SILVINO INTRA MOREIRA

ESTUDOS RELACIONADOS À REPRODUÇÃO SEXUADA DE *Pyricularia oryzae*

LAVRAS - MG

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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.

Orientador

Dr. Eduardo Alves

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APROVADA em 19 de Fevereiro de 2015.

Dra. Cristina Ferreira Silva e Batista	UFLA
Dr. Paulo Cézar Ceresini	UNESP
Dra. Sarah da Silva Costa Guimarães	UFLA
Dra. Cláudia Regina Gontijo Labory	UFLA

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“Porque Dele e por Ele, e para Ele, são todas as coisas; glória, pois, a Ele eternamente” (Romanos 11: 36).

RESUMO GERAL

A brusone é causada pelo fungo Ascomyceto *Pyricularia oryzae*, sendo a doença mundialmente mais importante do arroz, além de causar brusone em trigo no Brasil, no Paraguai e na Bolívia. A alta variabilidade genético-patotípica é possivelmente responsável pela baixa durabilidade da resistência de cultivares de arroz e trigo à brusone. A reprodução sexuada é a fonte mais provável da variabilidade nas populações do patógeno nos agro-ecossistemas brasileiros. São escassos os estudos sobre o papel da reprodução sexuada na estrutura genética de populações de *P. oryzae*, bem como na biologia reprodutiva do patógeno envolvendo morfogênese e fatores reguladores. Este trabalho teve por objetivo realizar estudos de indução, morfogênese das estruturas reprodutivas e expressão gênica durante a reprodução sexuada de *P. oryzae.* Num primeiro trabalho, isolados compatíveis de *P. oryzae* patogênicos ao arroz foram pareados em meio de aveia e mantidos por sete dias sob 25 ^{o}C e luz branca contínua, seguido de transferência para incubação sob 18 ºC e luz negra e branca contínuas, por 21 dias. Amostras para análises de qRT-PCR e de microscopia foram coletadas semanalmente. Foram identificadas três fases no desenvolvimento dos ascocarpos: fase inicial de enovelamento ao 14º dia; protoperitécio ao 21º dia; maturação do peritécio ao 28º dia. A expressão de todos os genes *MAT* avaliados foi maior com presença do parceiro compatível e apresentou o mesmo padrão ao longo do tempo, com maior expressão no 14º dia, durante o enovelamento micelial para formar o protoperitécio, seguido de redução até o 28º dia. No segundo trabalho, o objetivo foi avaliar o tamanho e a densidade de peritécios de *P. oryzae* infectivo ao trigo em diferentes espécies de Poaceae, em diferentes restos vegetais e, em meio de cultura, utilizando microscópios de luz e eletrônico de varredura. Foi observado que os peritécios de *P. oryzae* variam em tamanho e densidade, de acordo com o hospedeiro e variam em tamanho de acordo com o estado da planta e o meio de cultura. O reconhecimento dos indivíduos compatíveis pode ocorrer externa ou internamente nos tecidos vegetais. Além disso, verificou-se maior desenvolvimento dos ascocarpos em tecidos necrosados, indicando que possivelmente o ciclo sexual de *P. oryzae* ocorra na natureza, ao menos preferencialmente, em restos vegetais. Estes resultados podem orientar trabalhos de morfogênese e expressão genética, durante a reprodução sexual de *P. oryzae,* em meio de cultura e em plantas, contribuindo para a descrição do ciclo reprodutivo do patógeno e possivelmente para o desenvolvimento de novas medidas de manejo da brusone baseadas em resistência durável.

Palavras-chave: Peritécios. Expressão gênica. Microscopia. Brusone. Idiomorfos.

GENERAL ABSTRACT

Blast disease is caused by the Ascomycetous fungus, *Pyricularia oryzae*, which is the most important rice disease worldwide, also causing wheat blast in Brazil, Paraguay and Bolivia. High genetic-pathotypic variability is probably responsible for the low resistance durability to blast in cultures such as rice and wheat. Sexual reproduction is the most probable source of variability in population of the pathogens in Brazilian agro-ecosystems. Studies on the role of sexual reproduction over the genetic structure of *P. oryzae* populations, as well as on the reproductive biology of the pathogen involving morphogenesis and regulating factors are scarce. This work aimed conducting studies on induction, morphogenesis of the reproductive structures and gene expression during the sexual reproduction of *P. oryzae*. In a first assay, compatible *P. oryzae* isolates pathogenic to rice, were paired on Oatmeal agar and maintained for seven days at 25ºC and continuous white light, followed by the transference to incubation under 18ºC with continuous white and dark light, for 21 days. Samples for qRT-PCR and microscopy analyses were collected weekly. We identified three phases in the development of the ascocarp: initial hyphae folding phase, at the 14th day; proto-perithecia, at the 21st day; perithecia maturation, at the 28th day. The expression in all *MAT* genes evaluated was higher when the compatible partner was present, and it showed the same pattern over time, with higher expression at the 14th day, during the mycelial folding to generate the proto-perithecia, followed by the a reduction until the 28th day. In the second study, the objective was to evaluate the size and density of the *P. oryzae* wheat-infective perithecia in different Poaceae species, on different plant residue, and in culture medium, using light and scanning electron microscopy. We observed that the *P. oryzae* perithecia varied in size and density, according to the host, and varied in size according to the state of the plant and culture medium. The recognition of compatible individuals may occur externally or internally to plant tissues. In addition, we verified higher ascocarp development on tissues in necrosis, possibly indicating that the sexual cycle of the *P. oryzae* occurs in nature, at least preferentially, in plant residue. These results could orient works on morphogenesis and gene expression during the sexual reproduction of *P. oryzae*, in culture media and in plants, contributing for the description of the reproduction cycle of the pathogen, and possibly for the development of new blast disease management measures based on durable resistance.

Keywords: Perithecia. Gene expression. Microscopy. Blast disease. Idiomorphs.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A brusone é causada pelo fungo Ascomyceto *Pyricularia oryzae* Cavara, sendo a mais importante doença do arroz no mundo (WANG et al., 2014). Por outro lado, a brusone do trigo é recente, tendo emergido no Brasil no Estado do Paraná em 1985, causando também muitos danos (DUVEILLER; HODSON; TIEDMANN, 2010; IGARASHI et al., 1986). A brusone do trigo ocorre também no Paraguai e na Bolívia (DUVEILLER; HODSON; TIEDMANN, 2010). O fungo é hemibiotrófico e causa sintomas em todas as partes das plantas, como manchas elípticas em folhas (WANG et al., 2014) e branqueamento de espigas de trigo (MACIEL et al., 2013).

A alta variabilidade genética do fungo contribui para a frequente perda de resistência em cultivares de arroz e trigo (PRABHU; FILIPPI; SILVA, 2006) e, em conjunto com mudanças nos agro-ecossistemas, também, contribui com o fenômeno de 'mudança de hospedeiro'. O trabalho de Couch et al. (2005) aponta evidências de que linhagens infectivas ao arroz emergiram baseadas em isolados de *Setaria*, na China, seguido de especialização em plantas daninhas comuns ao arroz. Stuckenbrok e McDonald (2008) sugeriram que o mesmo tenha ocorrido para favorecer a emergência da brusone do trigo no Sul do Brasil. Existem evidências de que a reprodução sexuada seja a principal fonte de variabilidade de *P. oryzae* (MACIEL et al., 2014; SALEH et al., 2014).

A indução do desenvolvimento de peritécios pode ser realizada por pareamento de indivíduos compatíveis *in vitro* ou em plantas, em tecidos senescentes (HAYASHI et al., 1997; SILUE; NOTTEGHEM, 1990). Esta observação pode levar à hipótese de que a fase sexual ocorra em restos de cultura. No entanto, esta e outras questões permanecem ainda não esclarecidas.

2 REFERENCIAL TEÓRICO

2.1 A brusone e seu agente etiológico

A brusone é causada por espécies de *Pyricularia*, nomeados em sua fase sexual como *Magnaporthe* spp. (COUCH; KOHN, 2002; HEBERT, 1971) por muitos anos. No entanto, foi resolvida recentemente a utilização apenas do nome de sua fase assexuada (KLAUBAUF et al., 2014; MURATA et al., 2014; TOSA; CHUMA, 2014). *Pyricularia oryzae* é a espécie capaz de causar de brusone em arroz e trigo (KLAUBAUF et al., 2014). No entanto, é conhecido que isolados de *P. oryzae* de trigo e de arroz possuem diferente especificidade quanto aos hospedeiros que podem infectar (MACIEL et al., 2014).

Esta é a mais importante doença do arroz no mundo, detectada em 85 países produtores e pode causar perdas de até 100 %. Os primeiros relatos ocorreram em 1637, na China (WANG et al., 2014), e sua primeira constatação no Brasil foi em 1912 (AVERNA-SACCA, 1912).

A brusone do trigo foi relatada posteriormente, tendo emergido no Brasil no Estado do Paraná em 1985 (IGARASHI et al., 1986). Ocorre, também, no Paraguai e na Bolívia (DUVEILLER; HODSON; TIEDMANN, 2010). A severidade da doença varia muito de acordo com a região, condições climáticas e das cultivares de trigo utilizadas (GOULART; SOUSA; URASHIMA, 2007). Ensaios de campo em Indápolis – MS resultaram numa média de 88 % de espigas com brusone, chegando a 98 % para as linhagens de trigo IPR 87, Alcover e ES 046.

O fungo utiliza uma estratégia hemibiotrófica de colonização, invadindo as células do hospedeiro antes de matá-las, e os sintomas podem ser encontrados em todas as partes das plantas, incluindo folhas, bainhas, panículas, pedicelos, sementes e até mesmo raízes (TEBEEST; GUERBER; DITMORE, 2012). O

processo se inicia com a germinação do conídio formando um tubo germinativo, que se diferencia em um apressório, o qual se adere à superfície da planta utilizando uma mucilagem (HOWARD et al., 1991). A hifa de penetração (peg) desenvolve uma forte pressão sobre a folha, fazendo com que a cutícula e paredes celulares das células sejam rompidas e ocorra a penetração (DE JONG et al., 1997; HOWARD et al., 1991). Após a penetração, o *peg* se dilata formando bulbos ou lóbulos, de onde se desenvolvem hifas que colonizam o hospedeiro inter e intracelularmente (HEATH et al., 1990). A passagem da hifa de uma célula para outra é via plasmodesmas (KANKANALA; CZYMMEK; VALENT, 2007). Deste processo surgem os sintomas que vão de pequenos pontos de coloração castanha, que evoluem para manchas elípticas, com extremidades agudas, as quais, quando isoladas e completamente desenvolvidas, variam de 1-2 cm de comprimento por 0,3-5 cm de largura. Estas manchas crescem no sentido das nervuras, apresentando centro cinza e bordos marrom-avermelhados, às vezes, circundado por um halo amarelo. Grande parte do limbo pode ser afetada causando redução da área fotossinteticamente ativa (BEDENDO; PRABHU, 2005). Pode ser eficientemente disperso a curtas e longas distâncias, pelo vento (URASHIMA; LEITE; GALBIERI, 2007), e por meio de sementes infectadas (GOULART; PAIVA, 1995).

2.2 Compatibilidade sexual em populações de *Pyricularia oryzae*

Uma vez que *P. oryzae* é heterotálico (ou auto-incompatível) (KANG; CHUMLEY; VALENT, 1994), sua fase sexuada só é possível quando ocorre o cruzamento entre dois indivíduos de *mating types* compatíveis e que sejam férteis. Isso ocorre, quando a estrutura receptora (‘feminina’) chamada ascogônio está apta a receber o(s) núcleo(s) do indivíduo compatível doador (‘macho’), por meio de conídios ou hifas (Figura 1 A e B).

A fase sexuada de *P. oryzae* é um evento normalmente não observado naturalmente (HAYASHI et al., 1997; SALEH et al., 2012). No entanto, muitos estudos sugerem que isolados de *P. oryzae* patogênicos ao arroz podem se reproduzir sexuadamente, em razão da presença de ambos *mating types* e de fêmeas férteis em populações da Índia (DAYAKAR; NARAYANAN; GNANAMANICKAM, 2000), de Bangladesh (SHAHJAHAN, 1994), e do sudeste da Ásia (SALEH et al., 2012, 2014), centros de origem do arroz. O primeiro relato de elevados níveis de fertilidade (24 a 52%) em isolados de arroz, em diferentes regiões da Índia, foi feito por Dayakar, Narayanan e Gnanamanickam (2000).

Saleh et al. (2014) investigaram a estrutura populacional de isolados de *Pyricularia oryzae* patogênicos ao arroz, da Ásia (China, Indonésia, Laos, Nepal e Tailândia), Europa (França, Grécia, Hungria, Marrocos, Espanha e Turquia), Américas (Colômbia, Guiana Francesa e EUA), e África (Madagascar) utilizando para as análises dez marcadores microssatélites. Seus resultados revelaram dois centros de origem da diversidade genética de *P. oryzae*, nos sopés dos Himalaias (sul da China-Laos-norte da Tailândia) e no Nepal ocidental. Foi observado, para as populações avaliadas, que a reprodução sexuada ocorre apenas na região do sul da China-Laos-norte da Tailândia, identificada como provável centro de origem de todas as populações de *P. oryzae* do arroz. Três clusters genéticos foram identificados. No cluster A havia proporções equilibradas dos dois *mating types* e a presença de isolados fêmeas-férteis e fêmeas estéreis; no cluster B, predomínio de Mat1-1; e no cluster C, predomínio de Mat1-2. Raros isolados fêmeas-férteis foram encontrados nos clusters B e C. Os autores sugeriram que populações clonais, encontradas fora da Ásia, originaram-se de outras populações clonais pré-existentes antes das migrações mundiais. No entanto, isolados de arroz de origem brasileira, bem como de trigo e de outras Poaceae não foram avaliados.

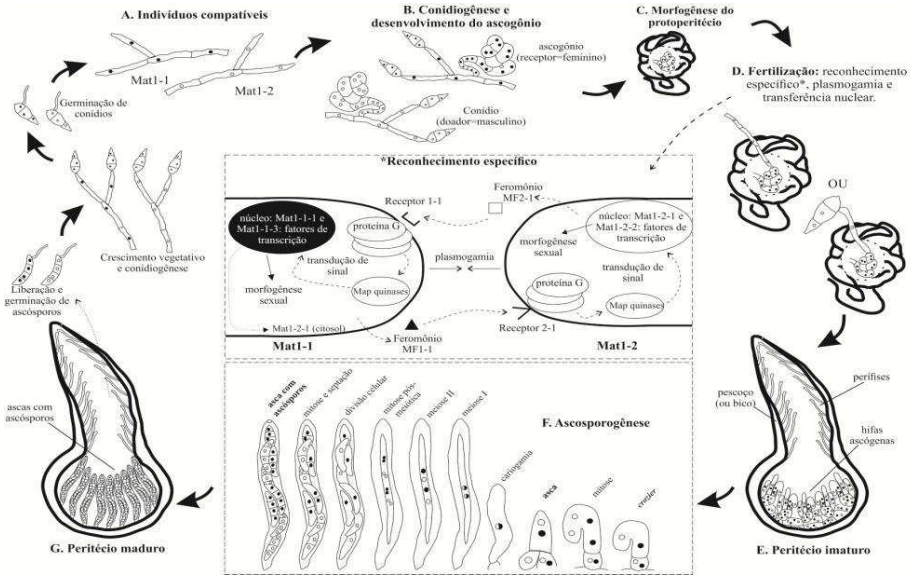

Figura 1 Esquema ilustrativo do ciclo sexual de *Pyricularia oryzae* com etapas da morfogênese e a sua regulação gênica em *P. oryzae* e/ou outros Ascomycota

Legenda: **A**, Presença de indivíduos compatíveis, Mat1-1 e Mat1-2 (KANG; CHUMLEY; VALENT, 1994); **B**, Desenvolvimento das estruturas 'masculinas' (hifas e/ou conídios, 'doadores') e 'femininas' (ascogônios, 'receptores'); **C**, Envelopamento do ascogônio, originando o protoperitécio (LICHIUS et al., 2012; LORD; READ, 2011); **D**, Fertilização: plasmogamia e transferência nuclear precedidos de reconhecimento por meio de feromônios MF1-1 e MF2-1 (SHEN; BOBROWICZ; EBBOLE, 1999) em receptores específicos de indivíduos compatíveis (JOHNSON, 1995; KESZTHELYI et al., 2007), desencadeando uma transdução de sinais (KIM; METZENBERG; NELSON, 2002; LEE et al., 2006) por meio de proteínas G (FANG; DEAN, 2000; SEO; HAN; YU, 2005), MAP quinases (LICHIUS et al., 2012), transferases, proteases, e fatores de transcrição, entre estes, genes *MAT* (KANAMORI et al., 2007; TURGEON, 1998), para a indução da expressão de genes necessários à morfogênese sexual. **E**, Desenvolvimento do protoperitécio em peritécio imaturo com hifas ascógenas fertilizadas (LICHIUS et al., 2012; LORD; READ, 2011) muitas vezes dependente da expressão de genes *MAT* (COPPIN; DE RENTY; DEBUCHY, 2005; KLIX et al., 2010), de feromônios (COPPIN; DE RENTY; DEBUCHY, 2005; FERREIRA et al., 1998) e/ou de proteínas G (FANG; DEAN, 2000). **F**, Ascosporogênese: cariogamia e meiose em hifas ascógenas, seguido de mitoses pós-meióticas, divisão celular, novas mitoses, e septação, para origem de ascósporos tetra-celulares (YAEGASHI; HEBERT, 1976b); evento muitas vezes dependente da expressão de genes *MAT* (FERREIRA et al., 1998), de feromônios (TURINA; PRODI; ALFEN, 2003), e/ou de proteínas G (FANG; DEAN, 2000), ou seja, sem a expressão destes genes pode ocorrer esterilidade. **G**, Peritécio maduro e liberação dos ascósporos por meio de deliquescência de ascas, com origem de novos indivíduos pela germinação de células terminais (TREDWAY; STEVENSON, 2003).

No Brasil, isolados de arroz avaliados por Urashima, Igarashi e Kato (1993) desenvolveram peritécios *in vitro*, mas não produziram ascósporos e não apresentaram compatibilidade sexual com isolados de trigo. Quanto aos isolados patogênicos a plantas de trigo, ambos os *mating types* foram detectados no campo (BRUNO; URASHIMA, 2001; URASHIMA; IGARASHI; KATO, 1993), compatíveis sexualmente com outros isolados de trigo, *Brachiaria plantaginea*, capim pé-de-galinha (*Eleusine indica*) e painço (*Setaria itálica*) (URASHIMA; IGARASHI; KATO, 1993), e cevadinha (*Bromus catharticus* Vahl), alpiste (*Phalaris canariensis*) e triticale (*X. triticosecal* Wittmack) (GALBIERI; URASHIMA, 2008). O idiomorfo Mat1-1 predominou nas populações amostradas nos Estados do Mato Grosso do Sul, Paraná e São Paulo. Por outro lado, Maciel et al. (2014) encontraram populações de isolados de trigo com diferentes distribuições dos idiomorfos Mat1-1 e Mat1-2: no Centro-Oeste (4:1), no Triângulo Mineiro (30:1), em São Paulo (1:0) e no Paraná (15:1). Tendo analisado a estrutura populacional do patógeno (com base em onze microssatélites e testes de patogenicidade) estes autores propuseram que as populações de *P. oryzae* possuem um sistema reprodutivo misto, onde a reprodução sexuada é seguida da dispersão clonal. O favorecimento da dispersão clonal de *P. oryzae* pela seleção de indivíduos Mat1-1 com um gene de resistência à estrobilurina foi recentemente descrito para populações do Distrito Federal e dos Estados de Goiás, Minas Gerais, Mato Grosso do Sul, Paraná, Rio Grande do Sul e São Paulo (CASTROAGUDÍN et al., 2014).

2.3 Morfogênese do peritécio de *Pyricularia* spp.

A fase sexuada em *Pyricularia* spp. foi primeiramente descrita como *Ceratosphaeria grisea*, por Hebert (1971), por meio de cruzamentos entre

isolados de capim-colchão, em meio Sachs´ Agar com grãos de cevada e bainhas de arroz e incubados a 25 °C sob luz fluorescente contínua.

Os peritécios de *Pyricularia* spp. podem estar isolados ou em grupos, fundidos ou não, parcialmente ou totalmente imergidos no substrato, com longos pescoços salientes com base na superfície, hialinos a escurecidos com a idade (Figuras 2 e 3), contendo longas perífises filiformes e deliquescentes. As ascas se originam na base do pescoço, cilíndricas a subclavadas, unitunicadas e com uma fina parede e um opérculo na extremidade superior. Os ascósporos são hialinos, fusiformes, triseptados, moderadamente curvados, com glóbulos de óleo usualmente presentes e, provavelmente, a liberação ocorre pela deliquescência da asca. Podem germinar a partir de uma, ou de ambas as células terminais (Figura 1 G) (HEBERT, 1971).

Hifas vegetativas uninucleadas dão origem a hifas ascógenas binucleadas, que formam o *crozier* (Figura 1 F). Eventualmente, é possível também observar dois núcleos em células de hifas vegetativas mais velhas de *Pyricularia* spp. (YAEGASHI; HEBERT, 1976b).

A fertilização ocorre quando o(s) núcleo(s) do indivíduo doador chega(m) aos ascogônios do indivíduo receptor, estruturas localizadas no interior de protoperitécios. Muitas espécies de Ascomycota possuem tricógines, prolongamentos que saem dos ascogônios e se projetam para o exterior dos protoperitécios. Os tricógines recebem núcleos de indivíduos doadores por meio de plasmogamia com o tubo germinativo de conídios ou de hifas. No entanto, esta estrutura ainda não foi caracterizada em *Pyricularia* spp., necessitando maior elucidação sobre como ocorre sua fertilização, talvez pela entrada de tubos germinativos e/ou hifas nos protoperitécios, onde estão as hifas ascógenas (Figura 1 D), ou ainda chegando ao ascogônio antes mesmo de seu envelopamento. O núcleo doado migra para o ascogônio (Figura D e E), onde poderá se recombinar com núcleo do indivíduo receptor na meiose (Figura 1 F),

o que caracteriza o início do ciclo de reprodução sexuada (COPPIN et al., 1997). Após, cada um dos quatro núcleos gerados sofrem mitose pós-meiótica, resultando assim nos oito núcleos dos oito ascósporos encontrados em cada asca (Figura 1 F). Novas mitoses ocorrem dentro dos ascósporos, seguido de septação e, ao final, ascósporos com quatro células uninucleadas, à medida que ocorre a maturação final do peritécio (Figura 1 G) (YAEGASHI; HEBERT, 1976b).

O protoperitécio é uma estrutura esférica a subesférica que se desenvolve envolvendo os ascogônios (Figura 1 C) e resulta na origem do peritécio maduro (Figura 1 G). Sua morfogênese em *Neurospora crassa* (descrita por meio de microscopia eletrônica de varredura (LICHIUS et al., 2012) foi dividida nas fases de formação da hélice ascogonial, expansão do protoperitécio e emergência do tricógine. O anel ascogonial é estabilizado de forma helicoidal por meio matriz extracelular (MEC). Ramificações de novas células ascogoniais envelopam a hélice, com um envelopamento adicional de hifas ramificadas do envelope original e hifas vizinhas, originando o protoperitécio, com uma capa compacta estabilizada por meio de MEC. Este se expande e origina elongações de 'tricógines', marcando a maturação do protoperitécio. Para a morfogênese do peritécio de *N. crassa*, a fusão com células *mating-type* específica é requerida. Foi possível caracterizar para *Sordaria macrospora* as fases ascogonial, protoperitecial e peritecial (LORD; READ, 2011). Ao menos treze tipos de células especializadas e morfologicamente distintas foram classificados em três classes, hifas, células conglutinadas e esporos. Ainda não existe uma análise descritiva em classes de células para os tecidos sexuados de *P. oryzae*.

2.4 Estrutura e função de genes relacionados ao ciclo sexual em *Pyricularia oryzae* e em outros Ascomycota

O *locus* onde estão localizados os genes *mating type* (*MAT*) em *P. oryzae* é chamado Mat1, localizado no cromossomo três. Possui dois idiomorfos, Mat1-1, que codifica os genes *MAT1-1-1*, *MAT1-1-2* e *MAT1-1-3*, e *MAT1-2*, o qual codifica os transcritos *MAT1-2-1* e *MAT1-2-2* (KANAMORI et al., 2007; TURGEON, 1998). Os genes *MAT1-1-3* e *MAT1-2-2* possuem, respectivamente, as ORFs *MAT1-1-3A* e *MAT1-1-3B*, e *MAT1-2-2A* e *MAT1-2-2B*, determinadas por *splicing* alternativo (KANAMORI et al., 2007).

A proteína deduzida do transcrito *MAT1-1-1* inclui um motivo α-box, que é conservado como produto de Mat1-1 em vários fungos Ascomycota (TURGEON, 1998). Este gene define o idiomorfo Mat1-1, e evidências suportam que sua proteína α é um fator de transcrição que se liga ao DNA via domínio α conservado. Seu gene correspondente em *Saccharomyces cerevisae* é *MATα1P*, um co-ativador transcricional essencial para a expressão de genes *mating type*-específicos, bem como de feromônios e receptores de feromônios (JOHNSON, 1995).

Por outro lado, a proteína deduzida de *MAT1-2-1* contém o motivo HMG-box de ligação ao DNA, que é conservado em produtos Mat1-2 de vários Ascomycota, e caracteriza o idiomorfo Mat1-2 (TURGEON, 1998). O domínio HMG (Grupo de Alta Mobilidade, *High Mobility Group*) possui uma sequência com afinidade a DNA encontrada em proteínas cromossomais não-histonas e fatores de transcrição. As ORFs *MAT1-1-3A* e *MAT1-2-2A* (KANAMORI et al., 2007) também codificam para o domínio HMG. Análises estruturais e filogenéticas de HMG-box de *MAT1-1-3* e *MAT1-2-1* de *P. anserina* mostraram que estas proteínas são muito dissimilares entre si e que provavelmente se ligam

ao DNA de maneira muito diferente (ARNAISE; DEBUCHY; PICARD, 1997; ARNAISE et al., 2001; ZICKLER et al., 1995).

Quanto ao transcrito de *MAT1-1-2*, *SMR1* de *P. anserina*, foi verificado que seu domínio conservado HPG possui resíduos de histidina, prolina e glicina. Mutações de substituição de triptofano para alanina promoveram completa inibição do desenvolvimento de peritécios, nos estádios iniciais. Estudos de localização celular de Smr1 com GFP indicaram sua localização no citoplasma, mas sua função permanece não elucidada (COPPIN; DE RENTY; DEBUCHY, 2005).

A interação entre os transcritos dos genes *MAT* pode ocorrer (JACOBSEN; WITTIG; POGGELER, 2002) ou não (ARNAISE; DEBUCHY; PICARD, 1997) em diferentes Ascomycota, sem função conhecida para o ciclo sexual.

A importância do *locus* MAT na reprodução sexuada parece ser diferente em diferentes fungos (COPPIN et al., 1993). O nocaute combinado de *MAT A-1* (*MAT1-1-1*) e *MAT A-3* (*MAT1-1-3*) em *N. crassa* reduziu a fertilidade, sem efeitos no corpo de frutificação e na compatibilidade vegetativa (FERREIRA et al., 1998). Já o nocaute de *MAT1-1-1* e *MAT1-2-1* afetou a fertilidade de *P. anserina* (DEBUCHY; TURGEON, 2006). Em *S. macrospora*, o nocaute de SMTA-2 (*MAT1-1-2*) ou a dupla-deleção SMTA-2/3 (deleção de *MAT1-1-1* e *MAT1-1-3*), teve como consequência o subdesenvolvimento do ascocarpo (KLIX et al., 2010). As análises de qRT-PCR nestes mutantes revelaram que a proteína SmtA-1 atua como um regulador positivo da expressão de genes de precursores de feromônios *PPG1* e *PPG2*, e *SMTA-2* teve um efeito negativo na expressão de ppg2. Já isolados *ΔMAT1-2-1* de *Fusarium verticillioides* tiveram regulação negativa na expressão de precursores e receptores de feromônio (KESZTHELYI et al., 2007), e a importância do *locus* Mat1-2 no ciclo sexual de *Gibberella zeae* foi verificada por Lee et al. (2006),

quando observaram que sua deleção reduziu significativamente a quantidade de transcritos de inúmeros genes relacionados direta ou indiretamente com o ciclo sexuado.

Kang, Chumley e Valent (1994) transformaram isolados de *P. oryzae* de um *mating type* com um cosmídeo linearizado contendo o gene do *mating type* oposto, resultando assim num isolado que continha os dois *mating types*, ou seja, um transformado homotálico, tanto para isolado Mat1-1 quanto para isolado Mat1-2. Todavia, tanto ascósporos quanto conídios destes indivíduos transformados não reproduziram a característica homotálica, apresentando um *mating type* ou outro.

Além dos genes *MAT*, a regulação do reconhecimento específico dos indivíduos de *mating type* oposto ocorre por meio de feromônios e seus receptores específicos, cuja interação desencadeia uma transdução de sinais para estimular a expressão de outros genes importantes para o ciclo sexual (SHEN; BOBROWICZ; EBBOLE, 1999). Com base neste reconhecimento, é iniciada uma rota de sinalização celular que envia sinais ao núcleo, onde, juntamente com diversos fatores de transcrição, induzem a expressão de genes relacionados à reprodução sexuada (Figura 1 D*). Os principais componentes da *rota MAP* quinase de resposta a feromônios já elucidados para *S. cerevisae*, *Schizosaccharomyces pombe* e *N. crassa*, foram citados por Kim, Metzenberg e Nelson (2002): proteínas regulatórias *mating type*; fatores *mating* (precursores de feromônios); proteínas G; proteínas MAP quinase; fatores de transcrição; transferases; endoproteases; e aminopeptidases. Após o sequenciamento do genoma de *P. oryzae,* verificou-se que diversos destes componentes possuem genes de alta similaridade com genes relacionados em *S. cerevisae* (DEAN et al., 2005). A reprodução sexuada de *Aspergillus nidulans* foi correlacionada com o aumento da expressão dos genes *MAT* e de genes-chave da rota de sinalização de feromônios MAP quinase (PAOLETTI et al., 2007).

Genes precursores de feromônios já foram caracterizados para *S. cerevisae* (CROSS et al., 1988), *Cryphonectria parasitica* (ZHANG; BAASIRI; ALFEN, 1998), *P. oryzae* (SHEN; BOBROWICZ; EBBOLE, 1999), *S. macrospora* (POEGGELER et al., 2000) e *N. crassa* (BOBROWICZ et al., 2002).

Shen, Bobrowicz e Ebbole (1999) identificaram os genes *Mating factors*, *MF1-1* e *MF2-1* como precursores de feromônios em *P. oryzae*. O gene *MF1-1* codifica para um polipeptídio de 26 aminoácidos e possui terminação CAAX, encontrado em genes de feromônios de *S. cerevisae*. O gene *MF2-1* contém sítios potenciais de protease Kex2 e sequências repetidas dipeptídicas na região N-terminal, como em *S. cerevisae* e *C. parasitica*.

A deleção de *MF1-1* em *C. parasitica* causa esterilidade em 'doadores' (machos), sem efeitos no ciclo vegetativo (TURINA; PRODI; ALFEN, 2003). No entanto, foi verificado que o gene precursor de feromônio *MFA-1* de *N. crassa* também pode afetar o crescimento filamentoso (KIM; METZENBERG; NELSON, 2002). A regulação da reprodução sexual pode sofrer influência das condições do ambiente, mas, além disso, existe um controle intrínseco do organismo, que obedece a um 'relógio molecular'. Foi verificado que a expressão de *MF1-1* em *C. parasitica* pode variar, de acordo com estímulos do ambiente, como a composição do substrato onde o fungo se encontra, e com a idade do isolado (TURINA; PRODI; ALFEN, 2003). A expressão de precursores de feromônios de *N. crassa* apresentou padrão circadiano (BOBROWICZ et al., 2002).

As proteínas G são mediadoras em respostas a sinais do ambiente. São compostas pelas subunidades α, β e ϒ, e seus genes são requeridos para formação de corpos de frutificação, como observado para *A. nidulans* (SEO; HAN; YU, 2005). O gene *MAGB* em *P. oryzae* codifica a subunidade *α* da

proteína G, cujo silenciamento levou a defeitos na formação de peritécios, apressórios e conídios e no crescimento celular (FANG; DEAN, 2000).

2.5 Métodos para a indução do ciclo sexual de *Pyricularia oryzae*

Frequentemente os estudos sobre a fase sexuada de *Pyricularia* spp. são conduzidos *in vitro*, desde sua primeira caracterização (HEBERT, 1971) até os dias atuais (SALEH et al., 2014; URASHIMA; SILVA, 2011) (Tabela 1). Utilizando isolados de *P. oryzae*, Itoi et al. (1983) desenvolveram um método, ainda bastante utilizado (Tabela 1), o "three-point-culture". Baseia-se no pareamento equidistante entre 'plugs' de micélio de três indivíduos em uma mesma placa, onde dois são testadores (conhecidamente férteis), um de cada *mating type*, e o outro isolado, avaliado quanto à compatibilidade com um dos dois testadores. Linhas duplas de peritécios com ascósporos viáveis na interface entre duas colônias indicam fertilidade, e o lado onde isso ocorre indica a qual *mating type* pertence o isolado avaliado. No entanto, nem todos os isolados de *P. oryzae* apresentam este comportamento. Em pareamento de isolados de arroz (linhagem 70-15) e de cevada (linhagem 40-91), linhas de peritécios foram observadas, como no método de ITOI et al. (1983) (Figura 2 B), mas quando isolados de trigo são pareados (linhagens 50-46 e 33-1), os peritécios se originaram dispersos de forma irregular nas placas (Figura 2 A, C e D). Provavelmente isto não deva ser uma regra para todos os isolados de arroz ou de trigo e não são conhecidos os fatores que determinam este comportamento. Assim, sugere-se, neste caso, primeiramente identificar o *mating type* dos isolados, por meio de PCR (TREDWAY; STEVERSON, 2003), seguido de pareamentos com duplas de indivíduos compatíveis, para localização de testadores (hermafroditas férteis) de Mat1-1 e de Mat1-2.

Tabela 1 Métodos para indução da produção de peritécios de *Pyricularia* spp.

Hospedeiro(s)	País(es) de origem dos isolados	Método	Testador(es)	Condições de luz/temperatura	Referência
Digitaria saguinalis	EUA	Pareamento em Sachs'agar com grãos de cevada e palha de arroz	-	25°C/luz fraca/21 dias	Hebert (1971)
Eleusine coracana	Japão	2 mL suspensão com fragmentos miceliais sobre bainhas de arroz desinfestadas, em Sachs'agar	-	20°C/ luz 330-780nm/fotoperíodo 12hrs (550erg/cm2/seg) /20-30 dias	Kato, Yamaguchi e Nishihara (1976)
Eleusine coracana	Japão	"Three-point-culture" com plugs de micélio em meio de aveia	G10-1, P2-6 (Mat1-1), Z2-1, Z5-1, Z7-1 (Mat1-2)	28°C/luz branca/10dias; Seguindo de 20 dias sob luz negra (perto da UV)	Itoi et al. (1983)
Oryza sativa	Guiana Francesa e Mali	Suspensão mista de conídios injetada em plantas de arroz	Guy11(Mat1-2) e ML25 (Mat1-1)	Plantas inoculadas sob 20ºC/luz branca/15 dias; bainhas com lesões transferidas para câmara úmida a 20ºC/luz branca/15dias	Silue e Notteghem (1990)

"Tabela 1, continuação"

Hospedeiro(s)	País(es) de origem dos isolados	Método	Testador(es)	Condições de luz/temperatura	Referência
E. coracana, Brachiaria plantaginea, Setaria italica, Triticum aestivatum	Japão, Brasil	"Three-point-culture" com plugs de micélio em meio de aveia (distância de 4cm) (22)	G10-1 (Mat1-1), Z2-1 (Mat1-2)	25°C/escuro/7 dias; seguido de 20 dias sob luz branca contínua/20°C	Urashima, Igarashi e Kato (1993)
O. sativa	China	Suspensão mista de conídios injetada em plantas de arroz (43)	CHNOS0101-3B-2 (Mat1-2), 3B-4 (Mat1-1) e 3B-5 (Mat1-2)	Diferentes protocolos testados, conforme Silue e Notteghem (43)	Hayashi et al. (1997)
E. coracana, O. sativa, paragrass	Índia, Guiana Francesa	"Three-point-culture" com plugs de micélio em meio de aveia (distância de 5cm) (22)	Guy11, KA-3, KA-9, KA-7	27°C/escuro/7 dias; seguido de 20 dias sob luz branca contínua/22-24°C	Viji e Gnanamanickam (1998)
E. coracana, O. sativa	Índia, Guiana Francesa	plugs de micélio em meio de aveia (distância de 2cm)	Guy11, KA-3, KA-9, KA-7	20-22°C/luz branca contínua (40W)/20 dias	Dayakar, Narayanan e Gnanamanickam (2000)
T. aestivatum	Brasil	"Three-point-culture" com plugs de micélio em meio de aveia (distância de 4cm) (22)	Bp3a e Br118.2D; Br7 e Br8; Br35 e Br48	22°C/luz branca contínua /30 dias	Bruno e Urashima (2001)

"Tabela 1, continuação"

Hospedeiro(s)	País(es) de origem dos isolados	Método	Testador(es)	Condições de luz/temperatura	Referência
E. coracana, O. sativa, T. aestivatum	Uganda, Índia, Guiana Francesa, Brasil	plugs de micélio em meio de aveia (distância de 4cm)	KA-3, KA-7, KA-9, Guy-11, BR-48, e BR-116.5	27°C/fotoperíodo 12hrs/7 dias; seguido de 20 dias sob luz branca contínua (76µE m^{-2} s^{-1})/20°C	Shen, Bobrowicz e Ebbole (1999)
Stenotaphrum secundatum, *Festuca arundinacea*	EUA, Guiana Francesa	Placa com meio de aveia dividida em 8 setores radiais com plugs alternados em testadores e avaliados	Guy11 (Mat1-2), 2539 (Mat1-1)	25°C/luz branca contínua /28 dias	Tredway e Stevenson (2003)
T. aestivatum, Hordeum vulgare, X. triticosecale, Brachiaria plantaginea, O. sativa e várias outras Poaceas	Brasil: MS, PR e SP	"Three-point-culture" com plugs de micélio em meio de aveia (distância de 4cm) (22)	Isolados parentais	22°C/luz branca contínua /28 dias	Galbieri e Urashima (2008)

"Tabela 1, conclusão"

Hospedeiro(s)	País(es) de origem dos isolados	Método	Testador(es)	Condições de luz/temperatura	Referência
Lolium multiflorum, *O. sativa*	Tailândia, China	plugs de micélio em meio de aveia (distância de 3cm)	Hermafroditas: TH12 (MAT1-1) e TH16 (MAT1-2); CHL43 (MAT1-1) e CHL42 (MAT1-2)	20°C/luz branca contínua /20 dias	Zeng et al. (2009)
T. aestivatum, xTricicosecale, L. multiflorum, O. sativa	Brasil	"Three-point-culture" com plugs de micélio em meio de aveia (22)	Br8	22°C/luz branca contínua /20 dias	Urashima e Silva (2011)

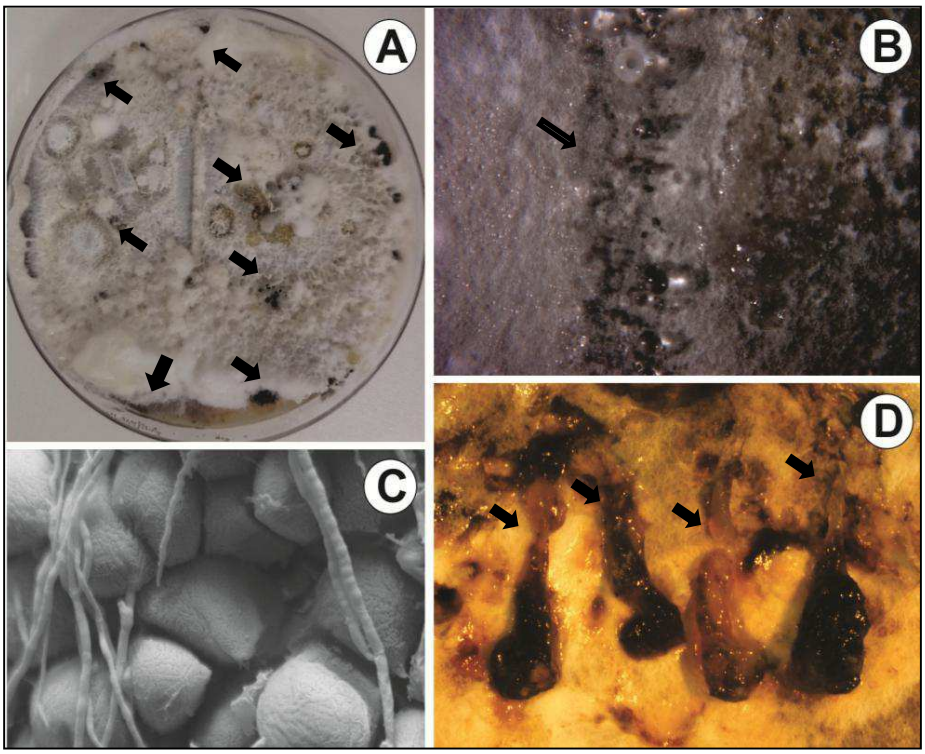

Figura 2 Indução *in vitro* de produção de peritécios de *Pyricularia oryzae* pelo pareamento de isolados compatíveis Mat1-1 e Mat1-2, patogênicos ao trigo (**A**, **C** e **D**) e ao arroz (**B**), em meio de aveia incubada sob iluminação fluorescente e negra (próxima à UV) combinadas de forma contínua a 22 ºC por 30 dias. **A**, imagem em esteriomicroscópio de peritécios dispersos em várias regiões da placa (setas); **B**, imagem em esteriomicroscópio de peritécios alinhados na região de encontro das colônias (seta); **C**, imagem em microscópio eletrônico de varredura: vários protoperitécios se desenvolvendo de forma agrupada; **D**, imagem em microscópio de luz de peritécios encontrados imersos no meio de cultura, apresentando longos pescoços (setas), removidos por meio de escarificação.

Métodos para a indução do ciclo sexual de *P. oryzae* em plantas, também, já foram desenvolvidos. Hayashi et al. (1997) e Silue e Notteghem (1990) observaram a formação de peritécios em bainhas senescentes de folhas de

arroz por meio da injeção de conídios em suspensões mistas com linhagens Mat1-1 e Mat1-2, patogênicas ao arroz. Repetimos este procedimento (HAYASHI et al., 1997) com sucesso em plantas de arroz (Figura 3 A) e trigo (Figura 3 B) inoculadas com isolados de trigo. Foram observados peritécios superficiais, parcialmente submersos (Figura 3 A) e internos (Figura 3 B) aos tecidos senescentes de hastes cortadas e incubadas em câmara úmida sob 20 ºC e luz fluorescente contínua por 30 dias. O desenvolvimento dos ascocarpos em tecidos senescentes pode levar à hipótese de que a reprodução sexuada de *P. oryzae* ocorra, durante a fase necrotrófica, provavelmente em restos de cultura. No entanto, esta questão permanece ainda não esclarecida, sendo potencialmente fundamental em termos de manejo da brusone.

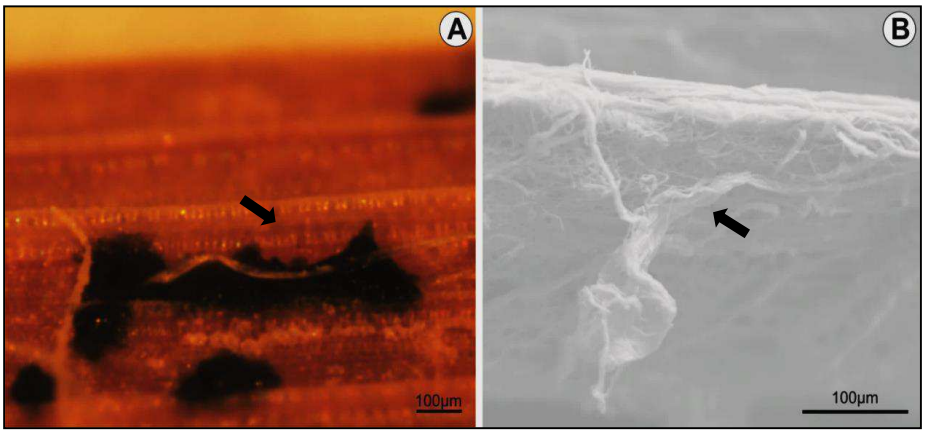

Figura 3 Indução da produção de peritécios de *Pyricularia oryzae* isolados de trigo em hastes senescentes de plantas de arroz (**A**) e trigo (**B**), por meio de inoculação com suspensão conidial mista contendo isolados compatíveis Mat1-1 e Mat1-2. **A**, imagem em microcópio de luz de peritécios desenvolvidos agrupados e parcialmente submersos nos tecidos vegetais (seta); **B**, imagem de microscopia eletrônica de varredura: corte transversal de haste, com colonização micelial interna e peritécio com pescoço longo (seta).

Diversas técnicas de indução do ciclo sexual já foram desenvolvidas (Tabela 1), e as condições ideais de luz, temperatura e nutrientes variam para isolados de diferentes origens geográficas e hospedeiros. Maior número de peritécios foi observado com temperatura de incubação de 20 °C para isolados de capim-colchão (*Digitaria horizontalis*) dos EUA, e 22 a 25 °C, para isolados de capim-pé-de-galinha (*Eleusine indica*) do Japão. A luz é essencial para a produção consistente de peritécios, e baixos comprimentos de onda (próximo à UV) são mais eficientes. O meio de aveia, comparado aos meios batata-sacarose e Sach's agar com bainhas de arroz, apresentou maior número de peritécios (YAEGASHI; HEBERT, 1976a).

Cruzamentos *in vitro* com obtenção de descendentes podem ser úteis em estudos genéticos. Valent, Farrall e Chumley (1991) realizaram pareamentos com isolado de arroz e de capim-chorão (*Eragrostis curvula*), tendo utilizado o isolado de arroz como o parental recorrente em seis gerações. Por meio de estudos de patogenicidade e virulência da progênie, foi observado que os parentais possuem diferentes controles genéticos quanto à habilidade de infectar arroz.

3 CONSIDERAÇÕES GERAIS

Muitas são as questões ainda não esclarecidas no ciclo sexuado de *Pyricularia oryzae*, tais como: distribuição de idiomorfos em diversas populações; estudos populacionais com obtenção de progênies da reprodução sexuada; o evento de fertilização; as funções específicas dos genes MAT e feromônios no reconhecimento dos indivíduos, na morfogênese sexual e na fertilidade; ocorrência da fase sexuada em tecidos vegetais; diferentes condições de indução *in vitro*. Assim, este objeto de estudo é desafiador, com amplas possibilidades a serem exploradas.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 Mating type and pheromone genes expression in *Pyricularia oryzae* during ascocarp development

Fungal Genetics and Biology – Artigo para ser submetido

Mating type and pheromone genes expression in *Pyricularia oryzae* during ascocarp development

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Abstract

The sexual reproduction of *Pyricularia oryzae* is regulated by mating type genes, which are organized in idiomorphs Mat1-1, that encodes *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*, and the Mat1-2, that encodes *MAT1-2-1* and *MAT1-2-2*. Another gene very important to recognition of partners and sexual development are pheromones and pheromone precursors, as the *MF1-1*. Our target was to verify if the mating type and pheromone-related genes have an expression on a defined pattern using qRT-PCR and microscopy analysis, during the time-course of sexual cycle in relation to asexual cycle, and how this pattern can be related with ascocarp morphogenesis. Sexually compatible lines

were crossed on Oatmeal Agar at 25°C under continuous fluorescent white light for seven days and then transferred to 18°C under continuous black light (near-UV). Samples for qRT-PCR and microscopic analyses were harvested weekly. At least three phases in ascocarp development were identified: the initial hyphae folding to form proto-perithecia at the 14th day; proto-peritecia at the 21st day; and mature perithecia at 28th day. The gene expression at the 7th day for every mating type transcripts was higher on couples in contrast with single strains, showing the recognition influence on the *MAT* genes expression. The higher expression was from *MAT1-2-1*, with a level around 19 fold increase, followed by *MAT1-2-2*, with 7 fold, *MAT1-1-1* and *MAT1-1-2*, with around 4 fold, and *MAT1-1-3*, with 2 fold increase in comparison with the single strains treatment. We observed a very strong increase on the couples at the 14th day, compared with the singles, in the mating type transcripts and *MGG07733* gene, supposed to be a pheromone, showing an influence of different factors on expression, as partner, light, temperature, and age. The higher expression was from *MAT1-2-1*, with around 1000 fold than the single, following the *MAT1-1-1* and *MAT1-1-3*, with 200, *MAT1-2-2*, 100, *MGG07733* gene, 75, and *MAT1-1-2*, 2 fold. This increase was followed by a decrease at 21st and 28th days for all mating type genes studied, generally without significant difference in expression among couples and singles. The *MF1-1* gene, had no difference in time-course among sexual and asexual conditions, probably due the strain Mat1-1 used to be female-sterile.

Keywords: qRT-PCR, perithecia, rice blast, heterothallic, idiomorphs.

1. Introduction

Pyricularia oryzae is the causal agent of blast disease on rice, wheat and on other grasses, causing serious damages to the crops around the world. The fungus is hemibiotrophic and causes symptoms in all part of plants, as the elliptic spots in leaves (TeBeest et al. 2014) and spikes bleaching (Maciel et al. 2013). *Pyricularia* spp. were named on its sexual phase as *Magnaporthe* spp. (Couch and Kohn 2002; Hebert 1971) for many years, however, was resolved recently the use of its asexual name only (Klaubauf et al. 2014; Tosa and Chuma, 2014).

The high fungus genetic variation is considered responsible for the lack of durable resistance on either rice and wheat cultivars (Prabhu et al. 2006). Sexual recombination is the most probable source of genetic variation on the fungus (Saleh et al., 2014; Maciel et al., 2014).

The sexual reproduction of the heterothallic *P. oryzae* is regulated by mating type genes, which are organized in two idiomorphs (Mat1-1 and Mat1-2), and require two compatible strains from opposite mating types (Mat1-1 and Mat1-2), and at least one fertile female and one fertile male from the opposite mating type.

The locus where the mating type genes is located is Mat1, at the chromosome 3. Its idiomorphs Mat1-1 codifies the transcripts *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3*, and the idiomorph Mat1-2 codifies the transcripts *MAT1-2-1* and *MAT1-2-2*. Turgeon (1998) characterized the *MAT1-1-1* and *MAT1-1-2* from idiomorph Mat1-1, and the *MAT1-2-1* from idiomorph Mat1-2. Later, the genes *MAT1-1-3* and *MAT1-2-2* was described by Kanamori et al. (2007). These genes have respectively, the

MAT1-1-3A and *MAT1-1-3B*, and *MAT1-2-2A* and *MAT1-2-2B* ORFs, determined by alternative *splicing*. The deduced protein from *MAT1-1-1* include a *α*-box motif, conserved as a Mat1-1 product in many Ascomycota (Turgeon, 1998). Evidences support that the α protein is a transcription factor that bind to DNA, which is required for transcription mating type-specific pheromones and pheromones receptors (Johnson, 1995). The correspondent gene in *Saccharomyces cerevisae* is *MATα1P*, which is an essential transcriptional co-activator for gene expression of mating type-specific pheromones as well as pheromone receptor (Johnson, 1995; Tsong et al., 2007). The deduced protein from *MAT1-2-1* contains the HMG-box DNA binding, known as a DNA-binding sequence within non-histones chromosomal proteins and transcription factors (Debuchy and Turgeon, 2006), which is conserved in Mat1-2 idiomorph of many Ascomycota, such as *Fusarium oxysporum* and *Alternaria alternata* (Arie et al., 2000). Lee et al. (2006a) observed that a locus Mat1-2 deletion in *Fusarium graminearum* induced a significant reduction of numerous genes directly or indirectly related to the sexual cycle. The genes *Mat1-1-3a* and *Mat1-2-2a* also codifies to HMG domain (Kanamori et al., 2007). Studies with GFP marked *SMR1* gene in *Podospora anserine*, correspondent to *Mat1-1-2* in *P. oryzae*, localized its product in the cytosol, but the function remained unclear (Coppin et al., 2005). In *Sordaria macrospora*, the *SmtA-2* (*Mat1-1-2*) was considered essential to sexual reproduction, characterized by a PPF domain, whose proteins have highly conserved domains with proline and phenyalalanine (Klix et al., 2010).

The importance of MAT loci in sexual reproduction regulation appears to be different in different fungi (Coppin et al., 1993). The combined knockouts of *MatA-1* (*Mat1-1-1*) and *MatA-3* (*Mat1-1-3*) in *Neurospora crassa* resulted in a strong decrease in fertility, without any effect on fruit body development, and vegetative heterokaryon compatibility with both A and a strains (Ferreira et al., 1998). In *S. macrospora*, the *SMTA-2* (*MAT1-1-2*) knockout or the double-deletion *SMTA-2/3* (*MAT1-1-1* and *MAT1-1-3* deletions) resulted in no fruit body development and no mature perithecia produced (Klix et al., 2010). The qRT-PCR analyses in these mutants revealed that the SmtA-1 protein acted as a positive regulator of the expression of both pheromone precursor genes *PPG1* and *PPG2*, while *SMTA-2* had negative effect on the expression of *PPG2*. Similarly, the *ΔMAT1-2-1 Fusarium verticillioides* strains had a down-regulation of pheromone precursor and pheromone receptor gene expression (Keszthelyi et al., 2007). The expression of the *MAT1-1-3* gene in *P. oryzae* was higher to strains in sexual conditions, comparing with a asexual strain (Kanamori et al., 2007).

Shen et al. (1999) identified the *MF1-1* and *MF2-1* genes as the pheromone precursors in *P. oryzae*. The *MF1-1* gene codifies for a polypeptide with 26 amino acids, ending in CAAX motifs, found in pheromone genes a factor from *S. cerevisae*. The *MF2-1* gene contains potential sites of protease Kex2, repetitive sequences in N-terminal region, like the ones identified in *S. cerevisae* and *Cryphonectria parasitica*. The *MF1-1* has high similarity in nucleotide sequence compared with the gene found in *C. parasitica*, which was observed to be

essential for fertility in strains used as spermatia (male), besides affecting another characteristics, such as vegetative growth and conidia production (Turina et al., 2003). However, their specific functions remain unclear. The expression of pheromone precursors of *N. crassa* showed definite pattern in time, under constant environmental conditions, suggesting that they follow a 'molecular clock' (Bobrowicz et al., 2002).

Many studies about mating type and pheromone genes were conducted for other Ascomycota. These studies used gene knock-out techniques, and further observations of cellular, morphological and biological activities were common to evaluate the most probable functions of these genes. However, very little is known about the dynamics of mating type and pheromone gene expression in *P. oryzae* during the sexual cycle. Thus, the objective of this work was to verify if the mating type and pheromone genes have a definite expression pattern during the course of sexual reproduction in comparison with the expression on asexual cycle, and how this pattern is related with specific steps of the sexual cycle. For this study we used both real-time qRT-PCR and microscopy analysis.

2. Material and Methods

Pyricularia oryzae isolates and Crossings

The compatible lines 70-15 (Mat1-1) from rice and 40-91 (Mat1-2) from barley, were used in all assays. The crosses were made following the protocol from Itoi et al. (1983), with modifications. Agar plugs of these

two isolates were placed approximately four cm apart on a 90 mm diameter Petri dishes containing Oatmeal agar (oatmeal 50 g/L, agar 20 g/L). The crosses were kept at 25 °C for seven days under continuous fluorescent white light until the plates were then transferred to 18 °C under continuous fluorescent white and near-UV light, for 21 days. The samples were harvested weekly, and three assays were made, for bright field microscopic, confocal laser microscopy, and qRT-PCR analysis.

Dissecting microscopy

Bright field images were taken with a Zeiss M2BIO Stereoscopic Dissecting microscope housed at the BioImaging Center in the Delaware Biotechnology Institute (bioimaging.dbi.udel.edu), and the image acquisition was made using the AxioVision software. Images of the crossings between the 70-15 and 40-91 strains were obtained weekly, until the 28th day. The images were edited by Image J and CorelDraw softwares.

Confocal microscopy

Confocal images were taken with the Zeiss 5 Live DUO Highspeed Spectral confocal microscope (LSM 5 DUO) housed at BioImaging Center in the Delaware Biotechnology Institute (bioimaging.dbi.udel.edu). Plugs of the fungal cultures at the joint of the 70-15 and 40-91 strains were sampled weekly, until the 28th day. The

samples were fixed with 1x Phosphate-buffered saline (PBS) with paraformaldehyde 4% for 30 min, followed by a wash with PBS for 2 min (three times). The samples were then treated with Calcofluor White 10 µL/mL for 10 min, and washed for three times with PBS. The microscopic observation were made at 405 nm emission laser line, and a 25 x water immersion objective was immersed in to the PBS suspension with the treated plugs. The Zen 2009 software was used for image acquisition, and the images were edited by Image J and CorelDraw softwares.

RNA extraction

Total RNA extraction was performed using Trizol reagent (Sigma Chemical, St. Louis, MO) following manufacturer's instructions. Plugs of the fungal cultures at the joint of the 70-15 and 40-91 strains were sampled weekly, until the 28th day, as well as plugs from edges of single colonies, from 70-15 and from 40-91 strains. The mycelia sampled was immediately ground to a fine powder in liquid nitrogen, placed in Trizol, and the pellet obtained was re-suspended in 15 µL of autoclaved nuclease free water (Qiagen Sciences, Valencia, CA). RNA was extracted from two replicates in time, which each replicate consisting of three repetitions. Isolated RNA was analyzed in integrity and concentration was assessed by using 1 % agarose gel and the ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), respectively.

Real time qRT-PCR

cDNA was obtained by reverse transcription reaction using the total RNA extracted and the QuantiTect-Reverse-Transcription kit (Qiagen Sciences, Valencia, CA). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using Real Master Mix SYBR ROX 2.5 x (5 PRIME, Gaithersburg, MD) for SYBR Green fluorescence detection on a Realplex2 Mastercycler (Eppendorf, Westbury, NY), housed at Molecular Plant-Pathogen Interactions lab in University of Delaware. All qRT-PCR primers were tested with RT-PCR before their use. The qRT-PCR reactions were performed in a final volume of 20 µL containing 10 µL of 2.5 x Master Mix, 0.06 µL of 100 µM of each forward and reverse primers, and 1 µL of cDNA. The reactions occurred at 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 3 min, and 72 °C for 10 min. After real-time PCR, specificity of the amplicons was checked by melting curve analysis at 95 °C for 15 sec, 60 °C 15 sec, 20 min, 95 °C for 15 sec. Relative expression levels of the genes *MAT1-1-1*, *MAT1-1-2* and *MAT1-1-3* were estimated from the junction area (both isolate's tissue) using the expression levels from the 70-15 single culture (Mat1-1) as a calibrator. For *MAT1-2-1* and *MAT1-2-2* genes, the levels from the 40-91 single culture (Mat1-2) was the calibrator. The following pheromone related genes studied were: *MGG07733* (XM_003712891.1), the 'clock controlled pheromone ccg-4', in the 70-15 strain; and *MGG07736* (XM_003712895), a hypothetical protein, in the 70-15 strain, described as mating type 1 pheromone precursor *MF1-1* in *P. oryzae* (Shen et al., 1999). The calibrator to

MGG07733 and *MGG07736* genes was the 70-15 strain (Mat1-1). Relative expression levels were determined by the $\Delta\Delta C_t$ method using glycerol-3phosphate dehydrogenase (GAPDH; MGG_01084.6) as the endogenous control. All qPCR reactions were performed at least twice yielding similar results. The sequences of qPCR primers are listed in Table 1.

Table 1: Primers used in relative expression analysis by cDNA amplification

Primer	Sequence (5'-3')	Genes	bp number	References
B156	GCAGGCAACTCGCAGGAATC	*MAT1-1-1*	19	GenBank: AB080670.2 (Kanamori et al., 2007)
111F2	CAGAGCAAATGACGAGAAAGAGCG	*MAT1-1-1*	23	
B199	TAGGATGACTGTGCTCTC	*MAT1-1-2*	17	
112R2	TTTACACCGAGCCCGATG	*MAT1-1-2*	17	
B205	TACGAGAACAGCATAGTGG	*MAT1-1-3*	18	
B208	GCGGTTTGGAGGCTTGGAA	*MAT1-1-3*	18	
A201	CTGCGAATGCCTACATCCTG	*MAT 1-2-1*	19	GenBank: AB080671.2 (Kanamori et al., 2007)
A202	GCCGACGAGGAGAGTAGCGA	*MAT 1-2-1*	19	
A24	CTCGAAATACCCTCTCAAT	*MAT 1-2-2*	18	
B208	GCGGTTTGGAGGCTTGGAA	*MAT 1-2-2*	18	
7733F*	CTACCTCGACCGCTACTTTGAC	clock-controlled pheromone ccg-4 (*MGG07733*)	22	GenBank: XM_003712891.1 (Ma et al., 2012, Unpublished) identical to CM001233 (Dean et al., 2005)
7733R*	AGAGTTGCAGTGGCGCTTGT		20	
7736F*	CAGGTCCCAAACGACTATTT	hypothetical protein similar to pheromone precursor Mf1-1 (*MGG07736*)	20	GenBank: XM_003712895 (Ma et al., 2012, Unpublished) identical to CM001233 (Dean et al., 2005); (Shen et al., 1999)
7736R*	GATTCTAGGCTTGGGTGAAG		20	
HKGF*	CCTCGTCCATCTTCGACGC	GAPDH (*MGG01084.6*)	19	GenBank: XM_003717805.1
HKGR*	GACACGACGGCTGTAGCC		19	

*Primers designed by IDT Primer Designer Tool (https://www.idtdna.com/primerquest/Home/Index).

3. Results

Through microscopic analysis we observed the morphology and the differentiation of fungal tissues during the sexual cycle. At day 7, we observed aerial mycelium growth at the jointing colonies (Fig. 1A and 1B), and the conidiation in course (Fig. 1B, arrows). The beginning of proto-perithecia development was observed at day 14 (Fig. 1C), following the presence of proto-perithecia at the day 21 (Fig. 1D and 1E), and finally, the formation of mature perithecia at the day 28 (Fig. 1F and 1G), with long beaks (Fig. 1H and 1I), while had a low fertility, with rare ascospore formation (not showed). The *in vitro* perithecia development had a typical localization, widespread at the joint region of the paired colonies, at the center of plates (Fig. 1F), on the 40-91 strain side (Fig. 1F, arrow), therefore, considered a female strain. In contrast, the 70-15 strain has not developed perithecia, and thus considered female sterile. The proto-perithecia was initially hyaline (Fig. 1D) and subglobose shaped (Fig. 1D and 1E), with 60.62 (34.31-95.20) µm diameter. Between the 21st and 28th day, we observed the melanization of the fruiting body, and the presence of mature perithecia with a dark brown to dark color (Fig. 1F). Many times, was wrapped with white mycelia (Fig. 1G), at the surface media or frequently found immersed on media, with long and hyaline emerged beaks (Fig. 1H and 1I). The mature perithecia had 222 (148.8-274.3) µm length and 204.7 (132.3-264.2) µm width. The beaks had variable sizes (Fig. 1G and 1H). The short beaks (Fig. 1G, arrow) were around, in average, 35 µm length and 25 µm width. The medium size beaks were 73.1 (45.5-130.5) µm length and 32.7 (20.4-68.1) µm

width (Fig. 1H). In contrast, the larger beaks (Fig. 1I) were 379.1 (331.6-442.6) µm length and 92.2 (61.2-131.1) µm width. At the day 28 new proto-perithecia in formation were detected (Fig. 1I, arrows).

The gene expression measured by qRT-PCR at the day 7 for every mating type transcripts studied was higher in sexually compatible pairings in comparison with the single strains (Fig. 2 and 3). The higher expression was from *MAT1-2-1*, with a level around 19 fold increase (Fig. 3A), followed by *MAT1-2-2*, with 7 fold, *MAT1-1-1* and *MAT1-1-2*, with around 4 fold, and *MAT1-1-3*, with 2 fold increase in comparison with the single strains treatment (Fig. 3B and Fig. 2A, 2B and 2C). We observed a much higher gene expression increase in the couple at the day 14, compared with the singles, in the mating type transcripts (Fig. 2 and 3). The higher expression was from *MAT1-2-1*, with a level around 1000 fold than the single strains, followed the *MAT1-1-1* and *MAT1-1-3*, with around 200, *MAT1-2-2*, with 100, and *MAT1-1-2*, with 2 fold. A general decrease in gene expression was observed at day 21 and day 28 for all mating type genes studied, generally without significant difference in expression between the strains pairing and the singles.

Similarly, the relative expression in the 'clock-controlled pheromone ccg-4' (*MGG07733*, or '*7733*') and in the 'Pheromone precursor *MF1-1*' (*MGG07736*, or '*7736*') had an increase on the couples between the 7th and 14th days (Fig. 4A and 4B). The *7733* gene had an increase on expression in relation to single strain, with 75 fold (Fig. 4A), and the *7736* gene, 1.11 fold, not significant (Fig. 4B). A non-expected expression in singles was slightly higher than in couples in the *7733* gene

at the 7th, 21st and 28th days (Fig. 4A), and about the *7736* gene, apparently, the expression among singles and couples was not different between 7th and 28th days (Fig. 4B).

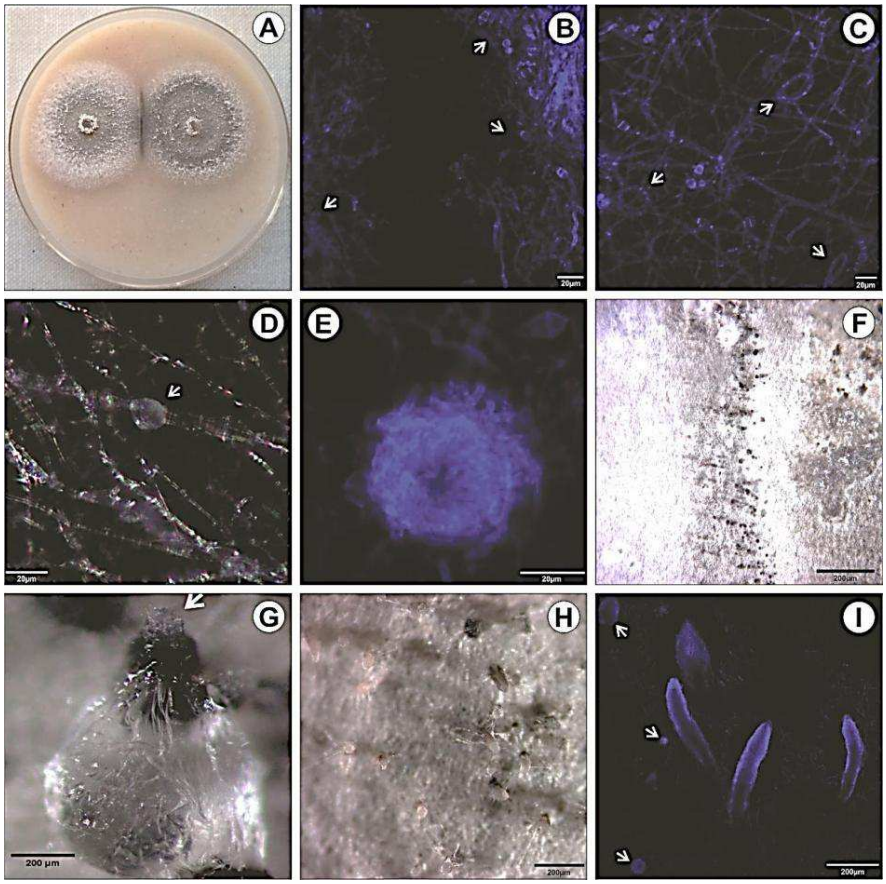

Fig. 1: Sexual Reproduction Time-course of *Pyricularia oryzae* 70-15 vs. 40-91 strains at the 7th day, with jointing colonies (A and B) and conidia production (arrows) (B); 14th day, when hyphae were folding to form proto-perithecia (B); 21st day, with proto-perithecia formation (D and E), initially hyaline (D, arrow); and 28th day, with dark mature perithecia developed at the center of Plate (F), at the media surface, short beaked and wrapped by aerial mycelia (G, arrow), or long-beaked and immersed on media (H and I), besides presenting new proto-perithecia (I, arrows). The B, C, E and I images was acquired by Confocal laser scanning microscope, where the cell walls were stained with Calcofluor White. The D, F, G and H images were acquired by Bright field Stereoscopic microscope.

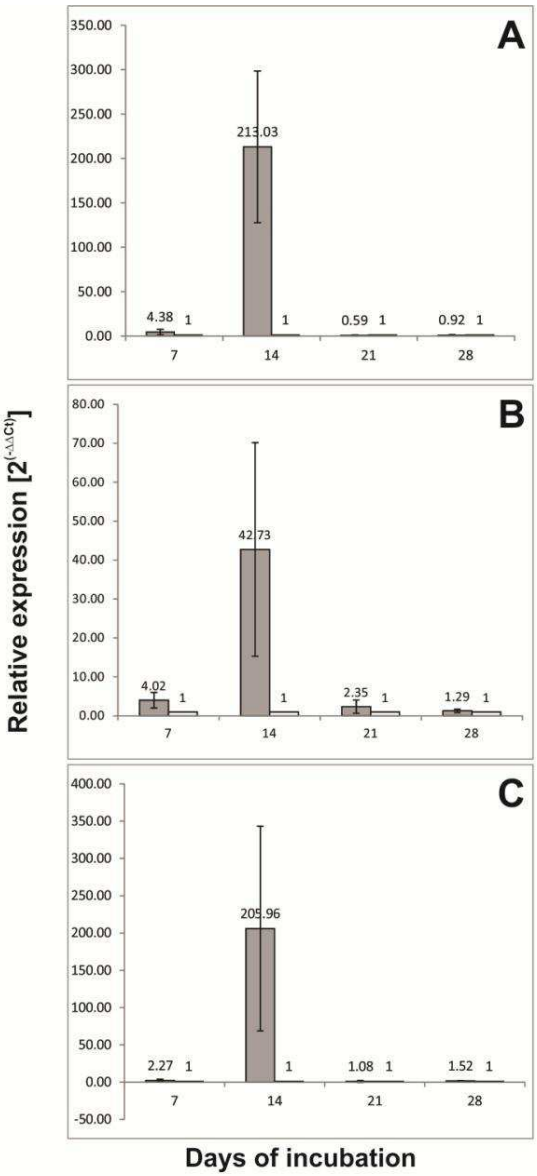

Fig. 2: Relative expression of *MAT1-1-1* (A), *MAT1-1-2* (B) and *MAT1-1-3* (C) in couples (grey bars), comparing with the single Mat1-1 strain (white bars) of *Pyricularia oryzae*, in time. Each data point represents the mean values, and the vertical lines indicates the standard deviations.

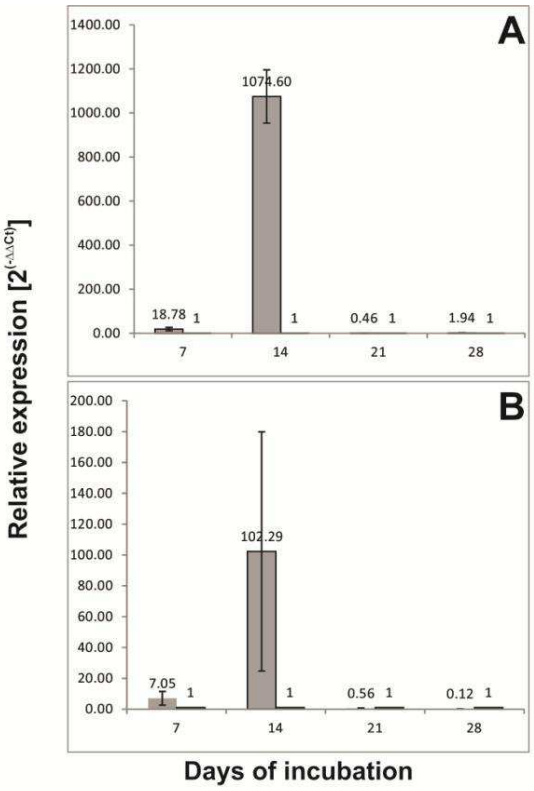

Fig. 3: Relative expression of *MAT1-2-1* (A) and *MAT1-2-1* (B) in couples (grey bars), comparing with the single Mat1-2 strain (white bars) of *Pyricularia oryzae* in time. Each data point represents the mean values, and the vertical lines indicates the standard deviations.

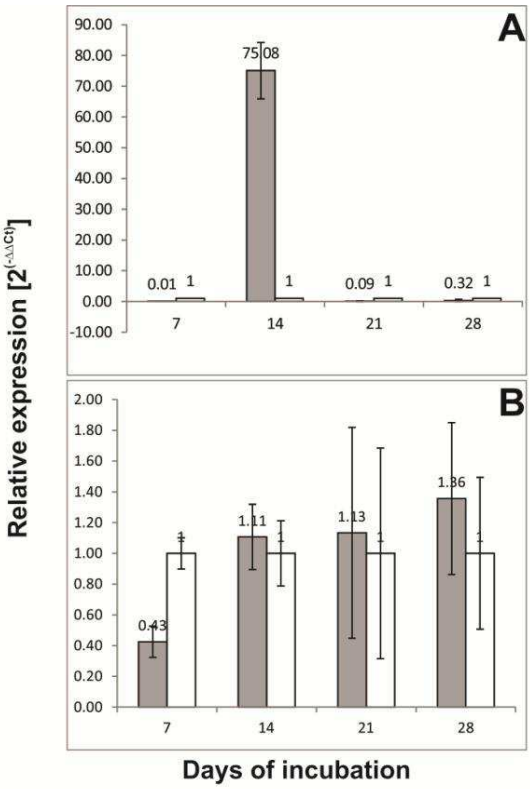

Fig. 4: Relative expression of 'Clock-controlled pheromone ccg-4' (*MGG07733*) (A) and 'Pheromone precursor *MF1-1*' (*MGG07736*) (B) in couples (grey bars), comparing with the single Mat1-1 strain (white bars) of *Pyricularia oryzae* in time. Each data point represents the mean values, and the vertical lines indicates the standard deviations.

4. Discussion

The time-course observations of sexual reproduction of *Pyricularia oryzae* following an initial incubation at 25 ºC under fluorescent white light for seven days and 21 days at 18 ºC and fluorescent white and near-UV lights permited us identify at least three phases in ascocarp development: the initial hyphae folding to form proto-perithecia at the

14th day (Fig. 1C), proto-perithecia at the 21st day (Fig. 1D and 1E), and mature perithecia at 28th day (Fig. 1F, 1G, 1H and 1I). We identified some of the stages of proto-perithecia morphogenesis described by Lichius et al. (2012) for *Neurospora crassa*, using scanning electron microscopy. The authors identified the stages of ascogonial helix formation (hyphae folding), proto-perithecia expansion, and trichogyne emergency. This last stage was not observed in our study.

At the day 7, we observed that the relative expression of *MAT* genes were higher in sexual conditions (Fig. 2 and 3), showing the influence of compatible strain. It was observed previously with the *MAT1-1-3* gene in *P. oryzae*, when strains in sexual conditions had higher expression in comparison with asexual strain (Kanamori et al., 2007). Concerning the increasing of relative expression of *MAT* genes between the 7th and 14th days (Fig. 2 and 3), it was probably a response of the few distinct factors combined, such as the presence of the partner, the changes in temperature and light, as well as the stage of development. The influence of the compatible partner was observed since the 7th day, but became stronger at the 14th day, once the couples had higher expression patterns in contrast with singles. These genes can regulate the mating type-specific genes related to the recognition mechanisms and other genes related with the sexual cycle, included pheromones and pheromone receptors (Johnson, 1995; Tsong et al., 2007). Apparently, the increase of expression until the 14th day was favored by the recognition between the strains by the induction of the differentiation of sexual tissues, later this high expression pattern was no longer needed. The knock-outs of *MAT1-1-1* and *MAT1-1-*

3 correspondents in *Neurospora crassa* results in a strong decrease in fertility, without effect on fruiting body development (Ferreira et al., 1998). However, in *Sordaria macrospora*, the *MAT1-1-2* correspondent knocked-out or the double-deletion of the *MAT1-1-1* and *MAT1-1-3* compromised the fruiting body development (Klix et al., 2010), and it down-regulated the pheromone precursor genes expression. At the same way, the *ΔMAT1-2-1 Fusarium verticillioides* strains had a down-regulation on pheromone precursor and pheromone receptor gene expression (Keszthelyi et al., 2007), showing once again the interdependence of *MAT* and pheromone genes during the sexual morphogenesis in Ascomycota. Thus, we can hypothesize that this kind of interdependence among the *MAT* and the pheromone genes expression in *P. oryzae* are possible.

At the 14th day, with changes of temperature, from 25 to 18ºC, and light quality, from white light to near-UV and white lights combined, a great increase of expression in couples was observed, showing that the environment conditions had an important contribution on these increase, and associated with the stage of development. In this case, are need of more tests to prove the independent influence of temperature and light in *MAT* genes. Since the first studies with the teliomorph induction of *P. oryzae*, different combinations of temperature, light and media are used, and varies according the strains origin and, obviously, the lab resources. Yaegashi and Hebert (1976) demonstrate the importance of light and temperature in perithecia production of *P. oryzae*. The higher amount of perithecia was observed about 20ºC for the crabgrass isolates and 22 to

25ºC for the goograss isolates. Moreover, they observed that the light was necessary for consistent perithecial production, and that short wavelengths (below 500nm, near-UV) were most effective. *Pyricularia oryzae* showed to be sensible to different light intensity, quality and photoperiod on your asexual spore-release kinetics, regulated by a blue-light receptor *MGWC-1* (Kim et al., 2011; Lee et al., 2006b), and perhaps this may happen in sexual cycle.

On the Mat1-1 strain, all *MAT* genes were expressed at the same pattern in time-course observed in Mat1-2 transcripts on its respective strain, which had normal ascocarp differentiation. Was not possible to evaluate the pheromone genes related to Mat1-2 strain, but is possible that the low expression of *7733* and *7736* genes in Mat1-1 strain (Fig. 4) influenced the fertility on the Mat1-2 strain, which was very low, maybe due to failures in molecular recognition mechanisms.

The isolate Mat1-1 used (the 70-15) was considered sterile female. Thus, the low expression observed for the *7733* and the *7736* genes in relation to the singles is possibly related to the sterility, once the pheromones are essential to ascocarp development in some species of Ascomycota, and essential to fertility in another ones. The *7736* gene, or *MF1-1*, was essential to fertility in strains used as spermatia (male) in *Cryphonectria parasitica* (Turina et al., 2003). On the other hand, is possible that the levels of expression among strains in asexual conditions are really close comparing with the strains in sexual, even if they are fertile.

About the expression of *MAT* genes during the vegetative condition, used as calibrator, we can't analyze details in time-course, in this case, but are possible to observe that its expression occurs along the time, lower than in sexual conditions initially, and at the same level in 21st and 28th days. Paoletti et al. (2007) observed the expression of *MAT* genes and pheromone-response pathway genes of *Aspergillus nidulans* in sexual conditions, and not in asexual condition, during a time-course RT-PCR analyzes. However, Wang et al. (2012) observed the expression of pheromone and receptor genes, and the increase of *MAT* genes during the asexual development of *Neurospora crassa*, by qRT-PCR analyses. Maybe it was occurred at the 21st and 28th day, the difference among singles and couples was almost zero. Wang et al. (2012) studies with *N. crassa* revealed expression of pheromone and receptor genes in strains of both mating types in all developmental stages, and revealed that the mating type genes were increasingly expressed over the course of asexual development. The transcripts from Mat A (correspondent to Mat1-1) exhibited a higher expression level than Mat a.

Knockout experiments with *MAT* genes, pheromone and pheromone precursor genes of *P. oryzae* are necessary to observe its importance in ascocarp development and fertility, as well in other biological functions. Moreover, more studies about sexual reproduction of *P. oryzae* are required to better understanding the influence of environment factors, as light, temperature and nutritional sources, on gene expression and on morphological levels.

More studies with the *MGG07733* and *MGG07736* genes in *P. oryzae* are needed to confirm the putative pheromone or pheromone precursor functions.

Our data could help the guidance of further studies on morphogenesis and gene expression during sexual reproduction of *Pyricularia oryzae*.

5. Conclusions

We identified three phases in ascocarp development of *Pyricularia oryzae*: initial hyphae folding, proto-peritecia, and mature perithecia.

The *MAT* genes expression in *P. oryzae* was influenced by the presence of the partner.

All *MAT* genes in *P. oryzae* had the same pattern of expression during the entire sexual reproduction process, with higher expression at the 14th day of incubation, during the hyphae folding to form the proto-perithecia.

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ARTIGO 2 Perithecia development of *Pyricularia oryzae* from wheat by different methods and in different plants

Journal of Plant Pathology – Artigo para ser submetido

PERITHECIA DEVELOPMENT OF PYRICULARIA ORYZAE FROM WHEAT BY DIFFERENT METHODS AND IN DIFFERENT PLANTS

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SUMMARY

Pyricularia oryzae causes blast disease, the most important disease of rice in the world, and causes wheat blast in Brazil, Paraguay and Bolivia, thus making important to disease quarantine. Sexual reproduction is probably the main source of its high genetic-pathotypic variability, responsible for the low durability in blast resistance in rice and wheat cultivars, and

maybe for the pathogen ‘host shift’ event. Much evidence indicates the possibility of *P. oryzae* sexual reproduction in nature, and our objective was study the morphology, measure the size and the density of perithecia of the wheat-infecting *P. oryzae* isolates occurs on distinct living plants species, on different types of plant debris, and on culture media, using light and scanning electron microscopy. The *P. oryzae* perithecia vary in size according to the state of the plant, host species and culture medium; the recognition of compatible isolates can occur on the outside or inside plant tissues; and necrotic tissues favor the developing of ascocarps.

Keywords: Blast disease. Life cycle. Sexual reproduction. Microscopy.

INTRODUCTION

Blast disease is caused by *Pyricularia oryzae* Cavara and is the most important rice disease worldwide (Wang *et al.*, 2014) and an emerging disease on wheat in South America (Duveiller *et al.*, 2010). The wheat blast disease has emerged firstly in southern Brazil in 1985 (Igarashi *et al.*, 1986). Currently, its range has expanded to Paraguay and Bolivia and blast disease has become an important quarantine disease for

major wheat-producing countries such as the United States and the European Union countries (Duveiller *et al.*, 2010).

Pyricularia oryzae is a hemibiotrophic fungus that can cause symptoms such as elliptic spots on leaves (TeBeest *et al.*, 2014) and bleaching on spikes (Maciel *et al.*, 2013). Historically, *Pyricularia* spp. had been associated with *Magnaporthe* spp. as its sexual stage (Couch and Kohn, 2002; Hebert, 1971). However, very recent phylogenetic studies following a modern taxonomic classification code has proposed a simple name for the genus *Pyricularia* for both the asexual and sexual stages (Klaubauf *et al.*, 2014; Murata *et al.*, 2014; Tosa and Chuma, 2014).

Because *Pyricularia* is a heterothallic fungus (self-compatible), the occurrence of its sexual phase is only possible when mating occurs between two individuals from compatible mating types (Mat1-1 and Mat1-2) and fertile. Population studies outside the origin centers have shown the predominance of only one mating type or the infertility, when the both are present. Perhaps this explains why the sexual stage of the rice-adapted *P. oryzae* pathogen has not been observed in agroecosystems outside the center of origin in Southern Asia (Saleh *et al.*, 2014). Thus,

the reports for both mating types and fertile-female were only for populations from the rice origin centers, as India (Kumar *et al.*, 1999; Dayakar *et al.*, 2000; Rathour *et al.*, 2004), Bangladesh (Shahjahan, 1994), and Southeast Asia (Saleh *et al.*, 2012; 2014).

In Brazil, the story is not different for the rice pathogen, and strains were classified by Urashima *et al.* (1993) as non-fertile and were not compatible with lines from other hosts. However, the fungus populations pathogenic to wheat are considered sexed, running fully for the rice *P. oryzae* paradigm established in the world. Wheat strain populations showed both mating types (Bruno and Urashima, 2001; Maciel *et al*., 2014; Urashima *et al.*, 1993), and were sexually compatible with strains from wheat, *Brachiaria plantaginea*, goosegrass (*Eleusine indica*) and finger-millet (*Setaria italica*) (Urashima *et al.*, 1993), and rescuegrass (*Bromus catharticus*), bird seed (*Phalaris canariensis*) and triticale (*X triticosecale*) (Galbieri and Urashima, 2008). In the fertility evaluation with testers, only crosses between wheat and *Brachiaria plantaginea* strains produced perithecia with asci and ascospores (Bruno and Urashima, 2001). In these populations, coming from Mato Grosso do Sul, Paraná and São Paulo States, there was a predominance of Mat1-1

idiomorph. However, Maciel *et al.* (2014) found wheat populations in different distributions for Mat1-1 and Mat1-2, in the Midwest (4:1), Triangulo Mineiro (30:1), São Paulo (1:0) and Paraná (15:1), respectively. After pathogen populational structure analysis, on the basis of eleven microsatellites and pathogenicity tests, these authors proposed that populations of *P. oryzae* have a mixed reproduction system, where the sexual cycle is followed by clonal dispersion. The fostering of *P. oryzae* clonal dispersion by selection of Mat1-1 lines with the strobilurin resistance gene was recently described for populations from the Distrito Federal, and Goiás, Minas Gerais, Mato Grosso do Sul, Paraná, Rio Grande do Sul and São Paulo States (Castroagudín *et al.*, 2014).

Often studies on the sexual stage of *P. oryzae* are conducted through *in vitro* assays from their first reports (Webster, 1965; Hebert, 1971) to the present day (Saleh *et al.*, 2014). However, Silue and Notteghem (1990), and Hayashi *et al.* (1997) observed perithecial development in leaf sheaths of rice by injection of conidial suspension with both compatible strains mixed, using rice strains. The perithecia were observed in senescent detached sheaths, maintained in a moistened chamber (Hayashi *et al.*, 1997). The development of ascocarps in

senescent tissues can lead to the hypothesis that the *P. oryzae* teliomorph phase occurs during the necrotrophic phase, probably in crop residues. However, this question has not yet been elucidated, and it is a potentially fundamental point in terms of blast disease management.

The objective of this study was to study the morphology, measure the size and the density of perithecia of the wheat-infecting *P. oryzae* on distinct living plants species, on different types of plant debris, and on culture media, by light and scanning electron microscopy.

MATERIAL AND METHODS

The follow assays were done at the Electron Microscopy and Ultrastructural Analysis Lab in the Federal University of Lavras.

***In vitro* induction on culture medium.** Isolates 50-38, 33-1, 50-07, 22-2 (Mat1-1) and isolates 50-46, 30-2, 50-03, and 50-45 (Mat1-2) from wheat (Maciel *et al.*, 2014) were paired to form couples of Mat1-1 and with Mat1-2.

The *in vitro* pairings were done according to the Itoi and collaborators protocol, with modifications (Itoi *et al.*, 1983): plugs of compatible isolates were placed approximately 4 cm apart in 90 mm Petri dishes containing oatmeal agar (OA, oatmeal, 50g/L; agar, 20g/L). The pairings were kept at 25^{0}C under fluorescent white light with 12 h photoperiod, for 7 days. Later on, plates were transferred to 20^{0}C under fluorescent white light with 12 h photoperiod, for another 21 days incubation for perithecial development. This assay was replicated once with two repeats of each experimental unit each time.

The combination of 33-1 (Mat1-1) with 50-46 (Mat1-2) produced the highest number of perithecia and were chosen for the further assays using living plant material or plant debris.

***In vitro* induction on autoclaved wheat stems using fungal mycelia.** Pieces of stems from healthy wheat plants (*Triticum aestivum*) cv. MGS Brilhante, contained in 700 mL pots with substrate, grown for one month at 25 ºC in a growth chamber, were collected and autoclaved at 121 ºC for 20 min. The pieces were then placed at the center of a 90 mm Petri dish containing OA and plugs of mycelia from the compatible

strains 33-1 and 50-46 placed at the opposite sides. This assay was replicated once with five repeats of the experimental unit each time.

Incubation for perithecial development was done similarly as described in the former method.

***In vitro* induction with injection of spore suspension in autoclaved wheat stems.** Pieces of stems from healthy wheat plants were obtained and autoclaved as described above. Three stem pieces were placed in 90 mm Petri dishes with water-agar 1.5 %. The pieces were inoculated by injection of 0.3 mL of a mixed spore suspensions at 10^4 conidia/mL from the strains (33-1 and 50-46), which were grown separately to sporulation on OA at 25 ^{0}C under fluorescent white light at 12 h photoperiod for 14 days (Hayashi *et al.*, 1997, modified).

These plates were incubated as in the first method described. This assay was replicated once with five repeats of the experimental unit each time.

***In vitro* induction within living stems from different plant species.** *P. oryzae* 33-1 and 50-46 strains were grown separately to sporulation on OA as described above. Later on, 10^4 conidia/mL

suspensions from each strain were mixed. The co-inoculation was made by injection of 0.3 mL suspension in living stems of 1-month-old healthy standing plants planted in 700 mL pots with substrate and grown at 25 ºC in a growth chamber. The plant species used were: Barley (*Hordeum vulgare*) cvs. BR Elis and MN 743; Bird seed (*Phalaris canariensis*); Black Oat (*Avena strigosa*) cv. Embrapa 29 Garoa; Hybrid *Brachiaria* cv. Mulato; Foxtail millet (*Setaria italica*); Red rice (*Oryza sativa*) cv. Yin Lu 30; Rice (*O. sativa*) cvs. BRS Primavera and BRSMG Relampago; Rye (*Secale cereale*) cv. BR1; Triticale (x*Triticosecale*) cv. IAC Caninde; Wheat (*Triticum aestivum*) cvs. BRS 264 and MGS Brilhante. With the exception of wheat "MGS Brilhante" (classified as moderately tolerant) all the other wheat cultivars, barley and oats are considered susceptible to wheat blast (Lima and Minella, 2007; Urashima and Silva, 2011). The wheat pathogen cannot cause blast on rice cultivars (Maciel *et al.* 2014).

After the co-inoculation, the pots with transferred to a humid chamber in moistened dark plastic bags for 21 h. The pots were removed from the humid chamber and placed in a growth chamber for 15 days at 25 ºC. The stems were collected and cut in 3 cm pieces, and placed in 90 mm sterilized Petri dishes within moistened plastic bags and kept at 20 ºC

for 1 month (Hayashi *et al.*, 1997, modified). On this assay we estimated also the density of perithecia on plant surface, analyzing at least three areas of approximate 0.5 mm^2 looking for ascocarp occurrence in each plant species.

***In vivo* induction within standing rice and wheat plants.** The mixed fungal spore suspension was prepared as described earlier. The rice plants from cvs. BRS Primavera and BRSMG Relâmpago, and the wheat plants from cvs. BRS 264 and MGS Brilhante were grown similarly as described above. After co-inoculation, a humid chamber was made with moistened dark plastic bags for 21 h, followed by 15 days incubation at 25 ºC in the growth chamber. The standing rice and wheat plants were then placed at 20 ºC in a growth chamber for one month (Hayashi *et al.*, 1997, modified).

Microscopy analysis. The perithecial development was documented and measured using light and scanning electron microscopes, housed at the Electron Microscopy and Ultrastructural Analysis Lab in the Federal University of Lavras.

The bright field image acquisition and measurements were taken with a Zeiss Axio Observer Z.1 microscope using AxioVision Rel. 4.7 software, and a Nikon SMZ1500 stereoscope microscope using NIS Elements D 3.2 software. The images were edited on Corel Draw X7 software.

The scanning electron microscope images and measurements were acquired on a Zeiss LEO EVO 40 microscope using Smart SEM Zeiss software. The samples were fixed by Karnovsky solution pH 7.2 for a minimum of 24 hours, followed by transfer to 0.05 M cacodylate buffer solution for a 10 min wash, three times. The fixed samples were then transferred to 0.1% osmium tetroxide solution for 1 hour, washed in distilled water three times, and dehydrated in a graded acetone series (25, 50, 75, and 90 %, once for each grade, and three times for 100% grade). Later, the samples were treated in a Bal-Tec CPD 030 Critical point dryer, to replace the acetone by CO_2. The specimens obtained were mounted on aluminum stubs with double sided carbon tape on an aluminum foil sheet, and covered with gold by a Bal-Tec SCD 050 sputter coater (Bozzola and Russell, 1999). The SEM images were

acquired operating at 10 kV and 10 to 30 mm work distance, and edited using Corel Draw X7 software.

RESULTS

We observed the development of *Pyricularia oryzae* proto-perithecia and/ or perithecia in the induction methods evaluated using the compatible strains 33-1 and 50-46 (Figures 1 and 2), with the exception for the *in vivo* induction within standing plants. The ascocarps were in black color (not showed), and the mature perithecia generally presented long beaks. The measurements and/or density of the fruiting bodies are shown in Table 1.

The method with induction on culture medium we observed the *P. oryzae* development of proto-perithecia and perithecia spreading along the surface of the medium or immersed in it (Figure 1 A and B). The proto-perithecia were dark in color and subglobose shaped (Figure 1 B), and the mature perithecia, were black and generally presented long beaks (Figure 1 B). The beaks often came from immersed perithecia, emerging from the medium surface (Figure 1 B). Asci and ascospores were not

produced. The dimensions of proto-perithecia, perithecia and beaks are listed in Table 1.

Many proto-perithecia and perithecia were observed in the induction on autoclaved wheat stems using fungal mycelia (Figure 1 C to F). The fruiting bodies were formed superficially and immersed in media (Figure 1 E), as well as on the plant surface (Figure 1 D) and immersed in the necrotic stems, inside the internode pith cavity (Figure 1 F).

As regards method injection of spore suspension in autoclaved wheat stems, we observed the colonization of the strains from the site of injection to the internode pith cavity (Figure 1 H, arrows). The proto-perithecia (Figure 1 I) and perithecia developing were within stems (Figure 1 J), sometimes with a long beak emersion (Figure 1 K), as well as partially immersed ascocarps in internode culm (Figure 1 K and L). The ascocarps were also observed on the epidermal plant surface (Figure 1 M and N).

The black *P. oryzae* proto-perithecia and /or perithecia were observed in the internode stems of all plant species evaluated for the

induction within living stems from different plant species (Figure 2). The sizes and density are shown in Table 1.

Compared the method induction on culture medium, without plants, the dimensions of *P. oryzae* perithecia were higher than in the other methods, with plants (Table 1). In the other hand, the induction methods with culture media and autoclaved stems, we observed fruit bodies with higher dimensions than in the method with living stems from different plant species (Table 1).

For the induction within living stems from different plant species, the bird seed was the plant in which the greatest size of ascocarps observed, with around 180 µm in length and 100 µm in width, and necks with around 150 µm in length and 10 µm in width (Table 1). In this method, only for bird seed did the ascocarp of *P. oryzae* develop, reaching mature size, and in the all other plants, we observed just proto-perithecia (Table 1). The plants with the highest density of ascocarps were triticale, red rice, and wheat cv. MGS Brilhante, with around 200, 180, and 130 proto-perithecia per mm^2, respectively (Table 1).

Table 1: Different methods to induce ascocarp development of *Pyricularia oryzae*, with average values of dimensions and/or density, with respective amplitude values in parentheses.

Method	**Body*** (µm)		**Neck** (µm)		**Density (number/mm²)**
	Length	**Width**	**Length**	**Width**	
***In vitro* induction on culture medium**	552.4 (527-570.6)	582.6 (561.4-596.5)	409.6 (143-819.8)	86.9 (71.7-108)	-
***In vitro* induction on autoclaved wheat stems using fungal mycelia**	176.7 (104.2-217.7)	119 (102.6-143.6)	204.3 (125-255.7)	32.5 (21.9-52)	-
***In vitro* induction with injection of spore suspension in autoclaved wheat stems**	196.1 (91.6-482.2)	128.2 (75.2-272.7)	242.8 (83-852.4)	27.1 (15.3-73.4)	-
***In vitro* induction within living stems from different plant species:**					
Barley (*Hordeum vulgare*) cv. BR Elis	27.2 (18.3-38.5)	27.2 (18.3-38.5)	-	-	66.8 (25.1-108.9)
Barley (*Hordeum vulgare*) cv. MN 743	50.3 (31.8-77.2)	56.1 (44.3-80.2)	56.4 (17.6-95.2)	8.7 (5.8-11.5)	68.5 (65-74.1)
Bird seed (*Phalaris canariensis*)	182.3 (101.2-263.5)	111.9 (108-115.8)	150.4	9.4	47.3 (23.7-77.6)
Black Oat (*Avena strigosa*) cv. Embrapa 29 Garoa	39.4 (30.7-45.5)	40.9 (28.8-51.4)	40.3 (33-47.7)	7.4 (5.9-8.9)	24.9 (10.6-38.5)
Hybrid *Brachiaria* cv. Mulato	32.6 (10.9-66)	32.6 (10.9-66)	-	-	42 (14.5-94.7)
Foxtail millet (*Setaria italica*)	31.8 (21.1-47.3)	31.8 (21.1-47.3)	-	-	72.5 (27.6-111.3)
Red rice (*Oryza sativa*) cv. Yin Lu 30	33.2 (18.8-62.3)	33.2 (18.8-62.3)	-	-	176.6 (156.5-190.6)
Rice (*O. sativa*) cv. Primavera	55.1 (27.5-113.7)	55.1 (27.5-113.7)	-	-	56 (17.2-81.9)
Rice (*O. sativa*) cv. Relampago	34 (21.5-61.9)	42.2 (28.9-67)			18.5 (8.9-25.8)
Rye (*Secale cereale*) cv. BR1	27.9 (22.2-33.2)	25.4 (21.9-29.1)	-	-	22.8 (10.9-35.9)
Triticale (x*Triticosecale*) cv. IAC Caninde	32.8 (10.1-89.3)	32.8 (10.1-89.3)	-	-	205.4 (133.2-276.4)
Wheat (*Triticum aestivum*) cv. BRS 264	40.4 (7.17-127.17)	39 (7.17-111.79)	-	-	113.5 (109.9-115.8)
Wheat (*T. aestivum*) cv. MGS Brilhante	28.9 (13.3-69.7)	30.2 (13.3-80.2)	-	-	126 (80.5-148.1)

*Globose ascocarps had just taken the measure of its diameter once the length and width were considered equals.

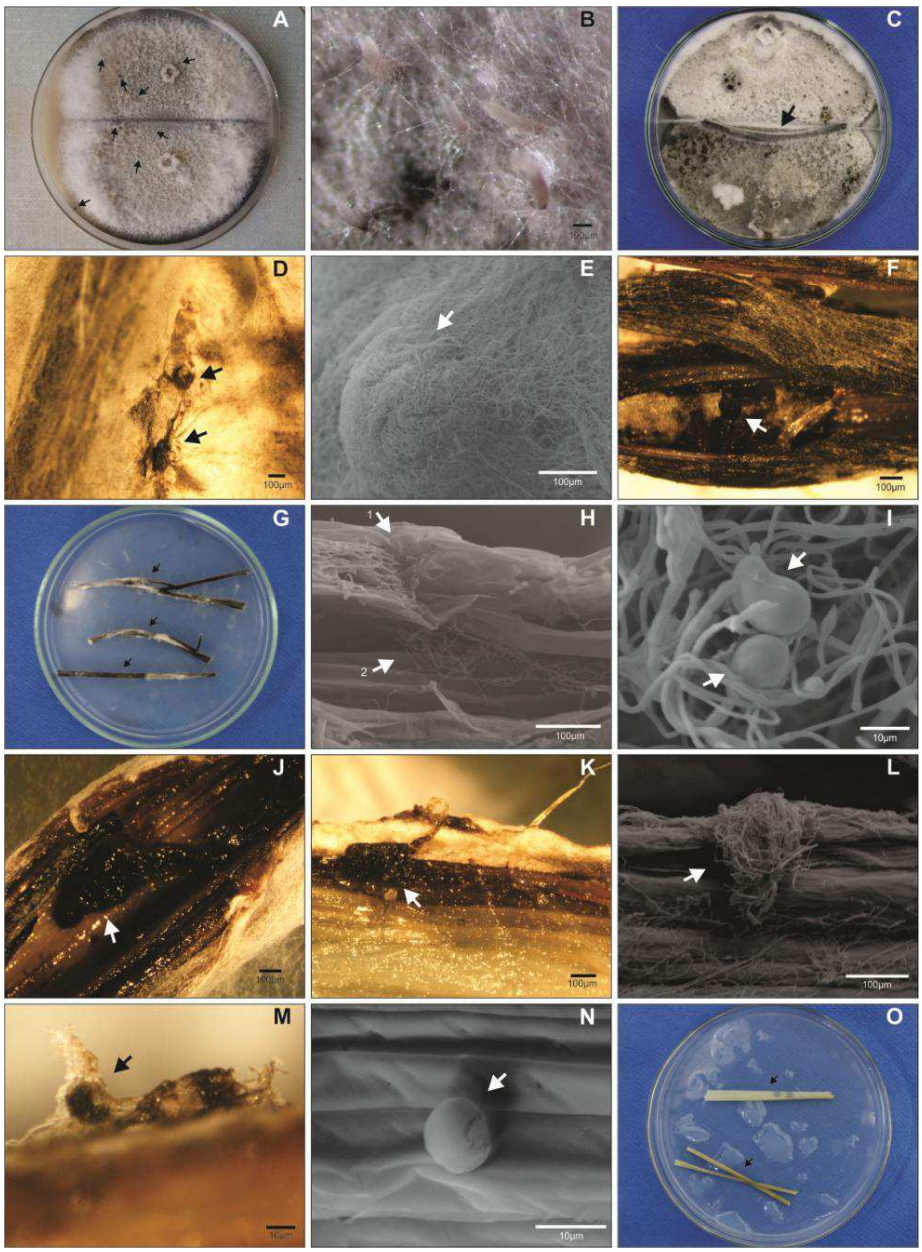

Figure 1: *In vitro* induction of the development of *Pyricularia oryzae* perithecia by different methods using Mat1-1 and Mat1-2 wheat strains: placing mycelial plugs in OA (**A and B,** induction on culture medium); placing mycelial plugs in OA in presence of autoclaved stems pieces (arrow) of wheat (*T. aestivum*) cv. MGS Brilhante (**C to F**, induction on autoclaved wheat

stems using fungal mycelia); injection of mixed conidia suspension in autoclaved stems pieces (arrow) of wheat (*T. aestivum*) cv. MGS Brilhante (**G to N,** induction with injection of spore suspension in autoclaved wheat stems). ***In vitro*** **induction on culture medium,** proto-perithecia and perithecia developing, spread through the medium (arrows), superficial or immersed in medium (**A and B**). ***In vitro*** **induction on autoclaved wheat stems using fungal mycelia,** perithecia development observed on plant surface (**D**), above the medium surface (**E**), and in internal tissues of stems (**F**). ***In vitro*** **induction with injection of spore suspension in autoclaved wheat stems,** site of injection (**H,** arrow 1) and fungal colonization within plant tissues (**H,** arrow 2); development of proto-perithecia (**I**) and perithecia (**J**) inside stems; **K,** perithecia developing from the internal plant tissues to beak emersion; proto-perithecia at interface (**L**) and on surface of plant tissues (**M and N**). **O**, Control composed of autoclaved stems without inoculation. The images B, D, F, J, K and M were acquired by light microscope, and the images E, H, I, L and N were acquired by scanning electron microscope.

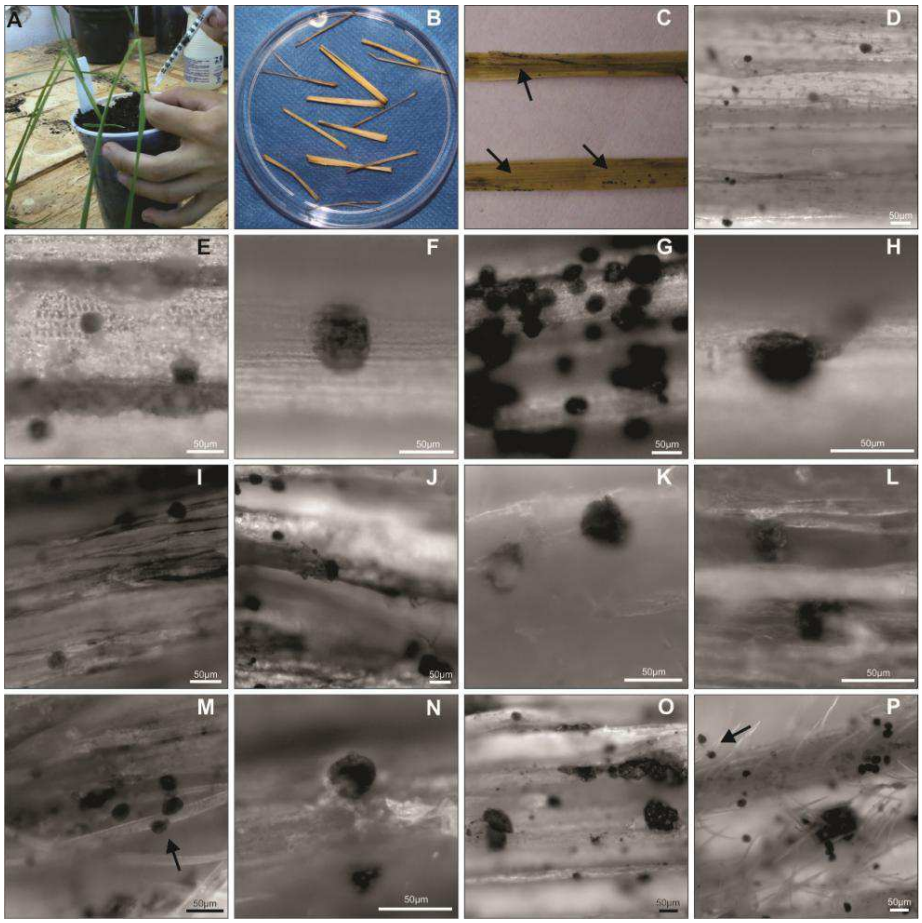

Figure 2: *In vitro* induction of the development of *Pyricularia oryzae* ascocarp by injection of conidia from Mat1-1 and Mat1-2 strains from wheat within stem pieces (**A**) of different plant species. **B**, Pieces of stem placed in 120 mm Petri dishes for incubation in humid chamber 1 month after inoculation. **C**, Stems with proto-perithecia and/or perithecia developing after incubation in humid chamber (arrows). Fruiting body in different plant species: **D**, Bird seed (*Phalaris canariensis*); **E**, Rice (*O. sativa*) cv. Primavera; **F**, Rice (*O. sativa*) cv. Relampago; **G**, Red rice (*Oryza sativa*) cv. Yin Lu 30; **H**, Hybrid *Brachiaria* cv. Mulato; **I**, Barley (*Hordeum vulgare*) cv. BR Elis; **J**, Barley (*Hordeum vulgare*) cv. MN 743; **K**, Rye (*Secale cereale*) cv. BR1; **L**, Black Oat (*Avena strigosa*) cv. Embrapa 29 Garoa; **M**, Foxtail millet (*Setaria italica*); N, Wheat (*Triticum aestivum*) cv. BRS 264; **O**, Wheat (*T. aestivum*) cv. MGS Brilhante; **P**, Triticale (x*Triticosecale*) cv. IAC Caninde. Images D to P were acquired by light microscope.

DISCUSSION

Our findings support the evidence that the sexual reproduction of *Pyricularia oryzae* can occur in nature during the necrotrophic phase, probably in the wet crop residues, after harvest. The methods for inducing perithecia evaluated in media and/or in autoclaved or senescent plant tissues, with compatible wheat isolates of *P. oryzae,* were efficient, with the exception of the method with living standing plants. Probably the senescence and/or necrosis of plant tissues is necessary to stimulate sexual reproduction in *P. oryzae*. Similarly, in studies with co-inoculation of sexually compatible isolates in living rice plants, Hayashi *et al.* (1997) observed perithecial formation only in senescent leaf sheaths and in senescent detached sheaths.

Another important observation is that the *P. oryzae* fruiting bodies were frequently found immersed inside the medium as well as inside the plant internode pith cavity (Figure 1 F, I and J), within the culm (Figure 1 K and L), or on superficial epidermal tissues (Figure 1 M and N). This fact is very important for future studies in order to find the *P. oryzae* sexual phase in nature, indicating that it requires a thorough examination, especially inside plant tissues. Other Ascomycota, *Fusarium*

graminearum, colonize the wheat plants systemically moving from the inoculated heads to vascular system and pith cavity radially (Guenther and Trail, 2005). The mycelia in chlorenchyma support the perithecium development, that erupting from row of stomata on culm, in the leaf base, near the node (Guenther and Trail, 2005). We observed here that the *P. oryzae* perithecia development in wheat probably erupted by the same way described above, in internodes, with internal colonization followed an emerging of perithecia at the surface. However, more anatomically studies are necessary to determine exactly how this sexual differentiation occurs.

Evidences in the literature indicate strongly probability of wheat *P. oryzae* sexual reproduction in Brazil, with presence of the both mating types on field, fertile strains, and microsatellite studies with wide-genome analysis indicating a very high genetic variability from sexual meiosis (Castroagudín *et al.*, 2014; Galbieri and Urashima, 2008; Maciel *et al.*, 2014).

We suggest that the ways to find *P. oryzae* sexual reproduction in nature would be to check inside the senescent or dead vegetal tissues of plant crop residues of wheat, rice or other hosts (Poaceas), in the field

where were blast disease occurs, with both *mating type* idiomorphs present in the populations.

With regard to the size of the ascocarp, we observed larger perithecia than ascocarps in media, when comparing methods with plants, probably simply there was no physical restriction on growth (Table 1). Comparing the methods in which autoclaved stems were used, the sizes were greater than in the method with senescent stems (Table 1). This may indicate that the dead tissues were most favorable to perithecial development, compared with tissues reaching the senescence phase. In the induction within living stems from different plant species, the better development was on bird seed, indicating that its tissues are favorable to inducing perithecia by this method.

This size variation according the substrate observed in *P. oryzae* did not occur in *Fusarium pseudograminearum*, for exemple, once its perithecial dimensions found in wheat residues were the same comparing with the ascocarps developed during induction by *in vitro* pairing (Summerell *et al.*, 2001).

Comparing the induction method on autoclaved wheat stems using fungal mycelia (Figure 1 C to F) and the method with injection of spore

suspension in autoclaved wheat stems (Figure 1 G to N), we observed that the meeting and recognition of the compatible *P. oryzae* strains may occur both on the plant surface and the interior. It is interesting that in method with mycelia and stems, in which the mycelia from both isolates had first grown outside, arriving on the stems (Figure 1 C), colonizing the tissues, meeting, and forming perithecia superficially (Figure 1 D and E) or internally inside the stems (Figure 1 F). The meeting and development of perithecia inside plant tissues was also observed in method with living stems from different plant species, by injection co-inoculation (Figure 2). Thus, since both *mating types* Mat1-1 and Mat1-2 *P. oryzae* strains are present in the field, if sexual reproduction occurs, it can begin by the recognition either on the plant surface or inside.

The Ascomycota sexual phase is influenced by many factors like substrate, host, light, temperature and origin (Trail, 2013). Yaegashi and Hebert (1976) demonstrate the efficiency of white and black (below 500 nm, near-UV) light, temperature (ranged from 20 to 25 ºC depending on isolate origin), and OA medium on the *P. oryzae* perithecial induction. It has been also shown that exist optimum conditions of temperature and

moisture to induce the sexual stage of *F. graminearum*, between 16 to 24 ºC, and -0.45 to -1.30 MPa, respectively (Dufault *et al.*, 2006).

Therefore, future works may be performed to determine the best conditions of temperature and light to the *P. oryzae* sexual phase development in plants, as well as the influence of these conditions on the expression of important genes for sexual recognition and morphogenesis, as the mating type and pheromone genes (Kanamori *et al.*, 2007; Shen *et al.*, 1999). We observed in this work better ascocarp development in necrotic tissues compared to tissues in early senescence and live tissues. It is possible that important genes for the *P. oryzae* sexual cycle have a higher expression stimuli in necrotic tissue.

Our work has shown that the wheat *P. oryzae* perithecia vary in size according to the state of the plant, when the senescence is required to induce the perithecia development, and the dead tissues had larger perithecia than the senescent. The *P. oryzae* perithecia vary in size and density according the plant species used in the induction. The recognition of compatible isolates can occur on the outside or inside the plant tissues.

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