LA DISSERTAZIONE DEL DOTTORATO DI RICERCA DI ANTONIO DI PIETRO

Forse nel vedere questo libro vi meraviglierete. Cosa ci fa in una pagina web dedicata a Morra un libro scritto in inglese dirà qualcuno.

Questo libro, invece, ha molto da dire ai morresi, anche a chi non capisce né l'inglese, né il tema scientifico di cui tratta.

Antonio Di Pietro è mio figlio e, benché nato e cresciuto a Binningen, ha sempre voluto bene al paese d'origine di suo padre, così come sua sorella Jolanda.

Quando a Morra ci fu il terremoto loro due andarono per un paio d'anni sulle strade di Binningen a raccogliere roba usata che la gente buttava, per poi rivenderla al mercatino delle pulci. Con quel mercatino raccogliemmo 5000 Franchi.

Quando Antonio, per i morresi Toni, scrisse questa dissertazione, in collaborazione tra la Ciba Geigy e l'Università di Basilea, Toni era membro del Comitato Centrale dell'Associazione Morresi Emigrati fin dalla sua fondazione. Fu lui a doppiare in italiano il film in inglese, che il professore Del Priore aveva commissionato sul terremoto a Morra.

Conosceva il nostro paese, dove ci recavamo ogni anno con tutta la famiglia e sapeva del grande critico e patriota Francesco De Sanctis, che era nato a Morra. Ci tenne, perciò a scrivere sulla copertina della sua dissertazione: Di Pietro Antonio **aus** (di) Morra De Sanctis. Credo che pochi studenti italiani in Svizzera l'avrebbero fatto, conosco alcuni che evitavano di dire o scrivere di essere italiani.

L'importanza della sua dissertazione sta nel fatto che durante la ricerca su un fungo che sopprimeva un altro fungo patogeno per le piante, grazie all'identificazione di un mutante (con un cambiamento genetico spontaneo) si accorse che il mutante del fungo buono non era più capace di sopprimerere quello cattivo.

Ricercando e isolando i vari elementi trovò che il mutante del fungo buono non produceva più la chaetomina, una sostanza tossica per il fungo cattivo, e per questo aveva perso la facoltà di sopprimere l'altro fungo patogeno.

La scoperta dovette essere di una certa importanza, visto che a quel mutante spontaneo diedero il nome di ADP13 (Antonio Di Pietro 13).

Dopo la dissertazione la Ciba Geigy e il Fondo Nazionale Svizzero per le Ricerche gli diedero uno stipendio di un anno per ricerche presso l'Università di Cornell University nei USA.

Adesso Toni si trova in Spagna, dove è sposato con una ricercatrice spagnola, la dottoressa Anna Maria Torres Romero ed è ricercatore e professore ordinario di Genetica all'Università di Cordoba. Recentemente ha editato insieme ad un altro Professore di Salamanca, un libro scientifico pubblicato dalla Editoriale Springer.

È editore di varie riviste scientifiche e autore di numerose pubblicazioni scientifiche su diverse riviste specializzate. Insieme ad altri scienziati ha contribuito a decifrare il genoma di *Fusarium*, un fungo patogeno che distrugge i raccolti su moltissime piante coltivate nel mondo. È coordinatore di diversi progetti di ricerca finanziati dal governo spagnolo e dalla Unione Europea.

Questa dissertazione è stata richiesta da ricercatori in tutto il mondo, per esempio dall'Ungheria, dall'Italia e dal Brasile, ciò dimostra l'importanza della scoperta in essa contenuta.

Di questo libretto ne stampai 125 copie, 100 andarono alla biblioteca dell'Università di Basilea, delle altre ne son rimaste solo poche copie.

SUCCESSO INTERNAZIONALE DI PRESTIGIO PER IL MORRESE Prof. Dr. ANTONIO DI PIETRO

UN MORRESE CHE ONORA IL SUO PAESE D'ORIGINE

Sulla prestigiosa rivista scientifica "SCIENCE", del 7 settembre 2007" è stato pubblicato lo studio sulla decifrazione del genoma del "Fusarium graminearum", responsabile di una malattia del grano che procura molto danno all'agricoltura. Al sesto posto nella lista dei ricercatori che hanno decifrato il genoma c`è il morrese Antonio Di Pietro. Segue la lista pubblicata da "SCIENCE"

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The *Fusarium graminearum* Genome Reveals a Link Between Localized Polymorphism and Pathogen Specialization

Christina A. Cuomo, Ulrich Güldener, Jin-Rong Xu, Frances Trail, B. Gillian Turgeon, Antonio Di Pietro, Jonathan D. Walton, Li-Jun Ma, Scott E. Baker, Martijn Rep, Gerhard Adam, John Antoniw, Thomas Baldwin, Sarah Calvo, Yueh-Long Chang, David DeCaprio, Liane R. Gale, Sante Gnerre, Rubella S. Goswami, Kim Hammond-Kosack, Linda J. Harris, Karen Hilburn, John C. Kennell, Scott Kroken, Jon K. Magnuson, Gertrud Mannhaupt, Evan Mauceli, Hans-Werner Mewes, Rudolf Mitterbauer, Gary Muehlbauer, Martin Münsterkötter, David Nelson, Kerry O'Donnell, Thérèse Ouellet, Weihong Qi, Hadi Quesneville, M. Isabel G. Roncero, Kye-Yong Seong, Igor V. Tetko, Martin Urban, Cees Waalwijk, Todd J. Ward, Jiqiang Yao, Bruce W. Birren, and H. Corby Kistler *Science* 7 September 2007 317: 1400-1402 [DOI: 10.1126/science.1143708] (in Reports)

.....Report Reports GENETICS The Fusarium graminearum Genome Reveals a Link Between...the genome of the filamentous fungus Fusarium graminearum, a major pathogen of cultivated...graminearum with its plant hosts. Fusarium, a genus of plant pathogenic fungi, causes.....

Abstract » Full Text » PDF » Supporting Online Material »

I COMMENTI DELLA STAMPA SPAGNOLA

Diario Córdoba

Fecha: 18/09/07
Sección: Opinión
Página: 10

CÓRDOBA

Posicion en la pagina

Colaboración

UNA GRAN NOTICIA PARA LA UCO

ENRIQUE
Aguilar *

La prestigiosa revista *Science* publica en su número de 7 de septiembre un artículo en el que se descifra el genoma de un hongo patógeno para los cereales: Fusarium graminearum.

Se trata de un trabajo en el que han participado 45 investigadores de 8 países liderados por el doctor **Corby Kistler**, de la Universidad de Minnesota, en colaboración con el Instituto Tecnológico de Massachussets, la Universidad de Harvard y la Universidad de Córdoba. Los profesores **M. Isabel González Roncero** y **Antonio Di Pietro**, del Departamento de Genética de nuestra Universidad, figuran entre los investigadores que han contribuido a descifrar y analizar el genoma de este hongo que produce microtoxinas dañinas para el consumo humano y animal.

La noticia es especialmente positiva para la Universidad de Córdoba por las siguientes razones: (1) la participación de los científicos españoles en el desciframeinto del genoma humano y de otras especies ha sido muy escasa, por lo que debemos felicitarnos de estar en algunas ramas de la ciencia en la vanguardia mundial; (2) frente al falso dilema planteado por muchos gestores de la política de nuestra Comunidad Autónoma y nuestro país sobre el interés de la investigación básica, debemos alegrarnos de que este estudio fundamental pueda tener tremendas repercusiones económicas, máxime en unos momentos en que el precio de los cereales parece dispararse por su demanda para la elaboración de biocombustibles; (3) el número de trabajos publicados por las diferentes universidades del mundo en tres revistas científicas de altísimo valor (*Science*, *Nature* y *Cell*) es uno de los elementos que se barajan en la elaboración de los rankings de las universidades. El trabajo de nuestros colegas genéticos es una satisfacción para ellos, producto de 15 años de trabajos sobre hongos patógenos, y para todos los que queremos colocar la investigación de nuestra Universidad a la cabeza del panorama nacional y europeo; (4) el trabajo publicado indica la necesidad imperiosa de la internacionalización de la actividad investigadora.

No es casual que la doctora González Roncero se ocupe actualmente del Vicerrectorado de Internacionalización y Cooperación. Finalmente, pero no menos importante, hay que señalar que el doctor Antonio Di Pietro, hoy profesor de nuestra Universidad, se reincorporó a la misma a través del programa Ramón y Cajal y se estabilizó gracias a la política decidida del equipo de Gobierno de la Universidad de Córdoba de captar y retener a los mejores investigadores. Todos, y especialmente los autores, estamos de enhorabuena. ■

* Vicerrector de Política Científica

ENRIQUE
AGUILAR

UNA GRANDE NOTIZIA PER LA UCO

La prestigiosa rivista Science pubblica nel numero 7 di settembre un articolo nel quale si decifra il genoma di un fungo patogeno dei cereali: Fusarium graminearum

Si tratta di un lavoro al quale hanno partecipato 45 ricercatori di 8 paesi diretto dal dottor Cory Kistler, dell'Università del Minnesota, in collaborazione con l'Istituto Tecnologico del Massachutes, l'Università di Harvard e l'Università di Cordoba. I professori **Isabella Gonzales Romero e Antonio Di Pietro,** del dipartimento di Genetica della nostra Università, figurano tra i ricercatori che hanno contribuito a decifrare e analizzare il genoma di questo fungo che produce microtossine dannose per il consumo umano e animale.

La notizia è specialmente positiva per l'università di Cordoba per le ragioni seguenti: (1) la partecipazione degli scienziati spagnoli nella decifrazione del genoma umano e d'altra specie è stato molto scarsa perciò dobbiamo congratularci di stare in alcuni rami della scienza nell'avanguardia mondiale: (2) difronte al falso dilemma esposto da molti gestori della politica della nostra Comunità Autonoma e il nostro paese sull'interesse della ricerca di base, dobbiamo rallegrarci che questo studio fondamentale può avere enormi ripercussioni economiche, massimamente in un momento in cui il prezzo dei cereali sembra precipitare per la sua domanda per l'elaborazione a biocombustibile; (3) il numero di lavori pubblicati dalle differenti Università del mondo in tre riviste scientifiche di altissimo valore (Science, Nature, e Cell) è uno degli elementi che si tiene di conto nell'elaborazione del rankings delle università. Il lavoro dei nostri colleghi di genetica è una soddisfazione per loro, prodotto di 15 anni di lavoro su i funghi patogeni, e per tutti quelli che desideriamo collocare la ricerca della nostra Università alla testa del panorama nazionale e europeo; (4) il lavoro pubblicato indica la necessità imperiosa della internazionalizzazione dell'attività di ricerca.

Non è casuale che la dottoressa Gonzalez Rocero si occupa attualmente del Vicerettorato di Internalizzazione e Cooperazione. Finalmente, però non meno importante, bisogna segnalare che il dottor Antonio Di Pietro, adesso professore della nostra Università, venne assunto attraverso il programma

Ramon e Cajal e si stabilì grazie a la politica decisa dallo staff del Governo dell'Università di Cordoba di captare e ritenere i migliori investigatori. Tutti, e specialmente gli autori ci congratuliamo.

Vicedirettore di Politica Scientifica

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CORDOBA (periodico)
Portada > Local
INVESTIGACION.
Descifran el genoma responsable de la `fusariosis´ del trigo
La aportación de la Universidad cordobesa ha sido muy importante.
07/09/2007 REDACCION
Un equipo de 45 investigadores de 8 países, incluida España, ha descifrado el genoma completo del *Fusarium graminearum* , un hongo patógeno de los cereales responsable de la *fusariosis* de la espiga del trigo, que provocó solo en Estados Unidos 3.000 millones de dólares de pérdidas en la década de los 90.
La iniciativa de la secuenciación y el análisis del genoma ha sido liderada por el profesor Corby Kistler de la Universidad de Minnesota en colaboración con el Broad Institute del Massachusetts Institute of Technology (MIT) y la Universidad de Harvard, todas en Estados Unidos.
La aportación española ha sido a través del departamento de Genética de la Universidad de Córdoba (UCO), en el que trabajan Roncero y el profesor Antonio Di Pietro. El grupo de Córdoba lleva investigando más de 15 años en los mecanismos de patogénesis de *Fusarium* y han contribuido con su experiencia al análisis y la interpretación de las secuencias genómicas del hongo patógeno. Según María Isabel García Roncero, de la UCO, con su trabajo, que publica hoy la revista *Science* , se facilita el desarrollo de nuevos métodos para el control sostenible y eficaz de las enfermedades de las plantas.

CORDOBA (giornale)
(traduzione dallo spagnolo)
Pagina> Locale

RICERCA.
Decifrano il genoma responsabile della ` fusariosis´ del grano
L'apporto dell'Università cordovese è stato molto importante.
07/09/2007 REDAZIONE
Una squadra di 45 investigatori di 8 paesi, compresa Spagna, ha decifrato il genoma completo del Fusarium graminearum, un fungo patogeno dei cereali responsabile della fusariosis della spiga del grano che provocò solo negli Stati Uniti 3.000 milioni di dollari di perdite nella decade dei 90.
L'iniziativa della sequenza e l'analisi del genoma è stata pubblicata dal professore Corby Kistler dell'Università del Minnesota in collaborazione col Broad Institute della Massachusetts Institute of Technology, MIT, e l'Università di Harvard, tutte negli Stati Uniti.
L'apporto spagnolo è stato dato attraverso il dipartimento di Genetica dell'Università di Cordova (UCO), dove lavorano Roncero ed il professore Antonio Di Pietro. Il gruppo di Cordova sta investigando da più di 15 anni nei meccanismi di patogenesi di Fusarium e ha contribuito con la sua esperienza all'analisi e l'interpretazione delle sequenze genomiche del fungo patogeno. Secondo Maria Isabel García Roncero, dell'UCO, col suo lavoro, che pubblica oggi la rivista Science, si facilita lo sviluppo di nuovi metodi per il controllo sostenibile ed efficace delle malattie delle piante.

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28 novembre 2007

FELIPE, IL PRINCIPE DELLA ASTURIE E SUCCESSORE DI JUAN CARLOS AL TRONO DI SPAGNA CON LA MOGLIE LETIZIA VISITANO L'UNIVERSITÀ DI CÓRDOBA E PARLANO, TRA L'ALTRO, CON TONI DI PIETRO, PER INFORMARSI DELLA RICERCA SULLA GENETICA ALL'UNIVERSITÀ, CONGRATULANDOSI PER I RISULTATI.

Toni ha informato i Principi delle Asturie degli eccellenti risultati ottenuti nel suo campo, che potrebbero ancora migliorare se i

fondi a disposizioni fossero aumentati. Il Principe si è congratulato con lui.
L'articolo che segue è preso dal Sito Web ufficiale dell'Università di Cordoba uco.es. Pubblico uno stralcio prima in spagnolo e poi la traduzione in italiano:

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Due giovani si stringono la mano. Il Principe Felipe di Borbone, erede al trono di Spagna e il prof. dr. Antonio di Pietro di Morra De Sanctis, professore e ricercatore all'Università di Córdoba, Spagna. Accanto al Principe la principessa donna Letizia. Accanto a Toni due ricercatrici.
(la foto è stata messa gentilmente a disposizione dall'Università di Córdoba

Noticias: Los Príncipes de Asturias inauguran el Rectorado de la Universidad de Córdoba

Los Príncipes de Asturias, Don Felipe y Doña Letizia, han inaugurado esta mañana la sede del nuevo Rectorado de la Universidad de Córdoba en una ceremonia a la que han asistido alrededor de 300 autoridades y representantes de la sociedad

cordobesa y andaluza. Antes del acto de inauguración, que ha consistido en el descubrimiento de una placa conmemorativa, sus altezas reales han tenido la oportunidad de visitar el salón de actos del nuevo Rectorado, así como el yacimiento arqueológico que existe en la parte trasera del edificio.

Además, Don Felipe y Doña Letizia han tenido la oportunidad de conocer de primera mano la actividad científica de la Universidad de Córdoba en una reunión con 12 jóvenes investigadores de la institución, en la que han estado presentes el rector, José Manuel Roldán Nogueras, así como el consejero de Innovación y Ciencia, Francisco Vallejo, y el secretario de Estado de Universidades, Miguel Ángel Quintanilla. Los investigadores presentes en la reunión han sido Carmen Galán, directora del departamento de Botánica, Ecología y Fisiología Vegetal, Antonio Di Pietro, profesor titular de Genética, Eva María Vázquez Gómez, profesora contratada doctora y vicedecana de Estudiantes y Programas de Intercambio de la Facultad de Derecho y Ciencias Económicas y Empresariales, María José Polo Gómez, profesora titular de Ingeniería Hidráulica, María del Mar Malagón Poyato, profesora titular de Universidad- Habilitada para Cátedrática del Área de Biología Celular, Manuel Tena Sempere, catedrático de Fisiología, Octavio Salazar Benítez, profesor titular de Derecho Constitucional, Emilio Fernández Reyes, Catedrático de Bioquímica y Biología Molecular, Pablo Pérez Martínez, médico Interno Residente e investigador Doctor, María Felipe Colodrero, becaria de FPU del Ministerio de Educación en el Departamento de Arqueología, Historia del Arte y Música, Alberto Marinas Aramendia, doctor contratado Programa de Retorno de la Junta de Andalucía del área de Química Orgánica, y Rosario Mérida Serrano, directora de Espacio Europeo de Educación Superior y especialista en Educación Infantil y Formación Docente en Educación Superior.

Tras la reunión, los Príncipes de Asturias han saludado a los miembros del Consejo de Gobierno para pasar posteriormente al despacho del rector donde esperaba la alcaldesa de Córdoba, Rosa Aguilar, donde han firmado en el libro de honor de la institución.

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Traduzione

I PRINCIPI DELLE ASTURIE INAUGURANO LA SEDE DEL NUOVO RETTORATO DELL'UNIVERSITÀ DI CORDOVA

I Principi delle Asturie, Don Felipe e Signora Letizia, hanno inaugurato
questa mattina la sede del nuovo Rettorato dell'Università di Cordova in una cerimonia alla quale hanno assistito circa 300 autorità e rappresentanti della società cordovana ed andalusa. Prima dell'atto di inaugurazione che è consistito nella scoprimento di una targa commemorativa, le sue altezze reali hanno avuto l'opportunità di visitare l'aula magna del nuovo Rettorato, come il giacimento archeologico che esiste nella parte posteriore dell'edificio.

Inoltre, Don Felipe e Signora Letizia hanno avuto l'opportunità di conoscere di prima mano l'attività scientifica dell'Università di Cordova in una riunione con 12 giovani investigatori dell'istituzione, nella quale sono stati presenti il rettore, José Manuel Roldán Nogueras, come il consigliere di Innovazione e Scienza, Francisco Vallejo, ed il sottosegretario di Università, Miguel Quintanilla. Gli investigatori presenti nella riunione sono stati Carmen Galán, direttrice del dipartimento di Botanica, Ecologia e Fisiologia Vegetale, Ingegneria Idraulica, María del Mar Malagón Poyato, insegnante titolare di Università - Abilitata per Cátedrática dell'Area di Biologia Cellulare, Manuel Tena Sempere, professore universitario di Fisiologia, Octavio Salazar Benítez, professore titolare di Diritto Costituzionale, Emilio Fernández Reyes, Professore universitario di Biochimica e Biologia Molecolare, Pablo Pérez Martínez, medico Interno Residente ed investigatore Dottore, María Felipe Colodrero, borsista di FPU del Ministero di Educazione nel Dipartimento di Archeologia, Istoria dell'Arte e Musica, Alberto Marini Aramendia, dottore contrattato Programma di Ritorno della Giunta dell'Andalusia dell'area di Chimica Organica, e Rosario Merida Montanaro, direttrice di Spazio Europeo di Educazione Superiore e specialista in Educazione Infantile e Formazione

Docente in Educazione Superiore. Dopo la riunione, i Principi delle Asturie hanno salutato i membri del Consiglio di Governo per passare poi all'ufficio del rettore dove aspettava il sindaco di Cordova, Rosa Aguilar, dove hanno firmato nel libro di onore dell'istituzione.....

Antonio Di Pietro, professore titolare di Genetica, Eva María Vázquez Gómez, insegnante contrattata dottoressa e vicedecana di Studenti e Programmi di Scambio della Facoltà di Diritto e Scienze Economiche ed Imprenditoriali, María José Polo Gómez, insegnante titolare di insegnante contrattata dottoressa e vicedecana di Studenti e Programmi di Scambio della Facoltà di Diritto e Scienze Economiche ed Imprenditoriali, María José Polo Gómez, professoressa titolare di Ingegneria Idraulica, María del Mar Malagón Poyato, insegnante titolare di Università - Abilitata per Catedrática dell'Area di Biologia Cellulare, Manuel Tena Sempere, professore universitario di Fisiologia, Octavio Salazar Benítez, professore titolare di Diritto Costituzionale, Emilio Fernández Reyes, Professore universitario di Biochimica e Biologia Molecolare, Pablo Pérez Martínez, medico internista ed investigatore Negli ultimi mesi abbondano i riconoscimenti internazionali per Antonio Di Pietro.

Dopo l'avanzamento a professore titolare di Genetica all'Università di Córdoba in Spagna e la pubblicazione del suo nome nella lista dei ricercatori che hanno decifrato il genoma di una malattia del grano, sulla prestigiosa rivista scientifica Science, dopo il suo incontro col principe delle Asturie Felipe e la principessa donna Letizia, ora gli è arrivata la nomina come membro del comitato scientifico della prestigiosa e rinomata Società British Society of Microbiology. Nel Comitato di questa Society fino ad ora c'erano solo membri che abitano in Inghilterra, Toni è l'unico che abita fuori della Gran Bretagna e questo è un grande onore per lui.

Se continua di questo passo certamente avrà altri riconoscimenti importanti.

Noi dell'AME, di cui Toni fa parte, gli auguriamo tutto il bene per il suo futuro scientifico e l'additiamo a Morra come uno dei tanti morresi emigrati che si son fatti strada in terra straniera grazie alla loro intelligenza e al loro lavoro.

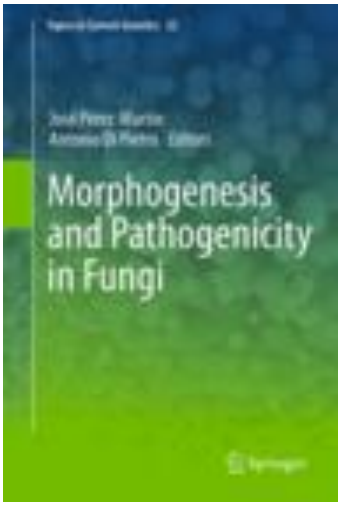
Anche la moglie dottoressa Anna Maria Torres è stata citata ultimamente su un giornale di Córdoba per le sue ricerche sulle rose. In sostanza stanno cercando di creare delle rose senza spine. Anche a lei auguri per il futuro nel suo Istituto di Ricerca e Formazione Agraria e della Pesca della Giunta di Andalusia.

Foto: La dr. sa Anna Torres-Di Pietro giornale di Cordoba ABC.

Questa foto è stata presa da un lungo articolo sul giornale spagnolo ABC

La Spring Verlag Tedesca (Casa Editrice Spring) ha pubblicato un libro scientifico un capitolo di Toni da loro richiesto sulla sua ricerca. Il libro costa 400 Euro. Il professore che cura la stesura del libro gli ha detto che il suo capitolo è il migliore. Già anni fa Toni fu contattato dall'India per scrivere un capitolo su un libro scientifico, che poi fu pubblicato in India. Infatti Toni è uno dei massimi esperti in questo campo. È anche membro di redazione di alcuni giornali scientifici. Adesso è professore titolare di genetica all'Università di Cordoba. Ci congratuliamo con lui, augurandogli una grande carriera scientifica, come genitori, ma anche come AME. Toni è iscritto alla nostra Associazione fin dalla sua fondazione ed era membro del Comitato Centrale AME fino a quando rimase in Svizzera.

(Gli articoli sopra sono stati presi in Internet dai rispettivi giornali ondine.)



Morphogenesis and Pathogenicity in Fungi Series: Topics in Current Genetics, Vol. 22 Pérez Martín, José; Di Pietro, Antonio Eds.) 2012 Preis ab **CHF 165.00**

Studies on the biology of *Chaetomium globosum* Kunze and its mode of action as an antagonist of *Pythium ultimum* Trow

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Antonio Di Pietro

aus Morra De Sanctis, Italien

Basel, 1990

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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag der

Herren Professoren Prof. Dr. F.J. Schwinn und Prof. Dr. T. Boller

Basel, den 18. 12. 1990

Dekan

Prof. Dr. M. Frey

To my parents Gerardo and Rosa Di Pietro

Acknowledgements

I thank Prof. Dr. F.J. Schwinn for allowing me the freedom to explore and for his support and guidance during this work.

I also thank Prof. Dr. T. Boller for his valuable suggestions and critical review of this dissertation.

I am particularly grateful to Dr. Manuela Gut-Rella for her enthusiastic encouragement, our fruitful discussions, and for introducing me into "real laboratory life".

Special acknowledgements are in order for Dr. J.P. Pachlatko, for his valuable help in the isolation and identification of the fungal metabolites.
I also thank Prof. Dr. H. Fritz for performing NMR and mass spectroscopy.

I am very grateful to H. Dahmen and to Dr. Ole Becker for their suggestions and the supply of fungal and bacterial isolates.

Special thanks to Guido Lüond for his help with scanning electron microscopy.

I also thank Snezana Neff, Bertrand Birlinger and all the members of the Microbiology Basic Research group in Ciba-Geigy for their help and sense of humor. Special thanks to Traute Bader and Lidija Jerkovic for their assistance to my work.

Thanks to Dr. Jenny Smith for sharing lab and music with me, and for her constructive corrections on my English.

Finally I thank my parents Gerardo and Rosa Di Pietro and my sister Jolanda for their confidence, patience and support.

Abstract

The importance of the three potential modes of action competition, mycoparasitism, and antibiosis in the antagonism of *Chaetomium globosum* against *Pythium ultimum*, the causal agent of damping-off, was investigated. Furthermore, studies on the optimum application method of *C. globosum* were conducted.

Different strains of *C. globosum* reduced Pythium damping-off of sugarbeet in the growth chamber. *C. globosum* grown on wheat bran (wheat bran inoculum; WBI) was the more effective application method than seed coating with ascospores of *C. globosum*. The optimum application rate for fresh WBI in sterile soil was 0.5% (w/v). Efficacy of dried WBI was equal to that of fresh WBI. In-furrow application of dry WBI of *C. globosum* strain Cg-13 at a rate of 2 g per row meter in Pythium-infested nonsterile soil resulted in 77% healthy plants (compared to 4% healthy plants in the Pythium check).

Three strains of *C. globosum* with differing efficacies in reducing Pythium damping-off were selected for further studies on the mode of action against *P. ultimum*. Strain Cg-13 (*C. globosum* var. *ochraceoides*) was chosen as highly effective, strain Cg-43 (*C. globosum* var. *flavo-viride*) as medium effective, and ADP-13, a spontaneous variant of strain Cg-13, as completely ineffective biocontrol strain.

Extensive colonization of cucumber and sugarbeet roots by *C. globosum* was observed with the light microscope using the fluorescent stain Calcofluor White. Population density (colony forming units) on cucumber seedlings did not differ significantly between the *C. globosum* strains during the first 4 days after planting.

No hyphal interactions were observed in soil between *C. globosum* and *P. ultimum*. Conversely, *C. globosum* hyphae coiled around the hyphae of the plant pathogen *Rhizoctonia solani*, forming appressoria-like structures. Alterated zones were visible on hyphae of *R. solani* at the interaction sites with *C. globosum*. These zones showed strong fluorescence upon staining with Calcofluor White, indicating the presence of polysaccharide oligomers due to partial lysis of the *R. solani* cell walls.

Strains Cg-13 and ADP-13 produced equal levels of ß-1,3-glucanase when grown in liquid culture on *P. ultimum* cell walls as sole carbon source. Only Cg-13 produced detectable quantities of chitinase.

Culture filtrates of *C. globosum* strain Cg-13 grown in 1% malt extract broth (ME) at temperatures from 10°C to 25°C were inhibitory to hyphal growth of *P. ultimum* and *R. solani*. The fungitoxic metabolite 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT) was isolated from the filtrate. BHT was not present in the culture filtrates of ADP-13 and of Cg-43, but the filtrate of Cg-43 contained a different unidentified fungitoxic metabolite. Eight out of eleven tested *C. globosum* strains produced BHT in 1% ME.

The fungitoxic metabolite chaetomin was isolated from culture filtrates of Cg-13 grown in 1% corn steep powder broth (CSP). Chaetomin was not present in the filtrates of ADP-13 and Cg-43. Six out of eleven tested *C. globosum* strains produced chaetomin in 1% CSP. A positive correlation between chaetomin production in liquid culture and the efficacy of the *C. globosum* strains in reducing Pythium damping-off was observed.

Chaetomin was extracted from sterile soil inoculated with WBI of strain Cg-13, but not from soil inoculated with WBI of ADP-13. Chaetomin, but not BHT was isolated from soil inoculated with Cg-13 on a vermiculite/ME substrate.

In a bioassay in microtiter plates, BHT showed only a weak inhibitory activity against hyphal growth and sporangial germination of *P. ultimum*. The minimal inhibitory concentration (MIC) was higher than 50 ppm. Conversely, chaetomin was extremely inhibitory to *P. ultimum*. MICs were 2.5 ppm and 0.5 ppm for mycelial growth and sporangial germination respectively. The inhibitory activity of chaetomin was in the same range as that of metalaxyl, and five to ten times higher than that of the chemically related antibiotic gliotoxin from *Gliocladium fimbriatum*.

The results of the present work suggest that chaetomin production in soil by *C. globosum* plays an important role in reduction of damping-off of sugarbeet caused by *P. ultimum*. Competition and mycoparasitism are probably not important mechanisms in this antagonistic system, but mycoparasitism may be a potential mode of action of *C. globosum* against *R. solani*.

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Abbreviations

a.i.	active ingredient
BHT	2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran
cfu	colony forming units
Cgselmed	semiselective medium for *Chaetomium globosum*
CMA	corn meal agar
CSP	corn steep powder
EC_{50}	concentration at which 50% inhibition is achieved
EC_{250}	emulsifiable concentrate
ELISA	enzyme linked immunosorbent assay
EtOAc	ethyl acetate
exp.	experiment
HPLC	high performance liquid chromatography
hr	hour(s)
L	liter
ME	malt extract
MEA	malt extract agar
MIC	minimal inhibitory concentration
min	minute(s)
NMR	nuclear magnetic resonance
PDA	potato dextrose agar
PDB	potato dextrose broth
PEG	polyethyleneglycol
Pselmed	semiselective medium for *Pythium ultimum*
r.h.	relative humidity
rF	relative to front
rpm	rounds per minute
sec	second(s)
SM	synthetic medium
TLC	thin layer chromatography
U	unit(s)
UV	ultra violet
v/v	volume/volume
w/v	weight/volume
WA	water agar
WBI	wheat bran inoculum
WP	wettable powder

Introduction

Considerable losses are due to plant diseases caused by soilborne fungal pathogens. Currently the use of fungicides is the only reliable and economic means to prevent diseases in large scale agriculture. Concerns about environmental hazards, the risk of development of resistant pathogen biotypes (COHEN & COFFEY, 1986), and the increasing trend towards the integrated use of different control measures have promoted research on alternative control methods. Biological control has gained considerable attention and appears to be a promising supplement or in some cases an alternative to chemical control (COOK & BAKER, 1983; PAPAVIZAS, 1985). However, biocontrol agents are frequently unreliable and expensive, and have not represented a truly feasible alternative to chemicals so far (LISANSKY, 1989).

To improve efficacy of biological control, a better understanding of mechanisms of action, nutrition and ecology of biocontrol agents is needed (COOK & BAKER, 1983). Such knowledge will lead to substantial progress in selection of superior strains, mass production and appropriate formulation of biocontrol organisms. The creation of new superior biocontrol strains by genetic engeneering, for example, seems very promising but requires extensive knowledge on specific gene products important in biological control and thus, on the mode of action of a particular antagonist.

Principal mechanisms of biological control are competition, antibiosis, and hyperparasitism (COOK & BAKER, 1983). Only in a few cases the mode of action of fungal antagonists of soilborne plant pathogens has been conclusively elucidated. A major problem lies in the difficulty of proving that results obtained in vitro are equally relevant in the in soil system (HORNBY, 1983). Furthermore, the same antagonist can exhibit different modes of action against different pathogens and under different conditions. Extensive studies conducted on the genus *Trichoderma* showed that, depending on the *Trichoderma* strain and on the pathogen, rhizosphere competence (AHMAD & BAKER, 1988) as well as antibiosis (CLAYDON & ALLAN, 1987; GHISALBERTI et al., 1990) and mycoparasitism (ELAD et al.,

1983; SIVAN & CHET, 1989) may be determinant factors in biocontrol.

The oomycete *Pythium ultimum* Trow is an important pathogen involved in seed rots and preemergence damping-off of a wide variety of plant species (HENDRIX & CAMPBELL, 1973). The disease is widely distributed all over the world (AGRIOS, 1988). The pathogen survives in soil as dormant oospores and sporangia during intersubstrate periods (STANGHELLINI & HANCOCK, 1971; STANGHELLINI, 1974; AYERS & LUMSDEN, 1975). Propagules germinate rapidly in response to nutrient stimuli such as soluble or volatile exudates from germinating seeds (NELSON, 1987). Spore germ tubes or saprophytic mycelium quickly penetrate the seed coat or the seedling through mechanical pressure and dissolution by means of enzymes. Colonization of sugarbeet seeds occurs within 4 hr under favorable conditions (OSBURN et al., 1989). Seeds fail to germinate, become soft and mushy, turn brown and finally disintegrate. Young seedlings are attacked at any point of the tissue. The infected area enlarges rapidly, the invaded cells collapse, and the seedling is overrun by the fungus and dies shortly after the beginning of infection. The severity of disease is greater when the soil is kept wet for prolonged periods; when the temperature is unfavorably low for the host plant; when there is an excess of nitrogen in the soil; and when the same crop is planted in the same field for several consecutive years. Control measures include cultural practices such as soil drainage and crop rotation, soil sterilization, and seed treatment with chemicals (AGRIOS, 1988).

In recent years research on biological control of seed rot and damping-off by *P. ultimum* has received increasing attention. These diseases are suitable targets for biological control because the susceptible period for the host is relatively short and therefore high populations of antagonists are not required for extended periods of time (SCHROTH and HANCOCK, 1981). Many reports describe different fungal antagonists reducing Pythium damping-off under controlled environmental conditions (MARTIN & HANCOCK, 1987; WALTHER & GINDRAT, 1988; LUMSDEN & LOCKE, 1989; PAULITZ et al., 1990). However, only few studies exist on the successful use of fungal biocontrol agents against *P. ultimum* under field conditions (HADAR et al., 1984; HARMAN et al., 1989).

Like *P. ultimum*, the basidiomycete *Rhizoctonia solani* Kühn causes damping-off of many different crops. The pathogen survives in soil or on plant material as black hard sclerotia or pigmented resistant mycelia. Under favorable conditions it can rapidly colonize the host plant by formation of adherent pigmented infection cushions or hyaline appressoria and infection pegs (AGRIOS, 1988). *R. solani* is subject to attack by several mycoparasites in soil and numerous attempts have been made to control the pathogen with various fungal antagonists (WALTHER & GINDRAT, 1988; LUMSDEN & LOCKE, 1989; LEWIS et al., 1990).

The saprophytic ascomycete *Chaetomium globosum* Kunze is a potential antagonist of several soilborne and seedborne plant pathogens. The fungus occurs very commonly in organic matter and soil and is especially favored by cellulosic substrates (DREYFUSS, 1975). Antagonistic activity of *C. globosum* was described against *Fusarium nivale* on oat (TVEIT & WOOD, 1955); *F. roseum* on corn (KOMMEDAHL & CHANG MEW, 1975); *F. solani* f.sp. *pisi* on pea (HUBBARD et al., 1982); *Alternaria raphani* and *A. brassicicola* on radish and cabbage respectively (VANNACCI & HARMAN, 1987); *P. ultimum* and *R. solani* on sugarbeet (WALTHER & GINDRAT, 1988). Several antagonistic mechanisms have been proposed to play a role in disease reduction by *C. globosum*. Coiling of *C. globosum* hyphae around hyphae of *Rhizoctonia solani* (WALTHER & GINDRAT, 1988) and *Alternaria brassicicola* (VANNACCI & HARMAN, 1987) was observed in dual culture, indicating potential mycoparasitism. However, the importance of mycoparasitism in the biological control of these pathogens by *C. globosum* has not been demonstrated. Furthermore, hyphal coiling was never observed around hyphae of *P. ultimum* (HUBBARD et al., 1982; WALTHER & GINDRAT, 1988) although *C. globosum* effectively reduced Pythium damping-off in the greenhouse (WALTHER & GINDRAT, 1988). A non-diffusable antibiotic was recovered from pea seeds treated with *C. globosum* ascospores (HUBBARD et al., 1982). Control of *P. ultimum* was therefore attributed to antibiosis, but definitive evidence is still lacking.

This study was conducted to gain further knowledge on the mode of action of *C. globosum* against *P. ultimum*. The following approach was used: in a first step, the ability of different *C. globosum* strains to

reduce Pythium damping-off of sugarbeet was determined in growth chamber experiments. The aim was to select *C. globosum* strains with strongly differing efficacies against *P. ultimum*. These strains were then compared with regard to different criteria related to the three potential antagonistic mechanisms competition, antibiosis, and mycoparasitism. Factors such as rhizosphere colonization, hyphal interactions, excretion of lytic enzymes, and production of inhibitory metabolites were evaluated for the selected strains. Thereby, the importance of each mechanism in antagonism against *P. ultimum* was assessed. Furthermore, studies on the optimum application method of *C. globosum* were conducted in the growth chamber.

Materials and Methods

1. Microorganisms

TABLE 1. Strains of *Chaetomium globosum*

Strain	Variety	Source
Cg-1	n.d.[a]	D. Gindrat[b]
Cg-3	n.d.	D. Gindrat
Cg-13	var. *ochraceoides* Dreyfuss[c]	D. Gindrat
ADP-13	var. *ochraceoides* Dreyfuss[c]	A. Di Pietro, spontaneous variant of strain Cg-13
Cg-14	n.d.	D. Gindrat
Cg-20	n.d.	D. Gindrat
Cg-22	n.d.	D. Gindrat
Cg-28	n.d.	H. Dahmen[d]
Cg-29	n.d.	D. Gindrat
Cg-30	n.d.	D. Gindrat
Cg-39	n.d.	D. Gindrat
Cg-40	n.d.	D. Gindrat
Cg-43	var. *flavo-viride* Novac[c]	D. Gindrat
Cg-41	n.d.	D. Gindrat
Cg-57	n.d.	D. Gindrat
Cg-98	n.d.	D. Gindrat
Cg-200	n.d.	H. Dahmen
C-1	n.d.	A. Di Pietro, tomato stem, Bari, Italy
C-3	n.d.	A. Di Pietro, strawberry, Cesena, Italy
C-4	n.d.	A. Di Pietro, tomato leaf, Bari, Italy
C-5	n.d.	A. Di Pietro, tomato root, Bari, Italy

[a]not determined

[b]Swiss Federal Agricultural Research Station of Changins, CH-1260 Nyon, Switzerland

[c]Variety was determined by L. Petrini (personal communication)

[d]Ciba-Geigy AG, Basel, Switzerland

All fungal strains were stored in 10% glycerol / 8.5% skim milk at -196°C according to DAHMEN et al. (1983). Strains were grown on 1% MEA unless otherwise stated.

TABLE 2. Fungal strains

Strain	Species	Source
A-158	*Aphanomyces laevis*	H. Dahmen[a]
F-198	*Fusarium oxysporum* f.sp. *lycopersici*	H. Dahmen
P-71	*Pythium ultimum*	H. Dahmen
P-146	*P. ultimum*	H. Dahmen
P-161	*P. ultimum*	H. Dahmen
P-194	*P. debaryanum*	H. Dahmen
P-231	*P. aphanidermatum*	H. Dahmen
P-232	*P. aphanidermatum*	H. Dahmen
P-312	*P. arrhenomanes*	H. Dahmen
P-318	*P. graminicola*	H. Dahmen
R-160	*Rhizoctonia solani*	H. Dahmen
X-1	*Rhizopus sp.*	A. Di Pietro, soil from Stein, Switzerland

[a]Ciba-Geigy AG, Basel, Switzerland

2. Growth media

The standard growth media listed here were used throughout the work. Composition of special growth media, and alterations in the composition of the standard media are detailed in the materials and methods section of the determined experiments. All quantities are given per L distilled water. The pH was adjusted to the appropriate value with 0.1 M HCl or 0.1 M NaOH, and the media were autoclaved at 121°C for 20 min.

Corn meal agar (CMA)	**Supplier[a]**	
Corn meal	(5)	17 g
Bacteriological agar No 1	(5)	15 g
pH 6.0		

1% corn steep powder broth (1% CSP)

Corn steep powder	(2)	10 g
pH 5.6		

1% malt extract broth (1% ME)

Malt extract	(6)	10 g
pH 5.6		

1% malt extract agar (1% MEA)

Malt extract	(6)	10 g
Bacteriological agar No 1	(6)	15 g
pH 5.6		

2% malt extract agar (2% MEA)

Malt extract	(6)	20 g
Bacteriological agar No 1	(6)	15 g
pH 5.6		

3% malt extract agar (3% MEA)

Malt extract	(6)	30 g
Bacteriological agar No 1	(6)	20 g
pH 5.6		

Potato dextrose agar (PDA)

Potato dextrose broth	(3)	24 g
Bacteriological agar No 1	(6)	15 g
pH 5.6		

Potato dextrose broth (PDB)

Potato dextrose broth	(3)	24 g
pH 5.6		

Semiselective medium for *C. globosum* (Cgselmed)

CGA 173506	(2)	10 mg a.i.
Ridomil	(2)	10 mg a.i.
Botran	(2)	1 mg a.i.
Tetracyclin	(7)	10 mg a.i.
Ampicillin	(7)	100 mg a.i.

Semiselective medium for *P. ultimum* (Pselmed)

Benlate	(2)	10 mg a.i.
Botran	(2)	10 mg a.i.
Tetracyclin	(7)	10 mg a.i.
Ampicillin	(7)	100 mg a.i.

Synthetic medium (SM) (OKON et al., 1973)

$MgSO_4.7H_2O$	(4)	0.2 g
KH_2PO_4	(4)	0.9 g
KCl	(4)	0.2 g
NH_4NO_3	(4)	1.0 g
$FeSO_4$	(4)	0.01 g
$ZnSO_4$	(4)	0.01 g
$MnSO_4$	(4)	0.006 g

V-8-agar

V-8 juice	(1)	200 ml
$CaCO_3$	(5)	3 g
Bacteriological agar No 1	(6)	15 g
Distilled water		800 ml
pH 6.2		

V-8-broth

V-8 juice	(1)	200 ml
$CaCO_3$	(5)	3 g
Distilled water		800 ml
pH 6.2		

For the preparation of cleared V-8-medium, V-8-juice was centrifuged at 12,500 g for 10 min at 4°C, and the clear supernatant was used.

Water agar (WA)

Bacteriological agar No 1	(6)	15 g
pH 5.6		

[a]Supplying chemical companies

(1) Campbell's Soups, Felegara, Italy
(2) Ciba-Geigy AG, Basel, Switzerland
(3) Difco Laboratories, Detroit, USA
(4) Fluka Chemie AG, Buchs, Switzerland
(5) Merck AG, Darmstadt, FRG
(6) Oxoid Ltd, Basingstoke, England
(7) Sigma Chemicals, St. Louis, USA

3. Buffers

Phosphate buffer

To obtain 10 mM phosphate buffer, pH 7.0, the following were dissolved in 1000 ml distilled water:

KH_2PO_4	1.36 g
K_2HPO_4	2.28 g

4. Soil

The soil used in all experiments was a sandy loam from Stein, Switzerland. Soil characteristics were: pH 7.8; 55.6% sand, 20.1% silt, 24.2% clay, 2.4% organic matter (analysis performed at Ciba-Geigy AG, Stein, Switzerland). For sterilization, the soil was heated in a drying oven to 120°C for 2 hr on two consecutive days, and stored in metal containers for at least 14 days before use.

5. Determination of growth rates and perithecium formation

Petri plates with the following growth media were prepared:

1% MEA, 2% MA, WA, CMA, PDA, V-8-agar.

The plates were inoculated with agar plugs from 7 day old colonies of the test fungi on 1% MEA. Three plates per strain, growth medium and temperature were inoculated. Plates were incubated at the following temperatures:

10°C, 15°C, 20°C, 25°C, 30°C.

Radial growth was measured 1, 2, 3, 4, and 8 days after inoculation and growth rates were calculated in mm per day. Perithecium formation of *C. globosum* was recorded five weeks after inoculation.

6. Growth chamber experiments

6.1. Production of *C. globosum* ascospores

3% MEA plates were inoculated with an ascospore suspension from a 4-week-old *C. globosum* culture on 1% MEA. Cultures were incubated for 4 weeks at 25°C. The plates were then left in a fume hood at room temperature under a continous air flow, until the agar was completely dry. Perithecia were harvested by scraping them from the agar with a sterile spatula, and transferred into a sterile glass vial. Ascospores were liberated by agitating the perithecia with a spatula. Ascospore powder was stored at 4°C until use. Ascospore suspension was prepared by adding an appropriate volume of sterile tap water to a determined quantity of ascospores in a sterile vial and vortexing. The concentration of spores was determined by counting in a hemacytometer, and sterile tap water was used to adjust the suspension to the desired end concentration.

6.2. Preparation of *C. globosum* wheat bran inoculum (WBI)

50 g wheat bran and 130 ml distilled water were mixed in autoclavable plastic bags and autoclaved for 30 min. The bran was inoculated with an ascospore suspension of *C. globosum* (see 6.1.) providing 10^6 spores per bag. The bran was mixed and incubated at 25°C for 7 days. Control bran was not inoculated. Bran colonized by the antagonist will be referred to as wheat bran inoculum (WBI). The number of cfu of *C. globosum* in the WBI at time of planting was approximately 10^5 cfu per g of WBI for all strains of *C. globosum*, as determined by vortexing 1 g of WBI in 10 ml of sterile phosphate buffer and plating serial dilutions on 1% MEA plates.
For the production of dry WBI the colonized bran was spread on plastic plates and dried in a ventilated drying oven at 35°C and 100% aeration for 3 days. After each day the bran was ground with a pestle to obtain a dry powder after 3 days. Dry WBI was stored in glass bottles at room temperature until use.

6.3. Seed coating with *C. globosum* ascospores

Ascospores of *C. globosum* were produced as described in 6.1. For seed coating of sugarbeet seeds, 30 mg of ascospore powder were suspended in 400 µl 20% vinamul (Vinyl Products, England) and transferred into a small plastic bag. 5 g of

sugar beet seed (*Beta vulgaris* L. cv. KW, monogerm) were added. For seed coating of cucumber seeds, 50 mg of ascospore powder were suspended in 500 µl 20% vinamul and transferred into a plastic bag. 15.5 g of cucumber seed (*Cucumis sativus* L. cv. Wisconsin SMR-58) were added. The closed bag was shaken vigorously to assure an even distribution of the ascospores on the seed surface, providing 1.5 x 10^5 spores per sugarbeet seed or 2 x 10^5 spores per cucumber seed. The seeds were subsequently dried overnight under a continous air flow and stored in petri plates at room temperature until use.

6.4. Soil inoculation with *P. ultimum*

Biocontrol activity of *C. globosum* against damping-off of sugar beet caused by *P. ultimum* was assayed in a soil from Stein, Switzerland. Soil characteristics and heat treatment of soil are described in chapter 4. *P. ultimum* strain P-71 was grown according to DAHMEN et al. (1983): 20 g of peeled, grated carrots and 150 ml of distilled water were autoclaved in Roux-bottles. Each bottle was inoculated with 3 agar plugs from a colony of *P. ultimum* on 1% MEA. Bottles were incubated at 20°C in the dark for 10 to 14 days. After the incubation, the content of a bottle was added to an equal amount of tap water and homogenized in a blender (Turmix AG, Switzerland) for 60 sec. The homogenate was mixed with 5 L soil. Control soil was mixed with a corresponding amount of tap water. The soil was incubated in plastic bags for 3 days under the growth chamber conditions described below. Inoculum density of *P. ultimum* at the time of planting was 500-1500 propagules per g of soil as determined by the method of STANGHELLINI & HANCOCK (1970).

6.5. Growth chamber assay for biocontrol activity of against Pythium damping-off

C. globosum strains were applied either as ascospore seed coating (see 6.3.) or as wheat bran inoculum (see 6.2.). WBI or uninoculated control bran was mixed with the soil before planting. The quantities of WBI added to the soil varied from 0.0625% to 2% (w/v) according to the experiment. The quantities used in each experiment are detailed in the results section.

To determine whether control of *P. ultimum* was due exclusively to fungitoxic metabolites produced by *C. globosum* during incubation on the bran, WBI of Cg-13 and uninoculated control bran were subjected to different killing treatments before adding to heat treated soil. Treatments were: autoclaving WBI for 30 min,

immersing WBI in liquid nitrogen for 10 min, or mixing WBI with 100 ppm Benlate (corresponding to a final concentration of 2 ppm in soil that would not affect growth of *P. ultimum*). All treatments killed the antagonist on WBI completely, as determined by the absence of growth after plating suspensions of treated WBI in sterile water on 1% MEA. Control WBI was not treated.

In certain experiments, metalaxyl WP 25 (Ciba-Geigy AG, Switzerland) was used as a chemical control standard. Metalaxyl was applied as a soil drench at the time of planting, at the concentrations detailed in the results section. Soil was filled in rectangular plastic pots (100 x 120 x 45 mm). 15 sugar beet seeds per pot were planted in 3 rows, with 5 seeds per row. Five replicates per treatment were performed.

The experiments with in-furrow application of *C. globosum* were carried out in rectangular plastic trays. 5 L of soil were filled in each tray (340 x 290 x 50 mm). Sugarbeet seeds were planted in four rows with 15 seeds each row. *C. globosum* was applied as dry WBI. A determined quantity of WBI (detailed in the descriptions of the single experiments) was strewed into the furrow of the row before planting the seeds. For rows per treatment were performed.

The growth chamber assay with cucumber (*Cucumis sativus* L. cv. Wisconsin SMR-58) was performed in plastic pots as described above, except that 10 cucumber seeds per pot were planted in 2 rows, with 5 seeds per row.

Pots were arranged in a randomized complete block design and incubated in a growth chamber (BBC-York GmbH., Mannheim, FRG) under the following conditions:

light: 14 hr, 23°C, 65% r.h.
dark : 10 hr, 18°C, 75% r.h.

For the experiments with application of dry WBI, the growth chamber conditions were as follows:

light: 14 hr, 17°C, 75% r.h.
dark : 10 hr, 12°C, 85% r.h.

The pots were watered as needed to maintain a moist surface. The number of healthy plants in each pot was recorded 14 to 17 days after planting.

6.6. Analysis of data

All experiments were at least performed twice. Data were subjected to analysis of variance and means were separated with Duncan's multiple range test (P=0.05). Results were expressed in % healthy plants compared to the non-inoculated check (100%).

7. Studies on colonization ability of *C. globosum*

7.1. Development of a semiselective medium for *C. globosum*

For the developement of a semiselective medium for the isolation of *C. globosum* from soil, the effect of several fungicides on growth of different soil fungi was determined. All fungicides tested were obtained from Ciba-Geigy AG, Basel, Switzerland. The following compounds were used.

TABLE 3. Fungicides used for the in vitro activity tests.

Product	Active ingredient	Formulation
Tilt	Propiconazole	EC 250
Tachigaren	Hymexazole	
Benlate	Benomyl	WP 50
Orthocid	Captan	
Botran	Dichloran	WP 75
Ridomil	Metalaxyl	WP 25
Score	Diphenoconazole	EC 250
Saphir	CGA 173506	WP 25

Stock solutions (100 mg per ml and 1 mg per ml) of the fungicides in sterile distilled water were prepared. 1% MEA was autoclaved in bottles and cooled to 60°C. Appropriate quantities of the stock solutions were added and mixed by gently shaking the bottle. The following concentrations were tested:

0 / 0.01 / 0.1 / 1 / 10 / 100 / 1000 ppm a.i.

Plates were poured and inoculated in the center with an agar plug from a colony of the test fungus on 1% MEA. Plates were incubated at 20°C and radial growth was measured at 24 hr intervals. The growth rates between day 2 and 3 (or between day 1 and 2 for *P. ultimum*) were calculated and used for determination of the EC_{50} values. The minimal concentration at which no visible growth occurred was designated as MIC.

7.2. Population density of *C. globosum* on cucumber seedlings

Sterile soil was inoculated with *P. ultimum* as described in chapter 6.4. *C. globosum* was applied as ascospore seed coating (see 6.3.) or 2% (w/v) WBI (see 6.2.). Planting procedures were as described in 6.5. Pots were incubated at the following growth chamber conditions: 14 hr light, 24°C, 60% r.h.; 10 hr dark, 18°C, 75% r.h., and regularly watered with sterile tap water. One, 2, and 4 days after planting, 3 seeds or seedlings were removed from each of 3 pots (9 seeds in total) per treatment. Of the 9 removed seeds, 3 remained untreated. The other 6 seeds were pooled in a vial containing 10 ml sterile tap water and vortexed for 10 sec. 3 of these seeds were then washed in 20 ml of sterile tap water. The remaining 3 seeds were surface sterilized (3 min in 4% bleach, 15 sec in 100 % ethanol) and washed in 20 ml of sterile tap water. One each of the untreated, the washed, and the surface sterilized seeds was placed on 1% MEA, on Cgselmed, and on Pselmed. The suspension in the vials was vortexed again for 10 sec and serial dilutions were prepared with sterile phosphate buffer. Three 10 µl drops from each dilution step (10^0, 10^{-1}, 10^{-2}, 10^{-3}) were pipetted on the 3 media described above. All plates were incubated at 24°C and the presence of Cg-13 and Cg-43 was determined. Population density of Cg-13 and Cg-43 was expressed in log cfu per seedling.

7.3. Population density of *C. globosum* on cucumber seedlings at different temperatures

The procedures of soil inoculation with *P. ultimum* were as described in chapter 7.2., except that soil sieved through a 3 mm mesh was used. Cucumber seeds were surface sterilized (10 min in 4% bleach, 30 min in sterile tap water). Plastic petri plates (Ø = 90 mm) were filled half with sterile soil or with soil inoculated with *P. ultimum*. In the WBI treatments, the WBI of *C. globosum* was mixed with the *P. ultimum* infested soil at a ratio of 2% (w/v). Sterilized seeds or seeds coated with *C. globosum* ascospores were placed on the soil layer in the petri plates and covered with additional soil. Eight seeds per petri plate were planted. 10 ml of sterile tap water were added to each petri plate. Plates were incubated at 100% r.h. at the following temperatures: 10°C, 15°C, 20°C, and 25°C.

One, 2, 3, and 4 days after planting, seeds or seedlings were removed aseptically from the petri plates (see Fig. 1). Per day, treatment, and temperature, seedlings from 2 petri plates (16 seeds in total) were removed. When the root length of the seedling exceeded 10 mm, only the root was used.

Eight of the seedlings (4 from each petri plate) were transferred individually to glass tubes containing 1 ml of sterile phosphate buffer and vortexed for 10 sec. Serial dilutions with sterile tap water were prepared in sterile microtiter wells. Two 8 µl drops from each dilution step (10^0, 10^{-1}, 10^{-2}, 10^{-3}) were pipetted on 1% MEA and on Cgselmed. The plates were incubated at room temperature. The presence of *C. globosum* was recorded after 2-4 days. Population density of *C. globosum* was calculated and expressed in cfu per seedling.

The remaining 8 seedlings were washed under running tap water. Three of them were placed on WA, 3 on Pselmed, and 2 on Cgselmed (controls). Presence *P. ultimum* on the seedlings was determined and expressed in % seedlings colonized by *P. ultimum*.

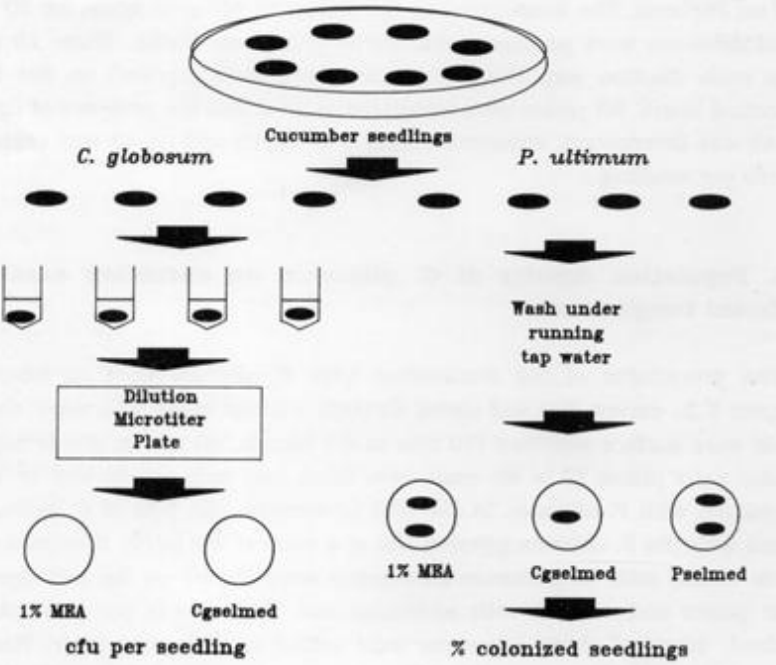


Figure 1. Procedure used to determine population density of *C. globosum* on cucumber seedlings.

7.4. Microscopical observation of *C. globosum* on roots of sugarbeet and cucumber

Sugarbeet or cucumber seeds were planted in heat treated soil inoculated with WBI of *C. globosum*. Plants were incubated in the growth chamber (see 7.2.). After 1-4 weeks, plants were recovered from soil. The soil particles were carefully removed, and roots were washed by gentle shaking in tap water. Samples were stained with a drop of a 0.05% (w/v) solution of the fluorescent stain Calcofluor White (Sigma) and observed with a Zeiss Axioplan microscope (excitation filter BP 350-440, chromatic beam splitter FT 460, barrier filter LP 470).

8. Hyphal interactions between *C. globosum* and *P. ultimum* or *R. solani*

8.1. Observation of hyphal interactions between *C. globosum* and *R. solani* using fluorescent staining

A method according to ELAD et al. (1983) was used to study hyphal interactions between *C. globosum* strain Cg-13 and *R. solani* strain R-160. Agar plugs from colonies of the two fungi on 1% MEA were placed on glass slides at a distance of about 1 cm. The slides were incubated at 100% r.h. in the dark at room temperature. After 6 days the agar plugs were carefully removed without disturbing the zone of hyphal interaction between the plugs. Hyphae were stained for 2 min in a 0.05% (w/v) solution of Calcofluor White (Sigma) and subsequently washed two times for 5 min in phosphate buffer. Hyphal interactions were observed with a Zeiss Axioplan microscope, using phase contrast or fluorescence (excitation filter BP 350-440, chromatic beam splitter FT 460, barrier filter LP 470).

8.2. Observation of hyphal interactions in soil

Hyphal interactions in soil were studied using a model system in petri plates. Heat treated soil was sieved through a 3 mm mesh and filled in petri plates. Soil was covered with an autoclaved cellophane membrane. An agar plug from a 1% MEA-colony of *C. globosum* strain Cg-13 was placed at one edge of the membrane and a plug from a colony of *P. ultimum* or *R. solani* was placed at the opposite

edge. The membrane was covered with additional soil and the petri plate was closed. Plates were incubated at 20°C and 100% r.h. After 7 days, the cellophane membranes were recovered from the soil and small pieces were cut out of the zones where colonies overlapped. Hyphal interactions were observed with a Zeiss Axioplan microscope and a scanning electron microscope.

8.3. Preparation for scanning electron microscope

Small pieces of cellophane membranes from the hyphal interaction area were placed on coverslips (Thermanox, Ø = 12 mm; Miles Laboratories Inc., USA). Samples were fixed in 2% glutaraldehyde (Fluka) in phosphate buffer for 60 min at room temperature, and post-fixed in the vapour phase of 1% OsO_4 (Fluka). Samples were washed two times 10 min in phosphate buffer and two times 10 min in distilled water. Specimens were dehydrated in a graded ethanol series (70%, 90%, 2 times 10 min; 100 %, 2 times 20 min). Critical point dried samples were then coated with gold palladium in a SCD-040 sputter coater (Balzers Union, Liechtenstein) and viewed in a scanning electron microscope (Cambridge S-200; Cambridge Instruments, England).

9. Production of lytic enzymes by *C. globosum*

9.1. Production of cellulase

100 ml SM in Erlenmeyer flasks (500 ml) was supplemented with one of the following carbon sources: glucose (Fluka); chitin (Fluka), cellulose (native; Merck); cellulose (microcrystalline; Merck), each at 50 g per L; and laminarin (Sigma) at 20 g per L. Each flask was inoculated with 2 plugs from a colony of the *C. globosum* strains Cg-13 or Cg-43. Controls were not inoculated. Cultures were incubated on a rotary shaker for 14 days at 25°C and 150 rpm, then centrifuged at 12,500 g for 10 min at 4°C. The supernatant was sterile filtrated through a syringe filter (X-60, 0.45 µm; Gelman Sciences, USA). The filtrate was concentrated tenfold, either by ultrafiltration (>10 kD, Novacell-Omegacell system; Filtron Corporation, USA) or lyophilization, and assayed for cellulase activity.

9.2. Assay for cellulase activity

The activity of cellulase was assayed using cellulose-azure (Type I; Sigma) as a substrate in a modification of the method of RINDERKNECHT et al. (1967). Cellulose-azure was ground in a mortar to a fine powder and a 2% suspension was prepared in 0.02 M sodium-phosphate buffer (pH 6.5). The reaction mixture containing 100 µl of crude enzyme and 900 µl of the substrate suspension was incubated at 35°C for 60 min in an Eppendorf reaction tube. The reaction was stopped by adding 300 µl of 1 M acetic acid. The mixture was centrifuged at 1500 g for 20 min and the absorption at 595 nm was measured. Blanks contained distilled water instead of enzyme or instead of the substrate suspension. Activity was calculated using a standard curve (Figure 2) obtained with the following concentrations of commercial cellulase from *Basidiomyces* sp. (20 mU/mg; Merck): 0.01 U; 0.05 U; 0.1 U; 0.2 U; and 0.5 U per ml. Activity was expressed in units.

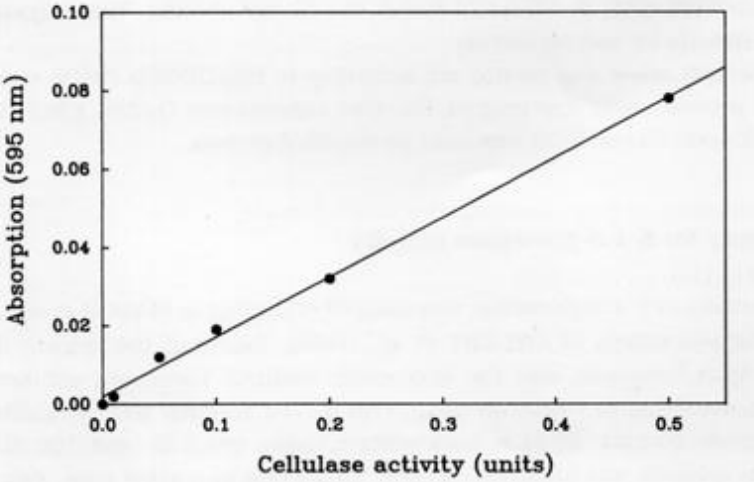


Figure 2. Standard curve for the cellulase activity assay.

9.3. Production of ß-1,3-glucanase and chitinase

Cell walls of *P. ultimum* were prepared as follows (according to SIVAN & CHET, 1989): *P. ultimum* was grown in Erlenmeyer flasks (2 L), each containing 400 ml 2.4% potato dextrose medium, on a rotary shaker at 22°C and 100 rpm for 5

days. The mycelium was harvested by centrifugation at 10,000 g and 4°C for 20 min, resuspended in sterile distilled water and homogenized in a blender (Sorvall Inc., USA) at the highest speed for 2 min. The mycelial suspension was centrifuged at 12,500 g at 4°C for 30 min. The pellet was resuspended in sterile distilled water, homogenized again and centrifuged. The procedure was repeated three times. The walls were then deep-frozen, lyophilized and kept as powder in a sealed container until use.

20 ml SM in Erlenmeyer flasks (100 ml) was supplemented with one of the following carbon sources (each at 10 g per L): laminarin (Sigma), or the *P. ultimum* cell wall preparation. After autoclaving, 0.1% glucose was added to the medium from a stock solution to promote ascospore germination. Flasks were inoculated with an ascospore suspension of the *C. globosum* strains Cg-13 or ADP-13, providing 2.5 x 10^4 spores per ml of medium. Controls were not inoculated. Cultures were incubated on a rotary shaker at 25°C and 100 rpm for 7 days, then centrifuged at 12,500 g and 4°C for 10 min. The supernatant was sterile filtered through a syringe filter (X-60, 0.45 µm; Gelman Sciences, USA) and dialysed against distilled water at 4°C for 24 h with one change of water. The dialysate was assayed directly for enzyme activity.

The protein assay was carried out according to BRADFORD (1976) using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories GmbH, FRG). Bovine serum albumin (Serva, FRG) was used as standard protein.

9.4. Assay for ß-1,3-glucanase activity

The activity of ß-1,3-glucanase was assayed according to MAUCH et al. (1988), using the procedures of DYGERT et al. (1965), following the release of free glucose from laminarin with the neocuproin method. Laminarin solution was prepared according to DENAULT et al. (1977). The reaction mixture containing 100 µl crude enzyme, 50 µl sodium acetate buffer (pH 5.0), and 100 µl 0.5% laminarin solution was incubated at 37°C for 20 min in a glass tube. Two ml of reagent A containing (g per L of distilled water): Na_2CO_3, 40; glycine, 16; $CuSO_4.5H_2O$, 0.45; and 2 ml of reagent B, containing 0.12 g neocuproine.HCl in 100 ml distilled water, were added. The reaction mixture was incubated at 100°C for 12 min and diluted with 3 ml of distilled water. The absorbance at 450 nm was measured in a spectrophotometer (Beckman DU-30, Beckman Instruments Inc., USA). Substrate and enzyme blanks contained distilled water instead of laminarin or instead of crude enzyme respectively. A standard containing 150 nm glucose was used to calculate the quantity of free glucose. Specific activity was expressed in nkatal per mg protein.

9.5. Assay for chitinase activity

The activity of chitinase was assayed according to BOLLER et al. (1983) following the release of soluble oligosaccharides from ^{3}H-marked chitin. ^{3}H-marked chitin was kindly supplied by T. Boller (Institute of Botany, University of Basel). The reaction mixture containing 100 µl crude enzyme, 50 µl sodium acetate buffer (pH 5.0), and 100 µl ^{3}H-marked chitin (250,000 cpm) was incubated in an Eppendorf test tube at 37°C for 30 min. The reaction was stopped by adding 250 µl of 10% trichloro-acetic acid, and the mixture was centrifuged at 4000 g for 10 min. 250 µl of the supernatant were transferred to a scitillation vial. 4 ml of scintillation cocktail (OptiPhase "highSafe 3", LKB Scintillation Products, Netherlands) were added, and radioactivity was determined in a counter (Beckman LS 1801; Beckman Instruments Inc., USA). Blanks contained distilled water instead of crude enzyme. The quantity of free N-acetyl-glucosamine equivalents in the reaction mixture was calculated (1000 cpm corresponded to 14 nmol N-acetyl-glucosamine equivalents). Results were expressed in nkatal per mg protein.

10. Isolation and characterization of fungitoxic metabolites of *C. globosum*

10.1. Effect of culture filtrates of *C. globosum* on mycelial growth of different fungi

100 ml volumes of 1% (w/v) malt extract (ME) were autoclaved in 500 ml Erlenmeyer flasks and inoculated with 3 agar plugs from a colony of *C. globosum* on 1% MEA. Control flasks were not inoculated. The flasks were incubated on a rotary shaker at 175 rpm for 12 days at the determined temperature.

After the incubation time, cultures were centrifuged at 15,000 g and 4°C for 10 min. The supernatant was sterile filtered through a syringe filter (X-60, 0.45 µm; Gelman Sciences, USA) and added at a ratio of 1 : 4 (v/v) to a malt extract agar medium that had been autoclaved and cooled to 60°C previously, providing a final concentration of 0.5% malt extract and 1.5% agar. The medium was poured into petri plates and the plates were inoculated in the center with an agar plug from a 4-day-old culture of the test fungus on 1% MEA. 3 plates were inoculated per treatment and test organism. Plates were incubated at 20°C and the colony radius was measured daily. Growth rates between day 1 and 2 after inoculation

were calculated. Growth inhibition was expressed as percent compared to control (0%).

10.2.Effect of size fractionated culture filtrate on mycelial growth of different fungi

Culture filtrate of *C. globosum* strain Cg-13 grown in 1% ME at 25°C was prepared, and a quarter of the filtrate was sterile filtered as described in 10.1. The second, third, and fourth quarter were subjected to ultrafiltration in a Novacell-Omegacell system (Filtron Corporation, USA), obtaining the following fractions: <100 kD; <10 kD; and <3 kD. Control filtrate containing uninoculated 1% ME was subjected to the same treatments. The inhibitory effect of the size-fractionated culture filtrates on different test fungi was assayed as described in 10.1.

10.3.Time course of production of fungitoxic metabolites by *C. globosum*

Erlenmeyer flasks (300 ml) containing 100 ml 1% ME were inoculated with Cg-13 as described in 10.1. Control flasks were not inoculated. Cultures were incubated at 25°C on a rotary shaker at 175 rpm. After 48 hr of incubation, half of the flasks were transferred to stationary culture at 25°C, while the other half remained at 175 rpm. One flask from each treatment was removed 48 hr, 96 hr, 168 hr and 336 hr after inoculation. The cultures were filtered as described in 10.1., and the filtrates were stored at -20°C until use. Inhibition of *P. ultimum* and *R. solani* by the culture filtrates was assayed as described in 10.1.

10.4.Extraction of fungitoxic metabolites from liquid cultures

The metabolite extraction and purification procedures described in the following sections were carried out in collaboration with the laboratory of J.P. Pachlatko (Ciba-Geigy AG, Basel). *C. globosum* strains were grown in liquid culture for 7 days under the conditions described in 10.1. Growth media were: 1% ME; 1% corn steep powder (CSP); or 0.5% ME + 1% CSP. Controls consisted of uninoculated medium. Cultures were filtered through two layers of filter paper (MN 713, Macherey-Nagel, FRG).

The filtrates were extracted twice with equal volumes of ethyl acetate (EtOAc). The solvent was evaporated on a rotary evaporator, and the solid extracts were dissolved in 1 ml of EtOAc.

10.5. Extraction of fungitoxic metabolites from soil

WBI of *C. globosum* was prepared as described in 6.2. WBI was added to 500 ml of sterile soil at a ratio of 5% (w/v). Soil characteristics and soil sterilization procedure are detailed in chapter 4. Control soil was mixed with 5% uninoculated wheat bran.

Vermiculite inoculum of *C. globosum* strain Cg-13 was prepared as follows: 30 g vermiculite (Vermica SA, Switzerland) and 120 ml 2% ME were mixed in a plastic bag and autoclaved for 30 min. Inoculation and incubation procedure were the same as described for WBI in 6.2. Vermiculite inoculum was added to 500 ml of sterile soil at a ratio of 5% (w/v).

The soil samples were incubated in sterile bottles for 6 days at 20°C. After the incubation period, the soil samples were extracted twice with an equal volume of EtOAc. The extracts were filtered through two layers of filter paper (MN 713; Macherey-Nagel, FRG) and the solvent was distilled off on a rotary evaporator. Solid extracts were dissolved in 1.5 ml EtOAc.

10.6. TLC-bioassay with *P. ultimum*

The presence of fungitoxic metabolites in the extracts from *C. globosum* liquid cultures or from soil was determined by a TLC-bioassay with *P. ultimum*. Different quantities of the extracts were applied to silica gel plates for thin layer chromatography (60 F_{254}; Merck AG, FRG). As a reference, 100 µg of pure chaetomin (kindly supplied by C. Tamm, Institute of Organic Chemistry, University of Basel) dissolved in EtOAc was also applied to the plates. The plates were developed in dichlormethane/methanol 95:5. For additional separation of chaetomin and BHT, the plates were subsequently developed in hexane/EtOAc 3:2. *P. ultimum* was grown in carrot medium as described in 6.5. After 2 weeks of incubation the mycelial mat was transferred to a sterile blender (Sorvall Inc., USA). Sterile V-8 broth supplemented with 0.5% glucose was added providing 100 ml of solution per 15 g of mycelium. To prevent bacterial growth, ampicillin (Sigma) was added at a concentration of 500 ppm. The mixture was homogenized for 2 min at maximum speed and transferred to a sterile spraying bottle. The developed TLC-plates were sprayed with the *P. ultimum* suspension and incubated in a plastic box with moist filter paper for 48 hr at 20°C. In order to visualize

zones of growth inhibition, the plates were dipped in an aqueous 4% carbon powder suspension. Carbon sticked to the mycelium while the inhibition zones remained clear.

10.7. Purification and identification of 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT)

51 Erlenmeyer flasks with 100 ml 1% ME were inoculated with Cg-13 and incubated for 14 days under the conditions described in 10.1. To check production of the inhibitory metabolite, the bioassay in petri plates was performed as described in 10.1. Cultures were filtered and extracted with EtOAc as described in 10.4., and the solvent was removed on a rotary evaporator. The solid extract (759 mg) was dissolved in 2 ml of EtOAc. 900 µl of the solution were applied to 2 preparative TLC plates (PSC 60 F_{254}, layer thickness 2 mm; Merck AG, FRG) on a line 2 cm from the bottom of the plates. Plates were developed three times in hexane/EtOAc 3:2 and subsequently once in pentane. The relevant band was localized under UV-light (254 nm) and scraped from the plate with a spatula. The scraped silica gel was filled into a glass filter (pore size 40 µm), that had been previously rinsed with EtOAc. The filter was filled with EtOAc, the silicagel was stirred with a spatula and eluted several times with EtOAc. The solvent was then evaporated from the filtrate on a rotary evaporator. The solid extract (177 mg) was dissolved in EtOAc providing a final concentration of 40 mg per ml. The following quantities of this solution were applied to silica gel plates (60 F_{254}; Merck AG, FRG): 1 µl (40 µg), 5 µl (200 µg), and 20 µl (800 µg). Plates were developed twice in hexane/EtOAc 3:2, and examined under UV-light. Two quenched spots were visible. They were marked with a pencil, and the plate was sprayed with a suspension of *P. ultimum* as described in 10.6. The zone of growth inhibition appeared at the second spot (rF = 0.75). To separate the 2 remaining compounds, the extract was dried in a rotary evaporator, and then redissolved and recrystallized in a minimal volume of hexane. Recrystallization gave 123 mg of pure 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT). The identification of the metabolite was carried out with NMR and mass spectroscopy (kindly performed by H. Fritz, Ciba-Geigy AG, Basel).

10.8. Purification and identification of chaetomin

Extracts of liquid cultures or of soil colonized by *C. globosum* were applied to TLC plates, and plates were developed as described in 10.6. Since the inhibitory metabolite was found to be visible as quenched spots under UV-light (254 nm),

this method of localization was used for the further purification steps. Zones containing the relevant metabolite were scraped from the plates with a spatula and eluted several times with EtOAc. The solvent was evaporated on a rotary evaporator. The identification of the fungitoxic metabolite was carried out with NMR and mass spectroscopy.

10.9. HPLC analysis of purified chaetomin

Purified chetomin was dissolved in acetonitrile (0.5 mg per ml). A 10 µl sample was subjected to HPLC analysis (facilities were kindly made available by M. Brandl, Ciba-Geigy AG, Basel). The analytical system consisted of a liquid chromatograph HP 1090 series M with a diode array detector (Hewlett Packard, Switzerland).

The parameters of analysis were as follows:

Precolumn: Ultrasphere ODS 5u, 4.6 mm x 4.5 cm; Beckman, USA
Column: Ultrasphere ODS 5u, 4.6 mm x 15 cm; Beckman, USA

TABLE 4. Gradient system for HPLC analysis of chaetomin:

Time (min)	Solvent A[a] (%)	Solvent B[b] (%)
1.0	100	0
10.0	0	100
15.0	0	100
20.0	100	0

[a]Solvent A: acetonitrile (Fluka); flow rate: 1.5 ml per min
[b]Solvent B: water (Fluka) flow rate: 1.5 ml per min

10.10. Effect of *C. globosum* metabolites on mycelial growth and on sporangial germination of *P. ultimum*

A bioassay in microtiter plates according to LUDWIG & BOLLER (1990) was used to test the inhibition of hyphal growth and of sporangial germination of *P. ultimum* by the purified metabolites from *C. globosum*. Sporangia of *P. ultimum* strain P-71 were prepared according to ROBERTS & LUMSDEN (1990). The sporangia were suspended in cleared V-8 medium at a concentration of 2.5 x 10^3

sporangia per ml. 90 µl aliquots of the suspension were pipetted into wells of sterile microtiter plates (96-well-plates, A/S Nunc, Denmark). Since the metabolites chaetomin and BHT were not readily soluble in water, the following dispersing procedure was used: 2 mg of the determined metabolite were dissolved in a minimal volume of EtOAc. 150 µl PEG 200 (Fluka) were added, and the EtOAc was evaporated on a rotary evaporator. 2 ml sterile distilled water were added and the sample was sonicated for 5 min (Branson 3200; Branson Europa B.V., Netherlands) providing an aqueous dispersion containing 1 mg a.i. per ml and 7.5% PEG 200. The dispersion was diluted to the appropriate concentrations and 10 µl aliquots were added to the sporangial suspension in the wells providing the desired end concentration of metabolite. The following concentrations were tested:

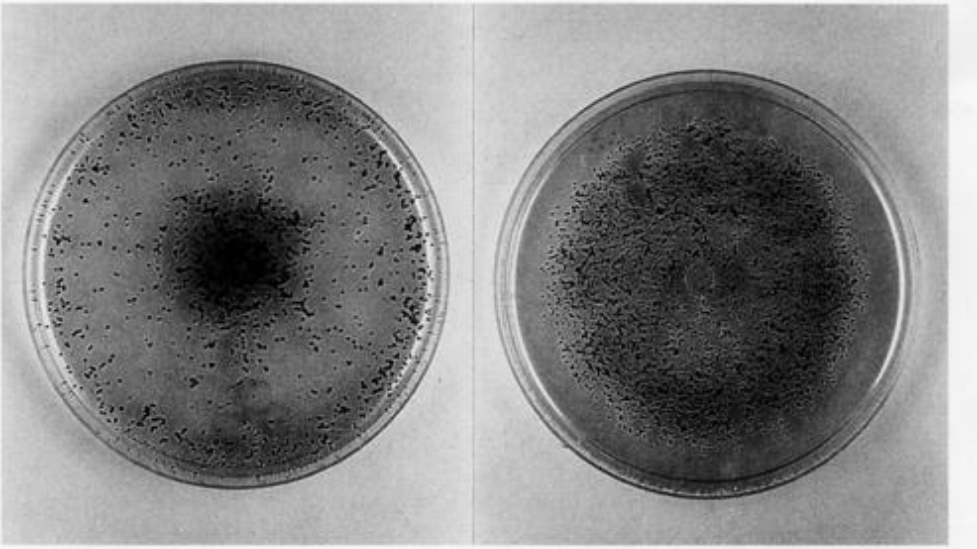
0.1; 0.25; 0.5; 0.75; 1; 2.5; 5; 7.5; 10; 50 ppm a.i.

As a blank control, a corresponding quantity of PEG 200 was added to the sporangial suspension. As inhibitory standards, gliotoxin (Sigma) and metalaxyl WP 25 (Ciba-Geigy AG, Switzerland) were applied in the same way. To assess the effect of metabolites on sporangial germination, the test compounds were added immediatly to the sporangial suspension. To determine the inhibition of mycelial growth, the substances were added to the suspension after 7 hr of incubation at 20°C, when at least 95% of the sporangia were germinated as determined by microscopical observation. Five wells with *P. ultimum* and two wells with cleared V-8 medium (blanks) were used for each treatment. Microtiter plates were incubated at 20°C in a plastic box with moist filter paper. Mycelial growth of *P. ultimum* was determined quantitatively by measuring the optical density in the wells after different times of incubation (LUDWIG & BOLLER, 1990). Measurements were performed in an ELISA-plate reader (Titertrek Multiskan plus MK II, Skan Laboratories, Switzerland) using a 578 nm filter. EC_{50} values for mycelial growth were determined by calculating the growth rates between the measurements at 24 hr and 48 hr after the start of the experiment. The minimal concentration where no measurable growth occurred was designated as MIC value. Depending on whether the inhibitor was added immediatly or 7 hr after the start of the experiment, MIC refers to inhibition of sporangial germination or of mycelial growth respectively. Failure of sporangial germination was additionally checked by microscopical examination.

Results

1. Isolation and phenotypic characterization of ADP-13, a spontaneous variant of *C. globosum* strain Cg-13

During the work with *C. globosum* strain Cg-13, a spontaneous variant with a different colony habitus was isolated. The variant, showing enhanced production of aerial mycelium and a different pattern of perithecial formation (Figure 3), was designated ADP-13. On the original colonies of ADP-13, reverting sectors, showing the morphotype of the parental strain, were observed. However, when hyphae from the sectors with the altered morphotype were subcultured, the variant remained stable during the following generations. For the phenotypic characterization of ADP-13, growth rate and perithecial formation on different media at different temperatures were compared with the parental strain Cg-13.



Cg-13 **ADP-13**

Figure 3. Colonies on 1% MEA of the *C. globosum* strain Cg-13 and its spontaneous variant ADP-13.

1.1. Radial growth rates and perithecium formation

Radial growth rates and perithecium formation of the *C. globosum* strain Cg-13 and the variant ADP-13 differed considerably, depending on growth medium and temperature. Growth rates of ADP-13 were generally lower than those of Cg-13 (Figure 4). However, on some growth media (WA, CMA) at non-optimum temperatures (10°C, 30°C), the growth rate of ADP-13 exceeded that of Cg-13.

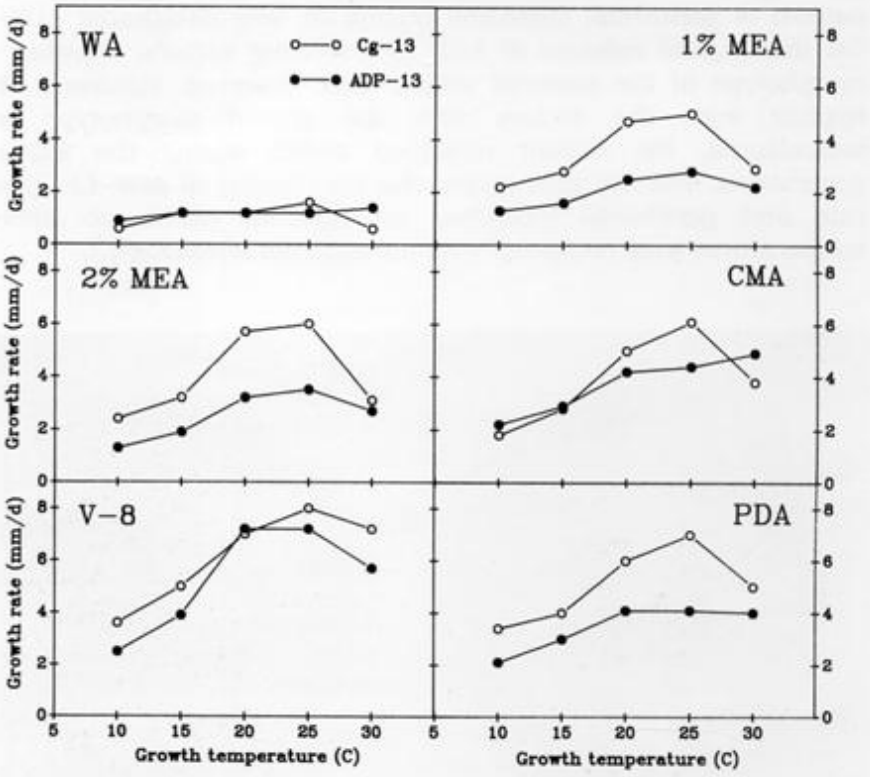


Figure 4. Radial growth rates of *C. globosum* strains Cg-13 and ADP-13 at different temperatures on different media.

Perithecium formation of ADP-13 was slightly poorer than that of Cg-13 (Tables 5 and 6). Also the pattern of perithecial distribution differed between the two strains (see Figure 3), and the size of

perithecia of ADP-13 was smaller than of those of Cg-13. On the other hand, size and shape of the mature ascospores were identical in both strains. ADP-13 generally showed an enhanced production of aerial mycelium.

TABLE 5. Perithecium formation of *C. globosum* strain Cg-13 on different media at different temperatures.

Medium	Growth temperature (°C) 10	15	20	25	30
WA	-	-	(+)	(+)	-
1% MEA	(+)	++	++	++	++
2% MEA	-	+	+	++	++
CMA	-	+	+	+	(+)
V-8	+	++	+++	+++	++
PDA	-	-	+++	+++	++

Perithecium formation (5 weeks after inoculation)

- \- absent
- (+) very weak
- \+ weak
- ++ medium
- +++ strong

TABLE 6. Perithecium formation of *C. globosum* strain ADP-13 on different media at different temperatures.

Medium	Growth temperature (°C) 10	15	20	25	30
WA	-	-	-	-	-
1% MEA	-	-	+	+	+
2% MEA	-	+	++	++	+
CMA	-	-	-	-	-
V-8	-	++	+++	+++	++
PDA	-	++	++	+++	-

Radial growth rates of *P. ultimum* strain P-71, the damping-off pathogen used in the growth chamber experiments (see section 2.), were 3 to 7 times higher than those of *C. globosum* strain Cg-13, depending on the growth medium and temperature (Table 7). The optimum temperature for growth of *P. ultimum* was 25°C-30°C. Note that the present data reflect only radial growth of the fungus, but not dry weight (representing mycelial production).

TABLE 7. Radial growth rates (mm/day) of *P. ultimum* strain P-71 on different media at different temperatures.

Medium	Growth temperature (°C)				
	10	15	20	25	30
WA	5	8	14	16	17
1% MEA	10	22	29	35	26
2% MEA	10	22	29	40	35
CMA	10	16	35	42	42
V-8	9	19	33	51	56
PDA	11	19	38	42	40

2. Growth chamber experiments

The efficacy of *C. globosum* in reducing Pythium damping-off was determined in growth chamber experiments. A system with sugarbeet as host plant, that gave reproducible results in previous work (DI PIETRO, 1986) was used in most experiments. In a first step, sixteen *C. globosum* strains were assayed for their efficacy in reducing Pythium damping-off. The aim was to select strains with differing efficacies against Pythium damping-off.

2.1. Efficacy of different *C. globosum* strains against Pythium damping-off of sugarbeet

All 16 *C. globosum* strains tested were highly effective in reducing Pythium damping-off in non-sterile soil inoculated with *P. ultimum* (Table 8). High efficacy was due in part to the low pathogenic pressure of *P. ultimum* in this experiment (shown by the relatively high number of healthy plants in the Pythium check where only the pathogen was applied).

Cg-43 was the only strain with a significantly lower efficacy than the other *C. globosum* strains. Cg-13 was one of the most effective strains against Pythium damping-off. Therefore, these two strains were selected for further experiments. ADP-13, a spontaneous variant of Cg-13, that had been previously isolated and phenotypically characterized (see section 1.), was also included in the further experiments. Different application methods of the three strains were tested in sterile and non-sterile soil.

The results are shown in Table 9. *C. globosum* strain Cg-13 applied as WBI effectively reduced damping-off of sugar beet in sterile soil inoculated with *P. ultimum*. Efficacy of Cg-13 treatment was comparable to 10 ppm metalaxyl. Strain Cg-43 showed a low efficacy as WBI. Results were consistent with those from the previous experiment (see Table 8), but the difference between the two strains was more pronounced, probably due to the higher pathogenic pressure of *P. ultimum*. WBI of the variant strain ADP-13 was completely ineffective. Ascospore seed coating as an application method of *C. globosum* was generally less effective than WBI. Only strain Cg-13 gave a low protection from Pythium damping-off when

applied as ascospore seed coating. The two other strains had no beneficial effect.

Protection in non-sterile soil was generally poorer and results were more inconsistent compared to sterile soil (Table 9). This was probably due to the presence of minor soilborne pathogens other than *P. ultimum*. Plants showing damping-off symptoms occurred even in the non-inoculated check, and *Fusarium* sp. was isolated from diseased plants. Therefore, heat treated soil was used in the following experiments.

TABLE 8. Efficacy of different *C. globosum* strains against Pythium damping-off of sugarbeet.

Treatment	Percent healthy plants[a]
Non-inoculated check	100 ab[b]
Pythium check	34 d
Pythium + bran[c]	22 d
Cg-1[d]	95 ab
Cg-3	95 ab
Cg-9	93 ab
Cg-12	99 a
Cg-13	**98 a**
Cg-14	96 ab
Cg-17	100 a
Cg-22	85 abc
Cg-28	76 bc
Cg-29	96 ab
Cg-43	**61 c**
Cg-200	87 abc
C-1	89 abc
C-3	88 abc
C-4	95 ab
C-5	100 a

[a]Data are expressed in % compared to the non-inoculated check.

[b]Values followed by the same letter are not significantly different (P=0.05) according to Duncan's multiple range test.

[c]Uninoculated control bran.

[d]WBI was added to the soil at a ratio of 2% (w/v).

TABLE 9. Efficacy of *C. globosum* strains Cg-13, ADP-13, and Cg-43 against Pythium damping-off of sugarbeet in sterile and non-sterile soil.

Treatment		Percent healthy plants	
		sterile soil	non-sterile soil
Non-inoculated check		100 a[a]	100 a
Pythium check		1 b	0 c
Pythium + bran		3 b	1 c
Metalaxyl	10 ppm	87 a	100 a
Cg-13	WBI 2%	90 a	24 b
ADP-13	WBI 2%	3 b	2 c
Cg-43	WBI 2%	18 b	23 b
Cg-13	seed coating	28 b	15 b
ADP-13	seed coating	6 b	0 c
Cg-43	seed coating	4 b	2 c

[a]Values in a column followed by the same letter are not significantly different (P=0.05) according to Duncan's multiple range test.

2.2. Efficacy of *C. globosum* strains Cg-13, ADP-13, and Cg-43 against Pythium damping-off of cucumber

The same *C. globosum* strains as in the previous experiment were assayed against Pythium damping-off of cucumber. In contrast to the experiment with sugarbeet, ascospore seed coating was more effective than WBI against Pythium damping-off of cucumber (Table 10). Protection, however, was not significant because of the relatively high number of healthy plants in the Pythium check. Cg-13 was the most effective of the tested strains, but did not reach efficacy of metalaxyl, that gave 100% protection. WBI treatments with all three strains did not reduce damping-off compared to the Pythium check. However, the order of efficacy of the three strains was the same as in the experiment with sugarbeet. This suggests that in the system with cucumber the beneficial effect of WBI was nullified by a stimulation of *P. ultimum* following addition of the bran. Thus, the most ineffective strain ADP-13 increased the incidence of damping-off compared to the Pythium check, while the most effective one, Cg-13, kept damping-off at the level of the Pythium check.

TABLE 10. Efficacy of *C. globosum* strains Cg-13, ADP-13, and Cg-43 against Pythium damping-off of cucumber.

Treatment		Percent healthy plants
Non-inoculated check		100 ab
Pythium check		42 cd
Metalaxyl	100 ppm	100 a
Cg-13	WBI 2%	30 cd
ADP-13	WBI 2%	2 e
Cg-43	WBI 2%	12 de
Cg-13	seed coating	70 bc
ADP-13	seed coating	52 cd
Cg-43	seed coating	52 cd

2.3. Effect of killed WBI of *C. globosum* on Pythium damping-off

This experiment was conducted to determine whether the presence of living *C. globosum* in the soil was required for a reduction of Pythium damping-off, or if this reduction was due exclusively to metabolites produced by *C. globosum* during the time of incubation on the bran. When *C. globosum* strain Cg-13 was killed on the WBI by different treatments before adding to soil, no reduction of damping-off was achieved in non-sterile soil (Table 11). This suggests that an active antagonistic mechanism in soil is responsable for the beneficial effect of *C. globosum*.

2.4. Different application rates of WBI of *C. globosum*

To optimize the application rate of *C. globosum*, the efficacy of different rates of fresh WBI of strain Cg-13 in reducing damping-off of sugarbeet in sterile soil inoculated with *P. ultimum* was determined. Efficacy was optimal when WBI was added to the soil at a w/v ratio of 0.5% (Figure 5). Increasing the ratio until 2% did not further improve efficacy. At WBI ratios lower than 0.5% the efficacy decreased gradually.

TABLE 11. Efficacy of living or killed *C. globosum* on WBI against Pythium damping-off of sugarbeet.

Treatment		Percent healthy plants
Non-inoculated check		100 a
Pythium check		5 c
Pythium + bran	2%	0 c
Cg-13 WBI	2%	45 b
Cg-13 WBI	2% autoclaved	0 c
Cg-13 WBI	2% liquid N_2	0 c
Cg-13 WBI	2% Benlate	0 c

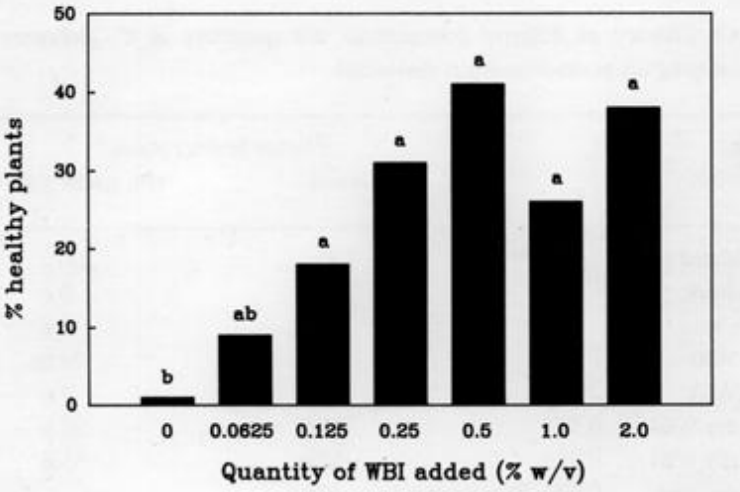


Figure 5. Efficacy of different application rates of *C. globosum* WBI against Pythium damping-off of sugarbeet in sterile soil.

2.5. Efficacy of dry and fresh WBI of *C. globosum*

Since fresh WBI is not a suitable formulation for the application of *C. globosum* under field conditions, dry WBI of strain Cg-13 at different application rates was compared to fresh WBI in efficacy against Pythium damping-off. Efficacies of both formulations did not differ significantly (Table 12). Microscopical examination showed that *C. globosum* survived in the dry WBI in the form of dormant hyphae. Fresh and dry WBI applied at a ratio of 2% and 0.5% (w/v) respectively gave the best protection in sterile soil inoculated with *P. ultimum*. Seed coating, dry WBI (0.25%), and vermiculite inoculum of Cg-13 gave lower protections. In non-sterile soil, dry WBI was slightly more effective than fresh WBI. Like in the previous experiment (see Table 9), protection in non-sterile soil was generally lower and more inconsistent than in sterile soil. Fresh WBI of strain ADP-13 (applied as negative control) was ineffective in either sterile or non-sterile soil.

TABLE 12. Efficacy of different formulations and quantities of *C. globosum* against Pythium damping-off in sterile and non sterile soil.

Treatment			Percent healthy plants	
			sterile soil	non sterile soil
Non-inoculated check			100 a	100 a
Pythium check			6 c	0 c
Pythium + bran			2 c	1 c
Cg-13	WBI	2%	81 ab	24 bc
ADP-13	WBI	2%	3 c	2 c
Cg-13	dry WBI	0.5%	71 b	35 b
Cg-13	dry WBI	0.25%	52 b	35 b
Cg-13	dry verm.	0.5%	40 b	-
Cg-13	seed coating		48 b	15 bc

2.6. In-furrow application of dry WBI of *C. globosum*

In-furrow application of dry WBI is a potential method for application of *C. globosum* under field conditions. Different formulations and application rates of strain Cg-13 were tested against damping-off of sugarbeet in sterile soil inoculated with *P. ultimum*. In-furrow application of dry WBI gave a good protection from damping-off (Table 13). Efficacy decreased when the quantity of WBI per row (34 cm long) was reduced from 2.72 g to 0.14 g. Dry vermiculite inoculum gave results comparable to the corresponding rate of dry WBI. Ascospore seed coating gave only low protection. Combined application of dry WBI and ascospore seed coating did not result in an increase in efficacy compared to WBI alone.

TABLE 13. Efficacy of different formulations and quantities of *C. globosum* applied in-furrow against Pythium damping-off.

Treatment		Percent healthy plants
Non-inoculated check		100 a
Pythium check		4 e
Pythium + bran		4 e
Cg-13 dry WBI	2.72 g[a]	84 ab
Cg-13 dry WBI	0.68 g	77 abc
Cg-13 dry WBI	0.14 g	40 cd
Cg-13 dry verm.	2.72 g	66 bc
Cg-13 seed coating		11 de
Cg-13 s.c.+ dry WBI	0.68 g	73 abc

[a]Amount of dry WBI per row (34 cm) with 15 seeds

3. Studies on the colonization of cucumber seedlings by different *C. globosum* strains

3.1. Inhibition of mycelial growth of soil fungi by different fungicides

For the development of a semiselective medium for the isolation of *C. globosum* from soil, different fungicides were tested in 1% MEA against the *C. globosum* strains Cg-13, ADP-13, and Cg-43, and against the three soil fungi *P. ultimum* strain P-71, *R. solani* strain R-160, and *Rhizopus* sp. strain X-1. Results are shown in Tables 14 and 15. Growth of *P. ultimum* was suppressed by 1 to 10 ppm metalaxyl. Growth of *R. solani* was suppressed by 1 ppm CGA 173506. *C. globosum* still grew well on 1000 ppm of both fungicides. Interestingly, *C. globosum* showed a typical growth pattern of concentric circles on CGA 173506 (Figure 6).

Growth of *Rhizopus* sp. was strongly suppressed by 1 ppm dichloran, while *C. globosum* still grew well at that concentration.

TABLE 14. MIC values (ppm a.i.) for different fungicides in 1% MEA against different fungi.

Fungicide(s)	Fungal strain					
	Cg-13	ADP-13	Cg-43	R-160	P-71	X-1
Propiconazole	100	100	10	1000	1000	1000
Hymexazole	100	-	100	1000	100	-[a]
Captan	>1000	>1000	1000	>1000	1000	-
Diphenoconazole	1000	-	10	>1000	>1000	1000
CGA 173506	>1000	>1000	>1000	1	>1000	>1000
Diphenoc.+Propic.(1ppm)	>10	-	1	>10	-	-
CGA 173506+Propic.(1ppm)	>10	-	1-10	1	-	-
Benomyl	1	1	1	10	>1000	>1000
Metalaxyl	>1000	>1000	>1000	>100	1-10	>100
Dichloran	100	100	100	>100	>100	10

[a]not determined

TABLE 15. EC_{50} values (ppm a.i.) for different fungicides in 1% MEA against different fungi.

Fungicide	Fungal strain					
	Cg13	ADP-13	Cg43	R-160	P-71	X-1
Propiconazole	3	5	2	0.3	100	3
Hymexazole	10	-	20	10	30	-[a]
Captan	50	50	70	5	200	-
Diphenoconazole	5	-	5	100	100	8
CGA 173506	>1000	>1000	>1000	0.01	500	5
Diphenoc.+1 ppm Propic.	0.5	-	<0.01	<0.01	-	-
CGA 173506+1 ppm Propic.	n.d.	-	n.d.	0.01	-	-
Benlate	0.1-1	0.1-1	0.1-1	2	800	10
Metalaxyl	>1000	>1000	>1000	>100	1-10	>100
Dichloran	5	5	5	10	200	0.5

[a]not determined

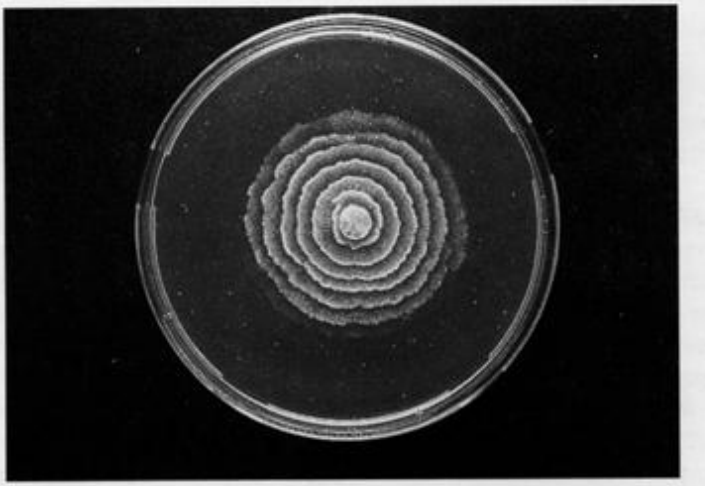


Figure 6. Typical growth aspect of *C. globosum* on 1% MEA supplemented with 100 ppm CGA 173506 (saphir).

3.2. A semiselective medium for the isolation of *C. globosum* from soil

The following optimum composition for a semiselective medium (Cgselmed) for the isolation of *C. globosum* from soil was chosen on the basis of the MIC and EC_{50} values of the previous experiment:

Semiselective medium for *C. globosum* (Cgselmed)	
CGA 173506	10 ppm
Metalaxyl	10 ppm
Dichloran	1 ppm
Tetracyclin	10 ppm
Ampicillin	100 ppm

C. globosum strains Cg-13, ADP-13, and Cg-43 grew well on Cgselmed, and were easily identified by the typical growth pattern in concentric circles (see Figure 6), due to the presence of CGA 173506 in the medium. Cgselmed suppressed growth of all other fungi present in Stein soil and was therefore suitable for the isolation of *C. globosum* from non-sterile soil. The two antibiotics tetracyclin and ampicillin were added to prevent growth of soil bacteria.

3.3. Population density of the *C. globosum* strains Cg-13 and Cg-43 on cucumber seedlings

Population density of the two *C. globosum* strains Cg-13 and Cg-43 on cucumber seeds and seedlings was determined. These two strains have different efficacies in reducing Pythium damping-off of sugarbeet and cucumber (see Tables 9 and 10, pp. 33 and 34). No correlation between biocontrol ability and population density was found: numbers of colony forming units did not differ significantly between Cg-13 and Cg-43 (Figure 7, Table 16). However, the population dynamics of *C. globosum* was significantly different between the two used application methods: with ascospore seed coating, concentration of *C. globosum* on the first day was higher than with WBI, but declined until day 4, while with WBI the concentration increased to a maximum value at day 4 (Figure 7). Presence of *P. ultimum* in soil had no influence on *C. globosum* colonization (Table 16).

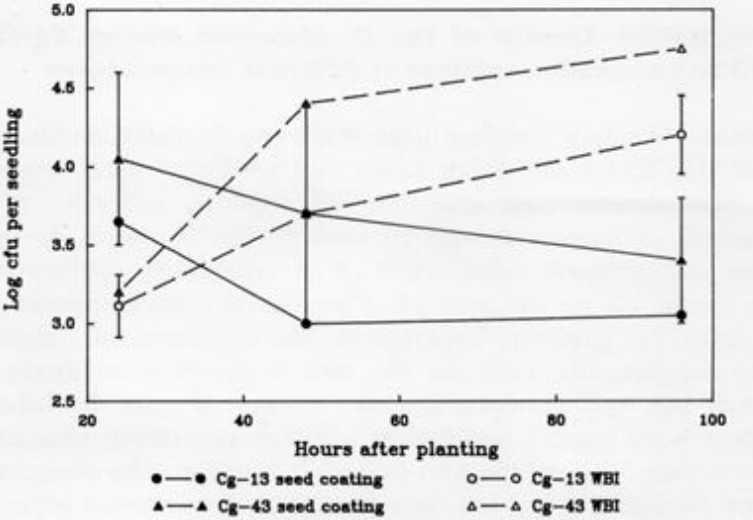


Figure 7. Population density of the *C. globosum* strains Cg-13 and Cg-43 on cucumber seedlings. Values are the means of data from Pythium-inoculated and from non-inoculated soil.

TABLE 16. Population density of the *C. globosum* strains Cg-13 and Cg-43 on cucumber seedlings.

Treatment	Population density of *C. globosum*[a] day 1[b]	day 2	day 4
Non-inoculated check	0	0	0
Pythium check	0	0	0
Cg-13 seed coating	3.4 ± 1.5	3.0 ± 1.0	2.9 ± 0.5
P+Cg-13 seed coating	3.9 ± 0.8	3.0 ± 0.4	3.2 ± 0.0
Cg-13 WBI	3.2 ± 0.0	3.4 ± 0.7	3.9 ± 0.5
P+Cg-13 WBI	3.0 ± 0.4	4.0 ± 0.7	4.5 ± 0.0
Cg-43 seed coating	3.9 ± 0.5	3.9 ± 0.5	3.4 ± 0.4
P+Cg-43 seed coating	4.2 ± 0.6	3.5 ± 1.0	3.4 ± 0.4
Cg-43 WBI	3.0 ± 0.7	4.9 ± 0.5	5.0 ± 0.0
P+Cg-43 WBI	3.4 ± 0.4	3.9 ± 0.8	4.5 ± 0.0

[a]Population density of *C. globosum* is expressed in log cfu per seedling

[b]Day after planting

3.4. Population density of the *C. globosum* strains Cg-13 and ADP-13 on cucumber seedlings at different temperatures

Numbers of colony forming units of the two *C. globosum* strains Cg-13 and ADP-13 on cucumber seeds and seedlings were determined. These two strains have different efficacies in reducing Pythium damping-off of sugarbeet and cucumber: Cg-13 effectively reduces Pythium damping-off, while ADP-13 is completely ineffective (see Tables 9 and 10, pp. 33 and 34). The results were comparable with those from the previous experiment: the dynamics of colonization differed significantly between the two application methods of *C. globosum* but not between the two strains (Figure 8, Table 17). Ascospore seed coating resulted in a higher population density of *C. globosum* than WBI on the first day after planting. The concentration declined on day 2 and 3 but, in contrast to the previous experiment, increased again on day 4. With WBI, concentration increased constantly to a maximum value on day 4. The population density of ADP-13 was slightly, but not significantly lower than that of Cg-13. Interestingly, the numbers of colony forming units did not differ significantly at different incubation temperatures (Table 17).

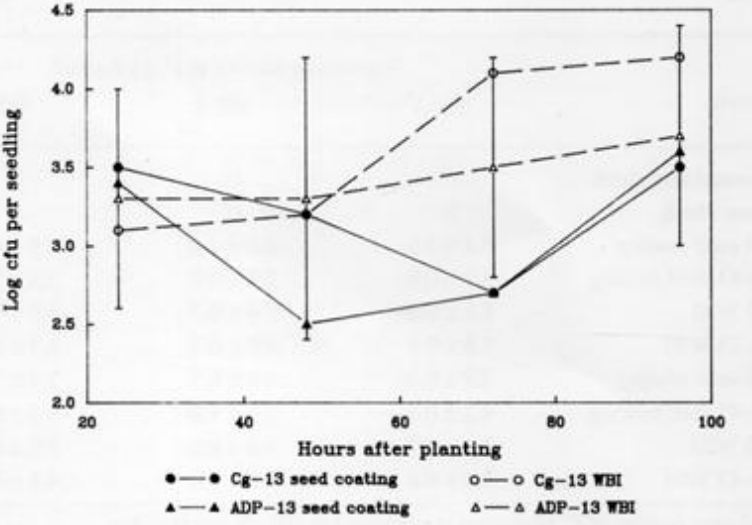


Figure 8. Population density of the *C. globosum* strains Cg-13 and ADP-13 on cucumber seedlings at 20°C in heat treated soil inoculated with *P. ultimum*.

TABLE 17. Population density of the *C. globosum* strains Cg-13 and ADP-13 on cucumber seedlings at different temperatures.

Treatment / Temperature	Population density of *C. globosum*[a] day 1[b]	day 2	day 3	day 4
Non-inoculated check				
10°C	0	0	0	0
15°C	0	0	0	0
20°C	0	0	0	0
25°C	0	0	0	0
Pythium check				
10°C	0	0	0	0
15°C	0	0	0	0
20°C	0	0	0	0
25°C	0	0	0	0
P + Cg-13 seed coating				
10°C	4.0 ± 0.7	3.5 ± 1.0	3.6 ± 0.7	3.7 ± 0.8
15°C	3.8 ± 0.9	3.7 ± 1.1	3.5 ± 0.8	3.3 ± 0.7
20°C	3.5 ± 0.8	3.2 ± 1.1	2.7 ± 0.7	3.5 ± 1.1
25°C	3.8 ± 2.0	2.9 ± 0.9	3.4 ± 1.0	3.5 ± 0.9
P + Cg-13 WBI				
10°C	2.4 ± 0.5	2.7 ± 0.8	3.0 ± 0.8	4.1 ± 0.9
15°C	2.7 ± 0.5	2.8 ± 0.5	3.7 ± 0.8	3.8 ± 0.8
20°C	3.1 ± 0.9	3.2 ± 1.0	4.1 ± 0.6	4.2 ± 0.8
25°C	2.8 ± 0.8	3.2 ± 0.7	3.0 ± 0.7	4.0 ± 1.0
P + ADP-13 seed coating				
20°C	3.4 ± 0.7	2.5 ± 0.7	2.7 ± 0.6	3.6 ± 1.0
P + ADP-13 WBI				
20°C	3.3 ± 0.7	3.3 ± 0.9	3.5 ± 0.7	3.7 ± 0.7

[a]Population density of *C. globosum* is expressed in log cfu per seedling
[b]Day after planting

Presence of *P. ultimum* on the seedlings was different according to the application method of *C. globosum* (Table 18, Figure 9): Cg-13 ascospore seed coating slightly reduced the incidence of Pythium colonization compared to the Pythium check. With WBI treatment, however, presence of *P. ultimum* was higher than in the Pythium

check. This fact is probably due to a stimulation of *P. ultimum* growth by the bran and may explain the poor efficacy of WBI treatments compared to seed coating in the biocontrol experiment with cucumber (see Table 10, p. 34).

TABLE 18. Presence of *P. ultimum* on cucumber seedlings at different temperatures.

Treatment / Temperature	Incidence of *P. ultimum* colonization[a] day 1[b]	day 2	day 3	day 4
Non-inoculated check				
10°C	0	0	0	0
15°C	0	0	0	0
20°C	0	0	0	0
25°C	0	0	0	0
Pythium check				
10°C	37	50	50	50
15°C	25	25	63	100
20°C	12	62	50	83
25°C	17	62	100	100
P + Cg-13 seed coating				
10°C	0	0	50	0
15°C	0	50	33	50
20°C	17	50	50	67
25°C	33	33	67	83
P + Cg-13 WBI				
10°C	17	50	67	100
15°C	50	100	100	100
20°C	33	100	100	100
25°C	67	100	100	100
P + ADP-13 seed coating				
20°C	17	60	100	100
P + ADP-13 WBI				
20°C	17	67	83	50

[a]Incidence of *P. ultimum* colonization is expressed in % colonized seedlings

[b]Day after planting

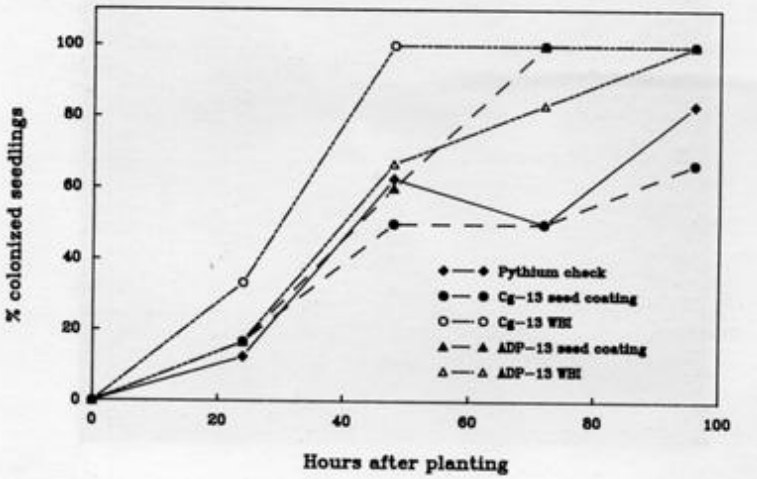


Figure 9. Presence of *P. ultimum* on cucumber seedlings in heat treated soil inoculated with the *C. globosum* strains Cg-13 or ADP-13.

3.5. Microscopical observation of *C. globosum* on roots of sugarbeet and cucumber

Colonization of sugarbeet and cucumber roots by *C. globosum* was examined with the light microscope. The fluorescent stain Calcofluor White was used to detect fungal hyphae on the roots from sterile soil inoculated with WBI of *C. globosum*. Extensive colonization of the roots by hyphae of *C. globosum* was observed. Although the root hairs showed some faint fluorescence, hyphae of *C. globosum* were usually well distinguished from the root parts by the brighter staining (Figure 10). Ascospores of *C. globosum* were frequently observed on the root surface (Figure 11). Although not fluorescent, they were easily detectable by observation with phase contrast due to their intense dark-brown color.

No difference in intensity of root colonization between the *C. globosum* strains Cg-13, ADP-13 and Cg-43 was observed. However, the density of colonization differed considerably between different root segments on the same root.

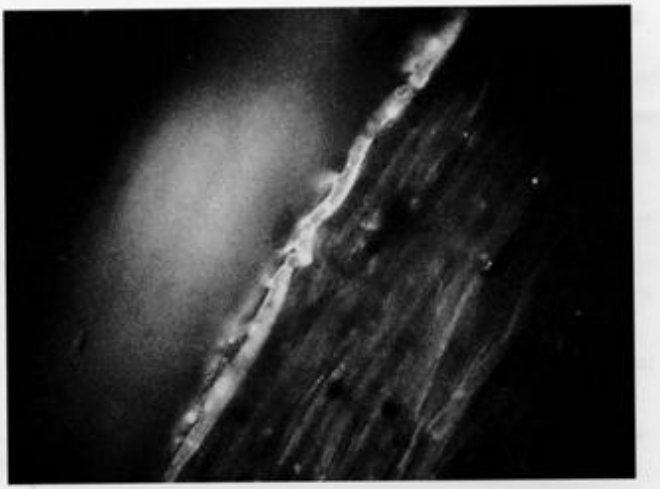


Figure 10. Hyphae of *C. globosum* strain Cg-13 on the surface of a sugarbeet root (400 x).

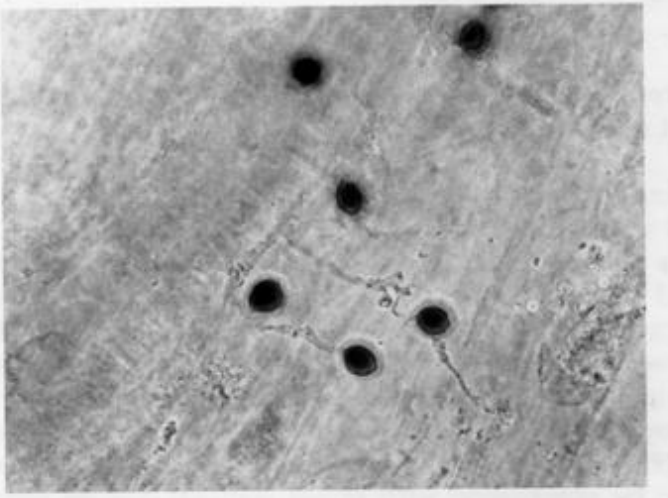


Figure 11. Ascospores of *C. globosum* strain Cg-13 on the surface of a cucumber root (1000 x).

4. Evidence for mycoparasitism

Mycoparasitism was evaluated as a potential antagonistic mechanism of *C. globosum*. The occurrence of hyphal interactions between the antagonist and the pathogens *P. ultimum* or *R. solani* in vitro and in the soil was investigated. Furthermore, the production of lytic enzymes by *C. globosum* was determined.

4.1. Hyphal interactions between *C. globosum* and *P. ultimum* or *R. solani*

Hyphal interactions in soil between *C. globosum* and *P. ultimum* or *R. solani* were studied with light microscope or scanning electron microscope. No hyphal interactions were observed between *C. globosum* and *P. ultimum*, indicating that mycoparasitism did not occur between the two fungi. However, intense coiling of *C. globosum* hyphae around hyphae of *R. solani* was observed (Figure 12). Hyphae of *C. globosum* attached to *R. solani* by forming hooks and appressorium-like structures. No penetration of the host mycelium could be observed, but light microscopy revealed alterations in the cell wall structure of *R. solani* hyphae at the interaction sites (Figure 13). When hyphae were stained with the fluorescent dye calcofluor, intense fluorescence was observed at these sites, indicating partial degradation of the cell walls of *R. solani*. Calcofluor binds to ß-glucans and *N*-acetyl-D-glucosamine oligomers in regions of incomplete cell wall polymers (KRITZMAN et al., 1978).

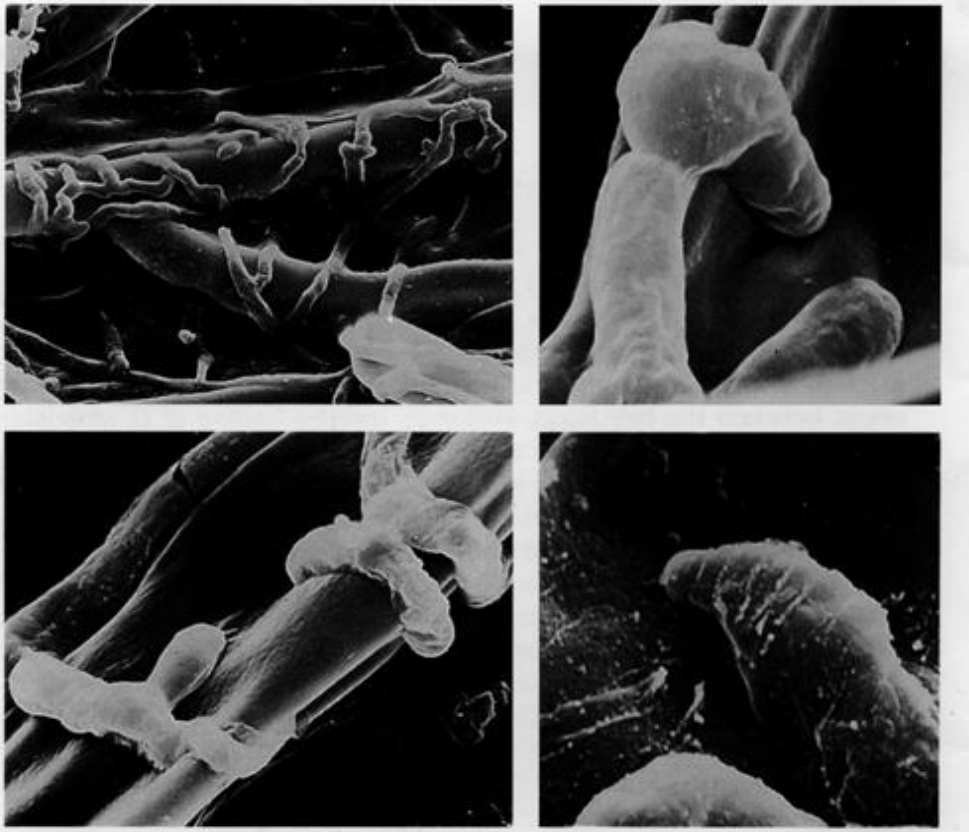


Figure 12. Scanning electron micrographs of hyphal interactions between *C. globosum* strain Cg-13 and *R. solani* strain R-160 in the soil. **1.** Coiling of *C. globosum* around hyphae of *R. solani* (1700 x). **2.** Appressorium-like structure formed by *C. globosum*. Note partial degradation of host cell wall. (11,800 x). **3.** Appressorium-like structure formed by *C. globosum* (5500 x). **4.** Hyphal tip of *C. globosum* attached to a hypha of *R. solani* (18,000 x).

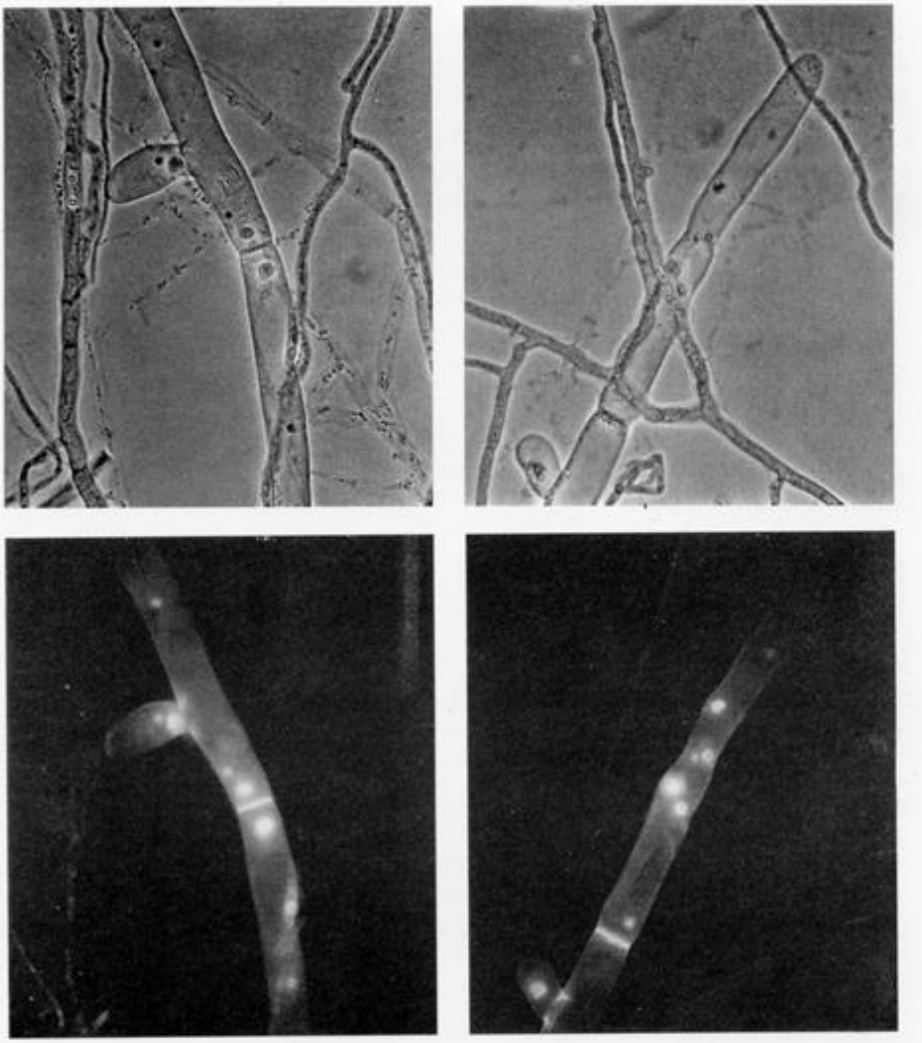


Figure 13. Light micrographs of hyphal interactions between *C. globosum* strain Cg-13 and *R. solani* strain R-160. **1.-2.** Hyphal interactions between *C. globosum* and *R. solani* observed with phase contrast. Note circular zones of alteration in cell wall structure of *R. solani* hyphae at the sites of interaction (1000 x). **3.-4.** The same interaction zones as in Figures 1. and 2., stained with the fluorescent dye Calcofluor. Note fluorescence at the sites of interaction.

4.2. Production of lytic enzymes by *C. globosum*

No hyphal coiling of *C. globosum* around hyphae of *P. ultimum* was found in the previous experiments. Nevertheless, *C. globosum* may be able to attack the cell walls of *P. ultimum*, even in the absence of direct physical contact, by means of hydrolases. To study this hypothesis, the production of lytic enzymes by *C. globosum* grown on different carbon sources, including cell walls of *P. ultimum*, was determined.

Cellulase activity was measured in tenfold concentrated culture filtrates of the *C. globosum* strains Cg-13 and Cg-43 grown on different carbon sources. Activity was calculated using a standard curve of commercial cellulase from *Basidiomyces* sp. (see Figure 2, materials and methods section).

Cellulase activity was only detected in culture filtrates of Cg-13 grown on cellulose as sole carbon source, but not on glucose, laminarin or chitin. Activities were 0.22 ± 0.01 units and 0.41 ± 0.02 units on native and microcrystalline cellulose respectively. No cellulase activity >0.006 units was detected in culture filtrates of Cg-43.

Activities of ß-1,3-glucanase, chitinase, and cellulase were determined in culture filtrates of the two *C. globosum* strains Cg-13 and ADP-13, grown either on *P. ultimum* cell walls or on laminarin as sole carbon source with an initial addition of 0.1% glucose to promote ascospore germination. Comparable activities of ß-1,3-glucanase were detected in dialyzed culture filtrates of Cg-13 and ADP-13 grown on *P. ultimum* cell walls (Table 19), indicating that both *C. globosum* strains were equally capable to exploit the cell walls as a carbon source. When laminarin was used as carbon source, glucanase was only detected in filtrates of ADP-13. Either the quantity of glucanase in filtrates of Cg-13 was below the detection level of the enzyme assay (>0.01 nkatal), or the growth of Cg-13 was due to the initial addition of glucose to the medium, since growth on laminarin as sole carbon source would require production of glucanase.

Chitinase activity was found in culture filtrates of Cg-13 grown on *P. ultimum* cell walls (Table 19). This result is rather surprising because Pythium cell walls do not contain chitin. It may be caused by a combined induction of ß-1,3-glucanase and chitinase. In filtrates of ADP-13 no chitinase activity >0.02 nkatal could be detected.

No cellulase activity >0.002 units was detected in the filtrates.

TABLE 19. Production of ß-1,3-glucanase and chitinase by *C. globosum* strains grown on different carbon sources.

C. globosum strain / carbon source	Activity ß-1,3 glucanase[a]	Activity chitinase[a]
Cg-13 / P-cell walls	0.14 ± 0.02	0.09 ± 0.03
ADP-13 / P-cell walls	0.18 ± 0.01	0
Cg-13 / Laminarin	0	0
ADP-13 / Laminarin	0.05 ± 0.02	0

[a]Enzyme activities are expressed in nkatal per mg protein

5. Evidence for antibiosis: isolation and characterization of fungitoxic metabolites of *C. globosum* and their role in reduction of Pythium damping-off.

Previous studies have provided evidence for the production of metabolites toxic to plant pathogenic fungi by *C. globosum* (HUBBARD et al., 1982; DI PIETRO, 1986). In the present work, the attempt was made to isolate and characterize the inhibitory compounds. Furthermore their role in reduction of Pythium damping-off of sugarbeet was evaluated.

5.1. Effect of size fractionated culture filtrate of *C. globosum* on mycelial growth of different fungi

Inhibition of different fungi by culture filtrate of the *C. globosum* strain Cg-13 grown in 1% ME was evaluated. Furthermore, the presence of the inhibitory compound(s) in different size fractions of the filtrate was determined. Results are shown in Table 20. All the size fractions from culture filtrate of Cg-13 inhibited mycelial growth of the test fungi. Even the <3 kD fraction had an inhibitory effect comparable to the nonfractionated filtrate. This indicates, that the molecular weight of the inhibitory compound(s) produced by Cg-13 in 1% ME is less than 3 kD.

TABLE 20. Effect of size-fractionated culture filtrates of *Chaetomium globosum* strain Cg-13 grown in 1% ME on mycelial growth of different fungi.

Preparation	Growth inhibition (%)			
	P.u.[a]	*A.i.*[b]	*R. s.*[c]	*F.o.*[d]
Nonfractionated	100	100	77	81
< 100 kD	100	100	76	81
< 10 kD	100	100	70	81
< 3 kD	92	100	69	74

[a]*P. ultimum* strain P-71
[b]*Aphanomyces laevis* strain A-158
[c]*R. solani* strain R-160
[d]*Fusarium oxysporum lycopersici* strain F-198

5.2. Time course of production of fungitoxic metabolites by *C. globosum*

To investigate the time course and the optimum culture method for production of the inhibitory metabolite, *C. globosum* strain Cg-13 was grown in 1% ME at 175 rpm for 48 hr, and subsequently incubated either at 175 rpm or at 0 rpm. Inhibitory activity of the culture filtrates against *P. ultimum* and *R. solani* was determined after different incubation periods. In shaking culture, inhibitory activity of the filtrates increased until an incubation time of 100 hr and then remained constantly high until 336 hr incubation (Figure 14 **B.**). Filtrates from stationary culture were less inhibitory to the test fungi than filtrates from 175 rpm. Mycelial growth of Cg-13 was comparable in both culture methods as shown by the mycelial fresh weight (Figure 14 **A.**).

5.3. Inhibition of *P. ultimum* and *R. solani* by culture filtrates of *C. globosum* grown at different temperatures

The previous experiments indicated the presence of an inhibitory metabolite in the culture filtrate of Cg-13, a *C. globosum* strain effective in reducing Pythium damping-off. In a next step we determined if the inhibitory activity was also present in filtrates of strain ADP-13, a variant ineffective in biocontrol, and of Cg-43, a moderately effective strain. Furthermore, the influence of the growth temperature on the production of the inhibitory metabolites was investigated. All *C. globosum* strains were grown in 1% ME. The results presented in Figure 15 show that the inhibition of mycelial growth of *P. ultimum* and *R. solani* by the *C. globosum* culture filtrates differed considerably, depending on the strain and the growth temperature of *C. globosum*. Culture filtrates of strain Cg-13 from all growth temperatures were inhibitory to both test fungi except the filtrate from 10°C that was inhibitory to *P. ultimum* but not to *R. solani*. Filtrates of the variant ADP-13 from all temperatures caused only very low inhibition on both test fungi, indicating that no inhibitory metabolite was produced. Filtrates of Cg-43 from 25°C, 20°C, and 15°C were inhibitory to both test fungi. However, this strain produced no inhibitory metabolites at 10°C.

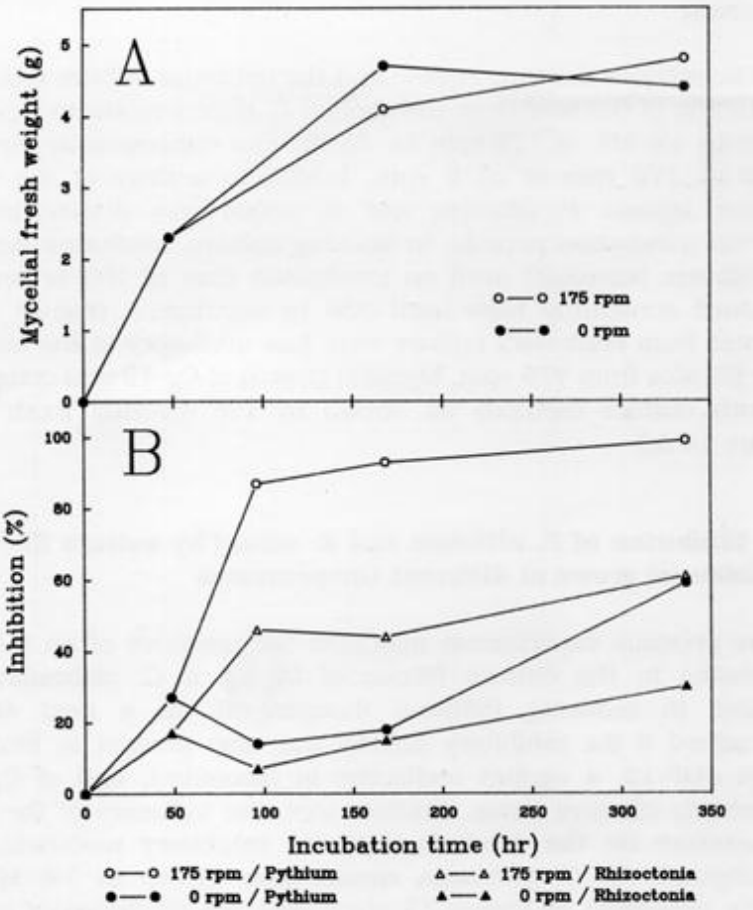


Figure 14. Time course of production of fungitoxic metabolites by *C. globosum* in 1% ME. **A.** Mycelial fresh weight of *C. globosum* strain Cg-13. **B.** Inhibition of *P. ultimum* and *R. solani* by culture filtrates of Cg-13. Cg-13 was incubated at 175 rpm for 48 hr, and subsequently at 175 rpm or 0 rpm for different time periods.

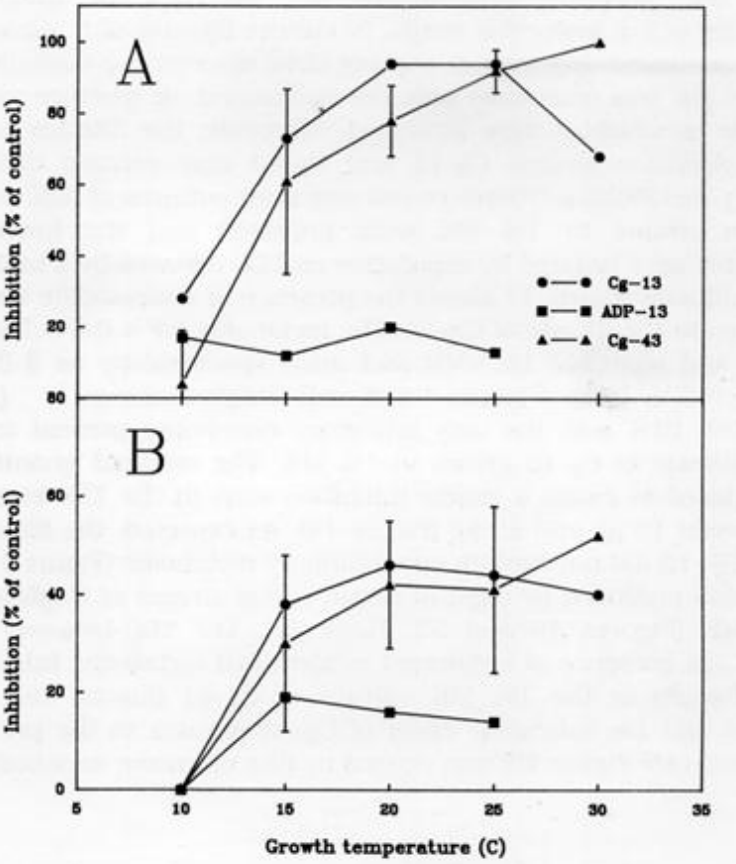


Figure 15. Inhibition of mycelial growth of **A.** *P. ultimum* and **B.** *R. solani* by culture filtrates of *C. globosum* strains Cg-13, ADP-13, and Cg-43 grown in 1% ME at different temperatures. Percent inhibition was calculated as follows: (G-g) / G x 100 (G = growth rate on control medium; g = growth rate on *C. globosum* culture filtrate).

5.4. Isolation of a fungitoxic metabolite from 1% ME cultures of *C. globosum*

In the previous experiments the presence of fungitoxic metabolites of low molecular weight in culture filtrates of *C. globosum* was demonstrated. However, it was not clear whether the inhibition of the test fungi was caused by only one compound, or whether several fungitoxic metabolites were produced. Moreover, the filtrates of the two *C. globosum* strains Cg-13 and Cg-43 may contain different inhibitory metabolites. Therefore extracts from cultures of different *C. globosum* strains in 1% ME were prepared and the fungitoxic metabolites were isolated by separation on TLC followed by a bioassay with *P. ultimum*. Figure 17 shows the presence of a metabolite toxic to *P. ultimum* in the filtrate of Cg-13. The metabolite (rF = 0.6-0.75) was purified and identified by NMR and mass spectroscopy as 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT; Figure 16). BHT was the only inhibitory metabolite present in the culture filtrate of Cg-13 grown in 1% ME. The minimal quantity of BHT required to cause a visible inhibition zone in the TLC-bioassay was between 10 µg and 20 µg (Figure 18). As expected, the filtrate of strain ADP-13 did not contain any inhibitory metabolite (Figure 17).

BHT was produced by eight of eleven tested strains of *C. globosum* in 1% ME (Figures 19 and 20, Table 22). The TLC-bioassay also revealed the presence of a different unidentified metabolite inhibitory to *P. ultimum* in the 1% ME culture of Cg-43 (Figure 19). This indicates that the inhibitory effect of Cg-43 filtrates in the previous experiment (see Figure 15) was caused by this unknown metabolite.

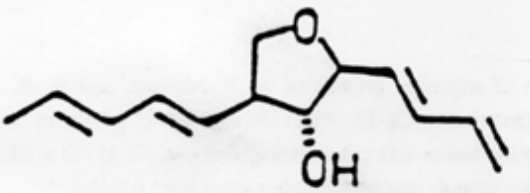


Figure 16. Structure of **2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT)**.

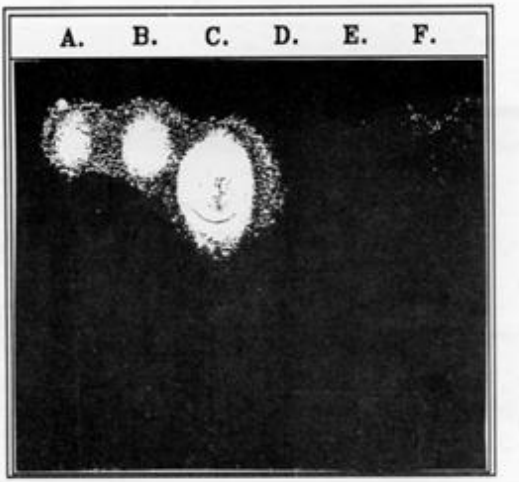


Figure 17. TLC-bioassay. Extracts of culture filtrates of the *C. globosum* strains Cg-13 and ADP-13 grown in 1% ME. Lanes: **A.-C.** Cg-13; **D.-F.** ADP-13; **A.** and **D.** 5 ml; **B.** and **E.** 10 ml; **C.** and **F.** 50 ml of extract solution.

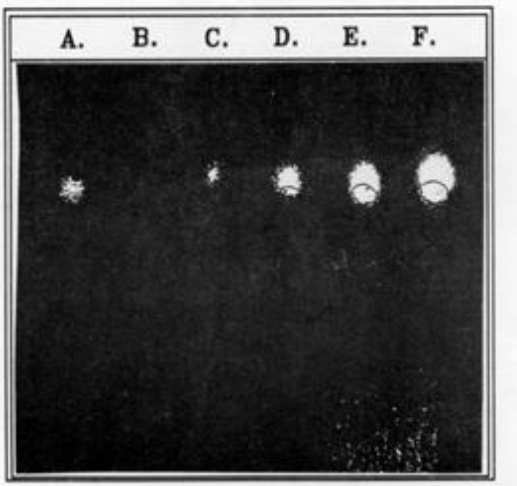


Figure 18. TLC-bioassay. Different quantities of BHT. Lanes: **A.** Metalaxyl, 0.1 µg; **B.** BHT, 10 µg; **C.** 20 µg; **D.** 30 µg; **E.** 50 µg; **F.** 100 µg.

Figure 19. Production of BHT by different *C. globosum* strains grown in 1% ME. Lanes: **A.** uninoculated ME; **B.** Cg-13; **C.** ADP-13; **D.** Cg-43; **E.** C-5; **F.** Cg-3. Note the presence of a different inhibitory metabolite in the filtrate of Cg-43.

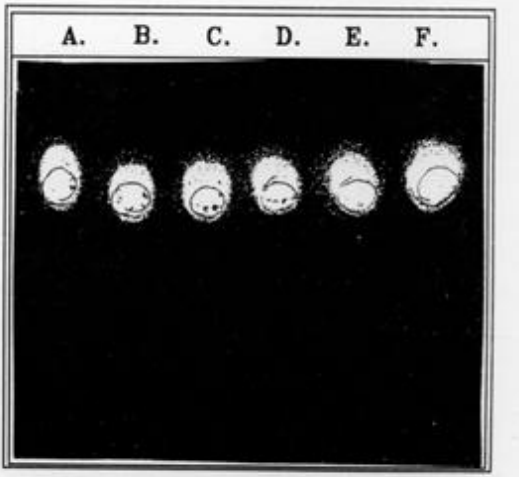


Figure 20. Production of BHT by different *C. globosum* strains grown in 1% ME. Lanes: **A.** Cg-14; **B.** Cg-1; **C.** Cg-20; **D.** Cg-13; **E.** Cg-29; **F.** Cg-200.

5.5. Inhibition of different Pythium species by different *C. globosum* strains

Inhibition of a range of *Pythium* species by culture filtrates of nine *C. globosum* strains grown in 1% ME was determined. Results are shown in Table 21.

TABLE 21. Effect of culture filtrates of different *C. globosum* strains grown in 1% ME on mycelial growth of different *Pythium* sp. and *R. solani*.

C. globosum strain	Growth inhibition (%)									
	71[a]	146[b]	161[c]	194[d]	318[e]	312[f]	231[g]	232[h]	R[i]	x[j]
Cg-1*	73	58	61	34	54	27	46	49	52	50
Cg-3*	83	85	90	46	58	70	52	51	61	66
C-5*	85	85	96	54	61	78	58	51	74	71
Cg-13*	75	87	90	46	54	73	60	41	58	65
ADP-13	23	17	10	10	8	13	33	12	19	16
Cg-14*	89	94	91	46	42	67	60	94	68	72
Cg-22[nd]	58	58	64	54	42	67	79	80	39	60
Cg-43	67	56	38	61	42	73	60	69	35	56
Cg-200*	100	100	100	100	92	100	96	98	81	96

[a]*P. ultimum* strain P-71
[b]*P. ultimum* strain P-146
[c]*P. ultimum* strain P-161
[d]*P. debaryanum* strain P-194
[e]*P. graminicola* strain P-318
[f]*P. arrhenomanes* strain P-312
[g]*P. aphanidermatum* strain P-231
[h]*P. aphanidermatum* strain P-232
[i]*R. solani* strain R-160
[j]Means of all inhibitions
*produces BHT in 1% ME (see Table)
[nd]prod. of BHT not determined

Although the *Pythium* sp. differed considerably in their susceptibility to the metabolites produced by the different *C. globosum* strains, the following general observations were made:

All three strains of *P. ultimum* tested (71, 146, 161) showed a very similar pattern of inhibition. They were strongly inhibited by the *C. globosum* strains producing BHT and to a lesser extent by the BHT-non-producing strains. *R. solani* showed the same pattern of inhibition.

The two strains of *P. aphanidermatum* (231, 232) and *P. arrhenomanes* strain 194 were less inhibited by the BHT-producing *C.*

globosum strains and more by the non-producing strains (Cg-43 and Cg-22). This suggests that they are less susceptible to BHT, and more susceptible to the unidentified metabolite produced by Cg-43 in 1% ME (see Figure 19).

Filtrates of *C. globosum* strain Cg-200 had a very strong inhibitory effect against all *Pythium* sp. Conversely, strain ADP-13 gave a very low inhibition against all test isolates.

5.6. Isolation of chaetomin

Production of secondary metabolites by *C. globosum* has been shown to be strongly dependant on the nutritional composition of the growth medium (BREWER et al., 1972). Therefore, we examined the production of inhibitory metabolites by the *C. globosum* strains Cg-13 and ADP-13 grown in a medium containing 1% corn steep powder. Cg-13 but not ADP-13 produced a fungitoxic metabolite (rF = 0.45-0.57) differing from BHT (Figure 22). This metabolite was also produced, additionally to BHT, when Cg13 was grown in a medium containing 0.5% ME and 1% CSP (Figure 25). The TLC-results suggested that the fungitoxic compound was chaetomin (see Figure 21), an antibiotic belonging to the group of the epidithiadiketopiperazines. This finding was confirmed by purification of the metabolite followed by NMR and mass spectroscopy. HPLC-analysis of purified chaetomin gave a distinct peak at a retention time of 9.17 min in the system used (Figure 24). Maximal absorption within the tested range occurred at 230 nm. The minimal quantity of chaetomin required to cause a visible inhibition zone in the TLC-bioassay was between 1 µg and 10 µg (Figure 23).

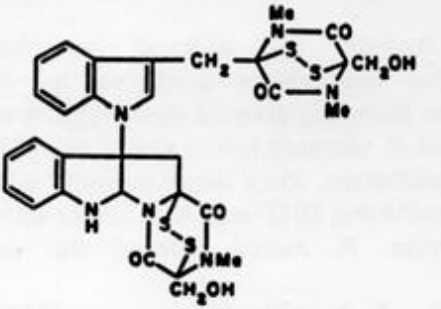


Figure 21. Structure of **chaetomin**.

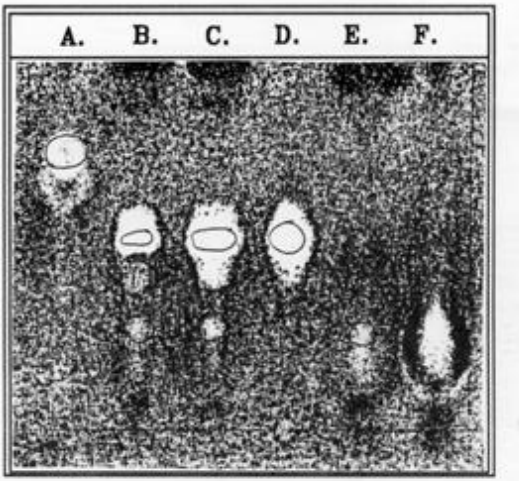


Figure 22. TLC-bioassay. Extracts from culture filtrate of *C. globosum* grown in 1% ME or 1% CSP. Lanes: **A.** Cg-13 / ME; **B.** Cg-13 / CSP; **C.** Cg-13 / CSP + chaetomin; **D.** chaetomin; **E.** ADP-13 / CSP; **F.** uninoculated CSP.

Figure 23. TLC-bioassay. Different quantities of chaetomin. Lanes: **A.** 0.01 µg; **B.** 0.1 µg; **C.** 1 µg; **D.** 10 µg; **E.** 50 µg; **F.** 100 µg.

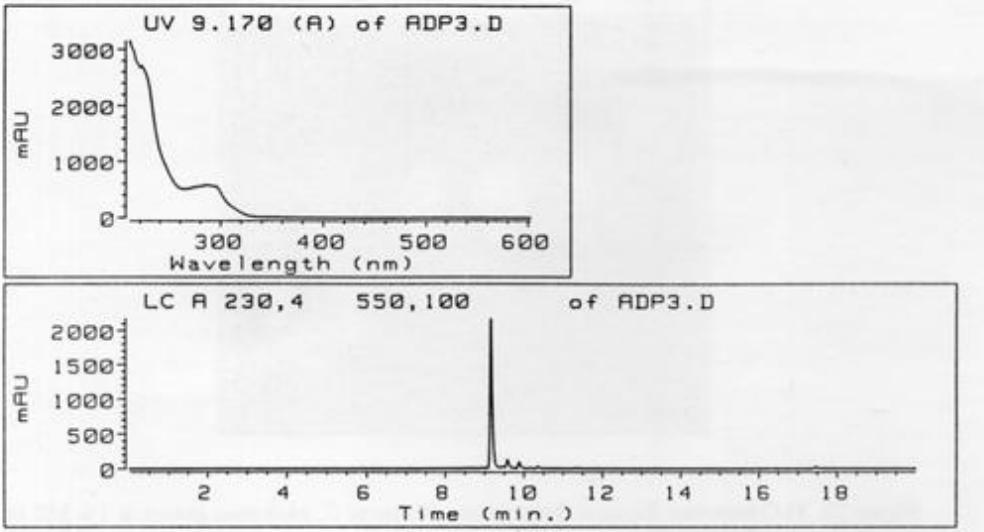


Figure 24. HPLC-chromatogram and UV/VIS-spectrum of chaetomin.

Chaetomin production by eleven *C. globosum* strains grown in 1% CSP-medium was determined. Results are shown in Figure 26 and Table 22. Six of the eleven tested strains produced chaetomin. Some of the strains that had previously been found to produce BHT in 1% ME (Cg-3, Cg-200), did not produce chaetomin. Strains ADP-13 and Cg-43 produced neither BHT nor chaetomin.

The results obtained so far suggested a potential role of BHT or chaetomin, or of both metabolites, in reduction of Pythium damping-off by *C. globosum*. However, it was still premature to extrapolate the in vitro data to the in vivo system. Therefore, in the following experiments we examined whether the fungitoxic metabolites were produced not only in liquid culture, but also in soil.

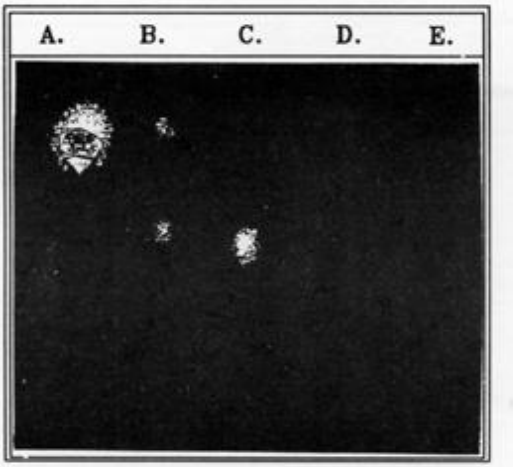


Figure 25. TLC-bioassay. Extracts of culture filtrates of the *C. globosum* strains Cg-13 and ADP-13 grown in 1% ME or 0.5% ME + 1% CSP. Lanes: **A.** Cg-13 / ME; **B.** Cg-13 / ME + CSP; **C.** chaetomin; **D.** ADP-13 / ME + CSP; **E.** ADP-13 / ME.

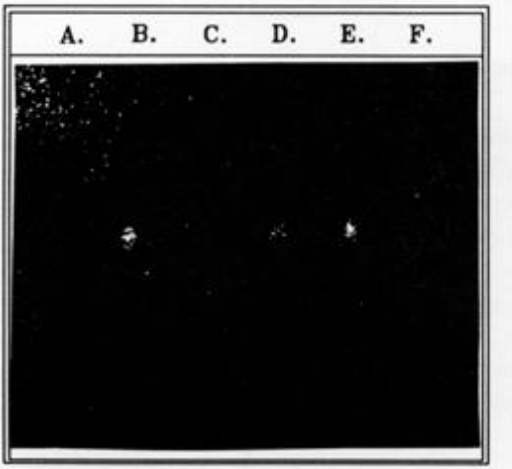


Figure 26. Production of chaetomin by different *C. globosum* strains grown in 1% CSP. Lanes: **A.** uninoculated CSP; **B.** Cg-1; **C.** Cg-3; **D.** C-5; **E.** Cg-13; **F.** ADP-13.

TABLE 22. Production of 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT) and chaetomin by different strains of *C. globosum*.

C. globosum strain	BHT[a]	Chaetomin[b]
Cg-1	+[c]	+
Cg-3	+	-
C-5	+	+
Cg-13	+	+
ADP-13	-	-
Cg-14	+	+
Cg-20	+	+
Cg-29	+	+
Cg-40	nd	-
Cg-43	-	-
Cg-200	+	-

[a]*C. globosum* strains were grown in liquid culture medium containing 1% ME.
[b]*C. globosum* strains were grown in liquid culture medium containing 1% CSP.
[c]Presence of fungitoxic metabolites was determined by a TLC-bioassay with *P. ultimum*.
[nd]not determined

5.7. Isolation of chaetomin from soil

To determine whether the production of fungitoxic metabolites by *C. globosum* also occurred in the soil, strains Cg-13 and ADP-13 were added to sterile soil as WBI at a ratio of 5% (w/v) and incubated in the soil for 6 days at 20°C. Growth conditions for *C. globosum* in the soil were comparable to those of the growth chamber experiments. TLC-analysis of the soil extracts showed that Cg-13, but not ADP-13, produced an inhibitory metabolite in the soil (Figure 27). The metabolite was purified and identified as chaetomin by NMR and mass spectroscopy. The metabolite BHT was not detected in the soil extract.

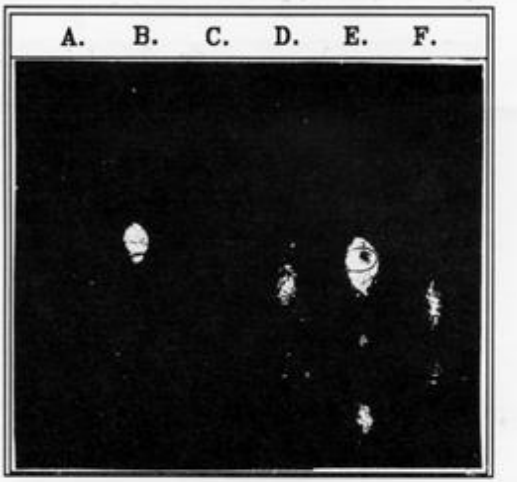


Figure 27. TLC-bioassay. Extracts of soil inoculated or non-inoculated with WBI of the *C. globosum* strains Cg-13 or ADP-13. Lanes: **A.** and **D.** Soil + ADP-13 WBI; **B.** and **E.** Soil + Cg-13 WBI; **C.** and **F.** Uninoculated soil. Lanes **A.-C.**: 10 µl; Lanes **D.-F.**: 20 µl of soil extract solution.

The absence of BHT in the soil extract of Cg-13 was rather surprising, since relatively high quantities of this metabolite were produced by Cg-13 in liquid culture (see Figure 17). However, production of BHT in liquid culture required the presence of ME in the growth medium, while in the present experiment wheat bran was used as a food base for *C. globosum* in soil. Therefore, in the following experiment we used a vermiculite/ME-inoculum of Cg-13 for soil inoculation. The TLC-bioassay with the soil extract is shown in Figure 28. Chaetomin was still the only inhibitory compound in the soil extract. BHT was not present. This indicates that the nutritional requirements for the production of metabolites by *C. globosum* in soil differ from those in liquid culture.

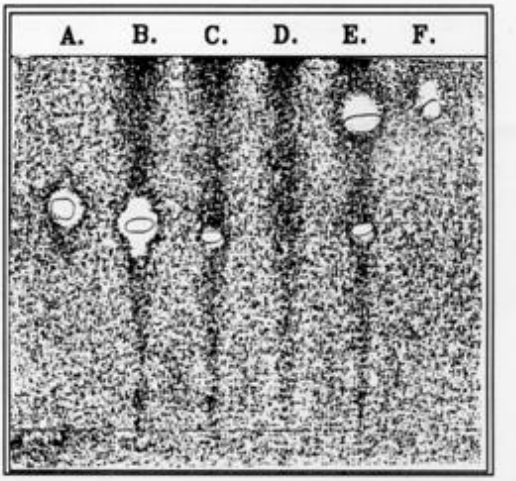


Figure 28. TLC-bioassay. Extracts of soil inoculated or non-inoculated with vermiculite/ME inoculum of the *C. globosum* strain Cg-13. Lanes: **A.** chaetomin; **B.** Soil + Cg13 WBI + chaetomin; **C.** Soil + Cg13 verm/ME; **D.** Uninoculated soil; **E.** Soil + Cg13 verm/ME + BHT; **F.** BHT.

5.8. Effect of *C. globosum* metabolites on mycelial growth and on sporangial germination of *P. ultimum*

The fungitoxic metabolites of *C. globosum*, in order to play an inmportant role in reduction of Pythium damping-off, should have a high inhibitory activity against *P. ultimum*. Inhibitory activities of BHT and chaetomin were found to be rather low in the TLC-bioassay (see Figures 18 and 22). However, these results may be misleading since the metabolites on the TLC-plates were exposed to air contact for a prolonged time period and may have been subject to degradation. A bioassay in microtiter plates was therefore used to determine inhibition of mycelial growth and of sporangial germination of *P. ultimum* by the purified metabolites from *C. globosum*. The fungicide metalaxyl, as well as gliotoxin, a epidithiadiketopiperazine from *Gliocladium fimbriatum*, were used as inhibitory standards. Results are shown in Figures 29 and 30.

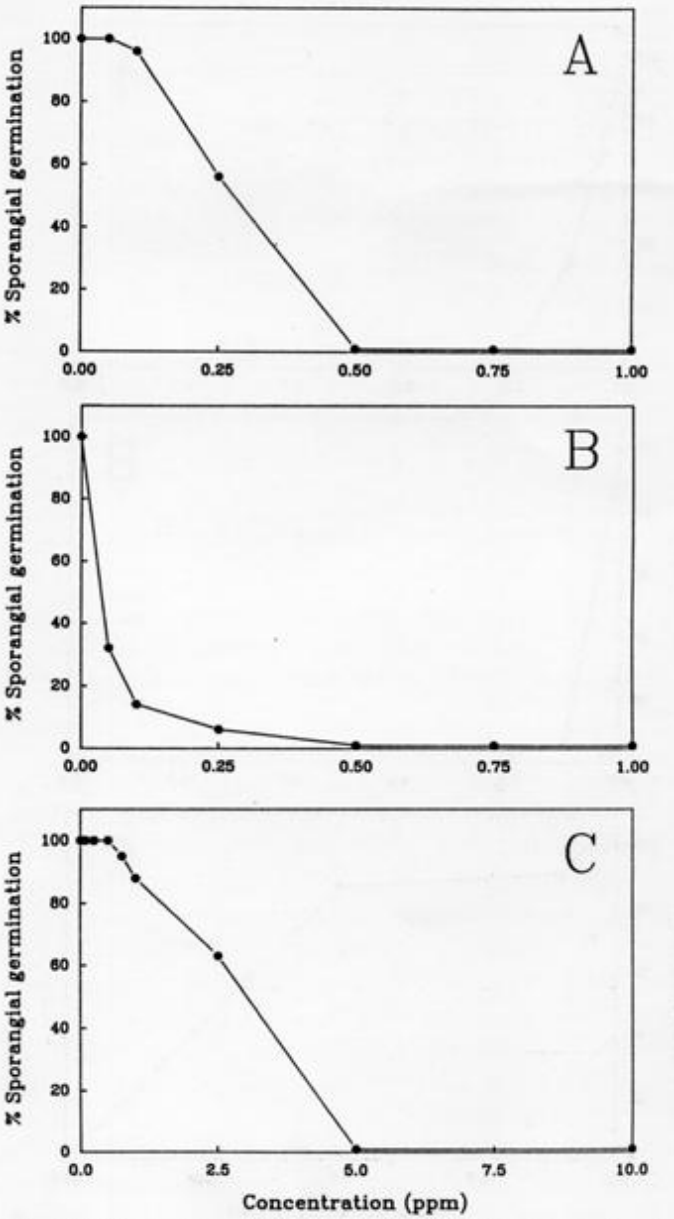


Figure 29. Inhibition of sporangial germination of *P. ultimum* by **A.** chaetomin; **B.** metalaxyl; **C.** gliotoxin. Note the x-axis scale is not the same in each graph.

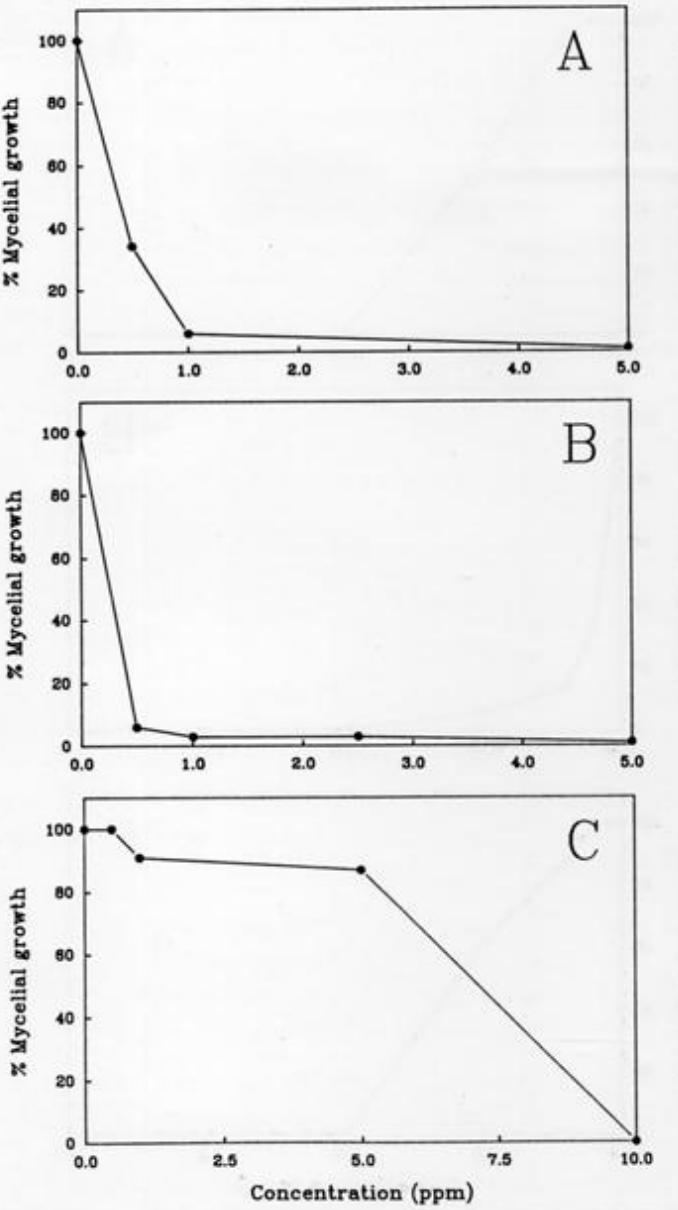


Figure 30. Inhibition of mycelial growth of *P. ultimum* by **A.** chaetomin; **B.** metalaxyl; **C.** gliotoxin. Note the x-axis scale is not the same in each graph.

Data from the microtiter plate bioassay are summarized in Table 23. BHT had a very low inhibitory activity against *P. ultimum*, confirming the results of the TLC-biotest. The MIC values of BHT for inhibition of mycelial growth and sporangial germination were both higher than 50 mg a.i./L. Conversely, the inhibitory activity of chaetomin against *P. ultimum* was considerably higher, namely in the same range as that of metalaxyl. Sporangial germination of *P. ultimum* was particularly sensitive to chaetomin. Germination was completely inhibited by 0.5 mg chaetomin/L (Figure 29). Corresponding MIC values for metalaxyl and gliotoxin were 0.5 mg/L and 5.0 mg/L respectively. Thus, chaetomin was 10 times more inhibitory to sporangial germination of *P. ultimum* than the closely related antibiotic gliotoxin. Inhibitory activity of chaetomin against mycelial growth of *P. ultimum* was slightly lower than against sporangial germination, but still considerably higher than that of gliotoxin (Figure 30).

TABLE 23. Effect of metabolites of *C. globosum* on sporangial germination and mycelial growth of *P. ultimum*.

Compound	Sporangial germination MIC[a]	Mycelial growth EC_{50}	Mycelial growth MIC
BHT	>50.0	50.0	>50.0
Chaetomin	0.5	0.5	2.5
Gliotoxin	5.0	7.5	10.0
Metalaxyl	0.5	0.25	1.0

[a]EC values are expressed in mg a.i./L

5.9. Efficacy of chaetomin-producing and non-producing *C. globosum* strains against Pythium damping-off of sugarbeet

The previous experiments have shown that chaetomin is produced by *C. globosum* in soil and that it is a potent inhibitor of sporangial germination and mycelial growth of *P. ultimum*. These results strongly suggest that chaetomin plays an important role in reduction of Pythium damping-off by *C. globosum*. To further test this hypothesis,

nine *C. globosum* strains, four of which did not produce chaetomin in liquid culture (see Table 22) were assayed for their efficacy in reducing damping-off of sugarbeet in sterile soil inoculated with *P. ultimum*. Results are shown in Figure 31: all of the chaetomin-non-producing strains (ADP-13, Cg-40, Cg-43, and Cg-3; marked with asterisks) were less effective in reducing Pythium damping-off than the chaetomin-producing strains, providing further evidence for the importance of this metabolite in control of *P. ultimum*. Cg-1 was the most effective of all the tested strains. Chaetomin-producing strains did not differ significantly in efficacy between each other.

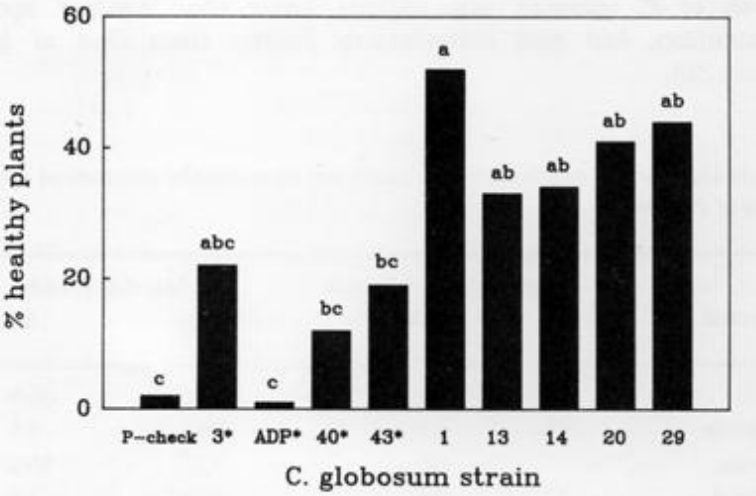


Figure 31. Efficacy of chaetomin-producing and non-producing *C. globosum* strains against Pythium damping-off of sugarbeet.

Discussion

The purpose of this study was to provide further knowledge on the mode of action of *C. globosum* against *P. ultimum*, the causal agent of damping-off. Strains of *C. globosum* with different abilities in reducing Pythium damping-off of sugarbeet were selected for comparative studies of factors related to the potential biocontrol mechanisms. Strain Cg-13 was selected as a highly efficient biocontrol strain, while Cg-43 was selected as less efficient strain. Ideally, the strains to be compared should differ in as few traits as possible to allow precise identification of the features important for biocontrol. Since Cg-43 belonged to a different subspecies (*C. globosum* var. *flavo-viride* Novac) than Cg-13 (*C. globosum* var. *ochraceoides* Dreyfuss) the two strains were not ideally suited for such a comparison. Therefore, a spontaneous variant of Cg-13, isolated during the work and designated ADP-13, was used for further experiments. It was assumed that the genotypes of Cg-13 and ADP-13 were closely related. The variant proved to be ideally suited for studies on the antagonistic mechanisms, because it was completely ineffective in reducing Pythium damping-off in the growth chamber. In vivo experiments were conducted in a controlled environment and mostly in sterile soil, in order to minimize the influence of disturbing factors such as soilborne pathogens other than *P. ultimum*. Subsequently, several features of the strains related to the potential modes of action competition, mycoparasitism, or antibiosis respectively were compared. The role of these mechanisms in the antagonism of *C. globosum* against *P. ultimum*, as suggested by the results of the present study, will be discussed in the following sections.

1. Competition

Competition for food or space is a potential mode of action of microbial antagonists. For example, PAULITZ & BAKER (1988) reported that the antagonist *Pythium nunn* competes saprophytically with *P. ultimum* for the colonization of organic substrate in soil. The ability of an antagonist to colonize the plant rhizosphere, the so-called

rhizosphere competence, was shown to be particularly important for its biocontrol efficacy in certain systems. Rhizosphere competent *Trichoderma harzianum* mutants increased the incidence of seedling emergence in *P. ultimum* infested soil more effectively than the non-rhizosphere competent wild type parents (AHMAD & BAKER 1988). In the present study, the *C. globosum* strains Cg-13, ADP-13, and Cg-43 colonized the rhizosphere of cucumber and sugarbeet. However, when the numbers of cfu were determined on the first 4 days after planting that are crucial for *P. ultimum* attack as demonstrated by NELSON (1987) and OSBURN et al. (1989), the three *C. globosum* strains did not differ significantly in their population density during this time period. Since the strains have different efficacies in reducing Pythium damping-off, the conclusion was drawn that competition is not a major mechanism in the antagonism of *C. globosum* against *P. ultimum*.

2. Mycoparasitism

Mycoparasitic activity is a major mechanism of fungal biocontrol agents, such as *Trichoderma* spp. (ELAD et al., 1983; SIVAN & CHET, 1989) or *Pythium oligandrum* (LUTCHMEAH & COOKE, 1984). Only few reports exist on potential mycoparasitic activity of *C. globosum*. VANNACCI & HARMAN (1987) showed coiling of *C. globosum* hyphae around hyphae of *Alternaria brassicicola*. Alterations of the cell wall structure of *A. brassicicola* at the sites of interaction, so-called "reaction zones", were observed. WALTHER & GINDRAT (1988) described coiling of *C. globosum* around hyphae of *R. solani*, but did not observe visible damage of wrapped hyphae. In the present work, extensive coiling of *C. globosum* around hyphae of *R. solani* in the soil is reported. Furthermore, formation of appressoria-like structures by *C. globosum* was observed upon contact with *R. solani*. Reaction zones similar to those described by VANNACCI & HARMAN (1987) were visible on the *R. solani* hyphae in the light microscope. Staining with Calcofluor revealed intense fluorescence of *R. solani* hyphae at these zones of interaction. This indicates the presence of polysaccharide oligomers (KRITZMAN et al., 1978; ELAD et al., 1983), suggesting lysis of *R. solani* cell walls by extracellular enzymes of *C. globosum*.

Probably lysis was only partial, since no penetration holes in the host cell walls were found by observation with scanning electron microscope. Hyphal interactions were observed in vitro and in soil. This indicates that mycoparasitic activity of *C. globosum* against *R. solani* occurs in the natural habitat of the two fungi. The importance, however, of mycoparasitism in control of *R. solani* by *C. globosum* remains unknown.

No interactions could be observed between hyphae of *C. globosum* and *P. ultimum*, in agreement with the results of HUBBARD et al. (1982) and WALTHER & GINDRAT (1988). Although *C. globosum* produced ß-1,3-glucanase and chitinase when it was grown on *P. ultimum* cell walls as sole carbon source, there is no evidence that mycoparasitism between the two fungi occurs in vivo. Furthermore, the levels of ß-1,3-glucanase excreted by the effective and the ineffective biocontrol strain of *C. globosum* were comparable. Although there were differences in chitinase production between the two strains, their relevancy in the interaction with *P. ultimum* is very doubtful since the cell walls of this oomycete do not contain chitin. These results lead to the conclusion that mycoparasitism is not a major mechanism in the antagonistic activity of *C. globosum* against *P. ultimum*.

3. Antibiosis

A role of antibiosis in the antagonistic activity of *C. globosum* against various plant pathogens has been suggested by different authors (TVEIT & WOOD, 1955; HUBBARD et al., 1982; CULLEN & ANDREWS, 1984; WALTHER & GINDRAT, 1988). The results of the present work provide strong evidence for production of antibiotics in soil as an important mechanism in the antagonism of *C. globosum* against *P. ultimum*. This conclusion is based on the following lines of evidence:

1) *C. globosum* strain Cg-13, which effectively reduced Pythium damping-off of sugarbeet in the growth chamber, produced at least two metabolites inhibitory to *P. ultimum* in liquid culture, 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-

tetrahydrofuran (BHT) and chaetomin. The metabolites were also produced by several other *C. globosum* strains.

2) Chaetomin was isolated from soil that had previously been inoculated with Cg-13.

3) ADP-13, a spontaneous variant of strain Cg-13 that failed to produce the fungitoxic metabolites in liquid culture and in soil, was ineffective in reducing Pythium damping-off of sugarbeet.

4) The *C. globosum* strains that produced chaetomin in liquid culture were more effective in reducing Pythium damping-off than the non-producing strains.

Production of antibiotics by *C. globosum* is well documented. Soil isolates of different *Chaetomium* spp. were shown to produce several antibiotic compounds in liquid culture (BREWER & TAYLOR, 1978). In the present study, BHT and chaetomin were isolated from liquid culture of *C. globosum*. BHT was first isolated by BURROWS (1967) from culture broth of *Chaetomium coarctatum*. No antibiotic properties of this compound have yet been described. The epidithiadiketopiperazine chaetomin was first described by WAKSMAN and BUGIE (1944). Its antibiotic properties have been reported by BREWER et al. (1966, 1972). The results of our work suggest that of the two metabolites only chaetomin is important in biocontrol of *P. ultimum*. First, chaetomin was about 100 times more inhibitory to *P. ultimum* than BHT in the microtiter plate bioassay. Second, chaetomin but not BHT, was extracted in detectable quantities from soil colonized by Cg-13.

The present study provides the first report on the production of chaetomin by *C. globosum* in soil. The chemically related antibiotic gliotoxin has been isolated from the coats of pea seeds in a soil colonized by a strain of *Trichoderma viride* (WRIGHT, 1956b). Furthermore, the extraction of a nondiffusable antibiotic from squash seeds coated with *C. globosum* ascospores has been described (HUBBARD et al., 1982). This antibiotic inhibited growth of a broad spectrum of soil-inhabiting fungi. The present results suggest that the compound isolated by these authors was probably chaetomin.

In the wheat bran system used in our study, antibiosis against *P. ultimum* may act in two different ways, since production of antibiotic was found to occur already during the incubation of *C. globosum* on the bran (data not shown). The question is whether the quantity of antibiotic in the WBI at the time of soil mixing is sufficient to control *P. ultimum* in the soil, or whether reduction of Pythium damping-off is due to metabolites produced by *C. globosum in situ* during proliferation in the soil. This question was investigated in the present study by killing *C. globosum* on the wheat bran inoculum before adding it to soil. Biocontrol activity was thereby completely eliminated, indicating that control of *P. ultimum* was not due to the metabolites already present in the WBI at the moment of soil mixing. It is unlikely that the antifungal metabolites in the WBI would be inactivated by all three different treatments used to kill the antagonist. These findings are consistent with the results of WALTHER & GINDRAT (1988) who found that seed coating with autoclaved ascospores of the *C. globosum* strain Cg-1 failed to control *P. ultimum* damping-off of sugarbeet. These results indicate that an active antagonistic mechanism in the soil is responsible for biocontrol of *P. ultimum*.

The antibacterial and antifungal properties of chaetomin (BREWER et al., 1966; BREWER et al., 1972) as well as its toxicity to mammals (BREWER et al., 1972) have been described. A possible role of this metabolite in the antagonism of *C. globosum* to the apple scab pathogen *Venturia inaequalis* has been proposed (CULLEN & ANDREWS, 1984; BOUDREAU & ANDREWS, 1987). Susceptibility of *P. ultimum* to antibiosis (STANGHELLINI & HANCOCK, 1971; HOWELL & STIPANOVIC, 1983) has as well been reported. JONES & HANCOCK (1988) described the high sensitivity of *P. ultimum* to gliotoxin, an epidithiadiketopiperazine chemically related to chaetomin. Since chaetomin has the same disulfide group as gliotoxin responsible for its biological activity (BREWER et al., 1966), the two antibiotics probably exhibit the same mechanism of action. JONES & HANCOCK (1988) suggested that the primary mechanism of action of gliotoxin involves selective binding to cytoplasmic membrane thiol groups. They found a rapid uptake of the toxin by *P. ultimum*. Furthermore, a deep rough mutant of *Salmonella typhimurium*, deficient in outer-membrane polysaccharide synthesis, was

hypersensitive to gliotoxin. The authors concluded that diffusion barriers play a role in relative sensitivity of an organism to gliotoxin. Therefore, partial lysis of the polysaccharide layer in fungal cell walls, e.g. by hydrolytic enzymes, may render the cells more susceptible to the action of the metabolite. ROBERTS & LUMSDEN (1990) suggested a synergistic effect of hydrolases and gliotoxin against *P. ultimum*. That may also occur in the case of chaetomin. In the present study, ß-1,3-glucanase and chitinase were induced by growing *C. globosum* on cell walls of *P. ultimum* as sole carbon source indicating that, at least in vitro, *C. globosum* is able to attack the cell walls of this pathogen.

ROBERTS & LUMSDEN (1990) reported strong inhibition of sporangial germination and mycelial growth of *P. ultimum* by the gliotoxin-containing culture supernatant from *Gliocladium virens*. In the present study, using a similar inhibition assay, we found that chaetomin was five to ten times more inhibitory to *P. ultimum* than commercial gliotoxin from *Gliocladium fimbriatum* (see Figures 29 and 30). This is true for mycelial growth as well as for sporangial germination of *P. ultimum*. Inhibitory activity of chaetomin was comparable to that of metalaxyl, a fungicide commonly used for the control of *P. ultimum*. These results indicate that even low levels of chaetomin in the soil may cause considerable inhibition or at least delay of sporangial germination and hyphal growth of *P. ultimum*. Since substantial colonization of the seed pericarps of sugarbeet by *P. ultimum* usually occurs within 8-12 hr under favorable conditions, and since the amount of damping-off is related directly to the incidence of pericarp colonization (OSBURN et al., 1989), a temporary reduction of *P. ultimum* growth may be sufficient for a successful control of the pathogen.

The positive correlation between the production of chaetomin by *C. globosum* strains in liquid culture or soil and their efficacy in reducing Pythium damping-off in the growth chamber provides further evidence for the relevance of this metabolite in antagonism of *C. globosum* against *P. ultimum*. A similar correlation was described by HOWELL & STIPANOVIC (1983). A mutant of *Gliocladium virens* lacking production of the fungitoxic metabolite gliovirin was obtained by UV-mutagenesis. The mutant also failed to protect cotton from Pythium damping-off. In the present study, all of the chaetomin-

producing *C. globosum* strains were superior to the non-producing strains in biocontrol efficacy. However, with the exception of the variant strain ADP-13, all the chaetomin-non-producing strains still gave a moderate degree of protection against Pythium damping-off. This indicates that other factors are involved in the reduction of Pythium damping-off by *C. globosum*.

4. Application method of *C. globosum*

The method of application of a biocontrol agent is an important criterion for its efficacy (HARMAN et al., 1989; PAPAVIZAS, 1985). In the present study, fresh WBI of *C. globosum* was very effective in reducing Pythium damping-off of sugarbeet in the growth chamber. However, its use as a biocontrol formulation is not feasible under conditions of practice, due to the impracticality of using freshly prepared cultures in agricultural systems (PAPAVIZAS & LEWIS 1989). Furthermore, the application quantity of fresh WBI required to achieve reduction of Pythium damping-off is too high. Drying the WBI and grinding it to a fine powder both improves the shelf life of the WBI and reduces the application quantity required. We showed that the drying procedure did not affect biocontrol activity of the WBI. In-furrow application of dry WBI at a rate of approximately 2 g per furrow meter still gave good protection against Pythium damping-off of sugarbeet. Dry WBI could be stored at 20°C for at least six months without loss of biocontrol activity (data not shown). However, when the room temperature increased to 30°C, the efficacy of the dry WBI decreased rapidly.

Undoubtedly, seed coating with ascospores is the application method best suited for commercial use because of its low requirement of material and its long shelf life. In the present study, however, efficacy of ascospore seed coating was considerably lower compared to the WBI treatment. Lack of an appropriate food base may lead to a a poor proliferation of the antagonist and have a negative impact on the production of antibiotics in soil. This may explain the reduced biocontrol activity of the seed coating.

Although absolute numbers of cfu of *C. globosum* in the rhizosphere of cucumber seedlings were comparable for WBI and

ascospore seed coating, the spatial distribution and the metabolic state of the antagonist may differ between the two application methods. WBI probably assures a better initial distribution of the antagonist in soil than the ascospore seed coating. With a seed treatment, the beneficial effect of the biocontrol agent can be limited to a restricted zone surrounding the seed (CHAO et al., 1986), leaving the growing seedling unprotected from the attack of soilborne plant pathogens. Indeed, KOMMEDAHL et al. (1981) were not able to detect rhizosphere colonization by *C. globosum*, when the antagonist was applied to corn seed as ascospore coating. Rhizosphere competent antagonist strains can overcome this limitation. AHMAD & BAKER (1988) found that rhizosphere competent *Trichoderma harzianum* mutants increased the incidence of seedling emergence in *P. ultimum* infested soil more effectively than the non-rhizosphere competent wild type parents. In the same study the antagonist was more effective when it was applied as conidia coated onto seeds than when it was added to soil in peat-bran. This observation apparently contrasts with the results presented here. The discrepancy may be due to superior rhizosphere competence and to different antagonistic mechanisms of the *Trichoderma* strains compared to the *C. globosum* strains used in the present work.

Although population proliferation is a major criterion for an effective biocontrol fungus (COOK & BAKER, 1983), the biological activities associated with antagonist proliferation are probably more critical than the proliferation itself (LEWIS & PAPAVIZAS, 1984). Wheat bran provides an excellent food base for *C. globosum*, promoting not only proliferation in soil, but also antibiotic production. Production of antibiotics by fungi in soil seems to depend on the availability of organic substrates. For example, high quantities of gliotoxin were produced by *Trichoderma viride* on wheat straws buried in soil, but only small amounts of the antibiotic were produced in the soil surrounding the straws (WRIGHT, 1956a).

Promotion of antibiotic production may be a major reason for the superior efficacy of wheat bran inoculum of *C. globosum* compared to ascospore seed coating. Although HUBBARD et al. (1982) were able to extract a nondiffusable antibiotic (probably chaetomin) from the seed coats of pea seeds treated with ascospores of *C. globosum*, the quantity of antibiotic produced is probably too low to ensure

consistent control of *P. ultimum*. Since chaetomin, like gliotoxin, is subject to bacterial degradation at pH levels less than 6.5 (BREWER & TAYLOR, 1967), it is likely that a continous high antibiotic production in soil is required to keep the quantity of chaetomin at a sufficiently high level. Incorporation of nutrients into microbial seed treatments has been shown to increase the efficacy of antagonists. NELSON et al., (1988) reported an greater than 40% increase of biocontrol activity of *Trichoderma koningii* and *T. harzianum* by addition of various compounds to seed treatments. Interestingly, organic acids were most promotive to the activity of *T. koningii* whereas polysaccharides were most promotive to *T. harzianum*. This indicates that the optimum nutrient adjuvants have to be determined for each specific antagonist. For example, polysaccharides such as chitin will enhance the activity of a mycoparasite (HARMAN et al., 1981) whereas an antibiotic producing antagonist may have specific nutritional requirements for the production of secondary metabolites. In the present study, production of chaetomin by *C. globosum* in liquid culture was strongly dependent on the nutritional composition of the growth medium. Chaetomin was produced in corn steep powder medium, but not in malt extract medium. Corn steep liquor has been described by BREWER et al. (1972) as particularly effective in enhancing chaetomin production in liquid culture. In soil, however, chaetomin was produced on both wheat bran and vermiculite imbibed with malt extract. Apparently, chaetomin production by *C. globosum* on both substrates was induced by the soil environment. This suggests that chaetomin production in soil is not as strictly dependent on nutritional composition of the food base as it is in liquid culture.

Both efficacy and reliability of biocontrol formulations may be increased by the combined application of different biocontrol organisms (COOK & BAKER, 1983). In the present study, combining in-furrow application of dry WBI of *C. globosum* with *Pseudomonas fluorescens* seed treatment reduced Pythium damping-off more effectively than each antagonist treatment alone (data not shown). Improved efficacy of the integrated treatment may be due to the contribution of different modes of action by the two biocontrol agents. Antagonistic mechanisms of *Pseudomonas fluorescens* against *P. ultimum* include production of antibiotics (HOWELL & STIPANOVIC,

1980) and siderophores (BECKER & COOK, 1988). The promising results of our preliminary experiments confirm the potential of applying *C. globosum* in combination with other microbial antagonists. However, more studies on the interactions between different biocontrol agents are needed. This knowledge will be necessary to optimize combined application of beneficial organisms in order to improve efficacy of biocontrol formulations.

5. Future directions

The evidence presented in this study, for antibiosis as the major mechanism in antagonism of *C. globosum* against *P. ultimum* is substantial but not fully conclusive. It is based in part on the comparative analysis of a parental biocontrol strain and a spontaneous variant deficient in biocontrol activity. However, the degree of homology between the genomes of parental and variant strain is not exactly known. Therefore, the strains should be analyzed by appropriate molecular techniques such as isozyme analysis (STASZ et al., 1989) and restriction fragment length polymorphism (RFLP) analysis to determine their genetic relatedness.

A different approach that may be useful in elucidating the particular role of chaetomin in reduction of Pythium damping-off by *C. globosum* is the creation of chaetomin-resistant mutants of *P. ultimum*. The mutants could then be tested in the growth chamber system and, provided that they are equally virulent to the parental strain, they are expected to be less susceptible to the biocontrol activity of *C. globosum*. In the present work the attempt was made to generate chaetomin-resistant mutants by UV-irradiation of *P. ultimum* sporangia. However, only few colonies with a slightly increased resistance to the metabolite could be isolated (data not shown). Moreover, these mutants were less virulent against sugarbeet in the growth chamber assay than the parental strain and were therefore not suited for further studies. Screening of a very high number of mutants is probably necessary to render this approach successful. Such a screening would require a large quantity of chaetomin, that is probably difficult to provide. The problem may be solved by using commercially available gliotoxin for primary screening. Gliotoxin-

resistant mutants could then be screened for resistance against chaetomin. Since the two metabolites have the same biologically active polysulfide region, there should be a high probability of cross resistance.

A very precise method to evaluate the importance of a specific product in biocontrol is to isolate the gene(s) coding for that product. An isolated gene can then be mutagenized in vitro, and used to exactly replace the wild type allele on the fungal chromosome (site directed mutagenesis). The resulting mutant will differ only in the mutated gene from the parental strain. Since the mutant will be deficient for the specific gene product, assessing its biocontrol activity will provide conclusive evidence on the role of this factor in biocontrol. Furthermore, removing the native promoter of the gene and replacing it with a series of promoters with different activities, allows to assess the role of a specific gene product quantitatively. Finally, genes useful in biocontrol may be transferred into other isolates resulting in superior biocontrol strains.

SHAPIRA et al. (1989) reported the cloning of a bacterial chitinase gene from *Serratia marcescens* and its transfer into *Escherichia coli*. The chitinase-producing *E. coli* strain was able to reduce disease incidence caused by *Sclerotium rolfsii*. THOMASHOW & WELLER (1988) used the genetic approach to demonstrate that phenazine antibiotics were required for control of take-all in wheat by *Pseudomonas fluorescens*. They not only generated phenazine-deficient mutants that failed to suppress take-all, but they isolated and cloned the specific genes and fully restored biocontrol activity by adding them back to the deficient strains. Similar studies have been used to analyze factors required for pathogenicity of fungal plant pathogens (SCHAEFER et al., 1989). However, such work has not yet been accomplished for fungal biocontrol agents. Recently, efficient transformation systems have become available for several fungi including the biocontrol agents *Gliocladium* sp. (THOMAS & KENERLY, 1989) and *Trichoderma harzianum* (GOLDMAN et al., 1990). HARMAN et al. (1989) obtained an improved biocontrol strain of *Trichoderma harzianum* by protoplast fusion of two parental strains. Production of protoplasts which is a prerequisite for transformation was readily accomplished with *C. globosum* by standard methods in the present study (data not shown). Lytic enzymes are primary candidates for assessment of relevance in

biocontrol by the cloning method, since they are controlled by a single gene. It will be more complicated to accomplish genetical analysis of products that are under multigenic control such as many secondary metabolites.

The methods mentioned above will provide further knowledge on the antagonistic mechanisms of biocontrol agents, and the conditions under which they are active. The application of this information may lead to the successful commercial use of microbial antagonists in disease management.

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