A FUNÇÃO DAS ANASTOMOSES ENTRE CONÍDIOS NA RECOMBINAÇÃO GENÉTICA EM *Colletotrichum lindemuthianum*

FRANCINE HIROMI ISHIKAWA

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-graduação em Genética e Melhoramento de Plantas, área de concentração em Biotecnologia, para a obtenção do título de "Doutor".

Orientadora

Profa. Dra. Elaine Aparecida de Souza

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LAVRAS
MINAS GERAIS - BRASIL

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ISHIKAWA, Francine Hiromi. **A função das anastomoses entre conídios na recombinação genética em *Colletotrichum lindemuthianum*.** 2009. 97 p. Tese (Doutorado em Genética e Melhoramento de Plantas) – Universidade Federal de Lavras, Lavras, MG.*

O *Colletotrichum lindemuthianum*, agente causal da antracnose do feijoeiro, apresenta ampla variabilidade patogênica e genética, o que tem dificultado o desenvolvimento de cultivares resistentes. Vários são os mecanismos responsáveis pela recombinação genética em fungos filamentosos. Este trabalho foi realizado com os objetivos de otimizar o protocolo para a obtenção e a regeneração de protoplastos, visando à manipulação genética; estudar a biologia dos tubos de anastomoses entre conídios (CATs) em *C. lindemuthianum* para análise *in vivo* e obter transformantes com núcleo marcado com proteínas fluorescentes, visando comprovar a ocorrência de recombinação genética na ausência do ciclo sexual utilizando imagens de células *in vivo*. Primeiramente, com o objetivo de transformar as diferentes raças de *C. lindemuthianum* para a marcação do núcleo com proteínas fluorescentes verde e vermelha (GFP e tdimerRed, respectivamente), foi realizado um trabalho de otimização nas condições de obtenção e regeneração de protoplastos desta espécie. Para a formação de protoplastos, foram testados diferentes tipos e concentrações de estabilizadores osmóticos, tempo de incubação, idade micelial e concentração de enzima de lise. Depois de otimizadas as condições para a obtenção dos protoplastos, foram testados diferentes estabilizadores osmóticos para a regeneração. As melhores condições para a obtenção de protoplastos do isolado de *C. lindemuthianum* foram obtidas utilizando-se micélio com 48 horas, em estabilizador osmótico NaCl 0,6 M, 30 $mg.mL^{-1}$ da enzima *Lysing Enzymes* e tempo de digestão de 3 horas. Sacarose 1,2M e 1M foi o estabilizador mais apropriado para a regeneração. Utilizando esta metodologia foram realizadas as transformações com os plasmídeos pMF357, que possui o gene da histona H1 ligada a GFP e o gene de resistência à higromicina e pGR02, que possui o gene de histona H4 em fusão com tdimerRed (vermelho) e o gene de resistência à fleomicina. Posteriormente, os aspectos relacionados à formação e à ocorrência de CATs foram estudados, utilizando-se quatro isolados de *C. lindemuthianum*, sendo dois pertencentes à raça 65 e um de cada uma das raças 73 e 81. Foram testados diferentes meios de cultivo, idade da cultura e tempo de incubação,

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demonstrando a necessidade de condições adversas à germinação para a fusão de CATs. Dessa forma, foi possível desenvolver uma metodologia fácil e prática para o estudo de CATs em *in vivo* e que facilitará estudos da função biológica dessas células em um fitopatógeno. Experimentos de compatibilidade vegetativa foram realizados, indicando a incompatibilidade do heterocário na colônia madura entre as raças transformadas 65b (verde) e 73 (vermelho). A incompatibilidade foi comprovada por meio da morte celular após a fusão de hifas. No entanto, foi possível visualizar a formação do heterocário e a passagem de núcleo entre esses isolados vegetativamente incompatíveis, por meio da fusão de CATs. A recombinação entre os isolados pode ser comprovada utilizando-se os marcadores para resistência aos antibióticos higromicina e fleomicina, além de *primers* específicos para os genes de interesse. Portanto, este mecanismo de fusão de CATs é uma fonte potencial de recombinação genética e/ou surgimento de novas raças e pode explicar a ocorrência da transferência horizontal de genes dentro dessa espécie entre isolados incompatíveis ou, mesmo, entre diferentes espécies, levando ao surgimento de patógenos mais virulentos.

GENERAL ABSTRACT

ISHIKAWA, Francine Hiromi. **Conidial anastomosis tubes function in the genetic recombination in *Colletotrichum lindemuthianum*.** 2009. 97 p. Thesis (PhD in Plant Genetics and Breeding) – Federal University of Lavras, Lavras, Minas Gerais, Brazil.*

The causal agent of common bean anthracnose, *Colletotrichum lindemuthianum*, presents a great genetic and pathogenic variability that has complicated the development of resistant cultivars. Several mechanisms are involved in the genetic recombination of filamentous fungi. The objectives of this work were to optimize a production and regeneration protocol for *C. lindemuthianum* protoplasts to use in genetic manipulation; to study live-cell imaging of conidial anastomosis tubes (CATs) in *C. lindemuthianum*; and to obtain nuclear labeled strains with fluorescent proteins to show the genetic recombination in the absence of sexual cycle, using live cell-imaging. First of all, to obtain *C. lindemuthianum* strains from different races labeled with different fluorescent proteins, green (GFP) and red (tdimerRed) respectively, an optimization study was performed on the conditions favourable for the formation and regeneration of protoplasts in this specie. Osmotic stabilizers, lytic enzymes, incubation time and mycellial age were evaluated in terms of their effects on protoplast yield. The optimal condition for protoplast production included the incubation of young mycelia (48 h) in 0.6 mol l^{-1} NaCl as osmotic stabilizer, with 30 mg ml^{-1} lysing enzymes from *Trichoderma harzianum* for 3 h-incubation. Sucrose concentrations of 1.2 mol l^{-1} and 1 mol l^{-1} were the most suitable osmotic stabilizers for the regeneration. Using this methodology transformations were performed with the plasmids pMF357 in which H1histone proteins were labelled with eGFP and had the hygromycin resistance genes, and pGR02 in which H4 histone proteins were labelled with tdimerRed and had phleomycin resistance gene. Later, CAT-related aspects of formation and occurrence were studied using four strains of *C. lindemuthianum* (races 65, 73 and 81). Different culture media, culture age and incubation time were tested and our results showed conditions against germination were necessary for CAT fusion. We developed a novel and robust protocol for CAT studies in order to perform live-cell imaging of CAT fusion which facilitates studies on biological

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functions of this cell type in a plant pathogen. Vegetative compatibility experiments were performed and indicated the existence of heterokaryon incompatibility in the mature colony between the races 65b (green) and 73 (red). Incompatibility reaction occurred and cell death was observed after hyphae fusion. However, the heterokaryon formation and nuclear migration could be observed between the vegetatively incompatible strains following CAT fusion. Recombination between these races could be shown using the selective markers for resistance to hygromycin and phleomycin. Furthermore, specific primers were designed for the interest genes. Therefore, this mechanism of CAT fusion is a potential source of genetic recombination and/or emerging of new races could explain the occurrence of horizontal gene transfer within species between incompatible strains or between different species arising virulent new pathogens.

CAPÍTULO 1

1 INTRODUÇÃO GERAL

Colletotrichum lindemuthianum é o agente causal da antracnose no feijoeiro comum (*Phaseolus vulgaris*). Perdas ocasionadas por este pátogeno podem ser totais quando são utilizadas sementes infectadas e as condições de ambiente são favoráveis. Entre as medidas de controle mais eficazes desse patógeno está a resistência genética, devido à redução nos custos de produção e à diminuição dos danos causados ao ambiente. No entanto, a obtenção de cultivares resistentes é dificultada pela ocorrência de várias raças fisiológicas do patógeno (Sartorato, 2002; Talamini et al., 2004; Silva et al., 2007; Ishikawa et al., 2008b).Mais de cinquenta raças já foram identificadas no Brasil, das quais as raças 65, 73 e 81 têm sido as mais frequentes nos últimos anos (Silva et al., 2007).

Vários são os mecanismos de recombinação que podem ocorrer na ausência do ciclo sexual em fungos filamentosos e, dentre eles, está o ciclo parassexual (Hastie, 1981; Roca, 1997; Castro-Prado et al., 2007). Roca et al. (2003a) descreveram um tipo de célula especializada encontrada em diferentes espécies de *Colletotrichum*, denominado tubos de anastomoses conidiais (Conidial Anastomosis Tubes - CATs). Em *C. lindemuthianum* foi demonstrado que os CATs são responsáveis pela fusão entre conídios em corpos de frutificação assexuais e que núcleos e organelas podem mover-se de um conídio para outro através das anastomoses. No entanto a biologia dos CATs e o processo de fusão não foram completamente estudados, visto que, na metodologia utilizada, os conídios já eram coletados fundidos. Os CATs parecem ser um fenômeno muito comum em fungos, mas poucos dados experimentais comprovam a sua importância biológica (Roca et al., 2005).

Resultados prévios em *Neurospora crassa*, fungo saprotrófico utilizado como modelo no estudo de CATs, indicam que respostas de incompatibilidade

que levam à morte celular são suprimidas em estágios iniciais do estabelecimento da colônia. Portanto, a fusão de CATs durante este período poderia proporcionar uma oportunidade da recombinação não meiótica (Read & Roca, 2005). Segundo esses autores, isto explicaria a grande variabilidade genética em espécies em que a reprodução sexual não ocorre ou é raramente encontrada na natureza. Além disso, os CATs mostraram-se morfologicamente e fisiologicamente distintos dos tubos germinativos, pois são mais finos, curtos e normalmente não ramificam. A indução dos CATs depende da densidade utilizada e eles, normalmente, crescem um em direção ao outro, enquanto os tubos germinativo se evitam e estão sob controle genético independente em *N. crassa* (Roca et al., 2005).

Apesar de as anastomoses entre conídios de *C. lindemuthianum* terem sido descritas por Roca et al. (2003a) e entre espécies de *Colletotrichum* (Roca et al., 2004), não foi comprovada a existência delas entre conídios de diferentes isolados ou raças, devido à necessidade de algum marcador que diferenciasse as duas raças, assim como a formação do heterocário e a recombinação não meiótica.

Entre as técnicas recentemente utilizadas para estudar a biologia e a genética de fungos, pode-se destacar a microscopia de fluorescência e confocal e a utilização de proteínas fluorescentes ligadas a genes de interesse. O desenvolvimento de plasmídeos contendo genes de histonas de fungos em fusão com genes de proteínas fluorescentes torna possível a realização de vários estudos envolvendo a dinâmica e a função nuclear em células *in vivo*.

A utilização de protoplastos é o procedimento mais apropriado para a transformação genética em *C. lindemuthianum* (Redman & Rodriguez, 1994). Apesar de a obtenção de protoplastos ter sido descrita anteriormente, existem vários fatores que podem afetar sua formação e regeneração. Dessa forma, o desenvolvimento de um protocolo visando otimizar a obtenção e a regeneração

de protoplastos de *C. lindemuthianum* é de extrema importância para a transformação genética. A utilização de histonas marcadas com proteínas fluorescentes vem sendo utilizada em vários fungos filamentosos e permite a identificação de fusões entre hifas, sendo uma metodologia eficiente para a detecção de eventos raros (Rech et al., 2007).

Diante do exposto, este trabalho foi realizado com os seguintes objetivos: 1) otimizar o protocolo para a obtenção e a regeneração de protoplastos, visando à manipulação genética; 2) estudar a biologia dos tubos de anastomoses entre conídios (CATs) em *C. lindemuthianum* para análise *in vitro*, determinando as condições mais adequadas para visualização e obtenção das fusões e 3) obter transformantes com núcleo marcado com proteínas fluorescentes, visando comprovar a ocorrência de recombinação genética na ausência do ciclo sexua,l utilizando análise de imagens de células *in vivo* e marcadores moleculares.

2 REFERENCIAL TEÓRICO

2.1 *Colletotrichum lindemuthianum* (*Glomerella cingulata f. sp. phaseoli*)

Glomerella cingulata (Stonem) Spauld & Schrenck f. sp. *phaseoli* (Ascomycotina) é comumente encontrada na sua forma mitospórica ou conidial *Colletotrichum lindemuthianum* (Sacc. & Magn.), causando a doença conhecida como antracnose no feijoeiro (Sutton, 1992).

A classificação dos Ascomicetos baseia-se em sua forma de reprodução sexual (forma meiospórica = forma sexual = forma telomórfica). Sob esta forma, o agente causal da antracnose do feijoeiro é raramente encontrado na natureza, tendo seu estado conidial sido descoberto primeiro (forma mitospórica = forma assexual = forma anamórfica) e o seu nome amplamente empregado e conhecido como *Colletotrichum lindemuthianum* (Sacc & Magn). Pertence à classe dos Coelomycetes e à ordem Melanconiales.

O *Colletotichum lindemuthianum* se reproduz, portanto, de forma assexual, produzindo os conídios num corpo de frutificação denominado acérvulo. Os conídios formam massas de cor salmão ou mel no meio de cultura, sendo unicelulares, hialinos e oblongos ou cilíndricos (Sutton, 1992). Por ocasião da germinação, um conídio pode emitir um ou mais tubos germinativos, que podem formar apressórios em seus ápices, ou continuar crescendo formando as hifas e micélios.

2.2 Variabilidade patogênica de *Colletotrichum lindemuthianum*

O fungo *C. lindemuthianum* possui alta variabilidade patogênica e está presente em diversas regiões produtoras de feijão do Brasil (Silva et al., 2007). Esta variabilidade pode ser devido a uma coevolução patógeno x hospedeiro (Pastor-Corrales et al., 1994).

A primeira comprovação da existência de variabilidade patogênica deste fungo foi realizada por Barrus (1911), citado por Silva (2004), que verificou reações de suscetibilidade diferenciada nas cultivares de feijoeiro, quando inoculadas com isolados de diferentes procedências, os quais foram denominados como Alfa e Beta. No Brasil, o trabalho pioneiro para a identificação de raças foi feito por Kimati (1966), citado por Silva (2004), a partir de isolados de São Paulo, identificando duas raças pertencentes ao grupo Alfa Mexicano II e Delta.

Vários levantamentos foram feitos em diversos países e regiões. No entanto, não havia um consenso quanto à nomenclatura das raças, o que dificultava a comparação de resultados (Talamini et al., 2004). Com a Primeira Reunião Latino Americana da Antracnose do Feijoeiro, realizada no CIAT (Cali, Colômbia), houve a padronização de doze cultivares diferenciadoras: 'Michelite', 'Michigan Dark Red Kideny' (MDRK), 'Perry Marrow, Cornell 49-242', 'Widusa, Kaboon', 'México 222', 'PI 207262', 'TO', 'TU', 'AB 136' e 'G 2333' e a utilização do sistema binário proposto por Habgood (1970) para classificar as raças do patógeno.

Vários estudos de levantamento de raças vêm sendo realizados desde então (Abreu et al., 1993; Sartorato, 2002; Talamini et al., 2004; Silva et al., 2007; Ishikawa et al., 2008b) comprovando a alta variabilidade patogênica. Silva et al. (2007) estudaram a distribuição e caracterizaram vários isolados provenientes de diversas regiões produtoras de Minas Gerais e observaram que as raças 65, 81 e 73 apresentaram maior estabilidade em relação às demais, devido às suas altas frequências, tanto no levantamento realizado por este autor quanto no que foi feito com os dados obtidos nos últimos dez anos. Estes mesmos autores observaram a ocorrência de raças complexas, como a 337 e a 593, que vencem alelos de resistência das cultivares TO (alelo Co-4) e TU (alelo Co-5), respectivamente e que vêm sendo utilizados como fontes de resistência

em alguns programas de melhoramento. Portanto, o surgimento dessas novas raças pode ter sido em consequência da pressão de seleção exercida pelo hospedeiro sobre o patógeno.

Vários mecanismos são responsáveis pela ampliação desta variabilidade em fitopatógenos. O estudo desses mecanismos é necessário para a elaboração de estratégias que possam ser utilizadas no melhoramento de plantas, visando à resistência a doenças (Souza, 2008).

2.3 Fontes de variação genética

Segundo Hastie (1981), a mutação é a fonte básica de variação genética e a heterocariose conduz à variação em fungos selvagens. A recombinação por meio do ciclo sexual é a forma mais comum de aumento da variabilidade genética, em que dois núcleos haploides (N) diferentes se juntam para formar o núcleo diploide (2N) denominado zigoto. Est,e posteriormente, divide-se por meiose e produz novas células haploides. A recombinação também pode ocorrer na divisão celular mitótica, durante o crescimento do indivíduo (Agrios, 2004). No entanto, em fungos filamentosos pode haver outros mecanismos de transferência de material genético, como a ocorrência do ciclo parassexual (Hastie, 1981; Roca, 1997; Castro-Prado et al., 2007) e a recombinação não-meiótica via fusão de CATs (Roca et al., 2003a, 2004), que podem levar ao polimorfismo cromossômico (Kistler & Miao, 1992; O' Sullivan et al., 1998; Roca et al., 2003b) e ao rearranjo do genoma (O' Sullivan et al., 1998).

2.3.1 Reprodução sexual em *Colletotrichum lindemuthianum*

C. lindemuthianum é comumente encontrado em campos de produção do feijoeiro. O teleomorfo (*Glomerella cingulata* f. sp. *phaseoli*) é raro na natureza. Embora a ocorrência do ciclo sexual em condições de laboratório tenha sido descrita por Shear & Wood (1913), sendo denominada como *Glomerella*

lindemuthiana, poucos trabalhos foram realizados desde então. Somente em 1970, Kimati & Galli realizaram novos estudos e propuseram o nome *Glomerella cingulata* f. sp. *phaseoli*.

Nos últimos anos, vêm sendo realizados vários estudos genéticos da fase sexual utilizando marcadores morfológicos e moleculares, evidenciando a formação de recombinantes (Batista & Chaves, 1982; Bryson, 1990; Mendes-Costa, 1996; Rodrigues-Guerra et al., 2005; Camargo Júnior et al., 2007; Luna-Martínez et al., 2007). O teleomorfo tem sido observado em isolamentos vindos do campo e mantidos em condições de laboratório (Camargo Júnior et al., 2007; Ishikawa et al., 2009). No entanto, o papel da reprodução sexual em populações naturais deste fitopatógeno ainda é discutido.

2.3.2 Ciclo parassexual

O ciclo parassexual foi descrito, pela primeira vez, por Pontecorvo & Roper (1952), citados por Azevedo (1998), em *Aspergillus nidulans* e também para outros fungos, principalmente os fungos imperfeitos, ou seja, aqueles desprovidos do ciclo sexual. Hastie (1981) cita que a recombinação genética durante o ciclo parassexual tem valor potencial na biologia de fungos conidiais e de outros fungos.

Segundo Azevedo (1998), para o início do processo parassexual é necessária heterocariose. A heterocariose é conseguida em fungos filamentosos, por meio das anastomoses entre hifas que permitem a troca ou passagem do núcleo, bem como parte do citoplasma. Anastomoses entre hifas foram encontradas em *C. lindemuthianum*, sem verificar a passagem de núcleo por Mendes-Costa et al. (1998), Rodriguez-Guerra et al. (2003) e Ishikawa et al. (2008a). O próximo passo deste processo seria a fusão de núcleos, obtendo-se diploides heterozigotos e, em seguida, haveria a produção dos recombinantes. O mecanismo de recombinação envolve permuta genética (mitótica) e

haploidização. A haploidização resulta de uma série de não-disjunções, que levam à perda de cromossomos, restaurando o estado haploide (Azevedo,1998).

Bos (1985) encontrou evidências de heterocariose utilizando mutantes auxotróficos de *C. lindemuthianum* e prováveis diploides, reconhecidos pela seleção em meio mínimo. Porém, não pode comprovar a cariogamia, devido à ausência de conídios híbridos ou recombinantes. Roca (1997) sugeriu que o ciclo parassexual poderia estar ocorrendo, supondo que o diplóide seria instável. Isto porque foram obtidas evidências citológicas das anastomoses entre conídios e variações no número de cromossomos, o que também foi observado por O'Sullivan et al. (1998).

Em *Colletotrichum sublineolum*, agente causal da antracnose do sorgo, Souza-Paccola et al. (2003) descreveram a ocorrência do ciclo parassexual utilizando mutantes auxotróficos. Os autores confirmaram a heterocariose nos cruzamentos, bem como a obtenção de aneuploides e recombinantes haploides, sugerindo que a parassexualidade pode, então, ser um mecanismo que gera variabilidade genética neste fungo e que explica o rápido aparecimento de novas raças fisiológicas.

Recentemente, Castro-Prado et al. (2007) realizaram um estudo em *C. lindemuthianum*, utilizando cinco isolados de diferentes raças e observaram a ocorrência do ciclo parassexual, utilizando mutantes auxotróficos (*nit*) e marcadores bioquímicos. Os autores relatam que, apesar de encontrar barreiras para a formação do heterocário em alguns dos cruzamentos, foi possível demonstrar a ocorrência do ciclo parassexual e a formação do diploide entre isolados vegetativamente compatíveis.

2.3.3 Heteroploidia: polimorfismo cromossômico

Heteroploidia é a existência de células ou tecidos, ou todo organismo com número de cromossomos por núcleo diferente do número básico descrito para um organismo particular. Heteroploides podem ser: haploides, diploides, triploides ou, mesmo, aneuploides, com um, dois ou mais cromossomos a mais ou perdidos (Agrios, 2004).

Ishikawa et al. (2003) e Roca et al. (2003b) por meio de estudos citológicos da variabilidade de *C. lindemuthianum* e da fase sexuada *G. cingulata* f. sp. *Phaseoli*, observaram que a fase sexuada é bastante estável com meiose normal e a ocorrência de polimorfismo cromossômico numérico de 4-8 na fase assexuada dos isolados avaliados.

A eletroforese de campo pulsado (Pulsed Field Gel Electrophoresis - PFGE) tem revolucionado o estudo genético em fungos por meio da separação e detecção de moléculas de DNA (Kistler & Miao,1992). Por meio dessa técnica é possível, de forma mais rápida, analisar várias amostras de diferentes isolados dentro de uma mesma espécie, em um único experimento, evidenciando rapidamente o polimorfismo.

Masel et al. (1993) utilizaram PFGE para detectar a presença de minicromossomos em várias espécies do gênero *Colletotrichum*, observando a ocorrência de minicromossomos em *C. gloesporioides*, *C. crassipes*, *C. dematum* e *C. truncatum*, com tamanho variando de 200 a 1200 Kb. No entanto, estes não foram encontrados nos isolados de *C. musae*, *C. lindemuthianum* e *C. trifolli.* Os mesmos autores dividiram os isolados de *C. gloesporioides* em duas classes, a primeira com menos de seis minicromossomos e a segunda com mais de seis, não observando qualquer correlação entre as duas classes e os sintomas da doença, raça, espécie do hospedeiro ou país de origem.

O'Sullivan et al. (1998) observaram duas classes de tamanhos distintos de cromossomos em *C. lindemuthianum*, das quais uma apresentava um número

variável de pequenos cromossomos, denominados minicromossomos (<2,5 Mb) e outra com cromossomos maiores. Apesar da constatação do polimorfismo cromossômico em *C. lindemuthianum* (O'Sullivan et al., 1998; Roca et al., 2003b), ainda não estão totalmente esclarecidos os mecanismos que efetivamente levariam a essa variação do genoma nesta espécie.

Em um trabalho com *C. gloesporioides*, He et al. (1995) observaram que a presença de um minicromossomo (1.2 Mb) não estava diretamente associada à raça, o que torna discutível a origem e a função de cromossomos dispensáveis nesta espécie.

Estudos demonstraram a existência de transferência horizontal interespecífica de vetores autônomos (Poplawski et al., 1997) e de um cromossomo específico (2Mb) (He et al., 1998) entre dois biotipos diferentes de *C. gloesporioides*, que são incompatíveis, tanto sexual quanto assexuadamente, o que explicaria a origem de cromossomos supranumerários nessa espécie. No entanto, o mecanismo que levou à transferência não foi identificado, já que os isolados eram vegetativamente incompatíveis. Os autores sugerem que o cromossomo de 2Mb poderia ter sido transferido por dois processos. No primeiro, ocorre a fusão nuclear e, em seguida, a perda de todo o genoma do biótipo A, deixando o cromossomo 2 Mb predominantemente no genoma do biótipo B. No segundo, o mecanismo de transferência existe em um interbiotipo em que o heterocário é instável (He et al., 1998).

2.4 Incompatibilidade vegetativa e seu controle genético

A fusão de hifas (anastomoses) e a formação do heterocário é um pré-requisito para a ocorrência do ciclo parassexual. Além disso, as anastomoses são importantes para comunicação intra-hifas e homeostase da colônia, durante o crescimento e a reprodução. Embora sejam óbvios os benefícios da formação do

heterocário, existem mecanismos genéticos que restringem a sua formação entre indivíduos geneticamente diferentes (Glass et al., 2000).

Em fungos filamentosos existem o reconhecimento sexual, que é controlado por locos *mat* ("mating types") e o reconhecimento vegetativo, que é controlado por um loco específico, denominado *het* (incompatibilidade de heterocário, também chamado *vig*, incompatibilidade vegetativa) (Saupe, 2000). Quando dois indivíduos se encontram, eles podem sofrer fusão celular, ou anastomose. Se os dois indivíduos tiverem o mesmo genótipo *het*, ocorre a heterocariose. Se eles diferirem geneticamente para o loco *het*, apesar da fusão de hifas, as células heterocarióticas são rapidamente destruídas ou têm o seu crescimento severamente inibido (Glass et al., 2004).

Acredita-se, então, que a incompatibilidade vegetativa funcione como um sistema de reconhecimento da fusão entre diferentes indivíduos para limitar a passagem de elementos infecciosos, prevenir a exploração por núcleos mal adaptados e/ou prevenir que recursos sejam retirados durante a reprodução sexual, sendo, portanto, um mecanismo de autodefesa em fungos filamentosos (Glass et al., 2000). Estes mesmos autores sugerem que a seleção atuaria sobre os locos *het* para a manutenção do polimorfismo em populações de fungos. Saupe (2000) comenta que a função dos genes *het* é preservar a individualidade genética.

Estudos mais detalhados sobre a fusão e o crescimento de hifas podem ser divididos em etapas. Leslie & Zeller (1996) propuseram um modelo simples, no qual distinguem quatro etapas diferentes na reação de (in)compatibilidade do heterocário. A etapa inicial é a pré-fusão e está sob o controle de genes, como aqueles envolvidos na produção do feromônio e receptores e genes que podem estar relacionados com a autoincompatibilidade do heterocário (*hsi*). A etapa da fusão é controlada por genes de reconhecimento *self* e *non-self*, como alguns genes de incompatibilidade do heterocário (*het*). Após a fusão, uma cascata de

reações ocorre desde o reconhecimento *non-self* até a morte celular, devendo ser influenciada por diversos genes, entre eles os genes supressores (*sup*), que modificam o sinal. Finalmente, genes apoptóticos levam à morte celular (Figura 1). O controle genético, molecular e fisiológico da anastomose de hifas ainda é pouco entendido.

Em *Neurospora crassa*, o uso de mutantes para fusão de hifas (*ham-1* e *ham-2*) e mutantes para reconhecimento *self* e *non-self* de fusão de hifas (*nrc-1 e mak-2*, por exemplo) tem auxiliado no entendimento dos mecanismos e da função das hifas na biologia de fungos filamentosos (Glass et al., 2000, 2004; Roca et al., 2005).

O conhecimento da genética dos sistemas de incompatibilidade vegetativa em ascomicetos tem se limitado a um número pequeno de espécies, como *Neurospora crassa*, *Podospora anserina*, *Aspergillus nidulans* e *Cryphonectria parasítica*. O número de locos *het* varia de espécie para espécie (Saupe, 2000). Para outras espécies, as quais o loco *het* não tenha sido identificado, têm-se definido grupos de compatibilidade vegetativa (VCGs).

A classificação de isolados em VCGs pode ser uma importante ferramenta para a análise de populações em fungos (Saupe, 2000).

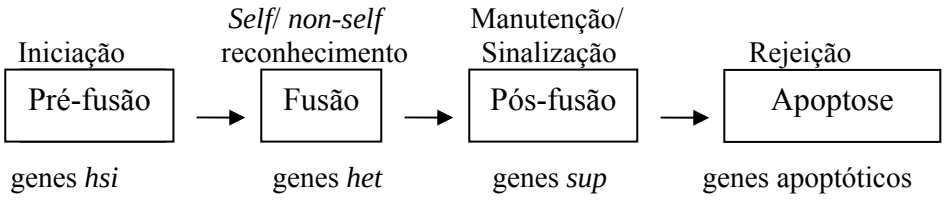

FIGURA 1 Modelo simplificado mostrando os diferentes passos no processo de incompatibilidade do heterocário e os genes associados a este processo, de acordo com Leslie & Zeller (1996).

Rodriguez-Guerra et al. (2003) realizaram um trabalho com isolados de *C. lindemuthianum* do México, coletados de diferentes partes de uma mesma planta em locais diferentes e determinaram padrões de variação no genótipo, no patótipo e nos grupos de anastomoses. Os resultados demonstraram que os padrões de variação no genótipo e grupos de anastomoses são complexos nos locais amostrados, não havendo relação entre genótipo, patótipo e grupos de anastomoses.

Ishikawa et al. (2008a) utilizaram treze isolados diferentes de *C. lindemuthianum*, todos classificados como raça 65 e os confrontaram dois a dois. A formação de anastomoses entre hifas de isolados diferentes foi observada, porém, ocorreram onze grupos diferentes de compatibilidade, de acordo com análise de agrupamento, evidenciando a variabilidade para esta característica dentro de raça.

Posteriormente, 47 isolados pertencentes a diferentes raças deste patógeno e a diferentes regiões produtoras de feijão do Brasil foram utilizados em um estudo de populações utilizando informações de grupos de compatibilidade vegetativa (VCG) e marcadores moleculares (Barcelos, 2007). A autora observou a formação de 45 grupos de compatibilidade vegetativa, tendo 44 isolados sido incompatíveis e em apenas uma combinação foi observada a formação do heterocário. Além disso, a autora comentou que alguns dos isolados que, pela análise de anastomoses de hifas, eram compatíveis (Ishikawa et al., 2008a), mostraram-se incompatíveis quando utilizados os mutantes auxotróficos, sugerindo que, apesar de os alelos de pré-fusão serem os mesmos e permitirem a fusão de hifas em um primeiro estágio, os alelos de incompatibilidade vegetativa eram diferentes, resultando na incompatilidade do heterocário. A alta incompatibilidade entre os isolados utilizados neste estudo pode ser explicada pela autoincompatibilidade, desde que a formação de

heterocários foi baixa, evidenciando a grande variabilidade existente nesta espécie.

2.5 Tubos de anastomoses entre conídios (CATs)

Roca et al. (2003a) descreveram um tipo de célula especializada encontrada em diferentes espécies de *Colletotrichum*, denominado tubos de anastomoses conidiais (Conidial Anastomosis Tubes - CATs). Em *C. lindemuthianum*, foi demonstrado que os CATs são responsáveis pela fusão entre conídios em corpos de frutificação assexuais e que núcleos e organelas podem se mover de um conídio para o outro através das anastomoses. Verificou-se que anastomoses realmente podem ser agentes promotores de variabilidade por meio da transferência de genes. Em *C. lindemuthianum*, os conídios são inicialmente uninucleados, porém, após 15 dias de desenvolvimento do acérvulo (corpo de frutificação assexual), e coincidentemente com a formação de CATs, há um incremento na fração de núcleos sofrendo divisão, migração e fragmentação. Algumas vezes, este fato é acompanhado da perda do núcleo de um dos conídios.

Por meio da microscopia eletrônica de varredura e de transmissão, foram detectadas anastomoses entre conídios e passagem de mitocôndrias e vacúolos em *C. lindemuthianum.* Com auxílio de análises genéticas e PCR, foi detectado que, durante a reprodução assexual, ocorrem mecanismos que permitem a troca de material genético (transferência horizontal) entre isolados incompatíveis de *C. lindemuthianum* e *C. gossippy*, produzindo novos fenótipos (Roca et al., 2004).

CATs acontecem durante o ciclo assexual de *C. lindemuthianum*, dando a oportunidade para genótipos diferentes da espécie encontrarem-se num complexo comum (complexo formado pelas anastomoses). Esse fato pode levar ao surgimento de fenótipos diferentes, com base em outros preexistentes (Roca

et al., 2004). Nesta espécie fúngica, os CATs têm sido observados emergindo dos conídios dentro do acérvulo, *in vitro*, após remoção do acérvulo e também *in vivo*, em lesões de antracnose de vagens de feijão ou sob a superfície de folhas inoculadas com o patógeno (Roca et al., 2003a; Ishikawa et al., 2009).

Os CATs parecem ser um fenômeno muito comum em fungos, mas sabe-se pouco sobre sua importância biológica experimentalmente (Roca et al., 2005). Estes autores sugerem que CATs podem ser importantes para incrementar as chances de estabelecimento da colônia e/ou para transferência de genes e esses possíveis papéis precisam ser rigorosamente analisados, experimentalmente. Foram encontrados conídios unidos por CATs germinando mais rapidamente do que conídios sozinhos (Roca et al., 2003a).

Em *Neurospora crassa*, os CATs mostram-se morfologicamente e fisiologicamente distintos dos tubos germinativos e estão sobre controle genético independente (Roca et al., 2005). Além disso, foram mostradas evidências da existência de um indutor extracelular de anastomoses derivado dos conídios, visto que, em baixas concentrações destes, havia uma redução na formação de CATs. Para identificar qual seria o quimioatrativo, utilizaram-se mutantes *cr-1* (que não produziam AMP cíclico - cAMP) e mutantes *ham*-2 (codificam uma proteína transmembrana), *mak*-2 e *nrc*-1 (proteínas quinases). Verificou-se que o cAMP não era o quimioatrativo, pois os mutantes *cr-1* produziam CATs normalmente e que os mutantes *ham*-2, *mak*-2 e *nrc*-1 não produziam CATs, mas produziam tubos germinativos, o que comprova o diferente controle genético. Utilizou-se uma técnica de pinças ópticas (‘Optical Tweezers’) para micromanipulação de conídios em que CATs estavam se formando para analisar a quimioatração entre eles. Neste mesmo trabalho, verificou-se a passagem de núcleos e microtúbulos por meio da fusão entre CATs, utilizando-se proteínas fluorescentes (GFP) ligados a histona H1 e β-tubulina, respectivamente.

Resultados prévios em *Neurospora crassa* indicam que respostas de incompatibilidade que levam à morte celular são suprimidas em estágios iniciais do estabelecimento da colônia. Portanto, fusão de CATs durante este período podem proporcionar uma oportunidade da recombinação não meiótica (Read & Roca, 2006). Segundo esses autores, isto pode explicar a grande variabilidade genética em espécies em que a reprodução sexual não ocorre ou é raramente encontrada na natureza.

2.6 Transferência horizontal de genes

A transferência horizontal de genes é a troca de genes específicos ou regiões genômicas entre espécies que são isoladas reprodutivamente. Este é um fenômeno bem documentado em procariotos, mas são poucos os relatos em eucariotos, especialmente animais e fungos (Stukenbrock & McDonald, 2008).

Existem quatro classes diferentes de elementos genéticos que podem ser transmitidos horizontalmente em fungos: mitocôndrias; plasmídeos, que em fungos normalmente são mitocondriais; elementos genéticos transponíveis e micoviroses (Diepeningen, 1999). Há vários casos bem documentados de transferência horizontal de genes mitocondriais, no entanto, são poucos os de genes nucleares (Walton, 2000).

Nos últimos anos, com o sequenciamento do genoma de vários organismos, incluindo os fungos, tem sido possível identificar essas transferências nucleares. Friesen et al. (2006) sugerem a transferência horizontal de um gene que produz uma toxina (*toxA*) entre duas espécies, *Stagnospora nodurum* e *Pyrenosphora tritici-repentis*, resultando na emergência de uma nova doença em trigo. Esses autores comentam que essa transferência ocorreu recentemente e que teria ocorrido de *S. nodurum* para *P. tritici-repentis* que, até 1941, não era considerado um patógeno muito sério e que a virulência teria surgido após a aquisição deste gene para *toxA*.

Embora os dados de sequenciamento e moleculares sugiram a transferência horizontal de genes em eucariotos, os mecanismos pelo quais essas transferências ocorrem são pouco entendidos (Sanders, 2006). Friesen et al. (2006) sugerem que a fusão de CATs interespecífico seja o mecanismo pelo qual essa transferência ocorra, já que ambas as espécies, *S. nodurum* e *P. tritici-repentis*, formam CATs. No entanto, não foram demonstradas evidências desta fusão, como foi demonstrado por Roca et al. (2004) entre espécies de *Colletotrichum.*

Sanders (2006) comenta que esforços devem ser concentrados na identificação dos primeiros passos deste processo e que a informação sobre como essa transferência ocorre é necessária.

2.7 Proteínas fluorescentes

Apesar de as anastomoses entre conídios de *C. lindemuthianum* terem sido descritas por Roca et al. (2003a) e entre espécies de *Colletotrichum* (Roca et al., 2004), não foi comprovada a existência delas entre conídios de raças diferentes, devido à necessidade de algum marcador que diferenciasse as duas raças, assim como a formação do heterocário e a recombinação não meiótica.

Entre as técnicas recentemente utilizadas para estudar a biologia e a genética de fungos, pode-se destacar a microscopia de fluorescência e confocal. O uso de fluorocromos tem vantagens sobre o microscópio de luz branca porque o uso de diferentes cores sobre um fundo preto permite uma melhor definição de algumas estruturas. Estudos genéticos envolvendo o movimento de organelas e migrações que ocorrem entre compartimentos celulares utilizando fluorocromos vitais vêm sendo realizados com sucesso em outros fungos (Hickey et al., 2005) e em *Colletotrichum* (Roca et al., 2003a; Roca et al., 2004).

A partir da clonagem do cDNA de um gene que codifica uma proteína fluorescente verde (green fluorescent protein – GFP) de *Aequorea victoria*

realizada por Prasher (1992), citado por Lorang et al. (2001), muitos trabalhos vêm sendo desenvolvidos utilizando este gene e seus derivados, inclusive em fungos filamentos. As proteínas fluorescentes são ferramentas importantes que têm revolucionado a biologia celular na última década, principalmente no estudo de células vivas (Freitag et al., 2004). Proteínas fluorescentes são excelentes marcadores moleculares, já que são geneticamente codificadas, não requerendo substratos ou cofatores para a sua atividade e podem ser empregadas em qualquer organismo que pode ser geneticamente modificado (Czymmek et al., 2005). Além disso, tem a vantagem em relação aos fluorocromos de que as células podem ser observadas por períodos prolongados.

Em *C. lindemuthianum*, o uso de proteína fluorescente (GFP) como gene repórter foi descrito por Dumas et al. (1999). Nesse trabalho, foi construído um vetor contendo o gene da endopoligalacturonase (*clpg2*) em fusão com o gene GFP e este foi introduzido no genoma de um isolado de *C. lindemuthianum.* Dessa forma, foi possível visualizar a expressão do *clpg2* em estágios precoces do desenvolvimento do conídio e formação do apressório.

Recentemente, foram desenvolvidos plasmídeos contendo genes de histonas de fungos (H1, H2A, H2B e H4) em fusão com os diferentes genes de proteínas fluorescentes, como *gfp*, *ecfp*, *eyfp* e *tdimerRed* (Ramon et al., 2000; Maruyama et al., 2001; Freitag et al., 2004; Li et al., 2007; Rech et al., 2007; Nesher et al., 2008). Estes plasmídeos que marcam o núcleo vêm sendo utilizados com sucesso em várias espécies de fungos filamentosos, como *Aspergillus nidulans* e *A. oryzae*, *Neurospora crassa*, *Coccidioides posadasii*, *Sordaria macrospora* e *Colletotrichum gloesporioides.* Dessa forma, é possível realizar vários estudos envolvendo a dinâmica e a função nuclear em células vivas.

Nesher et al. (2008) estudaram a mitose de *C. gloesporioides* durante a germinação de conídios e a formação de apressórios utilizando um isolado

transgênico que expressa a proteína sGFP ligado à histona H1 (hH1-sGFP). Estes autores observaram que a mitose não é requerida para a formação dos tubos germinativos e apressórios durante a infecção do hospedeiro. Além disso, foi possível observar que não ocorre a morte celular após a infecção e que as estruturas primárias de infecção permanecem viáveis, mesmo durante o ciclo de infecção, diferentemente de *Magnaporthe grisea*, em que a mitose e a morte celular do esporo são necessárias para infecção mediada por apressório (Veneault-Fourrey et al., 2006).

Rech et al. (2007) utilizaram o ascomiceto homotálico, *Sordaria macrospora*, para demonstrar a ocorrência rara de fusão de hifas e a formação do heterocário. Para distinguir os diferentes isolados estudados, o núcleo foi marcado com diferentes proteínas fluorescentes (azul - *ecfp* e amarelo - *eyfp*) que possuem marca de seleção para higromicina (*hph*). Segundo estes autores, estes vetores demonstraram-se úteis e podem ser utilizados em estudos com fungos que perderam estruturas reprodutivas especializadas ou marcadores auxotróficos.

2.8 Produção e regeneração de protoplastos

Protoplastos são células desprovidas de parede celular que permitem vários estudos em fungos filamentosos. A obtenção de protoplastos é uma ferramenta biológica importante para a manipulação genética de fungos, fusão e transformação, além do emprego para determinar o cariótipo molecular (Peberdy, 1995).

Nos últimos 20 anos, vários trabalhos vêm sendo realizados com *C. lindemuthianum*, envolvendo transformação genética via protoplastos (Rodriguez & Yoder, 1987; Redman & Rodriguez, 1994; Dufresne et al., 1998; Dumas et al., 1999; Parisot et al., 2002; Soares, 2007).

No entanto, vários fatores influenciam a obtenção de protoplastos, dentre eles preparação enzimática, estabilizador osmótico, idade micelial e microrganismo a ser utilizado (Peberdy et al., 1976). O meio de cultivo, o estado fisiológico da cultura, o tempo de digestão, o pH e a temperatura também são fatores importantes na liberação dos protoplastos. Os protoplastos, normalmente, são obtidos pelo tratamento do fungo com enzimas que destroem a parede celular em um meio com alta concentração de sais ou açúcares (estabilizadores osmóticos) que evitam o rompimento da célula.

Um dos fatores cruciais para a obtenção dos protoplastos é a preparação enzimática. Para fungos filamentosos, normalmente, são utilizadas enzimas derivadas do fungo *Thrichoderma harzianum* (Peberdy, 1995). Rodriguez & Yoder (1987) descrevem a obtenção de protoplastos de *C. lindemuthianum* utilizando como estabilizador osmótico $MgSO_4$ 1,2M em tampão fosfato 10 mM contendo Novozym 234 (Novo Industries Ltd), que é um coquetel de carbohidrolases derivados de *T. harzianum*, porém, relatam que poucos protoplastos foram produzidos e, por isso, substituíram Novozym 234 por β-glucoronidase Type H-2 (Sigma). Posteriormente, Redman & Rodriguez (1994) utilizaram a mistura de ambas as enzimas na obtenção de protoplastos, no entanto, Novozym 234 encontra-se hoje indisponível no mercado. Outra enzima descrita na obtenção de protoplastos de *C. lindemuthianum* é a Glucanex® (Novo Nordisk Ltd) (O'Sullivan et al., 1998; Roca et al., 2003c; Soares, 2007). A Lysing Enzymes de *T. harzianum* (Sigma) é equivalente à Glucanex®, que é o produto registrado pela Novozym Corp. e contém atividade de β-glucanase, cellulase, protease e chitinase.

Na literatura, os estabilizadores frequentemente utilizados na obtenção de protoplastos de *C. lindemuthianum* visando à transformação são $MgSO_4$ e KCl (Rodriguez & Yoder, 1987; Redman & Rodriguez, 1994; Dufresne et al., 1998; Parisot et al., 2002; Soares, 2007) e, para PFGE, citometria de fluxo e

PCR, o sorbitol (O'Sullivan et al., 1998; Roca et al., 2003c). Em geral, sais inorgânicos são eficazes na liberação de protoplastos em fungos filamentosos e açúcares em leveduras (Lalithakumari, 2000), apesar do uso de açúcares ser descrito também em fungos filamentos (TeBeest & Weidemann, 1987; O'Sullivan et al., 1998). Dias et al. (1997) observaram melhores resultados na obtenção de protoplastos em *Penicillium expansum* e *Penicillium griseorum*, quando utilizados sais. Resultados similares foram obtidos em estudos com *Sclerotium rolfsii* (Fariña et al., 2004) e *Aspergillus ochraceus* (Almeida et al., 2008).

Outro fator importante é o estado fisiológico do micélio utilizado para o isolamento do protoplasto. Tem sido relatada para várias espécies de fungos que o micélio jovem é mais suscetível à ação enzimática (Peberdy, 1995; Lalithakumari, 2000; Fariña et al., 2004).

Após o isolamento, quando em meio nutritivo apropriado, os protoplastos podem regenerar a parede celular, dando origem ao novo micélio, sendo que a taxa de regeneração e reversão varia de organismo para organismo (Marchi et al., 2006). De acordo com Dias et al. (1997), açúcares normalmente são bons estabilizadores para a regeneração de protoplastos.

2.9 Transformação genética em *C. lindemuthianum*

Estudos genéticos de fungos envolvendo a transformação mediada por DNA têm aumentado substancialmente nos últimos anos. Estes estudos têm revolucionado o entendimentos de fenômenos biológicos, como interação patógeno-hospedeiro, metabolismo secundário, respostas de organismos à estresses ambientais e biologia do desenvolvimento (Redman & Rodriguez, 1994).

Um dos primeiros trabalhos com *C. lindemuthianum* foi realizado por Rodriguez & Yoder (1987). Os autores obtiveram transformantes com marcas de

seleção para o gene *amdS*$^{+}$, o qual codifica acetamidase e permite o crescimento em meio acrescido de acetamida como única fonte de nitrogênio, e o gene *hygB*^{R}, que codifica higromicina B (Hy) fosfotransferase e permite o crescimento na presença do antibiótico Hy. No entanto, foi observada baixa eficiência de transformação (0,2 tranformantes/μg DNA).

Posteriormente, Redman & Rodriguez (1994) estudaram os fatores que afetam a transformação em espécies de *Colletotrichum* e relataram que, para *C. lindemuthianum*, o melhor método de transformação é por meio de protoplastos utilizando polietilenoglicol (PEG). Dessa forma, vários genes (*clk1, CLTA1, clap1, plcCl* e *msfCl*) relacionados à patogenicidade e/ou à virulência vêm sendo isolados utilizando a técnica de mutagênese insercional (Dufresne et al., 1998; Dufresne et al., 2000; Parisot et al., 2002; Soares, 2007).

No entanto, trabalhos envolvendo a transformação de *C. lindemuthianum* com proteínas fluorescentes são escassos. Dumas et al. (1999) obtiveram transformante de *C. lindemuthianum*, em que o gene GFP estava em fusão com o gene *clpg2*.

2.10 Análises do DNA por PCR

Identificar isolados diferentes, de forma confiável, é essencial em estudos genéticos. Análises genéticas têm sido feitas com diversos tipos de marcas morfológicas, químicas, etc. Atualmente, os marcadores moleculares são utilizados para fazer estudos genéticos em fungos filamentosos. Marcas simples, com bastante repetibilidade, são obtidas quando se utiliza o reconhecimento de sequências de DNA. Regiões homólogas são reconhecidas por iniciadores de DNA (*primers*). Com a utilização de condições de reações específicas para a replicação do DNA, podem ser realizadas amplificações e o produto pode ser observado posteriormente. Essas amplificações ocorrem em condições de aumento e redução de temperaturas em que a DNA polimerase atua. Na reação

ocorrem, então, desnaturação, anelamento e extensão do DNA (McPherson & Moler, 2000).

O PCR é uma ferramenta poderosa que permite a amplificação de sequências específicas de DNA. Os pré-requisitos para o sucesso no PCR incluem o desenho do par de *primers* apropriados, utilização de concentrações de *primer* e DNA adequadas, e otimização nas condições do PCR (Sambrook & Russel, 2001).

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CAPÍTULO 2

FACTORS AFFECTING THE PRODUCTION AND REGENERATION OF PROTOPLASTS FROM *Colletotrichum lindemuthianum*

1 RESUMO

No presente trabalho apresentam-se os fatores que afetam a produção e a regeneração de protoplastos de *Colletotrichum lindemuthianum*. O isolamento de protoplastos é muito relevante para diferentes aplicações, principalmente em procedimentos que envolvem a manipulação genética. Estabilizadores osmóticos, enzimas líticas, tempo de incubação e idade micelial foram testados com relação ao efeito na liberação de protoplastos. As condições otimizadas para produção de protoplastos foram incubação de micélio jovem (48 horas) em estabilizador osmótico NaCl 0,6 mol l^{-1}, acrescido de 30 mg ml^{-1} da enzima *Lysing Enzymes* de *Trichoderma harzianum*, incubado durante 3 horas. Sacarose nas concentrações de 1,2 mol l^{-1} e 1 mol l^{-1} foram os estabilizadores mais apropriados para a regeneração após 48 horas, sendo as taxas de regeneração de 16,35% e 14,54%, respectivamente. Este estudo produziu um método eficiente para produção e reversão de protoplastos à morfologia micelial típica de C*olletotrichum lindemuthianum* utilizando o isolado LV115.

2 ABSTRACT

The present work reports factors affecting the production and regeneration of protoplasts from *Colletotrichum lindemuthianum*. The usefulness of protoplast isolation is relevant for many different applications and has been principally used in procedures involving genetic manipulation. Osmotic stabilizers, lytic enzymes, incubation time and mycelial age were evaluated in terms of their effects on protoplast yield. The optimal condition for protoplast production included the incubation of young mycelia (48 h) in 0.6 mol l^{-1} NaCl as the osmotic stabilizer, with 30 mg ml^{-1} *Lysing Enzymes* from *Trichoderma harzianum* for 3 h of incubation. Sucrose concentrations of 1.2 mol l^{-1} and 1 mol l^{-1} were the most suitable osmotic stabilizers for the regeneration after 48 h, with rates of 16.35% and 14.54%, respectively. This study produced an efficient method for protoplast production and reverted them into a typical mycelial morphology using a C*olletotrichum lindemuthianum* LV115 isolate.

3 INTRODUCTION

Anthracnose is one of the most important diseases of the common bean (*Phaseolus vulgaris*). The causal agent of anthracnose is *Colletotrichum lindemuthianum.* There have been several studies with this pathogen involving genetic transformation by protoplasts (Rodriguez & Yoder, 1987; Redman & Rodriguez, 1992; Dufresne et al., 1998; Dumas et al., 1999). Protoplasts are cells obtained after complete removal of the cell wall, which is useful in many studies of filamentous fungi (Lalithakumari, 2000).

Protoplast release is an important biological tool for experiments addressing genetic transformation and other molecular approaches such as pulsed-field gel electrophoresis (PFGE) and flow cytometry. Molecular transformation has facilitated the understanding of biological phenomena such as host-pathogen interactions, secondary metabolism, environmental stress responses and developmental biology.

Although protoplast formation in *C. lindemuthianum* has been previously reported, any existing protocol should be assessed for each strain under study. Furthermore, most of these studies used *Novozym 234*, however, this enzymatic complex is not commercially available. Factors such as lytic enzymes, osmotic stabilizers, mycelium age and the type of microorganism to be used affect the maximum release of protoplasts (Peberdy, 1995).

Hence, it is important to optimize a protocol for the production and regeneration of protoplasts for each species of fungi. The main objective of this work was thus to study factors affecting the production and regeneration of *C. lindemuthianum* protoplasts using *Lysing Enzymes* from *Trichoderma harzianum* for use in genetic transformation.

4 MATERIAL AND METHODS

4.1 Fungal isolate and culture media

The LV 115 isolate of *C. lindemuthianum* belonging to race 65 was used in the study. This clone derived from the culture collection of the Department of Biology, Universidade Federal de Lavras (Lavras, MG, Brazil). The isolate was maintained on M_3 medium (Junqueira et al., 1984). To obtain high sporulation, the isolate was inoculated in sterilized green bean pods and was incubated at 22± 2ºC for 10-15 days in the dark.

4.2 Protoplast production of *C. lindemuthianum*

The following factors were evaluated: osmotic stabilizer (type and concentration), mycelium age, enzyme concentration, and incubation time. Initially, the following seven stabilizers were prepared in 0.01 mol l^{-1} phosphate buffer (pH 5.5) and tested: $MgSO_4$ (0.6 mol l^{-1}), Mannitol (0.6 mol l^{-1}), Sorbitol (0.6 mol l^{-1}), NaCl (0.37 mol l^{-1}), KCl (0.37 mol l^{-1}), Sucrose (0.56 mol l^{-1}), and NH_4Cl (0.6 mol l^{-1}). Later, the best osmotic stabilizers were evaluated at different concentrations. The enzyme concentration used for all experiments, except for the enzyme concentration experiment, was 10 mg ml^{-1}. The mycelium ages tested were 48 h, 72 h, 96 h, and 120 h, using NaCl 0.6 mol l^{-1} as the osmotic stabilizer. The enzyme concentrations tested were 10, 20, and 30 mg ml^{-1}, using mycelium at 72 h and NaCl 0.6 mol l^{-1} as the osmotic stabilizer.

A suspension of 10^6 spores ml^{-1} were inoculated into 250 ml Erlenmeyer flasks containing 30 ml M_3S medium (Tu, 1985) and incubated at 22ºC for 48-120 h. The mycelium produced was washed with an osmotic stabilizer and the weight was determined (100 mg of hydrated mycelium). The enzyme solution was prepared using *Lysing Enzymes* from *Trichoderma harzianum* (Sigma) in 3 ml of osmotic stabilizer and filter-sterilized. The mycelium and enzyme solution were

placed in a 25 ml Erlenmeyer flask and kept under agitation (75 rev min^{-1}) at room temperature for a period of 2-5 h. Three experimental measurements for each treatment were used. Protoplast release was determined by removing samples every hour and counting the protoplasts in a Neubauer chamber. The statistical significance of differences between mean values was assessed using an ANOVA and Tukey's student range test.

4.3 Protoplast regeneration

The digestion mixture was filtered through a slightly compacted cotton wool layer in a 5 ml syringe. The protoplasts were collected and washed twice in osmotic stabilizer by centrifugation at 4000 rev min^{-1} for 10 min. The final pellet was suspended in 1 ml of osmotic stabilizer and counted with a Neubauer chamber. The following osmotic stabilizers were tested in the regeneration medium (1% Yeast extract, 0.05% casein acid hydrolysate, 0.05% casein enzymatic hydrolysate, 1.5% agar): 0.6 mol l^{-1} NH_4Cl , 0.37 mol l^{-1} NaCl , 0.6 mol l^{-1} Mannitol , 0.6 mol l^{-1} Sorbitol, and 0.56 mol l^{-1} Sucrose. The osmotic stabilizer concentration was defined according Dias et al. (1997). The best osmotic stabilizers were evaluated at different concentrations (0.6 mol l^{-1}, 0.8 mol l^{-1}, 1.0 mol l^{-1}, and 1.2 mol l^{-1}). The regeneration medium with 0.5 % sucrose was used as a control. Before plating, purified protoplasts were diluted in order to obtain a 10^4 protoplasts ml^{-1} dilution. Subsequently, protoplasts from the dilutions were mixed with regeneration medium in 0.8% agar and poured into Petri dishes in order to have around 800 protoplasts per dish. These preparations were then incubated in a biochemical oxygen demand (BOD) incubator at 22ºC. In these experiments, 0.8% agar was used for plating the medium at low temperature to prevent protoplast damage.

Three replicates for each treatment were used and each replicate was constituted of 3 Petri dishes. After 48 h, colonies were counted and the regeneration rate

was calculated according to the following equation: Regeneration (%) = (A-B) /C x 100, where A was the number of colonies developed in the medium A (treatment), B was the number of colonies developed in the control medium, and C was the total number of protoplasts per dish.

5 RESULTS AND DISCUSSION

The efficiency of the provided osmotic support to the protoplasts following the removal of the cell wall was evaluated for seven different osmotic stabilizers. The best results were obtained with 0.6 mol l^{-1} NH_4Cl and 0.37 mol l^{-1} NaCl salts, which produced an average of 9.6 x 10^5 and 8 x 10^5 protoplasts ml^{-1}, respectively (Fig. 1). However, there was increased protoplast release at 4 h of incubation and the yields were 1.53 x 10^6 and 1.15 x 10^6 protoplast ml^{-1} for the respective stabilizers. Similar results were obtained in *Penicillium expansum*, *Penicillium griseorum* (Dias et al., 1997) and *Aspergillus ochraceus* (Almeida et al., 2008).

The use of $MgSO_4$ as an osmotic stabilizer has been utilized more frequently for protoplast isolation in protocols addressing the genetic transformation of *C. lindemuthianum* (Rodriguez & Yoder, 1987; Redman & Rodriguez, 1992; Dufresne et al., 1998; Parisot et al., 2002). Sorbitol has been used in experiments with molecular approaches like PFGE, flow cytometry and PCR (O'Sullivan et al., 1998; Roca et al., 2003). However, the results observed with these stabilizers were unsatisfactory (Fig. 1). Therefore, it is possible that $MgSO_4$ and sorbitol are not efficient when this enzymatic preparation (*Lysing Enzymes*) is used for this specie. Rodriguez & Yoder (1987) obtained few protoplasts when Novozym 234 was used as litic enzyme in $MgSO_4$. However, Fariña et al. (2004) obtained good results using *Trichoderma harzianum* enzymes in $MgSO_4$ as osmotic stabilizer for *Sclerotium rolfsii*.

When evaluating different stabilizer concentrations relative to their salts (NH_4Cl and NaCl), the best results were obtained with 1.2 mol l^{-1} NH_4Cl and 0.6 mol l^{-1} NaCl (Fig. 2a-2b). These experiments showed that high concentrations of NH_4Cl increased the number of released protoplasts (Fig. 2b). In contrast, for NaCl the best results were at the 0.6 mol l^{-1} and 0.8 mol l^{-1} concentrations (Fig. 2a). At a concentration of 0.6 mol l^{-1} NaCl, a higher number of protoplasts were released in a shorter incubation time (3 h).

NH_4Cl and NaCl, although not previously described in protocols for protoplast production in *C. lindemuthianum*, have been used with other species of fungi. Good results have been found with NaCl in *Colletotrichum capsici* (Lalithakumari, 2000) and *Colletotrichum graminicola* (Epstein et al., 1998; Thon et al., 2000).

A greater release of protoplasts was observed from the young mycelium (48 h) (Fig. 2c) as previously reported for other fungi. However, due to the slow growth of *C. lindemuthianum,* it was difficult to obtain enough mycelium within 48 h. For these experiments, it was necessary to inoculate a larger number of flasks to obtain enough mycelia. Satisfactory amounts of mycelium (0.3-0.35 g 100 ml^{-1}) had been collected after 72 h of growth.

According to Peberdy (1995), it is useful to understand of the growth kinetics of the strain being used for protoplast isolation, but some of the most recalcitrant species for protoplast isolation are the slow growing fungi.

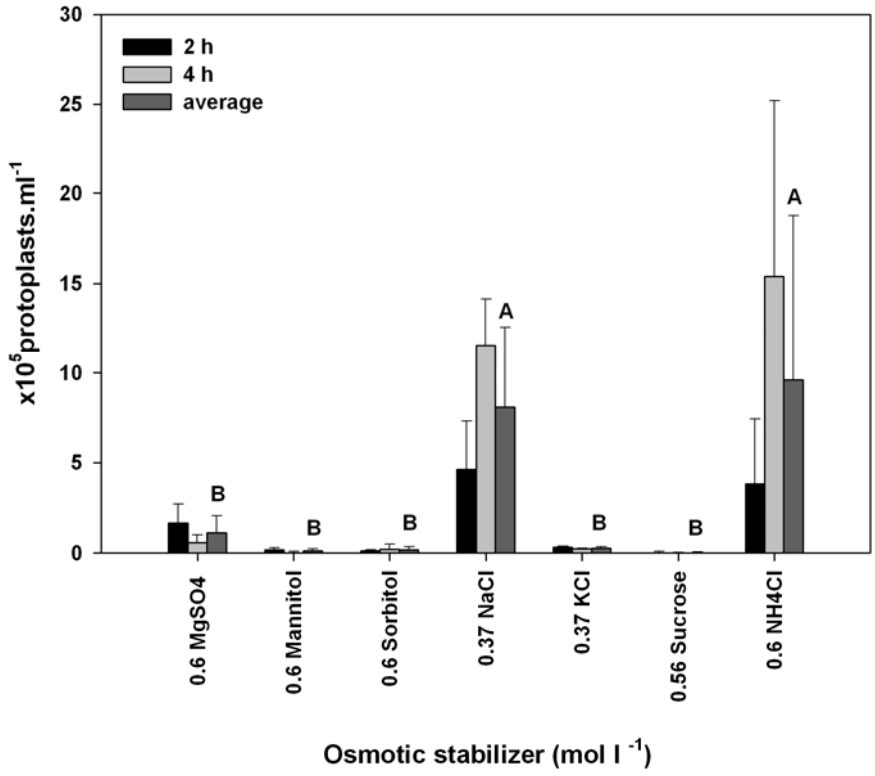

FIGURE 1 Production of *Colletotrichum lindemuthianum* protoplasts in different osmotic stabilizers at equivalent molar concentrations. The digestion mixture included the following: 100 mg of mycelium (72h old) into 3 ml osmotic stabilizer with 10 mg ml^{-1} enzyme. Incubation times were 2 and 4 h. Bars with the same capital letters are not significantly different according to a Tukey's test 5%. Error bar markers represent SD from mean values of three independent experiments

High enzyme concentrations (*Lysing Enzymes*, 30 mg ml^{-1}) resulted in the release of a larger number of protoplasts in a shorter digestion period (Fig. 2d). However, the number of protoplasts decreased with increasing digestion time. These findings were in agreement with those reported by Lalithakumari

(2000) about the effects of lytic enzyme concentration on the number of produced protoplasts. The number of protoplasts increases with lytic enzymes amendements, but high concentrations were sometimes harmful, resulting in the protoplasts lysis soon after their appearance, thereby indicating toxic levels of lytic enzymes. Incubation time was another critical factor for protoplast production (Fig. 2d). In addition, enzyme concentration and digestion time may have a strong effect on the regeneration of protoplasts (Dias et al., 1996).

Notably, all of the factors evaluated including osmotic stabilizer, lytic enzyme, incubation time, and mycellial age significantly influenced protoplast isolation. Therefore, the optimal condition for protoplast preparation was incubation of young mycelia (48 h) in NaCl 0.6 mol l^{-1} as the osmotic stabilizer with 30mg ml^{-1} *Lysing Enzymes* for 3 h.

Although protoplast formation in *C. lindemuthianum* has been reported before (Rodriguez & Yoder, 1987; Redman & Rodriguez, 1992; Dufresne et al., 1998; O'Sullivan et al., 1998; Dumas et al., 1999; Roca et al., 2003), any existing protocol should be assessed for each strain under study. This concern has been supported by the significant differences found between protocols, protoplast yields, and regeneration rates.

It is already known that protoplast formation often constitutes the starting point for downstream genetic manipulation. However, protoplast technology is frequently problematic and often not reproducible, particularly for filamentous fungi. Therefore, purification protocols should emphasize the necessity to determine the key factors leading to successful results (Fariña et al., 2004).

Finally, good results for protoplast regeneration were obtained with sugars. Preliminarily, protoplast regeneration was tested using 5 osmotic stabilizers and the best results were obtained with sucrose at 0.56 mol l^{-1} and mannitol at 0.6 mol l^{-1} (Fig. 3a). These were evaluated at different

concentrations and sucrose at 1.2 mol l^{-1} or 1.0 mol l^{-1} was the most suitable osmotic stabilizer for regeneration, with a rate of 16.35% or 14.54%, respectively (Fig. 3b).

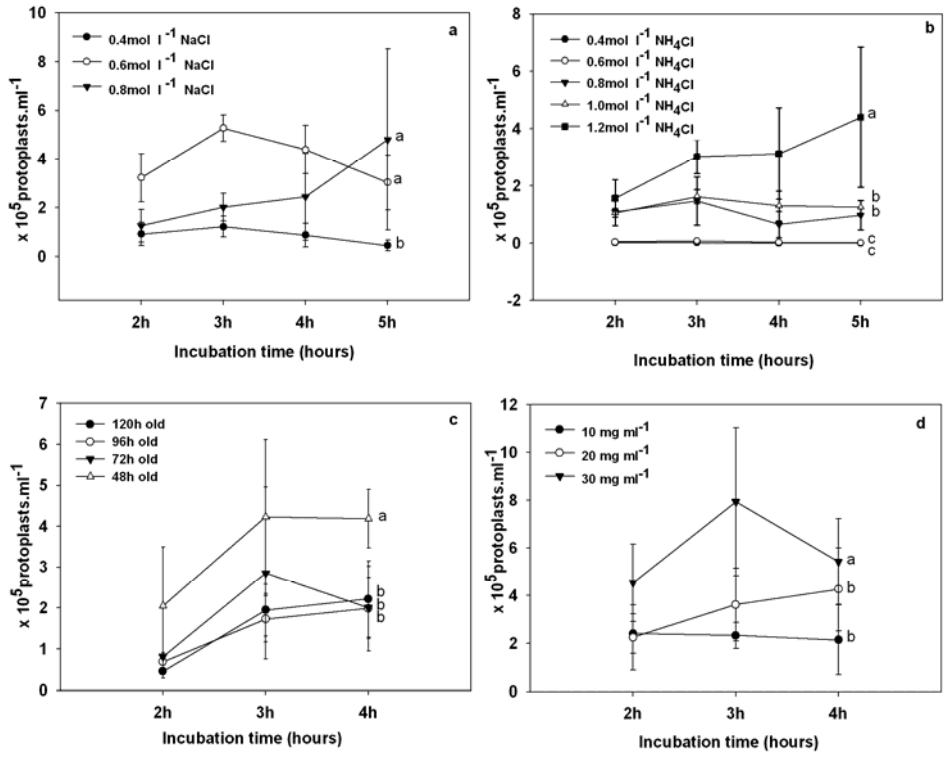

FIGURE 2 Production of *Colletotrichum lindemuthianum* protoplasts under the following conditions: different molar concentrations of NaCl (a) and NH_4Cl (b); mycelial age (c); enzyme concentration (*Lysing enzymes* from *Trichoderma harzianum)* (d). The digestion mixture included the following: 100 mg of mycelium into 3 ml of osmotic stabilizer. Means with the same letter are not significantly different according to a Tukey's test 5%

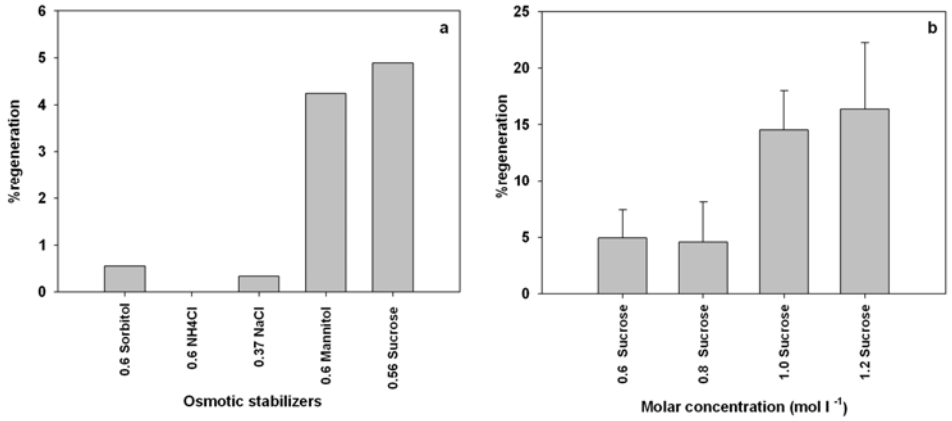

FIGURE 3 Regeneration percentage of *Colletotrichum lindemuthianum* protoplasts. (a) After 72 h incubation in regeneration medium with different osmotic stabilizers (at equivalent molar concentrations); (b) After 48 h incubation in regeneration medium at different sucrose concentrations.

According to Dias et al. (1997), sugars are sometimes inefficient for protoplast production but are good stabilizers for regeneration. This may occur because sugars favor the osmotic stability for protoplast viability, but negatively affect the enzymatic digestion of the cell wall.

6 CONCLUSION

In conclusion, this study demonstrated it is possible to optimize an efficient method for protoplast production and revert them into the typical mycellial morphology using the C. *lindemuthianum* LV115 isolate. The usefulness of protoplast isolation is relevant for many different applications and has been mainly used in procedures involving genetic transformation and

electrophoretic karyotype. Thus, this work may improve our knowledge about this important plant pathogen.

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CAPÍTULO 3

LIVE-CELL IMAGING OF CONIDIAL FUSION IN THE BEAN PATHOGEN, *Colletotrichum lindemuthianum*

1 RESUMO

A fusão entre conídios, conhecida como tubos de anastomoses conidiais (*conidial anastomosis tubes* - CATs), é um fenômeno comum em fungos filamentosos, incluindo muitos fitopatógenos. Os CATs têm numerosas funções e tem-se especulado que poderiam facilitar a recombinação parassexual, a qual contribuiria para a alta variação em fungos que não apresentam a reprodução sexual. O patógeno de feijoeiro, *Colletotrichum lindemuthianum*, produz a fusão de CATs naturalmente na superfície foliar e dentro de corpos de frutificação assexual em lesões de antracnose em seu hospedeiro. Foi desenvolvido um novo e robusto protocolo para análise de *C. lindemuthianum in vitro*. A porcentagem de germinação de conídios e fusão de CATs foi dependente da idade da cultura, do meio de cultivo e do isolado utilizado. O aumento na fusão de CATs está correlacionado com a redução na formação de tubos germinativos. Imagens capturadas ao longo do tempo mostraram todo o processo de fusão de CATs em *C. lindemuthianum* pela primeira vez e foi monitorada a migração nuclear através de CATs utilizando núcleo marcado com GFP. Foi encontrado que a fusão de CATs neste patógeno exibe diferenças significativas em relação ao sistema modelo *Neurospora crassa.* Ao contrário do observado em *N. crassa*, a fusão de CATs é inibida pela presença de nutrientes (ocorre apenas em água) e o processo leva um tempo consideravelmente longo.

2 ABSTRACT

Fusion of conidia and conidial germlings by means of conidial anastomosis tubes (CATs) is a common phenomenon in filamentous fungi, including many plant pathogens. It has a number of different roles, and has been speculated to facilitate parasexual recombination which may contribute to the high genetic variation in fungi lacking sexual reproduction. The bean pathogen *Colletotrichum lindemuthianum* naturally undergoes CAT fusion on the host surface and within asexual fruiting bodies in anthracnose lesions on its host. Here we report the development of a robust protocol using live-cell imaging techniques with *C. lindemuthianum in vitro.* The percentage of conidial germination and CAT fusion was found to be dependent on culture age, medium and fungal strain. Increased CAT fusion was correlated with reduced germ tube formation. We show time-lapse imaging of the whole process of CAT fusion in *C. lindemuthianum* for the first time and monitored nuclear migration through fused CATs using nuclei labeled with GFP. CAT fusion in this pathogen was found to exhibit significant differences to that in the model system *Neurospora crassa*. In contrast to *N. crassa*, CAT fusion in *C. lindemuthianum* was inhibited by nutrients (only occurred in water) and the process took considerably longer to occur.

3 INTRODUCTION

The conidial anastomosis tube (CAT) is a specialized type of hypha that allows fusion between conidia and conidial germlings (Read et al., 2009). CAT fusion is a common phenomenon in filamentous fungi and has been shown in 21 genera and 73 species (Roca et al., 2005b). CAT fusion is believed to allow interconnected germlings to act as a single, coordinated individual, the general homeostasis of which can be regulated by the interchange of nutrients, water, signal molecules, nuclei and other organelles through the fused CATs (Read et al., 2009). In some plant pathogens, hyphal fusion may be involved in pathogenicity by playing an important role in host colonization (Craven et al., 2008; Rosales & Di Pietro, 2008; Read et al., 2009). It has also been postulated that CAT fusion may facilitate parasexual recombination and contribute to the high level of genetic variation found in fungi that lack sexual reproduction (Roca et al., 2005b; Read & Roca, 2006).

In the last few years, CAT fusion has been analysed in most detail in the filamentous fungus, *Neurospora crassa*. The CAT system in *N. crassa* provides a simple, experimentally amenable and genetically tractable system which is being used as a model for the study of vegetative hyphal fusion in filamentous fungi (Read et al., 2009). The whole process of CAT fusion can be analysed within 6 h after adding conidia to growth medium making mutant screening, time-lapse, live-cell imaging and physiological experiments very easy to perform. In *N. crassa*, CATs have been shown to be morphologically and physiologically distinct from germ tubes and under separate genetic control (Roca et al. 2005a; Read & Roca, 2006; Read et al., 2009).

Conidial anastomosis tubes were originally identified as a cell-type distinct from germ tubes in *Colletotrichum lindemuthianum* (Roca et al., 2003),

a hemibiotrophic plant pathogen that causes anthracnose in the common bean, *Phaseolus vulgaris* (Sutton, 1992). In *C. lindemuthianum*, CAT fusion has been previously shown to occur on the host surface (Ishikawa et al., 2009) and within the acervulus (asexual fruiting body) either in anthracnose lesions on the host or in culture (Roca et al., 2003). Because CAT fusion only occurs within the acervulus walls it is very difficult to analyse the whole process *in situ* using live-cell imaging techniques which are now widely used for filamentous fungi (Hickey et al., 2005; Hickey & Read, 2009). Furthermore, the whole process of CAT fusion has not been analysed previously by live-cell imaging techniques in any other pathogenic fungus.

The first aim of the present study was to develop a robust technique for performing live-cell imaging *in vitro* to analyze the whole process of CAT fusion *Colletotrichum lindemuthianum* outside acervuli. The second aim was to image nuclear dynamics during the process of CAT fusion by using a strain in which we had labelled nuclei with GFP. The final aim was to compare the CAT fusion in this fungus with that in the saprotroph *N. crassa* in which the process has been extensively studied (Read et al., 2009).

4 MATERIALS AND METHODS

4.1 Strains and culture conditions

The *C. lindemuthianum* strains used are listed in Table 1. These strains were derived from the culture collection of the Department of Biology, Universidade Federal de Lavras (Lavras, MG, Brazil). All strains were grown and maintained on potato dextrose agar (PDA) (Sigma) at 22ºC in the dark. To stimulate sporulation, the strains were inoculated in glass tubes containing

autoclaved French bean pods submerged in 2% water agar (bean pod agar medium) and incubated in the dark at 22ºC for 7-16 days.

4.2 Preparation of conidia for quantification and live-cell imaging

Conidia were collected from 7-16 days-old cultures and suspended in distilled water. In order to quantify conidial germination and CAT fusion, conidia (at concentration 1 x 10^6 per ml) were used to inoculate potato dextrose broth (PDB) (Difco), Vogel's medium (Davis, 2000) containing 2% w/v sucrose, or distilled water. Conidial germination in M_3S medium (Tu, 1985) was also tested at concentration 1 x 10^5 per ml. 200 µl drops of conidial suspension were placed in an eight-well slide culture chamber (Nalge Nunc International, Rochester, NY) and incubated for an appropriate time in the dark at 22º C. They were then examined at room temperature on an inverted TE2000E microscope (Nikon, Kingston-Upon-Thames, United Kingdom) using bright-field or differential interference contrast optics with a 60x (1.2 NA) water immersion plan apo objective. Conidial germination was quantified as the percentage of conidia possessing one or more germ tubes that was not involved in conidial fusion and/or CATs whilst CAT fusion was quantified as the percentage of conidia or conidium germlings involved in fusion.

TABLE 1 Different *Colletotrichum lindemuthianum* strains used in this study.

Strain	**Race***	**Origin**
LV117	65[a]	Lavras (MG) – Brazil
LV115	65b	Patos de Minas (MG) –Brazil
LV51	73	Lavras (MG) – Brazil
LV77	81	Lavras (MG) – Brazil
tFI01 (hH1-GFP)	65b	Patos de Minas (MG) –Brazil

* Races were identified according Silva et al. (2007)

4.3 Nuclear labeling with GFP

Conidia were harvested from 10-15 day-old cultures of *C. lindemuthianum* race 65b, washed twice in water by centrifugation and used to inoculate 100 ml of M_3S medium (Tu, 1985). Protoplasts were obtained using young mycelium (from 48 h-old cultures) in 0.6 mol l^{-1} NaCl as the osmotic stabilizer, and were incubated for 3 h with 30 mg ml^{-1} *Lysing Enzymes* from *Trichoderma harzianum* (Sigma). Transformations were done using a modification of the methodology described by Thon et al. (2000). Protoplasts in STC (1.2 M sorbitol, 10 mM Tris base, and 50 mM $CaCl_2$, pH 7.5) at a concentration of 10^7 cells per 100 µl were combined with 3-5 µg of the plasmid DNA (pMF357) in a 50-ml polypropylene centrifuge tube (the plasmid pMF357 carried the *hH1-sgfp* gene under control of the *Neurospora crassa ccg-1* promoter and *hph* gene for hygromycin (Hyg) resistance, and was kindly provided by M. Freitag, unpublished data). This was incubated on ice for 20 min after which 1 ml of a PEG solution (40% PEG 3,350 w/v; 0.6 M KCl; 50 mM CaCl2; and 50 mM Tris, pH 8) was added. This mixture was incubated at room temperature for 20 min, then combined with 40 ml of regeneration agar (1 M sucrose, 1.25%, casein hydrolysate, 1.25% yeast extract, 1.0% agar), 100 $\mu g.ml^{-1}$ of hygromycin B and poured into two Petri dishes. The Petri dishes were incubated at 22°C, and hygromycin resistant colonies were transferred to PDA containing 50 $\mu g.ml^{-1}$ of hygromycin B after 7 days. Conidia from these primary transformants were spread onto PDA containing the antibiotic and after 48 h, a single hygromycin-resistant germling was recovered and transferred to PDA containing hygromycin B. Single conidia expressed hH1-sGFP were selected using a fluorescence stereomicroscope with a GFP filter set (Nikon model SMZ1500).

4.4 Live-cell imaging using confocal microscopy

Suspension in water of 10^6 conidia/ml conidia were collected from 15-18 day-old cultures. Samples were examined unstained, or their cell walls were stained with 0.12 M Calcofluor White M2R for 3-4 min (Sigma). 200 µl drops of the conidial suspensions were placed in an eight-well slide culture chamber (Nalg Nunc International) and incubated in the dark at 22º C for 24-48 h. Time-lapse widefield imaging was performed on the Nikon TE2000E microscope using bright-field optics with a 60x (1.2 NA) water immersion plan apo objective over a 12 h period. Confocal microscopy of Calcofluor White and hH1-GFP labelled samples was done on a Radiance 2100 system equipped with blue diode and argon ion lasers (Bio-Rad Microscience) and mounted on a Nikon TE2000U Eclipse inverted microscope. Calcofluor White and GFP were excited at 405 nm and 488 nm, respectively and fluorescence detected at 420/70 nm (for Calcofluor White) and 500/30 nm (for GFP) using a 60x (1.2 NA) water immersion objective lens. Images from the confocal microscope were captured using Lasersharp software (version 5.1; Bio-Rad) and were processed further in Imaris software (4.1 version) or Image J (freeware) and Paintshop Pro software (version 7.0; JASC).

5 RESULTS

5.1 Conidial germination was dependent on growth medium, culture age and strain

Conidial germination in *C. lindemuthianum* was close to 100% when 10^5 conidia per ml were incubated for 48 h in liquid M_3S media. When conidia were incubated at this concentration for 72 h with two different growth media (PDB and liquid Vogel's medium), no CAT fusion was observed (data not shown).

PDB was chosen because PDA has commonly been used for *C. lindemuthianum* (Roca et al., 2003) and liquid Vogel's medium which was selected because it has routinely been used for studies on CAT fusion in *N. crassa* (Roca et al., 2005a). We then incubated a higher conidium concentration (1 x 10^6 conidia per ml) in these media because this conidial concentration has been shown to be more-or-less optimal for CAT fusion in *N. crassa* (Roca et al., 2005a). Initially we assessed the conidia germination from four different races harvested from 7-, 10-, 13-, and 16-day old cultures and incubated them in PDB, liquid Vogel's medium, as well as in water alone. Surprisingly, the mean conidial germination was found to be low (consistently < 25%) 72 h after incubation and the amount of germ tube formation was dependent on the growth medium, the culture ages from which conidia were harvested, and the used race (Fig. 1). Shorter incubation periods (e.g. 48 h) resulted in even lower germination levels (data not shown). Conidia harvested from 10-day old cultures produced the highest germination levels in all three media of the four tested races (Fig. 1). PDB was the best medium for germ tube formation (< 25%) and water the worst (< 13%) (Fig. 1). Furthermore, germ tubes often differentiated into appressoria in water (Figs. 2A and 3A), but not in PDB or liquid Vogel's medium. Conidia incubated in Vogel's medium produced germ tubes that tended to be highly swollen, often exhibited aberrant growth (Fig. 3B) and sometimes underwent microsporulation (Fig. 3C). In addition, conidia immersed in Vogel's medium had a tendency to lyse.

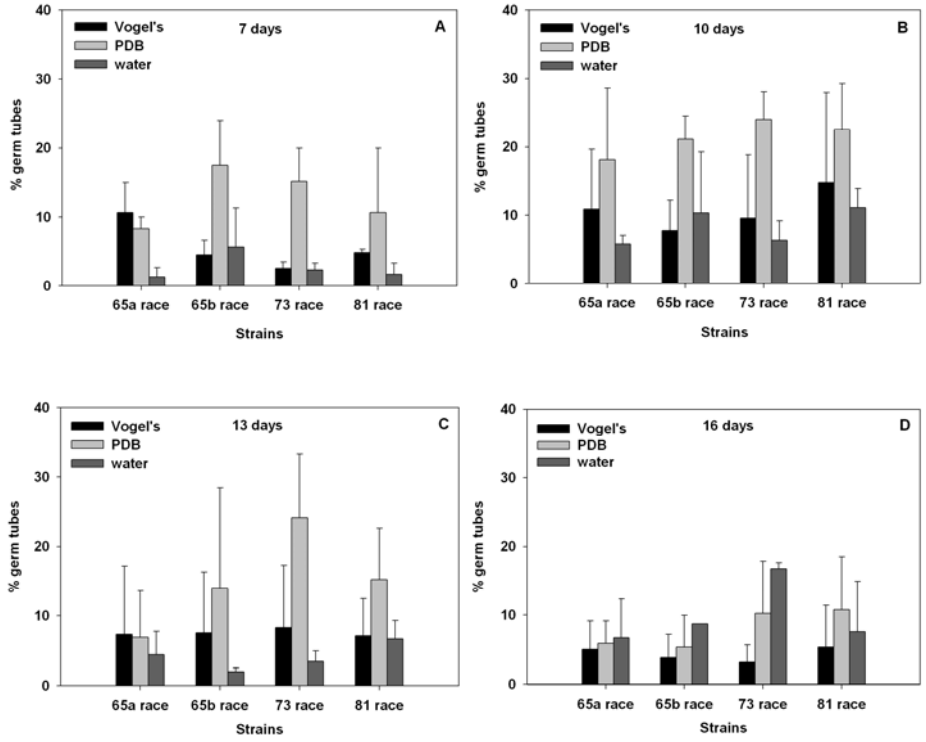

FIGURE 1 Percentage germination of conidia of 4 races (65a, 65b, 73 and 81) of *Colletotrichum lindemuthianum* harvested from 7, 10, 13 and 16 day-old cultures incubated in PDB, liquid Vogel's medium, and in water for 72 h. Conidial germination was quantified as the percentage of conidia possessing one or more germ tubes that were not involved in conidial fusion. Data from 3 replicates (n = 300).

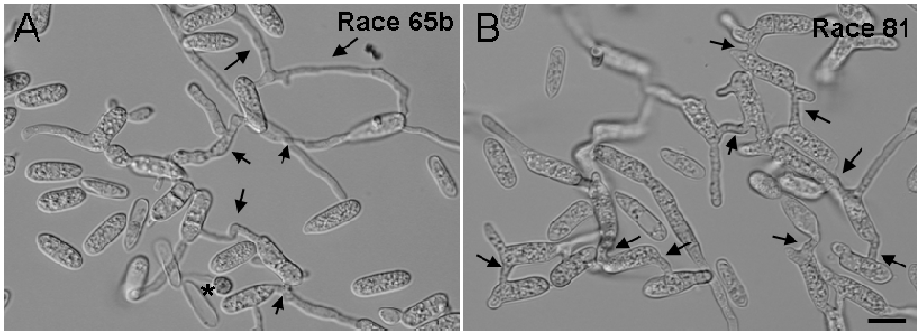

FIGURE 2 CAT fusion by different races of *C. lindemuthianum* in water after incubation for 72 h. The conidia were harvested from 16 day-old PDA cultures. Arrows indicated fusions between conidia/conidial germlings via CATs. (A) Race 65b. Note the appressorium (asterisk). (B) race 81. Bar: 10µm.

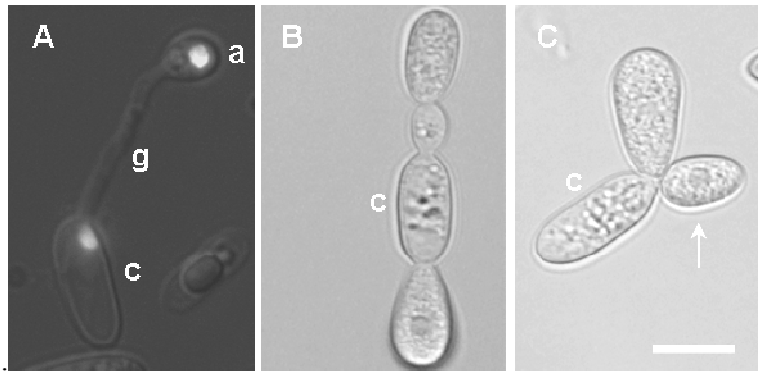

FIGURE 3 Different patterns of morphogenesis following conidial germination by *C. lindemuthianum* race 65b after 48 h incubation in water and liquid Vogel's medium. (A) Germ tubes formed in water often differentiated appressoria. Nucleus in conidium and nucleus in appressorium labelled with hH1-GFP and imaged by simulataneous confocal and brightfield imaging. (B) Germ tubes formed in liquid Vogel's medium showing aberrant patterns of polarized growth. (C) Conidia in liquid Vogel's medium that have undergone microsporulation (arrow). Abbreviations: a = appressorium; c = conidium; g = germ tube. Bar: 10 µm.

5.2 CAT fusion only occurs in water

At a concentration of 1 x 10^6 conidia per ml, CAT fusion was only observed to a very significant extent after incubation in water after 72 h (Figs. 2 and 4). Less than 0.01% of conidia underwent CAT fusion when incubated for 72 h in either PDB or liquid Vogel's medium (data not shown). Confocal microscopy of fused germlings stained with Calcofluor White and imaged in different optical planes followed 3D reconstruction clearly showed that multiple CAT fusion events resulted in the formation of chains of interconnected conidia (Fig. 5).

CAT fusion in water was dependent on the age of the cultures from which the conidia were harvested and the strain used (Fig. 4). CAT fusion was not observed between conidia from 7 day-old cultures. Although, all four races produced CATs that fused, it was only races 65b and 81 that fused at a significant level (Figs. 2 and 4). In each case, it was found that conidia from 16-day old cultures exhibited the highest level of CAT fusion (25.8% ± 6.8 for race 65b and 21.4% ± 22.5 for race 81) which was in contrast to the earlier finding that 10-day old cultures produce conidia that underwent optimal germination (Fig. 1).

The whole process of CAT fusion was best observed after conidia had been incubated for ~ 48 h (Fig. 2). After 72 h, the process was much less easy to visualize particularly because of the increased vacuolation of the cells involved. The whole process of fusion between CATs typically took an extremely long time (e.g. ~ 12 h) as is shown for race 81 in Fig. 6. Here one germ tube/CAT can be seen to be growing towards another conidium. At the 55 h time point it seemed to induce a very small bulge on the conidium it is growing towards. Between the 56 h and 60 h time points fusion occured.

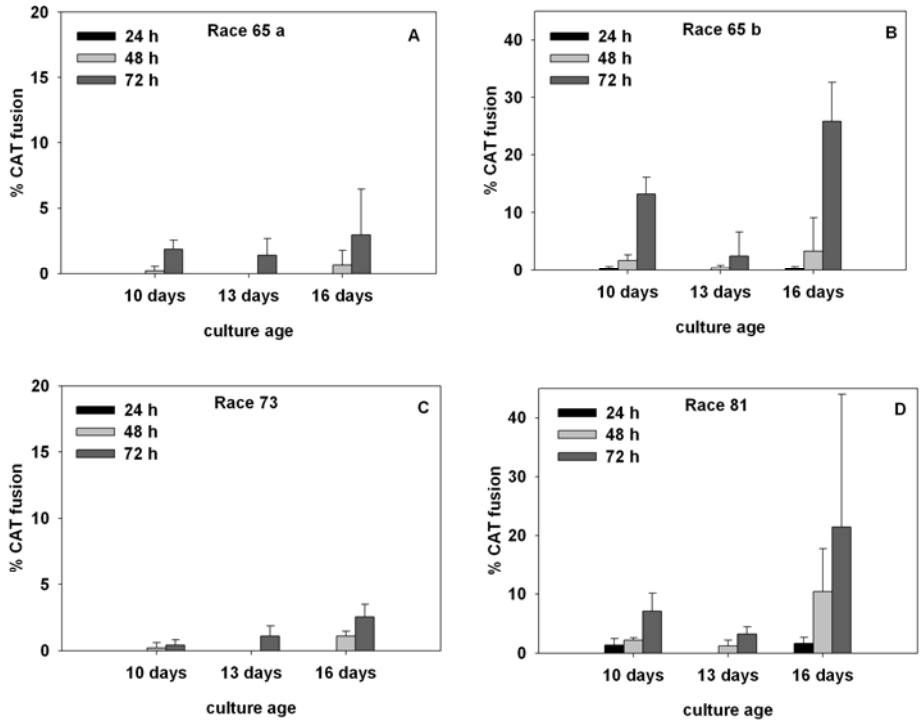

FIGURE 4 Percentage CAT fusion in water between conidia and conidial germlings of 4 races (65a, 65b, 73 and 81) of *Colletotrichum lindemuthianum* harvested from 10, 13 and 16 day-old cultures and incubated for 24 h, 48h and 72 h. CAT fusion was quantified as the percentage of conidia or conidia germlings involved in fusion. Data from 3 replicates (n = 300).

FIGURE 5 Projection of a 3D reconstruction of confocal images of an interconnected network of fused germlings of *Colletotrichum lindemuthianum* stained with Calcofluor White. The conidia were originally harvested from a 16 day-old PDA culture and incubated in water for 48 h. Bar: 10 µm.

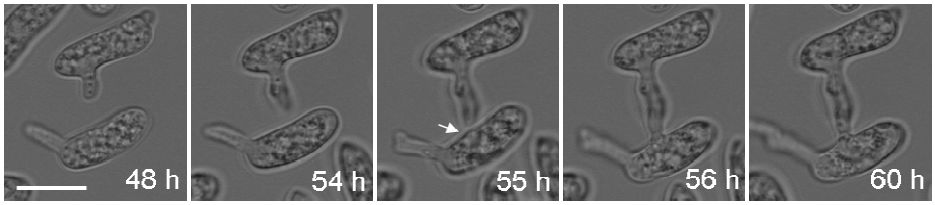

FIGURE 6 Time lapse sequence imaged by widefield, brightfield light microscopy showing a germ tube/CAT formed from the upper conidium growing towards another conidium below it. Note the formation of a slight bulge (arrow) on the surface of the lower conidium at the 55 h time point which has appeared at the site of subsequent fusion. Time-lapse imaging was performed on conidia harvested from a 10-day old culture of race 81. Bar: 10 µm.

5.3 Nuclei migrate through fused CATs

Nuclear dynamics were imaged during CAT fusion in a strain (tFI01) of race 65b in which the nuclei had been labeled with hH1-GFP (Fig. 7). Time lapse imaging by confocal microscopy of CAT fusion was performed after incubating the conidia in water for 40h. At this stage the conidia were found to be either uninucleate or binucleate. In Fig. 7, a sequence of two CATs, one derived from a unincleate conidum the other from a binucleate conidium, could be seen to be homing towards each other at the 40 h time point. After 1 h they have fused (41h time point in Fig. 7). Between the 42 h 30 min and 42 h 40 min time points, one of the left hand nucleus in the lower binucleate conidium underwent mitosis and then migrated through the fused CATs (42 h 40 min time point).

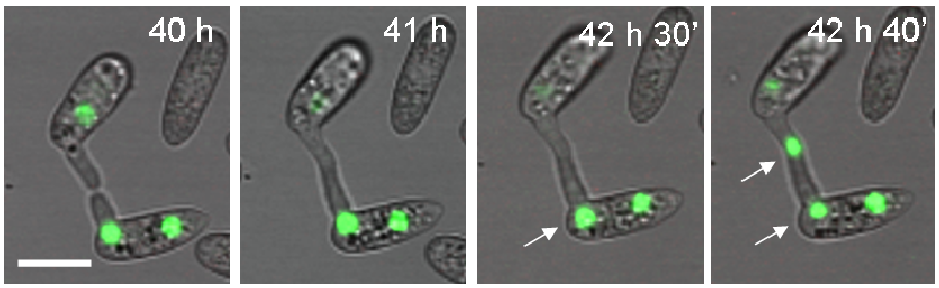

FIGURE 7 Time-lapse sequenced by combined confocal and brightfield imaging showing a sequence of two CATs homing towards each other, fusing and nuclei migrating through the fused CATs. Note that between the 42 h 30 min and 42 h 40 min time points, one of the left hand nuclei in the lower binucleate conidium underwent mitosis and one of the resultant nuclei (arrows) then migrated through the fused CATs (42 h 40 min time point). Time-lapse imaging was performed on conidia from a 16-day old cultue of race 65b in which the nuclei had been labeled with hH1-GFP. Bar: 10µm.

6 DISCUSSION

We have developed and optimized a robust protocol for CAT fusion in *C. lindemuthianum* in the absence of acervuli and host tissue, and we have shown how this could be used to successfully analyse the process by live-cell imaging. Our study has revealed interesting differences between CAT fusion in *C. lindemuthianum* compared with that in *N. crassa*.

Previously it has been shown 19% of conidia from acervuli developed on French bean pods submerged in water agar (bean pod agar medium) had undergone CAT fusion after incubation for 40 days (Roca et al., 2003). In the present study, it was possible to routinely get ~ 26% of conidia to undergo CAT fusion in water after 72 h incubation but this was dependent on using conidia of race 65b from 16-day old cultures grown on bean pod agar medium. Lower levels of CAT fusion were observed when conidia were incubated in other media (PDB or liquid Vogel's medium), harvesting conidia from younger bean pod agar cultures or when other races (65a, 73 or 81) were used.

Interestingly, older conidia (from 16-day old cultures grown on bean pod agar medium) were optimal for CAT fusion whilst younger conidia (from 10-day old cultures) were optimal for germ tube formation. It is not clear why there should be this difference. However, the inverse relationship of CAT fusion and germ tube formation may be related to the amount of internal resources that conidia can provide to support these two processes; thus increased CAT fusion may reduce these conidial resources that are available for germ tube formation. Alternatively, the reduced level of germ tube formation may be due to increased inhibition of germ tube formation by a germination self-inhibitor. In the acervulus no germ tubes are formed because of germination self-inhibitors present in the mucilage (Leite & Nicholson, 1992; Inque et al., 1996).

Neurospora crassa is a model organism which has been much used in

studies on CAT fusion (Roca et al., 2005a; Read et al., 2009). A number of notable differences were observed between CAT fusion undergone *in vitro* in *N. crassa* compared with that in *C. lindemuthianum*: (1) CAT fusion in *C. lindemuthianum* is inhibited by nutrients and only occurs in water. CAT fusion in *N. crassa*, on the other hand, requires the presence of nutrients to occur (Roca et al., 2005a). This may relate to the fact that there is little or no nutrient on the host surface where conidia of *C. lindemuthianum* normally germinate. CAT fusion has been previously observed on the surfaces of host leaves inoculated with *Colletotrichum spp*. (Latunde-Dada et al., 1999; Ishikawa et al., 2009). (2) Fewer *C. lindemuthianum* conidia were involved in CAT fusion in water (the maximum was ~25%) compared with *N. crassa* in which < 50% of the conidia can undergo CAT fusion in liquid Vogel's medium (M.G. Roca, unpublished). It is possible that higher levels of CAT fusion in *C. lindemuthianum* might be observed in new races studied in the future because the amount of CAT fusion observed in this study was very variable between the four races investigated. (3) In a population of conidia, CAT fusion occurs over a much longer period *in vitro* in *C. lindemuthianum* than in *N. crassa*. In *C. lindemuthianum* it occurred over a 72 h period after inoculation in water. In *N. crassa* it is completed within 5-6 h after inoculation in liquid Vogel's medium (M.G. Roca, unpublished). On the host leaf surface, *C. lindemuthianum* was found to grow and undergo CAT fusion for 48-72 h before there was any evidence of penetration into the leaf (Ishikawa et al., 2009). (4) Conidia from older cultures of *C. lindemuthianum* (optimally 16-day old cultures grown on bean pod agar) underwent the highest amount of CAT fusion. In *N. crassa*, much younger conidia (from 5-6 day old cultures grown on Vogel's agar medium) are optimal for undergoing CAT fusion. This difference may partly relate to the fact that *N. crassa* grows much faster than *C. lindemuthianum*. However, it is also clear that age of the bean pod agar culture from which conidia are harvested influences their propensity to

undergo CAT fusion versus germ tube formation (see above). The basis of conidial age-dependent CAT fusion is not understood.

Another difference between CAT fusion in *C. lindemuthianum* and *N. crassa* is that the whole process of CAT fusion seemed to take considerably longer in the former. However, this is complicated by the fact that it in some cases it is difficult to clearly distinguish CATs from germ tubes. In *N. crassa*, CATs can either form directly from conidia or from germ tubes (Roca et al., 2005a). Although CATs are short and commonly thinner than germ tubes they are not always very distinct from germ tubes, particularly if they are formed from the latter (Read et al., 2009). However, a strong indication of that the hypha in question is a CAT and not a germ tube is when they are growing towards (i.e. homing towards) each other (e.g. in Fig. 7). However, in other situations where a germ tube/CAT is growing towards the conidium, as shown in Fig. 6, it is unclear whether the hypha is a CAT because only one hypha is involved. What may be happening here is that a germ tube is non-tropically growing towards the conidium and when it gets very close its tip differentiates into a very short CAT that induces a very short CAT which can be detected as a bulge on the opposing conidium. This problem with interpretation should be improved when we have markers (e.g. proteins that can be labelled with GFP) that are specific for CATs.

Although CAT fusion seems to occur in the majority of ascomycete fungi (Roca et al., 2005b), number of species including *Aspergillus nidulans* and the plant pathogens *Magnaporthe oryzae* (Read et al., 2009) and *Alternaria brassicola* (Craven et al., 2008) have been reported not to undergo CAT fusion. It has been suggested that CAT fusion has not been observed in some fungi might be because CAT formation in these species may require specific conditions (e.g. nutritional factors or conidial density) that are not commonly used experimentally. Our results showed that the absence of nutrients, old

conidia, conidial density, long incubation times, and the race used are important factors which influence whether CATs are formed or not, and the extent to which they are formed.

Although CAT fusion is more difficult to analyse in *C. lindemuthianum* than in *N. crassa*, the present study should expedite future studies on CAT fusion in this organism. By having an experimentally robust method for analyzing CAT fusion *in vitro* this will facilitate further live-cell imaging, mutant screening, and physiological and pharmacological studies of the process. Of particular importance will be to establish whether CAT fusion: (1) plays important roles in pathogenesis (Read et al., 2009); (2) facilitates parasexual recombination and contribute to the high genetic variation in fungi lacking sexual reproduction (Roca et al., 2005b; Read & Roca, 2006); and (3) expedites horizontal gene transfer between pathogens (Roca et al., 2004; Friesen et al., 2006; Sanders, 2006).

7 CONCLUSIONS

- A robust protocol was developed for doing CAT assay in *Colletotrichum lindemuthianum* using live-cell imaging techniques *in vitro.*
- The percentage of conidial germination and CAT fusion was found to be dependent on culture age, media and the fungal strain used.
- Increased CAT fusion was correlated with reduced germ tube formation.
- It is possible to perform time-lapse imaging of the whole process of CAT fusion in *C. lindemuthianum* and monitored nuclear migration through fused CATs using nuclei labeled with GFP.
- CAT fusion in this pathogen was found to exhibit significant differences to that in the model system *Neurospora crassa*.

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CAPÍTULO 4

HETEROKARYON INCOMPATIBILITY IS SUPPRESSED FOLLOWING SPORE FUSION IN THE PLANT PATHOGEN *Colletotrichum lindemuthianum*

1 RESUMO

O patógeno do feijoeiro *Colletotrichum lindemuthianum* produz a fusão de esporos (conídios), um tipo de hifa especializada, conhecida como tubos de anastomoses conidiais (*conidial anastomosis tubes* - CATs), dentro do corpo de frutificação em seu hospedeiro. Utilizando-se a análise *in vivo*, o comportamento nuclear durante a fusão de CATs e hifas vegetativas foi analisado em isolados que expressam as histonas H1 e H4, marcadas com *eGFP* e *tdimerRed*, respectivamente. A incompatibilidade do heterocário foi observada entre as colônias de isolados vegetativamente incompatíveis, resultando em morte celular. No entanto, a fusão de CATs entre conídios uninucleados destes isolados incompatíveis não causou a reação de incompatibilidade, mesmo após 30 horas da fusão. Os núcleos vermelhos e verdes da combinação destes isolados mostraram migrar de um conídio para outro, formando o heterocário e, posteriormente, ocorreu a mitose. A formação de núcleos amarelos resultantes desta combinação H1-eGFP e H4-tdimerRed após a fusão foi observada em 27% dos heterocários formados. Há evidências de que alguns núcleos amarelos foram obtidos como resultado da importação das duas proteínas fundidas em um mesmo núcleo, ou foi formado pela fusão dos núcleos verde e vermelho. Novas fusões de hifas e divisões nucleares ocorrem com o crescimento do tubo germinativo e diferenciação em hifa vegetativa na colônia madura. A distribuição de núcleos verdes, vermelhos e amarelos na colônia heterocariótica não foi uniforme. A recombinação entre os diferentes isolados pode ser comprovada utilizando-se o meio seletivo com higromicina e fleomicina, além do uso de *primers* específicos de PCR. Os testes de patogenicidade mostraram que estes recombinantes podem perder a patogenicidade nas cultivares diferenciadoras ou, mesmo, gerar uma nova raça. Portanto, a fusão de CATs é um mecanismo potencial de recombinação não meiótica nesta espécie, a qual permite a transferência horizontal de genes entre isolados incompatíveis.

2 ABSTRACT

The bean pathogen *Colletotrichum lindemuthianum* undergoes spore (conidium) fusion by means of specialized hyphae called conidial anastomosis tubes (CATs) within asexual fruiting bodies on its host. Using live-cell imaging, nuclear behaviour during fusion in CATs and vegetative hyphae was analyzed in strains expressing the histone proteins H1 and H4 labeled with eGFP and tdimerRed, respectively. Heterokaryon incompatibility was observed between mature colonies of vegetatively incompatible strains and resulted in cell death. However CAT fusion between the uninucleate conidia of these incompatible strains did not cause an incompatible reaction within 30 h following fusion. Red and green labeled nuclei in these strain combinations were shown to migrate from one conidium to another to form the heterokaryon, and then to undergo mitosis. The formation of yellow nuclei resulting from the combination of H1-eGFP and H4-tdimerRed was observed in 27% of the heterokaryons formed following CAT fusion. Evidence was obtained for some yellow nuclei resulting from the import of the two fusion proteins into the same nuclei, and for other larger, yellow nuclei being formed by the fusion of green and red nuclei. Further hyphal fusion and nuclear division occurred as germ tubes grew and differentiated into the vegetative hyphae of the mature colony. The distribution of green, red and yellow nuclei in the heterokaryotic colony was non-uniform. Recombination between different strains could be proof using the selective medium with hygromycin and phleomycin, and using specific PCR primers. Pathogenicity tests showed these recombinants may be not pathogenic to differential bean cultivars or generate a new race. Therefore, CAT fusion is a potential mechanism of non-meiotic recombination in this specie and which allows horizontal transfer of genes between incompatible strains.

3 INTRODUCTION

Colletotrichum lindemuthianum is one of the most important plant pathogenic fungi causing anthracnose in common bean (*Phaseolus vulgaris*). The large degree of pathogenic and genetic variability observed in *C. lindemuthianum* strains (Mahuku & Riasco, 2004; Silva et al., 2007; Ishikawa et al., 2008) suggests the involvement of genetic recombination but how this is generated is unclear. Sexual reproduction in *C. lindemuthianum* is rarely observed in nature (Camargo Júnior et al., 2007) but evidence has been obtained for genetic variation in this phytopathogen arising by means of parasexuality (Castro-Prado et al., 2007) and non-meiotic recombination following spore fusion via conidial anastomosis tubes (CATs) (Roca et al., 2003, 2004, 2005).

Non-self hyphal fusion between mature, genetically different colonies of filamentous fungi commonly results in the rapid death of the heterokaryotic cell formed. Although the development of vegetative heterokaryons may advantageous for filamentous fungi, the genetic mechanism of vegetative incompatibility restricts the process between two genetically different individuals (Saupe, 2000; Glass & Dementhon, 2006). Very little is known about heterokaryon incompatibility genes in *Colletotrichum* species although it has recently been shown that genetic diversity in natural populations of *C. lindemuthianum* is considerable and that numerous vegetative compatibility groups are present (Barcelos, 2007).

Roca et al. (2004) observed CAT fusion between *C. lindemuthianum* and *C. gossypii* suggesting that genetic material had exchanged between these vegetatively incompatible species and that heterokaryons had been formed. However, these latter processes were not directly shown in this study. Using histone proteins (H1 and H4) fused with different fluorescent proteins (eGFP or tdimerRed), we observed the heterokaryon formation following CAT fusion

between two vegetatively incompatible strains in *C. lindemuthianum* and analyzed the nuclear behaviour in fused germling and hyphae in the mature colony.

This has been the first report demonstrating the incompatibility reaction and cell death in the mature colony in *Colletotrichum lindemuthianum*, as the heterokaryon formation and later recombination at early stages of development of the colony between two vegetative incompatible strains using live cell imaging.

4 MATERIALS AND METHODS

4.1 Strains and culture conditions

The *Colletotrichum lindemuthianum* strains used are listed in Table 1. These strains were deriving from the culture collection of the Department of Biology, Universidade Federal de Lavras (Lavras, MG, Brazil). All strains were grown and maintained on Potato Dextrose Agar (PDA) medium. For sporulation, the strains were inoculated in autoclaved French bean pods plus agar-water 2%.

TABLE 1 Different *Colletotrichum lindemuthianum* strains used in this study.

Strain	Race	Origin
LV117	65a	Lavras (MG) – Brazil
LV115	65b	Patos de Minas (MG) –Brazil
LV51	73	Lavras (MG) – Brazil
LV77	81	Lavras (MG) – Brazil

4.2 Transformation vectors

The plasmid pGR02 used here was obtained from Gabriela Roca. Vector was constructed from ApaI- and XbaI-digested pLC11 (ccg-1 promoter, tdimerRed fused to hH4-2 from *Fusarium graminearum*) ligated into ApaI- and XbaI-digested pBC-phleo, construct containing the *Ble* gene for phleomycin (Phleo) resistance controlled by the *A. nidulans* gpdA promoter and the *S. cerevisiae* CYC1 terminator (Silar, 1995). Plasmid pMF357 carries the *hH1-sgfp* gene under control of the *Neurospora ccg-1* promoter and *hph* gene for hygromycin (Hyg) resistance (M. Freitag, unpublished data). Cloning and propagation of plasmids was performed using standard laboratory conditions (Sambrook & Russel, 2001). All vectors were grown in *Escherichia coli* strains DH5α and purified using Qiagen columns.

4.3 Transformation for nuclei labeling

Conidia were harvested from 10-15 days-old culture of *C. lindemuthianum* strains (Table 1) and washed twice in water by centrifugation and inoculated in 100 ml of M_3S medium (Tu, 1985). The protoplasts were obtained using young mycelia (48 h-old cultures) in 0.6 mol l^{-1} NaCl as the osmotic stabilizer, and were incubated for 3 h with 30 mg ml^{-1} *Lysing Enzymes* from *Trichoderma harzianum* (Sigma). Transformations were done using the methodology described by Thon et al. (2000) with modifications. Protoplasts in STC (1.2 M sorbitol, 10 mM Tris base, and 50 mM CaCl2, pH 7.5) at a concentration of 10^7 protoplasts in 100 µl were combining with 3-5µg of the plasmid DNA (pMF357 and pGR02) in a 50-ml polypropylene centrifuge tube. The mixture was incubated on ice for 20 min. After was adding 1 ml of a PEG solution (40% PEG 3,350 wt/vol; 0.6 M KCl; 50 mM CaCl2; and 50 mM Tris, pH 8). This mixture was incubated at room temperature for 20 min, combined with 40 ml of regeneration agar (1 M sucrose, 1.25%, casein hydrolysate, 1.25%

yeast extract, 1.0% agar) added the antibiotic (100 μg.ml^{-1} of hygromycin B or 25 μg.ml^{-1} of phleomycin) and then poured into two Petri dishes. The dishes were incubated at 22°C, and hygromycin or phleomycin resistant colonies were transferred to PDA containing 50 μg.ml^{-1} of hygromycin B or 25 μg.ml^{-1} of phleomycin after 7 days. Conidia from these primary transformants were spread onto PDA containing the respective antibiotic and after 48 h, a single hygromycin-resistant germling was recovered from each transformant and transferred to PDA + Hyg or PDA + Phleo. Single conidia expressed H1-eGFP or H4-tdimerRed was selected using stereomicroscope (Nikon SMZ1500) with filter set dsRED Ultra (excitation 545/30 nm, barrier filter 620/60 nm) and GFP (excitation 460/500 nm, barrier 500/60 nm).

4.4 Preparation of conidia for quantification and live-cell imaging

Conidia were collected each from 10-16 days-old culture and suspended in water. In all experiments conidia were used at concentration 10^6 per ml. Water were used to evaluate the presence of CAT fusion. For imaging samples and for the quantification of conidial germination and fusion, 200 μl drops of conidial suspension were place in an eight-well slide culture chamber (Nalge Nunc International, Rochester, NY). The samples were placed in an incubator in the dark at 22º C until the first measurement. These were then examined at room temperature by using bright-field or differential interference contrast optics with 60x (1.2 numerical aperture [NA]) water immersion plan apo objective on an inverted TE2000E microscope (Nikon, Kingston-Upon-Thames, United Kingdom) and confocal laser scanning microscopy. CAT fusion was quantified as the percentage of conidia or conidium germlings involved in fusion.

4.5 Heterokaryon tests

All the wild type strains (Table 1) and the transformed strains (Table 2) were confronted in the mature colony using conventional methodologies (10 replicates) and microscopy analyses. To evaluated hyphae fusion between them cellophane was place in PDA Petri dishes in the contact area between them and after grow slides were mounted. These were then examined at room temperature by using bright-field or differential interference contrast optics with 40x (1.3 numerical aperture [NA]) oil immersion plan apo objective on an inverted TE2000E microscope (Nikon, Kingston-Upon-Thames, United Kingdom) and confocal laser scanning microscopy. Ten replicates were used in the first experiment which consist the pairing of wild type (73 or 81) with green nuclei labelled (65b). Five fields (195 x 195µm) were quantified for hyphae fusion for each repetition. In the second experiment, pairings were done using the red and green nuclei labelled strains in 12 repetitions.

Conidial suspension (10^6 conidia per ml) in water of wild type strains (races 73 and 81) were mixed with green nuclei labelled strain (tFI01) as both transformed strains (tFI01 and tFI04) at 10-16 days-old culture and incubation and quantification was done as described before.

TABLE 2 Transformed strains used:

Strain	Background	Race	Phenotype
tFI01	LV 115	65b	hH1-GFP/ hygromycin
tFI04	LV 51	73	hH4-tdimerRed/ phleomycin

4.6 Confocal live-cell imaging and imaging processing

For time lapse imaging of conidia and conidia germlings, the inverted agar block method of preparation (Hickey et al., 2005) was employed with modifications. Conidia were collected from 13 to 17 days-old culture and suspended in water. Conidia concentration was adjusted at concentration 10^6 per ml and the different strains were mixture in equal proportion (1:1). The samples were placed in an incubator in the dark at 22º C for 48 h. After this period, the suspension was diluted to 10^5 conidia per ml and plated in PDA during 24 h. Confocal live-cell imaging was performed over a 24 h period to follow different stages of CAT fusion and nuclei dynamic. Samples of the conidia mixture in water for 48h were plated in selective medium (PDA + hyg + phleo) to evaluate the heterokaryon colony development after 7-15 days-old. Confocal laser scanning microscopy was performed by using a Radiance 2100 system equipped with blue diode and argon ion lasers (Bio Rad Microscience) and mounted on a Nikon TE 2000U Eclipse inverted microscope. GFP and tdimerRed were image simultaneously by excitation with 488 and 543 laser lines with fluorescence detected at 500/30 nm (for GFP) and > 560 nm (for tdimerRed). A 40X (1.1 NA) oil immersion objective lens was used for imaging. Confocal images were captured using Lasersharp software (version 5.1; Bio-Rad). These images were transferred into Imaris software (4.1 version) or Image J (freeware) and Paintshop Pro software (version 7.0; JASC) for further processing.

4.7 DNA extraction and PCR

Conidia were collected from heterokaryotic colonies, resistant for both antibiotics, and plated in selective media again (PDA + 50 $\mu g.ml^{-1}$ of hygromycin B + 25 $\mu g.ml^{-1}$ of phleomycin). The putative recombinant was purified by isolation of single conidium that grew on selective medium. Wild type (65b and 73), transformed strains (tFI01 and tFI04) and the putative

recombinants were cultured in liquid M_3S (Tu, 1985) or PDB medium. DNA was extract using the methods of Raeder & Broda (1985). PCR primers (Table 3) were designed using the sequence from the plasmid pMF357, *hph* gene (hygromycin-resistance gene), *eGFP* gene, *ble* gene (phleomycin-resistance gene) and *tdimerRed* gene.

Amplification reactions were performed in thermocycler model Biocycler MJ96G (Biosystems) in a 12 µl final volume containing 4 µl water, 25 ng of genomic DNA and 50 µM of each dNTP, 0.4 µM oligonucleotide primer, 0.4 units Taq DNA polymerase enzyme. Amplification was programmed for one initial denaturation cycle (94ºC for 2 min), followed by 32 cycles of 20 s at 94ºC, 20 s at 65ºC and 20 s at 72ºC and a final extension step of 4 min. Amplification products were separated by electrophoresis in 1.0% agarose gel and visualized under UV light (Fotodyne Ultraviolet Trans-illuminator; Fotodyne Inc., New Berlin, WI, USA) before being photographed with a Kodak EDA– 290 camera (Eastman Kodak Company, Rochester, NY, USA).

TABLE 3 Primers designed using plasmid sequence pMF357, and sequence from the genes *hph* and *Ble* (hygromycin and phleomycin resistance genes, respectively), and GFP and tdimerRed genes (fluorescent proteins).

PCR Primers	Sequence 5'–3'	PCR product size (pb)
*pMF357*F	CTGACTTGAGCGTCGATTTT	999
*pMF357*R	GCTTTGCATTGTGGGTATTC	
*hph*F	TCGCGCATATGAAATCACGCCA	574
*hph*R	TGGTGCGTTTGTCAAGCAAGGT	
*egfp*F	ATGTGATCGCGCTTCTCGTT	589
*egfp*R	TAAACGGCCACAAGTTCAGCGT	
*ble*F	GCCAAGTTGACCAGTGCCGTT	353
*ble*R	ACGAAGTGCACGCAGTTGCC	
*tdimerRed*F	AAGAGTTCATGCGCTTCAAGGTGC	402
*tdimerRed*R	AGCCCATGGTCTTCTTCTGCAT	

4.8 Pathogenicity test

Colletorichum lindemuthianum transformed strains (tFI01 and tFI04) and the recombinants were inoculated in the 12 differential common bean cultivars (Centro Internacional de Agricultura Tropical, 1990) together with Pérola, which is a susceptible control. Seeds were cultivated in polystyrene trays (128 wells each) containing the commercial substrate Plantmax® (Eucatex, Paulina, SP, Brazil). After emergence of primary leaves, seedlings were sprayed with 200 ml of the suspension of fungal spores (1.2×10^6 spores/ml) that were harvested from 15-20 day-old culture and the trays were placed in a humid incubator at a temperature of 20 ± 2ºC with a photoperiod of 12 h dark / 12 h light. Seedlings were maintained in this controlled conditions for 10 days, after which the symptoms exhibited by the infected plants were evaluated on a scale from 1 to 9, in which scores 1 to 3 represented resistant plants and scores 4 to 9 represented susceptible plants (Schoonhoven & Pastor-Corrales, 1987). The Habgood (1970) binary system was employed for the purpose of race identification.

5 RESULTS

5.1 *Colletotrichum lindemuthianum* nuclei were labelled with different fluorescent proteins

The transformation efficiency *C. lindemuthianum* varied according to the strains and plasmids used. The highest transformation efficiency ($4x10^{-6}$) was obtained with the strain LV115 (race 65b) using the plasmid pMF357 in which the gene encoding eGFP protein was fused to H1 histone. A lower transformation efficiency (2.5×10^{-7}) was achieved with the strain LV51 (race 73) using the plasmid pGR02. Despite numerous attempts, it was not possible to transform race 65a and race 81 with these plasmids.

5.2 Heterokaryon incompatibility follows the fusion of mature colonies of incompatible strains

Mature colonies of races 65b (LV 115) and 81 (LV 77) were vegetatively compatible because when confronted with each other they exhibited no macroscopic evidence of an incompatible response (Fig. 1A). Confrontation between mature colonies of 65b (LV115) and 73 (LV51), however, showed that they were vegetatively incompatible because they stopped growing in the region of contact and formed a barrier at the line of contact (Fig 1B).

The region in which the colonies interacted in these two different responses was analyzed in more detail microscopically and the extent of fusion between hyphae of the same colony (self fusion) and between different colonies (non-self fusion) was quantified. To more clearly distinguish the parental hyphae in the compatible and incompatible combinations, nuclei were labeled with H1-GFP (race 65b), H4-tdimerRed (race 73) or were unlabeled (strain 81).

Time lapse imaging by confocal microscopy of fusion between incompatible hyphae of races 65b and 73 with green and red fluorescing nuclei, respectively, was followed over 18 h in a 15 day old culture (Fig. 1C). Following fusion the migration of green nuclei from a hypha of race 65b into a hypha of race 73 occurred (15 min time point in Fig 1C). 1 h 15 min after fusion, the heterokaryotic hyphae started to undergo cell death as the green and red fluorescence of the different nuclei started to disappear, presumably as result of nuclear breakdown. Unexpectedly, the green nuclei of the 65b strain disappeared faster than the red nuclei of the 73 strain (see 3 h 30 min time point in Fig. 1C). Thereafter, the red nuclei disappeared and eventually the hyphal compartments were completely dead and devoid of fluorescence in contrast to adjacent hyphal compartments which contained either red or green nuclei (see 18 h time point in Fig. 1C).

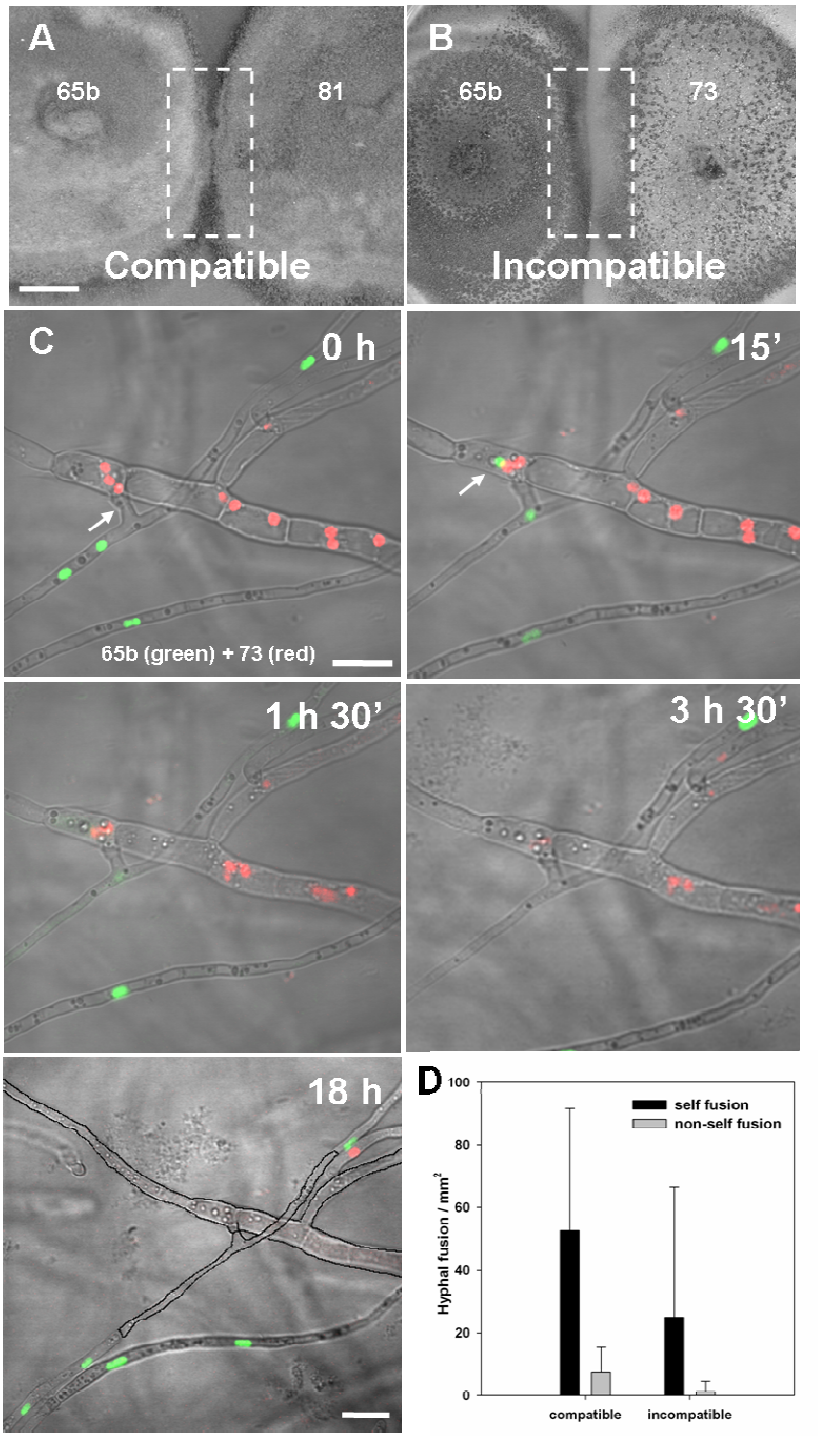
A
65b
81
Compatible
B
65b
73
Incompatible
C
0 h
65b (green) + 73 (red)
15'
1 h 30'
3 h 30'
18 h
D
self fusion
non-self fusion
Hyphal fusion / mm²
100
80
60
40
20
0
compatible
incompatible

FIGURE 1 Incompatibility reaction in the mature colony; A) Compatible reaction between race 65 and 81 in pairing done in PDA medium plate; B) Incompatible reaction between 65 and 73 with formation of barrier in the contact line (Culture age: 35 days; Bar: 1cm); C) Time lapse of hyphae fusion between different nuclei labelled strains (incompatible); After fusion, occurred the nuclear migration (15 minutes); After 1 h 30', cell vacuolization occurred and green nuclei started to disappear; At 3 h 30', green nuclei disappeared and hyphae seemed to be dead; Cell death in the compartment where the fusion occurred (after 18 h); D) Number of self-hyphae fusion and non-self fusion using different strains for compatible pairing (65 and 81 race) and incompatible pairing (65 and 73). (Number of fusion per mm^2 in a total of 10-12 pairings; standard deviation is high due data were not homogeneous).

Self fusion was relatively common in both compatible and incompatible reactions but non-self fusions were much rarer, especially with incompatible colony pairings in which it was less than 5% of the fusions observed (Fig. 1D). For incompatible strain combinations, we did not observe hyphal fusion in 50% of the 12 repetitions for both self and non-self fusion.

5.3 Heterokaryon incompatibility does not occur following CAT fusion

Pairing was done using conidia. It was possible to observe the fusion occurred between (non-self fusion) and within (self fusion) strains. Percentage of CAT fusion for each strain separately and in the mixture was shown in the Fig 2A. If we quantify just the fusion between different strains, at 72 h incubation time, the percentage of conidia involved in CAT fusion between compatible strains was 4.2% while between incompatible strains was 3.1% (2B).

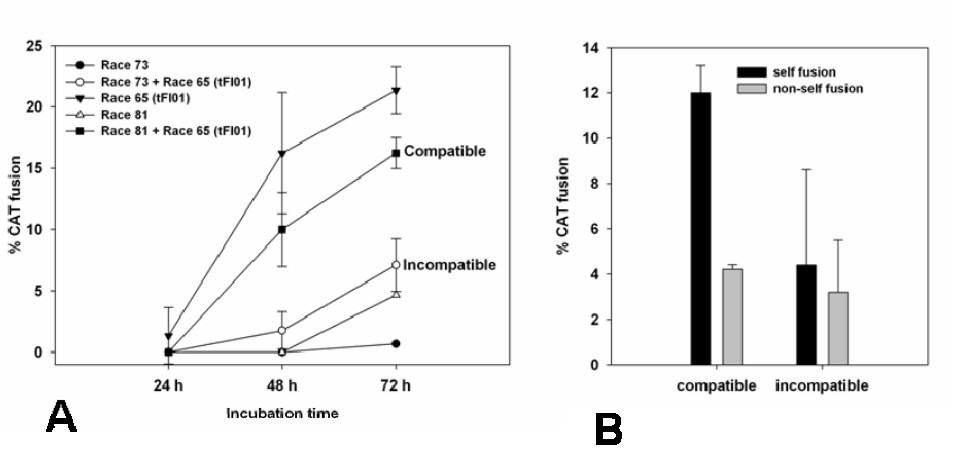

FIGURE 2 Percentage of CAT fusion: A) for each strain separately and in the mixture compatible or incompatible (self and non-self fusion was quantified) at 24, 48 and 72 h incubation time and conidial age 13 days old; B) Number detailed of CAT fusion at 72 h incubation time between different stains (non-self fusion).

Thus, the proportion of CAT fusions between incompatible strains (non-self fusion) was ~ 42%, while for the hyphae fusion in the mature colony was just 5% (Fig. 2B and 1D). Fused conidia were followed for more than 30 hours without cell death.

5.4 No obvious regulation of the cell cycle during CAT formation

Time lapse of CAT fusion and mitosis was visualized and nuclei migration could occur after mitosis in fused conidia (Fig. 3A). Nuclei behaviour after CAT fusion between different strains was evaluated. In general, CAT fusion could occur between uninucleate conidia of different strains (Fig 3B) and this was followed by mitosis, but not necessarily on both conidia (Fig 3C). Following, the fused conidia formed germ tubes and underwent further fusions. Nuclei were shown to migrate from one conidium to another to form the heterokaryon (Fig. 3D).

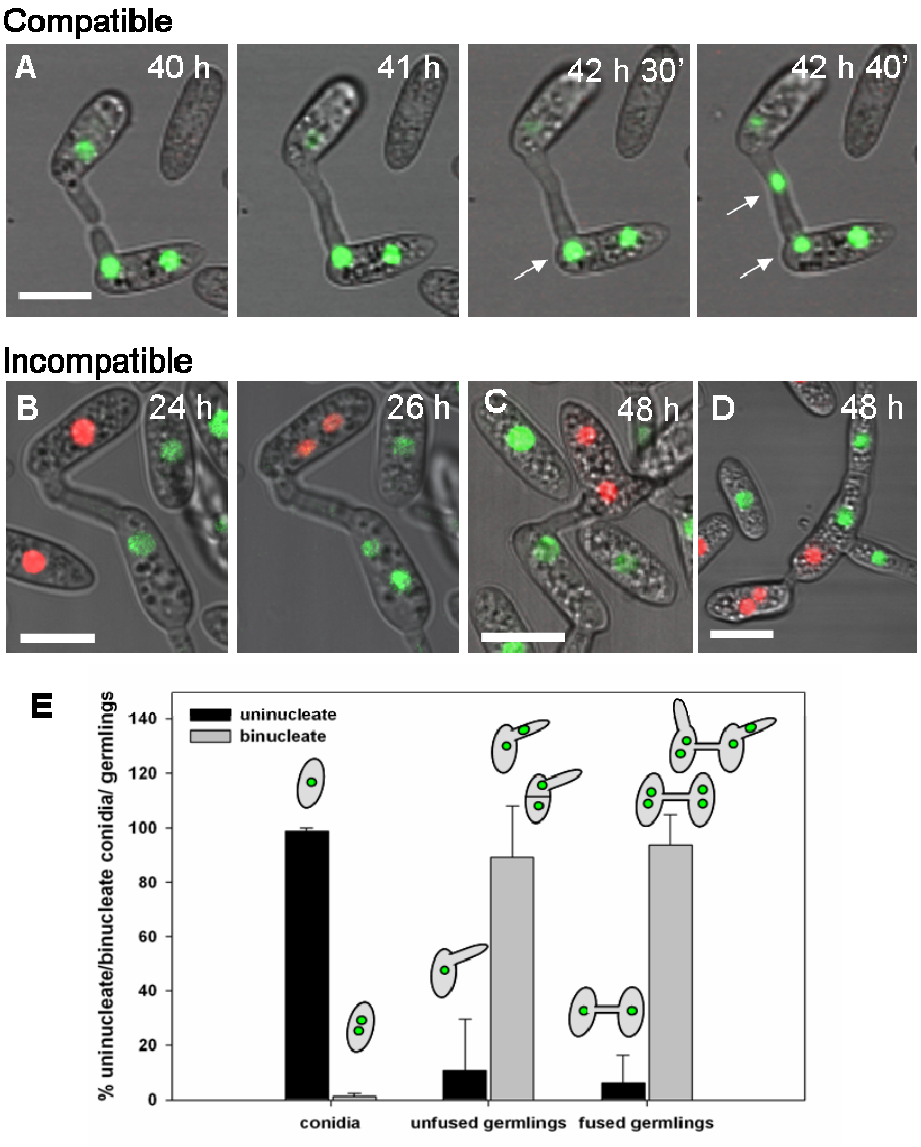

FIGURE 3 A) Time lapse of CAT fusion (tFI01) after 40h incubation time in water. After fusion, mitosis occurs in one of the nuclei (arrow) followed by migration in the CAT. B) CAT fusion occurred between uninucleate conidia of different strains (incubation time = 24 h); Mitosis occurred after 2 h (26 h); C) one conidia with one nuclei and other binucleate (48h incubation); D) Nuclei migrate from one conidia to another to form the heterokaryon (48h

incubation); E) Nuclear distribution at 48 h incubation time in water to different cell type (ungerminate conidia; unfused germlings and fused germlings). Three replicates (sample of 300 conidia per cell type per repetition). Bar: 10 μm.

5.5 Mitosis is not necessary for germination or CAT fusion.

Number of nuclei in different stages of germlings and CAT fused conidia was quantified at 48h incubation time (Fig 3E). According to our results, nuclei division is not essential for CAT fusion or germ tube formation since we observed uninucleate germlings and fused conidia.

5.6 Only few nuclei became yellow following CAT fusion between heterokaryons.

The formation of 'yellow' nuclei resulting from H1-eGFP and H4-tdimerRed was observed in 27% of the heterokaryotic fused cells (n = 60). Time of incubation was 72 h to observe these yellow nuclei. The results indicate two models to explain this phenomenon. The first one is based on the two proteins becoming incorporated into the same nucleus (Fig 4A). Time lapse during 24 h (after at least 48 h incubation) of fused conidia showed nuclei became yellow, and usually which was in the same cell compartment. In the second model proposed, two different haploid nuclei can fuse (Fig 4B). It was observed that is possible two different haploid nuclei share the same cell compartment and after one of these nuclei disappeared.

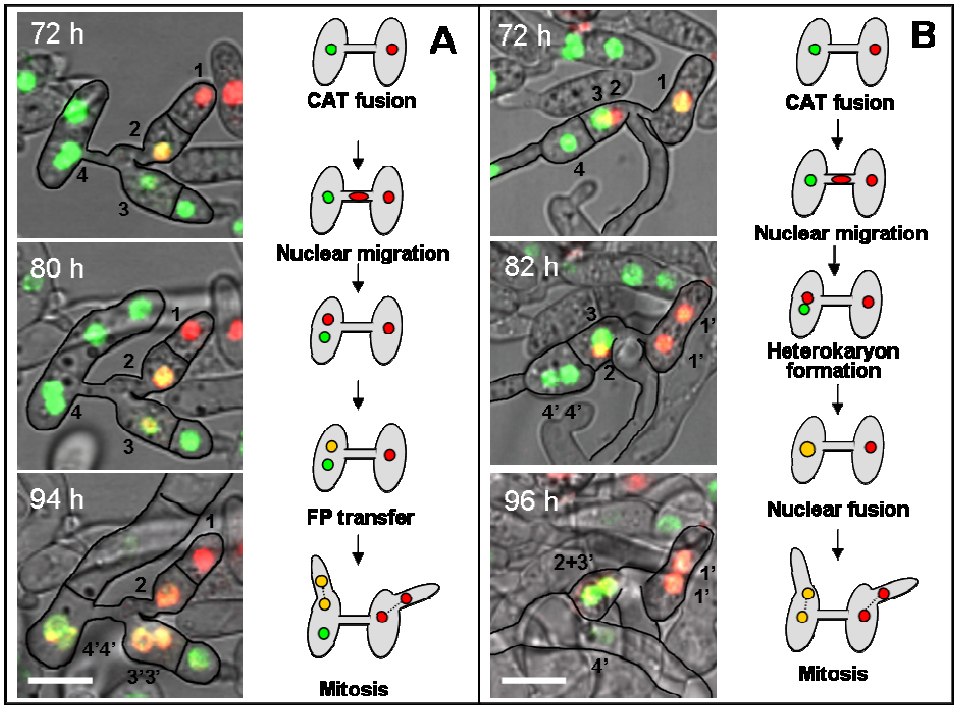

FIGURE 4 CAT fused conidia between different strains after 72 h incubation (48 h incubation in water and after plated in PDA incubated for 24h). Time lapse were performed during 24 h. Two models proposed: A). Model I proposed in which two proteins becoming incorporate into the same nucleus (3 and 4); B) Model II proposed that 2 different nuclei haploid (2 and 3) can fuse.

5.7 Nuclear distribution in the heterokaryotic colony is not uniform

Heterokaryotic colonies grow in selective medium were visualized at 7-15 days-old. Hyphae with both colours were found (Fig 5A), however the distribution of nuclei green, red and yellow was not uniform in the colony (Fig. 5A-B). However, it was rarely observed in some of hyphae from incompatible reaction or cell death, where anastomosis had occurred (Fig 5C).

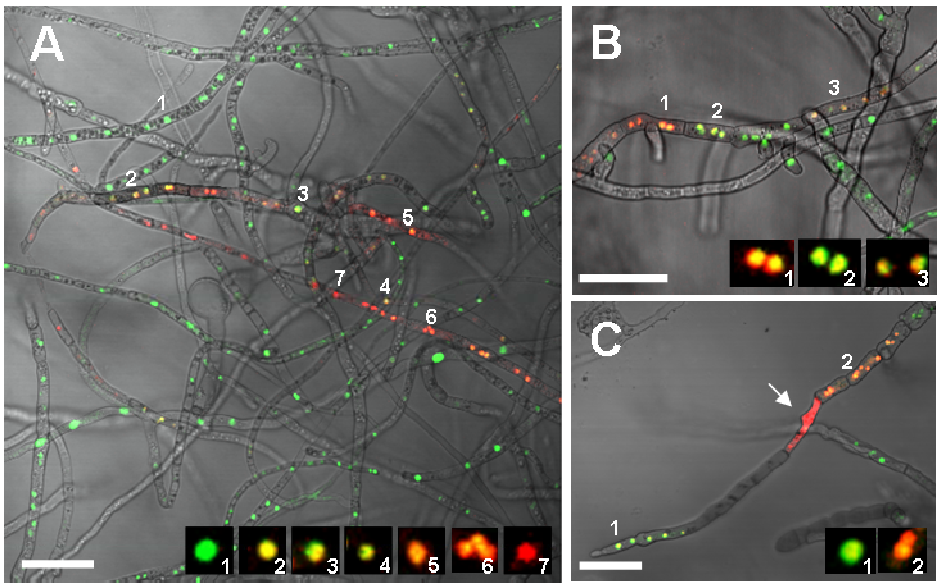

FIGURE 5 Heterokaryon colony grew in selective medium with 7-10 days culture old. A) Nuclear distribution in the colony was not uniform (in detail the different nuclei colours patterns observed in which numbers identified them in the colony). Images of nuclei are flatted image from Z stack and merged from the green and red channels); B) Detail of hyphae with nuclei showing different intensities of 'yellow'; C) Detail of hyphae that incompatible reactions were observed (arrow) and different nuclear colours patterns. Bar 40µm.

5.8 Recombinants are different from the parental strains

Conidia from the heterokaryotic colony were harvested and plated in medium containing both selective markers. Single conidia colonies were obtained with double resistance and sixteen double antibiotic-resistance colonies were tested to determine genetic exchange using PCR. Mycelium colour of these colonies were pink differently of the original strains that had black colony (Fig. 1A and 1B) and PCR results showed some of this putative recombinants were with both markers using different primers (Fig. 6).

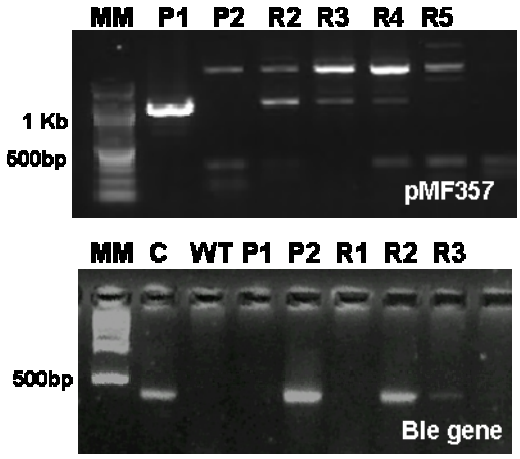

FIGURE 6 PCR amplification using primers pMF357 and Ble, MM = 100 bp and 1kb ladder, respectively; C = positive control for *Ble* gene (pGR02); WT = wild type used as negative control (race 73); P1 = tFI01 (65b-hH1-GFP + Hygromycin resistance); P2= tFI04 (73-hH4-tdimerRed + phleomycin resistance); R1-5 = Recombinants.

For GFP and tdimerRed genes the product was amplified just for the positive control (plasmid used for transformation) and respective parental strains in which the plasmid was inserted (data not shown). Pathogenicity tests showed parental strain tFI01 (race 65b) confirmed the race 65. However, parental strain tFI04 (race 73) was classified as race 72. Four recombinants (R2, R3, R15 and R19) were tested and just for the recombinant two (R2) symptoms were observed, however this was identified as race 8, for the others recombinants no symptoms were observed and they were classified as not pathogenic. Interestingly, R2 did not cause symptoms in Pérola cultivar used as susceptible control differently from the parental strains in which Pérola cultivar was completely susceptible.

5.9 Nuclear division in the mature colony showed different patterns.

A different type of mitotic behaviour occurred in vegetative hyphae in which we observed three types of mitosis: synchronous, asynchronous or in a wave down the hypha. These different patterns of nuclear division occurred in different regions of mycelium. In general, tip Type 1, showed the nuclei division occurs in a wave down the hypha (Fig 7A). In the tip Type 2, division occurs synchronously or from tip to middle (Fig 7B).

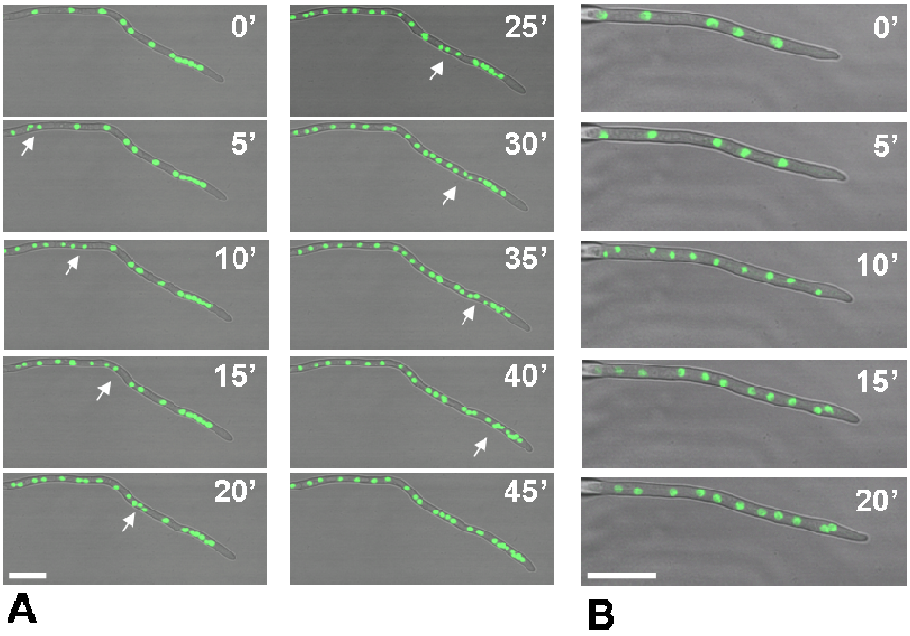

FIGURE 7 Different patterns of nuclei division on tip of hyphae in *Colletotrichum lindemuthianum;* A) Tip type 1 – nuclei division occurs in wave down the hyphae each 5 minutes; B) Tip type 2 – all nuclei divided at same time.

However, if we observed the intercalary region (between septum), all nuclei divided at same time in one cell, but not necessarily follow a pattern in

the following cells. Average time between mitosis on the tip was 3 h. After mitosis on the tips, there is hyphae extension and the septums arise. The patterns of nuclear division in mature hyphae are different to that in fused germlings, indicating that they are physiologically different.

6 DISCUSSION

This is the first report demonstrating the incompatibility reaction and cell death in the mature colony in *Colletotrichum lindemuthianum*, as the heterokaryon formation at early stages of development of the colony between two vegetative incompatible strains using live cell imaging and nuclei labelled with fluorescent proteins. These transformed strains facilitated CAT and hyphae fusion assays in the study of nuclear behaviour and the significance of this phenomenon in the genetic variation in this plant pathogen. Rech et al. (2007) used fluorescent-labelled histones for detection of hyphal fusion in *Sordaria macrospora* and described this is an efficient method to detect rare fusion event. *C. lindemuthianum* strains that showed vegetatively incompatible in the mature colony (Fig 1), did not show this behaviour during the early stages of development. In *N. crassa*, destruction of the heterokaryotic cell can be completed within 30 min after hyphae fusion (Glass & Kaneko, 2003).

Our results indicate that in *C. lindemuthianum* the hyphal compartmentation and cell death in the mature stage take more than 3 h (Fig 1C). Heterokaryosis and parasexual segregation were suggested among vegetative compatible nitrate non-utilizing mutants of *C. lindemuthianum*, although barriers to heterokaryon formation were detected due incompatible reactions (Castro-Prado et al., 2007). However, very little is known about heterokaryon incompatibility in this specie or heterokaryon formation between vegetatively incompatible strains.

The present study demonstrated that vegetative incompatibility was suppressed following CAT fusion how was suggested before (Roca et al., 2005). CAT fusion between incompatible strains occurred and fused conidia were followed for more than 30 hours without cell death. Moreover, heterokaryotic colonies resistant to both antibiotics developed without a pattern of nuclei distribution. These heterokaryotic hyphae were probably deriving as a result of CAT fusion since we observed the heterokaryons were not formed following fusion in the mature colony.

Fusion quantifications were performed and the percentage of CAT fusion between the incompatible strains was higher (~ 42%) compared to hyphae fusion in the mature colony (only 5%). Moreover, in general the number of fusion (self and non-self fusion) between incompatible strains was very small when compared to compatible strains. However, for CAT fusion between different strains (compatible and incompatible) not much difference was observed (Fig. 2B).

Nuclear behaviour during CAT fusion showed that there was no pattern for mitotic division, and nuclei showed migrate from one conidium to another to form the heterokaryon. This was not observed for hyphae fusion in the mature colony. For all fused hyphae between different incompatible strains, nuclei disappeared followed by cell death where the fusion occurred.

Time-lapse of fused heterokaryotic cells showed yellow nuclei can be formed for two different ways (Fig. 4). All these processes take a long time the mobility of nuclei in general was very slow. We observed the nuclear migration through the CAT after mitosis (Fig 3A), that is the time where the nuclei can move faster. Once two different nuclei were in the same compartment, we proposed that fusion occurs.

He et al. (1998) demonstrated the transfer of an entire dispensable chromosome between two incompatible isolates of *Colletotrichum*

gloesporieoides. However, the mechanism of this transfer was not determined, but if we observed the methodology used for them to obtain these recombinants, it is possible CAT fusion occurred because they mixed the spores of these different isolates and plated which could allow formation of CATs.

CATs have been described in several *Colletotrichum* species (Roca et al., 2003, 2005) and nuclear dynamic in *C. lindemuthianum* was analyzed using DAPI and propidium iodide. However, it was not possible identified which nuclei belong to one or other isolate. Roca et al. (2004) demonstrated that CAT fusion can occur between two different *Colletotrichum* species, vegetatively incompatible, which conidia were morphologically different (*C. lindemuthianum* and *C. gossypii*). Hybrids were obtained which suggest exchange of genetic material between them.

Recently, Friesen et al. (2006) presented evidence that a gene encoding a critical virulence factor was transferred from one species of fungal pathogen (*Stagonospora nodurum*) to another (*Pyrenophora tritici-repentis*) resulting in the emergence of a new disease of wheat. It was suggested CATs could be formed between these species, given that both co-occurs on wheat, facilitating genetic exchange (Sanders, 2006). According Walton (2000), horizontal gene transfer is the process by which genetic information of one organism is incorporated into the genome of another organism. The other organism could be the same or a different species and this process has importance of in the evolution. Our results demonstrated the transfer of genes between these incompatible strains and recombinants were formed by CAT fusion.

Finally, unlike germlings and fused conidia, nuclei division followed some kind of pattern. Nuclear behaviour was analysed on tip growth in the mature colony and interseptum showed interval between nuclear divisions is 3 hours. We observe two type of hyphae tip, the first one, some nuclei agglomerates on the tip (type 1) and the nuclear division occured in wave as

described for Hickey & Read (2009) in *Aspergillus*. In the second type, all nuclei are distributed equally inside the hyphae tip, and in this case, in general all nuclei divided at same time. In the region intercalary (interseptum), nuclei can divided all the same time in the same septum, or one first and after the others. However, not necessarily there are divisions in the cells neighbours. In *Neurospora crassa*, multinucleate organism, nuclear division is asynchronous (Freitag et al., 2004). Heterokaryotic colonies usually showed different patterns of nuclei distribution could be explained by this mitotic behaviour. Nuclei were not shown migrate from um compartment to another due presence of septum. In general all nuclei in the same compartment presented the same colour.

7 CONCLUSIONS

- *Colletotrichum lindemuthianum* nuclei could be labelled with different fluorescent proteins.
- Heterokaryon incompatibility occurred following fusion of vegetative incompatible strains in the mature colony.
- Heterokaryon incompatibility did not occur following CAT fusion.
- Mitosis was not necessary for germination or CAT fusion.
- Nuclear division in the mature colony showed different defined patterns
- Only few nuclei became yellow following CAT fusion between heterokaryons.
- Recombinants were formed after CAT fusion and showed differences from the parental strains.

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