 2 bp deletion leading to a frameshift, was found in two of the strains analyzed: one laboratory resistant strain (Abra20M) and one field resistant strain (Abra41). Among the field resistant strains, in the Abra40, it was located an amino acid substitution of a glutamic acid at position 753 by a lysine residue within the H box domain. This strain was highly resistant to fungicides and highly tolerant to osmotic stress. It's likely that this mutation affected significatively the enzyme activity even though it does not lead to any apparent perturbation in the response to osmotic stress. It is possible that a mutation in another locus could confer the fungicide resistant phenotype in Abra40 strain, which is probably the case for the unique strain with no mutation in the *AbNIK1* gene (AbraCP). Despite of the fact that both strains had a similar phenotype (resistant to fungicide and osmotic stress), the DNA sequence of its *AbNIK1* gene did not reveal any difference relative to the wild type.

The laboratory mutant Abra43M was found to have a point mutation that resulted in a stop codon within the repeat 2. This should be a null mutant, but unlike other *A. brassicicola* mutants, this strain was not found osmosensitive. The strain Abra43M had a Kozack sequence and a start codon 24 bases downstream from the mutation. The authors assume that this may give rise to a protein lacking the first two amino acid repeat. However, this truncated histidine kinase should still be active and able to regulate the downstream osmosensing pathway.

In addition, histidine kinases from *A. brassicicola* and *C. heterostrophus* were compared in this work[19]. It was found high similarities at the amino acid and nucleotide levels. The first five introns spanning these two coding regions, were found at the same location and only the length of the first intron was different between the two sequences. This difference was mainly due to the presence of a CA $(TA)_3$ $CA(TA)_3CA(TA)_2CA(TA)_3$ microsatellite in the sequence of *C. heterostrophus*. Two microsatellites $(GTT)_7$ and $(GTT)_8$, depending on the *A. brassicicola* strain, were also found in the 3´-untranslated region (3'UTR) of *AbNIK1*. The authors remarked that the relevance of the presence of these microsatellites in the non-coding region of the two fungal histidine kinase genes is currently unknown. It was hypothesized that the microsatellite expansions in 3´-UTR regions can cause transcription slippage and produce expanded mRNA which can disrupt splicing[44]. In agreement with this statement, in human cancer cells, a deletion of 1 bp in a $(T)_8$ microsatellite embedded in the 3´-UTR of CEACAM1 gene, was identified[45]. This gene does not contain any of the regulatory elements already classified. The authors observed that the wild type but not the mutated 3´-UTR, significantly decreased transgene expression at both mRNA and protein level

suggesting that a single mutation in the 3'-UTR short microsatellite, might affect gene expression.

Steel and Nair[46,47], proposed that dicarboximide resistance in *B. cinerea* and *Alternaria alternata* was mediated by altered levels of the antioxidant enzymes catalase (CAT) and superoxide-dismutase (SOD). Experimental evidences supporting this statement were generated by studying the activity levels of CAT and SOD, in a dicarboximide sensible (DS) and dicarboximide resistant (DR) isolate of *Botrytis cinerea*[48]. In the absence of the dicarboximide vinclozolin, the DR isolate had 1.5 and 3.0 folds higher CAT and SOD activities, respectively than the DS isolate. In this study, it was observed that the activities of CAT and SOD changed differently in the DS and DR isolates during incubation with vinclozolin. After 1 h of fungicide treatment, the CAT and SOD activities in the DR isolate increased 46 and 20%, respectively, while in the DS isolate, such increases of the enzyme activities were not obvious. This result implies that trough enhancement the CAT and SOD activities, the dicarboximide resistant isolate could have developed, at least partly, the resistance phenotype. In agreement with these results, Dry and coworkers[18] have speculated that mutations in the two components HK of fungal cells leads to an increase in the endogenous level of anti-oxidant enzymes protecting these cells from the deleterious effects of dicarboximide fungicides. Furthermore, it was hypothesized, based in the work of Choi and coworkers[48] that if the two components HK genes of the fungi above mentioned, carry out a regulatory function, mutations leading to an inactivation of the HK may result in the constitutive induction of stress response proteins such as catalase (CAT) and superoxide dismutase (SOD)[18]. In support of this hypothesis, a research carried out by Kovtun and coworkers[49], found the constitutive activation of oxidative stress pathways in high plant cells by a mutation of a specific mitogen activated protein kinase 3 (MAPK3) in *Arabidopsis* and tobacco cells which activates, in a constitutive manner, the expression of stress response genes such as the anti-oxidant enzyme glutathione-S-transferases (GST).

GSTs are a family of multifunctional enzymes playing an important role in cellular detoxification and excretion of a wide variety of xenobiotic substances, representing an integral part of phase II biotransformation enzymes[50]. These enzymes catalyze the sulfur conjugation between the thiol group of GSH (glutamylcysteinylglycine) and an electrophilic moiety in hydrophobic toxicants. Mammalian and plants GSTs have been well characterized, but little is known about fungal GSTs[51]. However, it had been suggested that fungal GSTs are more related to mammalian GSTs that to their bacterial counterparts[52]. Some of Fungal GSTs studied are GSTs of *Aspergillus fumigatus*[53], *Schizosacharomyces pombe*[54], *S. cerevisiae*[55], *Issatchenkia orientalis*[56], *Phanerochaete chrysosporium*[57], *Cunninghamella elegans*[52] *Streptomyces griseus*[58], *Botrytis cinerea*[59] and *Mucor circinelloides*[60]. Studies undertaken on these fungal GSTs revealed that they are potentially involved in protecting the cell against damage from oxidative stress, xenobiotics, heavy metals and antifungal compounds, thus highlighting the functional diversity of these enzymes[51]. Fungal GSTs exhibit differential expression patterns with in such a way that some isoforms had been shown to be induced in the presence of xenobiotics or oxidative stress. In *I. orientalis*, of two GSTs identified, only one was constitutive and both were induced in the presence of *o*-dinitrobenzene[55,56]. In *S. pombe*, three GSTs were induced by oxidative stress, but the transcription of these genes is subjected to differential regulation under various stress conditions[61]. Gst1, Gst2 and Gst3 have GST activity with the substrate 1-cloro-2, 4-dinitronenzene, while Gst3 has glutathione peroxidase activity. However, all three genes are required for normal cellular resistance to peroxides. Mutants lacking *gst1*, *gst2* or *gst3* were more sensitive to the presence of the antifungal drug fluconazole, thereby indicating a role of GST in mediating antifungal drug tolerance[54].

Resistant phenotype to natural fungicides: Among the natural compounds with fungicide activity that are receiving greater attention are the isothiocyanates, mainly due to their potent fungicide activity[62]. It has been reported the toxic effect of 3-methylsulphinylpropyl-isothiocyanate, benzil-isothiocyanate, 2-propenyl-isothiocyanate and (2*S*)-2-hydroxybut-3-enyl-isothiocyanate against eight phytopathogenic fungi: *Alternaria alternata*, *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Diaporthe phaseolorum*, *Fusarium oxysporum*, *Colletotricum coccodes* and *Phitium irregulare*[63]. Moreover, the effect of isothiocyanates on different developmental stages of isolates of *Fusarium oxysporum* was evaluated. It was found that both 2-propenyl- and ethyl-isothiocyanates inhibited 100 percent the mycelial growth as well as conidial and chlamydospores germination[64].

The activity *in vitro* and *in vivo* of benzyl-isothiocyanate (BITC) on *Alternaria alternata* strains was evaluated[65]. Results of the *in vitro* experiment showed that a BITC concentration of 0.05 mg ml^{-1}

inhibited 90% of mycelial growth, whereas concentrations of 0.1 mg ml^{-1} and higher (0.2-0.4 mg ml^{-1}) showed 100% inhibition. By other side, in the *in vivo* experiment, the BITC effect at two concentrations (0.28 y 0.56 mg ml^{-1}) during 18 and 36 hours on *Alternaria alternata* infecting tomato fruits was studied. The authors found that a concentration of 0.56 mg ml^{-1} reduced the fungi infection by *Alternaria alternata* on tomato fruits by 85%. Further, it was shown that this BITC concentration was more effective than a commercial fungicide in controlling the fungi infection with no negative effects on the postharvest quality of tomato fruit. Another set of experiments found that a concentration of 0.03 mg ml^{-1} of an isothiocyanate mixture inhibited 100% the growth of *Alternaria alternata* under *in vitro* conditions. Also, the utilization of this isothiocyanate mixture at concentration of 0.56 mg ml^{-1} showed a complete inhibition of *Alternaria alternata* infecting bell pepper fruit. Moreover, it was shown that this treatment with isothiocyanates mixture controlled better the fungi infection than a commercial fungicide without detrimental effects on the postharvest fruit quality[66].

Despite of the fact that the isothiocyanates had shown a clear inhibition activity on fungi growth in experiments carried out under *in vivo* and *in vitro* conditions, preliminary experiments carried out in our laboratory, showed that *Alternaria alternata* is able to grow after being chronically exposed to sublethal dosis of 2-propenyl-isothiocyanate. However, the elimination of the selective pressure along with inoculation in new media render *A. alternata* showing the same level of sensitivity as the unexposed control. Based on these data, the authors suggested that *A. alternata* developed an environmental resistance to the toxic effect of the isothiocyanates rather than a resistance developed by mutation, and therefore it dissapeared when the selective pressure was no longer present (R. Troncoso-Rojas, *personal communication*).

In order to study the genetic responses of the *A. alternata* to the isothiocyanates, the effect of fungus exposure to 2-propenyl-ITC (2p-ITC) and benzyl-ITC (BITC) on the inter simple sequence repeats regions (ISSR) of *A. Alternata* genome was analyzed. It was found ISSR polymorphism in the strains exposed to 2p-ITC and BITC when compared with the wild type. In one of the wild type strains, PCR analysis using the oligonucleotide $(GACA)_4$ amplified two fragments with sizes of 1.3 and 0.71 kb whereas in the exposed strain, the 0.71 kb fragment was not found. It is believed by the authors that the exposition to the isothiocyanates led to the elmination of the small ISSR suggesting an alteration in the microsatellite region GACA. In other strains exposed to 2p-ITC and BITC, it was found two DNA fragments of 603 y 350 bp, that were not present in the wild type. Furthermore, in the same experiment, a fragment of 2.17 kb was found only in the wild type (R. Troncoso-Rojas, *personal communication*).

The microsatellite regions are one of the most variable elements of the genome[67] and they have been found ubiquitously in the different fungi genome analyzed so far[68]. In average, the mutation rate in the microsatellite regions is various orders of magnitude higher than other DNA regions[69]. It had been demonstrated that mutations in the microsatellite regions can be induced by exposition to specific external factors like fungal infection[70], stress by genotoxic chemicals derived from environmental contamination or by climatic change[71], and ionizing radiation[72]. Therefore, the changes at the microsatellite regions can provide a mechanism for a rapid adaptation to environmental changes in both eukaryotic and prokaryotic organisms[44].

Recently, the effect of 2p-ITC and BITC isothiocyanates on *Alternaria brassicicola* gene expression was evaluated. Differential display analysis led to the isolation of the first GST gene from this fungus, designed *AbGSt1*, which was differentially expressed in the presence of 2p-ITC and BITC[51]. Furthermore, the authors observed that *AbGst1* was upregulated during the infection of *A. brassicicola* on *Arabidopsis thaliana*, a plant that shows resistance against this pathogen presumably by the presence of isothiocyanates as defense compounds. The deduced amino acid sequence of AbGSt1p showed significant identity to glutathione-S-transferase-I from *Saccharomyces cerevisiae* and glutathione-transferase-III from *Schizosaccharomyces pombe*. The results of this work showing upregulation of *AbGst1* by isothiocyanates and the increased activity of AbGSt1p in the presence of cyanogenic products strongly suggest that *AbGst1* enables this fungus to tolerate some plant defense compounds[51].

In a more extensive work, Sellam and coworkers[73] studied the changes in gene regulation of *A. brassicicola* to short exposure to 2p-ITC. The authors found upregulation of several genes such as glutathione transferases (GSTs), glutathione peroxidase, γ-glutamyl-cysteine synthetases, thioredoxins, thioredoxin synthetases, oxidoreductases, (all involved in the oxidative stress response). Moreover, the Allyl-ITC treatment induced one cytochrome P450-encoding gene, 10 genes for membrane transporters and one gene encoding a positive-acting sulphur regulatory protein.

The authors suggested that 2p-ITC treatment generates reactive oxygen species (ROS), leading to the activation of the conserved ITC-detoxification mechanism mediated by phase I and phase II enzymes.

Another gene shown to be involved in isothiocyanate detoxification in *Alternaria brassicicola* is *CyhAB*, which encodes a cyanide hydratase enzyme. It was shown the upregulation of this gene during the infection of *Arabidopsis thaliana* by *A. brassicicola*. Based on these experimental evidences, it was suggested a role for this gene in isothiocyanate detoxification[74].

CONCLUSION

Data available about the genetic and biochemical mechanisms leading to the development of fungi resistant phenotypes to natural and synthetic fungicides is an imperative need. Also, considering that this phenomenon is the major drawback for the use of fungicides to control fungal infections, it is urgent to conduct more research using techniques able to analyze the genetic responses with tools derived from the DNA recombinant technology. The development of knowledge about the fungal adaptation at genetic level will allow the designing of strategies to control fungi infections more effective and environmentally friendly.

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CAPÍTULO 2

Hypothetical Relationship between Changes in Gene Regulation and Inter Simple Sequence Repeats Polymorphism Induced by Isothiocyanates on *Alternaria alternata*. Báez-Flores ME, Troncoso-Rojas R, and Tiznado-Hernández ME.

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Se le envía el manuscrito 0807-2, titulado, "Hypothetical Relationship between Changes in Gene Regulation and Inter Simple Sequence Repeats Polymorphism Induced by Isothiocyanates on *Alternaria alternata*", autores: María Elena Báez-Flores, Rosalba Troncoso-Rojas, and Martín Ernesto Tiznado-Hernández, el cual ha sido condicionado a una revisión exhaustiva, para que haga las correcciones indicadas por los revisores de la Revista Mexicana de Fitopatología.

Le agradeceré me envíe por correo electrónico el manuscrito corregido, o una impresión del manuscrito, así como un CD con los archivos. De ser posible, envíe impreso los datos de las gráficas, si las hay. Favor de escribir los nombres completos de autor y coautores, así como las direcciones.

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Le agradezco el considerar nuestra Revista Mexicana de Fitopatología, para la publicación de sus actividades de investigación.

Atentamente

Dr. Guillermo Fuentes Dávila, Editor en Jefe

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Hypothetical Relationship between Changes in Gene Regulation and Inter Simple Sequence Repeats Polymorphism Induced by Isothiocyanates on *Alternaria alternata*

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Resumen. Los isotiocianatos (ITCs) son potentes fungicidas naturales que representan una alternativa viable para sustituir el uso de compuestos químicos en el control de enfermedades en productos agrícolas. Sin embargo, la exposición constante y prolongada de *Alternaria alternata* a ITCs, puede inducir un fenotipo tolerante a estos. Evidencia experimental indica la ocurrencia de polimorfismo en secuencias simples repetidas (SSRs) en el genoma de *A. alternata* tratada con ITCs. Otros estudios demostraron que la exposición de *A. brassicicola* a ITCs indujo genes codificantes para glutation-S-transferasas, transportadores ABC, citocromo P450, entre otros. Estos fitopatógenos infectan diferentes huéspedes; *A. brassicicola* es un patógeno especializado en brássicas, mientras que *A. alternata* es un patógeno generalista. El objetivo del presente trabajo fue evaluar si existe una relación entre el polimorfismo en SSRs y la tolerancia del hongo a ITCs. Se analizaron trabajos enfocados en la respuesta genética de hongos a ITCs y otros compuestos. Con base en ese análisis, se sugiere que el fenotipo tolerante a ITCs podría deberse a la activación de genes regulada por el polimorfismo en SSRs. Sin embargo se requiere realizar estudios experimentales que comprueben esa hipótesis.

Palabras clave adicionales: Brássicas, fenotipo de tolerancia, genes de estrés oxidativo, detoxificación, microsatélites.

Abstract. Isothiocyanates (ITC) are natural compounds produced by brassicas. They have antifungal activity at low concentrations and represent a viable alternative to substitute the use of synthetic chemicals in the control of diseases in agricultural products. However, the constant and prolonged exposure of *Alternaria alternata* to ITCs could induce a tolerant phenotype to these compounds. Experimental evidence indicates polymorphism in the inter simple sequence repeats (ISSR) in genomic DNA of *A. alternata* exposed to ITCs. Other studies had demonstrated the induction of genes encoding glutathione-S-transferase, cytochrome P450, and ABC transporter proteins, among others in *A. brassicicola*. These phytopathogens infect different hosts; *A. brassicicola* is fungus specialized in brassicas, while *A. alternata* is a generalist pathogen. The objective of the present review was to evaluate whether there is a relationship between the SSRs polimorphysm and the fungal ITC tolerance phenomena. As conclusion, it is suggested that ITC tolerant phenotype in *A. alternata* strains can be due to the activation of genes through changes in ISSR polymorphism. However, future experimental studies are required to probe that hypothesis.

Additional keywords: Brassicas, tolerance phenotype, oxidative stress genes, detoxification, microsatellites.

INTRODUCTION

One of the challenges to increase the food world supply is to reduce the postharvest losses of fruits and vegetables which can vary from 10 up to 50% of the

total production (Barbosa Canovas *et al.*, 2003). Phytopathogenic fungi are one of the main causes of postharvest diseases in fruits and vegetables. The genus *Alternaria* is commonly found since groups almost 100 species of dematiaceous hyphomycetes (Simmons, 1992), wich cause diseases considered among the most destructive for plants; in some parts of the world these fungi are responsible for about ~80% of foliar losses (Rotem, 1994).

The approach used to control the fungal diseases is based almost entirely on synthetic chemical products use. However, in the last decades, efforts had been directed to get a better understanding of the biological effects of compounds isolated from plants, which have a potential application in pest and disease control, minimizing dependence on artificial pesticides (Rosa and Rodrigues, 1999; Troncoso-Rojas and Tiznado-Hernández, 2007). The use of natural compounds has a minor impact on the ecosystems. Unlike synthetic chemicals, they are biodegradable and do not accumulate in the environment.

Among the compounds more used due to their potent fungicidal activity are the isothiocyanates (Tiznado-Hernández and Troncoso-Rojas, 2006), even though their toxicity mechanism is not well understood (Manici *et al.*, 1997). Indeed, it was reported that *Alternaria alternata* (Fr. : Fr.) Keissler is killed by low concentrations of 2-propenyl-isothiocyanate (Troncoso *et al.*, 2005; Troncoso-Rojas *et al.*, 2005). However, it was also observed that this fungus was able to grow in presence of this compound after a constant and prolonged exposition at sub lethal concentrations (Troncoso-Rojas *et al.*, 2008; submitted to Physiological and Molecular Plant Pathology). On the other hand, some other species of *Alternaria* genus are able to infect species of *Brassica* spp. with high content of isothiocyanates, e.g. *A. brassicicola*, and *A. brassicae* (Sigareva and Earle, 1999).

Experiments to elucidate the mechanism by which these fungi withstand the toxic effect of ITCs, found that *A. brassicicola* exposed to 2-propenyl-isothiocyanate (2p-ITC) and benzyl isothiocyanates (BITC) activates the transcription of genes

encoding glutathione-S-transferases (Sellam *et al.*, 2006), glutathione peroxidases, glutamylcysteine synthetases, thioredoxins, thioredoxins reductasas, oxidoreductases, cytochrome P450, ABC transporter proteins, and major facilitator superfamily (MFS) membrane proteins (Sellam *et al.*, 2007). However, in the case of *A. alternata* it does not belong to the group of pathogens of *Brassicas* (Giamoustaris and Mithen, 1997; Pua and Douglas, 2004) and still is able to grow under the ITC toxic effect, as mentioned above. Therefore, an important issue can be to elucidate what is the phenomena underlying the tolerance and resistance of this fungus to the ITC toxic effect.

The analysis of inter simple sequence repeats (ISSR) in the genomic DNA of *A. alternata* tolerant to 2-pITC and BITC found polymorphism in several inter simple sequence repeats (ISSR) *loci* (Troncoso-Rojas et al., 2008, submittet to Physiological and Molecular Plant Pathology). Mutations detected by microsatellite primers can identify several possible alterations in the genome. In fact, this tool had been used to assess the genotoxic effects of various chemical compounds (Leroy *et al.*, 2000). Studies in bacteria showed that unstable mutation-prone microsatellite *loci* is an useful resource to maintain the high level of phenotypic diversity required for successful exploitation of variable environments (Bayliss *et al.*, 2001). Furthermore, it had been suggested that microsatellite *loci* are a major factor in the ability of many bacterial species to respond rapidly and efficiently to environmental stressors, such as antibiotics (Rocha *et al.*, 2002).

The possibility of fungi developing resistance against ITCs makes important to understand the molecular basis of this phenomenon, e.g. what genes are expressed or overexpressed in response to these natural compounds and consequently, what kind of proteins are synthesized as an ITC response and further involved in the fungal defense mechanism.

Because of the above mentioned, the aim of this paper is to analyze in the literature the possible relationship between ISSR polymorphism and the activation of

genes encoding glutathione-S-transferase and transporter proteins type ABC and MFS as an adaptative response mechanism in fungi to the ITC toxic effect.

Effects of isothiocyanates on fungi.

The plants produce secondary metabolites not always involved in the vital metabolic processes, but playing roles in either the defense mechanisms against predators or as reserve substances. Among these metabolites are the glucosinolates (GLSs), natural products containing nitrogen and sulfur, present mainly in some species of the *Brassicaceae* family: mustard, broccoli, cabbage, cauliflower, radish, turnip, and canola (Fahey *et al.*, 2001). The GLSs itself are not toxic compounds, however the loss of cell compartmentalization by tissue damage can bring together the GLSs molecules with the myrosinase enzyme, which hydrolyze GLSs producing an unstable aglycone that spontaneously rearrange into biologically active compounds like thiocyanates, nitriles and isothiocyanates depending upon the cell iron concentration, side chain substitution and the cell pH (Wisttock and Halkier, 2002).

Out of these compounds, the isothiocyanates have a wide biocide activity that includes the inhibition of fungi (Mayton *et al.*, 1996), bacteria (Delaquis and Mazza, 1995; Tajima *et al.*, 1998), nematodes (Kermanshai *et al.*, 2001), insects (Ratzka *et al.*, 2002) and weed seeds (Brown and Morra, 1995). The mechanism by which ITCs inhibit microorganism and fungal growth is not well known. Some hypotheses propose that ITCs cause inactivation of intracellular enzymes by oxidative breakdown of disulfide bridges, inhibition of metabolic enzymes by thiocyanate radical and uncoupler action of oxidative phosphorylation.

Apparently, the high reactivity of ITCs is due mostly to the strong electrophilic nature of –NCS group (Kroll *et al.*, 1994). Experiments *in vitro* demonstrated that allyl-isothiocyanate can form covalent bonds with the disulfide bonds of the oxidized glutathione (Kawakishi and Kaneko, 1987), as well as with the

free amino and sulfhydryl group of amino acids (Cejpek *et al.*, 2000), suggesting that the ITCs can chemically react with almost any protein through the free amine and sulfhydryl groups present in the side groups of amino acids residues like lysine, arginine and cystein.

The antifungal activity of ITCs appear to be rather random than specific since it could react with any protein of the fungal cell. This unspecific nature of the ITC's action site makes unlikely a mutation that could induce a resistance phenotype in fungi, as it had been observed with some synthetic fungicides.

Microsatellites and fungi.

The studies based in protein polymorphism are of great utility in research, but with the development of the DNA recombinant technology, now there are a great number of markers based in the polymerase chain reaction (PCR): restriction fragments length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), variable number of tandem repeats or minisatellites (VNTR), simple sequence repeats or microsatellites (SSR) (Becerra and Paredes, 2000), and inter simple sequence repeats (ISSR) (Bussell *et al.*, 2005).

Due to their high variability, the microsatellites are valuable genetic markers useful for genome mapping of many organisms (Knapik *et al.*, 1998; Schuler *et al.*, 1996). Their applications comprise diverse areas like forensic studies of DNA, genetics of populations and conservation of biological resources (Jarne and Lagoda, 1996). The SSR, are shorts sequences from 1 to 6 base pairs, repeated in a continuous way. They are present in all the prokaryotic and eukaryotic genomes analyzed to date, either in coding and not coding regions. Microsatellites are characterized by their great variability in length, and apparently are originated due to the slippage phenomenon during DNA replication (Schlotterer and Tautz, 1992).

In fungal genomes the microsatellites are ubiquitous constituents. The ascomycetes and basidiomycetes rarely contain more than 5% of repetitive DNA, while the phylogenetically older division Zygomycota typically contains more than 30%. The effects of repetitive DNA in the expression of adjacent genes are scarcely understood and their role in the genomic flexibility on an evolutive scale is still enigmatic (Wöstemeyer and Kreibich, 2002). The mutation rate in microsatellites is on average, several orders of magnitude higher than in the rest of DNA, with differences between distinct species. Furthermore, within a species, it changes among *loci* with different repeat size, among alleles of one locus, and among individuals of different sex and age (Nikitina and Nazarenko, 2004).

The abundance of microsatellites ranging from 1 to 6 bp was evaluated in some fungal groups. It was found a taxa-specific variability in the frequency of distributions of simple sequences, suggesting that the theory of slippage of DNA strand itself is insufficient to explain completely the microsatellites distribution in the genome (Tóth *et al.*, 2000). Furthermore, there are strong evidences supporting a non-random distribution of microsatellites across protein coding regions, untranslated regions (UTRs) and introns.

Microsatellites instability promotes phenotypic changes in fungi and plants through alterations in gene regulation.

The mutational processes of microsatellites in the genome generate changes in DNA that are normally corrected by the DNA mismatch repair system (MMR System). The mutations not repaired would became new alleles at the microsatellite *loci* which can either alter the gene regulation or change gene products, leading eventually to phenotypic changes (Li *et al.*, 2004). Moreover, it was demonstrated endogenous microsatellite instability (expansion/contraction of long repeats) in plants in which the gene AtMSH2 encoding one protein of the MMR system was

disrupted (Leonard *et al.*, 2003). Furthermore, the effect of such microsatellite polymorphism, was studied in *Arabidopsis* MMR defective (AtMSH2-1) lines, which rapidly accumulated (five generations of seed-to-seed propagation) a wide variety of mutations leading to phenotypic changes e.g. aberrant form, size and color of leaves, siliques and seeds; abnormal germination, early flowering as well as dwarf and sterile flowers (Hoffman *et al.*, 2004).

This finding clearly showed a connection between microsatellite instability and alteration in phenotype due to mutations in plants. The rationale behind these experiment states that the errors escaping proofreading by replicative polymerase will be efficiently corrected by MMR system in wild-type cells, but not in MMR deficient cells, where rates of insertion-deletion mutations, especially at longer repeat sequences, increases dramatically, allowing the observation of the phenotypic effect by microsatellite instability in *Arabidopsis*.

In this case, the experimental MMR deficiency lead to negative consequences, but there are broad evidence showing that in cells with a wild-type MMR system, the appearance of SSRs polymorphism could represent an advantage for the organism survival. The effect of microsatellite will depend upon the place where it is located in a particular gene. SSR in 5′-UTRs could affect transcription and translation; SSR expansion in 3′-UTRs causes transcription slippage producing expanded mRNA which can disrupt splicing and other cellular functions.

Based on the above mentioned, microsatellites could provide a molecular basis for fast adaptation to sudden environmental changes in both prokaryotes and eukaryotes (Li *et al.*, 2004) with efficient MMR systems. To evaluate whether the microsatellite mutation can be directed by exposure to specific external cues, a system in which links between external cues and specific microsatellite *loci* have been previously established, was examined. The authors observed that previous

studies demonstrating the alterations of microsatellites by external cues used universal mutagens, which are able to induce mutation in both, the repetitive and the coding regions of the DNA. However, no evidence that those mutations represent a direct response to a specific stimulus was presented (Schmidt and Mitter, 2004).

Looking for that evidence, they carried out an experiment using wheat varieties resistant and susceptible to *Fusarium gramminearum* attack. Each variety of plants was inoculated in inflorescens, whereas control plants were uninoculated. The wheat varieties were genotyped in a number of microsatellite *loci* that map to chromosomes known to contain *Fusarium* head blight resistance/susceptibility *loci*. Genotyping analysis was carried out before and after pathogen exposition. The control and experimental plants of each variety showed identical genotypes at the locus *Xgwm112.1* before exposition. However, within 14 days of inoculation 58% of experimental plants had acquired an additional 81 bp allele at this locus. Such allele was not found in none of the control plants.

Moreover, this new allele was detected only in affected tissue; whereas the samples of healthy leaf from inoculated plants did not show the mutation. Cloning and sequencing of PCR products indicated that the new allele was generated by the reduction in the repetitions number of the dinucleotide (CT)n. This type of mutation was present in all treated varieties and absent in control plants not exposed to the head blight pathogen. The authors deduced that this microsatellital mutation was induced by the pathogen infection.

In our lab, we analyzed the effect of the 2p-ITC and BITC on the *A. alternata* genome by analyzing different ISSR *loci* (Troncoso-Rojas *et al.*, 2008, submittet to Physiological and Molecular Plant Pathology). It was found polymorphism in the exposed strains of *A. alternata* to the 2p-ITC and BITC, including variations in the number and size of alleles in different ISSR *loci*. In one experiment, the

amplification with the oligonucleotide $GACA_4$ found two alleles in the unexposed strains with sizes of 1.3 kb and 718 bp, whereas in the BITC exposed strain, it was not found the allele with 718 bp in size.

Another strains exposed to either 2p-ITC and BITC showed a different polymorphism profile as compared with the isogenic lines. For instance, two alleles with sizes of 603 y 350 bp were found in the exposed strain, but they were not present in the isogenic control lines. On the other hand, a new allele of 2.17 kb in size observed in the isogenic strain was not found in the exposed strains. The authors suggested that the ITC exposition induced the change in the alleles of the ISSR *loci* analyzed and described above.

Biochemical and genetic mechanisms involved in fungal resistance.

The ability of the necrotrophic pathogens to detoxify secondary metabolites may be an important factor in determining host specificity. This mechanism makes possible that species of *Alternaria* become specialized on a particular taxa. For example, *A. solani* are specific on tomato and potato, whereas *A. brassicae*, and *A. brassicicola* on *Brassicas*. In these fungi, ITCs may function as a stimulus to switch on genes required for pathogenesis, while more generalist specie such as *A. alternata*, lacks particular *Brassicas* compounds detoxifying enzymes (Giamoustaris and Mithen, 1997).

The tolerance of a particular pathogen to toxic compounds by detoxification result of degradative cellular activities that transform the defense compounds of the plant into less toxic substances (Osbourn, 1996). The degradative enzymes are very important for these fungi since they eliminate the effect of the host toxic compound allowing to the fungi infect the particular host. This was demonstrated in specific mutants generated by gene inactivation (Sandrock *et al.*, 1998). An example of those are mutants of the oat pathogen *Gaeumannomyces graminis*, unable to infect oat due

to a defect in the production of avenacinase, a detoxificant enzyme of the saponine avenacine, produced by that plant (Bowyer *et al.*, 1995).

Other mechanism of fungi tolerance to toxic compound is related with phase I and phase II enzymes (Sellam *et al.*, 2006), or with membrane integral proteins that transport toxic compounds, avoiding increased intracellular concentrations (Del Sorbo *et al.*, 2000). The effects of isothiocyanates had been studied in animals because of the evidence supporting their possible anticarcinogenic activity. In rats, the isothiocyanates induce and modulate the activities of Phase I and Phase II biotransformation enzymes which together catalyze a variety of hydrolytic, oxidative and reductive reactions (Phase I), whose products are available for conjugation reactions (Phase II) and excretion. The most important phase I enzyme are the cytochrome P450s which metabolize toxins, although it can also activate some carcinogens leading to tumours induction.

Glutation-S-transferases (GSTs) are highly conserved phase II enzymes implicated in pesticide resistance, xenobiotics tolerance and response to oxidative stress in plants, insects and fungi (Sheehan *et al.*, 2001; Burns *et al.*, 2005; Veal *et al.*, 2002). These enzymes catalyze the conjugation of glutathione to electrophilic substrates, producing compounds that are generally less reactive and more soluble, facilitating their removal from the cell via membrane-based glutathione conjugated pumps (Salinas and Wong, 1999). There is broad evidence from animal models, that certain isothiocyanates and their conjugates can inhibit the cytochrome P450 enzymes responsible for tumours induction. This is the reason by which the GST's induction is thougth to be protective (Mithen *et al.*, 2000). Evidence of coordinated induction of cytochrome P450 and GST in fungi was found treating *Streptopmyces griseus* with genistein (Dhar *et al.*, 2003).

The presence of glutathione conjugates could lead to the induction of transporters type ABC because they catalyze the elimination of these conjugates

from the cell. These transporters have an essential function in the secretion of specific and not specific host toxins, as a protection against the defense plant compounds and in fungicide resistance (Del Sorbo *et al.*, 2000). A typical fungal ABC transporter contains two intracytoplasmic regions, with nucleotide binding domains responsible for hydrolysis of ATP. Moreover they have two hydrophobic regions each including six transmembrane domains (TMD_6) (Higgins, 1992).

In yeast, there are a large number of genes regulating the resistance to toxic xenobiotics, and their products fall into the class of membrane transport proteins belonging to the ABC superfamily (Balzi and Goffeau, 1994). In *Sacharomyces cerevisiae*, the ABC transporters better characterized are those involved in multiple resistance to drugs. There are at least five ABC transporters located on the plasma membrane: Pdr5p, Snq2p, Pdr12p, Yor1p and Ycf1p (Bissinger and Kuchler, 1994). The overexpression of PDR5 causes resistance to hundreds of chemically not related drugs (Kolaczkowski *et al.*, 1996), including many classes of clinic antimycotic and agricultural fungicides like anilopirimidine, benzimidazols, dithiocarbamates, azols and strobilurin analogues.

In contrast, the inactivation of PDR5 causes hypersensibility to drugs and natural toxic compounds. Besides of the ABC, the Major Facilitator Superfamily (MFS) of transporters also prevents accumulation of toxic compounds, but their activity is driven by the proton motive force of membranes (Pao *et al.*, 1998). An MFS transporter usually has from 400 up to 800 amino acid residues arranged in 13 or 14 transmembrane domains with a molecular mass of 45 to 90 kDa (Del Sorbo *et al.*, 2000). There are several MFS families showing a high homology between members of the same family or among members from different families (Pao *et al.*, 1998).

Formerly, it was considered that the MFS transporters were only involved in the secretion of endogenous toxins like *Helminthosporium carbonum* toxin (Pitkin *et*

al., 1996), cercosporin of *Cercospora kikuchii* (Alexander, 1999) and trichotecenes of *Fusarium* spp. (Callahan *et al.*, 1999), protecting to the producing organism against these natural toxic compounds (Hayashi *et al.*, 2002). However, recent studies demonstrated that MFS transporters of *Candida albicans* and *S. cerevisiae* are also involved in protection against exogenous compounds like sterol demethylation inhibitors (Alarco *et al.*, 1997; Calabrese *et al.*, 2000).

Recently, studies in *Botrytis cinerea* found a MFS transporter, denominated Bcmfs1, playing a role in the tolerance towards both, natural toxic compounds and synthetic fungicides. Furthermore, this MFS transporter has a substrate specificity that overlaps with the ABC transporter BcatrD of *B. cinerea*. Adittionally, it was proposed that MFS transporters play a role not only as an efflux but also as influx transporters because of the increased sensitivity showed by Bcmfs1-overexpressing mutants to the presence of cycloheximide (Driessen *et al.*, 2000; Hayashi *et al.*, 2002).

Del sorbo *et al.* (2000), proposed that similar transporters are present virtually in all species of phytopathogenic fungi and stated that the regulation of genes encoding ABC and MFS transporters in filamentous fungi most likely will be an important topic of investigation in the future because of the possibility of treatment development based in the simultaneous utilization of transcription inhibitors of such genes and fungicides in strains showing multiple resistance. In fact, there are several blockers of mammalian and yeast ABC drugs pumps including FK506, propafenones and the antifungal drug terbinafine, able to reverse the transporter-mediated azole resistance in yeast and clinical isolates of *Candida albicans* (Schuetzer-Muehlbauer *et al.*, 2003).

Recently, it was found a potent inhibitor of the Pdr5p ABC transporter of *S. cerevisiae*, the isonitrile, a metabolite from a fungus belonging to the *Trichoderma* genus (Yamamoto *et al.*, 2005), which includes some important species in biological

control. This compound inhibited the Pdr5p-mediated efflux of cycloheximide and cerulenin in Pdr5p-overexpressing cells, without influencing the PDR5 gene expression and the amount of Pdr5 protein. However, the addition of isonitrile led to the intracellular accumulation of rhodamine 6G, a substrate for Pdr5p.

Detoxification of isothiocyanates by *Alternaria brassicicola*.

To identify candidate genes involved in pathogenicity of *A. brassicicola* on *Arabidopsis thaliana*, a suppression substractive hybridization (SSH) experiment was carried out, starting from conidia messenger RNA of the fungus germinated either on *Arabidopsis* leaf surface or in water (Cramer and Lawrence, 2004). It was found differentially expressed cDNAs encoding cyanide hydratase, arsenic ATPase, formate dehydrogenase and interestingly, a homologue of the major *A. alternata* allergene precursor alt a1, which was denominated Alt b1.

The fact that Alt b1 was preferentially expressed on spores germinating on the leaf surface of *Arabidopsis*, and not in spores germinating in water indicates that Alt b1 expression in *A. brassicicola* is controlled by specific environmental factors and may play a role in necrotrophic fungal pathogenesis of plant (Cramer and Lawrence, 2003; Cramer and Lawrence, 2004).

With the goal of investigate the mechanism involved in the detoxification of ITCs by *A. brassicicola*, germinated conidia of this fungus was exposed to 2p-ITC and BITC performing a differential expression protocol. The result was the isolation of the *AbGst1* gene, encoding a GST denominated AbGst1p (Sellam *et al.*, 2006). The expression of AbGst1p in *E. coli* showed high transferase activity with 2p-ITC and bencyl-ITC as substrates.

Additionally, the *AbGst1* upregulation during interaction with *A. thaliana* was demonstrated and it coincided with the beginning of the conidial germination, leaf tissues penetration and larger amount of fungal DNA, indicating that *A. brassicicola* germinating conidia were attempting to initiate the infection of the plant.

The promoter region of this *AbGst1* gene contains several putative cis-regulatory elements that are potentially activated by environmental stimuli to regulate transcription. Apparently, the isothiocyanate detoxification mechanism involving GSTs is remarkably conserved in fungi.

More recently, suppressive substractive hybridization (SSH) was carried out to generate a cDNA library from germinated conidia of *A. brassicicola* treated with 2p-ITC (Sellam *et al.*, 2007). The fungus response was an oxidative stress like, in such a way that 35% of genes transcriptionally induced by 2p-ITC are involved in the oxidative stress response: GSTs, glutathione peroxidases, glutamylcysteine synthetases, thioredoxins, thioredoxins reductasas, oxidoreductases and cytochrome P450. The response included also mechanisms oriented to limit the intracellular accumulation of the compound: 16% of cDNAs induced by ITC treatment encode transporter proteins, mostly PDR type ABC and MFS.

These results suggested that a redox imbalance occurs following the exposure to 2p-ITC, as a result of the depletion of the antioxidant GSH due to the reaction with ITCs. This leads to the activation of the conserved ITC detoxification mechanism mediated by phase I and phase II enzymes. With base on these results, it was suggested that the ITCs cause oxidative stress in fungus which could be the cause of the ITC toxicity.

CONCLUSIONS

Based on all the evidence reviewed, the polymorphism observed in the inter simple sequence repeats of *A. alternata* tolerant to the ITC could represent a mechanism to induce changes in gene regulation leading to the either increased or *de novo* expression of proteins involved in secretion and efflux of ITCs from the fungal cell as observed in *A. brassicicola*. The fact that both fungus belong to the same genus strongly suggest the presence of a similar mechanism for ITC detoxification.

Current studies of suppressive substractive hybridization carrying out in our lab will allow us to determinate what genes are being upregulated in *A. alternata* showing an ITC resistant phenotype. Scientific knowledge generated by studying the ITC detoxification mechanism of fungi will make possible to design treatments combining ITCs with inhibitors of such proteins, leading to a more effective fungi control.

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CAPÍTULO 3

Growth Kinetics of *Alternaria alternata* Tolerant to 2-Propenyl-Isothiocyanate on Potato Dextrose Broth.

María-Elena Báez-Flores, Rosalba Troncoso-Rojas, and Martín-Ernesto Tiznado-Hernández.

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RIAM Ms. 1215 - Recepción

De: **Martin Tiznado** (tiznado@ciad.mx)
Enviado: jueves, 22 de enero de 2009 07:30:48 p.m.
Para: MarÃa Elena BÃez Flores (elenabaezflores@hotmail.com)
CC: Maria Elena Baez Flores (ebaez@estudiantes.ciad.mx)

Mariel, te lo envio para tu información,

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Estimado **Dr. Tiznado**:

Muchas gracias por remitirnos su contribución titulada:

- **Growth kinetics of *Alternaria alternata* tolerant to 2-propenyl-isothiocyanate on potato dextrose broth**

Tan pronto como el comité editorial llegue a una decisión, recibirá más información.

Por favor, mencione el número de referencia de esta contribución **(1215)** en toda futura correspondencia relacionada con ella.

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Growth Kinetics of *Alternaria alternata* Tolerant to 2-Propenyl-Isothiocyanate on Potato Dextrose Broth

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RUNNING TITLE:

Alternaria alternata tolerant to 2-propenylisothiocyanate

SUMMARY:

The postharvest loses are mainly due to phytopathogenic fungi, being *Alternaria* spp. one of the most important. Isothiocyanates are being used to control fungal infections in horticultural products. However, *Alternaria alternata* can grow in

presence of 2-propenyl isothiocyanate (2p-ITC), but its growth kinetics is unknown. The objective of this work was to study the growth curve of *A. alternata* 2p-ITC treated and control fungus on potato dextrose broth to know the exponential growth phase occurrence. For both fungi, the exponential phase initiation was observed at day 2nd, whereas the stationary phase began at day 5th. Death phase in control fungus was observed by day 8th, whereas 2-pITC treated fungus continued growing.

KEY WORDS:

Alternaria alternata, 2-propenyl-isothiocyanate, growth curve, exponential phase

TÍTULO:

Cinética de crecimiento de *A. alternata* tolerant to 2-propenil-isotiocianato en caldo papa dextrosa

RESUMEN:

Las pérdidas poscosecha se deben principalmente a hongos fitopatógenos, siendo *Alternaria spp.*, uno de los mas importantes. En años recientes, los isotiocianatos se están utilizando para controlar infecciones fúngicas en productos hortícolas. Sin embargo, *Alternaria alternata* es capaz de crecer en presencia de 2-propenil isotiocianato (2p-ITC), aunque se desconoce su cinética de crecimiento y su tasa de crecimiento en medio sólido es controversial. El objetivo del trabajo fue estudiar la cinética de crecimiento de *A. alternata* tratada con 2p-ITC y del hongo control, en caldo papa dextrosa para determinar la ocurrencia de la fase exponencial. Para ambos organismos, la fase exponencial inició el segundo día y la fase estacionaria al día quinto. La fase de muerte para el control se observó al octavo día, mientras que el hongo tratado continuó su crecimiento.

PALABRAS CLAVE:

Alternaria alternata, 2-propenil isotiocianato, curva de crecimiento, fase exponencial

TEXT:

The postharvest infections on horticultural products are caused mainly by phytopathogenic fungi. Among them, the genus *Alternaria* spp is one of the most commonly found. This genus includes nearly 100 species of dematiaceous that occur worldwide [11]. Although several species are found in soil and dying plant tissue, the majority are plant pathogens that, collectively cause a range of diseases on a variety of crops [10]. In general, *Alternaria* species are foliar pathogens that cause a relatively slow destruction of host tissues by inducing necrotic lesions [1]. The main strategy to control agricultural fungal diseases is based in the use of synthetic chemicals compounds known to have adverse effects on the environment and consumer health.

In later years, with the objective to carry out a more environmentally friendly fungi control, it had been evaluated the use of natural compounds [9]. Among these, the isothiocyanates (ITCs) are the most promising alternative [6]. Manici et al. [5] reported a toxic effect of ITCs over several phytopathogenic fungi including *Alternaria alternata*. Smolinska et al. [12] studied the effect of 2-propenyl-ITC and ethyl-ITC on *Fusarium oxysporum* recording a 100% inhibition of mycelia growth as well as conidial and chlamydospores germination.

Similar results were found by Troncoso-Rojas et al. [14] using 2-propenyl-isothiocyanate (2-pITC) on *A. alternata*. However, in our lab it was observed that *A. alternata* can adapt and tolerate the 2p-ITC effect after a chronic exposition to sublethal concentrations, although showing a reduced growth rate. The growth of

filamentous fungi is a complex phenomenon which includes morphological, molecular and cellular changes depending on the organism, media composition, pH, incubation temperature and culture age [3].

Also, unusual culture conditions can lead to uncontrolled changes in the genome regulation [13]. Besides, the growth of fungi colonies on solid medium is complicated by gradients in substrate and product concentrations within the media and heterogeneity within the colonies. In contrast, liquid culture can provide well aerated and well mixed systems facilitating the estimation of biomass [8].

Morisseau and coworkers [7] reported a growth exponential phase for *A. alternata* fungus between days 2 and 6 of cultivation. However, there is not information available about the growth kinetics of *A. alternata* tolerant to 2-pITC in potato dextrose broth. We are planning to isolate by suppressive subtraction hybridization, the genes expressed in response to the ITC toxic effect in *A. alternata*. Because of this, it is important to study the growth curve of these fungi, to make sure that the treated and control fungi will be in a comparable physiological stage during sampling for genetic analysis. Otherwise, we could isolate genes induced by differences in developmental stage instead of genes induced by the 2p-ITC effect.

The objective of this work was to study the 2-pITC treated *A. alternata* growth behavior in potato dextrose broth to identify the exponential phase in both, the control and 2p-ITC treated fungus.

A. alternata was recovered from tomato (*Lycopersicum esculentum* Mill), purified by monospore isolation and maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). Conidial suspension with 3×10^3 conidium /mL was prepared from 15-day-old cultures. Erlenmeyer flasks containing 50 mL of

potato dextrose broth with 0.8% v/v of Tween 20 were inoculated with **a)** 44 µL of conidial suspension plus 50 µL of 2-pITC 9.7 µM (using acetone as solvent) or **b)** 44 µL of spore suspension plus 50 µL of pure acetone.

The flasks were covered with cotton plugs, gauze fabric and aluminum foil. Flasks were incubated in a shaker at 135 rpm during 10 days at 28°C and a light/dark cycle of 16:8 h. The mycelium was harvested separately from 8 flasks by filtration every 24 h, washed with distilled water and dried at 60°C for at least 24 h.

Statistical analysis was done based in a complete randomized design using the time as a blocking variable (SAS 8.01). The figure 1 shows the growth curve of 2p-ITC *A. alternata* treated and the control fungus. The results are the mean of 8 determinations in each sampling point with a standard error lower than 0.1%. No significant difference was found between the control and 2p-ITC treated fungus growth; both fungi showed a similar growth pattern, with an exponential phase going from 1-5 days.

The stationary phase for both fungi began at 5^{th} day, finishing at 9^{th} day for the control fungus, when it started the death phase. In the 2p-ITC treated fungus, we couldn't observe the death phase in the time elapsed in this experiment. The growth rate decrement is produced by the aging of the medium, the exhaustive consumption of nutrients and the accumulation of toxic subproducts [3]. The biomass eventually reaches a maximum value and the culture begins the stationary phase in which a relatively constant biomass is maintained through a balance between growth and autolysis. Thereafter, the autolysis dominates and the culture enters to the death phase [8].

During the growth time of *A. alternata* cultures, a great variability was observed in the number and size of the pellets formed in the culture. This can be ascribed to the hyphal growth behavior [3, 8]. Yanagita and Kogane [15], proposed the existence of four regions in the pellet: two external layers with viable hyphae, are surrounding the next one, which shows mycelium autolysis. In hollow pellets, there is a third layer with irregular walls, while the center contains unknowable mycelium. Dokmetzian and Rannalli [3], proposed that these factors lead to the formation of dissimilar areas of growing, affecting the biomass production.

Some authors have reported that in *A. alternata*, melanin is synthesized during the formation of conidia [2] to protect them from the UV light [4]. However, in the experiment melanin was observed without the presence of conidia in any culture.

The results of this experiment showed that the best sampling time for the 2-pITC tolerant and control fungus for genetics analysis could be between days 3-5, to make sure that the gene expression is due to the treatment, not to differences in the stage of fungal development.

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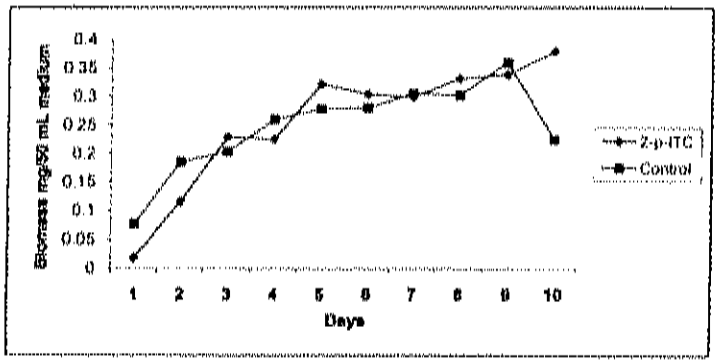
Figure 1.



Legend Figure 1

Figure 1. Growth curve of *A. alternata* treated with 2 propenyl-isothiocyanate and control fungus, in potato dextrose broth. Each point represents the mean of eight measurements with a standard error lower than 0,1%.

CAPÍTULO 4

cDNAs differentially Expressed in *Alternaria alternata* Tolerant to Lethal Concentrations of 2-Propenyl-Isothiocyanate.

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cDNAs from *A. alternata* ITC treated

cDNAs differentially expressed in *Alternaria alternata* tolerant to lethal concentrations of 2-propenyl-isothiocyanate

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Abstract: The development of resistant fungi strains to synthetic fungicides is an important problem in agriculture highlighting the urgent need of environmentally friendly alternatives to pest control. A promising alternative are natural products like

isothiocyanates (ITCs). Although ITCs have potent fungicide activity, it has been reported that *A. alternata* can tolerate lethal concentrations of 2-propenyl-isothiocyanate (2-pITC) after a chronic exposition. To isolate the differentially expressed genes in response to the ITC treatment, a forward suppressive subtractive hybridization (SSH) protocol was carried out using cDNA from *A. alternata* ITC treated as "tester" and cDNA from the untreated fungus as "driver". A total of 102 expresed sequence tags (ESTs) were obtained from the library, which generated 50 unigenes after the assembly (17 contigs and 33 singlets). Blastx analysis revealed that 38 and 40% of the unigenes had significant similarity to known and hypothetical proteins respectively, while 18% of the sequences did not show significant similarity to known genes. Clones with significant similarity to diverse fungal proteins like opsins, aromatic aminoacid transferases, nucleic acid binding proteins, helicases, ABC transporters, calmodulin, ATPases and S-nitroso glutathione proteins (SNOGs) were obtained. Additionally, the Blastn analysis results revealed significant similarity to clones of *A. brassicicola* induced under nitrogen starvation. Gene Ontology was applied to distribute the unigenes among the main GO categories. This tool provided GO terms for 46% from the unigenes set, assigning the 42% to biological processes, 40% to molecular functions and 18% to cellular component, which were mostly represented by cellular processes, binding activity and cytoplasmic component, respectively. Our results suggest that different processes and strategies integrate the defense response of *A. alternata* against ITCs. The ITC treatment induces the activation of genes encoding for proteins involved in a "non degradative" ITC remotion from the cell, in response to oxidative stress, in growth, and in repair and cellular structure maintenance.

Key words: 2-propenil- isothiocyanate, fungal response mechanism, natural fungicides, suppressive substractive hybridization.

INTRODUCTION

The genus *Alternaria* is widespread and of great economic importance, as it causes destructive leaf spots, blights (foliar and blossom), blemishes and damage to a great variety of fruits and seeds from numerous hosts. It belongs to the group of necrotrophic fungi, which represent about 4% of fungal diversity, but cause ~80% of foliar losses in some parts of the world (Rotem 1994). Like foliar pathogen, *Alternaria* spp. destroys the host tissues through the reduction of the photosynthetic potential (Agarwal et al 1997), while in stored products, this fungus causes quiescent infections penetrating the tissue in which it remains dormant until fruit conditions favour infection (Rotem 1994), being one of the most important potstharvest pathogens (Simmons 1992).

The indiscriminated use of synthetic agrochemicals to control agricultural fungal infections has led to the development of resistance in phytopathogenic fungal populations, which is one of the most important problems in agriculture. A good alternative to the chemical compounds utilization in fungal disease control are natural compounds like isothiocyanates. These compounds are part of the defense system of *Brassicas* plants and have inhibitory activity against bacteria, fungi, nematodes and insects. In the case of fungi, isothiocyanates have showed fungicide activity on different species under *in vitro*, *in vivo* and *in solum* conditions (Tiznado-Hernández and Troncoso-Rojas 2006).

Despite of the fact that the isothiocyanates have shown a strong inhibitory activity on fungi growth (Manici et al 1997, Mari et al 1993, Smolinska et al 2003, Troncoso-Rojas et al 2005, Troncoso et al 2005), including *Alternaria alternata*, preliminary experiments carried out in our laboratory, showed that this fungus is able to grow in presence of 2-propenyl-isothiocyanate (2p-ITC) after being chronically exposed to sublethal doses.

In order to study the response of *A. alternata* to the isothiocyanates, the effect of 2p-ITC and benzyl-isothiocyanate (BITC) on the inter simple sequence repeats regions (ISSR) of *A. Alternata* genome, was analyzed. There were found ISSR's polymorphisms in the strains exposed to 2p-ITC and BITC compared with the wild type. However, the microsatellite technique itself, do not reveals the specific genes or genetic regions that are being affected or changed, through ISSR polymorphism. Consequently, more information is needed to propose a possible defense mechanism used by the fungus to face the toxic effect of ITCs.

A suitable approach to gain insights into the molecular bases of the *A. alternata* defensive response is to study the transcriptomic response of the ITC tolerant fungus by looking for the differentially expressed genes in response to the ITC treatment. Genetic analyses of plant pathogen are important in understanding epidemiology, host-pathogen co-evolution, resistance management and control methods. It is expected that comparison of the genetic structure of fungicide-sensitive and resistant subpopulations within the same species could show the phenomena of evolution of fungicide resistance (Báez-Flores et al 2008, McDonald and McDermott 1993).

The development of knowledge about the fungi adaptation at genetic level will allow the designing of strategies to control fungi infections in a more effective and environmentally friendly manner.

Recently, the effect of 2p-ITC and BITC on *Alternaria brassicicola* (a specialized *Brassicas* pathogen) was evaluated. Differential display analysis led to the isolation of the first glutathione S- transferase (GST) gene from this fungus (Sellam et al 2006), designed *AbGSt1*, which was induced by the fungus in the presence of 2p-ITC and BITC. Furthermore, the authors observed that *AbGst1* was upregulated during the infection of *A. brassicicola* on *Arabidopsis thaliana*, a plant that shows natural resistance against this pathogen.

In a more recent work, suppressive subtractive hybridization technique (Chenchick et al 1998, Diatchenko et al 1996) was carried out to generate a cDNA library from germinated conidia of *A. brassicicola* treated with 2p-ITC (Sellam et al 2007). It was found that 35% of the genes transcriptionally induced by 2p-ITC are involved in the oxidative stress response: GSTs, glutathione peroxidases, glutamylcysteine synthetases, thioredoxins, thioredoxin reductases, oxidoreductases and cytochrome P450.

The response included also mechanisms aimed to limit the intracellular accumulation of the compound: 16% of cDNAs induced by ITC treatment encode transporter proteins, mostly pleitropic drug resistance type like ATP binding cassettes and major facilitator superfamily (MFS).

A. brassicicola is a specialized *Brassicas* (ITC's producer plants) pathogen, unlike *A. alternata,* which is a generalist pathogen, and consequently it was not expected that *A. alternata* could tolerate ITCs. In this regard, this organism

represents a good model to study the ITC effects in fungi not naturally exposed to these compounds. A good approach to gain insight in the *A. alternata* ITC tolerance mechanism is finding the differentially expressed genes in *A. alternata* treated with ITC by using subtractive suppressive hybiridization (SSH), a technique that allows find genes specifically expressed in response to a specific abiotic stress or treatment.

Because of the above mentioned, the present study was seeking to understand the molecular mechanism allowing *A. alternata* to grow in the presence of isothiocyanates.

MATERIALS AND METHODS

Fungal isolation and exposure to 2p-ITC.— *Alternaria alternata* was originally recovered from a field fruit of tomato (*Lycopersicum esculentum* Mill), purified by monospore isolation and maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 4°C. Fresh subcultures were made by transferring a disk of mycelium to a Petri dish containing 10 mL of PDA solid media covered with a cellophane membrane.

A disk of filter paper soaked with 9.7 µM 2p-ITC (Sigma-Aldrich Chemical, Co, Milwaukee, WI) was collocated in the lid of each dish. The dishes were then sealed with parafilm and incubated a 28°C during 5 days. The control fungus was inoculated and incubated in the same conditions except that the filter paper disk was soaked with distilled water. The mycelium of both fungi was harvested and maintained at -80°C until its use.

RNA extraction.— Whole-cell RNA from *A. alternata* ("tester" and "driver") was isolated according to an RNA extraction protocol specifically designed for

dematiaceous fungi (Islas-Flores et al 2006). This methodology avoided the coprecipitation of melanin with RNA. The method is a combination of the Trizol (Invitrogen, Carlsbad CA) and RNeasy Methods (Qiagen Biosciences, Maryland, USA).

Briefly, 0.25 g of mycelium was freezed in liquid N_2, crushed and suspended in 1 mL of trizol, incubated at 25°C for 5 min. Chloroform (0.2 mL) was added and the mixture was mixed by 15 seconds and incubated at room temperature during 5 min. Phases were separated by centrifugation at 12 000 *g* during 15 min at room temperature. The aqueous layer was removed into a fresh tube. After that, it was added 0.5 volumes of 100% etanol, mixed, transferred to a pink minicolumn (RNeasy kit, Qiagen), centrifuged at 8000 *g* for 15 seconds and the supernatant was discarded.

The column was washed three times with buffers provided by the manufacturer, and transferred to a new collection tube of 1.5 mL, and it was added with 30 µl of free RNase water, centrifugated to 8000 *g* for 1 min, and the eluate (30 µl), was recovered and passed again through the column by centrifugation at 8000 *g* for 1 min. The RNA was quantified in a Nano-Drop 1000 (Nano Drop Inc. Technologies, USA) and the RNA stability was checked by incubation at 42°C during two hours, and electrophoresed side by side with unincubated RNA and 0.24-9.5 kb RNA ladder (Invitrogen, Carlsbad CA).

Suppression subtractive hybridization protocol. – cDNAs were synthesized from 1 µg of total RNA isolated from *A. alternata* ITC treated and *A. alternata* control using the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA) following the recommendations of the manufacturer. The cDNA populations were then substracted

by the PCR-Select cDNA substraction kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The cDNAs from the treated fungus were used as "tester" and the cDNAs from the untreted fungus were used as "driver".

The efficiency of subtraction was evaluated by amplifying a fragment of the *A. alternata* β-tubulin gene after and before of the subtractive procedure using primers designed in our lab. The differentially expressed fragments obtained from SSH procedure were then ligated into pGEM-T vector (Promega, Madison, WI) and competent cells of *E. coli* JM109 were transformed to generate libraries of differentially expressed fragments. White/blue screening was done to identify recombinant clones.

Clones carrying DNA fragments were grown overnight in LB-Ampicillin to amplify the inserted clones. Plasmidic DNA extraction was performed by alkaline lysis method (Sambrook et al 1989). The presence and size of fragments in each putative recombinant clone was verified by digestion with *Rsa1* and electrophoresis in 1% agarose gel using the 1 kb plus DNA ladder (Invitrogen, Carlsbad CA) and ethidium bromide for DNA staining.

Clone sequencing, assembly and analysis.— The selected positive clones were all sequenced using the M13 Forward oligonucleotide in the Genomic Analysis and Technology Core Facility, University of Arizona, Tucson Arizona, U.S.A. The sequences obtained were analyzed to identify fragments of vector and adaptors origin, using the VecScreen program available in NCBI web site.

Foreign DNA fragments were eliminated from sequences, and assembled to eliminate redundancy by the CAP3 Sequence Assembler program (Huang and Maddan 1999). The resultant unigens and singlets were analyzed using the BLAST

program with the algorithms Blastn (Zheng Zhang et al 2000) and Blastx (Altschul et al 1990).

Gene Ontology annotation.— The annotation for the unigens and singlets was made with the Blast2GO v.2 program available at the Bioinformatics Department, Centro de Investigación Príncipe Felipe, Valencia Spain. Blast2GO is a bioinformatic tool for the automatic annotation of DNA or protein sequences data, mainly but not only, from non model species, based in GO vocabulary (Götz et al 2008). The primary goal of Blast2GO is to assign GO terms to nucleotide or protein sequences, grouping them in three basic categories: biological processes, molecular function and cellular component.

In this work, the initial blastx search was performed against the non redundant NCBI database with a minimum expectation value of 1×10^{-3} and a high scoring segment cut off of 33. The annotation step was made with default parameters: pre-evalue Hit filter was 1×10^{-6}, Annotation cut off was set in 55, and GO weight was 5. The annotation was expanded with ANNEX (Annotation Expander) and Blast2GO Iterpro scan was performed looking for additional GO terms associated with functional domains.

Gene expression level analysis by quantitative RT–PCR .— From the cDNAs differentially expressed in *A. alternata* 2p-ITC SSH library, six sequences putatively playing a role in the fungus tolerance to the ITC, were chosen to evaluate their expression levels at different times after 2p-ITC exposure. The RNA was isolated at 0.5, 1, 2 and 12 h after the treatment. This experiment was carried out with the Step One Real Time PCR System (Applied Biosystems, Foster City, CA) and the Full

Velocity SYBR Green QRT-PCR master mix 1 Step, according to the manufacturer's instructions.

The RT-PCR cycling program was: 50 °C for 30 min and 95 °C for 10 min (1 cycle); 95 °C for 10 s and 60 °C for 30s (40 cycles); 95 °C for 15 s and 60 °C for 1 min (1 cycle). A melting point analysis was carried out (cooling the samples to 60 °C and heating 0.3 °C S^{-1}) to assure that only the specific product was amplified. A single product at the specific melting temperature was found for each product. All samples were tested in triplicate. The β-tubulin gene was amplified in the same plate and used as endogenous control for normalizing the genetic expression level.

A relative comparison analysis was made using the comparative C_T method. Gene primers designed using the primer 3 software (Rozen and Skaletsky 2000) are shown in TABLE 1. The results of the expression experiments were analyzed by the software Rest©, which performs the pair wise fixed reallocation randomisation test, specifically designed for statistical analysis of relative expression results in RT-PCR real time (Pfaffl et al 2002).

RESULTS

As a result of the subtraction procedure, the differentially expressed fragments were in a range of 500 to 900 bp. After cloning them into pGEM-T vector, a total of 102 recombinant clones were obtained in a size range of 250 to 824 bp, with an average length of 477 bp. After sequencing, the assembly of sequences resulted in 50 fragments (17 contigs and 33 singlets). In a BLAST performed against the *A. brassicicola* ESTs collection (Blastn algorithm), 20% of our ESTs showed similarity

to genes induced under nitrogen starvation and, one sequence (Aaitcas10) was similar to clone altr304xn06 (GB|DN477570.1|) obtained from *A. brassicicola* mycelial culture infecting *Brassica napus*, an ITC producer plant (Cramer et al 2006). Additionally, one EST (Aaitc150) was similar to clone CmxP2E8 (gb|DY543110.1|) obtained from *A. brassicicola* conidia exposed to camalexin (Sellam et al 2007). Another EST was similar to cDNA clone P3G9 (gb|CA405384.1|) obtained from spores germinating on *Arabidopsis* leaf surface (Cramer and Lawrence 2004).

In the BLAST performed against the nonredundant GenBank CDS database (Blastx algorithm), 38% of unigenes represented in ESTs, showed significative similarity ($\leq$ to 1e-^{06}) to diverse known proteins, mostly from *Pyrenophora tritici repentis*; 40% were very similar to hypothetical SNOGs and PTRG proteins from *Phaeosphaeria nodorum* and *P. tritici repentis* respectively; 4% of the sequences showed similarity with proteins not reported in fungi and 18% correspond to unknown genes (results are summarized in TABLE 2).

Annotation and GO analysis.— The Blast2GO annotation provided $\geq$ 1 GO terms for 23 sequences (46%) from the total set. A total of 106 GO terms were retrieved, of which 45 (42.4% of the total) could be assigned to biological processes, 43 (40.5% of the total) to molecular functions and 18 (17%) to cellular components. Figure 1A shows the biological processes graph. The major categories (at level 2) were cellular process (GO: 0009987), metabolic process (GO: 0008152), developmental process (GO: 0032502) and multicellular organismal process (GO: 0032501).

Increasing the ontology level (to level 4), the cellular process was populated by ribonucleoprotein complex biogenesis and assembly (GO: 00226013), organelle

organization and biogenesis (GO: 006996), and gene expression (GO: 0010467), while metabolic process was integrated mostly by primary metabolic process (GO: 0044238), protein metabolic process (GO: 0019538), cellular macromolecule metabolic process (GO: 0044260), macromolecule biosynthetic process (GO: 0009059) and biopolymer metabolic process (GO: 0043883).

Developmental process was found represented by mycelium development (GO: 0043581). Interestingly, the EST Aaitc161 was annotated with GO terms related to xenobiotics response: GO: 009410 and GO: 0006805 (response to xenobiotic stimulus and xenobiotic metabolic process respectively), while another EST (Aaitc1A) was annotated with GO terms related to detection of abiotic stimulus (GO: 009582).

In molecular functions (FIG. 1B) the major categories were binding (GO: 0005488), catalytic activity (GO: 0003824) and structural molecule activity (GO: 0005198). Increasing the ontology level (to level 4, data not shown) the binding activity could be related mostly to, cation binding (GO: 0043169), metal ion binding (GO: 0046872), purine nucleotide binding (GO: 0017076), RNA binding (GO: 0003723), ribonucleotide binding (GO: 0032553), and in a small proportion to pyridoxal phosphate binding (GO: 0030170), and heme binding (GO: 0020037).

The catalytic activity was represented (in the higher ontology level 4) mostly by hydrolase activity (GO: 0016817, GO: 0016788), transferase activity (GO: 0016769, 0016741) and oxidoreductase activity (GO: 0016627).

RT-PCR analysis.— Among the six genes evaluated after the 2p-ITC treatment, four showed significant up-regulation ($p<0.05$; pair wise fixed reallocation randomisation

test): opsin, Ca^{++}ATPase, EF-hand protein (calmodulin), and ABC transporter (TABLE 3). The expression of the opsin gene was increased to 1.39 fold at 0.5 h after the treatment, while the expression of Ca^{++} ATPase was increased in all treatments, showing its maximum expression at 1 h with a fold change of 6.24, which decreased to 3.92 and 1.5 folds at 2 and 12 h, respectively. The cDNA corresponding to calmodulin showed significant increment at 0.5, 1, y 2 hours after the treatment (1.48, 2.29 and 1.92 fold change, respectively).

A marked increment of expression was observed in the ABC transporter with a notable fold change of 95.8 recorded at 0.5 h, which dropped to 25.6 and 3.8 fold at 1 and 2 hours, respectively. After 12 h of treatment, the ABC transporter cDNA expression level recorded a modest 1.3 fold change, compared with the expression recorded at 0.5 and 1 h. The ATPase level expression change is markedly minor to the ABC transporter fold change. However, it was constantly expressed in a significant way, without shows an abrupt decrement along different treatments. The ABC transporter decreased markedly its strong expression level after 2 hours of treatment. This could be related to the half life of these proteins. It had been reported that some ABC transporters have a half live of five days (Wakabayashi et al 2006). No significant expression changes were recorded for SNOG and 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase cDNAs.

DISCUSSION

In the *A. alternata* substractive cDNAs library we found sequences playing central roles in the post-transcriptional regulation of gene expression (RNA binding domains), and several cDNAS representing membrane integral proteins (e.g. amino

acid permeases, ABC multidrug CDR4 transporter, methotrexate protein resistance, opsin, ATPases and fumarate reductase). They are involved in amino acid transport (Hosie et al 2002); efflux of toxic natural and chemical compounds (Del Sorbo et al 2000); synthetic fungicide resistance (Brôco et al 1999); light-driven H^+ pumping action (Bieszke et al 1999) and in homeostasis's maintaining of the intracellular calcium concentration (Vanoevelen et al 2005).

The ITC treatment may perhaps directly impact nitrogen utilization in *A. alternata*, as suggested by the similarity (83-98% at nucleotidic level) showed by 20% of our ESTs with *A. brassicicola* clones obtained under nitrogen starvation conditions. Reinforcing this hypothesis, we found differentially expressed permeases and, it has been reported that *Saccharomyces cerevisiae* has permeases that are highly expressed under conditions of nitrogen limitation and are thought to scavenge amino acids to use them as nitrogen source (Magasanik and Kaiser 2002). Perhaps this can be related to the need of new amino acids or glutathione synthesis. However, more research should be conducted to firmly link the two processes.

An explanation for the induction of the ABC transporter in 2p-ITC *A. alternata* transcriptome is that in fungi, these transporters have an essential function in the secretion of specific and unspecific host toxins, as a protective strategy against the plant defense compounds, as well as in fungicide resistance (Del Sorbo et al 2000). In addittion, some ABC transporters seems to efflux xenobiotics using a cotransport mechanism with reduced glutathione (Leslie et al 2001).

The benomyl methotrexate resistance protein, is a MFS multidrug transporter conferring resistance against the antimitotic systemic benzimidazole fungicide benomyl and the antitumor agent methotrexate (Brôco et al 1999). Members of the

MFS family have been reported in 2p-ITC treated *A. brassicicola* together with ABC transporters. Furthermore, MFS and ABC transporters have been reported in camalexin treated *A. brassicicola* transcriptome besides alcohol dehydrogenase, oxidoreductases, and putative cation efflux transporter, as part of a defense mechanism against oxidative burst and stress caused by these compounds (Sellam et al 2007).

There is evidence that opsin gene expression has a light and conidiation-based regulation in *N. crassa*. Opsins bind to retinal to form the light absorbing pigments known as rhodopsins. Archael rhodopsins (which are different of visual rhodopsins) function in light activated ion pumping and phototaxis. In fungi, diverse stress conditions as anaerobic growth, and nutrient or water stress, affect the opsin gene expression (Bieszke et al 1999). We hypothesize that the ITC treatment impose an important stress to *A. alternata* mycelium, and as result, the conidiation could begins involving the opsine gene expression. However, a clear role for opsins in fungal biology remains to be elucidated (Idnurm and Howlett 2001), what is a statement further supported by the data generated in this work.

Fungal ATPases are all integral membrane proteins E_1-E_2 (P-type) pumps, that hydrolyzes ATP and translocate cations across the membrane (Bowman et al 1988). They working maintaining the adequate high calcium concentration in the lumen of the intracellular organelles, as a source of activator Ca^{++} for cytosolic processes; proper transcription, translation, translocation, folding, and processing of secreted proteins (Corbett and Michalak 2000). Also, ATPases play an important role removing the activator Ca^{++} from the cytosol and decreasing its cytoplasmic

concentration to basal levels after different external stimulus. Moreover, the ATPases function maintains the plasmatic membrane asymmetry (Vanoevelen et al 2005).

In addition, we found ESTs similar to protein participating in the control of different calcium signaling pathways (EF-hand Ca modulated protein, known as calmodulin superfamily). Calmodulin affects cellular events such as gene transcription, protein phosphorylation, nucleotide metabolism, and ion transport (Ikura and Ames 2006).

Our ESTs set included some sequences coding for proteins involved in protein transport between the endoplasmic reticulum and Golgi compartments (coatomer subunit alpha), (McMahon and Mills 2004), as well as for RNA helicase eIF4A, and for elongation factor-1 alpha, which are essential components of the eukaryotic translational apparatus involved in the binding of mRNA and aminoacyl-transfer RNAs to the ribosome, respectively (Rogers et al 2001, Tatsuka et al 1992). Helicases differentially expressed have been reported in *Plasmodium falciparum* resistant to chloroquine; indeed, it was observed that the strains able of produce high helicase levels, repaired the damage caused by chloroquine (Thelu et al 1994).

Moreover, we found ESTs that code for polypeptides similar to cellular protective enzymes against the toxic compound acetaldehyde (aldehyde dehydrogenase) (Perozich et al 1999); ESTs with similarity to proteins involved in the biosynthesis of amino acids (aromatic aminotransferases) (Ward et al 2002); ESTs similar to a protein that plays a critical role in gene-specific transcription regulation, DNA damage response and repair, as well as DNA replication (histone acetyltransferase MYST2) (Avvakumov and Côté 2007). Also, we found cDNAs with similarity to proteins involved in the methionine biosynthesis (Huang et al

2007). Finally, several of the ESTs and assemblies were highly similar to hypothetical SNOGs proteins.

The similarity of some isolated cDNAs to SNOG's proteins could imply the occurrence of stress oxidative. SNOGs proteins have an important role in diverse organisms as nitric oxide donors and are involved in protection against oxidative stress (Rosenberg et al 1999) and apoptosis (Itoga et al 1997). In mammals, NO plays a role as a neurotransmitter, physiological regulator and in host defense. Furthermore, NO regulates transcription factor activation, mutagenesis, apoptosis, protein acylation, glycolysis, mitochondrial electron transport chain and deoxynucleotide synthesis, among others (Chandran et al 1998).

In rat brain cells, there is evidence suggesting a protective role for nitric oxide in oxidative stress injury and exists the possibility that intracerebral nitric oxide production could be part of a defense mechanism against oxidative stress in these cells. Remarkable, nitric oxide appears have the potential to cause and protect against injury (Rosenberg et al 1999) at high and low concentrations respectively (Chandran et al 1998). Furhermore, the toxic or protective effect of nitric oxide appears to be regulated by the intracellular redox status.

Exists the possibility that nitric oxide causes the activation of a crucial protective factor or the inactivation of a crucial death factor in the setting of oxidative stress (Rosenberg et al 1999). Among the possible mechanisms suggested are the inhibition of glyceraldehyde 3-phosphate dehydrogenase (G3PDH), whose activity is necessary in one paradigm of neuronal apoptosis (Itoga et al 1997), or the induction of protective proteins (Kim et al 1997).

In *C. heterostrophus*, *S. nodorum*, and *A. nidulans*, orthologues of SNOGs genes are related to the synthesis of transport compounds and oxidative stress protection (Hane et al 2007). SNOGs of *C. heterostrophus* and *S. nodorum* appears to be directly related to *NPS4*, *psy1* (non ribosomal peptide synthase), from *A. brassicae* (Guillemette et al 2004) and *NPS2* from *A. brassicicola* (Kim et al 2007). *A. brassicicola* NPS2 gene is predicted to encode a component of the conidial wall.

Some SNOGs genes coding for G-alpha proteins are involved in the intracellular signals transduction (Solomon et al 2004). In mammals, there are SNOG consensus sequences downstream of the TATA box in genes involved in immune response, suggesting that in the context of these genes, the SNOG element may be used to modulate gene expression in cells under some conditions (Weber and Skene 1997). In *A. alternata*, the stress imposed by ITC may perhaps modulate the induction of SNOGs as a protective mechanism. Thus, in mammals the endothelial and the neuronal NO synthases are constitutively expressed enzymes that, upon stimulation, synthesize NO in a pulse controlled Ca^{+2}/Calmoduline mechanism (Kleinert et al 2000), which could explain the simultaneous induction of SNOGs, ATPases, and calmodulin proteins in *A. alternata* ITC treated.

Altogether, data generated in this work seems to suggest the involvement of Ca as second messenger during the signal transduction activated in response to ITC. Consistently with the mentioned results, the GO analysis revealed that cellular process, metabolic process and developmental process were the major categories corresponding to biological processes; while binding activity, catalytic activity and structural molecule activity were the major categories in molecular functions. The binding activity was mostly populated by cation, purine, and RNA binding activity,

reflecting the ATPase's, calmoduline and RNA binding domains activity. Moreover, some cDNAs coding membrane integral proteins and transcriptions factors, whose functions are related to the transport of diverse molecules across membranes and to transcription initiation or elongation, respectively.

These molecules perform their action by recognizing and binding to the target site. Because of this, binding activity and catalytic activity were the major classes in molecular function categories. The catalytic activity was mostly represented by hydrolase, transferase, and oxirreductase activity, reflecting the fungal response to the stress imposed by the toxic compound 2p-ITC.

CONCLUSSIONS

In our knowledge, the SSH protocol was used for first time to identify genes involved in the response to ITC treatment in *A. alternata*. This technique allowed us to isolate genes without previous knowledge of their sequences. Few ESTs of *A. alternata* have been reported and therefore it was not unexpected that some genes isolated in the present work were novel. Previous SSH studies on fungi had reported many unknown genes (Carpenter et al 2005, Wu et al 2008). The novel genes in the *A. alternata* SSH library represent an important contribution because these genes and their products are still unknown in *A. alternata* and other fungi.

By the other hand, it is remarkable that 20% of *A. alternata* ITC clones share high similarity to clones of *A. brassicicola* (a fungus whose genome sequence had just been completed) grown under nitrogen stress conditions (Cramer et al 2006). Both fungi belong to the same genus and it is logic to expect that these fungi share an

important part of their genetic structure, although marked genetic differences should also exist due to the intrinsic interspecies variation.

Our results suggest that different processes and strategies are working against the toxic ITC effect in *A. alternata*, whose concerted action contributes to the successful growth of *A. alternata* in presence of the toxic compound 2p-ITC. The fungal response seems to be focused in the induction of "non degradative" mechanisms for ITC remotion from the cell (integral proteins of membrane e.g. ABC and MFS transporters and, ATPases), in response to oxidative stress (SNOGs) and in DNA damage response and repair, structural maintenance and growth of hypha (e.g. permeases, calmodulin, histone acetil transferases, helicases, and ribosomal proteins). Thus, the remarkable increasing in the expression of the cDNA corresponding to the ABC transporter suggests the efflux as the major ITC detoxification mechanism in *A. alternata*.

Our unigens collection is small, but represents an important molecular resource providing valuable insights to gain a better understanding of the molecular mechanisms by which *A. alternata* and other fungi are successfully facing the challenge imposed by the use of natural fungicides.

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TABLE 1. Primer sets designed for quantitative RT-PCR experiments (Scale 100nM, purification grade PAGE).

Gene name	Forward primer sequence	Reverse primer sequence
B- tubulin	ACGCTTCTCATCTCCAAGATCCGT	AGAGAGCCTCGTTGTCAATGCAGA
Aaite1A	TGGGCGTCCAATCCCGTAACTATT	TACGCGGCATGTGTCTGTTTCCTA
Aaite65	ATTACCTTCATTGGGCTGGTGGGA	TAGGTAACCGTCGAACAATGGCGT
Aaite128	TTAGCCTCGAGGAACTCAGCAACA	AACTACCACTACGTCAAGCCCACA
Aaite 141	TGCGGCTTATCAGCTCTTCACTCA	TCCGACATCTTTGCCCTGACCTTT
Aaite168	AATGGCCGATGTGAAGTTCATGCC	TCTCCAAGTTCGCCAACGATGTCA
Aaitcas6	AACCGACGATGAGAGCGATGACAA	AATACACGACGAGCATACCAGCGT

TABLE 2. Sequence analysis of the SSH cDNA from *Alternaria alternata* 2p-ITC treated library. Blast results of searching in the nonredundant GenBank database (E-value < 1e – 05).

Contig/singlet	GenBank Accession Number	Size	Best Alignment Similar to/Organism/Accesion number	E value
Aaitcas1	EZ000322	496	Hypotetical protein PTRG_03541 *Pyrenophora tritici-repentis* XP_001933874.1	3e-35
Aaitcas2	EZ000323	636	**RNA binding domain** *P. tritici-repentis* XP_001938447.1	6e-83
Aaitcas3	EZ000324	475	hypothetical protein SNOG_00220 *Phaeosphaeria nodorum* XP_001790911.1	8e-66
Aaitcas4	EZ000325	453	**amino-acid permease inda1** *P. tritici-repentis* XP_001931109.1	9e-76
Aaitcas5	EZ000326	732	hypothetical protein *Vitis vinifera* CAN70790.1	9e-23
Aaitcas6	EZ000327	512	**ABC multidrug CDR4** *P. tritici-repentis* XP_001941554.1	4e-69
Aaitcas7	EZ000328	465	hypothetical protein PTRG_03541 *P. tritici-repentis* XP_001933874.1	1e-22
Aaitcas8	EZ000329	351	**coatomer subunit alpha** *P. tritici-repentis* XP_001932333.1	9e-49
Aaitcas9	EZ000330	585	hypothetical protein SNOG_00983 *P. nodorum* XP_001791644.1	1e-41
Aaitcas11	EZ000332	824	conserved hypothetical protein *P. tritici-repentis* XP_001934661.1	8e-32
Aaitcas12	EZ000333	656	conserved hypothetical protein *P. tritici-repentis* XP_001935771.1	1e-68
Aaitcas13	EZ000334	545	hypothetical protein SNOG_02797 *P. nodorum* XP_001793392.1	9e-47
Aaitcas15	EZ000336	721	conserved hypothetical protein *P. tritici-repentis* XP_001941363.1	8e-34
Aaitcas16	EZ000337	536	hypothetical protein PTRG_00335 *P. tritici-repentis* XP_001930668.1	1e-38
Aaitcas17	EZ000338	538	**elongation factor 1-alpha** *P. tritici-repentis* XP_001930631.1	7e-84
Aaitc1A	GE467965	508	**opsin-1** *P. tritici-repentis* XP_001937307.1	9e-39
Aaitc6A	GE467968	291	**benomyl/methotrexate resistance protein** *P. tritici-repentis* XP_001937580.1	2e-37
Aaitc11A	GE467971	729	**aldehyde dehydrogenase** *P. tritici-repentis* XP_001933942.1	2e-116
Aaitc15A	GE467974	345	**aromatic amino acid aminotransferase I** *P. tritici-repentis* XP_001941234.1	1e-57

TABLE 2 continued

Anitc27	GE467984	378	hypothetical protein SNOG_04093 *P. nodorum* XP_001794520.1	9e-45
Anitc29	GE467986	300	**60S ribosomal protein L18ae** *P. tritici-repentis* XP_001935145.1	8e-30
Anitc33	GE467988	332	hypothetical protein SNOG_03478 *P. nodorum* XP_001794041.1	8e-30
Anitc65	GE468002	318	**calcium-transporting ATPase 1** *P. tritici-repentis* XP_001942035.1	1e-46
Anitc68	GE468003	317	conserved hypothetical protein *P. tritici-repentis* XP_001941608.1	2e-11
Anitc101	GE468006	611	**histone acetyltransferase MYST2** *P. tritici-repentis* XP_001941960.1	6e-84
Anitc108	GE468013	609	**60S ribosomal protein L21-A** *P. tritici-repentis* XP_001937444.1	5e-63
Anitc110	GE468015	409	**40S ribosomal protein S18** *P. tritici-repentis* XP_001940789.1	2e-37
Anitc121	GE468021	532	hypothetical protein PTRG_06153 *P. tritici-repentis* XP_001936486.1	4e-48
Anitc126	GE468024	491	hypothetical protein Bm1_17870 *Brugia malayi* XP_001895031.1	3e-17
Anitc127	GE468025	486	hypothetical protein SNOG_05837 XP_001796232.1	8e-48
Anitc128	GE468026	343	**5-methyltetrahydropteroyltriglutamate-homocysteine** methyltransferase *P. tritici-repentis* XP_001934603.1	8e-54
Anitc141	GE468202	383	**EF-hand superfamily Ca2+-modulated protein** *P. tritici-repentis* XP_001936278.1	3e-27
Anitc143	GE468204	508	hypothetical protein SNOG_10318 *P. nodorum* XP_001800593.1	2e-07
Anitc145	GE468205	453	**ATP-dependent RNA helicase eIF4A** *P. tritici-repentis* XP_001938082.1	1e-64
Anitc148	GE468207	393	hypothetical protein SNOG_15237 *P. nodorum* XP_001805396.1	2e-48
Anitc150	GE468208	757	hypothetical protein SNOG_02797 *P. nodorum* XP_001793392.1	5e-54
Anitc152	GE468210	629	conserved hypothetical protein *P. tritici-repentis* XP_001932994.1	8e-86

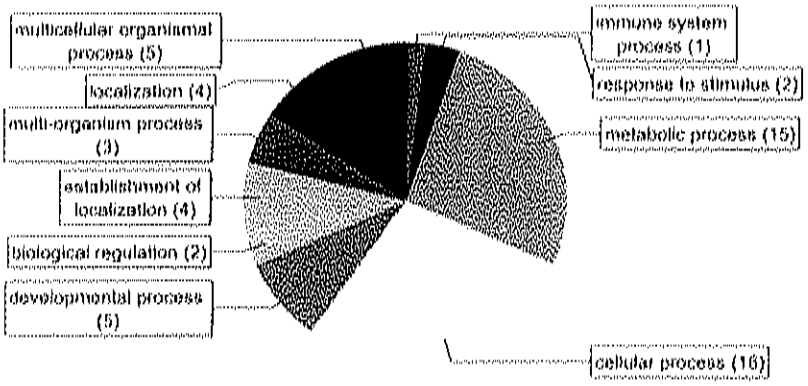
TABLE 2 continued

Aaltc154	GE468711	405	**aromatic amino acid aminotransferase I** *P. tritici-repentis* XP_001941234.1	2e-56
Aaltc161	GE468212	486	**fumarate reductase** *P. tritici-repentis* XP_001937985.1	5e-53
Aaltc168	GE468213	806	hypothetical protein SNOG_14270 *P. nodorum* XP_001804465.1	7e-111
AAltc227	GE468215	643	hypothetical protein PTRG_00774 *P. tritici-repentis* XP_001931107.1	1e-60

TABLE 3. Time-course expression of six gens in *A. alternata* 2 p-ITC treated measured by quantitative real time reverse transcription-PCR using the comparative C_T method. The gene transcription in each sample was normalized using the C_T values from the β-tubulin housekeeping gene. The fold change represents the number of times that one gene is expressed compared with the reference sample (untreated control). Results are the mean of three repetitions indicating the standard deviations. The ΔC_T values statistically different ($p < 0.05$; pair wise fixed reallocation randomization test), from that obtained for the control fungus, are shown in bold numbers.

	Fold change RQ ($2^{-\Delta\Delta CT}$) ± SD			
Genes	**30 min**	**1h**	**2h**	**12h**
opsin	**1.39** ± 0.05	0.64 ± 0.03	0.13 ± 0.12	0.62 ± 0.00
Ca^{++}ATPase	**2.67** ± 0.4	**6.24** ± 0.15	**3.92** ± 0.23	**1.50** ± 0.19
$5MeH_4$-PteGlu^n	0.22 ± 0.28	0.25 ± 0.20	0.76 ± 0.13	0.61 ± 0.04
EFhand protein	**1.48** ± 0.20	**2.29** ± 0.28	**1.92** ± 0.26	0.42 ± 0.13
SNOG protein	1.54 ± 1.0	0.81 ± 0.90	1.12 ± 0.93	1.87 ± 0.61
ABC transporter	**95.8** ± 0.00	**25.6** ± 0.04	**3.85** ± 0.04	1.38 ± 0.03

FIGURA 1. Distribution of Gene Ontology Terms (Level 2), for transcripts obtained from *Alternaria alternata* tolerant to 2p-ITC. The numbers in graph categories, show the frequency for each GO term in the unigenes set.
A. Biological Process. **B.** Molecular Function.



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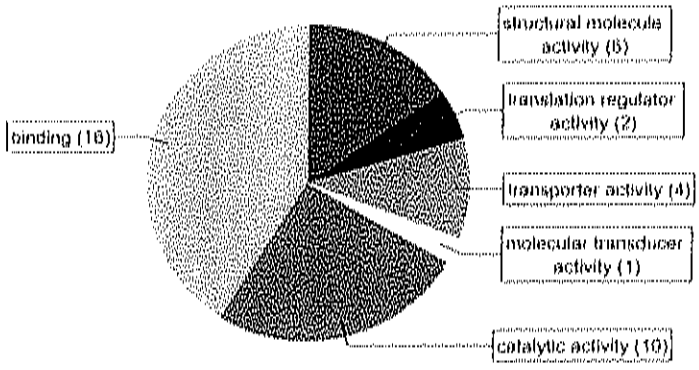

B

APÉNDICE I

National Center for Biotechnology Information. 2008. Expressed Sequence Tags. Differentially expressed cDNAs from *Alternaria alternata* treated with 2-propenyl-ITC.
Báez-Flores, M.E., Troncoso-Rojas,R., Tiznado-Hernandez, M.E., Islas-Osuna,M.A., Rivera-Dominguez, M. and Pryor, B.

Secuencias Expresadas y Números de Acceso en GenBank

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THORS     Baez-Flores,M.E., Troncoso-Rojas,R., Tiznado-Hernandez,M.E.,
          Islas-Osuna,M.A., Rivera-Dominguez,M. and Pryor,B.
TLE       Differentially expressed cDNAs from Alternaria alternata treated
          with 2-propenyl-ITC
URNAL     Unpublished (2008)
NT        On Oct 17, 2008 this sequence version replaced gi:209693395.
          Contact: Baez-Flores, ME
          Physiology and Molecular Biology of Plants Laboratory
          Centro de Investigacion en Alimentacion y Desarrollo A.C.
          Carretera a la Victoria Km 0.6, Hermosillo Sonora, CP 83000, Mexico
          Fax: +52-662-280 04 22
          Email: ebaez@estudiantes.ciad.mx; elenabaezflores@hotmail.com
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                    strains adapted to lethal levels of
                    2-propenyl-isothiocyanate according to the protocol
                    published by Islas-Flores et al., 2006 (Molecular
                    Biotechnology 34(1): 45-50). The cDNAs were prepared using
                    the SMART PCR cDNA synthesis kit and subtracted by SSH
                    procedure (Clontech, Palo Alto CA). The differentially
                    expressed cDNAs were cloned into p-GEM-T Easy vector and
                    cells of E. coli JM109 were transformed with them
                    (Promega, Madison, WI). Rsa I was used to digest the
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recombinant clones and verify the presence and sizes of the inserted fragments. Sequenciation of fragments was carried out using using the M13 forward primer in the Genome Analysis and Technologic Core Facility of the University of Arizona at Tucson, AZ."

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Last update: Mon, 12 Jan 2009 Rev. 149544

APÉNDICE II

DNA Data Bank of Japan. Transcriptome Shotgun Assembly. 2008. Genome Project: 32537. cDNAs from *Alternaria alternata* Tolerant to 2 Propenyl-ITC. Báez-Flores, M.E., Troncoso-Rojas,R., Tiznado-Hernandez, M.E., Islas-Osuna,M.A., Rivera-Dominguez, M. and Pryor, B.

Números de Acceso de Assemblies en Transcriptome Shotgun Assembly, DNA Data of Japan.

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  AUTHORS   Baez-Flores,M.Elena., Troncoso-Rojas,R.,
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            Tiznado-Hernandez,M.Ernesto. and Pryor,B.
  TITLE     cDNAs from Alternaria alternata tolerant to 2 propenyl-ITC
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 824)
  AUTHORS   Baez-Flores,M.Elena., Troncoso-Rojas,R.,
            Islas-Osuna,M.Auxiliadora., Rivera-Dominguez,M.,
            Tiznado-Hernandez,M.Ernesto. and Pryor,B.
  TITLE     Direct Submission
  JOURNAL   Submitted (31-OCT-2008) Coordinacion Tecnologia de Alimentos de
            Origen Vegetal, Centro de Investigacion en alimentacion y
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APÉNDICE III

Construcción y Resultados de la genoteca substractiva de cDNA's.

Construcción de la genoteca sustractiva de cDNA's

La genoteca substractiva se construyó a partir de 1 µg de RNA total de *Alternaria alternata* tratada con 2-propenil-isotiocianato (**Tester**) y 1 µg de RNA total de *A. alternata* testigo (**Driver**), utilizando los protocolos "SMART" ™ PCR cDNA Synthesis Kit" y el "PCR Select cDNA Subtraction Kit" (Clontech, Palo Alto, CA), siguiendo el procedimiento detallado a continuación.

Síntesis de la Primera Cadena de cDNAs de muestras de *Alternaria alternata* y el control "Smart" (Control para seguimiento protocolo SMART).

Se ajustó la concentración del RNA total de las muestras a 1.0 µg/µL y se prepararon 3 tubos (0.5 mL) para PCR etiquetándolos como "Tester", "Driver" y "control Smart" respectivamente, adicionando a cada uno los siguientes reactivos:

Reactivos	**Tubo 1 Tester**	**Tubo 2 Driver**	**Tubo 3 Control Smart**
RNA (1.0 µg/µ)	1 µl	1 µl	1 µl
3'SMART CDS primer IIA (12 µM)	1 µl	1 µl	1 µl
Oligonucleótido "SMART IIA" (12 µM)	1 µl	1 µl	1 µl
Agua destilada estéril	2 µl	2 µl	2 µl
Volumen total	5 µl	5 µl	5 µl

Se mezcló cada tubo por pipeteo y se incubaron a 70 °C por 2 minutos en el termociclador (Applied Biosystems; Gene Amp PCR system 9700). Posteriormente a cada tubo se le adicionó lo siguiente:

5X buffer primera cadena	2 µl
DTT (20 mM)	1 µl
Mezcla de dNTps (10 mM)	1 µl
Power Script RT (100 u/ µl)	1 µl

Nuevamente se mezcló por pipeteo cada muestra, se centrifugó y se incubó durante 1 h en el termociclador a 42 °C.

El producto de cada reacción de primera cadena fue diluído con 40 µl de buffer TRIS-EDTA (TE) y las diluciones se incubaron a 72 °C durante 7 min. Una vez terminada la reacción, las muestras fueron almacenadas a -20 °C.

Síntesis de la segunda cadena de cDNAs de muestras de *Alternaria alternata* y control "Smart".

Antes de sintetizar la segunda cadena de cada muestra y el control "Smart", se procedió a determinar el número óptimo de ciclos. La determinación del número óptimo de ciclos asegura que la reacción de síntesis de ADNc permanezca en la fase exponencial de amplificación. Cuando la intensidad de la banda de los productos de PCR no se incrementa al incrementar el número de ciclos, la reacción ha alcanzado su fase estacionaria. Un producto de PCR sobrecielado es un sustrato pobre para la substracción, mientras que un número escaso de ciclos ocasionará un bajo rendimiento del producto de PCR. El número óptimo de ciclos es un ciclo menos que aquél en que se logran distinguir claramente los productos de PCR y, la intensidad de la banda, ya no se incrementa al aumentar el número de ciclos. Si hay duda, es mejor usar menos ciclos que demasiados.

El número óptimo de ciclos se determinó mediante PCR a partir de la reacción de primera cadena recién sintetizada, retirando alícuotas a los 15, 18, 21 y 24 ciclos como se describe a continuación:

Con el termociclador a 95 °C se colocaron **3 tubos de PCR por cada muestra** (Tester, Driver, y control "Smart") conteniendo la siguiente mezcla maestra:

	1 Rxn	**10 Rxns**
Agua destilada estéril	83 µl	830 µl
Buffer 10X Advantage 2 PCR	10 µl	100 µl
dNTPs 50X (10 mM)	2 µl	20 µl
5' PCR primer IIA (12 µM)	2 µl	2 µl
Mezcla 50X advantage 2 Polimerasa (1.1 µg/µL)	2 µl	20 µl

A cada tubo conteniendo 99 µl de la mezcla maestra se le adicionó 1 µl del cDNA de primera cadena correspondiente. Las muestras se incubaron en el termociclador bajo las siguientes condiciones:

95 °C por 1 min

95 °C por 15 s
65 °C por 30 s
68 °C por 6 min
} 15 ciclos

Después de 15 ciclos, dos tubos de cada muestra se guardaron a 4 °C y con el tercer tubo de cada una de las muestras se hizo la determinación del número óptimo de ciclos como se describe a continuación:

Se tomaron 15 µl del tercer tubo de cada muestra, se etiquetaron y se almacenaron a 4 °C. Enseguida, se colocaron en el termociclador las mezclas restantes de cada tercer tubo, sometiéndolas a tres ciclos adicionales para completar 18 ciclos; nuevamente se reservaron 15 µl que se almacenaron a 4 °C. El incremento en los ciclos de PCR y la toma de alícuotas se repitió hasta alcanzar 24 ciclos (Fig. 1).

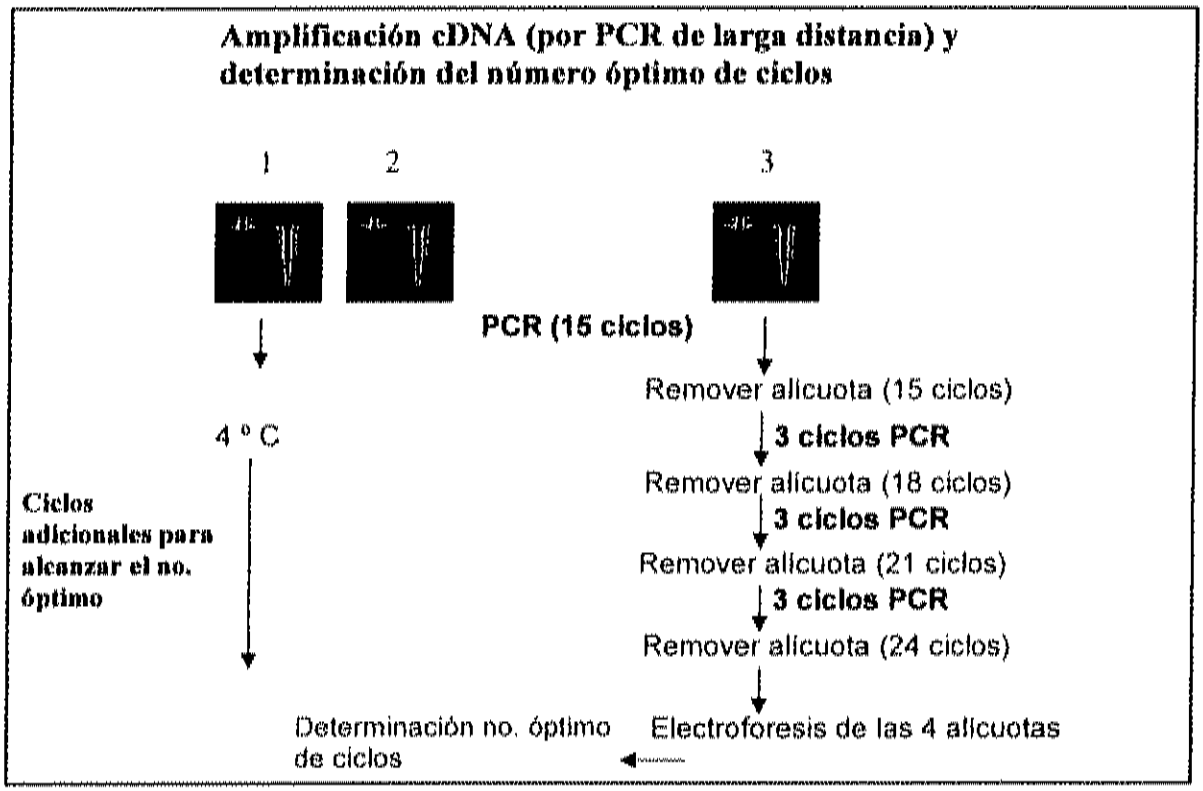

Fig 1. Esquema de la reacción para determinar del número óptimo de ciclos de PCR en la síntesis de la segunda cadena a partir de los cDNAs de cadena sencilla.

Posteriormente, se realizó electroforesis un gel de agarosa al 1.2 % en buffer TAE 1X cargando 5 µl de cada una de las cuatro alícuotas (15, 18, 21 y 24 ciclos) de cada muestra, usando como marcador de peso molecular el 1 Kb plus DNA ladder (Invitrogen). Los dos tubos de cada muestra que se habían almacenado a 4 °C fueron sometidos a los ciclos adicionales necesarios para alcanzar el óptimo de ciclos determinados en el paso anterior.

Nuevamente se realizó electroforesis en gel de agarosa al 1.2 %, con 5 µl de cada una de las muestras sometidas a los ciclos adicionales, para verificar la presencia de los productos de PCR y la eficiencia de la síntesis de la segunda cadena. Una vez verificada la presencia de los cDNAs de doble cadena por electroforesis se procedió con lo siguiente: A

cada tubo se le adicionaron 2 µl de EDTA 0.5M para detener la reacción y de cada una de las tres muestras ("Tester", "Driver" y control "Smart") se transfirieron 7 µl a un tubo nuevo, etiquetándolo como muestra A de cada uno y almacenándolo a -20 °C.

Cromatografía en columna del "Tester", "Driver" y control "Smart"

Se combinó el contenido de los dos tubos de cada muestra en un solo tubo eppendorf (1.5 mL) y se agregó un volumen igual (180 µl) de fenol/cloroformo/alcohol isoamílico (25:24:1).

Se mezcló completamente en vórtex y se centrifugó a 14 000 rpm por 10 min.

Se recuperó la fase acuosa en un tubo nuevo, se le agregaron 700 µl de n-butanol, se mezcló en vórtex y se centrifugó a temperatura ambiente a 14 000 rpm por 1 min.

Con una micropipeta se descartó la fase superior (n-butanol) quedando un volumen de 40 a 70 µl de muestra en cada tubo.

Se tomó una columna CRHOMA SPIN TM 1000 por cada muestra y se invirtió varias veces hasta resuspender la matriz (evitando dejar burbujas). Luego, se retiraron las tapas superior e inferior y se colocó la columna en un tubo eppendorf (se eliminó el buffer de la columna que cayó en el tubo eppendorf).

Se agregaron 1.5 mL de buffer TNE 1X y se dejó escurrir el buffer por la columna hasta que la superficie de la matriz alcanzó la marca de 0.75 mL. Se desechó el buffer colectado.

La muestra se aplicó al centro de la superficie de la matriz y luego se aplicaron 25 µl de buffer TNE 1X que se dejaron escurrir completamente por la columna, aplicando después 150 µl de buffer TNE 1X que igualmente se dejaron escurrir.

Se transfirió la columna a un tubo eppendorf nuevo y se aplicaron 320 µl de buffer TNE 1X colectando el eluído como la fracción purificada. De esta fracción se transfirieron 10 µl a un tubo nuevo etiquetándolo como "muestra B" y se guardó a -20 °C.

Se aplicaron 75 µl de buffer TNE 1X colectando el eluído en un tubo nuevo. Se etiquetó como "muestra C" y se guardó a -20 °C.

Se realizó electroforesis en gel de agarosa al 1.2 % con las tres muestras etiquetadas como A, B y C, cargando los volúmenes que se indican a continuación:

Muestra A (sin purificar)	3.0 µl
Muestra B (1ª elución)	10.0 µl
Muestra C (2ª. elución)	10.0 µl

El objetivo de este análisis fue eliminar sales, nucleótidos, enzimas, primers, solventes, y fragmentos pequeños de cDNAs.

Digestión del "Tester", "Driver" y control "Smart" con *Rsa* I

De cada muestra de cDNA purificado en columna, se separaron 10 µl y se etiquetaron, correspondientemente, como "muestra D" para usarlos en la confirmación de la digestión. A cada muestra restante de cDNA (300 µl) se adicionaron los siguientes reactivos:

Buffer de restricción *Rsa* I 10X	33.5 µl
Rsa I (10 U/ µl)	1.5 µl

Se homogenizó por vortex, se centrifugó suavemente y se incubó a 37 por 3 h.

Para confirmar la digestión se corrió un gel de agarosa al 1.2 % con 10 µl de los ADNcs sin digerir (muestras D) y 10 µl de cDNAs digeridos en buffer TAE 1X. Se utilizó el marcador molecular el 1Kb plus DNA ladder. Confirmada la digestión, se adicionaron 8 µl de EDTA 0.5 M a cada tubo para detener la reacción. Se transfirieron 10 µl de cada cDNA a un tubo nuevo y se etiquetaron correspondientemente como "muestra E", almacenándolos a -20 °C.

Purificación de los productos de la digestión de cDNAs

La purificación de los productos de la digestión se realizó utilizando columnas GFX ™ PCR DNA and Gel Band Purification (Amersham Biosciences) tal como se hace para productos de PCR siguiendo las especificaciones del protocolo y eluyendo en 50 µl de buffer

TE, pH 8.0. Enseguida, se aplicó el eluído a la columna de microfiltración colocada en un eppendorf de 1.5 mL, se centrifugó por 5 min (11 700 rpm) y se desechó la columna.

Al volumen restante de cada muestra se le agregó glicógeno (para alcanzar una concentración de 0.2 a 0.4 µg/µl en base a dicho volumen), ½ volumen de Acetato de amonio 5M y 2.5 volúmenes de etanol al 95%. Se agitó en vórtex y se centrifugó durante 20 min a 14 000 rpm y temperatura ambiente. Se descartó el sobrenadante y se lavó el pellet con 500 µl de etanol al 80%. Las muestras se centrifugaron a 14 000 rpm durante 10 min, se descartó el etanol y se secó el pellet por 10 a 15 min.

Enseguida el pellet se disolvió en 10 µl de H_2O libre de DNasa. De cada muestra, se usó 1 µl para cuantificar el cDNA por espectrofotometría en Nano Drop (Nano Drop Inc. Technologies, USA). La concentración de las muestras digeridas y purificadas debió ser de al menos 300 ng/µl. De lo contrario, se habría tenido que repetir la construcción de la genoteca desde la primera cadena. Antes de proceder a la ligación, la concentración de todas las muestras se ajustó a 330 ng/µl.

Síntesis de la primera cadena de cDNas control para el protocolo "PCR Select"

Se preparó la siguiente mezcla de reacción en un tubo de PCR:

RNA poli A de músculo esquelético humano (1.0 µg/µl)	2 µl
cDNA Syntheis Primer	1 µl
Água destilada estéril	2 µl
Volumen total	5 µl

La reacción se incubó a 70 °C por 2 minutos, se enfrió en hielo por otros 2 min, se centrifugó y se le adicionó lo siguiente:

5X buffer primera cadena	2 µl
Mezcla de dNTPs (10 mM)	1 µl
Agua destilada estéril	1 µl
Enzima AMV RT (20 U/ µl)	1 µl

Nuevamente se mezcló por pipeteo, se centrifugó brevemente y se incubó a 42 °C por 1.5 h. Finalizada la reacción, la muestra se colocó en hielo y se procedió inmediatamente con la síntesis de la segunda cadena del control de músculo esquelético humano (control "PCR select").

Síntesis de segunda cadena de cDNAs del control "PCR Select"

Al tubo de PCR de la primera cadena del control se le agregaron los siguientes reactivos:

Agua destilada estéril	48.4 µl
Buffer 5X 2ª. cadena	16 µl
dNTPs (10 mM)	1.6 µl
Cóctel de enzimas 20X de segunda cadena	4.0 µl

Se mezcló por pipeteo (volumen total: 80 µl), se centrifugó y se incubó a 16 °C durante 2 h.

Concluída la incubación se adicionaron 2 µl de T4 polimerasa (3 U/ µl), se mezcló perfectamente y se incubó a 16 °C durante 30 min en el termociclador.

Enseguida se agregaron 4 µl de EDTA/glicógeno 20X para detener la reacción.

Se transfirió el contenido del tubo a un tubo eppendorf de 1.5 mL.

Se agregaron 100 µl de fenol/cloroformo/alcohol isoamílico (25:24:1) se agitó en vórtex y se centrifugó a 14 000 rpm por 10 minutos a temperatura ambiente.

Se recuperó la fase acuosa (superior), se transfirió a otro tubo de PCR y se le agregaron 45 µl de cloroformo/alcohol isoamílico (24:1). Se mezeló por vórtex y se centrifugó a 14 000 rpm durante 10 min a temperatura ambiente.

Se recuperó la fase acuosa, se transfirió a otro tubo y se le agregaron 1 µl de glicógeno (20 µg/µl; Invitrogen), 22.4 µl de acetato de amonio 5M y 168.7 µl de etanol al 95%.

Se homogenizó en vortex y se centrifugó a 14 000 rpm por 20 min a temperatura ambiente.

Se descartó el sobrenadante, se dejó secar el pellet de 10 a 15 min y se resuspendió en 50 µl de agua destilada estéril, se tomó una alícuota de 6 µl y se guardó a -20 °C (esta alícouta se usó para el análisis de la digestión con *Rsa* I).

Digestión y purificación del control "PCR Select"

En un tubo eppendorf se mezclaron los siguientes reactivos:

cDNA control PCR Select	43.5 µl
Buffer de restricción *Rsa* I 10X	5 µl
Rsa I (10 U/ µl)	1.5 µl

Se centrifugó e incubó a 37 °C durante 1.5 h. Transcurrida la digestión, se separaron 5 µl de la mezcla de cDNA digerido para analizar la eficiencia de la enzima.

Se agregaron 2.5 µl de la mezcla EDTA/glicógeno 20X para detener la reacción.

Se le agregaron 50 µl de fenol/cloroformo/alcohol isoamílico (25:24:1) se agitó en vórtex y se centrifugó por 10 min a 14 000 rpm.

Se transfirió la fase acuosa a un tubo nuevo y se le agregaron 50 µl de cloroformo/alcohol isoamílico (24:1); se agitó en vórtex y centrifugó a 14 000 rpm por 10 min.

Se transfirió el sobrenadante a un tubo nuevo y se agregaron 0.6 µl de glicógeno (20 µg/ µl), 15 µl de acetato de amonio 5M y 112.5 µl de etanol al 95%; se agitó en vórtex y centrifugó a 14 000 rpm por 20 min.

Se descartó el sobrenadante y se lavó el pellet con 200 µl etanol al 80%. Se agitó en vórtex y centrifugó a 14 000 rpm por 5 min. Se descartó el etanol y se secó el pellet 10 a 15 min.

Se disolvió el pellet en 10 µl de agua libre de DNasa, se utilizó 1 µl para cuantificar en Nanodrop y el resto se guardó a -20 °C. Antes de proceder a la ligación, la concentración de este control se ajustó a 330 µg/ µl.

Para confirmar la digestión de muestras y controles se realizó electroforesis en geles de agarosa al 1.2% y buffer TAE 1X de:

cDNA "Control PCR Select" sin digerir	5.0 µl
cDNA "Control PCR Select" digerido	5.0 µl
cDNA "Driver" sin digerir	10 µl
cDNA "Driver" digerido	10 µl
cDNA "Tester" sin digerir	10 µl
cDNA "Tester" digerido	10 µl
cDNA "Control SMART" sin digerir	10 µl
cDNA "Control SMART" digerido	10 µl

Ligación de adaptadores

Cada cDNA tester (Tester *A. alternaria*, "tester SMART" y tester "PCR Select") se alicuotó en dos tubos separados; una alícuota se ligó al adaptador 1(T-1) y la otra se ligó al adaptador 2R (T-2R). Después de cada "tester" se combinó un volumen ligado al adaptador 1 con otro volumen ligado al adaptador 2R, para obtener los "testers no sustraídos" (Tns) que sirvieron como control positivo de la ligación. Los adaptadores no se ligaron al cDNA del Driver.

Preparación del Tester experimental (*Alternaria alternata*)

Se diluyó 1 µl del cDNA del "Tester" (*Alternaria alternata*) en 5 µl de agua libre de DNasa.

Preparación de los "tester" de los controles para la substracción control (la que se realizó con los controles de los protocolos).

Se centrifugó brevemente el tubo de DNA control (DNA de ΦX174 digerido con *Hae* III) proporcionado en el kit. Se tomaron 2 µl y se diluyeron con 38 µl de agua.

Se mezeló:

1 µl del cDNA control "PCR Select" digerido y purificado con 5 µl de ADNc/*Hae* III diluído del paso anterior. Éste fue el "tester PCR Select"

1 µl del cDNA control "Smart" digerido y purificado con 5 µl de ADNc/*Hae* III diluído del paso anterior. Éste fue el "tester Smart"

Preparación de la reacción de ligación para testers y controles "Smart" y "PCR Select".

Se preparó la siguiente mezcla maestra:

	1Rxn	7Rxn
Agua	3 µl	21 µl
5X buffer de ligación	2 µl	14 µl
T4 DNa ligasa (400 U/ µl)	1 µl	7 µl
Volumen total	6 µl	

Para el cDNA "Tester" experimental y controles "tester" se combinaron los siguientes reactivos en tubos de PCR (Cuadro 1):

Cuadro 1.- Preparación de las reacciones de ligación para "testers" y controles. **T-1** (Tester ligado al adaptador 1);**T-2R** (Tester ligado al adaptador 2R); **S-1** (tester control "Smart" ligado al adaptador 1); **S-2R** (tester control "Smart" ligado al adaptador 2R); **E-1** (tester "PCR Select" ligado al adaptador 1); **E-2R** (tester "PCR Select" ligado al adaptador 2R).

	T-1	**T-2R**	**S-1**	**S-2R**	**E-1**	**E-2R**
cDNA "tester" diluido	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Adaptador 1	2 µl	-	2 µl	-	2 µl	-
Adaptador 2R	-	2 µl	-	2 µl	-	2 µl
Mezcla maestra	6 µl	6 µl	6 µl	6 µl	6 µl	6 µl

Enseguida, en 3 tubos nuevos de PCR se combinaron:

2 µl de "T-1" con 2 µl de "T-2" y se etiquetó como "Tns" ("Tester" no sustraído)

2 µl de "S-1" con 2 µl de S-2R y se etiquetó como "Sns" (control "Smart" no sustraído)

2 µl de "E-1" con 2 µl de E-2R y se etiquetó como "Ens" (control "PCR Select" no sustraído)

Se centrifugaron brevemente los nueve tubos y se incubaron a 16 °C toda la noche. Al finalizar la incubación se agregó 1 µl de EDTA/glicógeno 20X para detener la reacción.

Se calentaron las muestras a 72 °C por 5 minutos para inactivar la ligasa y se centrifugó brevemente.

Se tomó 1 µl de "Tns", y se diluyó en 1 mL de agua. Se hizo lo mismo con las muestras "Sns" y "Ens". Estas tres muestras se guardaron a -20 °C y se usaron posteriormente como controles negativos de la sustracción.

Análisis de la ligación

Este análisis se realizó para verificar que al menos 25% de los cDNAs tengan el adaptador correspondiente ligado. Este experimento se diseñó para amplificar un

fragmento que abarca la unión adaptador-cDNA. Los primers incluídos en el protocolo PCR Select para el análisis de la ligación amplifican un fragmento del gen G3PDH de humano, rata y ratón. Dichos primers funcionan bien con los controles de los protocolos SMART y PCR Select, pero no con muestras de *Alternaria alternata*, por lo que fue necesario diseñar primers específicos para amplificar un fragmento del gen de β-tubulina de *A. alternata* y poder realizar el análisis de ligación de esta muestra.

Primeramente se realizó una digestión *in silico* de un fragmento del gen β-tubulina de *A. alternata* con *Rsa I*. Se obtuvieron 4 fragmentos (12, 18, 109, 321 y 721 pb). Se diseñaron primers a partir del fragmento de 721 pb, utilizando el programa Primer 3 disponible en Internet. Estos primers amplifican un fragmento de 530 pb. La secuencia de los oligonucleótidos diseñados es la siguiente:

5' GAGAACTCAGACGAGACCTTCTGC 3'

5' TTACCGACGAAGGTAGAGGACATC 3'

Posteriormente se realizó una reacción de PCR *in silico* y se confirmó la amplificación del fragmento de 530 pb. Con la digestión *in silico* se obtuvieron los fragmentos de la muestra de *A. alternata* a los que se ligarían los adaptadores y se aseguró que el fragmento a amplificar se encontrara completo en la muestra digerida de *A. alternata*.

El seguimiento de la ligación en la muestra del "Tester" (*A. alternata*) se realizó con los primers específicos para β-tubulina, mientras que el seguimiento de la ligación en las muestras controles "Smart" y "PCR Select", se llevó a cabo con primers específicos para el gen de G3PDH de humano. Por lo tanto, primeramente se corrió la PCR para analizar la ligación en el "Tester" y enseguida las reacciones de PCR para analizar la ligación en los controles "Smart" y "PCR Select" ya que las condiciones de PCR eran distintas.

Se diluyó 1 µl de cada cDNA ligado en 200 µl de agua

Se combinaron los siguientes reactivos en cuatro tubos por cada muestra ("Tester, "Control "Smart", control "PCR Select" (Cuadro 2). Primeramente se procesó el "Tester" y posteriormente los dos controles juntos. Las unidades se expresan en µl.

Cuadro 2. Preparación de las reacciones para el análisis de ligación del "Tester" y los controles "Smart" y "PCR" Select para realizar el análisis de ligación, 4 tubos por cada muestra.

* Primers β-tubulina, • Primers G3PDH (proporcionados en protocolo).

	"Tester"				**"Smart"**				**"PCR Select"**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Ligado al adaptador 1	1	1	-	-	1	1	-	-	1	1	-	-
Ligado al adaptador 2R	-	-	1	1	-	-	1	1	-	-	1	1
Primer 3' (10 µM)	*1	*1	*1	*1	•1	•1	•1	•1	•1	•1	•1	•1
Primer 5' (10 µM)	-	*1	-	*1	-	•1	-	•1	-	•1	-	•1
PCR primer 1 (10 µM)	1	-	1	-	1	-	1	-	1	-	1	-
Volumen total	3	3	3	3	3	3	3	3	3	3	3	3

Se preparó la siguiente mezcla maestra:

	1Rxn	**12.5 Rxn**
Agua	18.5 µl	231.25 µl
Buffer 10XPCR advantage 2	2.5 µl	31.25 µl
Mezcla de dNTPs (10 mM)	0.5 µl	6.25 µl
Mezcal DNA polimerasa Advantage 50X (1.1 µg/ µl)	0.5 µl	6.25 µl
Volumen total	22.0 µl	

Se agragaron 22 µl de la mezcla maestra a cada uno de los 12 tubos mezclando bien

El análisis de ligación del "Tester" se incubó bajo las siguientes condiciones:

95 °C x 5 min

94 °C x 1 min
55 °C x 1 min — 24 ciclos
60 °C x 1 min

72 °C x 7 min

El análisis de ligación de los controles "Smart" y "PCR Select" se incubó bajo las siguientes condiciones:

75 °C por 5 min

94 °C x 30 s
65 °C x 30 s
68 °C x 2.5 min
} 24 ciclos

Se analizaron 10 µl de cada reacción en un gel de agarosa al 1.2 % en buffer TAE.

Primera hibridación

El buffer de hibridación se dejó descongelar a temperatura ambiente por 20 min y se preparó la mezcla de hibridación para el "Tester" (HT), y los dos controles (HS y HE); Un tubo por cada ligación, en total: 6 tubos (Cuadro 3).

Cuadro 3 . Preparación de reacciones para la primera hibridación.

	HT		**HS**		**HE**	
	1	2R	1	2 R	1	2R
cDNA "Driver" digerido	1.5 µl	1.5 µl	1.5 µl	1.5 µl	1.5 µl	1.5 µl
Ligación del adaptador 1	1.5 µl	-	1.5 µl	-	1.5 µl	-
Ligación del adaptador 2R	-	1.5 µl	-	1.5 µl	-	1.5 µl
Buffer de hibridación 4X	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Volumen total	4 µl	4 µl	4 µl	4 µl	4 µl	4 µl

Cada muestra se mezcló por pipeteo, se centrifugó y se incubó a 98 °C por 1.5 min y a 68 °C por 8 h.

Segunda hibridación

Se rotularon 3 tubos, agregándose a cada tubo:

cDNA "Driver" (correspondiente)	1 µl
Buffer de hibridación 4X	1 µl
Agua destilada estéril	2 µl
Volumen total	4 µl

Los tubos se incubaron a 98 °C por 1.5 min y luego, con la micropipeta ajustada a 15 µl, se tomó, para cada muestra, todo el contenido del tubo con el adaptador 1, dejando una burbuja de aire en la punta de la pipeta. Enseguida, se tomó todo el volumen de la muestra del "Driver" correspondiente a esa muestra, y se depositó en el tubo conteniendo la muestra del adaptador 2R respectivo mezclando bien por pipeteo y centrifugando. Las tres reacciones (HT, HS y HE de la segunda hibridación) se incubaron a 68 °C toda la noche. Al término de la incubación, se les agregaron 200 µl de buffer de dilución a cada tubo, se mezcló por pipeteo y se calentó en el termociclador a 68 °C por 7 min. Las muestras se almacenaron a -20 °C.

Primera y Segunda PCR

Durante la primera y segunda PCR los fragmentos diferencialmente expresados se amplifican selectivamente. Previo a los ciclos de PCR la parte faltante en los adaptadores es rellenada mediante una breve incubación a 75 °C creando el sitio de alineamiento para el "PCR primer 1". En la primera PCR, solamente los

cDNAs con diferente secuencia adaptadora en cada extremo son exponencialmente amplificados; en la segunda amplificación se usan primers anidados que se alinean en secuencias internas de los adaptadores con el fin de reducir el "ruido de fondo" y enriquecer aún más la amplificación de los fragmentos diferencialmente expresados. Para óptimos resultados deben realizarse un mínimo de 7 reacciones como se detalla a continuación:

Primera PCR

Se prepararon tubos de PCR con 1 µl de DNAc de la segunda hibridación (tubos HT, HS y HE) y de los correspondientes controles no sustraídos ("Tns", "Sns" y "Ens"). Además se preparó otro tubo con 1 µl del cDNA control sustraído proporcionado por el protocolo (sDNA). En total, 7 tubos de reacción.

HT	HS	HE	Tns	Sns	Ens	sDNA

Se preparó la siguiente mezcla maestra para la primera PCR:

	1 Rxn	7.5 Rxn
Agua estéril	19.5 µl	146.3 µl
Buffer 10X PCR	2.5 µl	18.8 µl
Mezcla de dNTPs (10mM)	0.5 µl	3.8 µl
PCr primer 1 (10 µM)	1.0 µl	7.5 µl
Mezcla DNA polimerasa Advantage 50X (1.1 µg/µl)	0.5 µl	3.8 µl
Volumen total	24.0 µl	

Se alicuotaron 24 µl de la mezcla maestra en cada tubo de reacción y se incubaron en el termociclador a 75 °C por 5 min para extender los adaptadores. Inmediatamente se corrió el siguiente programa de PCR:

94 °C x 30s
66 °C x 30s } 27 ciclos
72 °C x 1 min

Se analizaron 8 µl de cada tubo en un gel de agarosa al 2% con buffer TAE 1X.

Se diluyeron 3 µl de cada muestra de la PCR primaria en 27 µl de agua.

Segunda PCR

Se colocó en un tubo nuevo 1 µl de cada producto de la primera PCR diluido en el paso anterior y se le agregaron 24 µl de la siguiente mezcla maestra:

	1Rxn	**7.5 Rxns**
Agua estéril	18.5 µl	138.8 µl
Buffer 10X PCR Advantage	2.5 µl	18.8 µl
Nested PCR primer 1 (10 µM)	1.0 µl	7.5 µl
Nested PCR primer 2R (10 µM)	1.0 µl	7.5 µl
Mezcla de dNTPs (10mM)	0.5 µl	3.8 µl
Mezcla DNA polimerasa Advantage 50X (1.1 µg/µl)	0.5 µl	3.8 µl
Volumen total	24.0 µl	

Las reacciones se sometieron al siguiente programa de PCR:

94 °C x 30s
68 °C x 30s } 10 ciclos
72 °C x 1.5 min

Análisis de la sustracción

Se analizó la eficiencia de la sustracción mediante PCR, comparando la abundancia relativa del gen de β-tubulina en el "Tester" antes y después de la sustracción. Para el análisis de la sustracción en la muestra experimental de *A. alternata* se utilizaron primers para el gen de β-tubulina de este hongo, ya que los

primers para el gen G3PDH incluidos en el protocolo no funcionan con muestras de hongos; la sustracción del control "Smart" se llevó a cabo con los primers para el gen de G3PDH humano (Tabla 4). El procedimiento se detalla a continuación:
Se diluyó 1 µl de los productos de la PCR secundaria ("HT" y "Tns") en 9 µl de agua. Se preparó la siguiente mezcla maestra en un tubo de 0.5 mL:

Cuadro 4. Preparación de las reacciones para el análisis de la sustracción.
* Primers β-tubulina (*A. alternata*), • Primers G3PDH humana
Las unidades se expresan en microlitros.

	HT	**Tns**	**HS**	**Sns**
cDNA sustraído diluido (HT, producto 2ª PCR)	1	-	1	-
"Tester no sustraído" diluido (Tns, producto 2ª PCR)	-	1	-	1
Primer 3' (10 µM)	1.2*	1.2*	1.2•	1.2•
Primer 5' (10 µM)	1.2*	1.2*	1.2•	1.2•
Agua estéril	22.4	22.4	22.4	22.4
Buffer 10X PCR	3.0	3.0	3.0	3.0
Mezcla de dNTPs (10mM)	0.6	0.6	0.6	0.6
Mezcla DNA polimerasa Advantage 50X (1.1 µg/µl)	0.6	0.6	0.6	0.6
Volumen total	30	30	30	30

Los tubos se mezclaron en vórtex y se centrifugaron brevemente
Las muestras **HT** y **Tns** se sometieron a 18 ciclos del siguiente programa de PCR:

94 °C x 30 s
55 °C x 30 s
60 °C x 2 min
} 18 ciclos

Al finalizar los 18 ciclos, se removieron 5 µl de cada reacción y se colocaron en un tubo nuevo que se guardó a 4 °C. Al volumen restante de cada reacción se le

dieron 5 ciclos más de PCR, retirando nuevamente 5 µl de cada reacción (23 ciclos). Estos pasos se repitieron para obtener alícoutas de 28 y 33 ciclos que se guardaron a 4 °C.

Las muestras **HS** y **Sns** se sometieron a 18 ciclos del siguiente programa de PCR realizando el mismo procedimiento que con las muestras anteriores: retirar alícuotas a los 18, 23, 28 y 33 ciclos.

94 °C x 30s

60 °C x 30s

68 °C x 2 min

Se analizaron las alícuotas (retiradas a los 18, 23, 28 y 33 ciclos) de el "Tester" sustraído (HT) y no sustraído (Tns), así como del control "Smart" sustraído (HS) y no sustraído (Sns) en un gel de agarosa al 2% teñido con bromuro de etidio.

Clonación de los productos de la segunda PCR

Los productos de PCR secundaria (cDNAs diferencialmente expresados) del "Tester" se ligaron al vector p-GEMT Easy (Promega Corporation). Con el producto de la reacción de ligación se transformaron células de *E. coli* JM109 y se sembraron en LB-Ampicilina (LB-Amp) adicionado con isopropyl-β-(D)-thiogalactopyranoside (IPTG) and 5-bromo-4-cloro-3-Indolyl-β-D-galactopyranoside (X-GAL) para diferenciar clones recombinantes. Posteriormente, los clones recombinantes se sembraron en caldo LB-Amp para amplificar los fragmentos insertados. Se realizó extracción de ADN plasmídico por el método de lisis alcalina y digestión con la enzima de restricción *Rsa* I. Se utilizó esta enzima debido a que los fragmentos diferencialmente expresados tienen un adaptador en cada extremo; en cada adaptador existe un sitio de restricción para *Rsa* I, de manera que al llevar a cabo la digestión con *Rsa* I, se libera el fragmento completo. Adicionalmente el vector pGEM tiene un

sitio de corte *Rsa* I por lo al digerir se obtienen tres fragmentos, uno que corresponde al inserto y dos que provienen de la digestión del vector.

Los productos de la digestión se analizaron en gel de agarosa al 1% teñido con bromuro de etidio para verificar la presencia y el tamaño de los insertos. Posteriormente los fragmentos de interés se enviaron a secuenciar utilizando el oligonucleótido M13 Forward (Genomic Analysis and Technology Core Facility of the Arizona State University).

Secuenciación y análisis de secuencias

Las secuencias obtenidas se sometieron a ensamblaje utilizando el programa CAP3 Sequence Assembler. Los unigenes y singlets resultantes se analizaron mediante el programa BLAST utilizando los algoritmos Blastn y Blastx con el fin de identificar el tipo de genes por comparación con las secuencias depositadas en el banco mundial de genes (GenBank). La anotación de los unigenes y los singlets se realizó mediante el programa Blast2GO disponible en Internet (Bioinformatics Department, Centro de Investigación Príncipe Felipe, Valencia Spain).

Almacenamiento de la genoteca substractiva

Las colonias recombinantes (previa confirmación por digestión y secuenciación) fueron inoculadas por separado en 5 mL de medio LB/ampicilina y cultivadas a 37 ° C y 150 rpm toda la noche. Al término de la incubación, se prepararon tubos eppendorf (1.5 mL) con 500 µL de glicerol al 100% a los cuales se les adicionaron 500 µL del cultivo correspondiente. Se mezcló en vórtex hasta homogenizar completamente. Los tubos se almacenaron a -80 ° C.

Análisis en etapas críticas de construcción de la genoteca

Número óptimo de ciclos.

El número de ciclos apropiado para la síntesis de la segunda cadena a partir de la primera cadena de las muestras "Tester", control "Smart" y "Driver" fue de 18 ciclos (Figura 2). A los 15 ciclos, el barrido del producto de PCR todavía no fue observable en ninguno de los casos, mientras que a los 18 ciclos, se observa perfectamente el barrido correspondiente al producto de PCR en todas las muestras. Al aumentar los ciclos a 21, se observó diferencia en la intensidad del barrido con respecto a las alícuotas de 18 ciclos. Sin embargo, al incrementar los ciclos de 21 a 24, ya no se observó diferencia en la intensidad del barrido en las tres muestras. Esto significa que la reacción alcanzó la fase estacionaria desde los 21 ciclos, por lo que el número óptimo de ciclos de PCR fue 18.

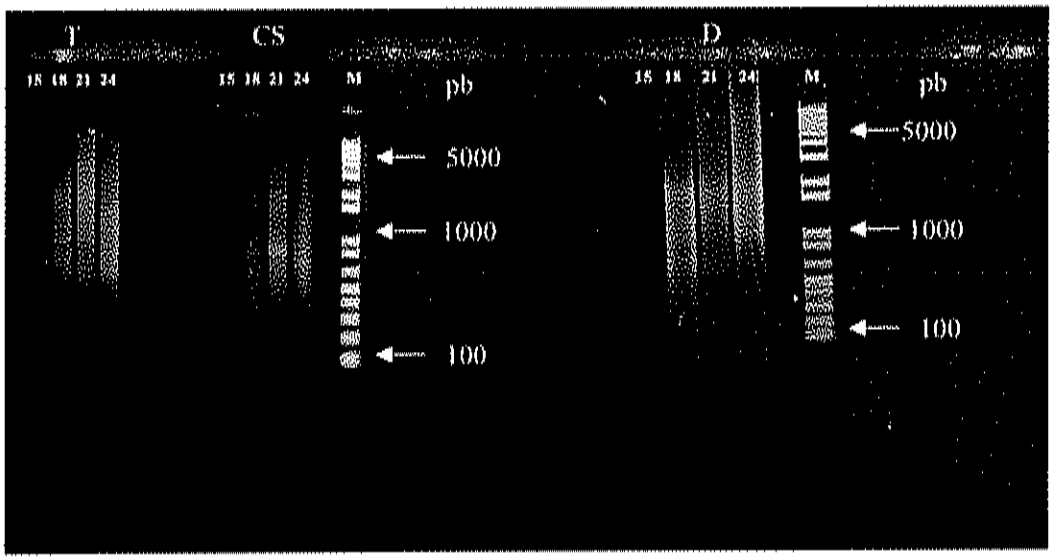

Figura 2 .- Electroforesis en gel de agarosa al 1.2 % de las alícuotas correspondientes a 15, 18, 21 y 24 ciclos de PCR para cada muestra. Las letras T, CS y D corresponden a las muestras "Tester", "Control Smart" y "Driver"; Los números sobre los carriles indican el número de ciclos a los que fue sometida la alícuota correspondiente y a letra M corresponde al marcador de peso molecular (DNA Marker 1 Kb plus). Se cargaron 5 µL de cada producto de PCR y 1 µL (0.1 µg) del marcador. Los números con flecha indican los tamaños en pares de bases de los fragmentos.

Análisis de la síntesis de la segunda cadena

Las muestras que se habían reservado a 4° C fueron amplificadas con el número de ciclos determinados en el paso anterior. Se llevó a cabo electroforesis en gel de agarosa al 1.2% y tinción con bromuro de etidio (Fig. 3), con lo que se verificó la presencia de los cDNAs de doble cadena, la eficiencia en la síntesis de la segunda cadena para cada muestra y el tamaño aproximado de los productos de PCR.

La síntesis de la segunda cadena se llevó a cabo exitosamente tanto en las muestras experimentales como en el control, obteniéndose productos de PCR de aproximadamente 4 kb para el "Tester" y el "Driver" y de 2 kb para el control "Smart".

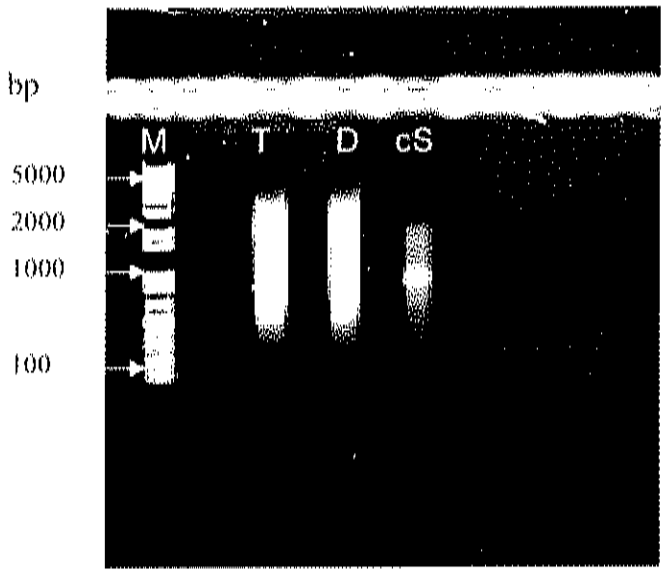

Figura 3. Electroforesis en gel de agarosa al 1.2% de la reacción de segunda cadena de las muestras "Tester" (T), "Driver" (D) y control "Smart" (cS) indicando la presencia y el tamaño aproximado de los productos de PCR de segunda cadena (aproximadamente 4 kb para las muestras de *Alternaria alternata* ("Tester" y "Driver") y 2kb para el control "Smart". La letra M corresponde al marcador de peso molecular 1 kb plus.

Análisis de la cromatografía en columna de los productos de PCR

Se llevó a cabo cromatografía en columna recogiendo las fracciones B y C. Posteriormente se corrió electroforesis de las fracciones A (cDNAs sin purificar), B (muestra purificada, primera elución), y C (muestra purificada, segunda elución).
Se compararon las intensidades de las bandas (A, B, C) de cada muestra en el gel (Fig. 4). La intensidad de las bandas B fue menor que la intensidad de las bandas A. Además los fragmentos más pequeños no aparecieron en las fracciones purificadas, por lo que la cromatografía en columna fue exitosa. Además se confirmó la presencia de los cDNAs en las fracciones purificadas y se estimó el peso molecular de los cDNAs en dichas fracciones.

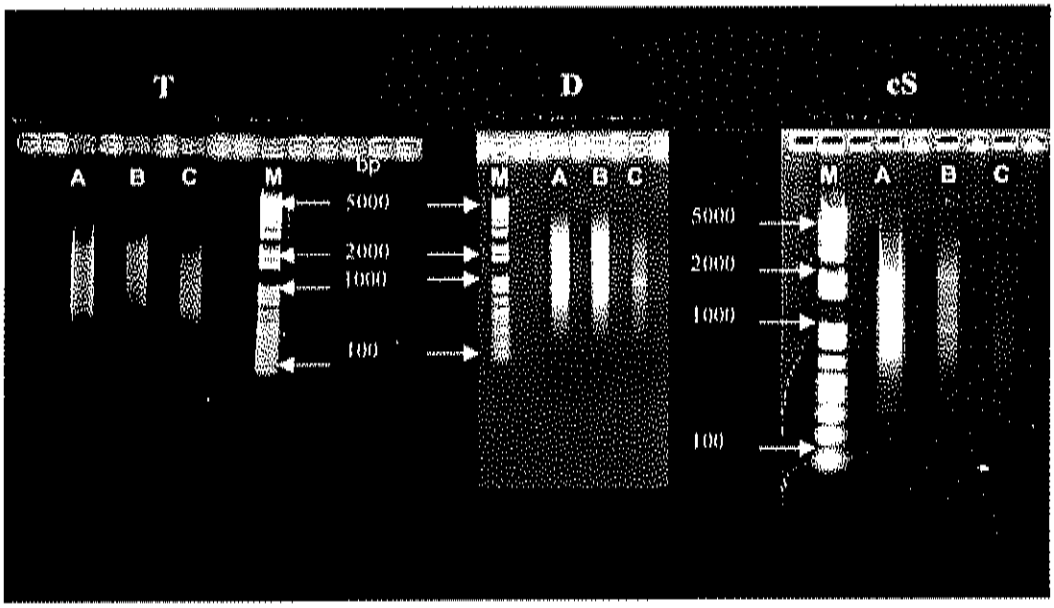

Figura 4. Electroforesis en gel de agarosa al 2% de la purificación de los productos de PCR del "Tester" (T), el "Driver" (D) y el control "Smart" (cS). La letra A representa la muestra sin purificar, B la muestra purificada (primera elución), C la muestra purificada (segunda elución), y M el marcador de peso molecular 1 kb plus.

Análisis de la eficiencia de la Digestión con *Rsa*I

Una vez confirmada la cromatografía en columna, se procedió con la digestión de todas las muestras. Se confirmó que la digestión fue exitosa, ya que el barrido de las muestras digeridas se observaron más abajo en el gel, que el barrido de la muestra sin digerir (Figs. 5 y 6).

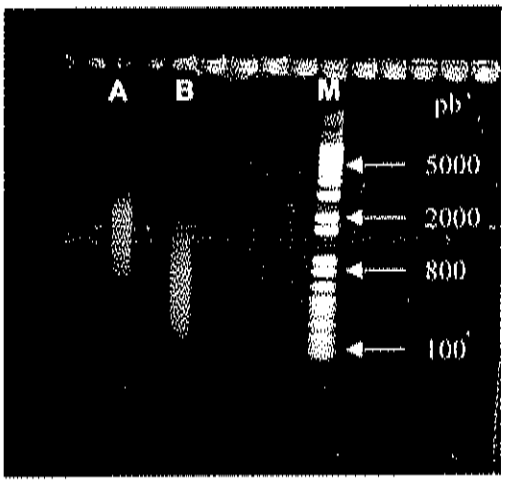

Figura 5. Digestión con *Rsa I* de cDNAs del "Tester". La letra A representa la muestra sin digerir. La letra B representa la muestra digerida. La letra M corresponde al marcador de peso molecular 1 kb plus.

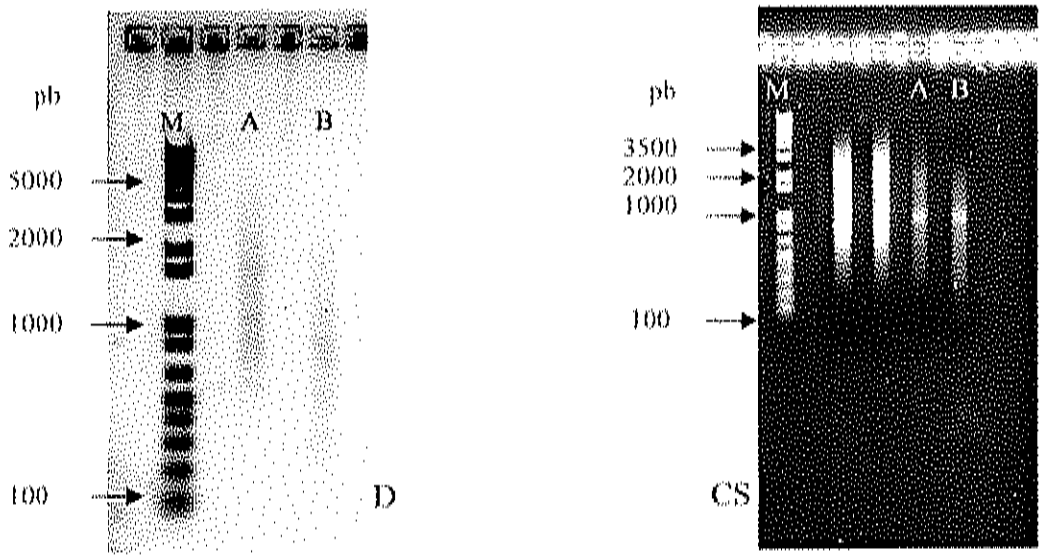

Figura 6.- Análisis de digestión con *Rsa I* de los cDNAs del "Driver" (D) y control "Smart" (cS). La letra M representa el marcador de peso molecular 1 kb plus, A representa la muestra sin digerir y B representa la muestra digerida.

Análisis de la eficiencia de la ligación de adaptadores

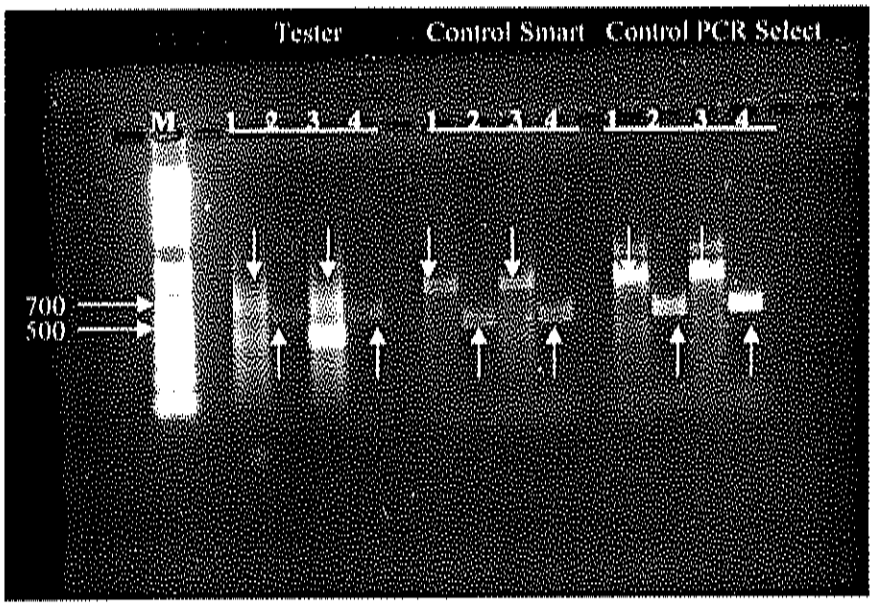

Figura 7.- Análisis por electroforesis (agarosa al 1.2%) de los productos de la ligación. 4 reacciones para el Tester, 4 para el Control Smart y 4 para el control PCR select. Las flechas indican los fragmentos de tamaño esperado: 500 y 700 pares de bases.
Tester
Carril 1: Tester 1-1, primer 3' β-tubulina y PCR primer 1
Carril 2: Tester 1-1, primers 3' y 5' de β-tubulina
Carril 3: Tester 1-2R, primer 3' β-tubulina y PCR primer 1
Carril 4: Tester 1-2R, primers 3' y 5' β-tubulina)
Control Smart y Control PCR Select
Carril 1: S-1, primer 3' G3PDH y PCR primer 1
Carril 2: S-1, primers 3' y 5' de G3PDH
Carril 3: E-2, primer 3' de G3PDH y PCR primer 1
Carril 4: E-2, primers 3' y 5' de G3PDH
M: Marcador de peso molecular 1 kb plus

Con este análisis se confirmó que más del 25% de los fragmentos poseen adaptador ligado en cada caso (1 y 2R), ya que la intensidad de las bandas que representan el producto de PCR con adaptador (700 pb) es similar a la intensidad de la banda que no contiene adaptador (500pb) en cada una de las 3 muestras (Fig. 7). Para que la ligación se considere exitosa la banda de 700 pb debe mostrar como

requerimiento mínimo, la cuarta parte de la intensidad mostrada por la banda de 500 pb. En este caso la ligación fue exitosa, ya que la intensidad de las bandas con y sin adaptador fue muy similar, indicando que el porcentaje de fragmentos con adaptador ligado fue superior al 25%.

Análisis de los productos de PCR secundaria

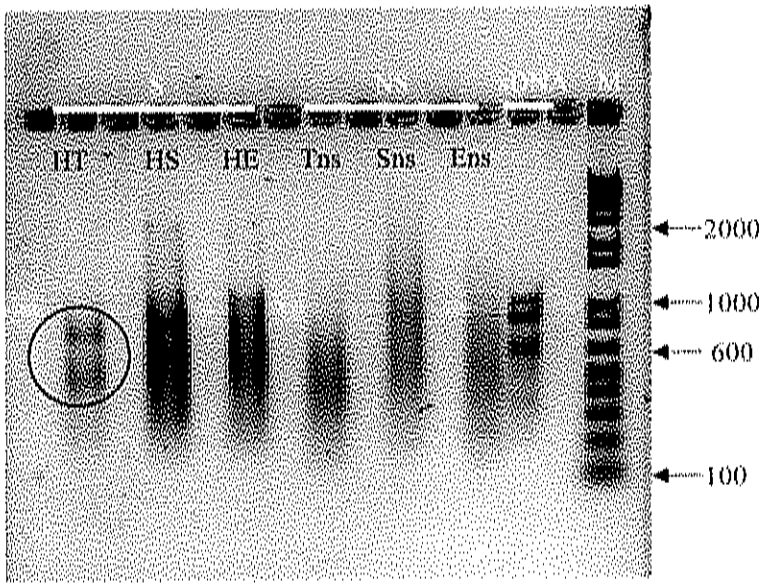

Figura 8.- Analisis mediante electroforesis en gel de agarosa al 2% de los productos de PCR secundaria.
S: Muestras sustraídas
HT: Tester sustraído (fragmentos diferencialmente expresados); **HS**: control "Smart" sustraído; **HE**: control "PCR Select" sustraído
NS: Muestras no sustraídas
Tns: Tester no sustraído; **Sns**: Control Smart no sustraído; **Ens**: Control "PCR Select" no sustraído
sDNA: Control sustraído comercial (no preparado en el laboratorio).
M: Marcador de peso molecular 1 kb plus

La amplificación de los fragmentos diferencialmente expresados fue exitosa (Fig. 8) y su tamaño osciló entre 300 y 900 pares de bases. En el gel puede apreciarse que el patrón de bandeo entre las muestras sustraídas y no sustraídas es diferente, con bandas discretas en las muestras sustraídas y un barrido difuso en las muestras no sustraídas. El DNA control sustraído (sDNA) muestra las bandas características correspondientes a los fragmentos de DNA φX174 digeridos con HAE III que deben aparecer después de la PCR secundaria. Esto confirma indirectamente que la sustracción de la muestra de *A. alternata* (HT) digerida con *Rsa*1 se llevó a cabo correctamente. Asimismo, en los controles "Smart" y "PCR Select" sustraídos (HS y HE respectivamente) se obtuvo un patrón de bandeo similar lo que indicó también una correcta sustracción.

Análisis mediante PCR de la eficiencia de la sustracción

Otra forma de verificar la eficiencia de la sustracción fue mediante el análisis de la abundancia del gen de β-tubulina antes y después del procedimiento sustractivo. Esta PCR se realizó en los productos de PCR secundaria sustraídos y no sustraídos a los 18, 23, 28 y 33 ciclos (Figs. 9 y 10). Después de la sustracción ocurrió una disminución en la concentración relativa del gen de β-tubulina; en la muestra sustraída de *A. alternata* el producto de amplificación, de 530 pares de bases fue apenas visible hasta los 33 ciclos, mientras que en la muestra no sustraída el producto de amplificación se observó desde los 23 ciclos (Fig. 9).

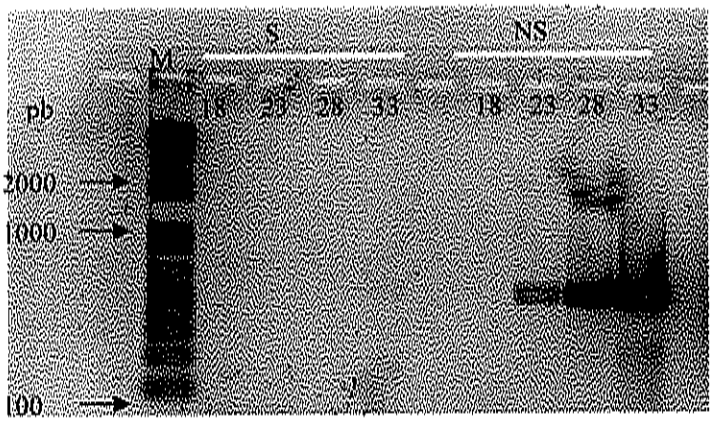

Figura 9 .- Electroforesis en gel de agarosa al 2% de las reacciones de PCR a los 18, 23, 28 y 33 ciclos de muestras sustraídas y no sustraídas de *A. alternata.*
S: Muestras sustraídas
NS: Muestras no sustraídas
M: Marcador de peso molecular 1 kb plus

En el análisis de la sustracción llevado a cabo con los productos de PCR secundaria del control "Smart" también se observó una disminución en la concentración relativa del gen de la G3PDH, ya que en la muestra sustraída el producto de amplificación de 600 pb se observó tenuemente a los 28 ciclos, mientras que en la muestra sin sustraer dicho producto pudo observarse desde los 23 ciclos (Fig. 10).

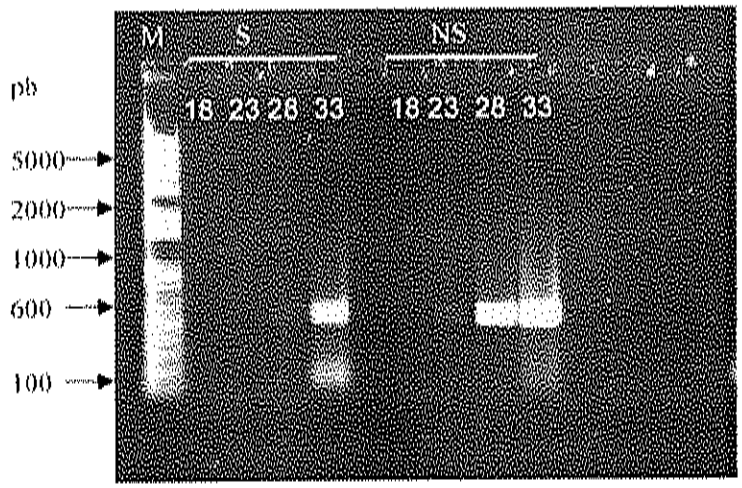

Figura 10 .- Electroforesis en gel de agarosa al 2% de las reacciones de PCR a los 18, 23, 28 y 33 ciclos de muestras sustraídas y no sustraídas del control "Smart". S: Muestras sustraídas, NS: Muestras no sustraídas, M: Marcador de peso molecular 1 kb plus.

Digestión de clones recombinantes con *Rsa*1

Las digestiones con enzima *Rsa*1 para verificar la presencia y el tamaño de los insertos fueron exitosas. Aquí se muestra un gel con el objeto de ilustrar el resultado de las reacciones de digestión de clones recombinantes de *A. alternata* (Fig.11). Los fragmentos que se obtuvieron en las diversas digestiones fueron de un tamaño de entre 300 y 900 pb.

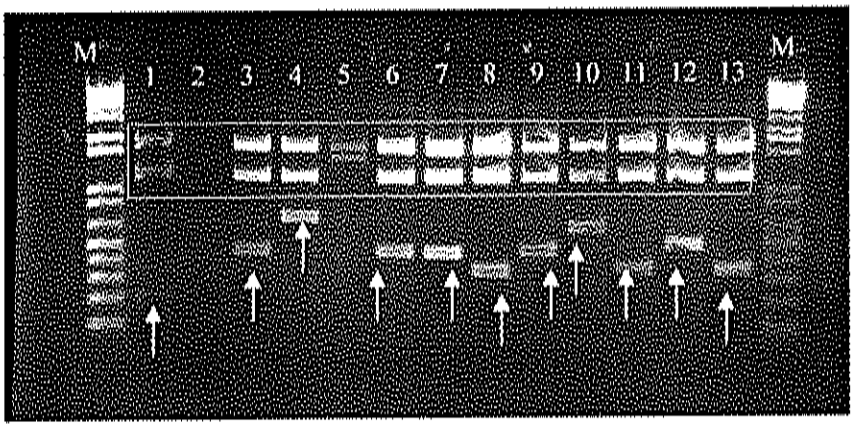

Figura 11. Digestión de clones recombinantes con *Rsa*I. Los números del 1-13 indican las muestras cargadas en el gel. La letra M corresponde al marcador de peso molecular 1 kb plus. En cada carril se aprecian tres bandas: las dos primeras, dentro del rectángulo, corresponden a la digestión del vector y la tercera, indicada con flecha, corresponde al inserto.

APÉNDICE IV

Participación en Congresos y Reconocimientos Obtenidos

Participación en congresos

1. Second International Biofumigation Symposium. University of Idaho, Moscow, Idaho USA. Junio 2006. Intersatellite DNA Polymorphism in *A. alternata* Following Exposure to LethalConcentrations of Isothiocyanates. Maria-Elena Baez-Flores, R.Troncoso-Rojas,B. Pryor, H.S. García, M.E. Tiznado-Hernandez. Centro de Investigación en Alimentación y Desarrollo, Mexico; Univ. of Arizona, USA.

2. Taller Teórico-Práctico de Introducción a la Bioinformática y Biología de Sistemas. Primera Reunión Nacional en Ciencias Microbiológicas. Noviembre 2007. Puebla, Puebla.

3. Third International Biofumigation Symposium. Commonwealth Scientific and Industrial Research Organization (CSIRO) CSIRO, Discovery Center. Canberra, Australia. Julio 2008. "Isolation of cDNAs upregulated in *Alternaria alternata* tolerant to lethal concentrations of 2-propenyl isothiocyanate".

Reconocimientos obtenidos

Durante la realización de este trabajo se obtuvieron dos distinciones. La primera, como resultado de la evaluación, en términos de contenido y presentación del trabajo en formato de póster, durante el Tercer Simposium Internacional de Biofumigación, realizado en la ciudad de Canberra Australia, donde se obtuvo el Primer Lugar.

La segunda distinción, fue otorgada por El Colegio de Sinaloa, al seleccionar nuestro proyecto como el trabajo ganador, entre los participantes en la convocatoria 2008, por la Beca de Disertación Doctoral en Ciencias Biomédicas y de la Salud "Dr. Hugo Aréchiga Urtuzuástegui", convocatoria que esta institución lanza anualmente en el estado de Sinaloa para aspirantes al grado de Doctor en Ciencias.