DÉRICA GONÇALVES TAVARES

MATING TYPE AND INFECTION-RELATED GENE ANALYSIS, SEXUAL REPRODUCTION, MICROCONIDIA, AND CONIDIAL ANASTOMOSIS TUBE EVENTS IN *Pyricularia oryzae*

LAVRAS – MG

2021

DÉRICA GONÇALVES TAVARES

MATING TYPE AND INFECTION-RELATED GENE ANALYSIS, SEXUAL REPRODUCTION, MICROCONIDIA, AND CONIDIAL ANASTOMOSIS TUBE EVENTS IN *Pyricularia oryzae*

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

Dr. Eduardo Alves

Orientador

Dr. Silvino Intra Moreira

Dra. Patrícia Gomes Cardoso

Coorientadores

LAVRAS – MG

2021

Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Tavares, Dérica Gonçalves.
Mating type and infection-related gene analysis, sexual reproduction, microconidia, and conidial anastomosis tube events in *Pyricularia oryzae* / Dérica Gonçalves Tavares. - 2021.
116 p. : il.

Orientador(a): Eduardo Alves.
Coorientador(a): Silvino Intra Moreira, Patrícia Gomes Cardoso.
Tese (doutorado) - Universidade Federal de Lavras, 2021.
Bibliografia.

1. Wheat blast. 2. Crossing. 3. Genetic transformation. I. Alves, Eduardo. II. Moreira, Silvino Intra. III. Cardoso, Patrícia Gomes. IV. Título.

DÉRICA GONÇALVES TAVARES

ANÁLISE DOS GENES *MATING TYPE* E GENES RELACIONADOS A INFECÇÃO, REPRODUÇÃO SEXUAL, MICROCONÍDIOS E TUBOS DE ANASTOMOSE DE CONÍDIOS EM *Pyricularia oryzae*

MATING TYPE AND INFECTION-RELATED GENE ANALYSIS, SEXUAL REPRODUCTION, MICROCONIDIA, AND CONIDIAL ANASTOMOSIS TUBE EVENTS IN *Pyricularia oryzae*

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

APROVADA em 30 de setembro de 2021.

Dr. Eduardo Alves	UFLA
Dr. Eustáquio Souza Dias	UFLA
Dr. Eduardo Seiti Gomide Mizubuti	UFV
Dr. Fabiano José Perina	EMBRAPA
Dr. Silvino Intra Moreira	UFLA

Prof. Dr. Eduardo Alves
Orientadora

Dr. Silvino Intra Moreira
Dra. Patrícia Gomes Cardoso
Coorientadores

LAVRAS-MG

2021

A Deus, que me dá força, que me guia e sem Ele nada seria possível.

À minha mãe, Hildenise Gonçalves Tavares, que sempre foi meu apoio e exemplo.

Às minhas irmãs, Débora e Makellen, pelo carinho, apoio e amizade.

Dedico.

AGRADECIMENTOS

Agradeço a Deus, por sempre me dar forças, para superar todos os obstáculos, por sempre estar comigo, ajudando-me, guiando-me e dando-me coragem. Agradeço-Lhe por tudo.

À minha mãe Hildenise Gonçalves pelo exemplo de vida, dedicação, força e sacrifícios. Por ter me permitido chegar até aqui, pelas orações, por ser meu apoio e por tudo que fez e faz por mim e minhas irmãs.

Às minhas irmãs, Débora Gonçalves Tavares e Makellen Gonçalves Tavares, por todo o carinho, amizade, apoio e pela disponibilidade de sempre poderem me ajudar.

A toda minha família que sempre está comigo me ajudando, apoiando e torcendo por mim, em especial em memória à minha avó Maria Dourado Tavares.

A todos os meus amigos, em especial, a Camila Botelho e Juliete de Lara por todo apoio, companheirismo e amaizade.

Ao meu orientador, Prof. Dr. Eduardo Alves, pelo apoio e orientação nas atividades acadêmicas.

Aos meus coorientadores Dr. Silvino Moreira, pelas ideias e orientações na condução dos experimentos, e a Prof. Dra. Patrícia Cardoso, pelo apoio e orientação não apenas nas atividades acadêmicas, mas pela amizade e disponibilidade.

Ao órgão de fomento, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão da bolsa de estudos no Brasil e durante os nove meses de bolsa no exterior pelo CAPS/PRINT.

Ao Programa de Pós-Graduação em Microbiologia Agrícola da Universidade Federal de Lavras, e aos professores do programa pelos ensinamentos e por contribuírem para a minha formação profissional.

Aos funcionários do Setor de Microbiologia Agrícola do Departamento de Biologia pela prestação dos servisos, boa vontade e gentileza.

As técnicas do Laboratório de Microscopia Eletrônica e Análise Ultraestrutural Aline Noberto e Elisa Castro por toda ajuda e amizade.

Aos amigos que fiz no Laboratório Adriano Dorigan, Aline Vieira, Gilmara Soares, Tamara Leite, Indiara Carol, Ginaini Moura, Yaya Kone, Luis Alberto, Gabriella Ramos, e as pós-docs Claúdia Labory e Patrícia Silveira pela amizade e contribuições nas atividades.

Muito Obrigada!

"A mente que se abre a uma nova ideia
jamais voltará ao seu tamanho original."
(Albert Einstein).

"A vida não é fácil para nenhum de nós. Mas
e daí? Temos que ter perseverança e, acima
de tudo, confiança em nós mesmos."
(Marie Curie)

RESUMO

A brusone, causada pelo fungo *Pyricularia oryzae,* é uma das principais doenças de plantas no mundo, atingindo várias espécies de gramíneas como arroz, trigo, triticale, cevada e centeio. Os genes que regulam a reprodução sexuada são denominados *mating type*. As vias Pmk1 MAPK, MPS1, e Osm1 MAPK regulam a formação de apressórios, penetração, conidiação, crescimento invasivo das hifas, e estresse hiperosmótico. Nesse trabalho foram avaliadas a reprodução sexuada utilizando isolados de *P. oryzae* patotipos *Triticum* e *Oryzae*, as sequências dos genes *mating type* e a expressão desses genes em condições de pareamento. Foi realizado o nocaute dos genes RGF1 e RGF2 do isolado Ku80 de *P. oryzae* pelo método *Split-Marker*. Para avaliar a interação desses genes com as vias Pmk1, Mps1 e Osm1 MAPK foram realizados os ensaios de fosforilação TEY (Pmk1 e Mps1) e TGY (Oms1). Foram avaliadas diferentes concentrações de β-glucanase e Lysing enzymes para obtenção de protoplastos de *P. oryzae* patótipo *Triticum* e microscopia eletrônica de transmissão (MET) foi utilizada para avaliar possíveis danos celulares nos protoplastos. Foi também avaliada a produção de microconídios e a formação de tubos de anastomose de conídios (CATs) desse patógeno. Um cruzamento fértil foi obtido a partir do isolado testador Guy11 e o isolado de trigo 12.1.053i. A análise dos genes *mating type* mostrou a presença de diferentes haplótipos e foram observadas que algumas *Single Nucleotide Polymorphisms* (SNPs) podem ter levado a alterações na sequência de aminoácidos e consequentemente na proteína predita de MAT1-1-3b. Uma região de repetição dos dinucleotídeos citosina e timina (CT) localizada a 5′- UTR de MAT1-1-3 mostrou que os isolados de *Triticum* apresentam uma região CT menor, o que pode ter influenciado na expressão do gene MAT1-1-3. A falta de expressão de MAT1-1-3 durante o ciclo sexual no par infértil, também mostrou que esse gene pode desempenhar um importante papel na fertilidade do isolado. Não houve alteração nos níveis de fosforilação em Oms1 nos mutantes *Δrgf1* e *Δrgf2*. Os níveis de fosforilação de Pmk1 e Mps1 estavam reduzidos no mutante *Δrgf1* e não foram reduzidos no mutante *Δrgf2*, indicando que apenas RGF1 está envolvido na ativação da sinalização de Pmk1 e Mps1 MAPK. O composto Lysing enzyme foi melhor para a obtenção dos protoplastos e concentrações acima de 30 mg/mL afeta a viabilidade dos mesmos. O armazenamento dos protoplastos não é ideal a -20 ºC mesmo com a utilização de glicerol como crioprotetor. Microconídios foram observados em meio de aveia agar e também em cultura submersa utilizando meio completo. Poucos eventos de CATs foram observados devido à alta taxa de germinação dos conídios de *P. oryzae*, no entanto esse é o primeiro relato de eventos de CATs em *Pyricularia* e tais eventos, poderiam ser empregados em estudos relacionados à parassexualidade desse fungo.

Palavras-chave: Brusone do trigo. Cruzamento. Expressão genética. Transformação genética. Nocaute de genes.

ABSTRACT

Blast disease caused by *Pyricularia oryzae* is one of the main plant diseases in the world, affecting several species of grasses such as rice, wheat, triticale, barley, and rye. The genes that regulate sexual reproduction are called mating type genes. The Pmk1 MAPK, MPS1, and Osm1 MAPK pathways regulate appressorium formation, penetration, conidiation, invasive hyphal growth, and hyperosmotic stress. In this study, sexual reproduction using isolates of *P. oryzae* pathotypes *Triticum* and *Oryzae*, the sequences of the mating type genes and the expression of these genes under pairing conditions were evaluated. Knockout of the RGF1 and RGF2 genes of the *P. oryzae* isolate Ku80 was performed using the Split-Marker method. The interaction of RGF1 and RGF2 genes with the Pmk1, Mps1, and Osm1 MAPK pathways was evaluated by TEY (Pmk1 and Mps1) and TGY (Oms1) phosphorylation assays. Different concentrations of β-glucanase and Lysing enzymes were evaluated to obtain protoplasts of *P. oryzae* pathotype *Triticum* and transmission electron microscopy (TEM) was used to assess possible cellular damage in the protoplasts. The production of microconidia and the formation of conidia anastomosis tubes (CATs) of this pathogen were also evaluated. A fertile cross was obtained from the tester Guy11 isolate and wheat isolate 12.1.053i. Analysis of the mating type genes showed the presence of different haplotypes, and it was observed that some Single Nucleotide Polymorphisms (SNPs) may have led to alterations in the amino acid sequence and consequently in the predicted protein of MAT1-1-3b. A repeat region of cytosine and thymine (CT) dinucleotides located at the 5'-UTR of MAT1-1-3 showed that *Triticum* isolates have a smaller CT region, which may have influenced the expression of the MAT1-1-3 gene. The lack of MAT1-1-3 expression during the sexual cycle in the infertile pair also showed that this gene could play an important role in the fertility of the isolate. There was no change in the phosphorylation levels of Oms1 in the *Δrgf1* and *Δrgf2* mutants. Phosphorylation levels of Pmk1 and Mps1 were reduced in the *Δrgf1* mutant and were not reduced in the *Δrgf2* mutant, indicating that only RGF1 is involved in the activation of Pmk1 and Mps1 MAPK signaling. The Lysing enzyme compound was better for obtaining protoplasts and concentrations above 30 mg/mL affected their viability. Storage of protoplasts is not ideal at -20°C even with the use of glycerol as a cryoprotectant. Microconidia were observed in oat agar medium and submerged culture using a complete medium. Few CAT events were observed due to the high germination rate of *P. oryzae* conidia; however, this is the first report of CAT events in *Pyricularia* and such events could be used in studies related to the parasexuality of this fungus.

Keywords: Wheat blast, Crossing, Gene expression, Genetic transformation, Gene knockout.

LISTA DE ILUSTRAÇÕES

PRIMEIRA PARTE

Figure 1 - Esquema do ciclo sexual de *Pyricularia* spp. com etapas da mofogênese e regulação gênica. 20

Figure 2 - Tipos de esporos e estruturas produzidas por *Pyricularia* spp. A. Macroconídios; B. Microconídios; C. Peritécio, ascas com ascósporos. 24

Figure 3 - O ciclo parassexual. a. Hifas de homocários geneticamente diferentes. b. Anastomose de hifas (heterocário). c. Cariogamia. d. Recombinação mitótica. e. Eventos de haploidização.....25

Figure 4 - Processo de fusão de CATs em *Neurospora crassa*. 27

Figure 5 - Vias de sinalização envolvidas na morfogênese relacionadas a infecção em *Pyricularia oryzae*. 28

Figure 6 - Esquema da abordagem *Split-marker* para substituição direcionada de genes. 37

SEGUNDA PARTE

ARTIGO 1

Figure 1 - Photograph (A) and Photomicrographs (others) of sexual reproduction in *Pyricularia oryzae*. 57

Figure 2 - Photographs of the induction of sexual reproduction in *Pyricularia oryzae* isolates. 58

Figure 3 - Perithecia from crossing between Guy11 *Oryzae* isolate and 12.1.053i *Triticum* isolate. 59

Figure 4 - Photomicrographs of asci and ascospores from crossing between Guy11 and 12.1.053i isolates. 60

Figure 5 - Photomicrographs of ascospores germination and colonies from ascospores of crossing between Guy11 *Oryzae* and 12.1.053i *Triticum* isolates.....61

Figure 6 - Phylogeny of mating type gene sequences of *Pyricularia oryzae* performend using Bayesian Monte Carlo Chain Markov (MCMC) method for ten million of interactions with sampling in each 1000. 63

Figure 7 - Comparison of CT-dinucleotide repeats (CT)n region upstream 5'-UTR (untranslated region - UTR) of MAT1-1-3 in *Pyricularia oryzae* isolates. 67

Figure 8 - Expression of mating type genes during sexual reproduction in *Pyricularia oryzae*.....69

ARTIGO 2

Figure 1 - Representation of split-marker-targeted replacement showing primers, genes, and recombination sites. 83

Figure 2 - Photographs of colony morphologies (A-D) and photomicrographs of conidia (E-H) from Ku80 (A, E), *Δrgf1*-1 (B, F), *Δrgf1*-2 (C, G), and *Δrgf2* (D, H). 88

Figure 3 - TEY and TGY phosphorylation assay. Anti-TEY detected phosphorylation levels of Pmk1 (42 kDa) and Mps1 (46 kDa) and Anti-TGY detected phosphorylation level of Oms1 (42 kDa). Proteins from Ku80 wild type, and *Δrgf1* and *Δrgf2* mutants.....89

ARTIGO 3

Figure 1 - *Pyricularia oryzae* pathotype *Triticum* protoplasts released using β-glucanase and Lysing enzymes at different concentrations and incubation times. 99

Figure 2 - Photomicrographs of *Pyricularia oryzae* pathotype *Triticum* protoplasts obtained with 30 mg/mL of Lysing enzymes in different times.....99

Figure 3 - *Pyricularia oryzae* pathotype *Triticum* protoplasts released using different concentrations of Lysing enzymes after 2 h of incubation. 100

Figure 4 - Number of viable protoplasts obtained with different concentrations of Lysing enzymes solution. A) Protoplasts recovered on the same day and B) after one week. 101

Figure 5 - Laser Confocal microscope images of *Pyricularia oryzae* pathotype *Triticum* protoplasts in the live-dead test. 102

Figure 6 - Transmission Electromicrograph of *Pyricularia oryzae* pathotype *Triticum* protoplasts released with Lysing enzymes concentrations. 103

ARTIGO 4

Figure 1 - Photomicrographs of microconidia from *Pyricularia oryzae* pathotype *Triticum* in false heads (A, B). C) Swollen cells and phialides. F, E) Microconidia. 112

Figure 2 - Photomicrographs of test CATs in *Pyricularia oryzae* using water as medium. 113

Figure 3 - Photomicrographs of probable CAT events in *Pyricularia oryzae*. 114

LISTA DE TABELAS

SEGUNDA PARTE

ARTIGOS 1

Table 1 - *Pyricularia oryzae* isolates used in the crosses and idiomorph evaluation analysis. 53

Table 2 - Isolates from which sequences were used to the analysis of idiomorphs evolution. 54

Table 3 - Primers used in this study for real-time RT-qPCR (KANAMORI et al., 2007). 56

Table 4 - Polymorphism in MAT1 locus in *Pyricularia oryzae* isolates.....62

Table 5 - Amino acid alteration in protein prediction due single nucleotide polymorphism in MAT1-1 idiomorph of *Pyricularia oryzae*.....65

Table 6 - Amino acid alteration in protein prediction due single nucleotide polymorphism in MAT1-2 idiomorph of *Pyricularia oryzae*.....66

ARTIGO 2

Table 1 - Primers useds in this study. 83

Table 2 - Combination of primers and templates for PCR reactions.....85

ARTIGO 4

Table 1 - Measurements of microconidia of *Pyricularia oryze* pathotype *Triticum*. 111

SUMÁRIO

PRIMEIRA PARTE 13

1 INTRODUÇÃO 13

2 REFERENCIAL TEÓRICO 14

2.1 A brusone do trigo 14

2.2 Agente etiológico da brusone do trigo 16

2.3 Variabilidade genética 17

2.3.1 Variabilidade genética em *P. oryzae* 18

2.4 Ciclo sexual 19

2.5 Ciclo assexual 23

2.6 Ciclo parassexual 24

2.7 Vias de sinalização no processo de infecção da planta por *P. oryzae* 27

2.8 Transformção genética em fungos 31

2.8.1 Transformação mediada por protoplastos 32

2.8.2 Procedimentos alternativos de transformação que não requerem protoplastos 35

2.8.3 Abordagem *Split-Marker* para substituição direcionada de genes 36

REFERÊNCIAS 37

SEGUNDA PARTE – ARTIGOS 49

ARTIGO 1 – MATING TYPE GENE ANALYSIS AND SEXUAL REPRODUCTION IN WHEAT BLAST FUNGI *Pyricularia oryzae* PATHOTYPE *Triticum* * 49

ARTIGO 2 – RELATIONSHIP OF RGF1 AND RGF2 GENES WITH THE PMK1, MPS1, AND OSM1 MAP KINASES PATHWAYS IN *Pyricularia oryzae* * 78

ARTIGO 3 – PROTOPLAST RELEASES OF *Pyricularia oryzae* PATHOTYPE *Triticum* UNDER DIFFERENT CONCENTRATIONS OF LYSING ENZYMES * 93

ARTIGO 4 – MICROCONIDIA AND CONIDIA ANASTOMOSIS TUBES IN *Pyricularia oryzae* PATHOTYPE *Triticum* * 107

PRIMEIRA PARTE

1 INTRODUÇÃO

A brusone do trigo é uma das principais doenças que afetam a produção dessa cultura. As perdas provocadas pela brusone podem variar de acordo com as condições climáticas e cultivares utilizadas, podendo chegar a 100 % da produção (IGARASHI et al., 1986; (GOULART; SOUSA; URASHIMA, 2007; KOHLI et al., 2011). O agente etiológico é o ascomiceto *Pyricularia oryzae* patotipo *Triticum* (AGHNOUM et al., 2019; PIECK et al., 2017; TOSA; CHUMA, 2014) podendo atacar toda a parte aérea das plantas. A doença causa grande severidade nas espigas, tornando sua porção superior branqueada (KOHLI et al., 2011).

Isolados de *Pyricularia* spp. produzem conídios e podem produzir também microconídios, conforme observado para *P. oryzae* (ZHANG et al., 2014). Os conídios são responsáveis pela infecção direta desse patógeno, e estudos indicam que os microconídios de *P. oryzae* podem estar relacionados com a disseminação da doença a partir de regiões infectadas para partes saudáveis da planta (ZHANG et al., 2014). A reprodução sexuada de *P. oryzae* em condições de campo, ainda não foi observada, embora existam populações com isolados sexualmente compatíveis tanto em teste *in vitro* quanto em plantas (HAYASHI et al., 1997; CASTROAGUDÍN et al., 2017).

Estudos com isolados de *P. oryzae* patotipo *Triticum* de diversas regiões do Brasil e diferentes Poaceas indicaram alta diversidade genética e diversidade na virulência em linhagens de trigo diferenciadoras (CASTROAGUDÍN et al., 2017). Essas populações exibiram estrutura genética consistente com um sistema reprodutivo misto em que ocorre a reprodução sexuada seguida por dispersão de clones adaptados localmente (CASTROAGUDÍN et al., 2017; MACIEL et al., 2014). Alguns autores estudaram a importância da recombinação parassexual como fonte de variabilidade em *Pyricularia* e juntamente com as mutações tem sido apontado como a maior causa de variação na sua patogenicidade, devido os relatos do ciclo sexual serem apenas em condicões de laboratorio (URASHIMA; IGARASHI; KATO, 1993; TSUJIMOTO NOGUCHI, 2011).

Várias vias de sinalização estão relacionadas à patogenicidade e já foram caracterizadas em *P. oryzae*. A via Pmk1 MAP quinase regula o estágio final da formação de apressórios, penetração e crescimento invasivo de hifas (LI; ZHOU; XU,

2012). A via Mps1 MAP quinase é importante para conidiação, integridade da parede celular e penetração em plantas (XU; STAIGER; HAMER, 1998) enquanto a via de sinalização Osm1 MAP quinase, regula a osmorregulação, resposta a estresse e sensibilidade a fungicidas (DIXON et al., 1999; LI; ZHOU; XU, 2012). Duas proteínas Ras, Ras1 e Ras2, foram identificadas funcionando *upstream* a via de sinalização Pmk1 em *P. oryzae* (PARK et al., 2006) e parecem estar envolvidas na regulação dessa via (ZHAO; MEHRABI; XU, 2007). Dois genes GEF (RGF1 e RGF2) foram identificados como reguladores do gene Ras2 em *P. oryzae*, em que RGF2 parece ser um gene dispensável em *P. oryzae* e RGF1 parece ser essencial para a formação de apressórios e patogenicidade.

Dessa forma, estudos sobre os ciclos sexual, assexual e parassexual podem ajudar a elucidar as estratégias de dispersão, bem como as potencias fontes de variabilidade genética do patógeno causador da brusone do trigo e outras poaceas, preocupação fitosanitária global. Enquanto a transformação genética, utilizando protocolos adaptados aos isolados em estudo pode facilitar o entendimento de processos específicos, e ser aplicado em estudos relacionados à infecção podendo ajudar a explicar processos que ainda não foram totalmente esclarecidos na relação patógeno-hospedeiro em *P. oryzae*.

2 REFERENCIAL TEÓRICO

2.1 A brusone do trigo

O trigo está entre os cereais mais cultivados, ocupando o segundo lugar na produção mundial e tendo a China e a União Europeia como os maiores produtores. O Brasil encontra-se como o décimo sexto país na produção mundial de trigo, apresentando maior número de importação que exportação (USDA, 2021). A produção brasileira de trigo na safra 2020 foi de 6,2 milhões de toneladas, com uma média anual de 11,4 milhões/ano (CONAB, 2021). O estado do Paraná é o maior produtor de trigo seguido pelo Rio Grande do Sul que juntos representam 89% da produção nacional, fazendo da região Sul a maior produtora desse grão (CONAB, 2021). O trigo é um dos cerais mais antigos e cultivados no mundo, sendo parte importante na alimentação e na economia mundial. No entanto, o surgimento de pragas, doenças e resistência aos defensivos agrícolas podem limitar a produção desse cereal.

A brusone do trigo foi identificada no Brasil em 1985 no Estado do Paraná, com seis municípios severamente afetados (IGARASHI et al., 1986) tornando-se uma grande ameaça a produção de trigo. Em 1986, a brusone se espalhou para o norte e oeste do Paraná, noroeste de São Paulo e sul do Mato Grosso do Sul (CRUZ; VALENT, 2017). Essa doença é provocada pelo fungo Ascomyceto *P. oryzae* patótipo *Triticum* e pode atacar todas as partes da planta acima do solo, com manchas elípticas nas folhas e com severidade maior quando ataca a espiga, onde a porção acima da infecção torna-se branqueada e impede a formação dos grãos, enquanto a porção abaixo do ponto de infecção, continua saudável e produz os grãos (KOHLI et al., 2011). As perdas provocadas pela brusone podem variar de acordo com as condições climáticas e cultivares utilizadas, podendo chegar a quase 100% de perda na produção (IGARASHI et al., 1986; GOULART; SOUSA; URASHIMA, 2007; KOHLI et al., 2011).

Em 1987 houve perdas no rendimento da produção de trigo nos estados do Paraná, Mato Grosso do Sul e São Paulo, com variação entre 10,5 e 53% (GOULART; PAIVA, 1992). A brusone também foi observada no Rio Grande do Sul, causando perdas variáveis (PICININI; FERNANDES, 1990). A brusone no trigo também atingiu a Bolívia em 1996, o Paraguai em 2002 e a Argentina em 2007. Na Bolívia, resultou em perdas de quase 80 % da produção em 1996 e de 100 % no ano seguinte (BAREA; TOLEDO, 1996; KOHLI et al., 2011). No ano de 2016, a brusone do trigo também foi relatada em alguns distritos de Bangladesh (MALAKER et al., 2016). Análises genômicas mostraram que os isolados fúngicos das regiões de Bangladesh estavam intimamente relacionados com isolados da América do Sul (FARMAN et al., 2017; MALAKER et al., 2016) e a análise de patogenômica confirmou que o fungo causador da brusone de trigo em Bangladesh provavelmente veio da América do Sul (ISLAM et al., 2016).

A brusone do trigo é uma das principais doenças que afetam a produção dessa cultura. O fungo reduz o rendimento e a qualidade do grão nas cultivares altamente suscetíveis, nas quais os grãos infectados, ficam pequenos, enrugados, deformados e com baixo peso (GOULART; SOUSA; URASHIMA, 2007). Quando a infecção ocorre durante a floração, a doença provoca perdas ainda maiores de rendimento (GOULART; SOUSA; URASHIMA, 2007), as quais podem chegar até 100% nas cultivares sensíveis (GOULART; PAIVA 1992, 2000). Na planta o branqueamento da espiga é o sintoma mais visível, que ocorre devido infecção no pedúnculo que pode bloquear a translocação

de fotossintatos, podendo resultar na morte das partes superiores da espiga (CRUZ; VALENT, 2017). No local da infecção observa-se uma coloração marrom a esbranquiçada, enquanto as glumas infectadas apresentam lesões elípticas com margens avermelhadas a cinza-escuro (CRUZ; VALENT, 2017). Durante a esporulação, as lesões apresentam centros acinzentados e quando liberados os esporos, as lesões mostram coloração branca a bronzeada (IGARASHI et al., 1986; IGARASHI, 1990). Em relação aos grãos, quando a infecção ocorre mais tarde a perda de rendimento é menor, entretanto pode aumentar as chances de transmissão por sementes desse patógeno (IGARASHI, 1990). As lesões maduras geralmente apresentam uma margem marrom escuro a marrom avermelhada, e também pode apresentar halos cloróticos ou amarelos (CRUZ; VALENT, 2017). As lesões individuais são, geralmente, em forma de olho ou elípticas, mas se juntam em infecções severas, podendo resultar na morte total da planta (CRUZ; VALENT, 2017).

Além do uso de cultivares resistentes, o controle químico é uma alternativa para ajudar a reduzir a gravidade da doença. Tratamentos de sementes com fungicidas podem diminuir a transmissão pela semente, mas não protegem a planta da infecção na espiga, devido à natureza não sistêmica do patógeno e a infecção na espiga ocorre principalmente por esporos dispersos pelo ar (KOHLI et al., 2011). Fungicidas que combinam triazóis com estrobilurinas foram utilizados efetivamente no estágio de espiga para controlar a doença em variedades moderadamente resistentes, mas em variedades susceptíveis não resultam em um bom controle da doença, não sendo rentáveis (KOHLI et al., 2011). E o uso extensivo de fungicidas a base de estrobilurina (QoI) no Brasil levou à mutações do citocromo b (*cyt b*) que confere resistência ao isolados de trigo e outras gramíneas a esse fungicida (CASTROAGUDÍN et al., 2014).

2.2 Agente etiológico da brusone do trigo

O gênero *Pyricularia* inclui espécies que são patógenos de uma ampla gama de plantas monocotiledôneas. Pertence ao reino Fungi, divisão Ascomycota, classe Sordariomycetes, ordem Magnaporthales e familia *Magnaporthaceae*. A fase sexual de espécies de *Pyricularia* foram nomeadas como *Magnaporthe* spp. (COUCH; KOHN, 2002). Entretanto, recentemente segundo o Novo Código Internacional de Nomenclatura para Algas, Fungos e Plantas, tem-se utilizado o nome *Pyricularia* para ambas as fases sexuada e assexuada (TOSA; CHUMA, 2014). O nome *Pyricularia* foi

denominado por Saccardo (1880), devido a forma piriforme dos conídios de *Pyricularia grisea*. Cavara (1892) designou isolados do arroz como *P. oryzae* e Sprague (1950) utilizou nomes de espécies de *Pyricularia* com base no hospedeiro, sendo *P. oryzae* para isolados de arroz e *P. grisea* para isolados de outros cereais e gramíneas.

Alguns autores consideram que o agente etiológico da brusone do trigo é um fungo que pertence a uma subpopulação dentro de *Pyricularia oryzae,* utilizando a classificação subespecífica de patótipos: *P. oryzae* patótipo *Triticum* (PoT, infectivo a trigo); patótipo *Oryza* (PoO, arroz); patótipo *Eleusine* (PoE, painço de dedo), patótipo *Setaria* (PoS, milho italiano), patótipo *Lolium* (PoL, azevém perene), patótipo *Panicum* (Pop, painço) e patótipo *Avena* (PoA, aveia) (AGHNOUM et al., 2019; CRUZ; VALENT, 2017; PIECK et al., 2017; TOSA; CHUMA, 2014). Essas linhagens compartilham um grau muito alto de similaridade de sequência do genoma, e foram agrupados como uma única espécie (COUCH; KOHN, 2002; KLAUBAUF et al., 2014; GLADIEUX et al., 2018). No Brasil foram encontrados dois grupos distintos do agente causal da brusone do trigo. Segundo Urashima et al. (1993) um grupo infecta arroz e o trigo, e o outro grupo infecta apenas o trigo.

Um trabalho com DNA 'fingerprinting' utilizando sondas de DNA repetitivo MGR563 e MGR586 demonstrou alto nível de diferenciação entre os patótipos *Oryza* (PoO) e *Triticum* (PoT) de *P. oryzae* do Brasil (FARMAN, 2002). Outro estudo mostrou que a população de *P. oryzae* do Brasil adaptada ao trigo era altamente distinta da população adaptada ao arroz e a análise de 69 isolados de trigo mostrou que nenhum desses fungos foi capaz de infectar o arroz (MACIEL et al., 2014).

2.3 Variabilidade genética

A recombinação genética diz respeito à troca de genes entre duas moléculas de DNA, formando novas combinações desses genes em um cromossomo. Em eucariotos, esse processo geralmente acontece como parte do ciclo sexual na formação das células gaméticas ou esporos, no caso dos fungos (TORTORA; FUNKE; CASE, 2000). A recombinação e as mutações contribuem para o aumento da diversidade genética de uma população, constituindo a fonte de variabilidade genética durante a evolução. Nesse contexto, a recombinação homóloga é um processo de troca de sequências de DNA que são praticamente idênticas, permitindo o pareamento das bases ao longo de uma extensão variável entre duas moléculas de DNA podendo ocorrer permuta de porções

dessas moléculas (ALBERTS et al., 2017). O processo de recombinação homóloga envolve a quebra e ligação das regiões pareadas, normalmente ocorre na reprodução sexuada sendo denominado *crossing-over* meiótico (TORTORA; FUNKE; CASE, 2000). Os processos de recombinação e mutação fornecem diversidade genética aos indivíduos descendentes, essa diversidade é o material para a evolução dos organismos. Nesse contexto, a seleção natural atuará nas populações assegurando a sobrevivência dos indivíduos adaptados a ambientes específicos. As mudanças adquiridas continuamente pelos microrganismos em seu genoma têm levado a rápidas adaptações e sobrevência em diferentes habitats.

2.3.1 Variabilidade genética em *P. oryzae*

Os efetores de avirulência (AVR) que são amplamente estudados como determinantes da especificidade da cultivar na doença da brusone do arroz (VALENT; KHANG, 2010), também são fatores importantes na especificidade das espécies hospedeiras de *P. oryzae* (TOSA et al., 2016; YAEGASHI; ASAGA, 1981). Os genes efetores *AVR* codificam as moléculas sinalizadoras do patógeno, que são reconhecidas pelos receptores codificados por genes da resistência vegetal (*R*), nas plantas, para desencadear a resistência hipersensível (CRUZ; VALENT, 2017). O gene *PWL2* foi o primeiro gene do tipo AVR clonado a partir de *P. oryzae* (SWEIGARD et al., 1995). O gene *AVR1CO39*, que funciona como um regulador do arroz, parece ter sido adquirido por um ancestral de *P. oryzae*, mas foi, posteriomente perdido por um evento de deleção mediado por transposons, a partir do patótipo *Oryza* (TOSA et al., 2005, 2006). Cinco genes *AVR* (*PWT1-5*) dos patótipos *Oryza*, *Setaria* e *Avena* bloqueiam a infecção do trigo (TOSA et al., 2006). Dois pares de genes bloqueiam a infecção de trigo, o gene MoL *AVR A1* e seu gene *R* correspondente no trigo, *Rmg6*, que confere forte resistência, e o gene *AVR A2* e o seu gene *R2* correspondente conferindo fraca resistência (TOSA et al., 2006).

No trigo, um gene *R* adicional, *Rmg1*, bloqueia isolados do patótipo *Avena* de infectar o trigo e dois genes, *Rmg4* e *Rmg5*, bloqueiam de forma independente os isolados do patótipo *Digitaria* de infectar o trigo (ANH et al., 2015). O surgimento de doenças como a brusone do trigo pode estar relacionado com a perda de alguns genes *AVR* (CRUZ; VALENT, 2017). Acreditava-se que a brusone do trigo no Brasil teria emergido a partir da brusone do arroz, porque houve endemia no arroz produzido no

norte do Paraná em 1985 (IGARASHI et al., 1986). Entretanto, devido à falta de infectividade entre os isolados de arroz e trigo e o alto nível de fertilidade sexual dos isolados de trigo em comparação com isolados inférteis de arroz, denota-se que provavelmente, a fonte de inóculo para a brusone do trigo não seria do arroz (PRABHU; FILIPPI; CASTRO, 1992; URASHIMA; IGARASHI; KATO, 1993).

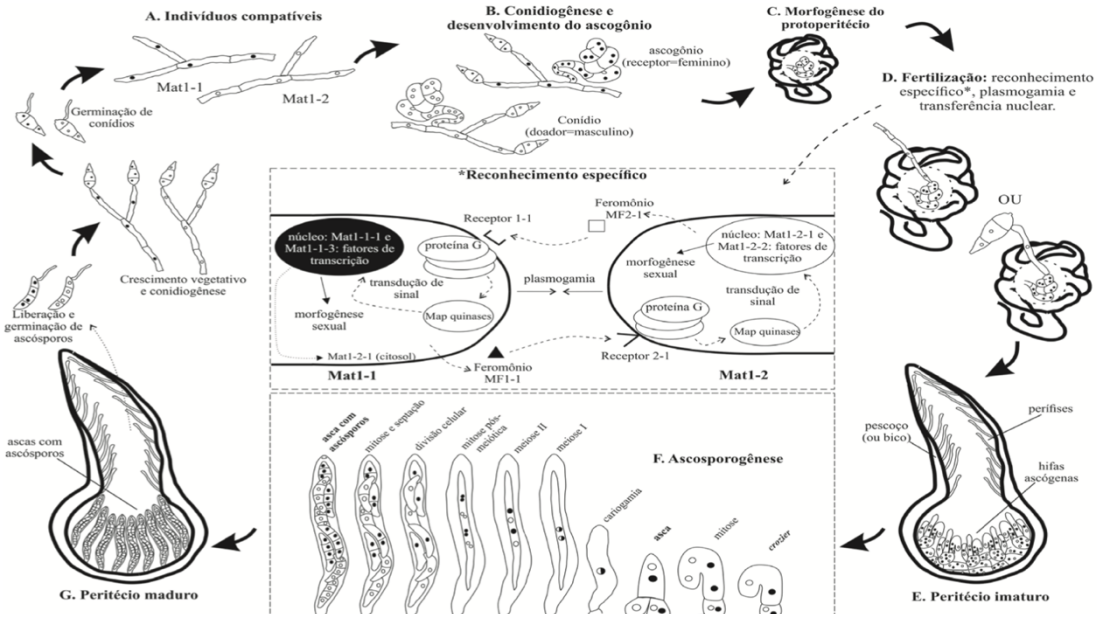
Os estudos genéticos dos isolados da brusone do trigo mostraram que se assemelham ao patossistema da brusone do arroz, seguindo a relação gene a gene (ANH et al., 2015) entretanto, dezenas de genes R foram identificados nos isolados da brusone do arroz enquanto parecem ser raros na brusone do trigo (LIU et al., 2014). Sete genes R da brusone foram identificados no trigo. Os genes *Rmg1* e *Rmg6* foram eficazes contra isolados de *Avena* e *Lolium*, respectivamente. Os genes *Rmg4* e *Rmg5* mostraram resistência a isolados de *Digitaria*. Mas esses 4 genes não apresentam resistência a isolados de *Triticum*. Os outros três genes foram eficazes contra isolados de um subgrupo de *Triticum*, em que os genes *Rmg2* e *Rmg3*, foram identificados na variedade 'Thatcher' de trigo comum e *Rmg7*, identificado nos tetraplóides, St24, St17 e St25 de trigo (ANH et al., 2015). Estes genes podem ser úteis para o melhoramento genético de trigo, mas ainda não há evidências de que foram mapeados.

2.4 Ciclo sexual

Pyricularia spp. constituem fungos heterotálicos (KANG; CHUMLEY; VALENT, 1994) e, para que aconteça o ciclo sexual, é necessário que ocorra o cruzamento entre dois indivíduos férteis de tipos sexualmente compatíveis (*mating types*). O ascogônio, estrutura receptora ‘feminina’, recebe núcleo(s) a partir de conídios ou hifas do indivíduo compatível doador ‘macho’ (Figura 1), sendo possível que os núcleos doados sejam provenientes de microconídios (MOREIRA; CERESINI; ALVES, 2015; ZHANG et al., 2014). Como pode ser observado na Figura 1, a fertilização ocorre quando o núcleo do indivíduo doador chega ao ascogônio, estrutura localizada no interior do protoperitécio, que pode ocorrer talvez pela entrada de tubos germinativos e/ou hifas, onde estão as hifas ascógenas, ou chegando ao ascogônio antes do seu envelopamento (MOREIRA; CERESINI; ALVES, 2015). O núcleo doado poderá se recombinar com núcleo do receptor por cariogamia, seguida pela meiose e, então, os quatro núleos formados sofrem mitose pós-meiótica, resultando em oito núcleos, os quais formarão os oito ascósporos em cada asco. Dentro dos ascósporos

ocorrem novas mitoses, seguido de septação, formando os ascósporos com quatro células uninucleadas (MOREIRA; CERESINI; ALVES, 2015; YAEGASHI; HEBERT, 1976).

Figure 1 - Esquema do ciclo sexual de *Pyricularia* spp. com etapas da mofogênese e regulação gênica.



Fonte: Moreira; Ceresini; Alves (2015).

O protoperitécio envolve o ascogônio e apresenta estrutura esférica a sub-esférica que resulta na formação do peritécio, que pode se encontrar isolado ou em grupo, fundido ou não, parcialmente ou totalmente imergido no substrato, apresentando longo rostro saliente a partir da superfície, sendo hialino e escurecendo com o tempo, e contém longa perífise filiforme e deliquescente (MOREIRA; CERESINI; ALVES, 2015). As ascas que se formam na base do pescoço, são cilíndricas a subclavadas, unitunicadas, com uma fina parede e um opérculo na extremidade superior e os ascósporos são hialinos, fusiformes, com três septos, moderadamente curvados, com glóbulos de óleo usualmente presentes e a liberação deve ocorrer pela deliquescência da asca (MOREIRA; CERESINI; ALVES, 2015; HEBERT, 1971).

Com relação aos genes que regulam a reprodução sexuada, são denominados *mating type* (MAT) e em *P. oryzae*, o locus é chamado Mat1 e está localizado no cromossomo três. Apresenta dois idiomorfos, o idiomorfo Mat1-1 codifica os transcritos Mat1-1-1, Mat1-1-2 e Mat1-1-3, e o idiomorfo Mat1-2 codifica os transcritos Mat1-2-1

e Mat1-2-2 (KANAMORI et al., 2007; TURGEON, 1998). As fases de leitura aberta ou ORFs (Open Reading Frame) MAT1-1-3a e MAT1-1-3b, e MAT1-2-2a e MAT1-2-2b, dos genes MAT1-1-3 e MAT1-2-2, respectivamente são determinadas por *splicing* alternativo. Segundo uma revisão feita por Moreira; Ceresini; Alves (2015), em vários fungos do filo Ascomycota, os genes MAT funcionam da forma descrita abaixo. Com relação ao idiomorfo MAT1-1, a proteína do transcrito Mat1-1-1 inclui um *motif* α-box, o qual leva à possibilidade de que esta proteína possa ser um fator de transcrição que se liga ao DNA. Em *Saccharomyces cerevisae*, o gene correspondente MATα1p funciona como coativador transcricional, essencial para a expressão de genes específicos *mating type*, para feromônios e receptores de feromônios (JOHNSON, 1995). Já o transcrito SMR1 de MAT1-1-2, em *Podospora anserina*, possui o domínio conservado HPG (com resíduos de histidina, prolina e glicina) e mutações de substituição do aminoácido triptofano para alanina levam à inibição do desenvolvimento de peritécios nos estádios iniciais (COPPIN; DE RENTY; DEBUCHY, 2005). A interação entre os transcritos dos genes MAT pode ser diferente em Ascomycota, sem função conhecida para o ciclo sexual, sendo que a importância do locus MAT na reprodução sexuada parece ser diferente em diferentes fungos. O nocaute combinado de MATA-1 (MAT1-1-1) e MATA-3 (MAT1-1-3) em *N. crassa* reduziu a fertilidade, sem efeitos no corpo de frutificação e na compatibilidade vegetativa (FERREIRA et al., 1998). No ciclo sexual de *Fusarium graminearum*, o idiomorfo Mat1-2, quando deletado, reduziu a quantidade de transcritos de vários genes relacionados com o ciclo sexual (LEE et al., 2006). Em *Fusarium verticillioides*, isolados ΔMAT1-2-1 apresentaram regulação negativa na expressão de precursores e receptores de feromônio (KESZTHELYI et al., 2007). Já em *Sordaria macrospora*, o nocaute de SMTA-2, que é referente a MAT1-1-2, ou a dupla-deleção SMTA-2/3, referente a deleção de MAT1-1-1 e MAT1-1-3, levou ao subdesenvolvimento do ascocarpo (KLIX et al., 2010). Foi observado em resultados de qRT-PCR que, em isolados mutantes, SMTA-1 apresenta uma regulação positiva da expressão dos genes precursores de feromônios PPG1 e PPG2, e SMTA-2 tem regulação negativa na expressão de PPG2 (KLIX et al., 2010). O grupo de alta mobilidade ou domínio GAM apresenta uma sequência de afinidade ao DNA, sendo que a proteína MAT1-2-1 tem o *motif* GAM-box de ligação ao DNA, e as ORFs de MAT1-1-3a e MAT1-2-2a também tem o domínio GAM (KANAMORI et al., 2007; MOREIRA; CERESINI; ALVES, 2015; TURGEON, 1998). Em *P. anserina*, análises

das proteínas dos domínios GAM-box de MAT1-1-3 e MAT1-2-1 são desiguais entre si e provavelmente se ligam ao DNA de maneira diferente e o nocaute de MAT1-1-1 e MAT1-2-1 afetou sua fertilidade (DEBUCHY; TURGEON, 2006).

Os feromônios e seus receptores específicos também atuam na regulação do reconhecimento específico dos indivíduos de tipo sexual oposto. Essa interação desencadeia uma transdução de sinais que estimulam a expressão de outros genes importantes para o ciclo sexual (SHEN; BOBROWICZ; EBBOLE, 1999). Depois desse reconhecimento, uma rota de sinalização celular, junto com vários fatores de transcrição, induz a expressão de genes relacionados à reprodução sexuada (MOREIRA; CERESINI; ALVES, 2015). A rota de sinalização, chamada rota MAP quinase, de resposta a feromônios apresenta proteínas regulatórias *mating type*, fatores precursores de feromônios, proteínas G, proteínas MAP quinase, fatores de transcrição, transferases, endoproteases e aminopeptidases (KIM; METZENBERG; NELSON, 2002). Genes precursores de feromônios já foram caracterizados para *P. oryzae* e foram identificados com genes *Mating factors*, MF1-1 e MF2-1 (SHEN; BOBROWICZ; EBBOLE, 1999). O gene MF1-1 codifica um polipeptídio de 26 aminoácidos, e possui terminação CAAX. O gene MF2-1 contém sítios potenciais de protease Kex2 e sequências repetidas dipeptídicas na região N-terminal (MOREIRA; CERESINI; ALVES, 2015). Em *Cryphonectria parasítica*, a deleção de MF1-1 levou a esterilidade em isolados 'machos', não tendo efeito no ciclo vegetativo (TURINA; PRODI; VAN ALFEN, 2003).

A regulação da reprodução sexual pode sofrer influência das condições ambientais, e existe um controle inerente ao organismo, que segue um 'relógio molecular' (MOREIRA; CERESINI; ALVES, 2015), como a expressão de MF1-1 em *C. parasitica* que pode variar com estímulos do ambiente, como a idade e composição do substrato (TURINA; PRODI; VAN ALFEN, 2003). Os genes das proteínas G são necessários para formação do ascocarpo em *Aspergillus nidulans*, também mediam respostas aos sinais do ambiente e apresentam as subunidades α, β e ϒ (SEO; HAN; YU, 2005). Em *P. oryzae*, o gene MAGB codifica a subunidade α da proteína G e o seu nocaute levou formação anormal de peritécios, apressórios, conídios e também no crescimento celular (FANG; DEAN, 2000).

A fase sexuada de *P. oryzae*, geralmente não é observada em condições de campo (HAYASHI et al., 1997), mas estudos indicam a presença de isolados

compatíveis em populações do fungo na Índia (DAYAKAR; NARAYANAN; GNANAMANICKAM, 2000), Bangladesh (SHAHJAHAN, 1994) e Ásia (SALEH et al., 2012). Urashima; Igarashi; Kato (1993), encontraram isolados brasileiros que formaram peritécios *in vitro*, mas não apresentaram ascósporos e nem compatibilidade sexual com isolados de outros hospedeiros. No trigo, foram encontrados em campo isolados de tipos compatíveis com outros isolados de trigo e outros hospedeiros (GALBIERI; URASHIMA, 2008; URASHIMA; IGARASHI; KATO, 1993).

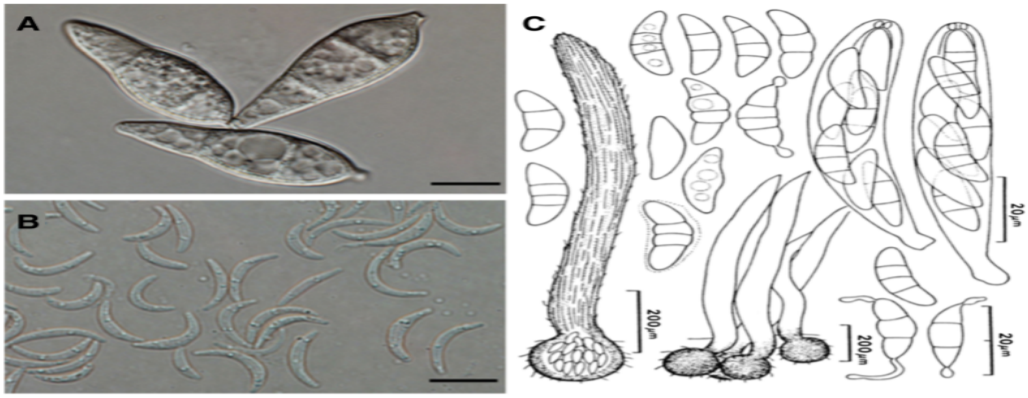
2.5 Ciclo assexual

P. oryzae na fase assexuada forma conidióforos solitários, eretos, retos ou curvos, não ramificados, septados, castanho médio e lisos, apresenta conidiogênese abundante, observada na metade superior do conidióforo (CASTROAGUDÍN et al., 2016). As células conidiogênicas são terminais e intercalares, castanho pálida, lisas, formando rachis com proliferação simodial, com vários dentículos salientes e os conídios são solitários, piriformes para obclavados, marrom pálidos, geralmente com dois septos (CASTROAGUDÍN et al., 2016).

Quando os isolados de *P. oryzae* de vários hospedeiros são cultivados em meios artificiais, como em meios de aveia, os microconídios podem ser observados concomitantemente com conídios em microscópio de luz (CHUMA et al., 2009). Ao passo que conídios apresentam três células e os ascósporos quatro, os microconídios são unicelulares e pequenos (0,7 μm de largura e 6 μm de comprimento), em forma de lua e hialinos (KATO et al., 1994) (Figura 2). Chuma e colaboradores encontraram um isolado de *P. oryzea* produzindo um aglomerado abundante de fiálides nas pontas das hifas, essas fiálides produzem muitos microconídios, não só sobre a superfície, mas também sob a superfície do ágar. Os microconídios, recém produzidos a partir de uma fiálide, formam uma massa globular coberta com uma matriz extracelular (CHUMA et al., 2009). Os microconídos podem ser produzidos em culturas submersas, sendo provável que sejam também produzidos por *P. oryzae* dentro de plantas de arroz doentes, em particular, no sistema vascular (ZHANG et al., 2014). Contudo, o papel dos microconídos no ciclo de vida de *P. oryzae* não é conhecido. Zhang et al. (2014) sugerem que os microconídios produzidos por *P. oryzae* dentro de plantas de arroz em condições de campo, podem ter papel na disseminação da região infectada para partes

saudáveis da planta, visto também que os microconídios apresentam baixa taxa de germinação, aproximadamente 10%.

Figure 2 - Tipos de esporos e estruturas produzidas por *Pyricularia* spp. A. Macroconídios; B. Microconídios; C. Peritécio, ascas com ascósporos.



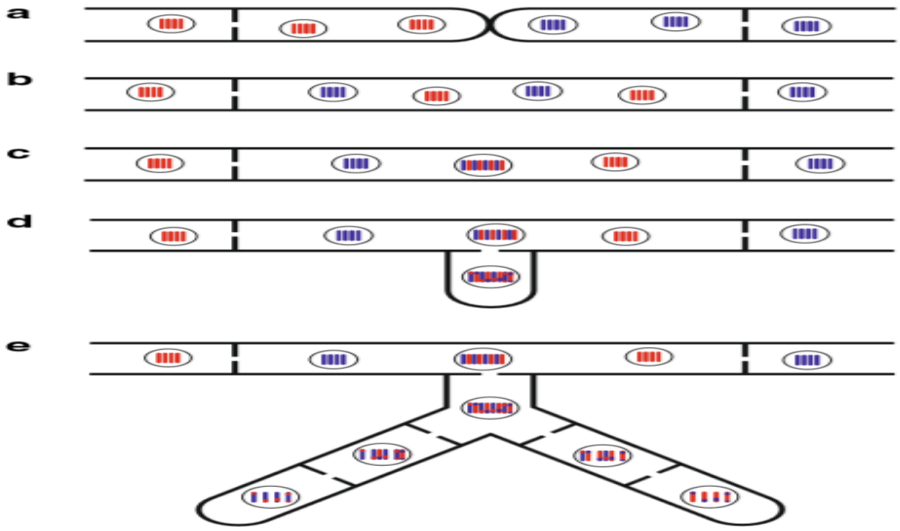
Fonte: Cruz; Valent (2017).

A reprodução assexuada é a forma mais comum de reprodução em Ascomycetos, e encontrada como responsável por grande parte da dispersão de esporos desses fungos. Estudos sobre *P. oryzae* patotípo *Triticum* sugerem que esse patótipo segue um sistema reprodutivo misto em que a recombinação sexual é seguida por dispersão assexuada de clones mais adaptados (MACIEL et al., 2014), o que contrasta com a brusone do arroz em que ocorre estritamente a reprodução assexuada (ZEIGLER, 1998).

2.6 Ciclo parassexual

A heterocariose que se refere à presença de dois ou mais núcleos geneticamente distintos dentro da mesma célula. Os filos Ascomycota e Basidiomycota são denominado por seus heterocários característicos (STROM; BUSHLEY, 2016). A formação do heterocário também ocorre durante o crescimento vegetativo como parte do ciclo parassexual (Figura 3), sendo um mecanismo para aumentar a diversidade genotípica em fungos nos quais o ciclo sexual não é conhecido ou ocorre raramente (STROM; BUSHLEY, 2016).

Figure 3 - O ciclo parassexual. a. Hifas de homocários geneticamente diferentes. b. Anastomose de hifas (heterocário). c. Cariogamia. d. Recombinação mitótica. e. Eventos de haploidização.



Fonte: Strom; Bushley (2016)

O ciclo parassexual pode resultar em descendentes haplóides geneticamente distintos sem sofrerem meiose. Hifas de dois indivíduos compatíveis crescem em direção ao outro por quimiotaxia (Figura 3. a) e se fundem, permitindo a troca de núcleos para formar um heterocário (Figura 3. b). Os núcleos podem sofrer cariogamia, resultando em células diplóides heterozigóticas (Figura 3. c). Após a cariogamia, pode ocorrer a recombinação mitótica (Figura 3. d), a qual contribuirá para a geração de recombinantes. Além disso, ocorre um processo de haploidização não meiótica, caracterizado por sucessivas perdas cromossômicas provocadas por erros de não-disjunção mitótica resultam na formação de células haploides bem como algumas aneuplóides, gerando genomas diferentes nos núcleos parentais (Figura 3. e) (STROM; BUSHLEY, 2016; TSUJIMOTO NOGUCHI, 2011). Os diploides heterozigotos são detectados pelo tamanho dos conídios, tamanho do núcleo e o conteúdo de DNA de um núcleo (NOGUCHI; YASUDA; FUJITA, 2006). Assim, mesmo na ausência de meiose e reprodução sexuada, o ciclo parassexual é efetivo no aumento da diversidade genotípica em fungos predominantemente assexuados (STROM; BUSHLEY, 2016). Portanto, variações na patogenicidade de isolados fúngicos podem ser geradas por reprodução sexual, mutação ou recombinação no ciclo parassexual.

Mutações e recombinação parassexual tem sido a maior causa de variação na patogenicidade de isolados de *P. oryzae*, devido não se ter relatos do ciclo sexual desses

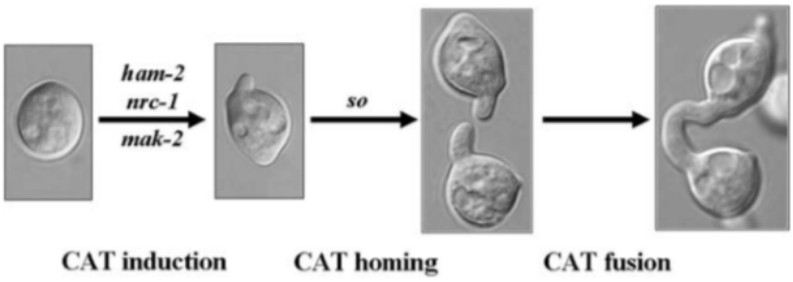
isolados (TSUJIMOTO NOGUCHI, 2011) ou apenas em condicões de laboratorio (URASHIMA; IGARASHI; KATO, 1993), havendo predominância da reprodução assexual. A recombinação parasexual em *P. oryzae* foi primeiramente sugerida por Yamasaki e Niizeki (1965), que observaram o comportamento nuclear na anastomose e obtiveram variantes associando duas cepas auxotróficas diferentes. Recombinantes auxotróficos podem ser produzidos ao associar diferentes isolados de parentais auxotróficos (GENOVESI; MAGILL, 1976). Resultados encontrados por Tsujimoto Noguchi (2011) sugerem que a variação na patogênese entre os recombinantes parasexuais de fungos da brusone do arroz corresponde ao que ocorre entre progênies sexuais.

Entretanto, podem ocorrer respostas de incompatibilidade de heterocário, também chamada de incompatibilidade vegetativa (GLASS; DEMENTHON, 2006). Se as células que sofreram anastomose possuírem alelos diferentes nos loci heterocário ou incompatibilidade vegetativa, eles serão incompatíveis vegetativamente e a fusão geralmente resulta em morte da célula heterocariótica formada (GLASS; DEMENTHON, 2006). Portanto, embora esse processo possa ser vantajoso para fungos filamentosos, o mecanismo genético de incompatibilidade vegetativa pode restringir a formação do heterocário entre dois indivíduos geneticamente diferentes (ISHIKAWA et al., 2012).

O tubo de anastomose de conídios (conidial anastomosis tube - CAT), que foi descrito pela primeira vez em *Colletotrichum lindemuthianum*, ocorre quando uma hifa especializada ou protrusão celular está envolvida na fusão de células somáticas durante a iniciação da colônia (ROCA et al., 2003). Esse processo é diferente do que ocorre na fusão de hifas em colônias maduras, e permite a formação de redes interconectadas de conídios (READ et al., 2010). Os CATs diferem do tubo geminativo nos seguintes aspectos: são finos e cutos; geralmente não são ramificados; a indução é dependente a densidade conidial; os CATs crescem em direção a outros CATs enquanto os tubos germinativos se evitam; e estão sob diferente controle genético (ROCA; READ; WHEALS, 2005). Em *Neurospora crassa* foram observados dois tipos de CATs, o primeiro emerge diretamente do conídio e o segundo desenvolve a partir do tubo germinativo (ROCA; READ; WHEALS, 2005).

O processo para que ocorra a fusão de CATs envolve três passos: indução do CAT, ‘CAT homing’ e fusão do CAT (Figura 4).

Figure 4 - Processo de fusão de CATs em *Neurospora crassa*.



Fonte: Roca; Read; Wheals (2005).

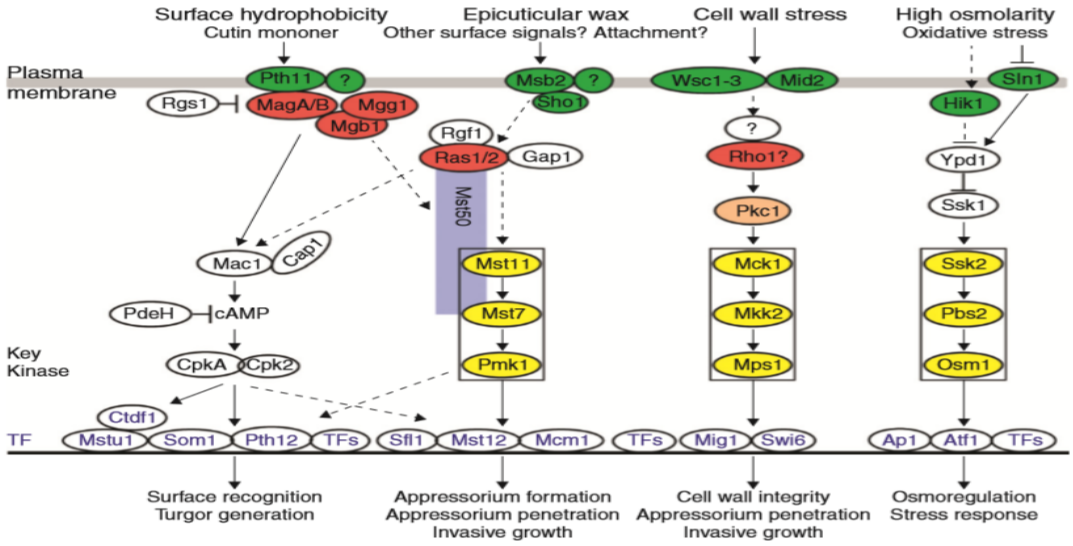
Os CATs têm sido comumente observados em fungos filamentosos e já foi relatado em 21 gêneros e 73 espécies (ROCA; READ; WHEALS, 2005), incluindo muitos agentes fitopatogênicos. Em *N. crassa* foram observados CATs em todos os três tipos de conídios: macroconídio, microconídio e artroconídio (ROCA; READ; WHEALS, 2005). A heterocariose a partir da fusão de hifas pode levar a uma resposta de imcompatibilidade levando a morte celular, caso as hifas apresentarem genes de incompatibilidade. Entretanto, há relatos de obtenção de heterocários via fusão de CAT sem uma resposta de incompatibilidade em *Penicillium notatum* (BAKER, 1994) e *C. lindermuthianum* (ISHIKAWA et al., 2012; ROCA et al., 2003). A heterocariose pode resultar em parassexualidade que geralmente ocorre por anostomose de hifas, mas é possível que a fusão de CAT possa ser um importante mediador desse processo (ROCA; READ; WHEALS, 2005). Roca et al. (2004) mostraram que pode ocorrer esse processo entre as duas espécies *C. lindemuthianum* e *C. gossypii*, e que as células heterocarióticas formadas possuíam características fenotípicas dessas duas espécies vegetativamente incompatíveis. Portanto, o CAT é uma possibilidade de avaliar isolados quanto a recombinação parassexual, mesmo em indivíduos incompatíveis vegetativamente.

2.7 Vias de sinalização no processo de infecção da planta por *P. oryzae*

Pela sua importância como patógeno, o fungo *P. oryzae* também é utilizado como modelo para estudar as interações planta-patógeno (VALENT; CHUMLEY, 1991; WILSON; TALBOT, 2009). Na Figura 5 estão ilustradas as principais vias de sinalização envolvidas na morfogênese relacionada à infecção em *P. oryzae*. Em que os genes do receptor/sensor, da GTPase trimérica ou pequena e da cascata MAP quinase

estão sombreados em verde, vermelho e amarelo, respectivamente, e todos os fatores de transcrição (TFs) putativos *downstream* estão escritos em textos azuis. A via Pmk1 MAP quinase regula o estágio final da formação de apressórios, penetração e crescimento invasivo de hifas (LI; ZHOU; XU, 2012). Os ortólogos *PMK1* são necessários para a formação de apressório em vários outros fungos formadores de apressório como *Colletotrichum orbiculare*, *Ustilago maydis*, *Pyrenophora teres*, and *Cochliobolus heterostrophus* (LI; ZHOU; XU, 2012). Vários genes funcionam *upstream* à via Pmk1, incluindo Mst11 (MAPKKK), Mst7 (MAPKK), proteína adaptadora Mst50 e Ras2 (ZHAO et al., 2005; ZHAO; MEHRABI; XU, 2007). Estudos moleculares confirmaram que a via do AMP cíclico (cAMP-PKA) desempenha um papel no reconhecimento de superfície, na iniciação da formação de apressório e na geração da pressão de turgor (XU; HAMER, 1996). Ambas as vias de sinalização Pmk1 MAPK e cAMP são essenciais para o crescimento invasivo e para a patogenicidade de *P. oryzae* (WILSON; TALBOT, 2009).

Figure 5 - Vias de sinalização envolvidas na morfogênese relacionadas a infecção em *Pyricularia oryzae*.



Fonte: Li; Zhou; Xu (2012).

A via MAPK está relacionada à integridade da parede celular e é conservada em patógenos de plantas e humanos na patogênese (LI; ZHOU; XU, 2012; ZHAO; MEHRABI; XU, 2007). Em *M. oryzae*, a via MPS1 (Figura 5) é dispensável para a formação de apressório, mas essencial para a integridade da parede celular, penetração

do apressório e crescimento invasivo (XU; STAIGER; HAMER, 1998). Mps1 regula o acúmulo de alfa-1,3-glucana, que é um dos componentes da camada externa da parede celular, responsável por fornecer proteção contra quitinases durante a infecção na planta e exerce interação com Mig1 e MoSwi6, seus prováveis fatores de transcrição localizados *downstream* (LI; ZHOU; XU, 2012). O gene *MIG1* é necessário para garantir que o patógeno passe pelas respostas de defesa da planta e para o crescimento invasivo (MEHRABI; DING; XU, 2008). Já o mutante do gene *MoSWI6* mostrou aumento da sensibilidade à parede celular e estresse oxidativo, e redução da virulência (LI; ZHOU; XU, 2012).

A via Osm1 MAPK (Figura 5) está relacionada a osmorregulação, resposta ao estresse e sensibilidade a fungicidas, mas é dispensável para a geração de turgor do apressório (DIXON et al., 1999; LI; ZHOU; XU, 2012). Os mutantes na via de osmorregulação são mais sensíveis às espécies oxidativas, no entanto, resistentes aos fungicidas fenilpirrol e dicarboximida (HAMEL et al., 2012; LI; ZHOU; XU, 2012). Embora a via Osm1 não seja necessária para a patogenicidade em *P. oryzae* e *Colletotrichum lagenarium*, seus ortólogos são importantes para a infecção em outros patógenos de plantas e de humanos, por exemplo, *Mycosphaerella graminicola* e *Cryptococcus neoformans* (LI; ZHOU; XU, 2012).

As proteínas Ras são necessárias para uma série de eventos fisiológicos e patogênicos, como proliferação, diferenciação, morte celular e capacidade de infecção (HANCOCK, 2003). Na levedura *Saccharomyces cerevisiae*, dois genes Ras, RAS1 e RAS2, são conhecidos por serem importantes para ativar a adenilato ciclase e estimular a síntese de cAMP (DAMAK et al., 1991). Além disso, as proteínas Ras são necessárias para a mitose e regulação do crescimento filamentoso em leveduras (YOSHIDA; ICHIHASHI; TOH-E, 2003). Essas proteínas atuam como interruptores, convertendo-se de formas "ativadas" para "desativadas" ao se ligar a GTP (RasGTP) ou GDP (RasGDP), que é regulado por fatores de troca de guanina (GEFs) e proteínas ativadoras de GTPase (GAPs), respectivamente (ROJAS; SANTOS, 2006). As proteínas Ras quando ativadas traduzem o sinal extracelular para regular os alvos *downstream* de maneiras específicas da via. O fungo *Ustilago maydis* possui duas proteínas Ras, mas com funções distintas. A Ras2 está relacionada ao crescimento filamentoso e a Ras1 está envolvida na regulação positiva da sinalização de feromônios. Sql2 é RasGEF

caracterizado em *U. maydis* e a sua deleção resulta em crescimento filamentoso e perda de virulência (MÜLLER et al., 2003).

Em um estudo anterior, duas proteínas Ras, Ras1 e Ras2, foram identificadas funcionando *upstream* às vias de sinalização de PmK1 e cAMP em *P. oryzae* (PARK et al., 2006). Ras1 e Ras2 têm interação com Mst50 que é uma proteína adaptadora na cascata MAPK, que pode interagir diretamente com Mst7 (MAPKK) e Mst11 (MAPKKK). Consequentemente, acredita-se que Ras1 e Ras2 estejam envolvidos na regulação da via de transdução de sinal Pmk1 MAPK (ZHAO; MEHRABI; XU, 2007). Um mutante do gene Ras1 (*Δras1*) forma apressório e infecta a planta assim como o isolado selvagem (PARK et al., 2006; ZHAO; MEHRABI; XU, 2007). Entretanto, o gene Ras2 parece ser essencial em *P. oryzae*. A expressão do alelo dominante $RAS2^{DA}$ no isolado selvagem resulta em um aumento no nível de cAMP intracelular e estimula a formação de apressórios com morfologia anormal em superfícies hidrofóbicas e hidrofílicas, sugerindo que Ras2 é funcionalmente relacionado à via de cAMP-PKA (LI; ZHOU; XU, 2012). Além disso, a fosforilação de Pmk1 é aumentada nos transformantes com $RAS2^{DA}$ (LI; ZHOU; XU, 2012). Dois genes GEF (RGF1 e RGF2) foram identificados como RasGEF em *P. oryzae*. RGF2 parece ser um gene dispensável em *P. oryzae* porque a deleção de RGF2 não teve efeitos significativos no crescimento e infecção da planta. No entanto, RGF1 é essencial para a formação de apressórios e patogenicidade, pois o mutante *Δrgf1*, produz conídios morfologicamente anormais e defeituosos na fixação em superfícies hidrofóbicas.

Proteínas quinases ativadas por mitógenos (MAPKs) são proteínas quinases com Ser/Thr que convertem estímulos extracelulares em uma ampla gama de respostas celulares. MAPKs estão entre as vias de transdução de sinal mais conhecidas e são utilizadas em vários processos biológicos (CARGNELLO; ROUX, 2011). Dentre as MAPKs convencionais estão as quinases reguladoras do sinal extracelular 1/2 ou ERK1/2 (extracellular signal-regulated kinases 1/2) e isómeros p38. ERK1/2 tem *motif* de ativação conservado em Thr-Glu-Tyr ou TEY (Treonina-Glutamato-Tirosina) em sua alça de ativação, já p38 tem *motif* de ativação conservado em Thr-Gly-Tyr ou TGY (Treonina-Glicina-Tirosina). Em *P. oryzae*, as vias Pmk1 e Mps1 tem MAPKs com ativação TEY e a via Osm1 tem MAPKs com ativação TGY (ZHANG et al., 2017). Ensaios de fosforilação TEY e TGY podem ser realizados para medir a quantidade de

MAPKs produzidas sob condições específicas, podendo, dessa forma, inferir a expressão dos genes de interesse que estão relacionados a essas vias.

2.8 Transformção genética em fungos

A transformação é uma transferência genética a partir da qual o DNA livre é incorporado em uma célula receptora, podendo promover alterações genéticas. A transformação genética facilitou a compreensão de fenômenos biológicos, como interações hospedeiro-patógeno, metabolismo secundário, respostas ao estresse ambiental e biologia do desenvolvimento. E para que haja sucesso nesse procedimento, vários fatores devem ser considerados como microrganismo alvo, o tipo de transformação, tipo de marcador, o objetivo da transformação, entre outros. Mesmo assim, muitas vezes a adequação do protocolo se faz necessária para a espécie microbiana em estudo.

O primeiro obstáculo encontrado quando se pretende transferir DNA exógeno para a célula fúngica é a parede celular. A maioria dos procedimentos utilizados para remoção da parede celular envolve a digestão enzimática. Os principais componentes da parede celular do fungo são quitina, 1,3-β-glucanas, 1,6-β-glucanas, proteínas, mananas e outros polímeros, que são reticulados para formar essa estrutura complexa (DEACON, 2006; RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). Os polissacarídeos cristalinos, quitina e β-glucanas, constituem a porção esquelética da parede, enquanto os polissacarídeos amorfos e os complexos proteínas-polissacarídeos são componentes da matriz da parede (DEACON, 2006). A forma, integridade e resistência mecânica do fungo são determinadas pela composição química da parede celular, que é responsável pela interação do fungo com seu ambiente, bem como por atividades biológicas (LESAGE; BUSSEY, 2006).

As enzimas hidrolíticas, que estão intimamente associadas à parede celular, são responsáveis pela manutenção da plasticidade da parede e funções durante o micoparasitismo (GRUBER; SEIDL-SEIBOTH, 2012). Entre as hidrolases identificadas até o momento, quitinases, glucanases e transglicosilases são responsáveis pela quebra e reforma das ligações dentro e entre os polímeros, levando à remodelagem da parede celular durante o crescimento e morfogênese (LESAGE; BUSSEY, 2006). Portanto, a remoção da parede celular fúngica, composta por glucanas ou celulose, quitina e proteinas envolve a presença de quitinases, proteases, celulases, β-glucanases etc.

Exemplos de preparações comerciais são as enzimas de lise de *Trichoderma harzianum* (Sigma-Aldrich), que é frequentemente usado para a preparação de protoplastos de fungos filamentosos (KELLY; NURSE, 2011).

2.8.1 Transformação mediada por protoplastos

A transformação de fungos filamentosos mediada por protoplastos depende da preparação eficiente dessas células. Protoplastos autênticos são definidos por três características: são células esféricas, sem parede celular, envoltos pela membrana plasmática; são liberados deixando um "fantasma" vazio de paredes celulares; e são viáveis, mas osmoticamente sensíveis e, portanto, requerem um estabilizador osmótico (VILLANUEVA; GARCÍA-ACHA, 1971). Protoplastos verdadeiros sem parede celular são às vezes difíceis de obter e o termo esferoplastos foi usado para se referir a células esféricas que ainda carregam restos de fragmentos de polímeros da parede celular (VILLANUEVA; GARCÍA-ACHA, 1971). Eles também são osmoticamente sensíveis e a presença de remanescentes da parede celular pode ser favorável para iniciar a regeneração da parede, atuando como *primers* de iniciação dos polímeros (MARTÍN, 2015). Em muitos casos, o excesso de enzimas líticas ou a presença de fosfolipases mal caracterizadas é claramente prejudicial para a estabilidade dos protoplastos, mesmo na presença de estabilizadores osmóticos (MARTÍN, 2015).

Os protoplastos podem ser preparados a partir de diferentes tipos de células, como micro e macroconídios em germinação e hifas (OLMEDO-MONFIL et al., 2004). Um importante fator a ser considerado para a remoção da parede celular é a fase de crescimento, de forma que a parede celular do fungo seja vulnerável ao ataque enzimático, como por exemplo, no estágio inicial após a germinação do esporo (RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). O tempo do crescimento micelial, na preparação dos protoplastos, desempenha um papel importante estando relacionado à fase de crescimento, devido aos componentes da parede celular serem variáveis durante os diferentes estágios dessa fase (NASEEMA et al., 2008). Os componentes e suas proporções na parede celular do fungo, também diferem entre as diferentes espécies e normalmente, o micélio é mais sensível às enzimas líticas na fase log (NASEEMA et al., 2008; RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). Em *Aspergillus ochraceus* com 24 h de incubação a 28 °C (ALMEIDA et al., 2008) e *Colletotricum lindemuthianum* com 48 h de incubação a 22 °C (ISHIKAWA et al., 2010) ocorreram

maiores liberação de protoplastos. Em geral, o ideal é que seja utilizado o micélio jovem de conídios germinados, podendo ser cultivados por 12 a 24 h a temperaturas de 25 a 30 °C, mas isso vai depender da espécie (RODRIGUEZ-IGLESIAS; SCHMOLL, 2015).

A estrutura da parede celular é distinta entre os fungos, exigindo diferentes combinações de enzimas junto com condições específicas para degradar parede celular e liberar protoplastos (RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). Nesse aspecto, a utilização de enzimas combinadas mostrou-se mais eficiente do que o uso de enzimas isoladas, devido a presença de diversos compostos na parede celular fúngica (GALLMETZER; BURGSTALLER; SCHINNER, 1999; RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). Portanto, a seleção das enzimas é um fator crucial na preparação de protoplastos e alguns formulados enzimáticos estão disponíveis comercialmente. Os compostos Glusulase e Novozym 234, uma mistura de enzimas de *T. viride,* eram comumente utilizados para produção de protoplastos (VOLLMER; YANOFSKY, 1986). No entanto, a produção de Novozym 234 (Novo Nordisk) foi descontinuada e o produto foi substituído por "Lysing enzymes" de *T. harzianum* (Sigma-Aldrich), também conhecido como Glucanex (RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). Esse novo formulado apresenta atividades de celulase, protease e quitinase e em *Trichoderma*, parece funcionar tão bem quanto Novozym 234 (STEIGER, 2013). Esses compostos apresentam principalmente 1,3-glucanases, quitinases, celulases e proteases, e deve ser avaliada a concentração necessária para a digestão da parede celular para cada combinação de enzimas e para cada fungo, geralmente estando entre 5 mg/mL e 20 mg/mL para diferentes espécies fúngicas (RODRIGUEZ-IGLESIAS; SCHMOLL, 2015).

Durante a digestão da parede celular, os fungos precisam manter o equilíbrio osmótico para evitar a ruptura das células. Paredes celulares rígidas são necessárias para que as células fúngicas sobrevivam em ambientes hipotônicos. Quando a parede celular é removida, um ambiente isotônico é necessário para manter a célula estável e evitar a lise (MARTÍN, 2015). Portanto, todas as soluções usadas para a preparação de protoplastos devem conter um estabilizador osmótico para prevenir a lise dos protoplastos. Exemplo de estabilizadores osmóticos são KCl 0,7 M (CANTORAL et al., 1987) e sacarose ou alguns álcoois de açúcar (por exemplo, sorbitol) com concentração de 0,8-1,0 M. O sorbitol, em concentrações entre 0,8 e 1,2 M, tem sido comumente usado e parece ser satisfatório para muitas espécies (RODRIGUEZ-

IGLESIAS; SCHMOLL, 2015). Para *Aspergillus* e *Penicillium*, cloreto de potássio em concentrações entre 0,6 e 0,7 M é usado como estabilizador osmótico padrão (BALLANCE; TURNER, 1985; DÍEZ et al., 1987).

O protocolo comum para a transformação de protoplastos começa com a mistura do DNA purificado, com a suspensão de protoplastos e uma solução contendo polietilenoglicol (PEG) (RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). O processo de transformação mediado por polietilenoglicol (PEG) na presença de íons Ca^{2+} é bem conhecido por causar fusão de protoplastos. Na presença de íons Ca^{2+}, o DNA é capturado e introduzido nos protoplastos, provavelmente por endocitose induzida por PEG (MARTÍN, 2015). Uma das soluções de transformação (PCM) usa KCl 0,7 M como estabilizador osmótico, $CaCl_2$ 50 mM e tampão MOPS 10 mM. O PEG líquido (MW 1.000-8.000) é misturado com o DNA em tampão KCM (MARTÍN, 2015). O PEG de peso molecular superior é muito viscoso ou sólido e não é adequado. Boa eficiência de transformação é obtida com 25% de PEG na mistura de transformação e maiores eficiências de transformação de *P. chrysogenum* são geralmente alcançadas aumentando a concentração de PEG até 50% (MARTÍN, 2015).

A regeneração da parede celular é realizada em meio complexo com ou sem pressão de seleção. A regeneração direta na presença do agente seletivo é adequada quando uma boa eficiência de transformação é alcançada (MARTÍN, 2015). No entanto, em alguns casos é preferível regenerar os protoplastos transformados na ausência do agente seletivo e depois transferir os transformantes para placas com os agentes seletivos (MARTÍN, 2015). Ao lidar com a complementação de cepas auxotróficas, a seleção direta dos transformantes prototróficos em meio mínimo pode ser desfavorável para a regeneração da parede celular (MARTÍN, 2015).

Os marcadores seletivos são necessários para seleção dos transformantes, sendo utilizados comumente marcadores nutricionais e os marcadores de resistência de seleção positiva. Os marcadores nutricionais conferem a capacidade dos transformantes crescerem em meio pobre ou de difícil captação de algum nutriente. As acetamidas que são uma fonte pobre em nitrogênio para várias espécies de *Aspergillus*, permitindo o crescimento muito limitado das cepas do tipo selvagem. Por isso, vetores carregando o gene *amdS*, podem ser usadas como marcadores de seleção para transformar cepas selvagens (auxotróficos), pois conferem crescimento mais rápido aos transformantes crescidos na presença desse componente (HYNES; CORRICK; KING, 1983). Uma

grande desvantagem das estratégias de transformação baseadas na complementação de auxotróficos é a necessidade de se obter primeiro os auxotróficos adequados; além disso, mutações auxotróficas podem afetar o crescimento (MARTÍN, 2015). Este problema é evitado com a utilização de marcadores de resistência positiva ou dominante. Um dos primeiros exemplos de marcadores de resistência dominantes foi o uso da resistência ao fungicida Benomyl em *N. crassa* e *Aspergillus niger* (MARTÍN, 2015). Outros marcadores de seleção, usados com menos frequência são a resistência à oligomicina em *Aspergillus nidulans* e a resistência a sulfonamidas em *Penicillium chrysogenum* (MARTÍN, 2015). Outros exemplos são marcadores de resistência à higromicina B, ao aminoglicosídeo G418 (gentamicina), à fleomicina. Uma vez que um marcador de resistência foi introduzido em fungos filamentosos, é difícil removê-lo para obter um "transformante limpo" para uma segunda rodada de manipulações genéticas (MARTÍN, 2015).

2.8.2 Procedimentos alternativos de transformação que não requerem protoplastos

Embora a introdução de DNA assistida por PEG em protoplastos seja um bom procedimento de transformação, vários outros métodos que não requerem protoplastos foram desenvolvidos (MARTÍN, 2015). A transformação de células inteiras, auxiliada com acetato de lítio (0,1 M) ou com sais de outros metais alcalinos, tem sido bem-sucedida em leveduras e foi relatada em vários fungos filamentosos (MARTÍN, 2015). Outros métodos alternativos incluem eletroporação, transformação mediada por *Agrobacterium tumefaciens* (DE GROOT et al., 1998) e transformação balística (também chamada de biolística) (RUIZ-DIEZ, 2002).

A eletroporação de células inteiras (ou protoplastos) foi alcançada com sucesso para vários fungos. O uso de células, geralmente esporos, evita a necessidade de obtenção de protoplastos. Os esporos dos fungos podem ser pré-germinados para facilitar a eletroporação (OZEKI et al., 1994). Atualmente, a eletroporação é um método confiável para a transformação de alguns fungos, entretanto os protocolos precisam ser otimizados para cada espécie de fungo (LAKROD; CHAISRISOOK; SKINNER, 2003).

Durante a infecção da planta, *A. tumefaciens* é capaz de transferir a região T-DNA do plasmídeo Ti para o genoma da planta infectada (MARTÍN, 2015). A região do T-DNA é delimitada por duas repetições invertidas e foi observado que o DNA

exógeno inserido entre as bordas esquerda e direita do T-DNA também é transferido durante a conjugação (MARTÍN, 2015). A conjugação de *A. tumefaciens* é mediada pelos produtos dos genes *vir*, de virulência, localizados no plasmídeo Ti e esses genes são induzidos pela acetoseringona e a adição deste indutor é necessária para o sucesso da transformação das células fúngicas nesse processo (MARTÍN, 2015). O T-DNA e os marcadores selecionáveis são integrados de forma aleatória no genoma dos transformantes.

A transformação biolística, usando partículas de tungstênio revestidas com DNA, que são introduzidas em alta velocidade em células fúngicas, é um método alternativo para transformar fungos que não podem ser transformados por outras ferramentas (HAZELL et al., 2000). No entanto, um equipamento especializado para esse processo é necessário e limita sua utilização em muitos laboratórios (MARTÍN, 2015).

2.8.3 Abordagem *Split-Marker* para substituição direcionada de genes

A eficiência de transformação na maioria dos fungos filamentosos é por vezes baixa e a integração de fragmentos de DNA de interesse, geralmente ocorre em locais não homólogos no genoma, sendo difícil, por exemplo, inativar genes específicos de interesse (MARTÍN, 2015). A substituição gênica direcionada é uma das principais estratégias para a caracterização funcional de genes em fungos e vários métodos já foram desenvolvidos (GOSWAMI, 2012). O aumento na disponibilidade de informações sobre sequências de genoma permitiu uma adoção ampla de protocolos baseados no conhecimento da sequência do gene de interesse e sua região circundante (GOSWAMI, 2012). Entre os métodos de substituição de genes de forma direcionada, a abordagem “Split-marker” tem ganhado popularidade para uso em fungos filamentosos (GOSWAMI, 2012). Este método envolve apenas duas rodadas de PCR e não requer subclonagem (Figura 6).

Figure 6 - Esquema da abordagem *Split-marker* para substituição direcionada de genes.

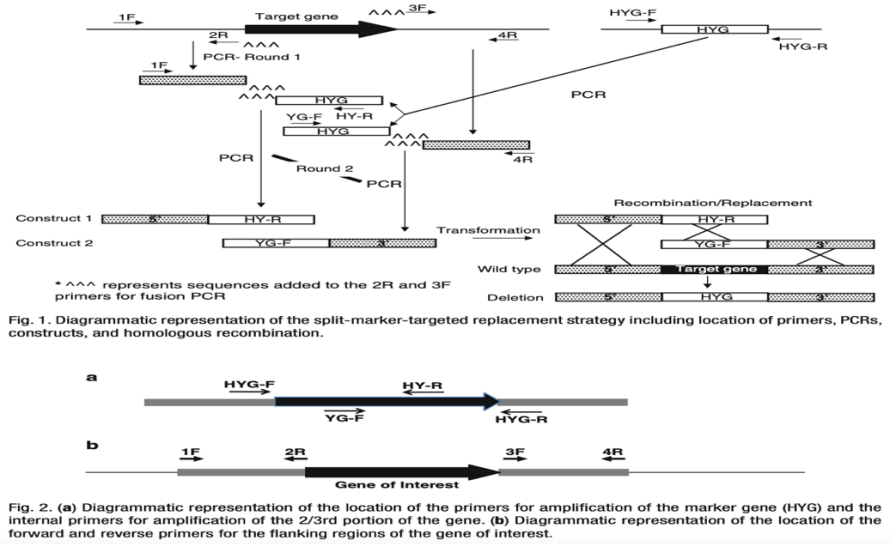


Fig. 1. Diagrammatic representation of the split-marker-targeted replacement strategy including location of primers, PCRs, constructs, and homologous recombination.

Fig. 2. **(a)** Diagrammatic representation of the location of the primers for amplification of the marker gene (HYG) and the internal primers for amplification of the 2/3rd portion of the gene. **(b)** Diagrammatic representation of the location of the forward and reverse primers for the flanking regions of the gene of interest.

Fonte: Goswami (2012).

Essa abordagem baseia-se na disponibilidade de um gene marcador, por exemplo, o gene de resistência a higromicina, e a sequência do gene de interesse, bem como regiões de cerca de 1 kb de comprimento que flanqueiam o gene em ambos os lados (GOSWAMI, 2012). Nesse método é realizada a amplificação por PCR das regiões flanqueadoras do gene de interesse e do gene marcador seguido por uma reação de PCR para a fusão desses amplicons que levam à criação de dois cassetes moleculares, cada um contendo uma parte do gene marcador fundido a uma região flanqueadora (GOSWAMI, 2012). Esses cassetes moleculares são então usados simultaneamente para a transformação de protoplastos. Três eventos de recombinação homóloga, um dentro de cada região flanqueadora e um no gene marcador, levam à substituição do gene de interesse por um gene marcador funcional (GOSWAMI, 2012). Os transformantes são então cultivados em meio seletivo e as colônias emergentes podem ser rastreadas quanto à presença do marcador e ausência do gene de interesse que foi substituído.

REFERÊNCIAS

ALBERTS, B. et al. **Biologia molecular da célula.** [s.l: s.n.].

ALEXANDER D JOHNSON. Molecular mechanisms of cell-type determination. **Current Opinion in Genetics and Development**, v. 5, p. 552–558, 1995.

ALMEIDA, A. P. M. et al. 1460-1462C. v. 3838, n. 55, p. 1460–1462, 2008.

ANH, V. L. et al. Rmg8, a new gene for resistance to *Triticum* isolates of *Pyricularia oryzae* in hexaploid wheat. **Phytopathology**, v. 105, n. 12, p. 1568–1572, 2015.

BAKER, G. E. Heterokaryosis in *Penicillium notatum*. **Bull. Torrey Bot. Club**, v. 71, p. 367-373, 1994.

BALLANCE, D. J.; TURNER, G. Development of a high-frequency transforming vector for *Aspergillus nidulans*. **Gene**, v. 36, n. 3, p. 321–331, 1985.

BAREA G.; TOLEDO J. Identificación y zonificación de piricularia o bruzone (*Pirycylaria oryzae*) en el cultivo del trigo en el dpto. de Santa Cruz. In: CIAT. Informe Técnico. Proyecto de Investigación Trigo, Santa Cruz, 76–86, 1996.

CANTORAL, J. M. et al. High-frequency transformation of *Penicillium chrysogenum*. **Bio/Technology**, v. 5, n. 5, p. 494–497, 1987.

CARGNELLO, M.; ROUX, P. P. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. **Microbiology and Molecular Biology Reviews**, v. 75, n. 1, p. 50–83, 2011.

CASTROAGUDÍN, V. L.; CERESINI, P. C.; DE OLIVEIRA, S. C.; REGES, J. T. A.; MACIEL, J. L. N.; BONATO, A. L. V.; DORIGAN, A. F.; MCDONALD, B. A. Resistance to QoI fungicides is widespread in Brazilian populations of the wheat blast pathogen *Magnaporthe oryzae*. **Phytopathology**, v. 105, p. 284–294, 2014.

CASTROAGUDÍN, V. L. et al. *Pyricularia graminis-tritici*, a new Pyricularia species causing wheat blast. **Persoonia: Molecular Phylogeny and Evolution of Fungi**, v. 37, n. DECEMBER, p. 199–216, 2016.

CASTROAGUDÍN, V. L.; DANELLI, A.; MOREIRA, S. I.; REGES, J. T. A.; CARVALHO, G.; et al. The wheat blast pathogen *Pyricularia graminis-tritici* has complex origins and a disease cycle spanning multiple grass hosts. **BioRxi,** 2017. doi: https://doi.org/10.1101/203455

CHUMA, I. et al. Cytological characteristics of microconidia of *Magnaporthe oryzae*. **Journal of General Plant Pathology**, v. 75, n. 5, p. 353–358, 2009.

CONAB - COMPANHIA NACIONAL DE ABASTECIMENTO. Séries históricas. Disponível em: https://www.conab.gov.br/info-agro/safras/serie-historica-das-safras. Acesso em: 20 de out. de 2021.

COPPIN, E.; DE RENTY, C.; DEBUCHY, R. The function of the coding sequences for the putative pheromone precursors in *Podospora anserina* is restricted to fertilization. **Eukaryotic Cell**, v. 4, n. 2, p. 407–420, 2005.

COUCH, B. C.; KOHN, L. M. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from M. grisea. **Mycologia**, v. 94, n. 4, p. 683–693, 2002.

CRUZ, C. D.; VALENT, B. Wheat blast disease: danger on the move. **Tropical Plant Pathology**, v. 42, n. 3, p. 210–222, 2017.

DAMAK, F. et al. SDC25, a CDC25-like gene which contains a RAS-activating domain and is a dispensable gene of *Saccharomyces cerevisiae*. **Molecular and Cellular Biology**, v. 11, n. 1, p. 202–212, 1991.

DAYAKAR, B. V.; NARAYANAN, N. N.; GNANAMANICKAM, S. S. Cross-compatibility and distribution of mating type alleles of the rice blast fungus *Magnaporthe grisea* in India. **Plant Disease**, v. 84, n. 6, p. 700–704, 2000.

DEACON, J. W. **Fungal Biology.** 4 ed. Malden, MA: Blackwell Publishing, 2006. 371 p. ISBN 9781405130660.

DEBUCHY, R.; TURGEON, B. G. Mating type in Euascomycetes. In: Kues, U., Fischer, R. **The Mycota I.** 2nd Ed. Berlin Heidelberg: Springer-Verlag, 2006.p.293-323.

DE GROOT, M. J. A. et al. *Agrobacterium tumefaciens* n1ediate d transforn1ation of filan1entous fungi. p. 839–842, 1998.

DÍEZ, B. et al. Selection and characterization of pyrG mutants of *Penicillium chrysogenum* lacking orotidine-5′-phosphate decarboxylase and complementation by the pyr4 gene of Neurospora crassaa. **Current Genetics**, v. 12, n. 4, p. 277–282, 1987.

DIXON, K. P. et al. Independent signaling pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection by *Magnaporthe grisea*.

Plant Cell, v. 11, n. 10, p. 2045–2058, 1999.

FANG, E. G. C.; DEAN, R. A. Site-directed mutagenesis of the magB gene affects growth and development in *Magnaporthe grisea*. **Molecular Plant-Microbe Interactions**, v. 13, n. 11, p. 1214–1227, 2000.

FARMAN, M. et al. The lolium pathotype of *Magnaporthe oryzae* recovered from a single blasted wheat plant in the United States. **Plant Disease**, v. 101, n. 5, p. 684–692, 2017.

FARMAN, M. L. Erratum: *Pyricularia grisea* isolates causing gray leal spot on Perennial ryegrass (*Lolium perenne*) in the United States: Relationship to P. grisea isolates from other host plants (Phytopathology (November 2002) 92 (245-254)). **Phytopathology**, v. 92, n. 11, p. 1253, 2002.

FERREIRA, A. V. B. et al. Characterization of mat A-2, mat A-3 and ΔmatA mating-type mutants of *Neurospora crassa*. **Genetics**, v. 148, n. 3, p. 1069–1079, 1998.

GALBIERI, R.; URASHIMA, A. S. Caracterização, compatibilidade e ocorrência de reprodução sexual entre isolados de *Pyricularia grisea* de diferentes hospedeiros. **Summa Phytopathologica**, v. 34, n. 1, p. 22–28, 2008.

GALLMETZER, M.; BURGSTALLER, W.; SCHINNER, F. An optimized method for the isolation of protoplasts from *Penicillium simplicissimum* to produce sealed plasma membrane vesicles . **Mycologia**, v. 91, n. 1, p. 206–212, 1999.

GENOVESI, A. D.; MAGILL, C. W. Heterokaryosis and parasexuality in Pyricularia oryzae Cavara. **Canadian Journal of Microbiology**, v. 22, n. 4, p. 531–536, 1976.

GLASS, N. L.; DEMENTHON, K. Non-self recognition and programmed cell death in filamentous fungi. **Current Opinion in Microbiology**, v. 9, n. 6, p. 553–558, 2006.

GOSWAMI, R. S. Targeted gene replacement in fungi using a split-marker approach. **Methods in Molecular Biology**, v. 835, p. 255–269, 2012.

GLADIEUX, P.; CONDON, B.; RAVEL, S.; SOANES, D.; MACIEL, J. L. N.; NHANI, A.; CHEN, L.; TERAUCHI, R.; LEBRUN, M. -H.; THARREAU, D.; MITCHELL, T.; PEDLEY, K. F.; VALENT, B.; TALBOT, N. J.; FARMAN, M.; FOURNIER, E. Gene flow between divergent cereal- and grass-specific lineages of the

rice blast fungus *Magnaporthe oryzae*. **mBio**, 9: e01219-17, 2018. https://doi.org/10.1128/mBio.01219-17.

GOULART, A. C. P.; SOUSA, P. G.; URASHIMA, A. S. Danos em trigo causados pela infecção de Pyricularia grisea. **Summa Phytopathologica**, v. 33, n. 4, p. 358–363, 2007.

GOULART, A. C. P.; PAIVA, F. A. Perdas no rendimento de grãos de trigo causada por *Pyricularia grisea*, nos anos de 1991 e 1992, no Mato Grosso do Sul. **Summa Phytopathol**, v. 26, p. 279–282, 2000.

GOULART, A. C. P.; PAIVA, F. de A. Wheat yield losses due to *Pyricularia oryzae* in the 1988–91 periods in Mato Grosso do Sul (Abstr.). **Fitopatología Brasileira**, v. 17, p. 171, 1992.

GRUBER, S. G.; SEIDL-SEIBOTH, V. Self versus non-self: Fungal cell wall degradation in *Trichoderma*. **Microbiology**, v. 158, n. 1, p. 26–34, 2012.

HAMEL, L. P. et al. Mitogen-activated protein kinase signaling in plant-interacting fungi: Distinct messages from conserved messengers. **Plant Cell**, v. 24, n. 4, p. 1327–1351, 2012.

HANCOCK, J. F. Ras proteins: Different signals from different locations. **Nature Reviews Molecular Cell Biology**, v. 4, n. 5, p. 373–384, 2003.

HAYASHI, N.; LI, Y. C.; LI, J. L.; NAITO, H. In vitro production on rice plants of perithecia of *Magnaporthe grisea* from Yunnan, China. **Mycological Research**, Cambridge, v. 101, n. 11, p. 1308-1310, 1997.

HAZELL, B. W. et al. Rapid transformation of high cellulase-producing mutant strains of *Trichoderma reesei* by microprojectile bombardment. **Letters in Applied Microbiology**, v. 30, n. 4, p. 282–286, 2000.

HYNES, M. J.; CORRICK, C. M.; KING, J. A. Isolation of genomic clones containing the amdS gene of *Aspergillus nidulans* and their use in the analysis of structural and regulatory mutations. **Molecular and Cellular Biology**, v. 3, n. 8, p. 1430–1439, 1983.

IGARASHI, S.; UTIAMADA, C. M.; KASUMA, A. H.; LÓPEZ, R. S. *Pyricularia* sp. em trigo. 1. Ocurrencia de *Pyricularia* sp. no Estado do Paraná. **Fitopatología Brasilera**, v. 11, p. 351–352, 1986.

IGARASHI, S. (1990) Update on wheat blast (*Pyricularia oryzae*) in Brazil. In: SAUNDERS, D. A. (ed) **Wheat for the nontraditional warm areas: a proceedings of the international conference**. Foz do Iguaçu, Brazil, pp 480–483.

ISHIKAWA, F. H. et al. Factors affecting the production and regeneration of protoplasts from *Colletotrichum lindemuthianum* | Fatores que afetam a produção e regeneração de protoplastos de *Colletotrichum lindemuthianum*. **Ciencia e Agrotecnologia**, v. 34, n. 1, p. 74–79, 2010.

ISHIKAWA, F. H. et al. Heterokaryon incompatibility is suppressed following conidial anastomosis tube fusion in a fungal plant pathogen. **PLoS ONE**, v. 7, n. 2, 2012.

ISLAM, M. T. et al. Emergence of wheat blast in Bangladesh was caused by a South American lineage of *Magnaporthe oryzae*. **BMC Biology**, v. 14, n. 1, p. 1–11, 2016.

KANAMORI, M. et al. Novel mating type-dependent transcripts at the mating type locus in *Magnaporthe oryzae*. **Gene**, v. 403, n. 1–2, p. 6–17, 2007.

KANG, S.; CHUMLEY, F. G.; VALENT, B. Isolation of the mating-type genes of the phytopathogenic fungus *Magnaporthe grisea* using genomic subtraction. **Genetics**, v. 138, n. 2, p. 289–296, 1994.

KATO, H. et al. Pathogenicity, Mating Ability and DNA Restriction Fragment Length Polymorphisms of *Pyricularia* Populations Isolated from Gramineae, Bambusideae and Zingiberaceae Plants. **Journal of General Plant Pathology**, v. 66, n. 1, p. 30–47, 2000.

KATO, H. Y. et al. Microconidium in *Magnaporthe*. [s.d.].

KELLY, F. D.; NURSE, P. De novo growth zone formation from fission yeast spheroplasts. **PLoS ONE**, v. 6, n. 12, 2011.

KESZTHELYI, A. et al. Tagging target genes of the MAT1-2-1 transcription factor in *Fusarium verticillioides* (Gibberella fujikuroi MP-A). **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 91, n. 4, p. 373–391, 2007.

KIM, H.; METZENBERG, R. L.; NELSON, M. A. Multiple functions of mfa-1, a putative pheromone precursor gene of *Neurospora crassa*. **Eukaryotic Cell**, v. 1, n. 6, p. 987–999, 2002.

KLAUBAUF, S. et al. Resolving the polyphyletic nature of *Pyricularia* (*Pyriculariaceae*). **Studies in Mycology**, v. 79, n. 1, p. 85–120, 2014.

KLIX, V. et al. Functional characterization of MAT1-1-specific mating-type genes in the homothallic Ascomycete *Sordaria macrospora* provides new insights into essential and nonessential sexual regulators. **Eukaryotic Cell**, v. 9, n. 6, p. 894–905, 2010.

KOHLI, M. M. et al. *Pyricularia* blast-a threat to wheat cultivation. **Czech Journal of Genetics and Plant Breeding**, v. 47, n. SPEC. ISSUE 1, p. 130–134, 2011.

LAKROD, K.; CHAISRISOOK, C.; SKINNER, D. Z. Expression of pigmentation genes following electroporation of albino *Monascus purpureus*. **Journal of Industrial Microbiology and Biotechnology**, v. 30, n. 6, p. 369–374, 2003.

LEE, S. H. et al. Identification of the down-regulated genes in a mat1-2-deleted strain of *Gibberella zeae*, using cDNA subtraction and microarray analysis. **Fungal Genetics and Biology**, v. 43, n. 4, p. 295–310, 2006.

LESAGE, G.; BUSSEY, H. Cell Wall Assembly in *Saccharomyces cerevisiae*. **Microbiology and Molecular Biology Reviews**, v. 70, n. 2, p. 317–343, 2006.

LI, G.; ZHOU, X.; XU, J. R. Genetic control of infection-related development in *Magnaporthe oryzae*. **Current Opinion in Microbiology**, v. 15, n. 6, p. 678–684, 2012.

LIU, W. et al. Novel insights into rice innate immunity against bacterial and fungal pathogens. **Annual Review of Phytopathology**, v. 52, n. May, p. 213–241, 2014.

MACIEL, J. L. N. et al. Population structure and pathotype diversity of the wheat blast pathogen *Magnaporthe oryzae* 25 years after its emergence in Brazil. **Phytopathology**, v. 104, n. 1, p. 95–107, 2014.

MALAKER, P. K. et al. First Report of Wheat Blast Caused by *Magnaporthe oryzae* Pathotype *Triticum* in Bangladesh . **Plant Disease**, v. 100, n. 11, p. 2330–2330, 2016.

MEHRABI, R.; DING, S.; XU, J. R. MADS-box transcription factor Mig1 is required for infectious growth in *Magnaporthe grisea*. **Eukaryotic Cell**, v. 7, n. 5, p. 791–799, 2008.

MARTÍN, F. J. Fungal transformation: from protoplasts to targeted recombination systems. In: VAN DEN BERG, M. A.; MARUTHACHALAM, K. (eds.), **Genetic Transformation Systems in Fungi, Volume 1, Fungal Biology**, DOI 10.1007/978-3-319-10142-2_2, Springer International Publishing Switzerland, 2015.

MOREIRA, S. I.; CERESINI, P. C.; ALVES, E. Reprodução Sexuada em *Pyricularia oryzae*. **Summa Phytopathologica**, v. 41, n. 3, p. 175–182, 2015.

MÜLLER, P. et al. Guanyl nucleotide exchange factor sql2 and ras2 regulate filamentous growth in *Ustilago maydis*. **Eukaryotic Cell**, v. 2, n. 3, p. 609–617, 2003.

NASEEMA, A. et al. Isolation and regeneration of protoplasts from the mycelium of *Fusarium pallidoroseum* - A potential biocontrol agent of water hyacinth [*Eichhornia crassipes* (Mart.) Solms]. **Journal of Tropical Agriculture**, v. 46, n. 1–2, p. 55–57, 2008.

NOGUCHI, M. T.; YASUDA, N.; FUJITA, Y. Evidence of genetic exchange by parasexual recombination and genetic analysis of pathogenicity and mating type of parasexual recombinants in mice blast fungus, *Magnaporthe oryzae*. **Phytopathology**, v. 96, n. 7, p. 746–750, 2006.

OZEKI, K. et al. Transformation of Intact *Aspergillus* mger by Electroporation. **Bioscience, Biotechnology, and Biochemistry**, v. 58, n. 12, p. 2224–2227, 1994.

PARK, G. et al. Multiple upstream signals converge on the adaptor protein Mst50 in *Magnaporthe grisea*. **Plant Cell**, v. 18, n. 10, p. 2822–2835, 2006.

PICININI, E. C.; FERNANDES J. M. C. Ocorrência da brusone (*Pyricularia oryzae*) em lavouras comerciais de trigo (*Triticum aestivum*) no estado do Rio Grande do Sul. **Fitopatología Brasilera**, v. 15, p. 83–84, 1990.

PRABHU, A. S.; FILIPPI, M. C.; CASTRO, N. Pathogenic variation among isolates of *Pyricularia oryzae* affecting rice, wheat, and grasses in Brazil. **Tropical Pest Management**, v. 38, n. 4, p. 367–371, 1992.

READ, N.; FLEIßNER, A.; ROCA, M.; GLASS, N. Hyphal Fusion. In: Borkovich K, Ebbole D, eds. Cellular and Molecular Biology of Filamentous Fungi: American Society of Microbiology. pp 260–273, 2010.

ROCA, M. G. et al. Conidial anastomosis tubes in *Colletotrichum*. **Fungal Genetics and Biology**, v. 40, n. 2, p. 138–145, 2003.

ROCA, M. G. et al. Conidial anastomosis fusion between *Colletotrichum* species. **Mycological Research**, v. 108, n. 11, p. 1320–1326, 2004.

ROCA, M. G.; READ, N. D.; WHEALS, A. E. Conidial anastomosis tubes in filamentous fungi. **FEMS Microbiology Letters**, v. 249, n. 2, p. 191–198, 2005.

RODRIGUEZ-IGLESIAS, A.; SCHMOLL, M. Protoplast transformation for genome manipulation in fungi. In: VAN DEN BERG, M. A.; MARUTHACHALAM, K. (eds.), **Genetic Transformation Systems in Fungi, Volume 1, Fungal Biology**, DOI 10.1007/978-3-319-10142-2_2, Springer International Publishing Switzerland, 2015.

ROJAS, J. M.; SANTOS, E. CHAPTER 2 RAS-GEF S AND RAS GAP S Abstract : n. Figure 1, p. 15–43, 2006.

RUIZ-DIEZ, B. Strategies for the transformation of ® lamentous fungi. **Journal of Applied Microbiology**, v. 92, p. 189–195, 2002.

SACCARDO, P. Fungorum extra-europaeorum Pugillus. **Michelia**, v. 2, p. 136–149, 1880.

SALEH, D. et al. Sex at the origin: An Asian population of the rice blast fungus *Magnaporthe oryzae* reproduces sexually. **Molecular Ecology**, v. 21, n. 6, p. 1330–1344, 2012.

SEO, J. A.; HAN, K. H.; YU, J. H. Multiple roles of a heterotrimeric G-protein γ-subunit in governing growth and development of *Aspergillus nidulans*. **Genetics**, v. 171, n. 1, p. 81–89, 2005.

SHAHJAHAN, A. K. M. Practical approaches to rice blast management in tropical monsoon ecosystems, with special reference to Bangladesh. In: ZEIGLER, R.S.;

LEONG, S.A.; TENG, P.S. Rice blast disease. Wallingford: CAB International, 1994. p.465-488.

SHEN, W. C.; BOBROWICZ, P.; EBBOLE, D. J. Isolation of pheromone precursor genes of *Magnaporthe grisea*. **Fungal Genetics and Biology**, v. 27, n. 2–3, p. 253–263, 1999.

SPRAGUE R (1950) Diseases of cereals and grasses in North America. Ronald Press Com, New York

STEIGER, M. G. Molecular tools in *Trichoderma* genetic studies. In: MUKHERJEE, P. K.; HORWITZ, B. A.; SINGH, U. S.; MUKHERJEE, M.; SCHMOLL, M. (eds) *Trichoderma*: biology and applications. CABI, Walllingford, 2013.

STROM, N. B.; BUSHLEY, K. E. Two genomes are better than one: History, genetics, and biotechnological applications of fungal heterokaryons. **Fungal Biology and Biotechnology**, v. 3, n. 1, p. 1–14, 2016.

SWEIGARD, J. A. et al. Identification, Cloning, and Characterization of. v. 7, n. August, p. 1221–1233, 1995.

TORTORA, G. J.; FUNKE, B. R.; CASE, C. L. **Anatomia functional de células procarióticas e eucarióticas.** [s.l: s.n.].

TOSA, Y. et al. Genetic constitution and pathogenicity of Lolium isolates of *Magnaporthe oryzae* in comparison with host species-specific pathotypes of the blast fungus. **Phytopathology**, v. 94, n. 5, p. 454–462, 2004.

TOSA, Y. et al. Evolution of an avirulence gene, AVR1-CO39, concomitant with the evolution and differentiation of Magnaporthe oryzae. **Molecular Plant-Microbe Interactions**, v. 18, n. 11, p. 1148–1160, 2005.

TOSA, Y. et al. Genetic analysis of host species specificity of *Magnaporthe oryzae* isolates from rice and wheat. **Phytopathology**, v. 96, n. 5, p. 480–484, 2006.

TOSA, Y. et al. Genetic and molecular analyses of the incompatibility between *Lolium* isolates of *Pyricularia oryzae* and wheat. **Physiological and Molecular Plant Pathology**, v. 95, p. 84–86, 2016.

TOSA, Y.; CHUMA, I. Classification and parasitic specialization of blast fungi. **Journal of General Plant Pathology**, v. 80, n. 3, p. 202–209, 2014.

TSUJIMOTO NOGUCHI, M. Parasexual recombination in *Magnaporthe oryzae*. **Japan Agricultural Research Quarterly**, v. 45, n. 1, p. 39–45, 2011.

TURGEON, B. G. Application of mating type gene technology to problems in fungal biology. **Annual Review of Phytopathology**, v. 36, n. 77, p. 115–137, 1998.

TURINA, M.; PRODI, A.; VAN ALFEN, N. K. Role of the Mf1-1 pheromone precursor gene of the filamentous ascomycete *Cryphonectria parasitica*. **Fungal Genetics and Biology**, v. 40, n. 3, p. 242–251, 2003.

URASHIMA, A. S.; IGARASHI, S.; KATO, H. **Host range, mating type, and fertility of Pyricularis grisea from wheat in BrazilPlant Disease**, 1993.

VALENT, B.; CHUMLEY, F. G. Molecular Genetic Analysis of the Rice Blast Fungus, *Magnaporthe Grisea*. **Annual Review of Phytopathology**, v. 29, n. 1, p. 443–467, 1991.

VALENT, B.; KHANG, C. H. Recent advances in rice blast effector research. **Current Opinion in Plant Biology**, v. 13, n. 4, p. 434–441, 2010.

VILLANUEVA, J. R.; GARCÍA-ACHA, I. Production and use of fungal protoplasts. In: Booth C (ed) **Methods in microbiology**, vol 4. Academic, New York, pp 665–718, 1971.

VOLLMER, S. J.; YANOFSKY, C. Efficient cloning of genes of *Neurospora crassa*. **Proceedings of the National Academy of Sciences of the United States of America**, v. 83, n. 13, p. 4869–4873, 1986.

WILSON, R. A.; TALBOT, N. J. Under pressure: Investigating the biology of plant infection by *Magnaporthe oryzae*. **Nature Reviews Microbiology**, v. 7, n. 3, p. 185–195, 2009.

XU, J. R.; HAMER, J. E. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. **Genes and Development**, v. 10, n. 21, p. 2696–2706, 1996.

XU, J. R.; STAIGER, C. J.; HAMER, J. E. Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. **Proceedings of the National Academy of Sciences of the United States of America**, v. 95, n. 21, p. 12713–12718, 1998.

YAEGASHI, H. Inheritance *Pyricularia* of Pathogenicity Isolates and from Finger in Crosses Weeping Millet of Lovegrass. [s.d.].

YAEGASHI, H.; ASAGA, K. Further studies on the inheritance of pathogenicity in crosses of *Pyricularia oryzae* with pyricularia sp. from finger millet. **Japanese Journal of Phytopathology**, v. 47, n. 5, p. 677–679, 1981.

YAEGASHI, H.; HEBERT, T. T. Perithecial Development and Nuclear Behaviour in *Pyricularia*. **Phytopathology**, St. Paul, v. 66, p. 122-166, 1976.

YAMASAKI, Y.; NIIZEKI, H. Studies on variation of the rice blast fungus, *Pyricularia oryzae* Cav. I. Karyological and genetic studies on variation. **Bull. Natl. Inst. Agric. Sci. Jpn.**, v. 13, p. 231–273, 1965.

YOSHIDA, S.; ICHIHASHI, R.; TOH-E, A. Ras recruits mitotic exit regulator Lte1 to the bud cortex in budding yeast. **Journal of Cell Biology**, v. 161, n. 5, p. 889–897, 2003.

ZEIGLER, R. S. Recombination in Telomeres. **Microbe Magazine**, v. 6, n. 3, p. 133–133, 2011.

ZHANG, H. et al. Germination and infectivity of microconidia in the rice blast fungus *Magnaporthe oryzae*. **Nature Communications**, v. 5, p. 1–9, 2014.

ZHANG, X. et al. Expression of HopAI interferes with MAP kinase signalling in *Magnaporthe oryzae*. **Environmental Microbiology**, v. 19, n. 10, p. 4190–4204, 2017.

ZHAO, X. et al. A mitogen-activated protein kinase cascade regulating infection-related morphogenesis in *Magnaporthe grisea*. **Plant Cell**, v. 17, n. 4, p. 1317–1329, 2005.

ZHAO, X.; MEHRABI, R.; XU, J. R. Mitogen-activated protein kinase pathways and fungal pathogenesis. **Eukaryotic Cell,** v. 6, n. 10, p. 1701–1714, 2007.

SEGUNDA PARTE – ARTIGOS

ARTIGO 1 – MATING TYPE GENE ANALYSIS AND SEXUAL REPRODUCTION IN WHEAT BLAST FUNGI *Pyricularia oryzae* PATHOTYPE *Triticum* *

ARTIGO FORMATADO DE ACORDO COM A NORMA NBR 6022 (ABNT 2018)

Dérica Gonçalves Tavares **

Prof. Dr. Eduardo Alves (Orientador) ***

ABSTRACT

Blast disease caused by *Pyricularia oryzae* affects several grass species such as rice, wheat, triticale, barley, and rye and is one of the main plant diseases in the world. Studies on genes related to the sexual cycle and infection pathways can help to elucidate potential sources of genetic variability in this pathogen. In this study, we performed sexual reproduction using the *P. oryzae* pathotypes *Triticum* and *Oryzae*, structural analysis of MAT1-1 and MAT1-2 idiomorph sequences, and expression of mating type genes under crossing conditions. No fertility crossing was observed between *Triticum* isolates only. However, fertile cross was obtained from the Guy11 *Oryzae* tester and 12.1.053i *Triticum* isolate. Mating type gene analysis showed the presence of different haplotypes separating isolates of *Triticum* and *Oryzae* into different groups. MAT1-1 idiomorph and MAT1-1-1 showed higher haplotype diversity. Single nucleotide polymorphisms (SNPs) may have led to alterations in the amino acid sequence and consequently in the predicted protein of MAT1-1-3b. A region of cytosine and thymine (CT) dinucleotides located at the 5'-UTR of MAT1-1-3 showed that sequences of *Triticum* isolates have a smaller CT region, which may have influenced the expression of MAT1-1-3. The lack of MAT1-1-3 expression during the sexual cycle in infertile isolates also showed that this gene plays an important role in the fertility of *P. oryzae*.

Keywords: Crossing. Locus MAT1, Single nucleotide polymorphism. Gene expression.

---

* Artigo Apresentado para a conclusão do curso de pós-graduação em Microbiologia Agrícola da Universidade Federal de Lavras.

** Doutoranda no curso de Microbiologia Agrícola, pela Universidade Federal de Lavras. Endereço eletrônico: dericatavares@gmail.com.

*** Professor da Universidade Federal de Lavras, Doutor em Fitopatologia.

1 INTRODUCTION

Wheat blast is one of the main diseases affecting crop production. The losses caused by wheat blast can vary according to the climatic conditions and cultivars used, reaching 100% of the production (IGARASHI et al., 1986; GOULART; SOUSA; URASHIMA, 2007; KOHLI et al., 2011). The etiological agent is the Ascomycete *Pyricularia oryzae* pathotype *Triticum*, which can attack the entire aerial part of the plant, causing great severity in the spike, causing its upper part to bleach (AGHNOUM et al., 2019; CRUZ; VALENT, 2017; KOHLI et al., 2011; PIECK et al., 2017; TOSA; CHUMA, 2014).

The sexual reproduction of *P. oryzae* pathotype *Triticum*, under field conditions, has not yet been observed, although there are populations with sexually compactible isolates (HAYASHI et al., 1997; CASTROAGUDÍN et al., 2017). Recently a study involving *P. oryzae* isolates from several regions and different grasses in Brazil, indicated high genetic marker diversity and virulence in differentiating wheat lines, with 198 multilocus microsatellite genotypes (MLMGs) and 25 virulence groups (CASTROAGUDÍN et al., 2017). These wheat populations exhibit a consistent genetic structure with a mixed reproductive system in which sexual reproduction occurs followed by locally adapted clones dispersal (CASTROAGUDÍN et al., 2017; MACIEL et al., 2014). The isolation of compatible strains is further evidence of the possibility of a sexual cycle in wheat blast and other grasses in Brazilian isolates (GALBIERI; URASHIMA, 2008; URASHIMA; IGARASHI; KATO, 1993).

Pyricularia spp. are heterothallic fungi (KANG; CHUMLEY; VALENT, 1994) it is necessary for the sexual cycle to cross between two fertile strains of compatible sexual types. Sexual reproduction is regulated by genes known as mating type (MAT). In *P. oryzae*, the locus is called MAT1 and is located on chromosome three (KANAMORI et al., 2007; TURGEON, 1998). The MAT1 locus contains the idiomorphs MAT1-1 and MAT1-2. The idiomorph MAT1-1 contains the genes MAT1-1-1, MAT1-1-2, and MAT1-1-3 which encode the transcripts Mat1-1-1, Mat1-1-2, and Mat1-1-3, respectively. The idiomorph MAT1-2 contains the genes MAT1-2-1 and MAT1-2-2 and encodes the transcripts Mat1-2-1 and Mat1-2-2, respectively (KANAMORI et al., 2007; TURGEON, 1998).

Two open reading frames (ORFs) in MAT1-1-3 called MAT1-1-3a and MAT1-1-3b, and in MAT1-2-2 called MAT1-2-2a and MAT1-2-2b were determined by alternative splicing with a frame change in the first intron (KANAMORI et al., 2007). The Mat-1-1 transcript

protein includes an α-box motif, which is conserved as a product of MAT1-1, and its α protein may be a transcription factor that binds to DNA via the conserved α domain (KANAMORI et al., 2007). The Mat1-2-1 transcript protein has a DNA-binding GAM-box motif, which is conserved and characterizes the idiomorph MAT1-2 (KANAMORI et al., 2007). However, the importance of the MAT locus in sexual reproduction seems to be distinct in different fungi and the interaction of mating type gene transcripts may be different and the function remain unknown for the sexual cycle (COPPIN; DE RENTY; DEBUCHY, 2005; FERREIRA et al., 1998).

Although, the perfect stage of *P. oryzae* has not been observed under field conditions, several studies have indicated the presence of compatible strains in populations of the fungus in India (DAYAKAR; NARAYANAN; GNANAMANICKAM, 2000), Bangladesh (SHAHJAHAN, 1994), Asia (SALEH et al., 2012), and Brazil (HAYASHI et al., 1997; CASTROAGUDÍN et al., 2017). Different distributions of the idiomorphs Mat1-1 and Mat1-2 in Brazilian regions were observed. A ratio of the idiomorphs Mat1-1 and Mat1-2 in the Midwest of 4:1, in the Triângulo Mineiro of 30:1, in São Paulo of 1:0, and in Paraná of 15:1 was observed, respectively (MACIEL et al., 2014). Urashima; Igarashi; Kato (1993) found wheat blast isolates from Brazilian fields that formed perithecia *in vitro* without asci and ascospores, and without sexual compatibility with isolates from other grasses. In this study we analyzed mating type genes in the *P. oryzae* pathotypes *Triticum* and *Oryzae* comparing this locus with sexual and asexual reference strains, showing the polymorphisms between them and possible changes in gene transcription and protein production. We also present the pairing and mating type gene expression at different times of the sexual cycle.

2 MATERIALS AND METHODS

2.1 CULTURE CONDITIONS AND CROSSES IN *P. oryzae* ISOLATES

Isolates of the *P. oryzae* pathotype *Triticum* and pathotype *Oryzae* used in this study are listed in Table 1. The *P. oryzae* isolates Guy11, CHNOS60-2-3, and CHNOS59-9-1, provided by Dr. Jin-Rong Xu from Purdue University, were used as testers to verify the isolates sexuality. All isolates were maintained in oatmeal (50 g/L oatmeal and 13.5 g/L agar) or complete medium (CM) agar (10 g/L glucose, 2 g/L peptone, 1 g/L de yeast extract, 1 g/L

casamino acids, 50 mL/L de 20x nitrate salts, 1 mL/L traces elements, 1 mL/L de vitamin solution, pH 6,5, 15 g agar) at 25 ºC in a photoperiod of 12 h for 7 or 10 days.

Table 1 - *Pyricularia oryzae* isolates used in the crosses and idiomorph evaluation analysis.

Isolates	Hosts	Pathotype	Mating-type (Kanamori et al., 2007)
12.1.204	*Triticum aestivum*	*Triticum*	MAT1-1
12.1.217	*T. aestivum*	*Triticum*	MAT1-1
12.1.234	*T. aestivum*	*Triticum*	MAT1-1
12.1.127	*T. aestivum*	*Triticum*	MAT1-1
12.1.169	*T. aestivum*	*Triticum*	MAT1-1
12.0.345i	*Avena sativa*	*Triticum*	MAT1-1
12.0.073	*A. sativa*	*Triticum*	MAT1-1
12.0.321	*A. sativa*	*Triticum*	MAT1-1
12.0.347	*A. sativa*	*Triticum*	MAT1-1
12.0.326	*Echinochloa crusgalli*	*Triticum*	MAT1-1
12.0.368	*Urochloa brizantha*	*Triticum*	MAT1-1
12.0.555i	*Digitaria sanguinalis*	*Triticum*	MAT1-1
12.1.032i	*T. aestivum*	*Triticum*	MAT1-2
12.1.014	*T. aestivum*	*Triticum*	MAT1-2
12.1.078	*T. aestivum*	*Triticum*	MAT1-2
12.1.181	*T. aestivum*	*Triticum*	MAT1-2
12.1.205	*T. aestivum*	*Triticum*	MAT1-2
12.1.225	*T. aestivum*	*Triticum*	MAT1-2
12.1.241	*T. aestivum*	*Triticum*	MAT1-2
12.1.053i	*T. aestivum*	*Triticum*	MAT1-2
12.1.117	*T. aestivum*	*Triticum*	MAT1-2
12.1.037	*T. aestivum*	*Triticum*	MAT1-2
12.1.291	*T. aestivum*	*Triticum*	MAT1-2
12.0.642i	*Cenchrus echinatus*	*Triticum*	MAT1-2
12.0.535i	*C. echinatus*	*Triticum*	MAT1-2
12.0.009i	*U. brizantha*	*Triticum*	MAT1-2
12.0.007i	*U. brizantha*	*Triticum*	MAT1-2
12.0.012i	*U. brizantha*	*Triticum*	MAT1-2
12.0.051i	*Rhynchelytrum repens*	*Triticum*	MAT1-2
12.0.534i	*Eleusine indica*	*Triticum*	MAT1-2
12.0.194	*Digitaria isularis*	*Triticum*	MAT1-2
658	*Oryza sativa*	*Oryzae*	MAT1-1
678	*O. sativa*	*Oryzae*	MAT1-1
364	*O. sativa*	*Oryzae*	MAT1-1
Guy11[t]	*O. sativa*	*Oryzae*	MAT1-1
CHNOS60-2-3[t]	*O. sativa*	*Oryzae*	MAT1-2
CHNOS59-9-1[t]	*O. sativa*	*Oryzae*	MAT1-1

[t]Mating type-tester isolates.
Source: Tavares (2021).

The crossing test was performed according to Itoi et al. (1983) by placing each isolate, listed in Table 1, in the apex of a triangle with Guy11 (MAT1-1) and CHNOS60-2-3 (MAT1-2) mating type-tester on oatmeal medium in a Petri dish of 9-cm-diam and incubated at 20 °C for 28 days under fluorescent light. Crossings only between the *Triticum* isolates were also performed. The isolates were identified for their respective mating types by polymerase chain reaction (PCR) with primers B15 (5'-CTCAATCTCCGTAGTAG-3') and B16 (5'-ACAGCAGTATAGCCTAC-3') for the Mat1-2 idiomorph and A1 (5'-AGCCTCATCAACGGCAA-3') and A5 (5'-GTAGCGTACAAGCACGG-3') for the Mat1-1 idiomorph (Xu and Hamer, 1995). PCR reaction was performed using Taq Pol Hot Start Cellco following the manufacturer's instructions. Cycling conditions were denaturation at 95 °C for 1 min; 30 cycles of 95 °C for 30 s/55 °C for 30 s/72 °C for 1 min; and final extension at 72 °C for 2 min. Crossings were performed according to Itoi et al. (1983) with modifications. Mycelium discs of 7-days-old oatmeal cultures of compatible mating types were placed about 4 cm apart, in Petri dishes containing oatmeal and incubated at 20 °C with constant fluorescent light associated with light close to UV for 28 days. Fertility was evaluated by observation of perithecia usually near the boundary of colonies, and by the presence of asci and ascospores inside the perithecia.

2.2 MATING TYPE GENE ANALYSIS OF *P. oryzae* IDIOMORPHS

The DNA sequences of the mating type locus of the isolates were provided by Dr. Daniel Croll of the Université de Neuchâtel from Pyricularia strain genomes. The correlation between the haplotypes of MAT1-1 and MAT1-2 idiomorphs was performed. The sequences were aligned with sexual and asexual isolates from Kanamori et al. (2007) and are listed in Table 2.

Table 2 - Isolates from which sequences were used to the analysis of idiomorphs evolution.

Isolates	Mating type	Origin	GenBank access number
70-6[a]	MAT1-1	USA	AB080670.2
70-14[a]	MAT1-2	USA	AB080671.2
Y93-164g-1[a]	MAT1-1	China	AB080672.2
Y93-164a-1[a]	MAT1-2	China	AB080673.2
Hoku-1[b]	MAT1-1	Japan	AB080668.2
P2[b]	MAT1-2	Japan	AB080669.2

[a]Sexual isolates. [b]Asexual isolates.
Source: Tavares (2021).

The sequences of the MAT1-1-1, MAT1-1-2, and MAT1-1-3 genes of idiomorph MAT1-1 and MAT1-2-1 and MAT1-2-2 genes of idiomorph MAT1-2 were aligned using CLUSTAL W, implemented in MEGA X software (KUMAR et al., 2018). The alignments were performed individually for the presence or absence of single nucleotide polymorphisms (SNPs), insertions and deletions (InDels), and the nucleotide (Pi) and haplotypic (Hd) diversities were calculated using DnaSP software (LIBRADO; ROZAS, 2009). The best replacement model was determined based on the lowest Akaike criteria performed using MEGA X and the JC model was selected. Phylogenetic relationships between the isolates were evaluated using the SNAPP tool implemented in BEAST v.2.6.3 software (BOUCKAERT et al., 2019) and the mutation rate was calculated using the Bayesian Monte Carlo Chain Markov (MCMC) method for ten million interactions with sampling in each 1000. Phylogenetic trees were observed using FigTree v1.4.4 software (http://tree.bio.ed.ac.uk/ software/figtree/) and assembled using CorelDraw 2020 software.

2.3 POLYMORPHISM OF THE CT-DINUCLEOTIDE REPEAT REGION UPSTREAM OF MAT1-1-3 GENE

The isolates were grown in CM broth and incubated for 3 days at 25 ºC and 105 rpm. Mycelium was separated from the supernatant and DNA was extracted using SDS-based method. Cytosine and thymine rich region CT $(CT)_n$ upstream of the MAT1-1-3 gene were amplified using the forward primer B113-800 (5'-GCTCGCCACTATGCTGTTCTCGTA-3') and reverse primer B113-008 (5'- GCTCCACTCCACGCTCCACATCTT-3') (KANAMORI et al., 2007). PCR was performed using Taq Pol Hot Start Cellco following the manufacturer's instructions. Cycling conditions were denaturation at 95 ºC for 1 min; 30 cycles of 95 ºC for 30 s/55 ºC for 30 s/72 ºC for 1 min; and final extension at 72 ºC for 2 min. The amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System Promega and the samples were sequenced by ACTGene Molecular Analysis in Porto Alegre, RS, Brazil. The CT-rich region sequences were aligned with the reference strains 70-6, Y93-164g-1, and Hoku-1 using MEGA X.

2.4 MATING TYPE GENE EXPRESSION DURING SEXUAL REPRODUCTION

To perform sexual reproduction conditions, two 10-day-old strains of different mating types were placed on opposite sides, approximately 4 cm from each other, in a Petri dish of 9-cm-diam with oatmeal agar medium and incubated at 20 ºC under fluorescent light. The strains used in the crossing were Guy11 (MAT1-1) and CHNOS60-2-3 (MAT1-2) as the fertile pair and CHNOS59-9-1 (MAT1-1) and CHNOS60-2-3 (MAT1-2) as infertile pair. Total RNA was extracted after 7, 14, and 21 days of incubation and the samples were obtained at the meeting point of the colonies. The samples taken were about 0.5 cm from each colony side in contact and as part of the perithecium was formatted inside the culture medium close to the surface the depth of cut was approximately 2 mm. The samples were macerated with liquid nitrogen, the RNA was extracted using the TRIzol™ Sigma, reagent following the manufacturer's instructions and the DNA remaining was removed with the DNA-free™ DNA Removal kit Invitrogen. Reverse transcriptase (RT)-PCR was performed using SuperScript® IV Reverse Transcriptase Invitrogen, following manufacturer's instructions and the RT-reaction containing up to 2 μg of total RNA or mRNA with a final volume of 20 μL.

Quantitative real-time RT-PCR was performed using the Bio-Rad Real-Time PCR System with iTaq Universal SYBR® Green Supermix Bio-Rad kit following the manufacturer's instructions. Real-time RT-PCR reaction mixture contained 20 ng of total cDNA with a final volume of 10 μL. Thermal conditions were as follows: polymerase activation and denaturation at 95 °C for 30s; denaturation at 95 °C for 5s; annealing/extension at 60 °C for 30 s; 40 cycles; final extension at 95 °C for 10 s; melt curve 65 °C to 95 °C with 5 °C for 5s increments. Elongation factor 1-α - MGG_Ef1 (MGG_03641) or 40S ribosomal protein subunit S27a MGG_40S (MGG_02872) were used as reference genes (OMAR et al., 2016) and the mating type genes were used as target genes (Table 3) in the real-time RT-PCR.

Table 3 - Primers used in this study for real-time RT-qPCR (KANAMORI et al., 2007).

Genes	Forward primer 5'-3'	Reverse primer 5'-3'
MAT1-1-1	AGAGCAAATGACGAGAAAGAGCG	GCAGGCAACTCGCAGGAATC
MAT1-1-2	TAGGATGACTGTGCTCTC	TTTACACCGAGCCCGATG
MAT1-1-3	TACGAGAACAGCATAGTGG	GCGGTTTGGAGGCTTGGAA
MAT1-2-1	CTGCGAATGCCTACATCCTG	GCCGACGAGGAGAGTAGCGA
MAT1-2-2	CTCGAAATACCCTCTCAAT	GCGGTTTGGAGGCTTGGAA
MAT1	CTGCATGGTTTGGATCTGGG	GCGGTTTGGAGGCTTGGAA
MGG_Ef1*	CATCTTAACGTCGTCGTCATC	AGTGGCCGGTAGTCGTGG
MGG_40S*	ACAAGCTCAAGACCCTCGTC	GGTGGTGATGGTGAAGCAG

*Reference genes (OMAR et al., 2016).
Source: Tavares (2021).

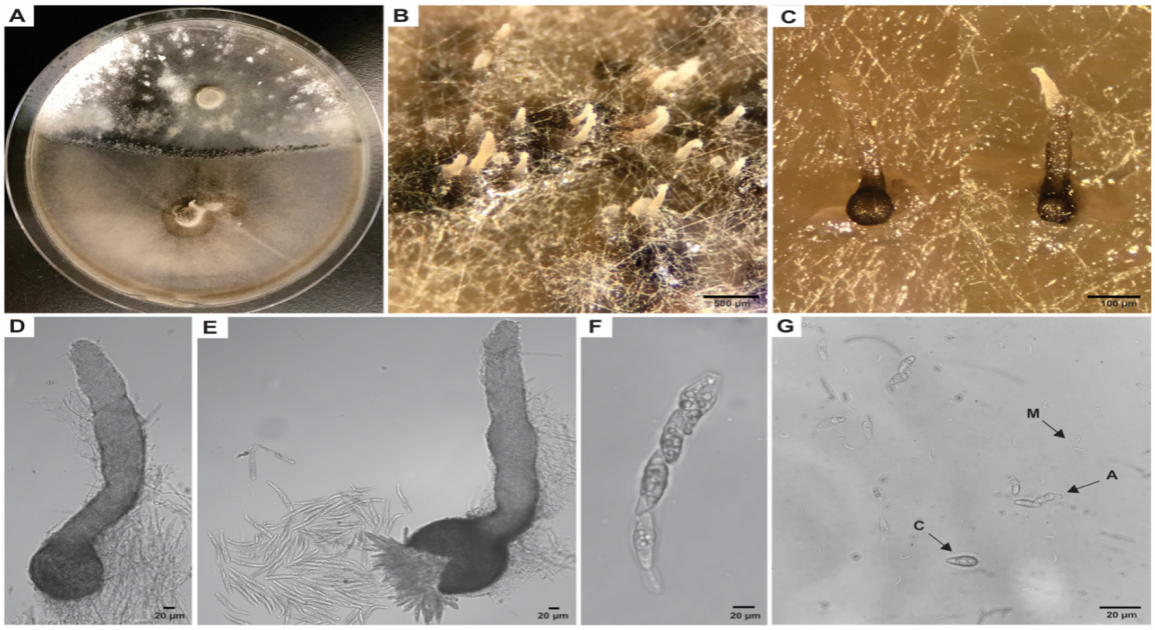
The specificity of the amplicons was checked by melting curve analysis and the relative normalized expression level of each gene was calculated using the reference genes.

3 RESULTS

3.1 SEXUAL REPRODUCTION IN *P. oryzae* PATHOTYPES *Triticum* AND *Oryzae*

Fertile crossing between Guy11 and CHNOS60-2-3 *Oryzae* testers, asci, and ascospores are shown in Fig. 1. Conidia and microconidia asexual spores were also seen with ascospores in Fig. 1G. The perithecia averaged were 147.5 µm high by 167.1 µm wide and long "necks" were observed with 545.2 μm long by 75.9 µm wide. The perithecia line (Fig. 1B) was present only on the side of the Guy11 isolate, which could therefore be considered a fertile female, and the CHNOS60-2-3 isolate as a donor (male).

Figure 1 - Photograph (A) and Photomicrographs (others) of sexual reproduction in *Pyricularia oryzae*.

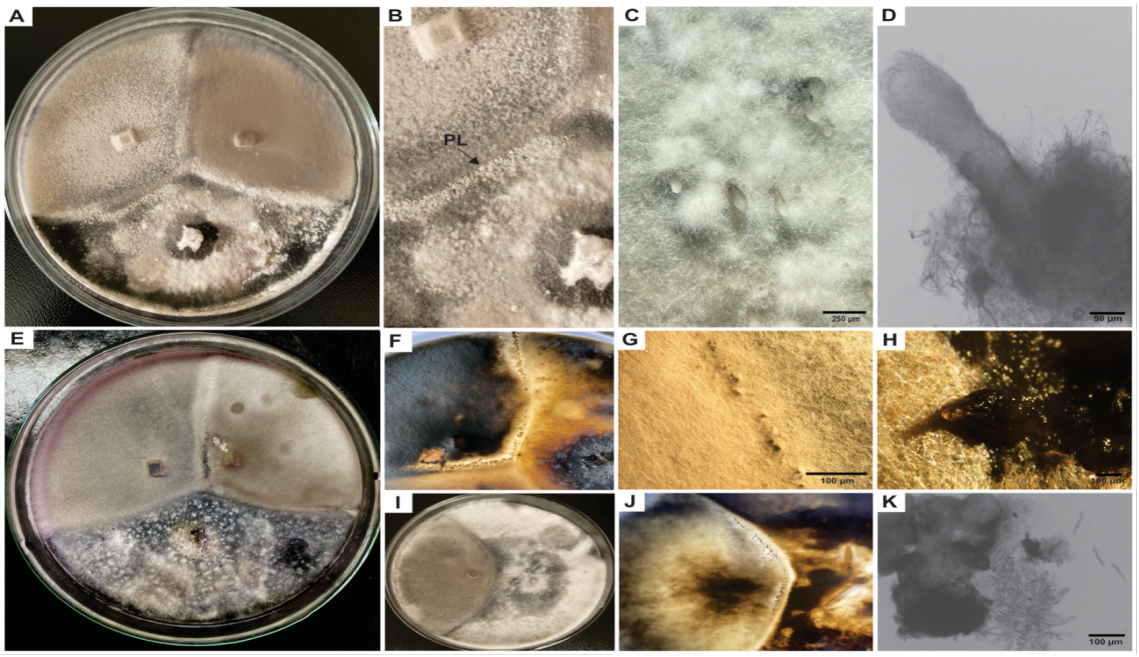


A) Crossing between MAT1-1 Guy11 (upper part of the picture) and MAT1-2 CHNOS60-2-3 (lower part of the picture). B) Line with perithecia "neck" outside of the medium. C-E) Perithecia. F) Asci. G) Ascospores (A), conidia (C), and microconidia (M).
Source: Tavares (2021).

The fertility of the 34 Brazilian isolates was tested. There were 31 *Triticum* lineage isolates, 12 were MAT1-1 and 19 were MAT1-2, and 3 *Oryzae* lineage isolates, all of which were MAT1-1. No fertility crossings were observed only among *Triticum* isolates. The

crossing in a triangle form placing the fertile isolates Guy11 and CHNOS60-2-3 in the apex with Brazilian isolates in the bottom is showed in Fig. 2A, E. Perithecia or protoperithecia were observed with the *Triticum* isolates 12.0.194, 12.1.117, 12.0.642i, and 12.1.181 in the Guy11 side, no asci or ascospores were observed. In the crossing between the 364 *Oryzae* isolate and CHNOS60-2-3 (Fig. 2A-D), a perithecia line (Fig. 2B) with long "neck" perithecia (Fig. 2C, D) on the 364 side which may indicate that this isolate is female, but infertile because of the absence of ascospores.

Figure 2 - Photographs of the induction of sexual reproduction in *Pyricularia oryzae* isolates.

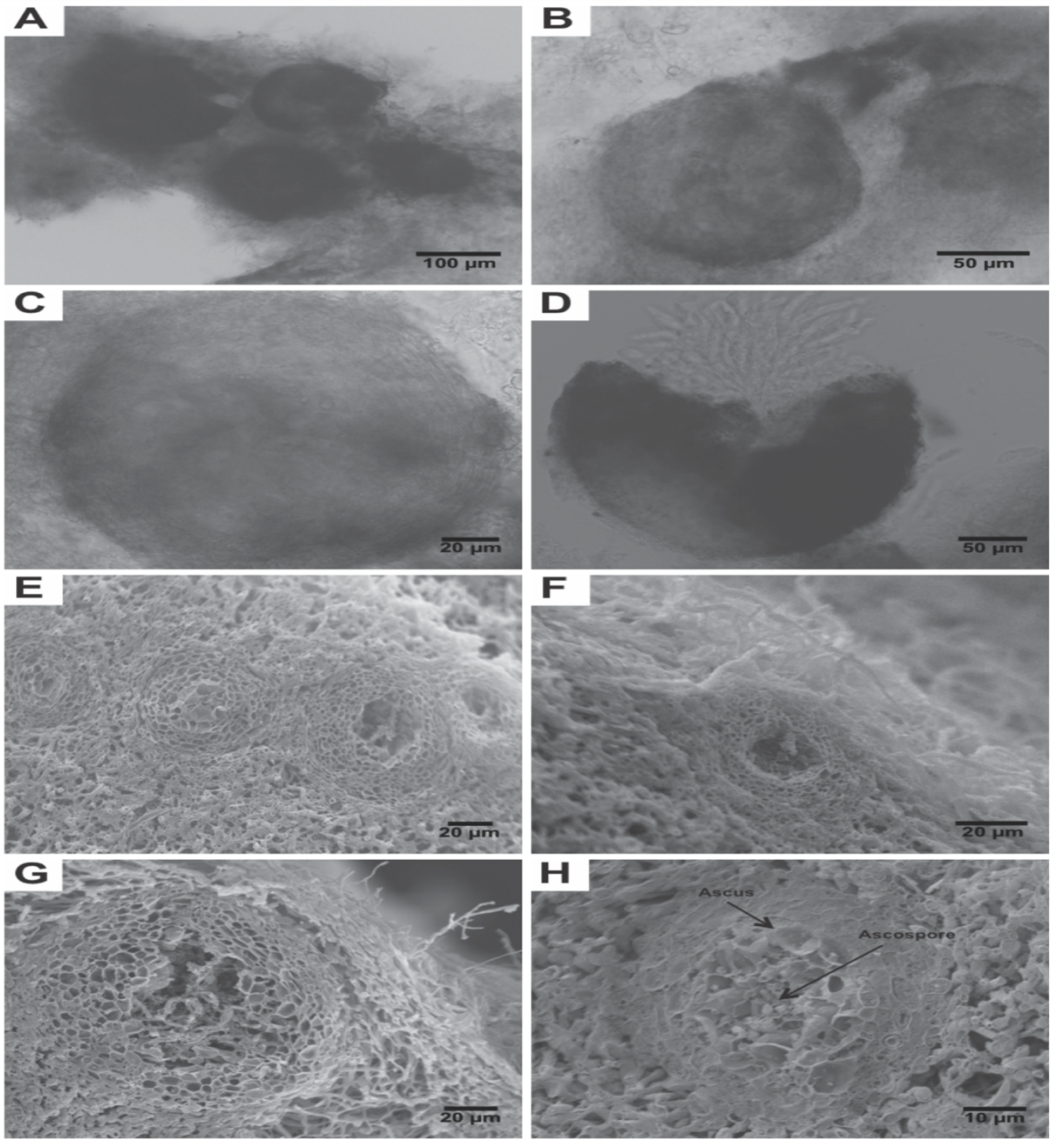


A) Crossing in triangle with the mating type-tester Guy11 (MAT1-1) in the right side and CHNOS60-2-3 (MAT1-2) in the left side, and the 364 isolate in the lower part of the Petri dish. B) Perithecia line (PL) between isolates CHNOS60-2-3 and 364 in 364 side. C) Perithecia "neck" outside of the medium. D) Infertile perithecium. E) Crossing in triangle with the mating type-tester Guy11 (MAT1-1) in the right side and CHNOS60-2-3 (MAT1-2) in the left side, and the 12.1.053i wheat isolate in the lower part of the Petri dish. F) Perithecia line between isolates CHNOS60-2-3 and Guy11, and 12.1.053i and Guy11. G) Perithecia line. H) Perithecia without long "neck". I) Crossing between Guy11 and 12.1.053i. J) Perithecia line in Guy11 side. K) Fertile perithecium with asci.
Source: Tavares (2021).

Fertile crossing was observed between Guy11, and the 12.1.053i *Triticum* isolate (Fig. E-J). The perithecia line was observed on the Guy11 side (Fig. 2F) from both CHNOS60-2-3 and 12.1.053i crossing (Fig. 2F) Perithecia did not have a long "neck" (Fig. 2G-I), but they were fertile. Asci and ascospores were observed in the perithecia (Fig. 2J). Perithecia wall were brown to dark brown when mature, its averaged were 259.1 µm (150.9-523.4 µm) high

by 237.0 µm (136.1-479.5 µm) wide (Fig. 3A-H). Perithecia are formed separately or together in the culture medium and can be seen in light (Fig. 3A) and scanning electron microscopy (Fig. 3E). Several layers of fungal cells were placed to form the perithecium wall (Fig. 3C, G). Asci from the perithecium are shown in Fig. 3D and a mature perithecium with a thick cell wall inside the asci and ascospores are shown in Fig. 3H.

Figure 3 - Perithecia from crossing between Guy11 *Oryzae* isolate and 12.1.053i *Triticum* isolate.

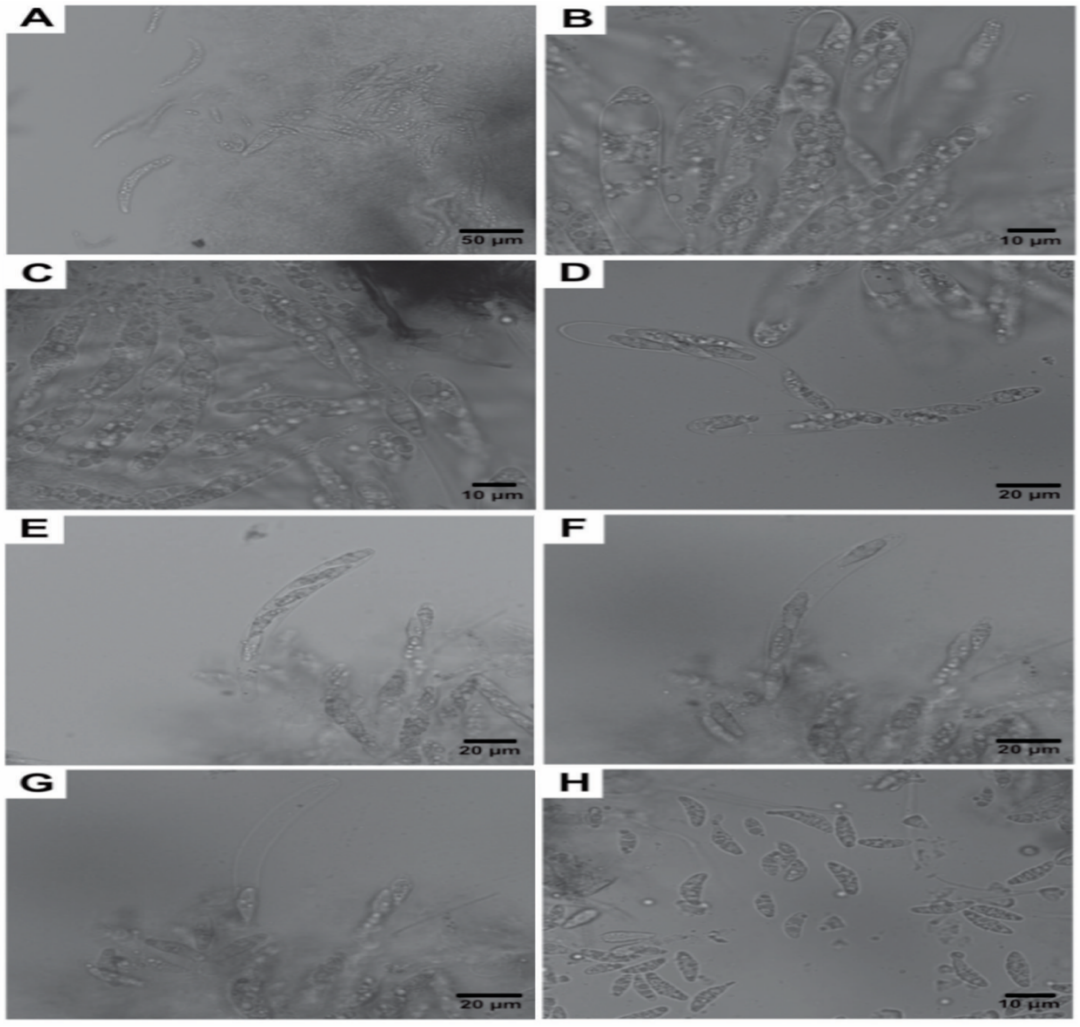


A-D) Photomicrographs. E-G) Scanning electron micrographs. A, E) Set of perithecia. B, F) perithecia in formation. C, G) Layers of cells to form the perithecium wall. D, H) Fertile perithecium with asci and ascospores.
Source: Tavares (2021).

The asci of 68.3-127.6 x 8.7-13.6 µm were cylindric-clavate (Fig. 4A-G), hyaline, unitunicate, and inoperculate (Fig. 4AG). The Sequence of ascospore release from the ascus

base is shown in Fig. E-G and a double-walled remaining ascus can be seen (Fig. 4G). Asci usually containing 8 hyaline ascospores range 9.9-14.1 μm large by 3.5-4.6 μm wide, and 1.9-2.8 μm in tip wide with three septa slightly curved (Fig. 4H).

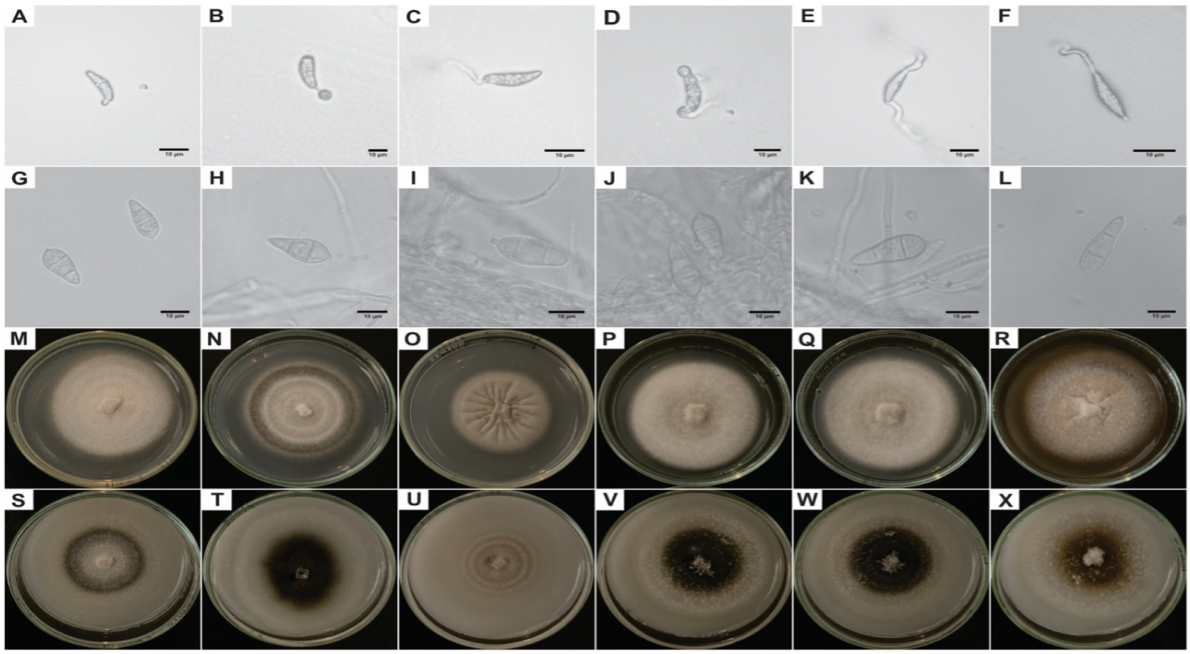
Figure 4 - Photomicrographs of asci and ascospores from crossing between Guy11 and 12.1.053i isolates.



A) Asci release from perithecium. B-D) Set of asci. E-G) Sequence of ascospore release from asci. H) Ascospores.
Source: Tavares (2021).

Ascospore germination was observed in 3% water agar after 24 h of incubation at 25 °C (Fig. 5A-F). Germination occurred from one (Fig. 5A-C) or both (Fig. 5D-F) of the ascospore tip cells. Six colonies were obtained, each from a single ascospore (Fig. 5M-X). *Pyricularia* piriform shape conidia were observed in the colonies (Fig. 5G-L) and colony morphologies were observed in CM (Fig. 5M-R) and oatmeal media (Fig. 5S-X).

Figure 5 - Photomicrographs of ascospores germination and colonies from ascospores of crossing between Guy11 *Oryzae* and 12.1.053i *Triticum* isolates.



A-C) Ascospores germination of an apical cell. D-F) Ascospores germination of two apical cells. G-L) Conidia of colonies from ascospores. Photographs (M-R) Colonies in complete medium. S-X) Colonies in oatmeal medium. G/1.053i-1 (G, M, S), G/1.053i-2 (H, N, T), G/1.053i-3 (I, O, U), G/1.053i-4 (J, P, V), G/1.053i-5 (K, Q, W), and G/1.053i-6 (L, R, X) isolates from ascospores.
Source: Tavares (2021).

The G/1.053i-1 isolate showed white aerial velvety-looking colony growing in concentric circles in CM medium (Fig. 5M) with little mycelial growth olivaceous in the center and white on the edges in oatmeal medium (Fig. 5S). The G/1.053i-2 isolate showed concentric circles growth with higher white mycelial density in the center and olivaceous growth on the edges in CM medium (Fig. 5N) and poor mycelial growth dark green in the center and white on the edges in oatmeal medium (Fig. 5T). The G/1.053i-3 colony grew slowly and showed soft gray velvety-looking colony in CM medium (Fig. 5O) and soft beige with little mycelial growth in concentric circles in oatmeal (Fig. 5U). The G/1.053i-4 and G/1.053i-5 isolates showed similar colony morphologies in both CM medium (Fig. 5P, Q) and oatmeal media with a white aerial cotton-like mycelium in CM (Fig. 5V, W) and little mycelial growth dark green in the center and white on the edges in oatmeal medium. The G/1.053i-6 isolate showed floccose surface soft beige in the center and white on the edges and produced rust color pigment in CM medium (Fig. 5R) and little mycelial growth ochraceous in the center and white on the edges in oatmeal medium (Fig. 5X).

3.2 MATING TYPE GENE ANALYSIS OF *P. oryzae* IDIOMORPHS

Primers A1/A5 and B15/B16 were used to amplify fragments of the idiomorphs Mat1-1 and Mat1-2, respectively according to Xu; Hamer (1996). Our isolates were aligned with sequences of fertile 70-6 and 70-14 (USA), fertile Y93-164a-1 and Y93-164g-1 (Chinese), and non-fertile Hoku-1 and P2 (Japanese) isolates. Based on the alignment the definition MAT1-1 for Mat1-2 and MAT1-2 for Mat1-1 was used because it identifies the idiomorphs according to Turgeon; Yoder (2000). This classification was adopted because the analyzed sequences are characteristic of those found in the MAT1 allele of ascomycetes (KANAMORI et al., 2007).

MAT1 locus analysis was performed with 19 sequences of idiomorph MAT1-1 and 23 sequences of idiomorph MAT1-2. The fertile 70-6 and 70-14, and non-fertile Hoku-1 and P2 were used as references for sexual and asexual isolates, respectively. The number of SNPs was 16 and 17 for MAT1-1 and MAT1-2 idiomorphs, respectively and both had five haplotypes (Table 4). MAT1-1-3 and MAT1-2-1 genes showed more SNP and mutation sites between the five genes analyzed separately (Table 4).

Table 4 - Polymorphism in MAT1 locus in *Pyricularia oryzae* isolates.

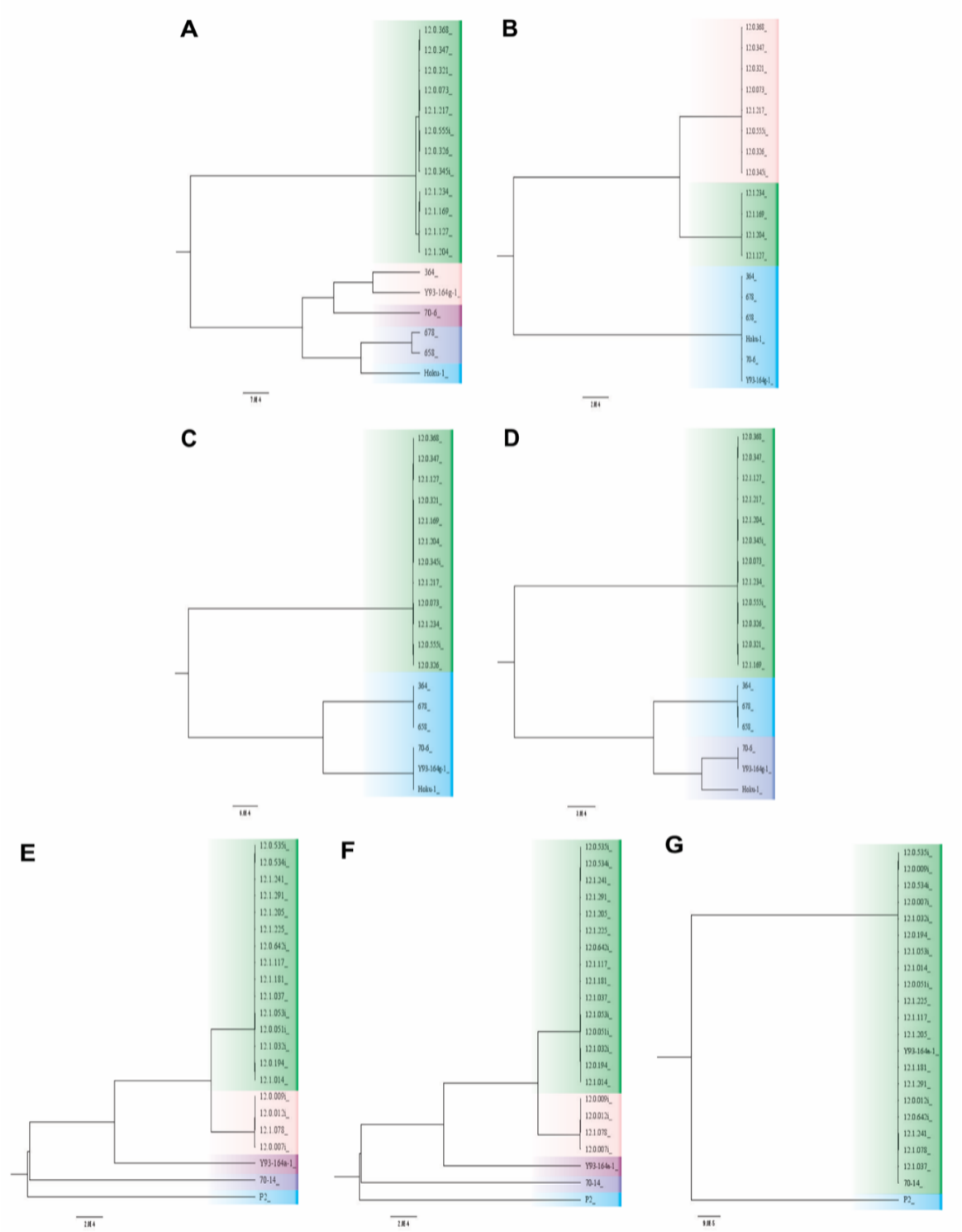
Genes	Sites	SNPs	Haplotypes	InDels	Hd	Pi
MAT1-1	4667	16	5	73	0,752	0,00152
MAT1-1-1	1037	4	3	0	0,680	0,00187
MAT1-1-2	909	2	2	1	0,471	0,00104
MAT1-1-3	1266	6	3	2	0,503	0,00195
MA1-2	3736	17	5	0	0,519	0,00058
MAT1-2-1	1504	10	5	0	0,519	0,00096
MAT1-2-2	1171	2	2	0	0,091	0,00016

SNPs: single nucleotide polymorphisms. InDels: insertions and deletions. Hd: haplotypic diversity. Pi: nucleotide diversity.
Source: Tavares (2021).

The highest number of 73 InDels was observed in the MAT1-1 idiomorph (Table 4). MAT1-1-2 and MAT1-1-3 genes showed one and two InDels while the others had no InDels. The MAT1-1 idiomorph showed a higher Hd with 0.752 than MAT1-2 with 0.519 (Table 4). The MAT1-1-1 gene had the highest Hd (0.680) value and MAT1-1-3 had the highest Pi (0.00195) value. MAT1-2-2 had the lowest Hd (0.091) and Pi (0.00016) values.

The phylogenetic tree was constructed based on the idiomorph sequence and each mating type gene separately (Fig. 6). Haplotypes for each gene in Table 4 are also shown in different colors in Fig. 6.

Figure 6 - Phylogeny of mating type gene sequences of *Pyricularia oryzae* performend using Bayesian Monte Carlo Chain Markov (MCMC) method for ten million of interactions with sampling in each 1000.



A) MAT1-1, B) MAT1-1-1, C) MAT1-1-2, D) MAT1-1-3, E) MAT1-2, F) MAT1-2-1, and G) MAT1-2-2 genes. Haplotype groups are highlight in different colors.
Source: Tavares (2021).

The MAT1-1 idiomorph showed five haplotypes, one for all *Triticum* isolates, and two different haplotypes for 70-6 and Hoku-1 reference isolates, and *Oryzae* isolates were placed in two different haplotypes (Fig. 6A). The MAT1-2 idiomorph also showed five haplotypes in which the *Triticum* isolates were separated into two groups and the 70-14 and P2 reference isolates and Y93-164a-1 had a haplotype for each one (Fig. 6E).

The MAT1-1-1 gene showed three haplotypes, one haplotype for *Oryzae* isolates, and two for *Triticum* isolates (Fig. 6B). The MAT1-1-2 gene presented two haplotypes separating *Oryzae* from *Triticum* isolates (Fig. 6C). Reference isolates were separated into one haplotype in MAT1-1-3, two different haplotypes, one for *Oryzae* and one for *Triticum* (Fig. 6D). The MAT1-2-1 gene presented similar results as its idiomorph MAT1-2 with five haplotypes showing high diversity when compared with MAT1-2-2 (Fig. 6F). MAT1-2-2 gene showed only two haplotypes and low diversity was observed between the *Triticum* isolates and the 70-14 and Y93-164g-1 sexual isolates that were grouped in the same haplotype (Fig. 6G). The asexual and reference P2 isolates formed one haplotype (Fig. 6G).

Alterations in protein predictions due to SNPs in the MAT1-1 idiomorph sequence of *P. oryzae* based on the 70-6 sequence are shown in Table 5. The Predicted MAT1-1-1 protein of the isolate sequences from 12.0.555i and 12.1.217 *Triticum* isolates (Table 5) showed a G in the nucleotide position 1113 of the 70-6 MAT1-1 sequence which changed asparagine (Asp) to glutamic acid (Glu). However, some SNPs caused silent mutations in the MAT1-1-1 gene, with no changes in the predicted protein in all *Triticum* isolate sequences. These sequences have a C, C, and T in the third nucleotide codon at positions 463, 885, and 1161 of the 70-6 MAT1-1 sequence whereas the *Oryzae* isolate sequences have a T, T, and A that did not change the phenylalanine, isoleucine, and threonine amino acids, respectively in the predicted protein.

Table 5 - Amino acid alteration in protein prediction due single nucleotide polymorphism in MAT1-1 idiomorph of *Pyricularia oryzae*.

Isolates		MAT1-1-1	MAT1-1-3b				
		1112	3575	3868	3900	3951	3987
	n.p.	-	-	-	-	-	-
		1114	3577	3869	3902	3953	3989
	a.p.	254	55	125	136	153	165
70-6*		gac (Asp)	cct (Pro)	atg (Met)	tcc (Ser)	gtg (Val)	cat (His)
Hoku-1*		gac (Asp)	cct (Pro)	atg (Met)	tcc (Ser)	gtg (Val)	c**g**t (**Arg**)
Y93-164b-1*		gac (Asp)	cct (Pro)	atg (Met)	tcc (Ser)	gtg (Val)	cat (His)
12.1.169^{T}		gac (Asp)	c**g**t (**Arg**)	a**c**g (**Thr**)	t**t**c (**Phe**)	g**g**g (**Gly**)	cat (His)
12.0.555i^{T}		ga**g** (**Glu**)	c**g**t (**Arg**)	a**c**g (**Thr**)	t**t**c (**Phe**)	g**g**g (**Gly**)	cat (His)

n.p.: Nucleotide position in MAT1-1 sequence of 70-6.
a.p.: Amino acid position in predicted MAT1-1-1 and MAT1-1-3b proteins.
*Reference isolates: 70-6 and Y93-164b-1 sexual isolates; Hoku-1 asexual isolate.
^{T}*P. Oryzae* pathotype Triticum isolates.
Nucleotide and amino acid substitution mutation in bold.
Source: Tavares (2021).

The MAT1-1-2 gene of all *Triticum* sequences has only two SNPs that cause silent mutations carried on a T and G in the third nucleotide codon at positions 2641 and 2128 of the 70-6 MAT1-1 sequence while the *Oryzae* sequences have a C and T that did not change valine and threonine amino acids, respectively. The MAT1-1-3a gene of all *Triticum* sequences also had SNPs that caused silent mutations in the predicted protein. These sequences carry a C, T, and G in the third nucleotide codon at positions 3867, 3900, and 3951 of the 70-6 MAT1-1 sequence whereas the rice isolate sequences have a T, C, and T that did not change asparagine, leucine, and arginine amino acids, respectively.

The asexual Hoku-1 sequence has a silent mutation in the third nucleotide codon G at position 3987 of the 70-6 MAT1-1 sequence while the other sequences have a A, which did not change proline amino acid in the predicted protein of MAT1-1-3a gene. The predicted MAT1-1-3b protein of the sequences from *Triticum* showed four SNPs that caused non-synonymous mutations in the amino acids when compared to *Oryzae* sequences. *Oryzae* isolate sequences have a C, T, C, and T in the second nucleotide codon at positions 3573, 3867, 3900, and 3951 of the 70-6 MAT1-1 sequence whereas *Triticum* sequences have G, C, T, and G which changed proline, methionine, serine, and valine to arginine, threonine, phenylalanine, and glycine, respectively (Table 5).

Alterations in protein predictions due to SNPs in the MAT1-2 idiomorph sequences of *P. oryzae* based on the 70-14 sequence are shown in Table 6. The predicted MAT1-2-1 protein of isolate sequences from *Triticum* showed SNPs that caused amino acid alterations

when compared to *Oryzae* isolates. *Oryzae* sequences have C in the first nucleotide codon at position 1009 of the 70-14 MAT1-2 sequence while *Triticum* sequences have a T that changed histidine to tyrosine (Table 6). The 70-14 sequence had A, G, and T while the other sequences had G, A, and C in the second nucleotide codon at positions 1241, 1532, and 1592 of the 70-14 MAT1-2 sequence, which changed aspartate, glycine, and leucine to glycine, aspartate, and proline, respectively (Table 6). The P2 isolate sequence had CT while the other sequences had GA at the positions 1955/1956 of the 70-14 MAT1-2 sequence, which changed leucine to glutamic acid (Table 6). The Y93-164a-1 sequence has G while the other sequences have C in the second nucleotide codon at position 2214 of the 70-14 MAT1-2 sequence, which changed arginine to proline in the MAT1-2-1 predicted protein (Table 6).

Table 6 - Amino acid alteration in protein prediction due single nucleotide polymorphism in MAT1-2 idiomorph of *Pyricularia oryzae*.

Isolates	MAT1-2-1					MAT1-2-2a		MAT1-2-2b	
n.p.	1010 - 1012	1241 - 1243	1533 - 1534	1592 - 1594	1955 - 1957	2214 - 2216	2951 - 2953	2952 - 2954	3057 - 3059
a.p.	77	154	251	271	374	438	90	99	134
70-14*	cac (His)	g**a**c (**Asp**)	g**g**c (**Gly**)	c**t**g (**Leu**)	gag (Glu)	ccg (Pro)	agc (Ser)	gcc (Ala)	cat (His)
P2*	cac (His)	ggc (Gly)	gac (Asp)	ccg (Pro)	c**t**g (**Leu**)	ccg (Pro)	a**a**c (**Asn**)	**a**cc (**Thr**)	c**g**t (**Arg**)
Y93-164a-1*	cac (His)	ggc (Gly)	gac (Asp)	ccg (Pro)	gag (Glu)	c**g**g (**Arg**)	agc (Ser)	gcc (Ala)	cat (His)
12.1.053i[T]	**t**ac (**Tyr**)	ggc (Gly)	gac (Asp)	ccg (Pro)	gag (Glu)	ccg (Pro)	agc (Ser)	gcc (Ala)	cat (His)

n.p.: Nucleotide position in MAT1-2 sequence of 70-14.
a.p.: Amino acid position in predicted MAT1-2-1, MAT1-2-2a, and MAT1-2-2b proteins.
*Reference isolates: 70-14 and Y93-164a-1 sexual isolates; P2 asexual isolate.
[T]*P. Oryzae* pathotype Triticum isolate.
Nucleotide and amino acid substitution mutation in bold.
Source: Tavares (2021).

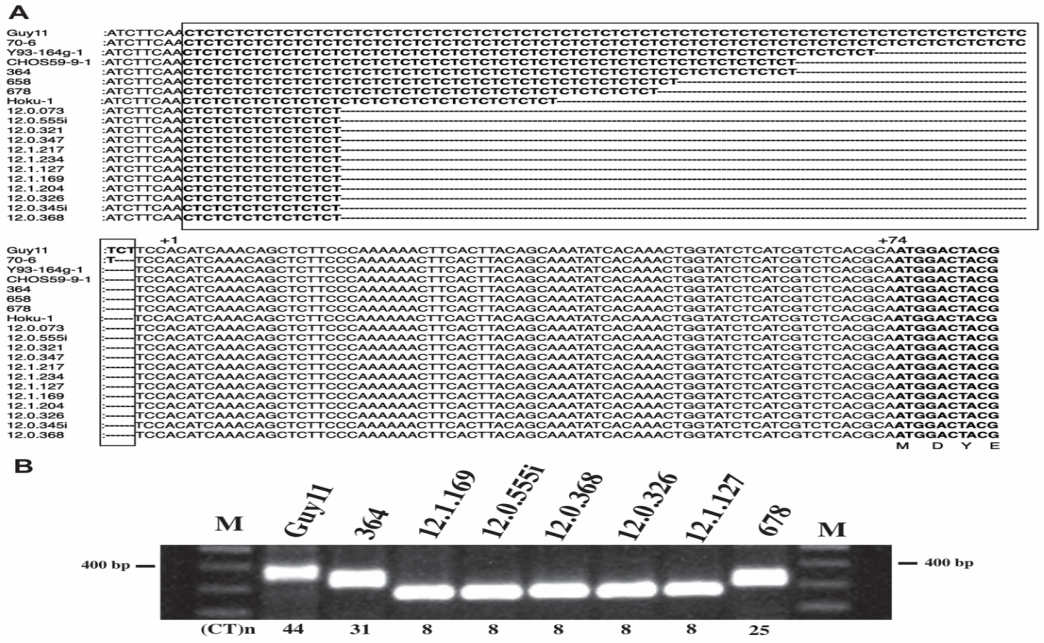
Some SNPs caused silent mutations in the MAT1-2-1 gene, with no changes in the predicted protein. The 12.0.007i, 12.0.009i, 12.0.012i, and 12.1.078 sequences have a T in the third nucleotide codon at position 824 in the 70-14 MAT1-2 sequence while the other sequences had a C however, it did not change the asparagine amino acid in the predicted protein. The 70-14, P2, and Y93-164a-1 sequences have an A in the third nucleotide codon and the other sequences have a G at position 2125 of the 70-14 MAT1-2 sequence, which did not change proline amino acid. SNPs in MAT1-2-2a were observed in P2 which has an A in

the third nucleotide codon at position 2951 of the 70-14 MAT1-2 sequence while the other sequences had a G, which changed asparagine to serine (Table 6). Two SNPs in the MAT1-2-2b gene of the P2 sequence caused an alteration of G and A to A and G in the first and second nucleotide codons at positions 2951 and 3057 of 70-14 MAT1-2A, which changed alanine and histidine to threonine and arginine, respectively (Table 6). A SNP in MAT1-2-2b caused a silent mutation in P2 isolate that had a G in the third nucleotide codon and the other sequences has A at position 3056 of the 70-14 MAT1-2 sequence which had no amino acid change.

3.3 POLYMORPHISM OF THE CT-DINUCLEOTIDE REPEAT REGION UPSTREAM OF MAT1-1-3 GENE

Within the MAT1-1 idiomorph, an upstream 5'-UTR (untranslated region - UTR) region of MAT1-1-3 has a cytosine and thymine-rich part - CT (CT)n, showing to be variable among sexual and asexual sequenes (Fig. 7A) as first observed by Kanamori et al. (2007).

Figure 7 - Comparison of CT-dinucleotide repeats (CT)n region upstream 5'-UTR (untranslated region - UTR) of MAT1-1-3 in *Pyricularia oryzae* isolates.



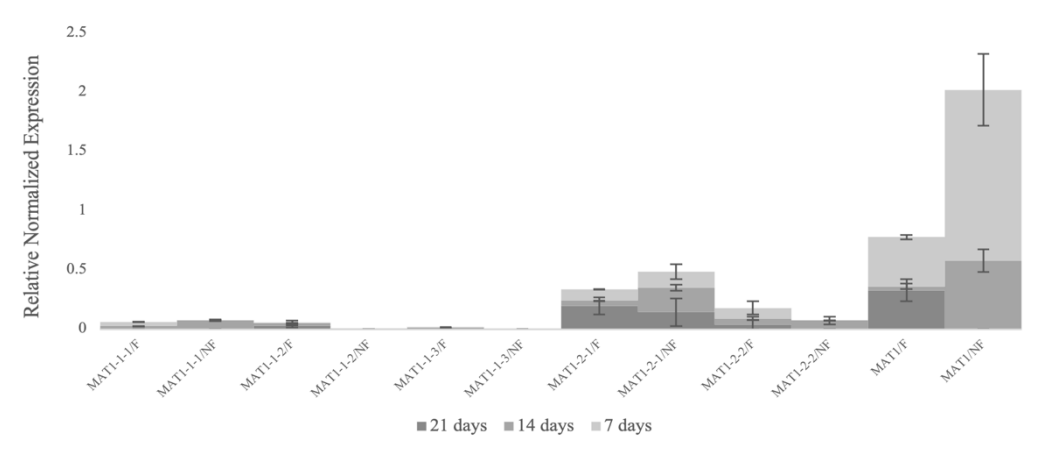
A) CT repeated sequences are shown inside the box delimited with black lines. The MAT1-1-3 mRNA transcription point, and translation start are indicated by +1 and +74, respectively. The first amino acids M, D, Y and E (methionine, aspartate, tyrosine, and glutamate, respectively) are shown below the last sequence. B) Amplification of CT-rich region from the genomic DNA with the primers B113-008 and B113-800. The size of each CT-rich region determinate by sequence are shown below each isolate.
Source: Tavares (2021).

Guy11 and 70-6 *Oryzae* lineage sequences and fertile isolates showed the largest dinucleotide region with $(CT)_{44}$ and $(CT)_{43}$, respectively followed by the Y63-164g1 *Oryzae* fertile isolate with $(CT)_{35}$. Isolates that showed no fertile crossing presented dinucleotide repeat regions less than 31 $(CT)_{31}$. The CHNOS59-9-1, 658, 678, and Hoku-1 non-fertile *Oryzae* isolates showed regions of $(CT)_{31}$, $(CT)_{25}$, $(CT)_{24}$, and $(CT)_{19}$, respectively. The 326 and 368 *Oryzae* isolates and *Triticum* isolates showed only $(CT)_8$ repeated sequences upstream of MAT1-1-3. Comparison between CT-rich region fragments can also be seen by agarose gel electrophoresis, Guy11 fertile isolate showed amplicons of approximately 400 bp, non-fertile *Oryzae* isolates showed amplicons between 400 and 300 bp and *Triticum* isolates showed amplicons less than 300 bp (Fig. 7B).

3.4 MATING TYPE GENE EXPRESSION DURING SEXUAL REPRODUCTION

The Normalized Relative Expression of the mating type genes was calculated based on the reference genes MGG_Ef1 (MGG_03641). MAT1-1-1 was expressed for 7 and 14 days in the fertile pair Guy11 and CHNOS60-2-3 and was expressed only for 14 days in the infertile pair CHNOS59-9-1 and CHNOS60-2-3 (Fig. 8). While the MAT1-1-2 and MAT1-1-3 genes were expressed, even in small amounts, only in the fertile pair at 7 and 14 days and were not expressed in the infertile pair. The MAT1-2-1 gene was expressed at 7, 14 and 21 days in both the fertile and infertile pairs, however the MAT1-2-2 gene was expressed at 7, 14 and 21 days in the fertile pair and only at 14 days in the infertile pair. The high expression of the MAT1 locus was observed in both the pairs. In the fertile pair, expression was observed at 7, 14 and 21 days and in the infertile pair for 14 days and 21 days (Fig. 8).

Figure 8 - Expression of mating type genes during sexual reproduction in *Pyricularia oryzae*.



F: Fertile pair. NF: Infertile pair.
Source: Tavares (2021).

4 DISCUSSION

The presence of both mating types in the fungal population indicates the possibility of sexual reproduction, although among *Triticum* isolates both mating types were present, the isolates did not show fertile crossing between them. In the crossing between 364 *Oryzae* isolate and CHNOS60-2-3 tester were observed long "neck" perithecia but they were not fertile. There were no asci and ascospores in the perithecia, which also indicate the low crossing capacity of rice isolates, as observed in other studies (BRUNO; URASHIMA, 2001; DAYAKAR; NARAYANAN; GNANAMANICKAM, 2000; GALBIERI; URASHIMA, 2008; SILUE; NOTTEGHEM, 1990; ZEIGLER, 1998). However, it is important to consider that some biological conditions are necessary for fertile crossing. The presence of opposite mating types is one of the requirements, but the sexuality of the isolates with fertile females and/or hermaphrodites must also be considered. Studies show a predominance of sterile females in *Pyricularia* isolates (BRUNO; URASHIMA, 2001; GALBIERI; URASHIMA, 2008) which also agrees with Zeigler (1998) who reported that most ascomycetes are sterile females. In addition, the predominance of males (sterile females) in fungal isolates shows that femininity can be lost through successive asexual cultures due to mutations, gene deletion, and chromosomal rearrangements (ITOI et al., 1983; XU, 2002). Fertility can also be lost through asexual reproduction as reported by Saleh et al. (2012) who observed a decrease in perithecia formation to loss of sexual reproduction capacity in fertile females of *P. oryzae*. Regulating the opposite sexual type recognition by pheromones that trigger signal

transduction to stimulate the expression of important genes in sexual reproduction, in addition to environmental stimulation, composition of the environment and the age of the isolate are also important factors to be considered (MOREIRA; CERESINI; ALVES, 2015).

In Brazil, two distinct groups of wheat blast were found, according to Urashima; Igarashi; Kato (1993) one group infects rice and wheat, and another group infects only wheat. DNA fingerprinting with repetitive DNA probes, MGR563 and MGR586, showed a high level of differentiation between the *Oryzae* (PoO) and *Triticum* (PoT) pathotypes of *P. oryzae* (FARMAN, 2002). Another study showed that the populations of *P. oryzae* from Brazil adapted to wheat was highly distinct from the population adapted to rice and the analysis of 69 wheat isolates showed that none of these fungi were able to infect rice (MACIEL et al., 2014).

However, fertile crossing was observed between the rice field isolate Guy11 and the 12.1.053i wheat isolate that even with high genetic differentiation between isolates from rice and wheat hosts they were able to cross. Genomic analyses of a worldwide collection of *P. oryzae* isolates from different host plants showed that these isolates are a single species, and strains adapted to rice, wheat, and other cereal crops are connected by significant genetic exchange and have a high degree of similarity in genome sequences corresponding to the same species (GLADIEUX et al., 2018). Furthermore, evidence of sexual fertility was found in *P. oryzae* isolates from different host lineages, including *Oryzae* and *Triticum*, producing viable ascospores in laboratory studies (KATO et al., 2000; TOSA et al., 2006, 2016). Crosses between a *P. oryzae* pathotype *Triticum* isolate from Brazil and an *Oryzae* isolate from Indonesia was performed to study the mechanisms of its infection specificity in wheat, and three loci were found to be involved in the avirulence of the *Oryzae* isolate on wheat (TOSA et al., 2006). Wheat blast was identified in Brazil in 1985 in the Paraná State (IGARASHI et al., 1986) and in 1986, it spread to the north and west of Paraná, northwest of São Paulo, and south of Mato Grosso do Sul (CRUZ; VALENT, 2017). Wheat blast also affected Bolivia (1996), Paraguay (2002), and Argentina (2007) (BAREA AND TOLEDO, 1996; KOHLI et al., 2011) has been reported outside of South America in Bangladesh (2016) (MALAKER et al., 2016). There is a risk that *Triticum* isolates in South Asia would recombine with fertile *Oryzae* isolates and the potential for evolution of new *P. oryzae* strains aggressive to both rice and wheat should not be underestimated (VALENT et al., 2019).

Analysis of mating type idiomorphs of *P. oryzae* isolates showed higher haplotype diversity (Hd) and InDels in MAT1-1 and higher SNPs in MAT1-2. The MAT1-1-3 and

MAT1-2-1 genes showed higher SNPs and Hd which were responsible for the diversity in the MAT1-1 and MAT1-2 idiomorphs, respectively. All mating type genes but MAT1-2-2 separate *Oryzae* from *Triticum* isolates. This corroborates the findings of Castroagudín et al., (2016) who showed great differences between *P. oryzae* pathotype *Oryzae* (isolated from rice) and *P. oryzae* pathotype *Triticum* and other grasses.

According to Kanamori et al. (2007), there are no obvious differences in gene structure between asexual and sexual isolates, except for differences in some amino acids in MAT1-2-1 and repeated upstream CT dinucleotide sequences of the MAT1-1-3 gene. However, structural differences in *Triticum* isolates mating type gene sequences may change the protein sequence and its action. Differences were observed mainly in the MAT1-1-3b and MAT1-2-1 sequences. The predicted protein of the MAT1-2-1 gene of *Triticum* isolates have glycine, aspartic acid, and proline at the 154, 251, and 271 positions of the predicted protein sequence of the 70-14 fertile isolate with aspartic acid, glycine, leucine, and glutamic acid, respectively. However, the 12.1.053i *Triticum* isolate that has the idiomorph MAT1-2 is a fertile isolate which implies that the amino acid changes did not affect the protein function of the MAT1-2-1 gene. Expression of the MAT1-2-1 gene was observed in both fertile and infertile pairs in this study.

The SMR1 transcript of MAT1-1-2 in *Podospora anserina*, has the conserved HPG domain (with histidine, proline and glycine residues) and tryptophan to alanine amino acid substitution mutations lead to inhibition of perithecia development in early stages (COPPIN; DE RENTY; DEBUCHY, 2005). The predicted protein of MAT1-1-3b genes has four amino acid sequence changes. Arginine, threonine, phenylalanine, and glycine at the 55, 125, 136 and 153 positions of the predicted protein sequence of the 70-6 fertile isolate with proline, methionine, serine, and valine, respectively. In addition, the comparison between the CT-rich regions at the 5'-UTR of MAT1-1-3 gene showed a large difference in the size of *Triticum* isolate sequences and the size of Guy11, 70-6, and Y93-164g-1 fertile isolates. The function of a CT-rich region is not clear, but it may act as a transcriptional signal that determines the initiation and increases the efficiency of MAT1-1-3 transcription depending on the number of CTs (KANAMORI et al., 2007; XU; GOODRIDGE, 1998). In the present study, we observed a small CT region sequence, especially in *Triticum* isolates, which may have influenced the expression of MAT1-1-3 gene, since no fertile MAT1-1 *Triticum* isolates were found. We also observed the expression of MAT1-1-3 gene in pair of fertile isolate but not in pair of infertile isolates. Kanamori et al. (2007) using RT-PCR showed strong expression of MAT1-

1-3 gene in isolates with a longer CT region, under conditions of sexual reproduction. This implies that the MAT1-1-3 gene plays an important role in sexual reproduction.

Early expression of MAT1-1-1 (7 days) in fertile pair may be involved in the activation of other genes, such as MAT1-1-2 and MAT1-1-3. MAT1-1-3 can be involved in fertility since its expression was not observed in the infertile pair and MAT1-1-2 may be involved in fruiting body production since its expression was observed in fertile pair at 14 and 21 days and no expression was observed in the infertile pair. The knockout of MATA-1 (MAT1-1-1) and MATA-3 (MAT1-1-3) in *Neurospora crassa* reduced its fertility, but without effects on the fruiting body and vegetative compatibility, in *Podospora anserina*, protein analyzes of the GAM-box domains of MAT1-1-3 and MAT1-2-1 are unequal to each other and likely bind to DNA differently and knockout of MAT1-1-1 and MAT1-2-1 affected its fertility (FERREIRA et al., 1998; DEBUCHY; TURGEON, 2006) . The importance of the MAT locus in sexual reproduction seems to be different in different fungi. In *Sordaria macrospora*, knockout of SMTA-2, which is related to MAT1-1-2, or the double-deletion of SMTA-2/3, which are related to MAT1-1-1 and MAT1-1-3, led to the underdevelopment of ascocarp (KLIX et al., 2010).

MAT1-2-2 may regulate late stages, such as production of ascus and ascospores since its expression was observed throughout the sexual cycle in fertile pair but was not observed at 7 and 21 days in the infertile pair. Kanamori et al. (2007) observed transcription of MAT1-1-1, MAT1-1-2, MAT1-2-1 and MAT1-2-2, genes were similar between sexual and asexual isolates and two ORFs identified in the locus mating type, MAT1-1-3a and MAT1-2-2a encode proteins that are potentially involved in sexual behavior. In the sexual cycle of *Fusarium graminearum*, the idiomorph MAT1-2 when deleted reduced the transcript levels of several genes related to the sexual cycle whereas in *Fusarium verticillioides*, ΔMAT1-2-1 mutant showed negative regulation in the expression of pheromone precursors and receptors (KESZTHELYI et al., 2007; LEE et al., 2006). High expression of the MAT1 locus in the fertile pair (Guy11 and CHNOS60-2-3) during the sexual cycle showed that mating type genes are expressed and are important during sexual reproduction. However, even in the infertile pair (CHNOS59-9-1 and CHNOS60-2-3) high expression in the MAT1 locus can be observed, which may indicate other roles of this locus in the fungal life cycle in addition to its role in mating (KANAMORI et al., 2007).

5 CONCLUSION

Fertile pairing was observed between the Guy11 *Oryzae* isolate tester and 12.1.053i *Triticum* isolate, and colonies were obtained from its ascospores. Infertile perithecia were observed in the crossing between the 364 *Oryzae* isolate and CHNOS60-2-3 tester, and protoperithecia in some crosses between the *Triticum* isolates and Guy11 tester. Analysis of SNPs separated in different haplotypes *Triticum* isolates from *Oryzae*, except for MAT1-2-2 which did not have enough diversity to separate them. Analysis of the predicted protein sequence, CT-rich region, and expression of mating type genes during sexual reproduction showed that MAT1-1-3 plays a critical role in the fertility of the *P. oryzae* pathotype *Triticum*.

REFERENCES

BAREA G.; TOLEDO J. Identificación y zonificación de piricularia o bruzone (*Pirycularia oryzae*) en el cultivo del trigo en el dpto. de Santa Cruz. In: CIAT. **Informe Técnico. Proyecto de Investigación Trigo**, Santa Cruz, 76–86, 1996.

BOUCKAERT, R. et al. BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. **PLoS Computational Biology**, v. 15, n. 4, p. 1–28, 2019.

BRUNO, A. C.; URASHIMA, A. S. Inter-relação sexual de *Magnaporthe grisea* do trigo e de outros hospedeiros. **Fitopatologia Brasileira**, v. 26, n. 1, p. 21–26, 2001.

CASTROAGUDÍN, V. L. et al. *Pyricularia graminis-tritici*, a new Pyricularia species causing wheat blast. **Persoonia: Molecular Phylogeny and Evolution of Fungi**, v. 37, n. DECEMBER, p. 199–216, 2016.

COPPIN, E.; DE RENTY, C.; DEBUCHY, R. The function of the coding sequences for the putative pheromone precursors in *Podospora anserina* is restricted to fertilization. **Eukaryotic Cell**, v. 4, n. 2, p. 407–420, 2005.

CRUZ, C. D.; VALENT, B. Wheat blast disease: danger on the move. **Tropical Plant Pathology**, v. 42, n. 3, p. 210–222, 2017.

DAYAKAR, B. V.; NARAYANAN, N. N.; GNANAMANICKAM, S. S. Cross-compatibility and distribution of mating type alleles of the rice blast fungus *Magnaporthe grisea* in India.

Plant Disease, v. 84, n. 6, p. 700–704, 2000.

DEBUCHY, R.; TURGEON, B. G. Mating type in Euascomycetes. In: Kues, U., Fischer, R. **The Mycota I**. 2nd Ed. Berlin Heidelberg: Springer-Verlag, p.293-323, 2006.

FARMAN, M. L. Erratum: *Pyricularia grisea* isolates causing gray leal spot on Perennial ryegrass (*Lolium perenne*) in the United States: Relationship to *P. grisea* isolates from other host plants (Phytopathology (November 2002) 92 (245-254)). **Phytopathology**, v. 92, n. 11, p. 1253, 2002.

FERREIRA, A. V. B. et al. Characterization of mat A-2, mat A-3 and ΔmatA mating-type mutants of *Neurospora crassa*. **Genetics**, v. 148, n. 3, p. 1069–1079, 1998.

GALBIERI, R.; URASHIMA, A. S. Caracterização, compatibilidade e ocorrência de reprodução sexual entre isolados de *Pyricularia grisea* de diferentes hospedeiros. **Summa Phytopathologica**, v. 34, n. 1, p. 22–28, 2008.

GEORGE, M. L. C. et al. Rapid population analysis of *Magnaporthe grisea* by using rep-PCR and endogenous repetitive DNA sequences. **Phytopathology**, v. 88, n. 3, p. 223–229, 1998.

GLADIEUX, P.; CONDON, B.; RAVEL, S.; SOANES, D.; MACIEL, J. L. N.; NHANI, A.; CHEN, L.; TERAUCHI, R.; LEBRUN, M. -H.; THARREAU, D.; MITCHELL, T.; PEDLEY, K. F.; VALENT, B.; TALBOT, N. J.; FARMAN, M.; FOURNIER, E. Gene flow between divergent cereal- and grass-specific lineages of the rice blast fungus *Magnaporthe oryzae*. **mBio**, 9: e01219-17, 2018. https://doi. org/10.1128/mBio.01219-17.

GOULART, A. C. P.; SOUSA, P. G.; URASHIMA, A. S. Danos em trigo causados pela infecção de *Pyricularia grisea*. **Summa Phytopathologica**, v. 33, n. 4, p. 358–363, 2007.

HAYASHI, N.; LI, Y. C.; LI, J. L.; NAITO, H. In vitro production on rice plants of perithecia of *Magnaporthe grisea* from Yunnan, China. **Mycological Research,** v. 101, p. 1308-1310, 1997.

ITOI, S.; MISHIMA, T.; ARASE, S.; NOZU, M. Mating behaviour of Japanese isolates of *Pyricularia oryzae*. **Phytopatologhy,** v. 73, p. 155-158, 1983.

KANAMORI, M. et al. Novel mating type-dependent transcripts at the mating type locus in *Magnaporthe oryzae*. **Gene**, v. 403, n. 1–2, p. 6–17, 2007.

KANG, S.; CHUMLEY, F. G.; VALENT, B. Isolation of the mating-type genes of the phytopathogenic fungus *Magnaporthe grisea* using genomic subtraction. **Genetics**, v. 138, n. 2, p. 289–296, 1994.

KATO, H. et al. Pathogenicity, Mating Ability and DNA Restriction Fragment Length Polymorphisms of *Pyricularia* Populations Isolated from Gramineae, Bambusideae and Zingiberaceae Plants. **Journal of General Plant Pathology**, v. 66, n. 1, p. 30–47, 2000.

KESZTHELYI, A. et al. Tagging target genes of the MAT1-2-1 transcription factor in *Fusarium verticillioides* (*Gibberella fujikuroi* MP-A). **Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology**, v. 91, n. 4, p. 373–391, 2007.

KLIX, V. et al. Functional characterization of MAT1-1-specific mating-type genes in the homothallic Ascomycete *Sordaria macrospora* provides new insights into essential and nonessential sexual regulators. **Eukaryotic Cell**, v. 9, n. 6, p. 894–905, 2010.

KOHLI, M. M. et al. *Pyricularia* blast-a threat to wheat cultivation. **Czech Journal of Genetics and Plant Breeding**, v. 47, n. SPEC. ISSUE 1, p. 130–134, 2011.

KUMAR, S. et al. MEGA X: Molecular evolutionary genetics analysis across computing platforms. **Molecular Biology and Evolution**, v. 35, n. 6, p. 1547–1549, 2018.

LEE, S. H. et al. Identification of the down-regulated genes in a mat1-2-deleted strain of *Gibberella zeae,* using cDNA subtraction and microarray analysis. **Fungal Genetics and Biology**, v. 43, n. 4, p. 295–310, 2006.

LIBRADO, P.; ROZAS, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. **Bioinformatics**, v. 25, n. 11, p. 1451–1452, 2009.

MACIEL, J. L. N. et al. Population structure and pathotype diversity of the wheat blast pathogen *Magnaporthe oryzae* 25 years after its emergence in Brazil. **Phytopathology**, v. 104, n. 1, p. 95–107, 2014.

MALAKER, P. K. et al. First Report of Wheat Blast Caused by *Magnaporthe oryzae* Pathotype *Triticum* in Bangladesh . **Plant Disease**, v. 100, n. 11, p. 2330–2330, 2016.

MOREIRA, S. I.; CERESINI, P. C.; ALVES, E. Reprodução Sexuada em *Pyricularia oryzae*.

Summa Phytopathologica, v. 41, n. 3, p. 175–182, 2015.

OMAR, S. C. et al. Validation of reference genes for robust qRT-PCR gene expression analysis in the rice blast fungus *Magnaporthe oryzae*. **PLoS ONE**, v. 11, n. 8, p. 1–18, 2016.

SALEH, D. et al. Sex at the origin: An Asian population of the rice blast fungus *Magnaporthe oryzae* reproduces sexually. **Molecular Ecology**, v. 21, n. 6, p. 1330–1344, 2012.

SHAHJAHAN, A. K. M. Practical approaches to rice blast management in tropical monsoon ecosystems, with special reference to Bangladesh. In: Zeigler RS, Leong SA, Teng PS **Rice blast disease.** Wallingford: CAB International, 1994. p.465-488.

SILUE, D.; NOTTEGHEM, J. L. Production of perithecia of *Magnaporthe grisea* on rice plants. **Mycological Research**, v. 94, n. 8, p. 1151–1152, 1990.

TOSA, Y. et al. Genetic analysis of host species specificity of *Magnaporthe oryzae* isolates from rice and wheat. **Phytopathology**, v. 96, n. 5, p. 480–484, 2006.

TOSA, Y. et al. Genetic and molecular analyses of the incompatibility between *Lolium* isolates of *Pyricularia oryzae* and wheat. **Physiological and Molecular Plant Pathology**, v. 95, p. 84–86, 2016.

TURGEON, B. G. Application of mating type gene technology to problems in fungal biology. **Annual Review of Phytopathology**, v. 36, n. 77, p. 115–137, 1998.

TURGEON, B. G.; YODER, O. C. Proposed nomenclature for mating type genes of filamentous ascomycetes. **Fungal Genetics and Biology**, v. 31, n. 1, p. 1–5, 2000.

URASHIMA, A. S.; IGARASHI, S.; KATO, H. Host range, mating type, and fertility of *Pyricularis grisea* from wheat in Brazil**. Plant Disease**, 1993.

VALENT, B. et al. *Pyricularia graminis-tritici* is not the correct species name for the wheat blast fungus: response to Ceresini et al. (MPP 20:2). **Molecular Plant Pathology**, v. 20, n. 2, p. 173–179, 2019.

XU, G.; GOODRIDGE, A. G. Act repeat in the promoter of the chicken malic enzyme gene is essential for function at an alternative transcription start site. **Archives of Biochemistry and Biophysics**, v. 358, n. 1, p. 83–91, 1998.

XU, J. Estimating the spontaneous mutation rate of loss of sex in the human pathogenic fungus *Cryptococcus neoformans*. **Genetics**, v. 162, n. 3, p. 1157–1167, 2002.

XU, J. R.; HAMER, J. E. Assessment of *Magnaporthe grisea* mating type by spore PCR. **Fungal Genet Newsl,** v. 42, p. 80, 1995.

XU, J. R.; HAMER, J. E. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. **Genes and Development**, v. 10, n. 21, p. 2696–2706, 1996.

ZEIGLER, R. S. Recombination in Telomeres. **Microbe Magazine**, v. 6, n. 3, p. 133–133, 2011.

ARTIGO 2 – RELATIONSHIP OF RGF1 AND RGF2 GENES WITH THE PMK1, MPS1, AND OSM1 MAP KINASES PATHWAYS IN *Pyricularia oryzae* *

ARTIGO FORMATADO DE ACORDO COM A NORMA NBR 6022 (ABNT 2018)

Dérica Gonçalves Tavares **

Prof. Dr. Eduardo Alves (Orientador) ***

ABSTRACT

The objective of this study was to obtain mutants of the RGF1 and RGF2 genes from the *Pyricularia oryzae* Ku80 isolate and to evaluate these genes interaction with the Pmk1, Mps1, and Osm1 MAPK pathways. Mutants were obtained using the Split-Marker method and their proteins were extracted. TEY (Pmk1 and Mps1) and TGY (Oms1) phosphorylation assays were performed. Only two and four mutants of RGF1 and RGF2 were obtained, respectively. The *Δrgf1* mutant showed little mycelial growth and abnormal conidia shape with mostly one septum. The *Δrgf2* mutant had colony morphology similar to that of wild type Ku80 and its conidia had a normal shape with 2 septa as wild type Ku80. Phosphorylation levels of Pmk1 and Mps1 were reduced in the *Δrgf1* mutant and were not reduced in the *Δrgf2* mutant indicating that only RGF1 is involved in the activation of Pmk1 and Mps1 MAPK signaling. Phosphorylation levels of Oms1 in *Δrgf1* and *Δrgf2* mutants were not reduced indicating that RGF1 and RGF2 are dispensable for activating the Oms1 MAPK pathway. Therefore, Rgf1 seems to function upstream of the Pmk1 and Mps1 MAP kinase pathways in *P. oryzae* and these results indicate that RGF1 is the dominant RasGEF gene in *P. oryzae* and RGF2 plays a minor role as RasGEF.

Keywords: Gene replacement, Pathogenicity, Western blot, TEY phosphorylation

---

* Artigo Apresentado para a conclusão do curso de pós-graduação em Microbiologia Agrícola da Universidade Federal de Lavras.

** Doutoranda no curso de Microbiologia Agrícola, pela Universidade Federal de Lavras. Endereço eletrônico: dericatavares@gmail.com.

*** Professor da Universidade Federal de Lavras, Doutor em Fitopatologia.

1 INTRODUCTION

Rice blast disease is caused by *Pyricularia oryzae* and is one of the most destructive agricultural diseases in the world. *P. oryzae* attacks all above-ground parts and causes significant economic losses in rice plants (VALENT; KHANG, 2010). *P. oryzae* is also a model fungus used to study plant-pathogen interactions (VALENT; CHUMLEY, 1991; WILSON; TALBOT, 2009).

Several signaling pathways are related to pathogenicity and have been characterized in *P. oryzae*. The Pmk1 MAP kinase pathway regulates the final stage of appressorium formation, penetration, and the invasive hyphae growth (LI; ZHOU; XU, 2012). The Pmk1 MAPK and cAMP pathways are essential for invasive growth and pathogenicity of *P. oryzae* (WILSON; TALBOT, 2009). Another MAP kinase essential for pathogenesis is MPS1, which is important for conidiation, cell wall integrity, and penetration into plant cells (XU; STAIGER; HAMER, 1998). The Osm1 MAPK signaling pathway regulates hyperosmotic stress responses, but it is not necessary for appressorium turgor generation (DIXON et al., 1999).

Several genes have function upstream of Pmk1 pathway, like Mst11 (MAPKKK), Mst7 (MAPKK), adapter protein Mst50, and Ras2 (ZHAO et al., 2005; ZHAO; MEHRABI; XU, 2007). In a previous study, two Ras proteins, Ras1 and Ras2, were identified to function upstream of the PmK1 and cAMP signaling pathways in *P. oryzae* (PARK et al., 2006). Ras1 and Ras2 interact with Mst50 which is an adapter protein in the MAPK cascade, which can directly interact with Mst7 (MAPKK) and Mst11 (MAPKKK). Consequently, Ras1 and Ras2 are believed to be involved in regulation of the Pmk1 MAPK signal transduction pathway (ZHAO; MEHRABI; XU, 2007). A mutant of the Ras1 gene (Δras1) forms appressorium and infects the plant as well as the wild isolate (PARK et al., 2006; ZHAO; MEHRABI; XU, 2007). However, Ras2 gene appears to be essential in *P. oryzae*. Expression of the dominant $RAS2^{DA}$ allele in the wild isolate results in increased levels of intracellular cAMP and stimulates the formation of appressoria with abnormal morphology on hydrophobic and hydrophilic surfaces, suggesting that Ras2 is functionally related to the cAMP-PKA pathway (LI; ZHOU; XU, 2012). Furthermore, phosphorylation of Pmk1 is increased in transformants with $RAS2^{DA}$ (LI; ZHOU; XU, 2012).

Ras proteins are required for a series of physiological and pathogenic events, such as proliferation, differentiation, cell death, and infective capacity (HANCOCK, 2003).

Furthermore, Ras proteins are required for mitosis and the regulation of filamentous growth in yeast (YOSHIDA; ICHIHASHI; TOH-E, 2003). These proteins act as switches, converting its form from "on" to "off" by binding to GTP (RasGTP) or GDP (RasGDP), that is regulated by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively (ROJAS; SANTOS, 2006). Ras proteins when activated translate the extracellular signals to regulate downstream targets. Two GEF genes (RGF1 and RGF2) were identified as RasGEF in *P. oryzae*. RGF2 appears to be a dispensable gene in *P. oryzae* because its deletion had no significant effects on growth and plant infection. However, RGF1 is essential for appressoria formation and pathogenicity, because the *Δrgf1* mutant produces conidia with an abnormal shape and is defective in fixation on hydrophobic surfaces (Unpublished work).

Mitogen-activated protein kinases (MAPKs) are Ser/Thr protein kinases that convert extracellular stimulus into a wide range of cellular responses. MAPKs are among the oldest signal transduction pathways and are used in several biological processes (CARGNELLO; ROUX, 2011). Exemples of conventional MAPKs are extracellular signal regulatory kinases 1/2 or ERK1/2 and p38 isomers. ERK1/2 has an activation motif conserved in Thr-Glu-Tyr or TEY (Threonine-Glutamate-Tyrosine) and p38 has an activation motif conserved in Thr-Gly-Tyr or TGY (Threonine-Glycine-Tyrosine) in its activation loops. In *P. oryzae*, the Pmk1 and Mps1 pathways have MAPKs with TEY activation and the Osm1 pathway has MAPKs with TGY activation (ZHANG et al., 2017). TEY and TGY phosphorylation assays were performed to measure the amount of MAPKs produced under specific conditions. In this study, we evaluated the expression of protein kinases in RGF1 and RGF2 mutants of *P. oryzae* by TEY and TGY phosphorylation assays to verify the functionality of these genes in the Pmk1, Mps1, and Oms1 pathways.

2 MATERIAL AND METHODS

2.1 CULTURE CONDITIONS AND DNA EXTRACTION

The Ku80 isolate of *P. oryzae* was used to obtain RGF1 and RGF2 mutants. Ku80 was cultivated in oatmeal medium (50 g/L oatmeal and 13.5 g/L agar) at 25 ºC. For DNA extraction, the isolate was grown in complete medium (10 g/L glucose, 2 g/L peptone, 1 g/L yeast extract, 1 g/L casamino acids, 50 mL/L of 20x nitrate salts, 1 mL/L of trace elements, 1 mL/L of vitamin solution, and pH 6.5) for 3 days with stirring at room temperature and 105

rpm. Mycelium was collected, dried, and macerated with liquid nitrogen and the DNA was extracted using the CTAB (Cetyl Trimethyl Ammonium Bromide) method.

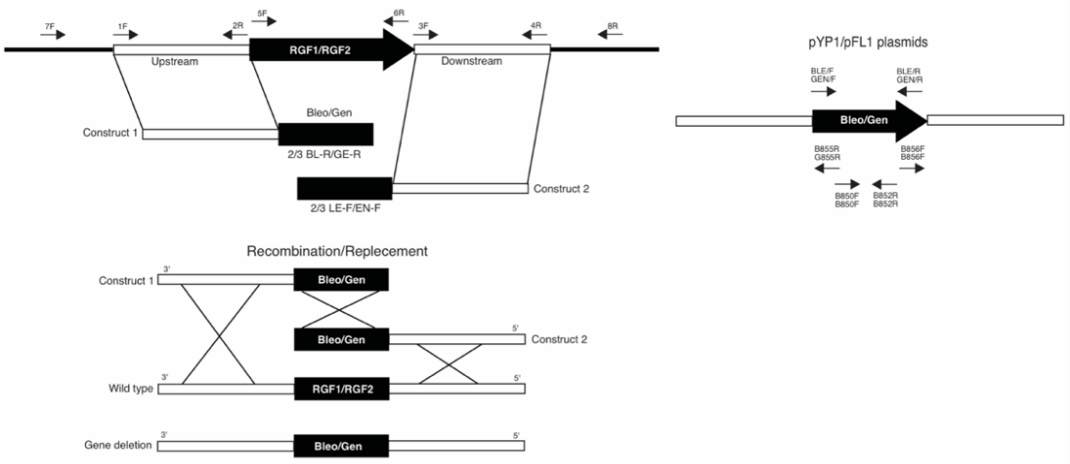
2.2 PROTOPLAST PREPARATION

Mycelium of 10-days-old Ku80 strain was blended with 50 mL of 5 x YEG (0.5% yeast extract, 2% glucose) containing 50 μg/mL ampicillin, with 3 pulses of 10 s each, and incubated for less than 24 h under stirring at room temperature at 105 rpm. On the second day, the culture was blended again with 3 pulses of 10 s each and 50 mL of complete medium containing 50 μg/mL ampicillin was added. The macerate was incubated for up to 12 h at 105 rpm and room temperature. On the third day, the mycelium was collected by filtration through a double layer of sterile Miracloth and washed with 1.2M sorbitol. The mycelium was resuspended in 20 mL of 1.2M sorbitol containing 10 mg/mL of Lysing enzymes from *Trichoderma* (Sigma L1412), incubated at 30 °C for 2h with shaking at 45 rpm. Protoplasts were collected by filtration through a double layer of sterile Miracloth, centrifuged at 4500 rpm for 6 min at room temperature and resuspended in 20 mL of 1 x STC (20% sucrose, 50 mM Tris-HCl pH 8.0, and 50 mM CaCl2). Protoplasts were centrifuged again and resuspended in 1 x STC at a concentration of 2 x 10^7 to 1 x 10^8/mL.

2.3 GENE REPLACEMENT BY USING SPLIT-MARKER APPROACH

The methodology used was based on Goswami, (2012). Two different marker genes were used. The marker gene is a contruct containing an antibiotic resistance gene. Resistance to zeocin (Bleo) and geneticin (Gen) marker genes were used to replace RGF1 and RGF2, respectively. The plasmids pYP1 and pFL1 contain Bleo and Gen marker genes, respectively, and were mantained into *Escherichia coli* strains. Fungal DNA and plasmids were used to obtain two linear constructs by fusing the PCR products. The first linear construct has an upstream flanking region of the target gene and 2/3rd of the marker gene from the 5' end, and the second linear construct has a downstream flanking region of target gene and 2/3rd of marker gene from the 3' end (Fig. 1). These linear constructs were used simultaneously for protoplast transformation and gene replacement occurs by homologous recombination at two sites, within the marker gene and between the flanking regions (Fig. 1).

Figure 1 - Representation of split-marker-targeted replacement showing primers, genes, and recombination sites.



Source: Tavares (2021).

The primers used in this study are listed in Table 1. Primers were designed for upstream (1F and 2R) and downstream (3F and 4R) regions of target genes and the center of target genes (5F and 6R) (Fig. 1). Primers were designed using sequences from RGF1(MGG_00371) and RGF2 (MGG_00199). Two primers were used to amplify the entire marker gene, for zeocin (BLE-F and BLE-R) and geneticin (GEN-F and GEN-R), two more primers to amplify 2/3rd of marker genes from the 5’ and 3’ ends (BL-R and LE-F; GE-R and EN-F, respectively) (Fig. 1). Complementary sequences to the marker primers BLE-F and BLE-R were added to the 5' ends of the Rgf1/2R and 5' ends of the Rgf1/3F primers, respectively, and the same was done for the RGF2 gene using the marker primers for geneticin. This extension is necessary for fusion of the flanks to marker sequences.

Table 1 - Primers useds in this study.

Name	5'→ 3' sequences
Rgf1/1F	AAGATATCATTCCAATGCAT
Rgf1/2R	CCAAGCCCAAAAATGCTCCTTCAGGTAACTTTGGACGCAATCACGTTCG
Rgf1/3F	GCACAGGTACACTTGTTTAGAGGTAATCCGCAAACGACTATACAACACG
Rgf1/4R	GTCTCCATTGTGTCTCGATCCGTTCTGGCT
Rgf1/5F	GGACTCTATGAACGGACAAC
Rgf1/6R	CTCCCTGATACTGGAGACAA
Rgf1/7F	AATAGGAAACCTGGGTACCT

Rgf1/8R	TGAATAACATGTATGGCTGA
BLE/F	TACCTGAAGGAGCATTTTTGGGCTTGG
BLE/R	GGATTACCTCTAAACAAGTGTACCTGTGC
BL/R	TGGATGCCGACGGATTTGCA
LE/F	TCAGCCCACTTGTAAGCAGTAGC
B850/R	TCGCCCTTCCTCCCTTTATTTC
B852/F	GCCAAGAGCGGATTCCTCAGTC
B855/R	ATGTCCTCGTTCCTGTCTGCTA
B856/F	TCTCAAGCCTACAGGACAC
Rgf2/1F	GGTTTGTACTGTGCAGTATT
Rgf2/2R	CAGATACGGCAGAGAAATCGCAACCTCCATGACCTTTGGGAAGAAGC
Rgf2/3F	GTTTAGATTCCAAGTGTCTACTGCTGGCTCTCAAGCAAGCCTCGGAA
Rgf2/4R	CTTGGTTGTTGCTGCTAGGAA
Rgf2/5F	ATCGCTATTGTCCGAGCTT
Rgf2/6R	ATCGCGGTAAGCATCAAGGAA
Rgf2/7F	GTGATCTACAGTTACTCGGT
Rgf2/8R	TCCTGGGTGTTCAAGAACGT
GEN/F	GAGGTTGCGATTTCTCTGCCGTATCTG
GE/R	CCACAGTCGATGAATCCAGAAAAGCG
GEN/R	GCCAGCAGTAGACACTTGGAATCTAAAC
EN/F	GGAAGGGACTGGCTGCTATTGG
G852/F	TCGGCTATGACTGGGCACAACA
G850/R	GAGCGGCGATACCGTAAAGCAC
G855/R	TGTTGGGTTTGAGCTAGGTGGG
G856/F	GAATGGTCAAATCAAACTGCTAGATAT

Source: Tavares (2021).

Split-marker-targeted replacement was performed in two rounds of PCR. The First round of PCR was performed to amplify the flanking regions of the RGF1 and RGF2 genes using DNA extracted from the Ku80 isolate (Table 2). And 2/3rd of the marker gene from the 5' end and 2/3rd of the marker gene from the 3' end 2/3 using pYP1 (Bleo) and pFL1 (Gen) plasmids extracted from *E. coli* strains (Table 2). PCR conditions were as follows: 95 °C for 1 min, 35 cycles of 30 s at 95 °C, 30 s at 56 °C and 2 min at 72 °C, with a final extension at 72

°C for 5 min. The final reaction volume was 100 μL. The PCR products were purified using the QIAquick Gel Extraction kit, Qiagen.

Table 2 - Combination of primers and templates for PCR reactions.

First PCR round	Primers*	Template*	Amplicon*
1	Rgf1-1F and Rgf1-2R	Ku80 genomic DNA	5' flank of gene
2	Rgf1-3F and Rgf1-4R	Ku80 genomic DNA	3' flank of gene
3	BLE-F and BL-R	Marker gene from pYP1 plasmid	2/3 of markr gene from 5' end
4	LE-F and BLE-R	Marker gene from pYP1 plasmid	2/3 of markr gene from 3' end
5	BLE-F and BLE-R	Marker gene from pYP1 plasmid	Entire marker gene (Bleo)
Second PCR round	Primers*	Template*	Amplicon*
6	Rgf1-1F and BL-R	5' flank of gene (1) and 2/3 of markr gene from 5' end (3)	Construction 1 for transformation with the 5' end flank and 2/3rd of the marker gene
7	LE-F and Rgf1-4R	3' flank of gene (2) and 2/3 of markr gene from 3' end (4)	Construction 2 for transformation with the 3' end flank and 2/3rd of the marker gene

*The same was done for the RGF2 gene, using the respective primers and plasmid pFL1.
Source: Tavares (2021).

In the second round of PCR, each flank of the first round was fused to the marker through PCR splicing by overlapping the homologous regions. The PCR product of 2/3rd of the marker gene was used as a template with gene-specific primers (Table 2). The PCR conditions were as follows: 95 °C for 1 min, 35 cycles of 30 s at 95 °C, 30 s at 58 °C and 2 min at 72 °C, with a final extension at 72 °C for 5 min. The final volume of each reaction was 50 μL. Two constructions were obtained (Fig.1 and Table 2) and used to transform the protoplasts.

Protoplasts were transformed using the polyethylene glycol (PEG) mediated method (LEUNG et al., 1990). PCR products from reactions 6 and 7 (Table 2) were used in a volume of 15 μL containing approximately 50 ng/μL of genetic material, which were incubated with 200 μL of protoplast solution for 20 min at room temperature. Then, 1 mL of 40% PEG (40%

PEG 8000 in 1 x STC) was added to the protoplast solution and incubated again at room temperature for 20 min. After this period, 5 mL of TB3 medium (3 g/L yeast extract, 3 g/L casamino acids and 20% sucrose) containing 50 μg/mL ampicillin was added and incubated at room temperature at 100 rpm using a shaker table overnight. The next day, TB3 with 0.70% agar containing 50 μg/mL of ampicillin and 100 μg/mL of bleomycin (Invitrogen) was added to the protoplast solution to complete 15 mL to select transformants of the RGF1 gene. Protoplast solution containing RGF2 constructs was proced as described above but 250 μg/mL geneticin (G418, Sigma-Aldrich) was added instead of bleomycin. Then, the protoplast solution containing TB3 was poured into a Petri dish and incubated at room temperature for 24 h. Then, another layer of TB3 with 0.70% agar containing 50 μg/mL ampicillin and 150 μg/mL bleomycin or 300 μg/mL geneticin, for RGF1 or RGF2, respectively was added to the plate and incubated at room temperature.

After approximately one-week colonies that grew were picked to oatmeal containing bleomycin or geneticin. DNA from these colones was extracted and PCR was performed using primers Rgf1/5F and Rgf1/6R or Rgf2/5F and Rgf2/6R were performed to verify RGF1 and RGF2 transformants, respectively. More PCRs were performed with Rgf1/7F and B855/R, B856/F and Rgf1/8R primers to confirm the insertion of the 5' and 3' flanking regions, respectively of the RGF1 added to 2/3rd of the Bleo marker, B850 and B852 primers to confirm insertion of the Bleo marker gene (Table 1). The same was done to confirm the presence of Gen marker gene using specific primers (Table 1).

2.4 TEY AND TGY PHOSPHORYLATION ASSAY

2.4.1 Protein extraction

Proteins were extracted from the mycelia of the transformants, which were grown in complete medium for 2 days at 105 rpm at room temperature. Approximately 3 g of partially dried mycelium was placed in microtubes containing 0.5 mm glass beads and the microtubes were filled with Lysis Buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol) containing 10 μL/mL protease inhibitors (Sigma-Aldrich) and Phosphatase Cocktails 2 and 3 (Sigma-Aldrich). Microtubes were shaken in a cell disruptor 4 times for 20 s each with an interval of 2 min. The samples were kept on ice at intervals and centrifuged at 13000 rpm for 20 min at 4 ºC. The supernatant was removed and centrifuged

again under the same conditions and 100 μL of the supernatant was mixed with 100 μL of Loading Buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8). Microtubes with samples were boiled (100 ºC) for 5 min, placed on ice and stored at 4 ºC.

2.4.2 Western Blot

Total proteins were separated on a 10% SDS-PAGE gel that consist in 10% resolving gel (30% acrylamide/bis; 1.5 mM Tris-HCl, pH8.8; 10% SDS; 15 μL of TEMED; 10% APS; ultrapure water) which is the bottom of the gel and 4% stacking gel (30% acrylamide/bis; 0.5 mM Tris-HCl, pH6.8; 10% SDS; 7.5 μL TEMED; 10% APS; ultrapure water) which is the top of the gel. Aliquots of 10 μL proteins were placed in gel wells and Precision Plus Protein Standard Dual Color, Bio Rad 10 – 250 kD was used as a protein marker. Gel electrophoresis was performed at 60V for 60 min and then at 120V for 2 h with Tris/Glycine/SDS running buffer (25 mM Tris; 190 mM glycine; and 0.1% SDS). The proteins in the gel were stained with Coomassie Blue for quantification. Two more gels prepared under the same conditions as above were used to transfer proteins to a nitrocellulose membrane. A “sandwichs” consisting of a layer of foam, filter paper, nitrocellulose membrane, gel, filter paper and foam was assembled inside a transfer buffer (25 mM Tris; 190 mM glycine; and 20% methanol).

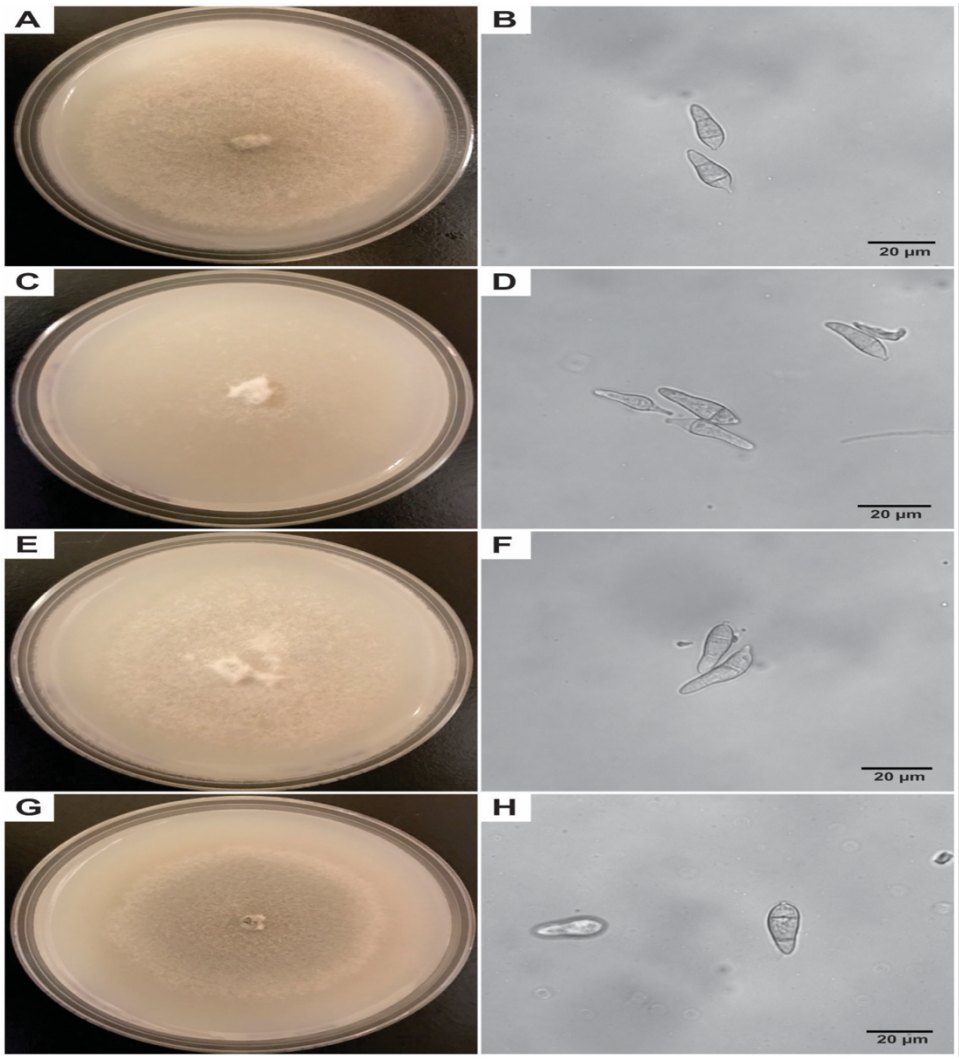
The “sandwichs” were placed in an electrophoresis chamber containing transfer buffer to run at 100 Amp for 2 h. The nitrocellulose membranes were stained with Ponceau S (0.2% Ponceau S and 5% glacial acetic acid) to check the transfer quality and then incubated with the primary antibody Phospho-ERK1/2 (Invitrogen) for TEY or Phospho-p38 MAPK for TGY overnight in TBST (20 mM Tris, pH 7.5; 150; mM NaCl; 0.1% Tween 20) with 5% non-fat milk at 4 ºC with agitation. The next day, membranes were washed with TBST 3 times with agitation for 5 min and incubated with anti-rabbit IgG secondary antibody, Invitrogen, in TBST with 5% non-fat milk for 1 h at room temperature with agitation. The membranes were washed again 3 times with TBST for 5 min and incubated with chemiluminescents for 1 min and antigen-antibody detection was performed in Sapphire Biomolecular Imager Laser Scanning, Azure Biosystems.

3 RESULTS

3.1 RGF1 AND RGF2 GENE REPLACEMENT BY SPLIT-MARKER APPROACH

Probable transformants were picked, 119 and 192 isolates which only 2 and 4 were confirmed by PCRs as mutants of RGF1 and RGF2 genes, respectively. Colonies and conidia from *Δrgf1* (Fig. 2B, C) and *Δrgf2* (Figure 2D) mutant isolates are shown in Fig. 2. The *Δrgf1* mutant showed little mycelial growth compared to wild type Ku80 (Fig. 2A). Conidia also showed an abnormal shape and mostly had only one septum (Fig. 2F, G). Conidia of *P. oryzae* present a piriform shape and 2 septa, as can be seen in wild type Ku80 (Fig. 2E). The *Δrgf2* mutant had a similar colony morphology (Fig. 2D) to wild type Ku80 and its conidia had a normal shape with 2 septa as Ku80 (Fig. 2H).

Figure 2 - Photographs of colony morphologies (A-D) and photomicrographs of conidia (E-H) from Ku80 (A, E), *Δrgf1*-1 (B, F), *Δrgf1*-2 (C, G), and *Δrgf2* (D, H).

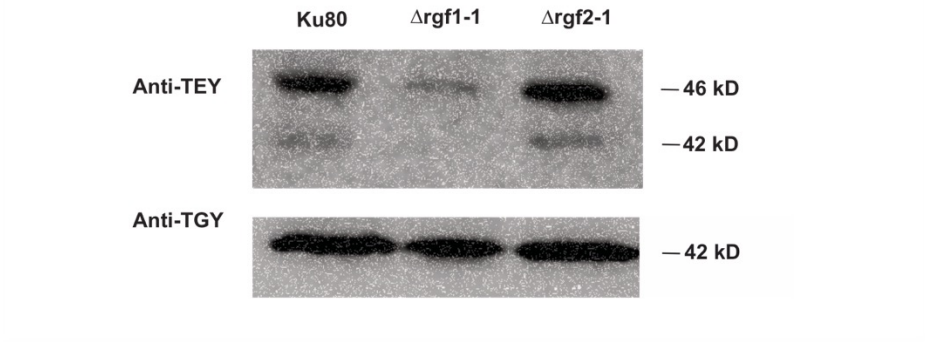


Source: Tavares (2021).

3.2 TEY AND TGY PHOSPHORYLATION ASSAY

The TEY assay showed that the phosphorylation level of Pmk1 (42 kD) in the *Δrgf1* mutant was drastically reduced compared to wild type Ku80 and the *Δrgf2* mutant (Fig. 3). The phosphorylation level of Mps1 (46 kD) was significantly reduced only in the *Δrgf1* mutant (Fig. 3).

Figure 3 - TEY and TGY phosphorylation assay. Anti-TEY detected phosphorylation levels of Pmk1 (42 kDa) and Mps1 (46 kDa) and Anti-TGY detected phosphorylation level of Oms1 (42 kDa). Proteins from Ku80 wild type, and *Δrgf1* and *Δrgf2* mutants.



Source: Tavares (2021).

The TGY assay showed that the phosphorylation level of Oms1 (42 kD) in *Δrgf1* and *Δrgf2* mutants was not reduced when compared to the wild type Ku80 (Fig. 3). These results indicate that RGF1 is related to activation of the Pmk1 pathway and may be involved in the activation of the Mps1 pathway, but it is not necessary for activation of the Oms1 pathway. However, RGF2 seems to be unnecessary for activation of these pathways.

4 DISCUSSION

In preliminary results *Δrgf1* mutants of *P. oryzae* showed reduced conidiation, with most conidia failing to germinate on hydrophobic surfaces, and failing to attach to the surface, as well as most failing in appressorium formation, and infection of rice plants. This shows that the RGF1 gene is important for mycelial growth, conidia germination, appressorium formation, and plant infection. However, *Δrgf2* mutants were normal in conidia germination,

appressorium formation, and plant infection. This indicates that RGF2 is dispensable for hyphal growth, germination, appressorium formation and infection by *P. oryzae*.

PMK1 orthologs are required for appressorium formation in several other appressorium-forming fungi such as *Colletotrichum orbiculare*, *Ustilago maydis*, *Pyrenophora teres*, and *Cochliobolus heterostrophus* (LI; ZHOU; XU, 2012). PMK1 is essential for appressorium formation and invasive hyphal growth; defects of the *Δrgf1* mutant in appressorium formation and plant infection indicate that the RGF1 gene functions upstream of the Pmk1 MAPK pathway. Mps1 regulates the accumulation of alpha-1, 3-glucan which is a component of the outer layer of the cell wall that provides protection against chitinases during plant infection (LI; ZHOU; XU, 2012). In our study, phosphorylation levels of Pmk1 and Mps1 were reduced in the *Δrgf1* mutant, indicating that RGF1 is involved in activation of Pmk1 MAP kinase signaling and may be involved in activation of Mps1 MAPK as its phosphorylation level was reduced. The Osm1 MAPK pathway regulates hyperosmotic stress responses, but it is not necessary for appressorium turgor generation (DIXON et al., 1999). Phosphorylation levels of Oms1 in *Δrgf1* and *Δrgf2* mutants of *P. oryzae* were not reduced which indicating that RGF1 and RGF2 may not play a role in regulating the Osm1 MAP kinase pathway in *P. oryzae*. Although the Osm1 pathway is not required for pathogenicity in *P. oryzae* and *Colletotrichum lagenarium*, its orthologs are important for infection by other plant and human pathogens, such as *Mycosphaerella graminicola* and *Cryptococcus neoformans* (LI; ZHOU; XU, 2012).

Therefore, Rgf1 appears to function upstream of the Pmk1 and Mps1 MAPK pathways in *P. oryzae*. These results indicate that RGF1 is the dominant RasGEF gene in *P. oryzae* and RGF2 plays a minor role in RasGEF. In *U. maydis*, the *Δrgf1* mutant is required for virulence, indicating that it is involved in signaling processes that are important for pathogenesis (MÜLLER et al., 2003). In *S. cerevisiae*, only Cdc25 (related to RGF1) is essential for the growth and activation of adenyl cyclase. In contrast, Sdc25 (related to RGF2) is dispensable for growth under normal culture conditions, but the *Δsdc25* mutant was defective in growth when cultivated under nutritionally restricted conditions or cultivated in unfermented carbon sources (BOY-MARCOTTE; IKONOMI; JACQUET, 1996; DAMAK et al., 1991).

5 CONCLUSION

The TEY phosphorylation assay showed that the RGF1 in *P. oryzae* is involved in regulating the Pmk1 MAP kinase pathway and has a role in the Mps1 MAPK signaling. RGF2 appers to be dispensable for activating these pathways. RGF1 and RGF2 are dispensable for activating the Oms1 MAPK pathway by TGY phosphorylation assay. Therefore, RGF1 seems to function upstream of the Pmk1 and Mps1 MAP kinase pathways in *P. oryzae* also, these results showed that RGF1 is the dominant RasGEF gene and RGF2 plays a minor role as RasGEF in *P. oryzae*.

REFERENCE

BOY-MARCOTTE, E.; IKONOMI, P.; JACQUET, M. SDC25, a dispensable Ras guanine nucleotide exchange factor of *Saccharomyces cerevisiae* differs from CDC25 by its regulation. **Molecular Biology of the Cell**, v. 7, n. 4, p. 529–539, 1996.

CARGNELLO, M.; ROUX, P. P. Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. **Microbiology and Molecular Biology Reviews**, v. 75, n. 1, p. 50–83, 2011.

DAMAK, F. et al. SDC25, a CDC25-like gene which contains a RAS-activating domain and is a dispensable gene of *Saccharomyces cerevisiae*. **Molecular and Cellular Biology**, v. 11, n. 1, p. 202–212, 1991.

DIXON, K. P. et al. Independent signaling pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection by *Magnaporthe grisea*. **Plant Cell**, v. 11, n. 10, p. 2045–2058, 1999.

GOSWAMI, R. S. Targeted gene replacement in fungi using a split-marker approach. **Methods in Molecular Biology**, v. 835, p. 255–269, 2012.

HANCOCK, J. F. Ras proteins: Different signals from different locations. **Nature Reviews Molecular Cell Biology**, v. 4, n. 5, p. 373–384, 2003.

LEUNG, H. et al. Transformation of the rice blast fungus *Magnaporthe grisea* to hygromycin B resistance. **Current Genetics**, v. 17, n. 5, p. 409–411, 1990.

LI, G.; ZHOU, X.; XU, J. R. Genetic control of infection-related development in *Magnaporthe oryzae*. **Current Opinion in Microbiology**, v. 15, n. 6, p. 678–684, 2012.

MÜLLER, P. et al. Guanyl nucleotide exchange factor sql2 and ras2 regulate filamentous growth in *Ustilago maydis*. **Eukaryotic Cell**, v. 2, n. 3, p. 609–617, 2003.

PARK, G. et al. Multiple upstream signals converge on the adaptor protein Mst50 in *Magnaporthe grisea*. **Plant Cell**, v. 18, n. 10, p. 2822–2835, 2006.

ROJAS, J. M.; SANTOS, E. CHAPTER 2 RAS-GEF S AND RAS GAP S Abstract : n. Figure 1, p. 15–43, 2006.

VALENT, B.; CHUMLEY, F. G. Molecular Genetic Analysis of the Rice Blast Fungus, *Magnaporthe Grisea*. **Annual Review of Phytopathology**, v. 29, n. 1, p. 443–467, 1991.

VALENT, B.; KHANG, C. H. Recent advances in rice blast effector research. **Current Opinion in Plant Biology**, v. 13, n. 4, p. 434–441, 2010.

WILSON, R. A.; TALBOT, N. J. Under pressure: Investigating the biology of plant infection by *Magnaporthe oryzae*. **Nature Reviews Microbiology**, v. 7, n. 3, p. 185–195, 2009.

XU, J. R.; STAIGER, C. J.; HAMER, J. E. Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. **Proceedings of the National Academy of Sciences of the United States of America**, v. 95, n. 21, p. 12713–12718, 1998.

YOSHIDA, S.; ICHIHASHI, R.; TOH-E, A. Ras recruits mitotic exit regulator Lte1 to the bud cortex in budding yeast. **Journal of Cell Biology**, v. 161, n. 5, p. 889–897, 2003.

ZHANG, X. et al. Expression of HopAI interferes with MAP kinase signalling in *Magnaporthe oryzae*. **Environmental Microbiology**, v. 19, n. 10, p. 4190–4204, 2017.

ZHAO, X. et al. A mitogen-activated protein kinase cascade regulating infection-related morphogenesis in *Magnaporthe grisea*. **Plant Cell**, v. 17, n. 4, p. 1317–1329, 2005.

ZHAO, X.; MEHRABI, R.; XU, J. R. Mitogen-activated protein kinase pathways and fungal pathogenesis. **Eukaryotic Cell**, v. 6, n. 10, p. 1701–1714, 2007.

ARTIGO 3 – PROTOPLAST RELEASES OF *Pyricularia oryzae* PATHOTYPE *Triticum UNDER* DIFFERENT CONCENTRATIONS OF LYSING ENZYMES *

ARTIGO FORMATADO DE ACORDO COM A NORMA NBR 6022 (ABNT 2018)

Dérica Gonçalves Tavares **

Prof. Dr. Eduardo Alves (Orientador) ***

ABSTRACT

Protoplast-mediated transformation is widely used to study the behavior and interactions of filamentous fungi. Although protoplast production of *Pyricularia oryzae* has been previously reported, some modifications may be performed for different strains. Therefore, the objective of this work was to standardize a methodology using Lysing enzymes or β-glucanases, and methods to conserve protoplasts obtained from wheat blast *P. oryzae*. The enzymatic products β-glucanase from *Trichoderma longibrachiatum* and Lysing enzymes from *Trichoderma harzianum* were tested at concentrations of 30 and 60 mg/mL. For the best enzyme product, the concentrations of 5, 10, 20, 30, 60 and 90 mg/mL were tested, and transmission electron microscopy (TEM) was used to assess cellular changes in protoplasts at concentrations of 60 and 90 mg/mL. Storage of protoplasts at -20 °C using 0, 10, 20, and 30% glycerol as cryoprotectant was also evaluated. Lysing enzymes was better for protoplast release and concentrations above 30 mg/mL affected its viability. Protoplast cell membranes were affected because of the high enzyme concentration observed by TEM. The cells were shrunk, and the cell membrane was thick with 90 mg/mL and very thin with 60 mg/mL of Lysing enzymes. Storage of protoplasts is not ideal at -20 ºC even with a cryoprotectant, which seems to have caused greater osmotic stress on the cells.

Keywords: Genetic transformation. Lytic enzymes. Transmission electron microscopy. Confocal microscopy.

---

* Artigo Apresentado para a conclusão do curso de pós-graduação em Microbiologia Agrícola da Universidade Federal de Lavras.

** Doutoranda no curso de Microbiologia Agrícola, pela Universidade Federal de Lavras. Endereço eletrônico: dericatavares@gmail.com.

*** Professor da Universidade Federal de Lavras, Doutor em Fitopatologia.

1 INTRODUCTION

Genetic transformation is a powerful tool that can aid in the study and confirmation of biological processes that occur in a variety of living organisms. The understanding of biological phenomena such as host-pathogen interactions, secondary metabolism, environmental stress responses, and developmental biology is facilitated by transformation (ISHIKAWA et al., 2010). Expression of fluorescent proteins such as GFP, RFP, YFP, CFP, and knockout genes, are examples of genetic transformation that allow us to observe gene expression, cell lineage markers, and monitor the location of cells to cell components.

One important step in transforming an organism is to make its receptor cells permeable to the DNA, and the use of protoplasts is one way to mediate this process. Protoplasts are cells that have their walls removed by enzyme action which facilitates the entry of genetic material into the cell. Protoplasts are widely used to transform filamentous fungi (PEBERDY, 1976) and their production is an important biological method for experiments aimed at transformation and other molecular approaches (ISHIKAWA et al., 2010).

The cell wall is the first obstacle in transferring exogenous DNA to fungal cells. The main components of the fungal cell wall are chitin, 1,3-β-glucans, 1,6-β-glucans, proteins, mannans, and other polymers, which are cross-linked to form this complex structure (DEACON, 2006; RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). Crystalline polysaccharides, chitin, and β-glucans, constitute the skeletal portion in the cell wall, while amorphous polysaccharides and protein-polysaccharide complexes are components of the cell wall matrix (DEACON, 2006). The form, integrity, and mechanical strength of the fungus are determined by the chemical composition of the cell wall, which is also responsible for the interaction of the fungus with its environment (LESAGE; BUSSEY, 2006).

The cell wall structure is distinct between different fungi, requiring different combinations of enzymes and specific conditions to degrade the cell wall and release protoplasts (RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). The use of combined enzymes was shown to be more efficient than the use of isolated enzymes because of the presence of several compounds in the fungal cell wall (GALLMETZER; BURGSTALLER; SCHINNER, 1999; RODRIGUEZ-IGLESIAS; SCHMOLL, 2015). Therefore, enzyme selection is a crucial factor in the preparation of protoplasts and enzyme formulations are commercially available.

The products Glusulase and Novozym 234, a mixture of enzymes from *Trichoderma viride* were commonly used to produce protoplasts (VOLLMER; YANOFSKY, 1986).

Although protoplasts in *Pyricularia* have been previously reported, some modifications must be made for each strain under study. In addition, most studies used the Novozym 234 lytic enzyme preparation; however, this enzyme complex is no longer commercially available. Factors such as the microorganism, osmotic stabilizer, age of the mycelium and enzyme preparation can influence the production of protoplasts (PEBERDY, 1976). In this study, we tested different concentrations of Lysing enzymes to obtain protoplasts of the blast fungus *P. oryzae* pathotype *Triticum* and ways to store the protoplasts at -20 ºC to maintain viable cells for a longer period.

2 MATERIALS AND METHODS

2.1 MYCELIUM PREPARATION

The Blast wheat isolate used in this study was *Pyricularia oryzae* CML3547. The isolate was grown in OA medium at 25 ºC under constant light for 10 days. Then, the mycelium was collected carefully to avoid collect medium, and were blended at three pulses of 10 s each with 50 mL of 5xYEG (0.5% yeast extract, 2% glucose) containing 50 μg/mL ampicillin, and incubated under shaking at 105 rpm for 24h at 25 °C. On the second day the macerate was blended again with three pulses of 10 s each, and 50 mL of complete medium (10 g/L glucose, 2 g/L peptone, 1 g/L de yeast extract, 1 g/L casamino acids, 50 mL/L de 20x nitrate salts, 1 mL/L trace elements, 1 mL/L vitamin solution, pH 6,5) containing 50 μg/ml ampicillin, and incubated under shaking at 105 rpm overnight for about 10 h at 25 ºC. On the third day the mycelium was collected by filtration through a double layer of sterile Miracloth and washed with NaCitrate/EGTA/NaCl buffer (50% 0.2 M NaCitrate, pH5.8; 2% 0.5 M EGTA; 10% 5 M NaCl; 38% distilled water). About 0.3 g of the mycelium was resuspended in 10 mL NaCitrate/EGTA/NaCl buffer containing different enzymes and concentrations, and incubated under shaking at 45 rpm and 30 °C.

2.2 PROTOPLAST RELEASE EVALUATION USING B-GLUCANASE OR LYSING ENZYMES

Lysing enzymes from *Trichoderma harzianum* (Sigma) and β-glucanase from *Trichoderma longibrachiatum* (Sigma) were tested at concentrations of 30 and 60 mg/mL and incubated for 2, 6, 9, and 12 h. Protoplasts were recovered by filtration through a double layer of sterile Miracloth and centrifuged at 4500 rpm at room temperature for 6 min. The supernatant was discarded, and the protoplasts were resuspended in 10 mL of STC solution (20% sucrose, 50 mM Tris-Cl, 50 mM CaCl2), and this process was repeated once more. Then, the protoplasts were centrifuged at 4500 rpm for 6 min, and the supernatant was discarded, and gently resuspended in 1 mL of STC solution. Protoplasts were counted using a Neubauer chamber and the experiment was performed with two repetitions.

2.3 PROTOPLAST RELEASE USING DIFFERENT CONCENTRATIONS OF LYSING ENZYMES AND ITS VIABILITY EVALUATION

Protoplast release was tested using 5, 10, 20, 30, 60, and 90 mg/mL of Lysing enzymes solution in NaCitrate/EGTA/NaCl buffer after 2 h of incubation. Protoplasts were counted using a Neubauer chamber and the experiment was performed with two repetitions. Protoplasts were diluted in STC medium to obtain 25 and 250 colonies for Petri dish and recovered in TB3 medium (3 g/L yeast extract, 3 g/L casamino acids and 20% sucrose) with 0.7% agar containing 50 μg/mL ampicillin, incubated at room temperature for 72h. The protoplasts recovered into colonies were counted and their viability was estimated after use of different enzyme concentrations. The viability of protoplasts obtained with 10 mg/mL of Lysing enzymes was also tested after storage. Two conditions were tested just STC medium and STC medium with glycerol as a cryoprotectant. Glycerol was used at concentrations of 10%, 20%, and 30% and viability was evaluated after one week and one month of storage at -20 ºC. Protoplast were checked using fluorescent microscopy and the cells were recovered in TB3 medium with 0.7% agar. Samples stored for one week and one month of storage were prepared using fluorochrome Syto9 (Thermo Fischer®) to mark living cells (ChS1 detector), and a solution of propidium iodide (PI) (Sigma®) to mark dead cells (Ch2 detector). For this purpose, 5 µL of each sample was placed on top of agar/water blocks of approximately 0.25 cm^2 and kept at rest for 10 min to allow absorption of the aliquot. Afterwards, 5 µL of Syto9 (20 µM) was applied on top of the previously absorbed aliquot, making a brief homogenisation, and leaving it to rest in the dark for 10 min. Then, 5 µL PI (1 µg/mL) was applied and a brief homogenisation was carried out, leaving it to rest in the dark for another

10 min. Fluorescence images were obtained using a Zeiss Axio-Observer Z1 LSM 780 confocal microscope with Zen 2010 software (Carl Zeiss Microscopy MBH). Image treatments were performed with Corel Draw 2020 software. The protoplast samples with one week and one month of storage were also recovered in TB3 medium as described above.

2.3 TRANSMISSION ELECTRON MICROSCOPE OF PROTOPLASTS RELEASED USING DIFFERENT CONCENTRATIONS OF LYSING ENZYMES

Transmission electron microscopy (TEM) was used to evaluate possible cellular damage due to enzymatic digestion by the 60 and 90 mg/mL treatments. For this purpose, Karnovisky fixative solution was added in a proportion of 1:1 with the protoplasts STC solution and left for 24 h. Protoplasts were then centrifuged at 6000 rpm for about 2 min to form a pellet. The supernatant was removed, and the protoplasts were resuspended in 1% low-melting agarose gel at 27 ºC, and after solidification the agarose containing protoplasts was cut into 1 mm x 1 mm strips (PERINA et al., 2015). The samples were washed in cacodylate buffer three times for 10 min each and post-fixed in 1% osmium tetroxide for 2 h at room temperature and transferred to 0.5% uranyl acetate solution for 12 h at 4 °C. Then, the samples were washed again in distilled water and dehydrated in acetone gradients of 30, 50, 70, and 90% for 10 min and 100% for 10 min repeated three times. After this process, the samples were included in an increasing gradient of Spurr/acetone 30% (8 h), 70% (12 h) and 100% twice for 24 h each. The samples were mounted in molds, polymerized at 70 ºC for 8 h. The molds with the samples were trimmed and cut into ultra-thin cuts of 70 nm and collected in copper grids of 300 mesh. The images captured using a Transmission Electron Microscope (TEM), Mod. EM-109, Carl Zeiss with a CCD camera to capture the images. Image treatments were performed using Corel Draw 2020 software.

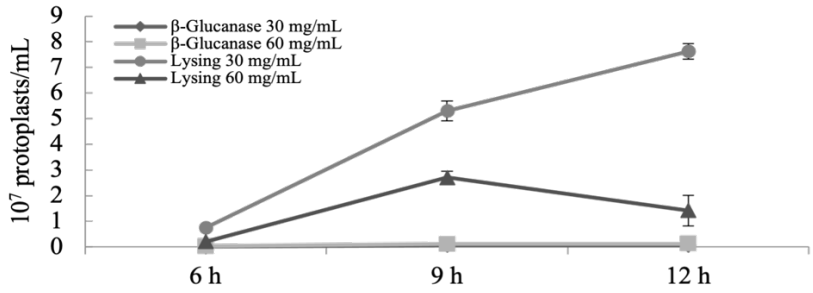
3 RESULTS

3.1 PROTOPLAST RELEASE EVALUATION USING B-GLUCANASE OR LYSING ENZYMES AT DIFFERENT CONCENTRATIONS AND INCUBATION TIMES

β-glucanase (Sigma) and Lysing enzymes (Sigma) were evaluated for their ability to remove the cell wall and release protoplasts. Figure 1 shows the number of protoplasts released during incubation with different enzymes concentrations. The largest number of

protoplasts released was 7.6 x 10^7 using Lysing enzymes at a concentration of 30 mg/mL after 12 h of incubation (Fig. 1). After 6 and 9 h of incubation, the highest release was also obtained with 30 mg/mL of Lysing enzymes with 7.5 x 10^6 and 5.3 x 10^7 protoplasts/mL, respectively.

Figure 1 - *Pyricularia oryzae* pathotype *Triticum* protoplasts released using β-glucanase and Lysing enzymes at different concentrations and incubation times.

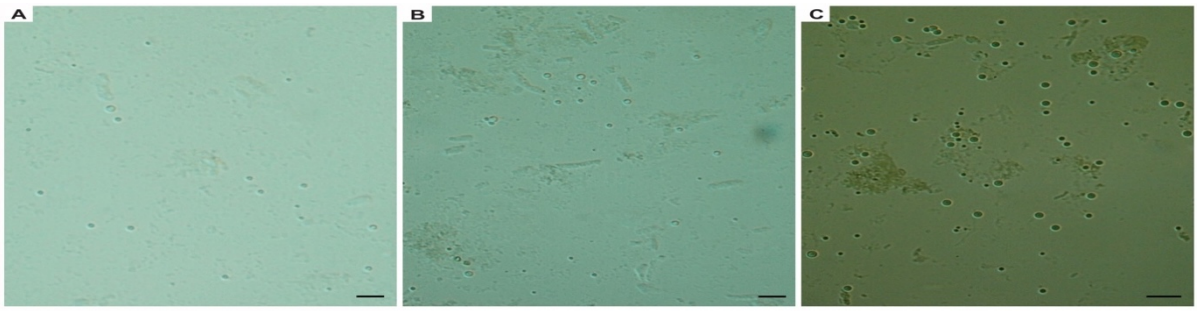


Source: Tavares (2021).

Number of protoplasts released with 60 mg/mL of Lysing enzymes do not increase over the time from 9 to 12h, showing numbers of 2.7 x 10^7, and 1.4 x 10^7 protoplasts/mL, respectively. The results showed that the number of protoplasts released using β-glucanase at both concentrations and incubation times were low when compared to Lysing enzymes, with values below 1.1 x 10^6 protoplasts/mL.

Although, high concentrations of protoplasts were achieved with 9 and 12 h of incubation using 30 mg/mL of Lysing enzymes, the solutions contained large amounts of material from the enzymatic digestion (Fig. 2). This material was small enough to pass through the filters used to separate the protoplast solution from the mycelium.

Figure 2 - Photomicrographs of *Pyricularia oryzae* pathotype *Triticum* protoplasts obtained with 30 mg/mL of Lysing enzymes in different times.

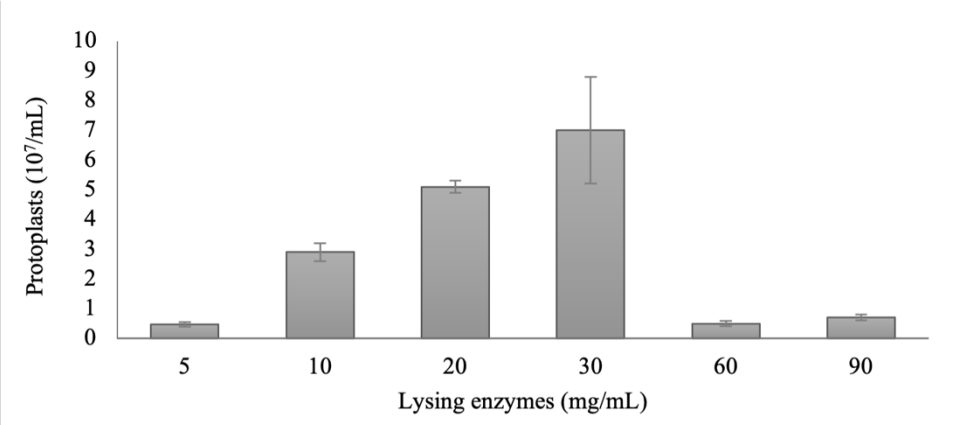


A) 6 h, B) 9 h, and C) 12 h of incubation. 10 μm scale bar.
Source: Tavares (2021).

3.2 PROTOPLAST RELEASE USING DIFFERENT CONCENTRATIONS OF LYSING ENZYMES AND ITS VIABILITY EVALUATION

Protoplasts released with different concentrations of the Lysing enzymes are shown in Fig. 3. The quantity increases from 4.7 x 10^6 to 7 x 10^7 protoplasts/mL using 5 to 30 mg/mL of enzyme solution. However, the protoplast number decreased to 5 and 7 x 10^6 protoplasts/mL with 60 and 90 mg/mL of enzyme solution, respectively. The results show that high enzyme concentrations over 60 mg/mL can damage protoplast release.

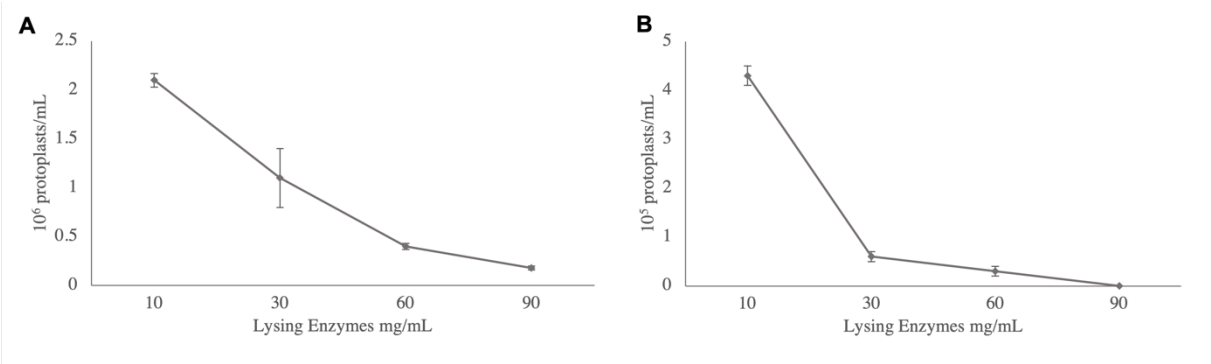
Figure 3 - *Pyricularia oryzae* pathotype *Triticum* protoplasts released using different concentrations of Lysing enzymes after 2 h of incubation.



Source: Tavares (2021).

Protoplasts obtained with 10, 30, 60, and 90 mg/mL of Lysing enzymes were recovered in TB3 medium and the number of colonies from the protoplasts is shown in Fig.4. A high number of viable protoplasts was obtained using 10 mg/mL of Lysing enzymes solution with 2.1 x 10^6 protoplasts/mL. The viable protoplasts released with 30, 60, and 90 mg/mL of the enzyme solutions decreased to 1.1 x 10^6, 4 x 10^5, and 1.8 x 10^5 protoplasts/mL (Fig. 4A).

Figure 4 - Number of viable protoplasts obtained with different concentrations of Lysing enzymes solution. A) Protoplasts recovered on the same day and B) after one week.

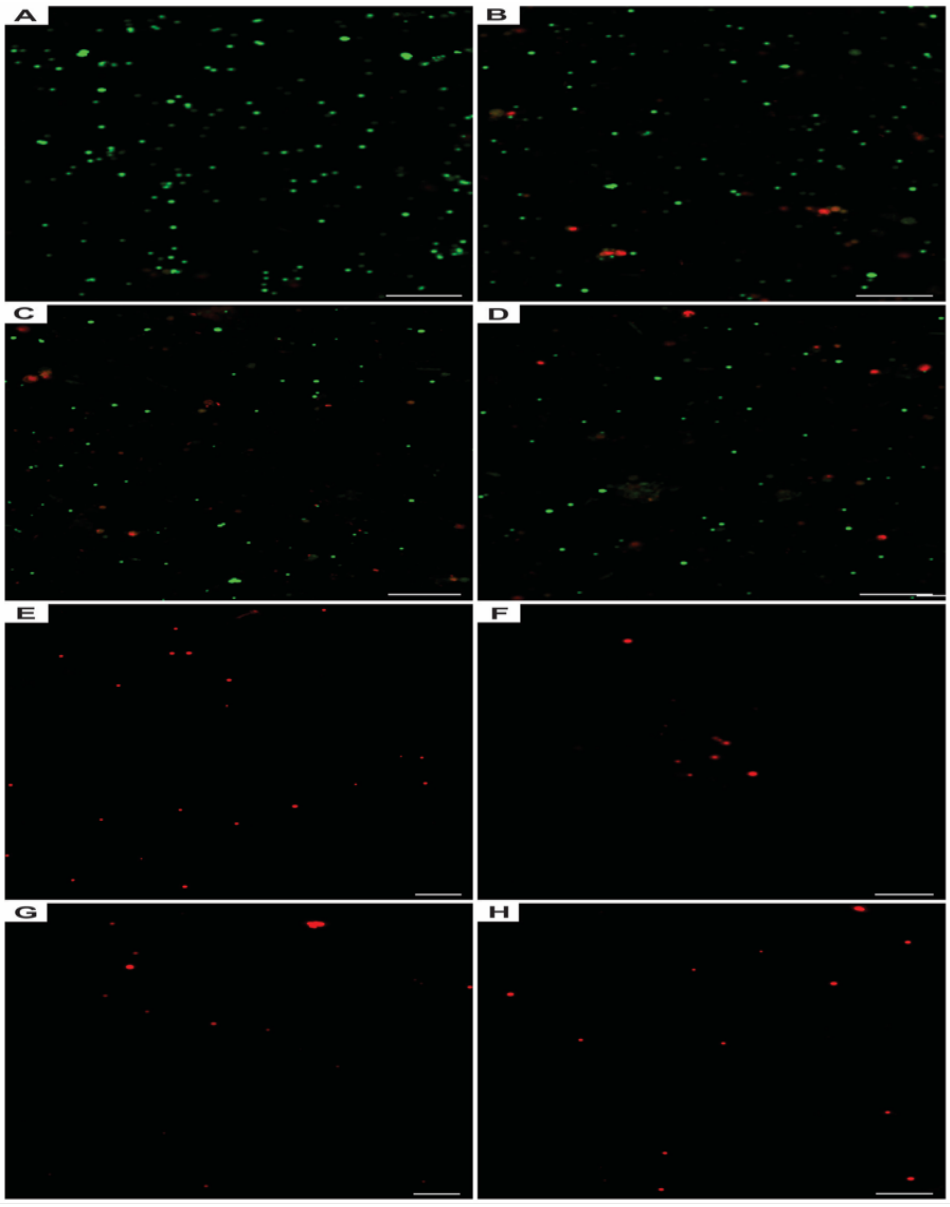


Source: Tavares (2021).

Protoplasts after one week of storage at -20 °C were also recovered in TB3 medium and the number of colonies from the protoplasts are shown in Fig. 4B. The number of viable protoplasts decreased to 4.3 x 10^5 protoplasts/mL with 10 mg/mL of Lysing enzymes after storage. This number decreased even more with over 30 mg/mL of enzyme solution which showed that high enzyme concentrations and storage at -20 °C caused damage to the cell which would imply the recovery of the protoplasts.

Protoplast was also evaluated in absence and presence of different concentrations of glycerol as cryoprotectant after one week and one month of storage. The live-dead test shows live cells in green and dead cells in red (Fig. 5). In the absence of glycerol, most protoplasts were alive, but with increasing glycerol concentration, living protoplasts decreased (Fig. 5A-D). After one month of storage, no live protoplasts were observed at any of the concentrations tested, even in the absence of glycerol, and the number of protoplast cells also decreased (Fig. 5E-H).

Figure 5 - Laser Confocal microscope images of *Pyricularia oryzae* pathotype *Triticum* protoplasts in the live-dead test.

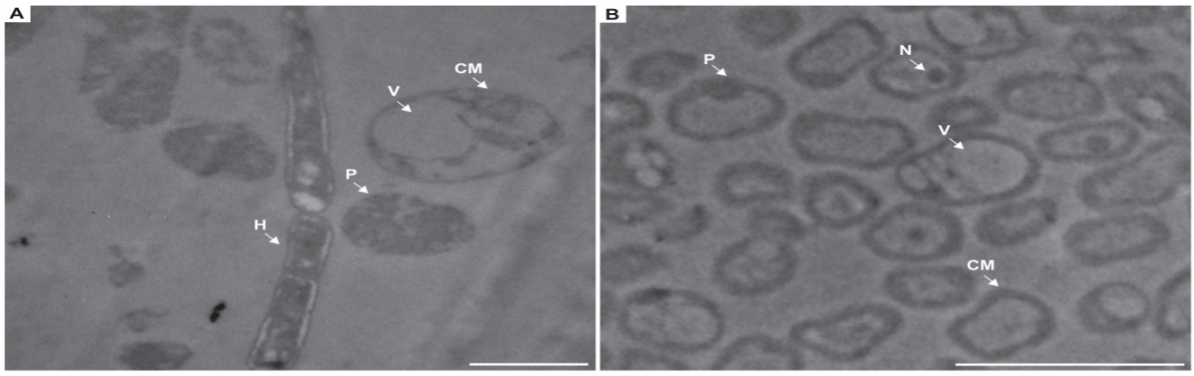


The fluorochrome Syto9 was used to mark living cells (ChS1 detector) and a solution of propidium iodide (PI) was used to mark dead cells (Ch2 detector). A-D) Evaluation after one week of storage. E-H) Evaluation after one month of storage. A and E) 0% glycerol. B and F) 10% glycerol. C and G) 20% glycerol. D and H) 30% glycerol. Scale bar of 20 μm.
Source: Tavares (2021).

3.3 TRANSMISSION ELECTRON MICROSCOPY OF PROTOPLASTS RELEASED USING DIFFERENT CONCENTRATIONS OF LYSING ENZYMES

Transmission electron microscopy of protoplasts released using 60 and 90 mg/mL of Lysing enzymes solution was performed to evaluate whether there was any damage related to the protoplast cell integrity (Fig. 6).

Figure 6 - Transmission Electromicrograph of Pyricularia oryzae pathotype *Triticum* protoplasts released with Lysing enzymes concentrations.



A) Protoplasts obtained with 60 mg/mL of Lysing enzymes. B) Protoplasts obtained with 90 mg/mL of Lysing enzymes. H: hypha; P: protoplast; V: vacuole; CM: cell membrane; N: nucleus. 2 μm scale bar. Source: Tavares (2021).

Protoplast cell membranes were altered because of the high enzyme concentrations observed by TEM. The cells shrunk and had a cell membrane thickness of 90 mg/mL (Fig.6B) and very thin with 60 mg/mL of Lysing enzymes (Fig.6A).

4 DISCUSSION

Lysing enzymes are a set of lytic enzymes from *T. harzianum* and comprise a mixture with β-1,3-glucanase, protease, and chitinase activity, which hydrolyzes oligosaccharides from the fungal cell wall. The major structural constituents in the fungal cell wall are chitin, β-1,3-glucan, and β-1,6-glucan with a matrix of mannoproteins and α-1,3-glucan (DEACON, 2006), which justified the highest activity found by the Lysing enzymes set. Otherwise, β-glucanase from *T. longibrachiatum* comprises carbohydrate enzymes that have β-xylosidase, α-L-arabinofuranosidase, amylase, and protease activities and β-glucosidase which breaks the glycosidic bonds of β-glucan. Although β-glucan is a part of the fungal cell wall, the enzyme complex with more lytic activities related to the fungal cell wall constituents is found in the Lysing enzymes set than in the β-glucanase compound.

Protocols for obtaining *P. oryzae* protoplasts have been reported mainly using Novozym 234 and a few using Lysing enzymes set, but these studies do not report the number of released protoplasts (KADOTANI et al., 2003; SWEIGARD; CHUMLEY; VALENT, 1992; TALBOT et al., 1993). Studies have been carried out to evaluate the efficiency of Lysing enzymes in obtaining protoplasts from different species of fungi. Ishikawa et al.

(2010) used concentrations of 10, 20, and 30 mg/mL of Lysing enzymes to obtain *Colletotrichum lindemuthianum* protoplasts, and a high protoplast release (8 x 10^6 protoplasts/mL) was achieved with 30 mg/mL. In this study, they also observed that after 4 h of incubation there was a loss in the number of protoplasts. Almeida et al. (2008) used Lysing enzymes, Glucanex (Sigma), and Meicellase (Karlan) at different concentrations and combinations and observed that the best conditions were 20 mg/mL of Lysing enzymes and 6 h of incubation to obtain protoplasts (3 x 10^7 protoplasts/mL) of *Aspergillus ochraceus*. In our study, we used 5, 10, 20, 30, 60, and 90 mg/mL of Lysing enzymes and a high number of protoplasts released were obtained with 30 mg/mL (7 x 10^7 protoplasts/mL). We also observed that the quantity of *P. oryzae* protoplasts did not decrease with time using 30 mg/mL of Lysing enzymes and after 9 h of incubation the number of released protoplasts was still increasing. However, the quantity of digest material present in the protoplast solution, such as hyphal remnants also increases with time.

Protoplasts obtained with 10, 30, 60, and 90 mg/mL of Lysing enzymes were recovered in TB3 medium. This test showed that the protoplast viability and high number of viable protoplasts were obtained using 10 mg/mL (2.1 x 10^6 protoplasts/mL) of Lysing enzymes. The number of viable protoplasts decreased with increasing enzyme concentration. The enzymes act synergistically to lyse cell wall in yeast, but only two are essential for cell disruption, specific lytic protease, which degrades the outer layer of mananaprotein, and β-1,3-glucanase, which degrades the inner glucan layer (FLEURI; SATO, 2005; ZLOTNIK et al., 1984). Proteases can also act in the hydrolysis of membrane cell proteins, which may be facilitated in high concentrations of the enzyme solution causing cell lysis. Although the number of protoplasts was greater with the use of 30 mg/mL of the enzymatic solution, the use of 10 mg/mL was more suitable because more viable protoplasts were obtained.

Freezing results in the formation of ice crystals which leads to severe damage to cells and membranes (NIEDERMEYER; PARISH; MOOR, 1977). Cryoprotectors are widely used to minimize the damage caused by freezing, which allows the morphological preservation of cells. Glycerol is one of the most commonly used cryoprotectants as it does not interfere with the metabolic activity of the cytoplasm and does not cause changes in the membrane ultrastructure. However, cryoprotectants can cause osmotic stress leading to changes in cell ultrastructure after treatment and during glycerol uptake into cells numerous vesicle-like structures can be seen in the cytoplasm suggesting metabolic activity (NIEDERMEYER; PARISH; MOOR, 1977), which seems to have occurred in our study.

In yeast cells, treatment with glycerol solutions from 17.4 to 26.1% produced extensive changes in the plasmalemma and freezing micrographs showed total disorganization of membranes and cells. Thus, incubation in glycerol solutions above 17.4% can be lethal to exponentially growing yeast cells (NIEDERMEYER; PARISH; MOOR, 1977). In addition, glycerol-treated yeast protoplasts shrink and then recover their original volume after prolonged incubation (NIEDERMEYER; PARISH; MOOR, 1977). In the present study, the addition of glycerol even at a concentration of 10%, does not seem to be the best form of storage, as the viability of the protoplasts was better in the absence of the cryoprotectant. Thus, osmotic gradients produced during the introduction and removal of the cryoprotectant can be harmful to the cell and membranes by excessive swelling and shrinking, as was also observed by Niedermeyer et al. (1977). Therefore, the results showed that the best preservation method was without glycerol.

5 CONCLUSION

According to the results obtained, the best conditions for obtaining *P. oryzae* pathotype *Triticum* protoplasts would be the use of 10 mg/ml of the Lysing enzymes compound for an incubation period of 2 h. Lower concentrations of the enzyme can be used however, a larger amount of mycelium should be used. The best condition for freezing/storing the protoplasts would be without adding glycerol as a cryoprotectant for a short period of time.

REFERENCES

ALMEIDA, A. P. M. M.; DIAS, E.; PEREIRA, R. T. G.; TOLEDO, R. C. C.; PFENNING, L. H. Obtenção de protoplastos do fungo filamentoso *Aspergillus ochraceus*. **Ciência Rural**, v. 38, n. 5, p. 1460-1462, 2008.

DEACON, J. W. **Fungal Biology**. 4 ed. Malden, MA: Blackwell Publishing, 2006. 371 p. ISBN 9781405130660.

GALLMETZER, M.; BURGSTALLER, W.; SCHINNER, F. An optimized method for the isolation of protoplasts from *Penicillium simplicissimum* to produce sealed plasma membrane vesicles . **Mycologia**, v. 91, n. 1, p. 206–212, 1999.

ISHIKAWA, F. H. et al. Factors affecting the production and regeneration of protoplasts from *Colletotrichum lindemuthianum* | Fatores que afetam a produção e regeneração de protoplastos de *Colletotrichum lindemuthianum*. **Ciencia e Agrotecnologia**, v. 34, n. 1, p. 74–79, 2010.

KADOTANI, N. et al. RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. **Molecular Plant-Microbe Interactions**, v. 16, n. 9, p. 769–776, 2003.

LESAGE, G.; BUSSEY, H. Cell Wall Assembly in *Saccharomyces cerevisiae*. **Microbiology and Molecular Biology Reviews**, v. 70, n. 2, p. 317–343, 2006.

NIEDERMEYER, W.; PARISH, G. R.; MOOR, H. Reactions of Yeast Cells to Glycerol Treatment. **Protoplasma**, v. 92, p. 177–193, 1977.

PEBERDY, J. F.; BUCKLEY, C. E.; DALTREY, D. C.; MOORE, P. M. Factors affecting protoplast release in some filamentous fungi. **Transactions of the British Mycological Society**, v. 67, n. 1, p.23-26, 1976.

PERINA, F. J. et al. Thymus vulgaris essential oil and thymol against Alternaria alternata (Fr.) Keissler: Effects on growth, viability, early infection and cellular mode of action. **Pest Management Science**, v. 71, n. 10, p. 1371–1378, 2015.

RODRIGUEZ-IGLESIAS, A.; SCHMOLL, M. Protoplast transformation for genome manipulation in fungi. In: VAN DEN BERG, M. A.; MARUTHACHALAM, K. (eds.), **Genetic Transformation Systems in Fungi, Volume 1, Fungal Biology**, DOI 10.1007/978-3-319-10142-2_2, Springer International Publishing Switzerland, 2015.

SWEIGARD, J. A.; CHUMLEY, F. G.; VALENT, B. Cloning and analysis of CUT1, a cutinase gene from Magnaporthe grisea. **MGG Molecular & General Genetics**, v. 232, n. 2, p. 174–182, 1992.

TALBOT, N. J. et al. Karyotypic variation within clonal lineages of the rice blast fungus, Magnaporthe grisea. **Applied and Environmental Microbiology**, v. 59, n. 2, p. 585–593, 1993.

VOLLMER, S. J.; YANOFSKY, C. Efficient cloning of genes of Neurospora crassa. **Proceedings of the National Academy of Sciences of the United States of America**, v. 83, n. 13, p. 4869–4873, 1986.

ARTIGO 4 – MICROCONIDIA AND CONIDIA ANASTOMOSIS TUBES IN *Pyricularia oryzae* PATHOTYPE *Triticum* *

ARTIGO FORMATADO DE ACORDO COM A NORMA NBR 6022 (ABNT 2018)

Dérica Gonçalves Tavares **

Prof. Dr. Eduardo Alves (Orientador) ***

ABSTRACT

The objective of this study was to evaluate the formation and production of microconidia, and to induce fusion of conidia anastomosis tubes (CATs) in the *Pyricularia oryzae* pathotype *Triticum*. Microconidia were evaluated from 16 isolates that were produced on glass slides covered with OA and PDA (potato dextrose agar) medium, incubated in a humid chamber for 10 days at 25 ºC under constant light. Microconidia were also produced in submerse culture using complete medium, PD broth, and 5 x YEG. Conidia anastomosis tubes (CATs) formations were evaluated using a suspension of 10^6 conidia/mL of 12.1.146 isolate in PD media, Vogel with 2% glucose, and distilled water. The same treatments were performed using 0.1% Benomil to inhibit gemination. The treatments were incubated for 24, 48, and 72 h at 22 ºC in the dark. Microconidia were observed in AO medium in 7 isolates they were unicellular with 7.0 x 4.1 μm long and formed a globular mass of microconidia (false heads) and were also produced in CM liquid medium. The fungicide Benomil inhibited conidia germination and CAT events, which were observed only in treatments without Benomil and in water. This is the first report of CAT events in *Pyricularia* and such events could facilitate and be used in studies related to the parasexuality of this fungus.

Keywords: Asexual reproduction. Heterokaryon. CAT fusion. Germ tube.

---

* Artigo Apresentado para a conclusão do curso de pós-graduação em Microbiologia Agrícola da Universidade Federal de Lavras.

** Doutoranda no curso de Microbiologia Agrícola, pela Universidade Federal de Lavras. Endereço eletrônico: dericatavares@gmail.com.

*** Professor da Universidade Federal de Lavras, Doutor em Fitopatologia.

1 INTRODUCTION

Asexual reproduction is responsible for a large part of spore dispersion in Ascomycetes while sexual reproduction is less frequent. Isolates of *Pyricularia oryzae* produce conidia and may also produce microconidia, as noted by Zhang et al. (2014). Conidia are largely responsible for the direct infection of this pathogen, and studies indicate that *P. oryzae* microconidia may be related to the spread of the disease from infected regions to healthy parts of the plant (ZHANG et al., 2014). However, the role of these spores is still unclear, and there are no reports of its production by the *P. oryzae* pathotype *Triticum*. Mutations and parasexual recombination have been the major causes of variations in the pathogenicity of *P. oryzae* isolates, because the reports of the sexual cycle are only under laboratory conditions (TSUJIMOTO NOGUCHI, 2011; URASHIMA; IGARASHI; KATO, 1993). The parasexual cycle occurs parallel to the sexual cycle resulting in genetically distinct haploids without meiosis events. Conidia anastomosis tubes (CATs) can be used to study fungal parasexuality. CATs were first described in *Colletotrichum lindemuthianum* and occurs when a specialized hypha or cell protrusion is involved in the fusion of somatic cells during colony initiation allowing the formation of networks interconnected with conidia and is different from hyphal anastomosis that occurs in mature colonies (ROCA et al., 2003; READ et al., 2010). Heterocariosis from hyphae fusion can lead to an incompatibility response leading to cell death if the hyphae have incompatible genes. However, heterokarya were obtained via CAT fusion without an incompatibility response in *Penicillium notatum* (BAKER, 1994) and *C. lindermuthianum* (ISHIKAWA et al., 2012; ROCA et al., 2003). Heterocariosis can result in parasexuality and usually occurs by hyphal anastomosis, but CAT fusion could be an important mediator of this process (ROCA; READ; WHEALS, 2005). Therefore, knowledge of such events in *P. oryzae* can help in studies of potential sources of genetic variability. Thus, the objectives of this work were microconidia production and determine the optimal conditions for CAT fusions in *P. oryzae* pathotype *Triticum*.

2 MATERIAL AND METHODS

2.1 MICROCONIDIA PRODUCTION

The *P. oryzae* pathotype *Triticum* isolates used in this study were 12.1.243, 12.1.291, 12.0.368, 012.0.366, 12.0.555i, 12.1.037, 12.1.119, 12.1.117, 12.1.127, 12.0.655i, 12.0.194, 12.1.179, 12.1.117, 12.1.053, 12.1.158, and 12.0.324. Discs of 8 mm oatmeal (30 g/L oatmeal and 20 g/L agar) 10-days-old cultures were transferred to glass slides covered with oatmeal or PDA (potato dextrose agar) medium, and a cover slide was placed on top of each disc and incubated in a humid chamber for 10 days at 25 ºC under constant fluorescent light (CHUMA et al., 2009). The slides were observed under an Epifluorescence microscope with an Apotome system. Production of microconidia in submerse culture was performed using two 5 mm discs of 10-days-old cultures in 40 mL of homemade PD broth (200 g/L potato, 20 g/L dextrose, and 15 g/L), CM (10 g/L glucose, 2 g/L peptone, 1 g/L de yeast extract, 1 g/L casamino acids, 50 mL/L de 20x nitrate salts, 1 mL/L trace elements, 1 mL/L de vitamin solution, pH 6,5, 15 g agar) medium or 5 x YEG (5g/L yeast extract and 10 g/L glucose), incubated at 25 °C for 3 days then or 20°C for 4 days. Microconidia were obtained by filtration using two layers of Miracloth (CalBiochem) (ZHANG et al., 2014).

2.2 INDUTION OF CATS EVENTS

The 12.1.146 isolate was used in this assay because of its high conidial production and resistance to azoxystrobin fungicide, which can be used in future studies of resistance transfer via conidia anastomosis. The isolate was grown in oatmeal for 15 days under constant fluorescent light to induce sporulation. Conidia were collected in water and a suspension of approximately 10^7 conidia/mL was adjusted. CAT formations were induced in PD broth, Vogel medium containing 2% glucose (VOGEL, 1956), and distilled water. The same treatments but with Benomil fungicide were performed to inhibit germination which would induce only CAT formations. To perform this experiment, 180 μL of the medium or water was added to an eight-well culture chamber with 20 μL of conidia suspension to obtain an optimal concentration of 10^6 conidia/mL. Treatments with Benomil were added 0.2 μL of 1000x Benomil solution, reaching a final concentration of 0.1% to avoid cytotoxic damage. The eight-well culture chamber was incubated for 24, 48, and 72 h at 22 ºC in the dark and the evaluation was performed using an inverted objective Epifluorescence microscope Zeiss Axio Observer Z.1 (AxioVision software) (ISHIKAWA et al., 2012).

3 RESULTS AND DISCUSSION

3.1 MICROCONIDIA PRODUCTION

Microconidia were observed only in oatmeal medium in 6 isolates and 3 of these isolates (12.0.368, 12.1.169, and 12.1.291) were used to measure the structures (Table 3). Ten units of the structure were used for the measurement.

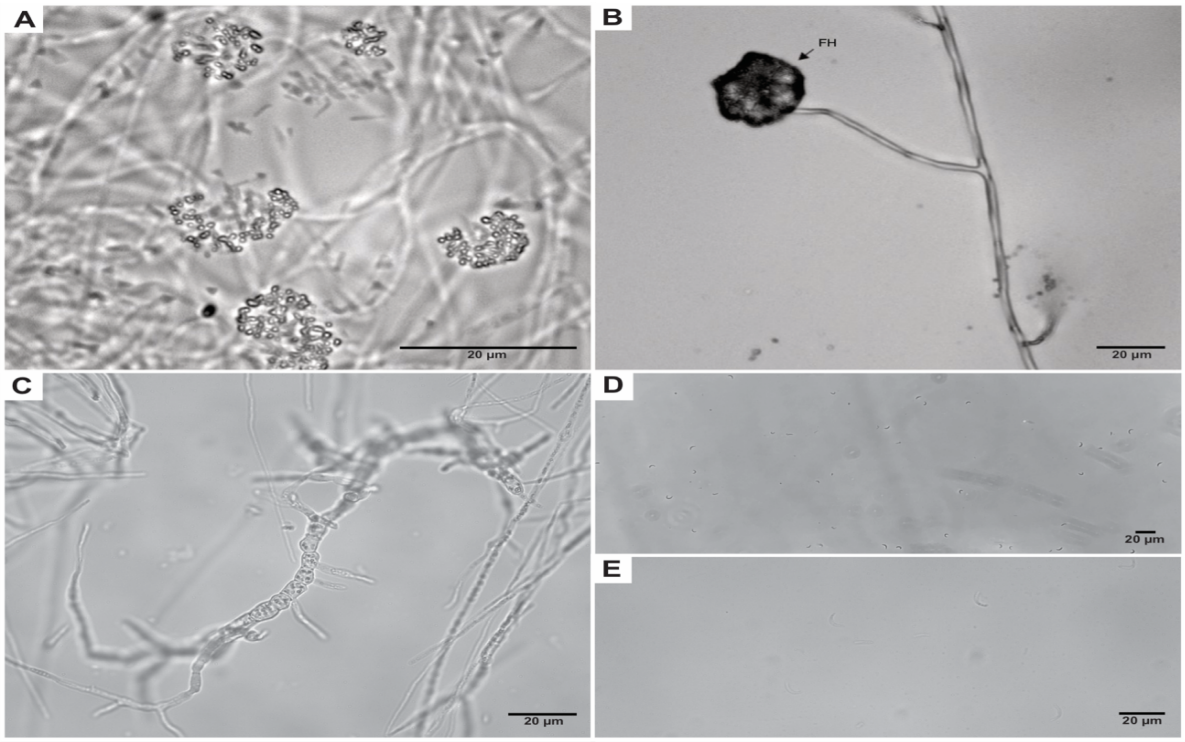
Table 1 - Measurements of microconidia of *Pyricularia oryze* pathotype *Triticum*.

Isolates	Microconidia (μm)	
	Length	Width
12.0.368	4.1-7.0	1.5-2.1
12.1.169	4.1-5.1	1.4-1.7
12.1.291	4.1-6.1	1.5-2.1

Source: Tavares (2021).

Unicellular microconidia were found with 2.1-2.1 μm wide to 7.0-4.1 μm long (Table 1). A globular mass of microconidia (Fig. 1A, B), also called false heads, was observed on the culture medium covered with an extracellular matrix. Microconidia have been reported in the literature produced by *P. oryzae* and were 0.7-2 μm wide and 5.6-11.2 μm long, being the third type of spore produced by *P. oryzae* and can be observed in culture medium with macroconidia and ascospores (CHUMA et al., 2009; KATO et al., 1994). Chuma et al. (2009) observed abundant microconidia from phialides on the medium surface and microconidia that had been released from a phialide formed a globular mass covered with an extracellular matrix. However, few microconidia were produced by *P. oryzae* isolates in the present study, unlike what was found by Chuma et al. (2009).

Figure 1 - Photomicrographs of microconidia from *Pyricularia oryzae* pathotype *Triticum* in false heads (A, B). C) Swollen cells and phialides. F, E) Microconidia.



FH: False head.
Source: Tavares (2021).

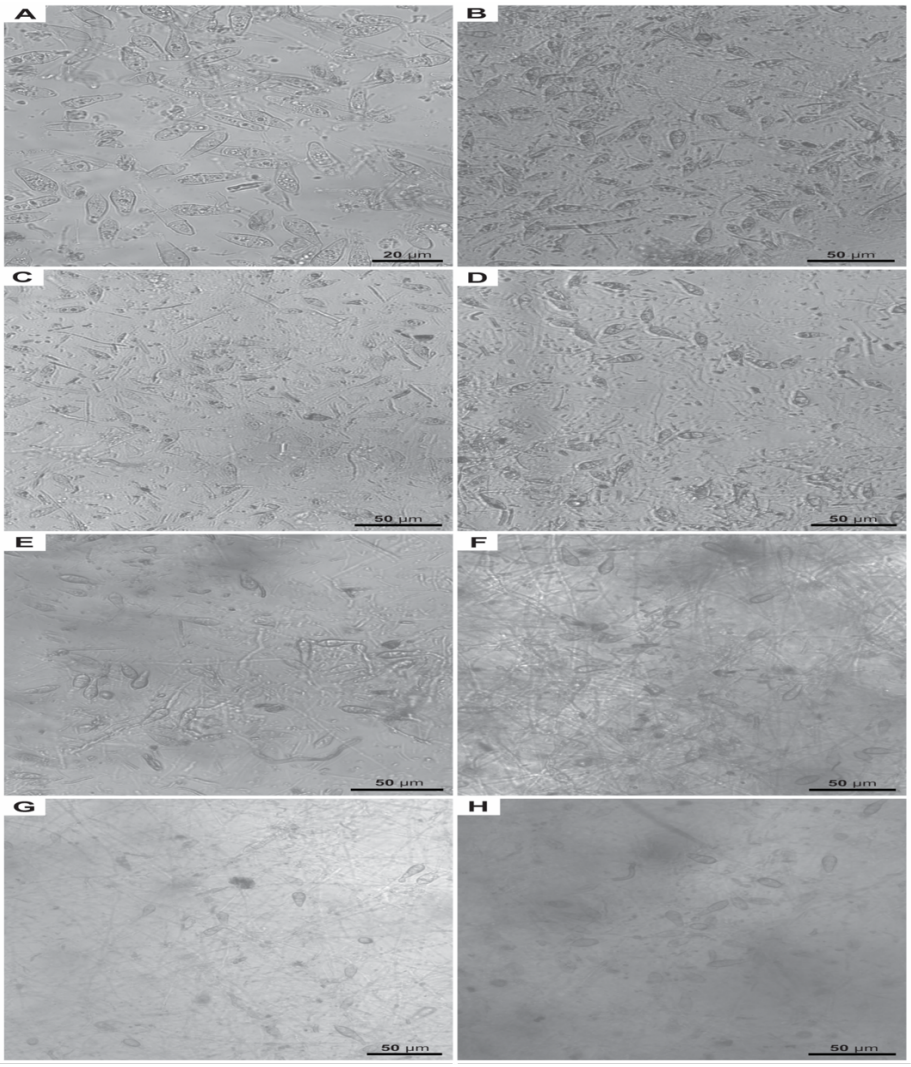
Microconidia in submerse culture were observed only in CM medium at 12.1.037 (Fig. 1D, E) and 12.1.169 isolates and swollen cells were observed from submerged fungal growth (Fig. 1C). Swollen cells were probably phialides in their formation. Zhang et al. (2014) observed higher microconida production in homemade PD than in CM medium which was different from our results.

Transmission electron microscopy images showed that *P. oryzae* microconidia have nuclei that occupy a large portion of the cell and have no nucleoli and may be in a dormant state, unlike conidia that showed smaller nuclei with the presence of nucleoli (CHUMA et al., 2009). Another study evaluated the germination and infectivity of *P. oryzae* microconidia and shown that microconidia have low germination capacity, about 10%, are not infective, but can colonize and develop lesions in injured leaves and stems of rice and barley (ZHANG et al., 2014). Although the importance of microconidia has not been fully elucidated, studies have shown that these structures may be important in the dissemination of rice blast through the plant and/or may be involved in sexual reproduction events (MOREIRA; CERESINI; ALVES, 2015; ZHANG et al., 2014).

3.2 INDUCTION OF CATS

Visualization of CAT events were not possible in PD medium because of the difficulty in passing light through the medium. Visualization was possible in Vogel medium and water. Benomil fungicide was added to inhibit germination and induce the formation of CATs. No conidia germination was observed in the Benomil treatments, even after 72 h of incubation but CAT events were also not observed (Figure 2A-D). However, after 6 h of incubation without the addition of Benomil, conidia germination and appressoria formation were already observed (Figure 2E) and from 24 h to 72 h mycelial growth prevented visualization through the samples in water (Figure 2F-H) and in Vogel medium.

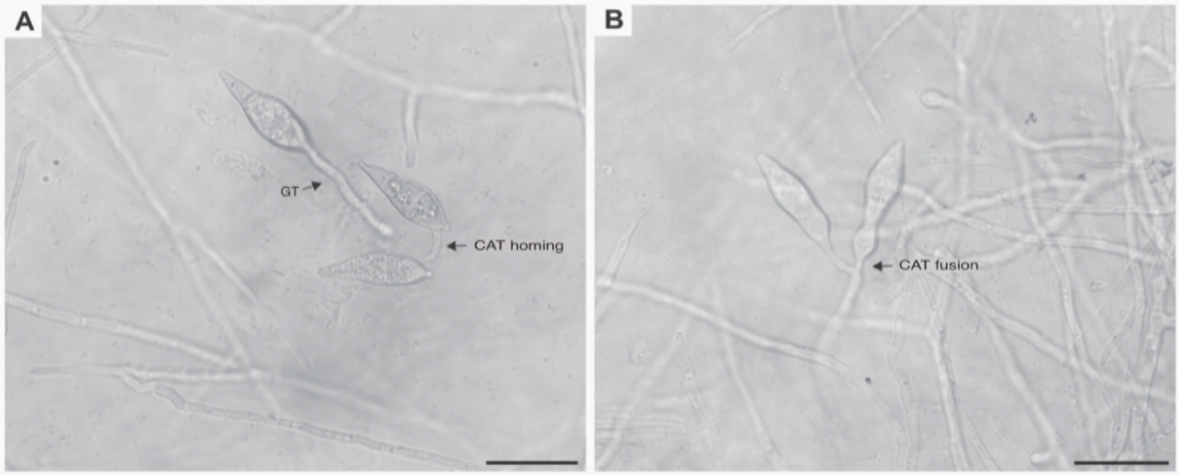
Figure 2 - Photomicrographs of test CATs in *Pyricularia oryzae* using water as medium.



A-D) Test with addition of 0.1% Benomil. E-H) Test without Benomil. Incubation times of 6 h (A, E), 24 h (B, F), 48 h (C, G) and 72 h (D, H).
Source: Tavares (2021).

The benomil concentration used is considered not to cause cytotoxic effects, but only to inhibit germination in *Neurospora crassa* (LICHIUS et al., 2010). In the present study there was no conidia germination, however, tests will be carried out to evaluate whether there are cytotoxic effects of 0.1% Benomil on *P. oryzae*, as well as the evaluation of different concentrations of the fungicide for the CATs test. In treatment without addition of Benomil and in water, probable CAT events were observed: induction of CAT (Fig. 3A) and fusion of CATs (Fig. 3B) after 24 h of incubation. In Vogel medium, no CAT events were observed in *P. oryzae*. Water is the best medium for CATs events in *C. lindemuthianum* (ISHIKAWA et al., 2010), however Vogel medium is better for *N. Crassa* (LICHIUS et al., 2010).

Figure 3 - Photomicrographs of probable CAT events in *Pyricularia oryzae*.



A) CAT homing. B) CAT fusion. GT: Germ tube. 20 μm scale bar.
Source: Tavares (2021).

Conidia produced both germ tubes and CATs (Fig. 3). Germ tubes are wider and tend to avoid each other while CATs are thin and short and grow toward each other usually resulting in fusion (ROCA; READ; WHEALS, 2005). The fusion of CATs shown in Fig. 3B appears to have occurred between the CAT tube and germ tube. Roca et al. (2005) observed three fusion patterns of conidia and conidia germinal tubes in *N. crassa*: (1) fusion between CATs from conidia, (2) fusion between germ tubes that narrow at the tips, and (3) fusion between CATs produced as subapical branches of germ tubes. The fusion of CATs in the present work may be a variation of the third type of fusion observed by Roca et al. (2005).

Studies with *C. lindemuthianum* have used incubation times of 24 to 72 h to assess CAT events (ISHIKAWA et al., 2010, 2012; ROCA et al., 2003). In *N. crassa* evaluations

were made with 2 h and 5 h of incubation and showed 98% germination and after 7 h there was a maximum of 58% CATs fusion (ROCA; READ; WHEALS, 2005). The incubation time is another factor that must be adapted for *Pyricularia* which may be related to the genus or species under study. Conidial density is an important condition for the induction and fusion of CATs, suggesting that some extracellular substance are responsible for inducing this event, such as a quorum-sensing system and can inhibit conidial germination (ROCA; READ; WHEALS, 2005). In the present study, we used a concentration of 10^6 conidia/mL, which is the recommended concentration for CAT studies (ROCA; READ; WHEALS, 2005; LICHIUS et al., 2010) and Roca et al. (2005) reported that concentrations below 10^5 conidia/mL reduced CAT formations. We tested 10^7 and 10^8 conidia/mL but conidia still had a high germination rate and appressoria formation. The challenge to get high fusion of CATs in *P. oryzae* seems to set up a protocol to decrease germination and appressoria formation rate.

4 CONCLUSION

Unicellular microconidia were produced in solid and liquid media by the *P. oryzae* pathotype *Triticum*. However, microconidia were produced in few quantities. The fungicide Benomil inhibited conidia germination and CAT events, which were observed only in treatments without Benomil and in water. This is the first report of CAT events in *P. oryzae* and such events could facilitate and be used in studies related to the parasexual cycle.

REFERENCES

BAKER G. E. Heterokaryosis in *Penicillium notatum*. **Bull. Torrey Bot. Club**, v. 71, p. 367-373, 1994.

CHUMA, I. et al. Cytological characteristics of microconidia of *Magnaporthe oryzae*. **Journal of General Plant Pathology**, v. 75, n. 5, p. 353–358, 2009.

ISHIKAWA, F. H. et al. Factors affecting the production and regeneration of protoplasts from *Colletotrichum lindemuthianum* | Fatores que afetam a produção e regeneração de protoplastos de *Colletotrichum lindemuthianum*. **Ciencia e Agrotecnologia**, v. 34, n. 1, p. 74–79, 2010.

ISHIKAWA, F. H. et al. Heterokaryon incompatibility is suppressed following conidial anastomosis tube fusion in a fungal plant pathogen. **PLoS ONE**, v. 7, n. 2, 2012.

KATO, H. Y. et al. Microconidium in *Magnaporthe*. [s.d.].

LICHIUS, A.; ROCA, M. G.; READ, N. D. How to distinguish conidial anastomosis tubes (CATs) from germ tubes, and to discriminate between cell fusion mutants blocked in CAT formation and CAT homing. **The *Neurospora* Protocol Guide**, 2010. http://www. fgsc. net/Neurospora/NeurosporaProtocolGuide

MOREIRA, S. I.; CERESINI, P. C.; ALVES, E. Reprodução Sexuada em *Pyricularia oryzae*. **Summa Phytopathologica**, v. 41, n. 3, p. 175–182, 2015.

READ, N.; FLEIßNER, A.; ROCA, M.; GLASS, N. Hyphal Fusion. In: Borkovich K, Ebbole D, eds. Cellular and Molecular Biology of Filamentous Fungi: American Society of Microbiology. pp 260–273, 2010.

ROCA, M. G. et al. Conidial anastomosis tubes in *Colletotrichum*. **Fungal Genetics and Biology**, v. 40, n. 2, p. 138–145, 2003.

ROCA, M. G.; READ, N. D.; WHEALS, A. E. Conidial anastomosis tubes in filamentous fungi. **FEMS Microbiology Letters**, v. 249, n. 2, p. 191–198, 2005.

TSUJIMOTO NOGUCHI, M. Parasexual recombination in *Magnaporthe oryzae*. **Japan Agricultural Research Quarterly**, v. 45, n. 1, p. 39–45, 2011.

URASHIMA, A. S.; IGARASHI, S.; KATO, H. Host range, mating type, and fertility of *Pyricularis grisea* from wheat in Brazil. **Plant Disease**, 1993.

VOGEL, H. J. A convenient growth medium for *Neurospora* (Medium N). **Microbial Genet. Bull.**, v. 13, n. 1970, p. 42–43, 1956.

ZHANG, H. et al. Germination and infectivity of microconidia in the rice blast fungus *Magnaporthe oryzae*. **Nature Communications**, v. 5, p. 1–9, 2014.