UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA
AGRÍCOLA E DO AMBIENTE

Marcadores moleculares e fenotípicos para avaliação da variabilidade genética em isolados monopóricos e polispóricos de *Bipolaris sorokiniana*

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Porto Alegre, Rio Grande do Sul, Brasil.
Março de 2014.

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Marcadores moleculares e fenotípicos para avaliação da variabilidade genética em isolados monopóricos e polispóricos de *Bipolaris sorokiniana*

Autor: Michele Bertoni Mann
Orientadora: Profa. Dr. Sueli T. Van Der Sand

RESUMO

A Mancha marrom é uma das principais doenças do trigo, causada pelo fitopatógeno *Bipolaris sorokiniana,* responsável por grandes perdas econômicas no cultivo do trigo em todo mundo. Este fungo apresenta uma grande diversidade morfológica, fisiológica e genética. O objetivo do trabalho foi utilizar marcadores moleculares e fenotípicos para avaliar a variabilidade genética em isolados monopóricos e polispóricos de *Bipolaris sorokiniana* de sementes de trigo oriundos do Brasil e outros países. A caracterização molecular envolveu as metodologias URP-PCR, PCR-RFLP, REP-PCR, BOX-PCR, ERIC-PCR assim como testes isoenzimáticos e de patogenicidade. A análise de patogenicidade revelou que isolados polispóricos são mais severos para as sementes que para as partes aéreas das plantas quando comparados com os monospóricos. A caracterização isoenzimática e molecular através das técnicas URP-PCR, PCR-RFLP, REP-PCR, BOX-PCR, ERIC-PCR exibiram uma grande diversidade intra-populacional. REP-PCR e ERIC-PCR revelaram maior diversidade entre os isolados, com uma similaridade inferior a 70%. No entanto, as amplificações realizadas com o BOX-PCR apresentaram um perfil com uma maior similaridade. Com os resultados obtidos a partir das amplificações com BOX-PCR um par de *primers* foi desenhado a partir de um fragmento comum a todos os isolados e que foi capaz de amplificar um produto único ao gênero *Bipolaris sp.* entre as amostras testadas. Com a realização de mais ensaios com outros microrganismos é possível que o mesmo possa ser utilizado como um marcador deste gênero. A técnica de PCR-RFLP utilizando as enzimas *Hae*III, *Hinf*I, *Hha*I, *Eco*RI e *Hind*III apresentaram perfis de restrição com variação no número de fragmentos e no peso molecular. Uma possível explicação para à variabilidade observada em todas as técnicas utilizadas pode ser atribuída à condição multinucleada das células de *B. sorokiniana* bem como a heterocariose, que pode levar a recombinação mitótica e ao polimorfismo.

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Molecular and isoenzymatic characterization of monosporic and polysporic *Bipolaris sorokiniana* isolates

Author: Michele Bertoni Mann
Advisor: Profa. Dr. Sueli T. Van Der Sand

ABSTRACT

The brown spot is a major disease of wheat caused by the pathogen *Bipolaris sorokiniana*, responsible for large economic losses in wheat cultivation worldwide. This fungus has a wide morphological, physiological and genetic diversity. The main objective of this work was to characterize monosporic and polisporic *B. sorokiniana* isolates of wheat seeds from Brazil and other countries. Pathogenicity tests were conducted to evaluate the feasibility of virulence genes as well as different molecular methodologies were tested as: URP-PCR, PCR-RFLP, REP-PCR, BOX-PCR, ERIC-PCR and isoenzyme patterns of the isolates. The pathogenicity assay reveals that the polysporic isolates caused a more severe disease to seeds than to aerial parts of the plants when compared to single spore isolates. Isoenzyme and molecular characterization through the URP-PCR, PCR-RFLP, REP-PCR, BOX-PCR, ERIC-PCR techniques showed a large intra-population diversity among the isolates. REP and ERIC-PCR revealed greater diversity among the isolates with a similarity below 70%. However, the amplification results using with BOX- PCR showed a highest similarity. The PCR-RFLP using *Hae*III, *Hinf*I , *Hha*I , *EcoR*I and *Hind*III restriction enzymes showed a profile variation between all isolates in relation to the number and molecular weight of the fragments. However the results obtained with BOX- PCR amplifications a higher similarity was obtained. With this result a primer was designed, based on a fragment common to all isolates, and the amplifications using these primers produced a unique fragment with the *Bipolaris* genus among others isolates tested. More phytopatogeneic isolates must be tested in order to confirme the specificity for *Bipolaris* sp. With the PCR-RFLP assay, using the enzymes *Hae*III, *Hinf*I, *Hha*I, *Eco*RI e *Hind*III, variability was observed among the restriction patterns realated to the number of fragments and molecular weight. One possible explanation for the observed variability in all techniques used may be attributed to the condition of multinucleated cells of *B. sorokiniana* and the heterokaryosis which can lead to mitotic recombination and polymorphis.

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RELAÇÃO DE ABREVIATURAS E SÍMBOLOS

%: Porcentagem
°C: Graus Celsius
µg: micrograma
µL: microlitro
µm: micrômetro
Blast: Basic Local Alignment Search Tool
CAPES: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico
DNA: Ácido Desoxirribonucleico
ERIC: Enterobacterial Repetitive Intergenic Consensus
GenBank: Banco de Dados do NCBI (National Center for Biotechnology Information)
ICBS: Instituto de Ciências Básicas da Saúde
KDa: Kilo Dalton
mg: miligrama
Min.: minutos
mL: mililitro
mM: milimolar
ng: Nanogramas
PCR: reação em cadeia da polimerase
PF: Oligonucleotídeo —Forward
PR: Oligonucleotídeo —Reverse
RFLP: *restriction fragment length polymorphism*
rep-PCR: repetitive element palindromic PCR

INTRODUÇÃO

O trigo é o segundo cereal mais produzido no mundo, com significativo peso na economia agrícola global. No Brasil, o trigo é cultivado nas regiões Sul, Sudeste e Centro-Oeste, com uma produção de 600 milhões de toneladas/ anuais. No entanto, a produção não é autossuficiente para atender a demanda interna, sendo necessária a importação para suprir as necessidades internas do país. Um dos fatores que interferem na baixa produção é a ocorrência de doenças fúngicas, que são favorecidas pelo clima quente e alta umidade. A presença de fungos patogênicos na agricultura tem sido descrita como um problema presente em diversas regiões do Brasil e do mundo. Nesse cenário essas variáveis impactam diretamente na quantidade produzida e aumentam o custo de produção.

Dentre as doenças fúngicas, destaca-se o fitopatógeno *Bipolaris sorokiniana* (Sacc. In Sorok) Shoem. que ataca gramíneas como centeio, cevada, aveia, trigo e etc. No Brasil este patógeno é de grande importância para a cultura do trigo, encontrando-se disseminado por todas as regiões tritícolas, ocasionando moléstias que recebem várias denominações conforme

o órgão afetado da planta: podridão comum da raiz, mancha marrom, carvão do nó e ponta preta dos grãos.

A mancha marrom é uma doença é causada por *Bipolaris sorokiniana* e várias outras espécies, que provocam perdas significativas nas culturas de inverno. A variabilidade morfológica que o fungo apresenta e o tempo necessário para o cultivo dificulta a sua identificação, impossibilitando que medidas de controle adequadas sejam adotadas para reduzir as moléstias, enquanto que a variabilidade fisiológica dificulta o estabelecimento de variedades de trigo resistentes a estas moléstias.

Fungicidas são bastante utilizados no controle do fitopatógeno devido a sua facilidade de aplicação e resultados imediatos. No entanto, o uso continuo destes produtos pode aumentar as chances de resistência de fungos fitopatogênicos. Extensas áreas tratadas com o mesmo ingrediente ativo aumentam a pressão de seleção, além disso, quanto menor o tempo de geração do patógeno, mais frequente é a necessidade de exposição ao fungicida e maior o risco de resistência (Ghini & Kimarti, 2002).

Segundo Martins Netto & Faiad (1995), a qualidade sanitária das sementes é um fator importante na germinação, pois pode ocasionar perdas através da deterioração, anormalidades e lesões em plântulas. Os fungos são os agentes causais mais importantes , e podem ser disseminados através de sementes, permanecendo viáveis por períodos prolongados de tempo.

Técnicas isoenzimáticas e de biologia molecular são utilizadas com o proposito de elucidar a variabilidade genética intra-populacional de populações diferenciando espécies próximas e morfologicamente semelhantes.

1.1 Justificativa e Objetivos Gerais

Entre os principais produtos agrícolas brasileiros, nenhum apresenta tantas nuances quanto o trigo, no aspecto tecnológico de produção, na inserção nos sistemas de produção regionais, na agregação de renda às propriedades agrícolas, no aspecto de abastecimento interno e no papel de produto relevante nas transações comerciais brasileiras com outros países. Por esta razão o trigo ocupa uma considerável área cultivada no Brasil, e pelo fato do País não ser autossuficiente, a tendência é de aumento na área de plantio.

Diante do exposto e da necessidade de conhecer este patógeno, buscou-se neste trabalho a caracterização de isolados de *Bipolaris sorokiniana* através de técnicas isonzimáticas e moleculares, para avaliar o perfil de isolados monospóricos e polispóricos. Este estudo buscou fornecer informações importantes sobre a variabilidade genética de *Bipolaris sorokiniana* para contribuir para rapidez e a correta identificação do fitopatógeno

1.2 Objetivos específicos

- Caracterizar isolados monospóricos e polispóricos de *Bipolaris sorokiniana*, quanto ao seu potencial patogênico;
- Analisar os padrões de polimorfismo entre isolados monospóricos e polispóricos através do PCR –URP;

- Avaliar os perfis de polimorfismo do fungo *B. sorokiniana* com base na análise das regiões ITS1, 5.8S, ITS2 do rDNA através PCR-RFLP;
- Correlacionar os padrões de fragmentos obtidos entre as técnicas de, BOX-PCR, ERIC-PCR e REP-PCR;
- Analisar os padrões de polimorfismo obtidos na diversidade molecular e sequenciar fragmentos relevantes produzidos BOX-PCR;
- Estudar a variabilidade dos perfis gerados por análise de Isoenzimas;
- Agrupamento de todos os dados com objetivo de caracterizar a espécie *Bipolaris sorokiniana*.
- Relacionar os padrões de polimorfismos com a região geográfica de origem dos isolados.

REVISÃO BIBLIOGRÁFICA

2.1 Trigo

Originário da Ásia, o trigo foi introduzido na Índia, na China e na Europa desde cinco mil anos A.C. (Picinini & Fernandes, 2000). Achados arqueológicos indicam que esse cereal foi o segundo grão cultivado, após a cevada, sendo de grande importância para o desenvolvimento da civilização ocidental, permitindo que a espécie humana abandonasse a caça e coleta para se fixar à terra e nela formar povoados e cidades (Cunha et al.,1999).

O trigo de panificação, *Triticum aestivum* L.*,* é a espécie mais cultivada, da qual são conhecidas mais de 20 mil variedades. Seu genoma é resultante da hibridização natural de três espécies diferentes, o que confere excepcional capacidade de adaptação às variadas condições ecológicas (Cunha et al. 1999).

O melhoramento cientificamente planejado do trigo teve início na França há cerca de 200 anos. A partir daí, os países que dominaram a economia mundial são os mesmos que mais investiram em pesquisas de melhoramento genético. No Brasil, a cultura de trigo que foi introduzida pelos colonizadores europeus, teve dificuldades de adaptação devido à acidez do solo, moléstias fúngicas, entre outras.

O trigo é o cereal mais comercializado mundialmente, sendo plantado em aproximadamente 17% do total das terras cultivadas do mundo. Estima-se que as populações dos países em desenvolvimento consumam hoje metade do trigo produzido no mundo e que nos próximos dez anos esse consumo alcançará 60%, podendo chegar em 2020 a uma demanda 40% maior do que a necessária atualmente (CIMMYT, 2000).

A China é o maior produtor mundial de trigo, com cerca de 17% do total. Os países da União Européia aparecem, segundo informações do Departamento de Agricultura dos Estados Unidos (USDA), em 2º lugar, porém individualmente, esta posição é da Índia. Os maiores exportadores são os EUA, Canadá, Austrália e Argentina. Este último é o quarto maior exportador mundial, disputando o lugar com a União Européia como um todo. Estão entre os maiores importadores os países do norte da África, Japão, Brasil e Indonésia.

O auge da triticultura brasileira foi alcançado em 1987, quando foram semeados mais de 3,9 milhões de hectares, com a produção atingindo 6,2 milhões de toneladas (Picinini & Fernandes, 2000). Atualmente, o Brasil importa 70% do trigo necessário para suprir as suas necessidades de consumo interno. Na última década o Brasil tem produzido trigo em uma área bastante limitada, estimada em cerca de 2,4 milhões de hectares segundo o CONAB (Conselho Nacional de Abastecimento). De acordo com dados das alfândegas brasileiras, de julho de 2012 a julho de 2013, as importações de trigo do Brasil somaram 7 milhões de toneladas, o maior volume dos últimos seis anos, e os gastos aumentaram 41%, somando US$ 2,26 bilhões. Com a escassez do grão

na Argentina, o maior fornecedor do Brasil tornou-se os Estados Unidos. De acordo com Abitrigo (Associação Brasileira da Indústria do Trigo) o volume importado aumentou em função da escassez de produção que deve-se a fatores climáticos no Paraná e no Rio Grande do Sul, estados onde a produção de trigo nacional está concentrada. A escassez de trigo para consumo doméstico, a qualidade, além de taxa de câmbio favorável aumentaram as importações dos EUA ao segundo nível mais alto nos últimos oito anos.

A produção projetada de trigo para 2020/2021 é de 6,2 milhões de toneladas, e um consumo de 11,7 milhões de toneladas no mesmo ano. O consumo interno de trigo no País deverá crescer em média 1,2% ao ano, entre 2010/11 e 2020/2021. O abastecimento interno exigirá importações de 6,7 milhões de toneladas em 2020/2021. Apesar da produção de trigo crescer nos próximos anos em ritmo superior ao consumo, mesmo assim o Brasil deve manter-se como um dos maiores importadores mundiais de trigo (CONAB, 2013).

2.2 Fatores limitantes para produção de trigo

Além dos fatores econômicos e políticos, a produção brasileira de trigo encontra obstáculos como a incidência de doenças, muitas delas fúngicas, que atacam a planta desde a raiz até o caule, folhas e sementes. Os patógenos que ocorrem com maior frequência na cultura de trigo gaúcha e do sul do país, citados pela Reunião da Comissão Sul-Brasileira de Trigo, são *Bipolaris sorokiniana, Drechslera tritici-repentis, Fusarium* e *Oidium* sp., que são responsáveis em grande parte pela perda ou menor rendimento na produção de grãos.

Áreas quentes e úmidas não são adequadas ao cultivo deste cereal. Estes dois fatores favorecem o desenvolvimento de muitas doenças, além de alterarem o desenvolvimento normal das plantas (Mundstock, 1998).

Picinini & Fernandes (2000) demonstraram que em doze anos de experimentação ocorreram em média, 44,61% de perdas na cultura do trigo causadas por doenças o que equivale a 1.152 kg de trigo por hectare. Entretanto, os autores ressaltam que atualmente o país tem condições de solo, clima, materiais genéticos, tradição agrícola e tecnologia disponível para cultivar mais de 10 milhões de hectares. Medidas técnicas e políticas buscam a recuperação da triticultura brasileira, para que em médio prazo, possa produzir 60% da demanda nacional (Reunião da Comissão Sul-Brasileira de Pesquisa de Trigo).

2.3 Fitopatógeno *Bipolaris sorokiniana*

O fungo causador da mancha marrom é denominado *Bipolaris sorokiniana* (Sacc. In Sorok) Shoemaker, 1959. (sinônimos *Helminthosporium sativum* Pammel, King & Bakke, Sacc.ex.Sorok, *D.sorokiniana* (Sacc.) Subram & Jain) que corresponde a fase anamórfica, é um fungo fitopatogênico de centeio, cevada, aveia, trigo e outras gramíneas. Os conídios germinam por um ou ambos os pólos, o primeiro septo é produzido delimitando 1/3 basal do conídio e o hilo é externo, porém truncado (Muchovej et al, 1988).

Na fase teleomórfica o fungo é denominado *Cochliobolus sativus* (Ito & Kuribayashi) Dresxhsler ex Dastur, um ascomiceto de rara ocorrência na natureza (Ellis, 1971, Mehta, 1978, Wiese, 1987).

O gênero *Bipolaris* contém aproximadamente 45 espécies a maioria das quais são parasitas de plantas, como*: B. maydis* (milho) *B. oryzae* (arroz), *B.saccari* (cana-de-açúcar). Entretanto, várias espécies, como *B. australiensis, B.hawaiiensis* e *B. spicifera*, são patogênicas para o homem.

Os fungos pertencentes a essa categoria taxonômica são chamados de fungos imperfeitos (ou mitospóricos), pois possuem apenas a reprodução assexuada (conidial) como forma de propagação da espécie.

Bipolaris sorokiniana é fungo saprófita que sobrevive principalmente no solo e sobre as culturas. O estágio sexual não é tão importante no ciclo da doença. A partir do inoculo primário que inclui a semente infectada, os conídios germinam na presença de hospedeiros suscetíveis e se iniciar a infecção primária no coleóptilo ou nas raízes primárias. O fitopatogeno penetra no tecido hospedeiro através da epiderme através das aberturas naturais ou lesões, através dos apressório formados antes da penetração. A infecção continua a partir da epiderme para a endoderme, resultando na desintegração do tecido. A colonização de partes de plantas infectadas progride pela propagação de conídios (Mathre, 1987). .

Qualquer movimento do solo pelo vento, água e implementos pode mover inóculo do patógeno. A semente também pode servir como um meio de disseminação do patógeno a longas distâncias (Mathre, 1987). O inóculo secundário não é importante para o desenvolvimento da doença por continuar abaixo do solo, mas fornece inóculo para as culturas subseqüentes (Murray et al, 1998). Os factores ambientais desempenham um papel importante na gravidade da doença causada por *B. sorokinana* e outros fungos da podridão

da raiz. Os sintomas iniciais da doença são a ocorrência de lesões necróticas pardas nas primeiras folhas, em virtude da transmissão a partir das sementes. Nas demais folhas, as lesões possuem formato elíptico, 0,5 a 1,0 cm de comprimento, e coloração variável, inicialmente cinza claro até negras (Reis et al., 2001). Quando a temperatura encontra-se entre 23 a 30^{0}C, o número das lesões causadas por *B. sorokiniana* é maior, fato que justifica a presença da doença ser mais frequente em regiões de clima tropical e subtropical. Os danos na produtividade podem ultrapassar a 30% e, a partir dos 53 dias após a emergência, podem ser superiores (Oliveira & Gomes, 1984).

Fonte: Mann, 2008

FIGURA 1: Sintomas da doença (a) folha de trigo sem lesão, (b) lesão do colmo, (c) mancha folha e (d) podridão da semente infectada por *B. sorokiniana*

O fungo pode infectar todos os órgãos das plantas suscetíveis, sendo que a fonte de inóculo primário são as sementes, os restos culturais

infectados, as plantas voluntárias, os hospedeiros secundários e os conídios livres dormentes no solo (Reis et al., 2001).

No processo de infecção, o fungo passa por uma fase biotrófica de crescimento sobre o hospedeiro e uma necrotrófica. A primeira fase é caracterizada pela penetração da cutícula e da parede celular, seguida do desenvolvimento das hifas dentro das células da epiderme. Ocorre a invasão do mesófilo na fase necrotrófica e a morte das células atacadas (Kumar et al., 2002). Outra característica do fitopatógeno é a produção de toxinas sesquiterpenóides sintetizadas a partir do farnesol. O prehelmintosporol é o mais abundante e ativo composto, exercendo seu efeito inibitório sobre ATPase (Kumar et al., 2002). Essa mesma toxina tem propriedade anfipática que pode funcionar como um detergente, auxiliando no amolecimento da camada cerosa da cutícula (Nilson et al., 1993). Outra toxina também importante é o helmintosporol que afeta a permeabilidade da membrana, fosforilação oxidativa, fotofosforilação e o bombeamento de prótons através da membrana plasmática.

2.4 Técnicas para o estudo de fungos fitopatogênicos

2.4.1 Métodos Enzimáticos

O termo isoenzima define um grupo de múltiplas formas moleculares da mesma enzima que ocorre em uma determinada espécie, como resultado da presença de um ou mais genes codificando para cada uma destas formas

(Selander *et* al., 1986). Este método detecta os diferentes alelos de genes diagnósticos analisando a mobilidade eletroforética das enzimas que codificam.

O princípio básico da técnica reside no uso de eletroforese em gel de amido ou poliacrilamida e na visualização do produto enzimático por métodos bioquímicos. Os padrões eletromórficos produzidos para certo número de enzimas são identificados como fenótipos multilocus ou tipos eletroforéticos (Eletrophoretic Types-ET's), os quais refletem o genótipo cromossômico. A difusão do uso desta metodologia deu-se através do desenvolvimento de metodologias eficientes para a visualização do produto enzimático, e da aplicabilidade imediata encontrada em diversas áreas da biologia (Ferreira & Grattapaglia,1995).

O estudo dos padrões de isoenzimas obtidos por eletroforese (MLEE – multilocus enzyme electrophoresis) tem sido amplamente aplicado nos estudos de diversidade genotípica cromossômica e da estrutura genética de populações bacterianas do solo, inclusive dentro do gênero *Rhizobium* (Denny et al., 1988; Piñero et al., 1988 Young, 1985).

Inúmeras investigações têm utilizado com a técnica de isoenzimas para estimar os níveis de variabilidade genética de populações naturais, estudar o fluxo gênico entre populações, a dispersão de espécies, tamanho efetivo da população etapas de cruzamento (Ferreira & Grattapaglia 1998).

2.4.2 Métodos moleculares

Ferron (1978) salientou que o uso de critérios morfológicos no estudo de fitopatógenos, deve ser o primeiro passo de identificação, mas que algumas

características morfológicas podem ser devidas à instabilidade do isolado ou às condições de cultivo. Portanto, as análises fenotípicas devem ser confirmadas por métodos baseados em características genéticas.

Para tanto, técnicas como reação em cadeia da polimerase com oligonucleotídeos universais do arroz (URP-PCR) têm sido utilizados para estudar a variabilidade genética inter e intra-específica entre microrganismos, e têm mostrado resultados rápidos e eficazes (Kang et al., 2002). Kang et al. (2003) utilizaram oligonucleotídeos URP para estudar 25 isolados de seis espécies diferentes de *Alternaria* que produzem toxinas específicas. Os resultados mostraram que oito oligonucleotídeos foram capazes de revelar polimorfismos em isolados de *Alternaria* em níveis de intra-e inter-especifico. No estudo realizado por Mann *et al*, (2014) utilizando 12 oligonucleotídeos URP para caracterização molecular de 60 isolados monospóricos de *B. sorokiniana* do Brasil e de outras partes do mundo houve um alto nível de polimorfirmos entre isolados. O estudo mostrou que os primers URP-2F, URP-6R, URP-17R, a URP-30F e URP-38F produziram um padrão de fragmentos monomórficos em 73% dos isolados. O *primer* URP-2F gerou um fragmento de 578-pb em 83,7% dos isolados; o URP-6R apresentou um fragmento de 548 em 89,1% e o *primer* URP-38F com o fragmento 650pb em 80% dos isolados.

O advento da biologia molecular tem causado mudanças significativas nas abordagens utilizadas para caracterizar e identificar patógenos de plantas, como também, na gestão de estratégias de doenças. Rep-PCR tem sido amplamente usado para identificar microrganismos, diferenciar cepas e acessar a diversidade genética de patógenos de plantas (Louws *et al.*1999) e

pode ser utilizado para determinar as relações genéticas dentro dos grupos. A técnica de rep-PCR faz uso de *primers* complementares de sequências de DNA repetitivas altamente conservadas e presentes em múltiplas cópias nos genomas da maioria das bactérias Gram-negativas e várias Gram-positivas. Três famílias de sequências repetitivas foram identificadas, incluindo a sequência REP (“Repetitive Extragenic Palindromic”) de 35-40 pb, sequência ERIC (“Enterobacterial Repetitive Intergenic Consensus”) de 124-127 pb, e o elemento BOX de 154 pb. Estas sequências parecem estar localizadas em posições intergênicas distintas no interior do genoma. Os elementos repetitivos podem estar presentes em ambas as orientações e os *primers* foram desenhados de modo a promover a síntese de DNA a partir da repetição invertida nos REP e ERIC, e da subunidade boxA nos BOX, amplificando assim regiões genômicas distintas localizadas entre os elementos repetitivos. Os protocolos correspondentes são designados REP-PCR, ERIC-PCR, BOX-PCR e rep-PCR coletivamente.

A utilização da técnica de rep-PCR permite a identificação dos microrganismos através de um perfil de amplificação e caracteriza-se como uma ferramenta com boa discriminação até mesmo entre estirpes. Diferentes estudos foram realizados para discriminar fungos fitopatogênicos como no estudo com caracterização de *Penicillium* por sequenciamento de rDNA e análise por BOX, ERIC e REP-PCR, que possibilitou discriminar *Penicillium* de outros gêneros (Redondo *et al.*2009) . No trabalho realizado por Arruda et al. (2003) com caracterização através de ERIC-PCR com isolados de *Crinipellis perniciosa* do Brasil observou correlação geográfica com distintos grupos.

Outras técnicas moleculares para o estudo de sistemática de fungos que incluem desde a análise por RAPD (Muller et al. 2005; Jaiswal et al., 2007), PCR utilizando oligonucleotídeos sítio específicos, "Restriction Fragment Lenght Polymorfism – RFLP" (Nascimento et al, 2008) " Amplified Fragment Lenght Polymorphism- AFLP" (Zhong & Steffenson, 2001). Todas estas técnicas que avaliam a variabilidade genética em fungos fitopatogênicos são capazes de revelar perfis de polimorfismos de DNA e têm sido utilizado para a identificação sistemática e estudos filogenéticos de plantas, animais e fungos (Lessa, 1992). Nascimento and Van Der Sand (2008), estudando 50 isolados de *B. sorokiniana* utilizando PCR-RFLP/ITS observaram polimorfismo intra-específico entre dois isolados de *B. sorokiniana* na região ITS1. Dados fornecidos pelo PCR-RFLP/ITS mostraram que os isolados oriundos do Brasil agruparam-se predominantemente entre eles e o mesmo resultado foi observado com os isolados oriundos de outros países. Com os resultados obtidos não foi possível aos autores diferenciar isolados de *B. oryzae* e *D. teres* que foram também utilizados no trabalho. Os métodos utilizados para a identificação molecular de fungos em nível de espécies têm sido baseados principalmente na região dos espaços transcritos internos (ITS) e os resultados têm sido altamente variáveis (Paul, 2001).

Estudo de RFLP (restriction fragment length polymorphism), PCR e sequenciamento possibilitam determinar a diversidade genética entre os fungos com o objetivo de caracterizar os microrganismos.

2.5 Patogenicidade

No inicio do século XX, surgiram as primeiras coleções de culturas microbianas. Inicialmente eram mantidas por repicagens periódicas e constantes, acarretando perda da variabilidade, alterações fisiológicas e declínio na patogenicidade. Estes fatores levaram à busca de testes para verificar a viabilidade dos isolados quanto à patogenicidade. Os testes de patogenicidade se fazem necessário porque visam verificar se um isolado desconhecido é patogênico para uma planta ou animal ou verificar se um isolado conhecido não perdeu sua capacidade de infectar e causar doenças em seu hospedeiro. Os isolados devem ser inoculados em seu hospedeiro e incubados sob condições que favoreçam a colonização (EMBRAPA, 2011). A maior ou menor capacidade de um patógeno causar doença é referida como virulência. A maior ou menor virulência de um patógeno dependerá: a) de sua capacidade de penetração no hospedeiro (agressividade); b) de sua capacidade de colonizar os tecidos hospedeiros (infecciosidade); e c) de sua capacidade de produzir substâncias tóxicas à planta hospedeira (poder toxígeno).

3. MATERIAIS E MÉTODOS

Os materiais e os métodos específicos para cada um dos experimentos desenvolvidos neste trabalho estão descritos nos respectivos artigos, apresentados nos capítulos 1, 2, 3, 4 e 5, que seguem desta Tese (RESULTADOS).

Este estudo foi realizado na cidade de Porto Alegre, Rio Grande do Sul no Laboratório de Micologia Ambiental (Lab. 209) do Instituto de Ciências Básicas da Saúde (ICBS) da Universidade Federal do Rio Grande do Sul (UFRGS).

RESULTADOS

Os resultados deste trabalho estão apresentados na forma de artigos científicos. Os subtítulos deste capítulo correspondem aos títulos do artigo publicado e do artigo formatado de acordo com as normas do periódico escolhido para submissão.

CAPÍTULO 1. ARTIGO CIENTÍFICO 1

Pathogenicity of monosporic and polysporic *Bipolaris sorokiniana* isolates under controlled conditions

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Pathogenicity of monosporic and polysporic *Bipolaris sorokiniana* isolates under controlled conditions

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ABSTRACT

Bipolaris sorokiniana may present considerable genetic diversity and highly variable pathogenicity and virulence. The pathogenicity of 99 *B. sorokiniana* isolates (27 polysporic and 72 monosporic isolates) from Brazil and other countries was assessed. The variables evaluated were aborted germination, seed black point, leaf spot, and coleoptile lesion. The Principal

Component Analysis (PCA) was used to evaluate the similarity patterns between isolates considering the variables of pathogenicity. Polysporic isolates presented higher virulence (over 60%), when compared with the monosporic isolates (43%) in all variables analyzed, except coleoptile injury. Of the isolates used to infect seeds, 8% presented the highest virulence value, with pathogenic action over 75% in all variables assessed. The correlation of *B. sorokiniana* isolates with pathogenicity variables demonstrated that polysporic isolates were more virulent, especially in seeds, compared with aerial plant parts.

Key words: Variability, virulence, *Triticum aestivum* L., spot blotch

INTRODUCTION

Wheat (*Triticum aestivum* L.) was one of the first plant species grown by man. Throughout history the species has had fundamental importance in mankind's food basis, and today it takes the first place in worldwide agricultural production figures (EMBRAPA, 2013). According to data published by the United Nations Food and Agriculture Organization, global wheat production is estimated to reach a record number of 600 million ton in the 2012/2013 harvest (FAO, 2012). In Brazil, wheat production is around 5,000 to 6,000 ton. The largest cultivated areas, accounting for 90% of the country's production, are in southern (States of Rio Grande do Sul, Santa Catarina and Paraná) and midwest Brazil (States of Mato Grosso do Sul, Goiás and Distrito Federal) (EMBRAPA, 2013).

As an agricultural product, wheat is subject to biotic and abiotic

limitations, such as adverse climatic conditions, soil, pests and diseases. Among the limiting conditions, the phytopathogen *Bipolaris sorokiniana* (Sacc.) Shoemaker (teleomorph, *Cochliobolus sativus*) stands out as the causal agent of common root rot, leaf spot, seedling blight and black point in seeds of both wheat and barley cultures, apart from diseases in rye, oat, triticale, sorghum and fescue (Tinline, 1961). However, the most severe symptoms of these diseases are observed in wheat and barley cultures in hot and humid regions, with significant production losses (Kumar et al., 2002).

Of a cosmopolitan nature, spot blotch caused by *B. sorokiniana* is estimated to affect 25 million hectares of wheat plantations worldwide to variable degrees, which accounts for 12% of the total area of the culture that is grown (Duveiller et al., 2005). Seeds infected with the pathogen are highly infectious and stand as the main survival mechanism of this fungus off season, representing the means by which hot spots of the disease remain active in the field (Forcelini, 1991). This fungus may also play a deleterious role in germination and in the establishment of cereal plantations, leading to aborted seed, seed rot, necrosis and discoloration of aerial parts of infected plants, reducing seed viability (Neergard, 1979).

Spot blotch has symptoms that usually appear on the aerial parts of plants and take the form of oval necrotic lesions surrounded by chlorotic halos. This infection may reduce photosynthetic area and eventually to premature plant senescence (Ghazviniand et al., 2007).

Combined strategies to control *B. sorokiniana* in wheat and barley cultures have been recommended by Mehta (1998). These strategies include

the use of resistant varieties, chemical control of soils, waste management and crop rotation. In spite of the fact that spot blotch is considered one of the most important diseases affecting wheat worldwide, control measures have not produced satisfactory results (Kumar et al., 2007).

In this scenario, the present study assesses the pathogenic potential of monosporic and polysporic *B. sorokiniana* isolates in wheat seeds and seedling under controlled conditions.

MATERIALS AND METHODS

Origin of microorganisms

The fungal polysporic isolates from different regions in Brazil were provided by Empresa Brasileira de Pesquisa Agropecuária - Trigo (EMBRAPA-Trigo, Passo Fundo), while the other isolates used in this study were kindly provided by the International Maize and Wheat Improvement Center (CIMMYT – México). All isolates used were obtained from seeds and tissues of wheat plants. The biological material was deposited in the collection of the Environmental Mycology Laboratory, DMIP, ICBS, UFRGS. Ninety-nine *B. sorokiniana* isolates were used in the pathogenicity assay, of which 27 were polysporic and 72 were monosporic isolates (Table 1).

Monosporic cultures

The monosporic cultures were obtained from the aerial mycelia of the polysporic cultures grown on plates with potato dextrose agar (PDA). A 0.85% saline solution was poured over the plated colonies, and the conidia were

transferred to microcentrifuge tubes. The contents of tubes were homogenized thoroughly to achieve complete conidia release. The suspension was transferred to a Petri dish with PDA and incubated at room temperature for 2 h. Using a stereomicroscope with optical magnification of ×40, the conidia were transferred using plates with PDA media. The plates were maintained at 24 ± 2ºC until the complete colonies had developed, and then were stored at 4ºC. Each spore culture was designated with a letter (A, B, and C).

Fungal inoculum preparation

Bipolaris sorokiniana isolates were multiplied in a culture medium prepared with vegetable broth and carrot agar specific for sporulation and incubated in a BOD stove for 10 to 15 days at 25ºC in a 12-h photoperiod. To standardize the fungal inoculum, 5 mL sterile saline (0.85%) containing Tween 80 (0.1%) were added to colonies. Then, colonies were lightly streaked using a Drigalski spatula, spores were removed and the suspension was transferred to sterile glass test tubes. Final spore concentration was adjusted to 10^6 spores/mL by counting conidia in a Neubauer chamber.

Pathogenicity assay

The pathogenicity assay was carried out using the 99 *B. sorokiniana* isolates. Samples of 100 wheat seeds, cultivar BRS Buriti, which is considered moderately susceptible to leaf spot, were disinfected using ethanol 70% for 2 min, sodium hypochlorite 2.5% for 2 min and then three washes with sterile distilled water. Samples were then placed in tubes containing the previously

adjusted spore suspension and left at room temperature for 24 h. After, seeds were incubated according to a modified version of the Blotter test method. Each 100-seed sample was divided in groups of 25 seeds that were placed one by one on wet filter paper sheets, accounting for four repeats. The sheets containing seeds were folded as a sachet, which was incubated in a seed germinator (JP-1000, J. Prolab) at 25ºC in a 12-h photoperiod with controlled temperature and humidity for 10 days. Then, 10 days after seeding, wheat seeds and seedlings were individually assessed as to germination aborted, leaf spot, black point of the seed and coleoptile lesion. The assay was carried out as 10 steps, each of which included a control group of seeds not challenged with the *B. sorokiniana* infection. After the lesions were analyzed, all the tissues of the organs were submitted to re-isolation of the phytopathogen using culture conditions on dishes contain PDA medium and after growth analysis of the structures under the microscope was perfomed.

Statistical analysis

A descriptive statistics of the pathogenic action of *B. sorokiniana* was carried out for the four variables, expressed as percent values: % germination aborted (GA), % black point of the seed (BP), % leaf spot/germinated seeds (LS), % coleoptile lesion/germinated seeds (CL).

The differences in pathogenicity degree between controls and groups of monosporic and polysporic isolates, for the variables analyzed, were assessed using the one-factor analysis of variance followed by an analysis of the differences between treatments using the randomization test, as described

by Pillar & Orlóci (1996).

The differences in pathogenicity patterns between isolates based on the four variables assessed (GA, BP, LS and CL) was evaluated using the Principal Component Analysis (PCA) (Person, 1901).

The statistical analyses were carried out using the application R (R Development Team 2008) and the action interface for Excel (Estacamp, 2013). Normality of variables was tested using the Shapiro-Wilk test. The analyses of variance and multivariate analyses were made in the MULTIV (Pillar, 1997).

RESULTS

The data analyses of the four variables assessed indicated that the monosporic and polysporic isolates of *B. sorokiniana* strongly induced the diseases in wheat seed and seedlings, when compared to controls (Table 2).

The comparison between treatment groups (monosporic, polysporic and control isolates) revealed a significant difference between groups, when one same variable is considered. The exception was observed for coleoptile lesion, for which no significant difference was observed between monosporic and polysporic isolates (Table 2).

The action of single spore isolates on germination, showed a median of 59.5 seeds with germination aborted, and the variation ranged from a minimum of 16 to a maximum of 100 seeds. The pathogenesis of polysporic isolates had a higher median, of 75 seeds with germination aborted, and the variation ranged from a minimum of 43 and maximum 110 seeds (Table 2 and Fig. 1).

The highest virulence towards germination (values above the third quarter) was exerted by 18 monosporic isolates, with values over 73.25% of seeds germination aborted, and by 6 polysporic isolates, with seed values over 84%.

The pathogenic action of *B. sorokiniana* isolates on wheat seeds led to high degree of deterioration, with a median value of 100 seeds with black point, both for monosporic and polysporic cultures. The data analyses showed statistically significant differences between all treatments. With polysporic cultures, a higher incidence of black point of the seed was observed. Mean number of seeds affected by black point after treatment with polysporic cultures was 97.4%, while monosporic cultures caused the disease in 83.9% and the black point of seed in controls was 13.2% (Table 2). On the other hand, 5.4% of monosporic isolates did not cause symptoms in seeds, and did not differ from controls. All polysporic isolates caused black point of seeds, with the lowest value of 61% of seeds.

Coleoptile lesion caused by polysporic and monosporic *B. sorokiniana* isolates presented medians of 82% and 67.9%, respectively (between zero and 100%). The analysis of variance to compare groups indicated a significant difference between the control and the treatment groups, though this difference was not observed between monosporic and polysporic isolates (Table 2 and Fig. 1).

Incidence of lesions on leaf blades presented medians of 44.6% and 65.4% for monosporic and polysporic isolates, respectively (Table 2 and Fig. 1). Among the most virulent isolates that caused the symptom of leaf spot

(above the third quarter), 18 were monosporic isolates, causing the effect in more than 69% of leaves, while six polysporic isolates triggered the effect in more than 79.8% of seeds.

Analysis of the pathogenic action of monosporic and polysporic isolates

The similarity pattern in the pathogenic action of isolates, based on the four variables evaluated simultaneously, is shown in Figure 2 and 3. Ordination axis I contains 64.9% of the total variation of pathogenicity data, in which all variables exhibited high, positive correlation (GA = 0.71, SR= 0.79, LS = 0.87, and CL = 0.83) with this axis and the major contribution was that of leaf spot with 29.5% of the total variation in this axis. The position of isolates on axis I allows identifying the most virulent isolates, on the right, namely CEV48P, 98042P, 98042C and CFO201B. Low pathogenicity isolates are at the far end of the axis, especially 98012C, 98023B, 98026C, CFO201A and CS1004A.

Axis II contains 20.9% of the total variation in pathogenicity data and allows differentiating the most pathogenic isolates to wheat seeds from the most pathogenic to the aerial parts of the plant. Germination aborted and black point of seed had positive correlation with axis II (0.58 and 0.36, respectively), while leaf spot and coleoptiles lesion presented negative correlation (-0.35 and -0.48 respectively). The highest contribution was given by the variable seed germination aborted, with 40.9%.

The ordination chart of isolates also reveals a clear distinction between control and monosporic and polysporic isolates (Figure 2). Also, single

spore isolates presented higher variation in pathogenicity degree, when compared to polysporic isolates. However, these were a little more specialized in terms of pathogenicity, affecting more seeds than the aerial parts.

Of the isolates used to infect seeds, 8% presented the highest virulence, with pathogenic action over 75% for all variables assessed. The isolates with the highest and lowest scores in axis I and II were the most virulent when the four variables are considered as a set 98004A, 98025C, 98034C, 98042P, 98042C, CEV48P and BS15M2A (Figure 3). Black point of seed was observed in all monosporic and polysporic isolates, except for isolates 98031P, 98012C, 98041A and CFO201A. On the other hand, monosporic isolates 98012C, 98041A and CFO201A presented low pathogenicity, with symptoms observed in less than 1% of wheat seedlings and seeds.

DISCUSSION

In general, microorganisms present high genetic diversity, leading to differences in morphology, physiology and pathogenicity. Variations in the use of substrates, tolerance to determined temperature and pH ranges, production of toxins and other metabolites are among the manifestations of physiological distinctions in one population, which often result in variation of pathogenicity of biotypes (Machado, 1980).

The pathogenic fungus *B. sorokiniana* uses all plant organs of winter cereals as substrate. For this reason, two distinct disease stages are discernible: the interference in photosynthesis, when the infection occurs in the aerial parts of the plant, and the interference in the search and absorption of

water and nutrients, which is the stage that affects underground parts (Forcelini, 1991). Here, the presence of symptoms and the wide variation in this pathogen's virulence patterns are reported based on the analysis of pathogenicity variables, which indicate that polysporic isolates are more virulent, with values over 60%, when compared with monosporic isolates, with values over 43% (Table 2). The monosporic cultures were used to reduce the effect of heterokaryosis, since one single conidium may be homokaryotic or present reduced variability, which makes it easier to identify pathotypes based on isolate virulence. This characteristic may be linked with the different genes present in heterokaryotic cells of monosporic isolates, which in turn may manifest in different ways, depending on the quality and quantity of nuclei contained in cells and on the roles played by the environment and the host (Tinline, 1961). This may explain the wider effect spectrum of monosporic isolates on the pathogenicity variables shown in Figure 2.

Pathogenicity tests carried out by Christensen (1925) using 37 monosporic *H. sativum* isolates indicated that 18 formed zones in BDA medium, which differed from the parental colony as to morphology and pathogenicity. In a previous study, the virulence, morphology and growth rate in culture medium of 10 *B. sorokiniana* isolates from different regions in Brazil were analyzed in wheat. Wide variations in morphology and growth rates were observed between parental and re-isolated isolates. However, no relationship between morphological variability and virulence was detected between these two types of isolates (Oliveira et al., 1998) or origin of isolates (Valim-Labres et al. 1997).

The results obtained in the present study show that polysporic

isolates exerted higher pathogenic action, predominantly in seeds, as compared to aerial parts. Often the pathogens that cause common root rot also cause different diseases in one single plant species; however, most specific symptoms in one plant are regulated by infection time and soil conditions, mainly temperature and humidity (Wheeler & Rush, 2001). Our results also reveal that polysporic isolates, which presented high pathogenicity levels in seed germination, did not show the same virulence indices in comparison with the respective monosporic isolates. For example, isolate 98041P inhibited germination in 97% of wheat seeds, while the monosporic isolates generated from the same polysporic strain reduced germination by approximately 50%. According to Mehta (1998), a likely source of variability may be inherent to the fungus itself, since its pathogenicity may vary with time.

Duveiller & García Altamirano (2000) showed that *B. sorokiniana* isolates from different parts of a plant did not cluster according to virulence, when they were reinoculated. In this sense, the authors discovered that the number of leaf spot varies with the isolate used for inoculation, and that this isolate does not depend on the organ from where it was isolated (Duvellier & García Altamirano, 2000). Fetch & Steffenson (1999) observed variation in virulence patterns of *Cochliobolus sativus* in relation to barley cultivars and to the development stage of plants.

In the present study, we observed that the most severe symptoms were associated with germination and black point of the seed, with reduced germination and high levels of rot (Table 2). This condition is due to mainly the hemibiotrophic nature of *B. sorokiniana* and to the complex enzymatic

apparatus it has, which is able to use any organ of a plant as nutritional substrate. The seed is considered one of the most efficient means of transmission and dissemination of phytopatogenic agents, mainly for long distances. In unaffected areas, seeds mediate the introduction of pathogens, which may be spread, selected and distributed by means of primary disease hotspots (Maffia et al., 1988).

The incidence of *B. sorokiniana* in wheat seeds is often observed negatively, affecting germination and triggering the occurrence of symptoms in plants and seeds, and even causing the death of plants (Lasca et al., 1983). The association of the pathogen to seeds is an efficient mechanism of survival and dissemination, and is the main reason behind the outbreaks of epidemics in wheat production regions in Brazil (Goulart et al., 1990). According to Kumar et al. (2002), infections may be so severe that the infected plants wither, without producing one single seed. Under conditions that favor the pathogen’s life cycle, spikelets may be affected, causing seeds to dry out.

The pathogenicity assays were carried out using the wheat cultivar BRS Buriti, which is moderately susceptible to leaf spot and is recommended for the establishment of wheat plantations in winter, in southern Brazil. In this sense, the use of *B. sorokiniana* isolates from different regions of Brazil and the world may indicate that pathogenicity levels differ, which in fact was not observed. The results obtained did not afford to cluster *B. sorokiniana* isolates by geographic origin or the definition of similarity patterns in pathogenic action. Maraite et al. (1998) analyzed 360 wheat leaf samples from 10 countries presenting symptoms of the disease, and did not observe specific relationships

between pathogenicity in terms of geographic origin and genotype.

The most interesting aspects observed in the present study are associated with the wide pathogenic variability of *B. sorokiniana* isolates. Pathogenic action patterns based on conidial origin were established using the correlation (r = 0.5) between the variable leaf spot and polysporic isolates for which we observed the highest values of pathogen virulence.

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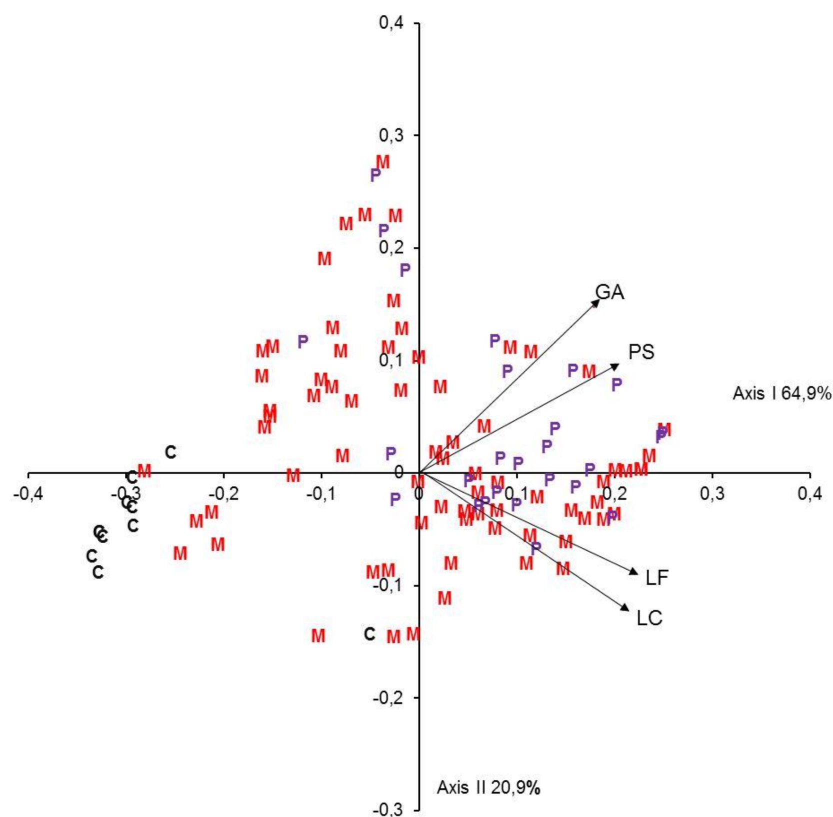

Figure 1 Ordination chart for *B. sorokiniana* isolates in terms of pathogenicity variables constructed based on the principal components analysis and correlation as a measure of similarity between the variables: (A) seed germination aborted (GA); (B) black point of seed (SR); (C) leaf spot/germinated seeds (LS); (D) coleoptile lesion/germinated seeds (CL). The percentage of variation in each axis and the variables that correlated with at least one of the two axes is indicated.

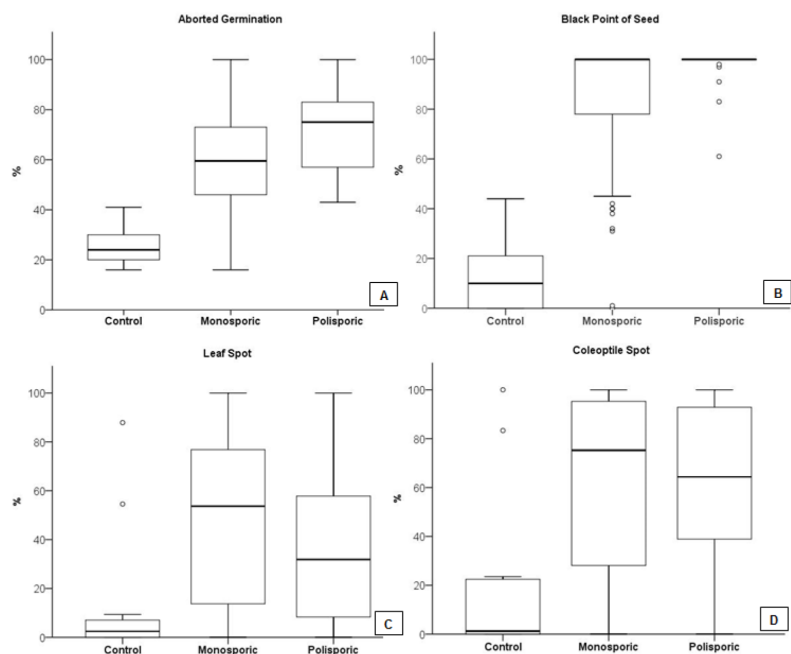

Figure 2. Boxplot of the variables germination aborted (GA), black point of seed (SR), leaf spot/germinated seeds (LS), and coleoptile lesion/germinated seeds (CL) in the pathogenicity test of the monosporic and polysporic isolates of *B. sorokiniana*. The point in the boxes represents the mean.

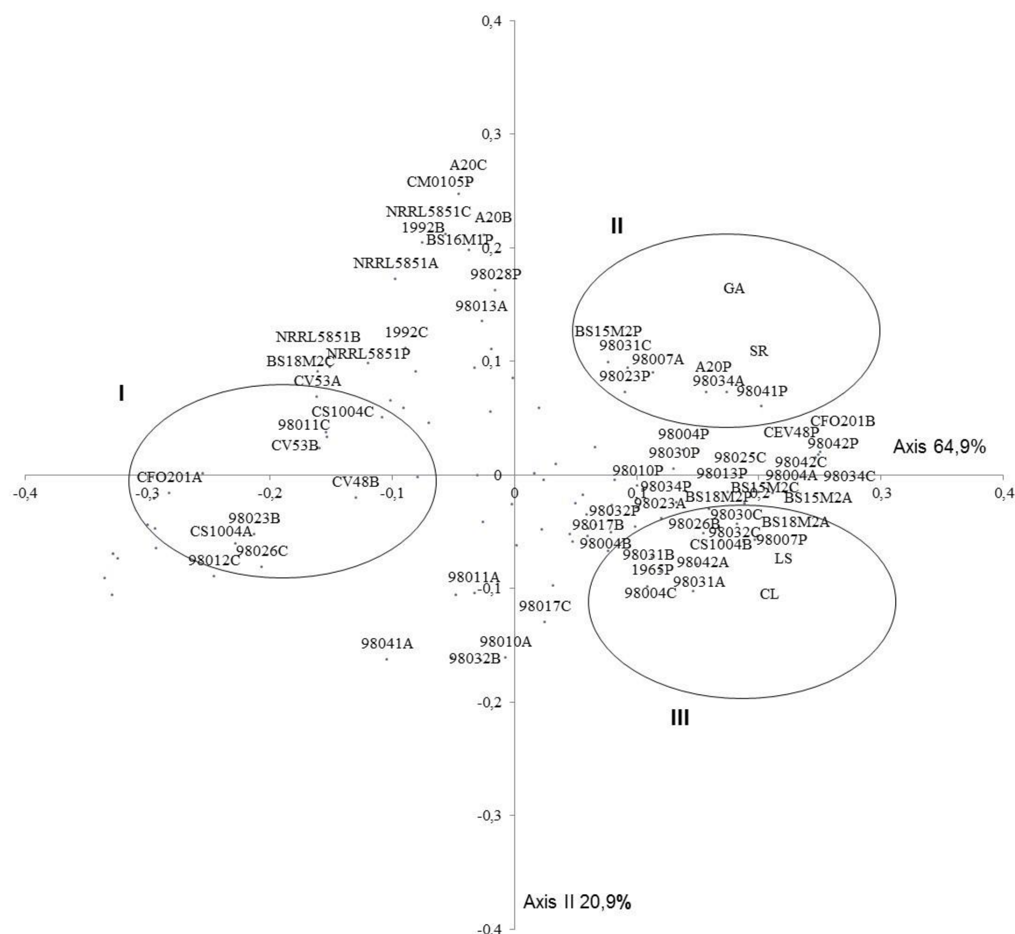

Figure 3 Ordination chart for *B. sorokiniana* isolates in terms of pathogenicity variables constructed based on the principal components analysis and correlation as a measure of similarity between the variables seed germination aborted (GA), black point of seed (SR), leaf spot/germinated seeds (LS), and coleoptile lesion/germinated seeds (CL). The identification codes of isolates with high and low values are shown in axes I and II. Isolates presenting intermediate values were not labeled, and are identified by dots. The ellipsis I indicates isolates with low pathogenicity, ellipsis II signals highly pathogenic isolates affecting mainly seeds, and ellipsis III indicates highly pathogenic isolates affecting mainly aerial parts.

Table 1 Origin of *B. sorokiniana* isolates from different regions in Brazil and other countries.

Isolates code	Origin
98004 P, A, B, C	Cruz Alta, RS –Brasil
98007 P, A, C	Cruz Alta, RS -Brasil
98030 P, A, C	Cruz Alta, RS –Brasil
98032 P, A, B, C	Engenheiro Beltrão, PR –Brasil
CEV53 A, B	Guarapuava, PR –Brasil
98011 P, A, C	Lagoa Vermelha, RS –Brasil
98012 P, A, C	Lagoa Vermelha, RS –Brasil
98031 P, A, B, C	Nova Estância, PR –Brasil
98028 P	Pelotas, RS –Brasil
98025 P, A, C	Piratini, RS –Brasil
98026 P, B, C	Piratini, RS –Brasil
98042 P, A, B, C	Piratini, PR –Brasil
1992 B, C	Planaltina, GO – Brasil
98010 P, A, B, C	Santa Rosa, RS –Brasil
98041 P, A, B, C	União da Vitória, PR –Brasil
98023 P, A, B, C	União da Vitória, PR –Brasil
98013 P, A, B, C	União da Vitória, PR –Brasil
98017 A, B, C	Samambaia, PR –Brasil
CEV48 P, A, B, C	Tapera, RS –Brasil
98034 P, A, B, C	Unknown
NRRL5851 P, A, B, C	África do Sul
CFO201 P, A, B, C	África do Sul
A20 P, A, B, C	Canadá
1965 P	Copenhague-Dinamarca
BS15M2 P, A, B, C	Delicias, Chihuahua – Mexico
BS16M1 P, C	Delícias, Chihuahua- Mexico
BS18M2 P, A, B, C	Poza Rica,Vera Cruz-Mexico
CMO105 P, A, B, C	Mexico
BS52M1 P, A, B,	Monterrey-Nuevo Leon
CS1004 P, A, B, C	Hanoi-Vietnam

P: Polysporic *B. sorokiniana* isolate; A, B, and C: Monosporic *B. sorokiniana* isolate originated from the respective polysporic isolate; CEV: *B. sorokiniana* isolate from barley.

Table 2 Analysis of variance between the treatment groups monosporic and polysporic isolates and control, for the variables seed germination aborted (GA), black point of seed (SR), leaf spot/germinated seeds (LS), and coleoptile lesion/germinated seeds (CL).

Variable	Treatment	N	Mean	Median	Min	Max	SD	Lower limit (LL mean)	Upper limit(UL mean)
Germination aborted (1)	C[a]	11	25.7	24	16	41	7.7	20.6	30.9
	M[b]	74	60.7	59.5	16	100	19.9	56.1	65.3
	P[c]	27	72.4	75	43	100	17.5	65.5	79.3
Black point of seed (2)	C[a]	11	13.2	10	0	44	16.1	2.3	24
	M[b]	74	83.9	100	0	100	27.0	77.6	90.1
	P[c]	27	97.4	100	61	100	8.1	94.2	100
Coleoptile lesion (3)	C[a]	11	9.3	0	0	83.3	24.8	0	26
	M[b]	72	60.7	67.9	0	100	34.3	52.6	68.8
	P[b]	26	69	82	0	100	34.1	55.3	82.8
Leaf spot (4)	C[a]	11	5.6	0	0	54.5	16.3	0	16.6
	M[b]	72	43	44.6	0	100	33.7	35.1	51
	P[c]	26	59.9	65.4	0	100	31.3	47.3	72.6

C: Control, M: Monosporic isolate, P: Polysporic isolate, N: Number of treatments, Min: Minimum seeds, Max: Maximum seeds, SD: Standard deviation, CI: Confidence interval. Groups followed by different letters differ significantly from one another based on the probabilities obtained by the pairwise randomization test: (1) P= 0.001 for C-M. P= 0.001 for C-P and P= 0.01 for M-P; (2) P= 0.001 for C-M. P= 0.001 for C-P and P= 0.009 for M-P; (3) P= 0.005 for C-M. P= 0.001 for C-P and P= 0.02 for M-P; (4) P= 0.001 for C-M.

P= 0.001 for C-P and P= 0.296 for M-P.

CAPÍTULO 2. ARTIGO CIENTÍFICO 2

Genetic diversity among monoconidial and polyconidial isolates of *Bipolaris sorokiniana*

Submetido no periódico:

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Genetic diversity among monoconidial and polyconidial isolates of *Bipolaris sorokiniana*.

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Short title: Genetic diversity of *B. sorokiniana*

Abstract: Spot blotch caused by *Bipolaris sorokiniana* is a destructive disease of wheat in warm and humid wheat growing regions of the world. This fungus shows a high genetic diversity and morphological and physiological variability. In this study 19 polysporic and 57 monosporic isolates of *B. sorokiniana* were characterized using universal rice primers - URP-PCR. The results obtained when the dendrogram was constructed with all the data produced with the amplification products showed very distinct clusters. However, the similarity among the isolates was low where 37% and 26.3% of the monosporic and polysporic isolates respectively showed similarity above 70%. All primers

amplified multiple DNA fragments of polysporic as well as the monosporic isolates. Isolates fingerprints were constructed based on binary characters revealed by the three primers. An amplified fragment of approximately 750bp was observed among 40% of the isolates, when primer URP-1F was used. When primers URP-4R and URP-2R were used a fragment of 450pb and 400pb were present in 31.5% and 29% of the isolates respectively. It was expected a higher similarity among the isolates since the monosporic cultures were originated from the polysporic. The dendrogram did not enable the separation of *B. sorokiniana* isolates by their geographic origin. This low correlation suggests that gene transfer may have occurred by parasexual combination in this fungus population. However, in spite of the research efforts for that end, it has not been possible to establish patterns that characterize the profile of *B. sorokiniana.*

Keywords: *Bipolaris sorokiniana*, URP-PCR, genetic variability, monosporic, polysporic culture

Introduction

Bipolaris sorokiniana Sacc. Shoemaker [teleomorph *Cochliobolus sativus* Ito & Kuribayash (Drechs.)] is a serious phytopathogenic fungus causing spot blotch, root rot, leaf spot disease, seedling blight, head blight, and black point in cereal crops [10,9]. Among these diseases, spot blotch is prevalent in the warm and humid countries, resulting in significant yield losses in wheat plantation [11]. In Brazil, the phytopathogen raises deep concerns especially as regards to wheat production because in all wheat-producing regions *B.*

sorokiniana is found. Both morphological and pathogenic variability in the population of *B. sorokiniana* had been reported by several authors [6,13,16,18].

Molecular methods such as PCR-RAPD [6,12,16] and PCR-RFLP [13], AFLP [19] were used in several studies to assess intra-population genetic diversity in *B. sorokiniana*. Aggarwal et. al [1] used URP-PCR to characterize *B. sorokiniana* from India. Universal rice primers (URPs), which can be used in PCR fingerprinting of various organisms including plants, animals and microorganisms were developed from repetitive sequence of rice genome [7]. The URP-PCR technique is a useful tool for the characterization and grouping of fungal species at the inter- and intra-specific levels [7].

The spot blotch is considered as one of the most important diseases affecting wheat worldwide. Combined strategies to control B. *sorokiniana* in wheat and barley cultures have been recommended. However, these control measures have not produced satisfactory results so far [8]. So it is important to work a way where the knowledge about this fungus will give us enough tools to detect it or to inhibit its growth before its dissemination in the field.

The present study assesses the genetic diversity of monosporic and polysporic *B. sorokiniana* isolates, from Brazil and different countries using URP-PCR and compare the diversity between these two groups of isolates.

Materials and methods

Origin of microorganisms

The fungal isolates from different regions in Brazil were provided by Empresa Brasileira de Pesquisa Agropecuária - Trigo (EMBRAPA- Trigo, Passo

Fundo), while the other isolates used in this study were kindly by provided the International Maize and Wheat Improvement Center (CIMMYT – México). All isolates used were obtained from seeds and tissues of wheat plants. The biological material was deposited in the collection of the Environmental Mycology Laboratory, of the Departamento de Microbiologia, Imunologia e Parasitologia, UFRGS. In the genetic diversity assay, 19 polyconidial isolates and 57 monoconidial *B. sorokiniana* isolates were used for inter and intra-population diversity (Table 1).

Monoconidial cultures

The monoconidial cultures were obtained from the aerial mycelia of the 19 polyconidial cultures grown on Petri dishes with potato dextrose agar (PDA). A 0.85% saline solution was poured over the grown colonies, and the conidia were transferred to microcentrifuge tubes. The tubes were vortexed thoroughly to achieve complete conidia release. The suspension was transferred to a Petri dish with PDA and incubated at room temperature for 2h. Using a stereomicroscope with optical magnification of 40x, the conidia were transferred using plates with PDA media. The plates were incubated at 24 ±2ºC until complete developed of the colonies, and then were stored at 4ºC From each polysporic culture three spores were selected and each spore culture was designated with a letter (A, B, and C). The criteria used to select the spores for monoconidial cultures was randomly since the spores were most distant one from another.

Genomic DNA extraction

Genomic DNA was extracted following the protocol developed by Ashktorab and Cohen [2] with adaptations for this fungus. The isolates were grown in conical flasks containing 300 mL potato-dextrose broth (PD). After inoculation, incubation took place at at 25°C ± 2 under orbital agitation of 120 rpm for 7–10 days in order to obtain 300mg of mycelia (wet weight). After incubation, the mycelium of each isolate was strained and washed three times with sterile distilled water, and excess water was removed using filter paper. Weighed mycelium was ground to fine powder using liquid nitrogen. The powder was transferred to a tube, and 1.5mL of extraction-lysis buffer (200mM Tris–HCl, pH 8.0; 250mM NaCl; 25mM EDTA, pH 8.0; 2% SDS) per gram of mycelium was added along with 10 µL/mL β-mercaptoethanol and 50µg/mL proteinase K. The tubes were incubated in a water bath at 65 °C for 1h, shaken every 15min, and then centrifuged at 2795 ×*g* for 20 min. The supernatant was transferred to clean centrifuge tubes and DNA was purified twice with 1 volume of phenol (pH 8.0) and three times with 1 volume of phenol–chloroform (0.5:0.5 v/v). Finally, 1 volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the supernatant. Pancreatic RNAse I (50µg/mL) was added to the aqueous phase resulting from the centrifugation and the mixture was incubated at 37°C for 20min. DNA was precipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of isopropanol at –20°C. DNA was collected with a glass rod, transferred to a falcon tube, washed with 70% ethanol (v/v), and centrifuged at 2795 × *g* for 10 min. DNA was allowed to dry at room temperature and was then resuspended in Milli-Q water and stored at –20°C.

DNA amplification by URP-PCR

Genomic DNA were amplified using the primers URP-1F: **5′**ATCCAAGGTCCGAGACAACC**3′**;URP-2R: **′**CCCAGCAACTGATCGCACAC**3′** and URP-4R: **5′**AGGACTCGATAACAGGCTCC**3′**. PCR reactions were performed in a 25µL final volume containing 50ng of genomic DNA, 1U Taq DNA *polymerase*, 20pmol primer, 0.2mM dNTP, 1× reaction buffer, 4mM $MgCl_2$, 200ng/µL bovine serum albumin, and sterile Milli-Q water. The amplification conditions were adapted according to the samples used in this work to obtain the optimal products. All amplifications were performed in an Eppendorf Mastercycler Personal thermal cycler (Canada) under the following conditions: one cycle at 94°C for 4 min followed by 35 cycles at 94°C for 1min, 55°C for 1min and 72°C for 2min, and a final extension at 72°C for 7min. Amplification products were separated by electrophoresis in 1.5% (w/v) agarose gel, stained with ethidium bromide, and photographed under UV light using a Vilber Lourmat Doc-Print II. Digital photos of the gels were taken using Kodak DC 120 digital camera and Kodak 1D (version 3.5.2) software. All amplifications were repeated at least twice for each isolate in separate experiments.

Statistical analysis

In order to indentify similarity patterns between isolates based on their genetic characterization and taking polysporic and monosporic isolates together, a clustering and ordination analysis was conducted using a binary matrix (0 for absence, 1 for presence of fragment in one isolate). The clustering analysis was done using Jaccard´s coefficient of similarity and the unweighted

pair-group method with arithmetic averages (UPGMA) algorithm. The data sets were considered clustered if they had a similarity coefficient above 70%. All amplifications were repeated at least twice for each isolate in separate experiments.

Results and discussion

B. sorokiniana in general present high genetic diversity, leading to differences in morphology, physiology and pathogenicity. A previous work done by our group to evaluate the similarity patterns between isolates considering the pathogenicity variability of *B. sorokiniana* isolates demonstrated that polysporic isolates were more virulent than monosporic isolates, preferentially over seeds, compared with the aerial parts of plant.

Mann et al. [10] used 12 URP primers in the molecular characterization of 60 monosporic *B. sorokiniana* isolates from Brazil and other parts of the world and the PCR amplification generated 232 different DNA fragments. Primers URP-4R, URP-2R, and URP-1F generated greater numbers of amplified fragments from the single-spore isolates, and polymorphism was observed among the isolates generated using these primers. Taking in consideration the results obtained by Mann et al. [10] using the monosporic cultures and the work done with the pathogenicity of all these isolates, the same three primers URP-1F, URP-2R e URP-4R were used in this work, in order to compare the DNA genetic diversity of the polysporic and the monosporic isolates. All primers amplified multiple DNA fragments with all isolates. A fragment of approximately 750bp was observed among 40% of the monosporic

and polysporic isolates, when amplified with primer URP-1F. In the same way when primers URP-4R and URP-2R were used a fragment of 450bp and 400bp were present in 31.5% and 29% of the isolates respectively. The results obtained in this work show a genetic diversity even among the monosporic isolates originated from the same polysporic isolate. Fig. 1A shows some of this behaviour when a fragment of approximately 350bp is present in isolates 98026B and 98032B however absent in in isolates 98026C and 98032C respectively. Also a fragment of approximately 1100bp is present in isolates 98041A and 98041C and absent in all the others. Isolate NRRL5851P from South Africa shows a completely different profile (Fig. 1B). No monomorphic fragment was observed among all isolates not even among the isolates from Brazil. Aggarwal et al. [1] assessed the genetic diversity of *B. sorokiniana*, using 12 URP-PCR primers to characterize isolates from different places in India. In their work one primer produced monomorphic fragments that could be used as molecular markers. Also, since all isolates were from different regions from India they were able to group these isolates by geographic origin.

The dendrogram constructed with the data generated from the amplifications of the monosporic and polysporic isolates using the primers URP-1F, URP-2R and URP-4R exhibit two very distinct clusters (Fig. 2). The dendrogram shows for the monosporic isolates 10 clusters with more than 70% of similarity what correspond to 37% of the isolates. Out of that 4 groups showed a coefficient of similarity of 100%. The monosporic isolates 98023, BS16M1, BS52M2, CMO105, NRRL5851, A20 from different geographic origins were the only ones to group with their conidia A, B e C, with a similarity upper to

70%. This result still shows the high diversity among this phytopathogen. In all the clusters isolates from Brazil did not group with the ones from other countries. On the other hand with the polysporic isolates two groups were formed with a cut off of 70% and one of this subgroup was formed by isolates from Brazil and Mexico (Fig. 2). Historical data about wheat in Brazil (Empresa Brasileira de Pesquisa Agropecuária - Trigo EMBRAPA, 2013) showed that when the crop culture was inserted in the State of Rio Grande do Sul tall cultivars showed strong losses due to flattened the wheat straws. This problem was solved with the introduction of cultivars developed by CIMMYT (Mexico), thus it might be possible to infer that the group formed by Brazil and Mexico, can be explained by the susceptibility profile of the seed used by both countries in relation to *B. sorokiniana.*

Analyzing the matrix constructed with the amplification data it is was observed a coefficient of similarity with values lower than 10% among the polysporic and monosporic isolates. These results suggest high genetic diversity for both monoconidial and polyconidial isolates. Oliveira et al. [16] studying the virulence, genetic diversity, morphology and growth rate in culture medium of 10 *B. sorokiniana* isolates, from different regions in Brazil found a wide variation in morphology and growth rate between parental and re-isolated isolates from the infected plants assay. However, no relationship between morphological and genetic variability was detected between these two groups of isolates.

The monoconidial cultures were used to reduce the effect of heterokaryosis, since one single conidium may be homokaryotic or present

reduced variability, which makes it easier to identify pathotypes based on isolate virulence. This characteristic may be linked with the different genes present in heterokaryotic cells of monoconidial isolates, which may manifest in different ways, depending on the quality and quantity of nuclei contained in cells and on the action of the environment and of the host [17]. Chand *et* al. [3] working with natural populations of *B. sorokiniana* from India concluded that the variability in *B. sorokiniana* was revealed in different combinations of the nuclei.

Poloni *et* al. [15] working with monoconidial and polyconidial cultures of *B. sorokiniana,* analyzing the morphological diversity of the isolates when different carbon source were used in the media observed a very high diversity, for polyconidial cultures, when grown in Sabouraud maltose these same differences were not observed for the monoconidial cultures. Accordingly Farias et al. [4] apart from the fact that *B. sorokiniana* has predominantly asexual, haploid and heterocaryotic reproduction, the variability in virulence suggests that extensive genetic exchange occurs in this species. This variability may be explained by the presence of nuclei in the mycelium and conidia and the variation can be generated when the exchange of nuclei is followed by nuclear fusion, somatic recombination, and consequently chromosomal rearrangement for the haploidization. In this work the genetic diversity of *B. sorokiniana* isolates is indicated by the formation of a reduced number of clusters, both of monosporic and polysporic isolates and by the presence of dissimilarity pattern for most isolates. Zhong and Steffenson [19] detected 95 AFLP polymorphic markers for *C. sativus* using eight pairs of primers, and reported that each isolate of the pathogen exhibited one single AFLP pattern.

Ghavini and Tekauz [5] investigated the genetic diversity of 93 *B. sorokiniana* isolates divided in eight different virulence groups by AFLP. The analysis revealed that the isolates, collected in different regions in Canada and other countries, presented high genetic diversity, but only two groups were discriminated using the technique. Nizam et al. [14] in order to study different stages of hyphal fusion and pathogenic mechanisms of *B. sorokiniana*, two fluorescence markers the red fluorescent protein (DsRed-Express) and the green fluorescent protein (EGFP1) were constitutively expressed. The transformation method showed to be reliable and suitable for the functional genomics studies of the fungus and for further investigations into the molecular mechanisms of pathogenesis in *B. sorokiniana*.

Based on previously published results it is possible to infer that the morphologic, physiologic and genetic diversity exhibited by *B. sorokiniana* has been studied with the aim of enlarging the knowledge on virulence and severity of this pathogen especially in wheat culture. However, in spite of the research efforts for that end, it has not been possible to establish patterns that characterize the profile of *B. sorokiniana*.

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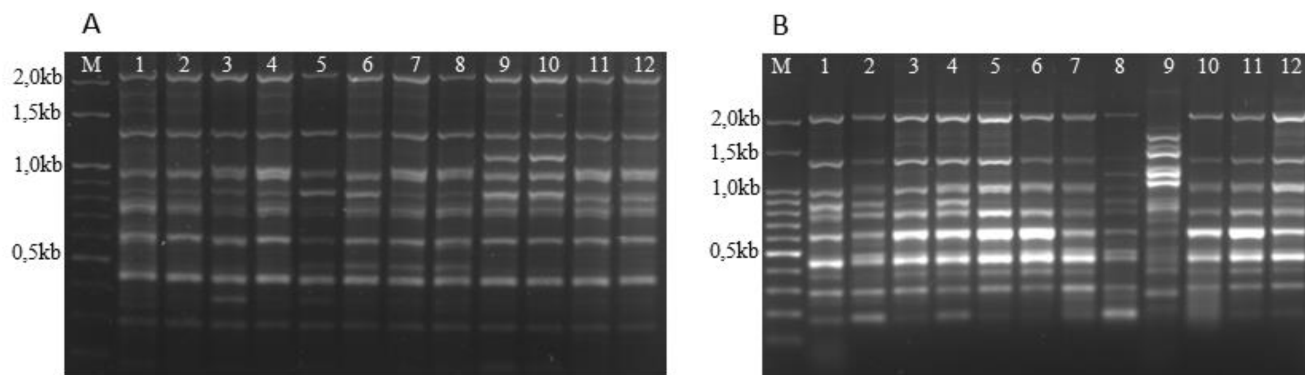

Figure 1 Genomic DNA amplification products of some *Bipolaris sorokiniana* obtained using primer URP-1F: (A) monosporic isolates: (M) DNA Marker Ladder 100 bp; (1) 98013A; (2) 98013B; (3) 98026B; (4) 98026C; (5) 98032B; (6) 98032C; (7) 98034B; (8) 98034C; (9) 98041A; (10) 98041C; (11) BS16M1A; (12) BS16M1C; (B) polysporic isolates: (M) DNA Marker Ladder 100 bp; (1) 98011P; (2) 98012P; (3) 98013P; (4) 98023P; (5) 98026P; (6) BS18M2P; (7) 98034P; (8) 98041P; (9) NRRL5851P; (10) BS15M2P; (11) BS52M2P; (11) CS1004P.

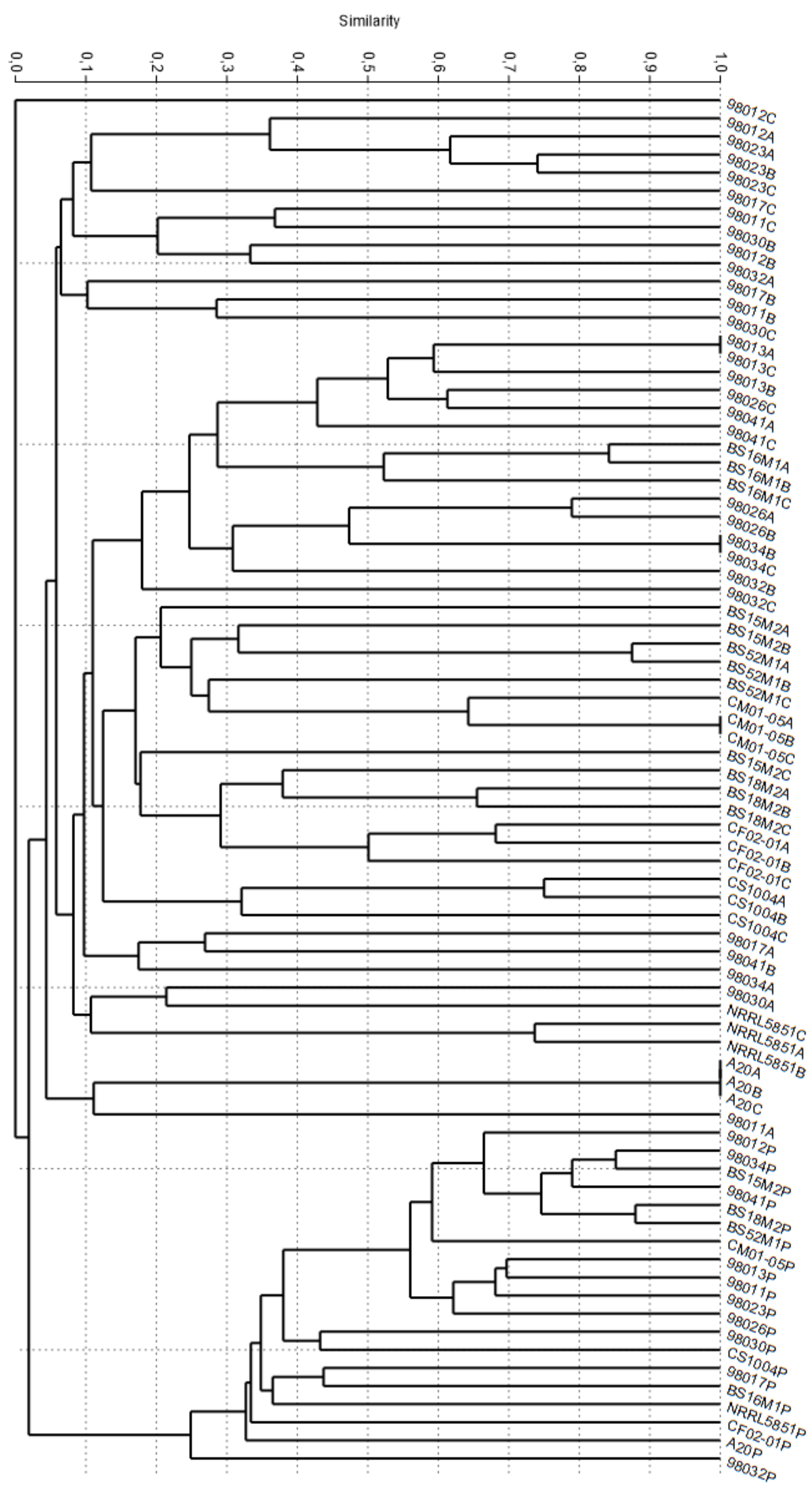

Figure 2 A dendrogram of monosporic and polysporic *Bipolaris*

sorokiniana isolates amplified with primers URP-1F, URP- 2R and URP-4R with a cut off above 70% of similarity. A data matrix was generated by scoring the presence or absence of a fragment as 1 and 0 respectively. With these data a similarity matrix was constructed with PAST (Paleontological Data Analysis) using Jaccard´s coefficient of similarity. The dendrogram was reproduced using UPMGA (unweighted pair-group method with arithmetic averages).

Table 1. Origin of *B. sorokiniana* isolates from different regions in Brazil and other countries.

Isolate code	Location, State, Country
98030 P, A, B, C	Cruz Alta, RS –Brasil
98032 P, A, B, C	Engenheiro Beltrão, PR - Brasil
98011 P, A, B, C	Lagoa Vermelha, RS -Brasil
98012 P, A, B, C	Lagoa Vermelha, RS -Brasil
98026 P, A, B, C	Piratini, RS –Brasil
98023 P, A, B, C	União da Vitória, PR -Brasil
98013 P, A, B, C	União da Vitória, PR -Brasil
98041 P, A, B, C	União da Vitória, PR -Brasil
98017 P, A, B, C	Samambaia, PR –Brasil
98034 P, A, B, C	unknown origin
NRRL5851 P, A, B, C	África do Sul
CFO201 P, A, B, C	África do Sul
A20 P, A, B, C	Canadá
BS15M2 P, A, B, C	Delicias, Chihuahua – Mexico
BS16M1 P, A[1], B[1], C	Delícias, Chihuahua- México
BS18M2 P, A, B, C	Poza Rica,Vera Cruz-México
CMO105 P, A, B, C	México
BS52M1 P, A, B, C	Monterrey-Nuevo Leon
CS1004 P, A, B, C	Hanoi-Vietnam

P: Polysporic *B. sorokiniana* isolates; A, B, and C: Monosporic *B. sorokiniana* isolate originated from the respective polysporic isolates.

CAPÍTULO 3. ARTIGO CIENTÍFICO 3

Análise do perfil isoenzimático de isolados do fitopatógeno de trigo *Bipolaris sorokiniana*.

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Análise do perfil isoenzimático de isolados do patógeno de trigo *Bipolaris sorokiniana*.

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RESUMO

O fungo filamentoso *Bipolaris sorokiniana* é um fitopatógeno que causa moléstias em cereais de inverno, tais como mancha marrom, ponta preta do grão e a podridão comum da raiz. Este fungo utiliza como estratégia de obtenção de nutrientes, o parasitismo necrotrófico e biotrófico, para colonizar a planta hospedeira. O seu controle é dificultado por este apresentar uma ampla variabilidade morfológica, fisiológica e genética. O objetivo deste trabalho foi investigar a presença de isoenzimas em isolados monospóricos e polispóricos de *Bipolaris sorokiniana* e avaliar a sua diversidade nas diferentes culturas. Foram avaliados quanto à presença de isoenzimas em 78 isolados de *B. sorokiniana* sendo destes 26 isolados policonidiais e 52 monoconidiais. Com os resultados obtidos foi observado que os todos isolados monospóricos, bem como, os polispóricos foram positivos para todos os sistemas enzimáticos testados, no entanto, se diferenciaram quanto à intensidade e número de bandas quando observados em eletroforese em gel de poliacrilamida. A enzima álcool desidrogenase, glutamato desidrogenase, superóxido dismutase, peroxidase apresentaram uma banda monomórfica, enquanto que a enzimas fosfatase ácida, esterase, glicose desidrogenase, glicerol 3 fosfato desidrogenase e malato desidrogenase apresentaram um perfil polimórfico. A análise do dendrograma permitiu observar dois grupos bem definidos entre isolados oriundos do Brasil e os isolados oriundos de outros países.

Palavras chaves: Isoenzimas, fungo fitopatogênico, cereais.

ABSTRACT

The filamentous fungus *Bipolaris sorokiniana* is a pathogen that causes diseases in winter cereals such as brown spot, black point of the grain and common root rot. The strategy of this pathogen to obtain nutrients is the, necrotrophic and biotrophic parasitism, to colonize the host plant.The control of this fungus is very difficult do to its wide morphological, physiological and genetic variability. The main objective of this study was to investigate the presence of isoenzymes in monosporic and polisporic isolates of *Bipolaris sorokiniana* and analyse the diversity os the different cultures. The presence of the isozymes was analysed for 78 isolates of *B. sorokiniana*, out of that 26 were polyconidial isolates and 52 monoconidial. With the results obtained it was observed that all monosporic isolates, as well as the polysporic were positive for all enzyme systems tested, however, differ regarding the intensity and number of bands as observed in the electrophoresis gel polyacrylamide. The alcohol dehydrogenase, glutamate dehydrogenase, superoxide dismutase, peroxidase showed a monomorphic band, while acid phosphatase enzymes, esterase, glucose dehydrogenase, glycerol 3 phosphate dehydrogenase and malate dehydrogenase had a polymorphic profile. The dendrogram shows two groups very distinct among the isolates from Brazil from the one’s of other countries.

Key words: Isoenzimes, phytophatogenic fungus, cereals

INTRODUÇÃO

Fungos patogênicos dependem da obtenção de nutrientes (vitaminas, aminoácidos e etc.) que são produzidos somente por células vivas ou a partir da morte e degradação dessas células, para sua sobrevivência. Dessa forma, dependendo da estratégia de obtenção desses nutrientes, o parasitismo pode ser necrotrófico ou biotrófico. Para o fungo patogênico colonizar a planta hospedeira, inicialmente ele precisa quebrar o sistema de defesa, produzir enzimas hidrolíticas, que quebram a cutícula e a parede da célula hospedeira, e toxinas que reduzem ou inibem completamente a atividade das células da planta a ser parasitada (Esposito & Azevedo, 2010). O fungo hemibiotrofico *Bipolaris sorokiniana* (teleomorph: Cochliobolus sativus) é um importante agente patogénico do trigo (*Triticum aestivum*) e da cevada (*Hordeum vulgare*) este patógeno é globalmente conhecido por causar danos significativos na agricultura (Kumar *et al*. 2002). Alta temperatura e umidade relativa do ar favorecem o aparecimento da doença (Aggarwal *et al.*2000).

Este fungo tem sido identificado através de marcadores morfológicos, biológicos e moleculares. Poloni *et al.*(2008) avaliou culturas monoconidiais e policonidiais de *B. sorokiniana*, quanto a diferentes fontes de carbono e observou uma diversidade muito elevada entre isolados. Müller *et al.*(2005), Jaiswal *et al*. (2007) utilizaram RAPD na caracterização deste microrganismo. Nascimento & Van Der Sand (2008) utilizou análise das regiões ITS para caracterizar este patógeno. A análise isoenzimática é uma técnica bioquímica com inúmeras aplicações em fitopatologia, tem sido muito utilizada pelos geneticistas para estudar a genética de diversas populações. As isoenzimas

são definidas como múltiplas formas moleculares de uma única enzima que possuem geralmente propriedades enzimáticas semelhantes ou idênticas, mas composições de aminoácido ligeiramente diferente devido às diferenças presentes na sequência de nucleotídeos do DNA que codifica para a proteína. Este trabalho teve por objetivo investigar a presença de isoenzimas em isolados monospóricos e polispóricos de *Bipolaris sorokiniana* e avaliar diversidade das diferentes culturas.

MATERIAS E MÉTODOS

Cultura fúngica

Neste trabalho foram utilizados isolados fúngicos de *B. sorokiniana* de diferentes regiões do Brasil, fornecidos pela Empresa Brasileira de Pesquisa Agropecuária - Trigo (EMBRAPA- Trigo, Passo Fundo), enquanto que os outros isolados internacionais foram gentilmente cedidos pelo Centro Internacional de Melhoramento de Milho e Trigo (CIMMYT – México). Todos os isolados utilizados foram obtidos a partir de sementes de tecidos de plantas de trigo, oriundo da coleção do Laboratório de Micologia Ambiental, DMIP, ICBS, UFRGS. Um total de 78 isolados de *B. sorokiniana* foram usados no ensaio isoenzimático, onde 26 foram policonidiais e 52 monoconidiais (Tab. 1).

Monoconidial cultures

As culturas monoconidiais foram obtidas a partir dos micélios aéreos das culturas policonidiais cultivadas em placas com ágar batata dextrose (BDA). Sobre as colônias verteu-se uma solução salina 0.85% e os conídios

foram transferidos para tubos de microcentrífuga. Os tubos foram cuidadosamente homogeneizados para conseguir a liberação completa dos conídios. A suspensão foi transferida para uma placa de Petri com BDA e incubada à temperatura ambiente durante 2h. Usando um microscópio estereoscópico com ampliação ótica de 40 x, os conídios foram transferidos para placas com meio BDA. As placas foram mantidas a 24 ± 2°C até o completo desenvolvimento das colónias em seguida foram armazenados a 4ºC. A cada cultura de esporos foi designado uma letra (A, B e C).

Preparação da amostra

Para analise isoenzimática, isolados foram crescidos em Erlenmeyer de 250mL contendo 100mL de caldo batata dextrose (20% batata, 2% dextrose (w/v)), por 10 dias a 24 ± 2°C. Após esse período, o micélio foi separado por filtração, lavado três vezes com água estéril e transferido para um gral, macerado utilizando nitrogênio liquido até ficar um pó fino.

Extração enzimática e PAGE

A extração das proteínas e eletroforese foi realizada conforme descritos por (Alfenas *et al.*(1991). Foi pesado 200mg de pó micelial e adicionado 1mL de solução tampão Tris- HCl 0,617M, pH 6,8, a mistura foi homogeinizada e mantida sob refrigeração a 4^0C, por 16 horas. Após este período a mistura foi centrifugada a 13.000g por 20min e 200 μL do sobrenadante foi coletado, o que constituiu o extrato protéico, a este foi acrescido 100 μL de tampão de amostra. A eletroforese foi realizada utilizando

um sistema descontinuo (Sambrook *et al.*1989) gel separador com concentração de 10% e um gel empilhador com concentração 4,5% e submetido a uma corrente de 100W e 50mA/ 3h. Posteriormente os géis foram revelados conforme tabela 2.

Sistemas Enzimáticos

Foram testados os seguintes sistemas isoenzimáticos: fosfatase ácida (ACP), esterase (EST), álcool desidrogenase (ADH), glicose desidrogenase (GDH), glutamato desidrogenase (GTDH), glicerol 3 fosfato desidrogenase (G3PDH), superóxido dismutase (SOD), peroxidase (PO) e malato desidrogenase (MDH).

Analise de dados

A interpretação dos resultados foi baseada na análise visual dos géis de eletroforese, levando-se em consideração a presença, a ausência e a intensidade e o número de bandas. Estes dados foram utilizados para a construção de uma matriz binária, considerando 1 para presença da banda e 0 para ausência. A matriz foi analisada utilizando PAST por meio do coeficiente de similaridade de Jaccard. O dendrograma foi produzido usando o método UPGMA (*Unweighted Pair Group Method with Arithmetic Mean)*.

RESULTADOS E DISCUSSÃO

Para avaliar a fisiologia dos isolados monospóricos e polispóricos de *B. sorokiniana* foram utilizados nove marcadores bioquímicos. Os marcadores bioquímicos e moleculares são considerados importantes ferramentas para o estudo da diversidade de patógenos em que as características morfológicas

não são adequadas para distinguir corretamente diferentes isolados (Sharma *et al.*2005). Além disso, características morfológicas também são influenciadas pelas condições ambientais e culturais.

A partir de cada cultura polispóricas, foram obtidas duas culturas monospóricas. A obtenção destas culturas teve como objetivo diferenciar isolados monospóricos e polispóricos e o comportamento fisiológico frente aos diferentes sistemas enzimáticos. Os resultados mostraram que todos os isolados monospóricos, bem como os polispóricos, foram positivos para todos os sistemas enzimáticos testados, no entanto, se diferenciaram quanto à intensidade e número de bandas. A análise do dendrograma com todos isolados e todas as enzimas, mostrou dez agrupamentos bem definidos entre isolados do Brasil e internacionais, com exceção do isolado 1992 de origem brasileira, que agrupou-se com os isolados internacionais (Fig. 1).

De todos os sistemas testados as enzimas álcool desidrogenase (E.C. 1.1.1.1), glutamato desidrogenase (E.C. 1.4.1.4), superóxido dismutase (E.C. 1.15.1.1), peroxidase (E.C. 1.11.1.7) apresentaram apenas uma banda, enquanto que variantes isoenzimáticas foram detectadas para as enzimas as enzimas fosfatase ácida (E.C. 3.1.3.2), esterase (E.C. 3.1.1.1.1), glicose desidrogenase (E.C. 1.1.1.118), glicerol 3 fosfato desidrogenase (E.C.1.1.1.8) e malato desidrogenase (E.C.1.1.1.37), que apresentaram um perfil polimórfico.

Todos isolados quando submetidos à revelação para a isoenzima fosfatase ácida obtiveram perfil positivo com uma variação de uma a duas bandas. Os isolados pertencentes ao Brasil apresentaram uma maior intensidade de bandas quando comparados com os isolados internacionais

formando um agrupamento que se diferenciou quanto à região geográfica. No entanto, o isolado 1992, oriundo de Planaltina- GO, exibiu um perfil semelhante aos isolados internacionais apresentando uma menor intensidade nas bandas. Lima & Menezes (2002) em um estudo com *Colletotrichum graminicola*, que observaram duas bandas para enzima fosfatase ácida com variação na intensidade e migração no gel em todos os isolados analisados.

A enzima esterase apresentou um fenótipo monomórfico para isolados pertencentes ao Brasil e para os isolados internacionais um perfil polimórfico havendo uma variação de 1 a 6 bandas. Novamente o isolado brasileiro 1992, mostrou um perfil polimórfico semelhante aos isolados internacionais. A enzima esterase tem um papel importante para fungos patogênicos por estar associada com fenômenos de penetração na cutícula, que estão presentes na mucilagem que cobre esporos, alteram a superfície da planta produzindo diferentes propriedades adesivas (Pascholati *et al.*1993). Diante deste fato, podemos inferir que variabilidade no perfil apresentado pelos isolados internacionais quando comparados com os brasileiros pode estar associada à patogenicidade dos isolados e sua origem geográfica. Neste estudo com esterase, observado que a enzima está presente em fungos patogênicos por estar associada com fenômenos de penetração na cutícula do grão.

Os resultados obtidos com a isoenzima álcool desidrogenase formaram dois agrupamentos, os isolados do Brasil com bandas com baixa atividade por consequência bandas menos intensas e o grupo internacional com maior atividade e bandas mais visíveis. Na análise dos dendrogramas para a isoenzima álcool desidrogenase (ADH), os isolados internacionais agregaram-

se com os isolados CEV 48P, A e C da região de Tapera-RS e 1992P, B e C com 100% de similaridade. Avaliado o resultado obtido para a enzima álcool desidrogenase foi possível observar que o isolado (CEV48), de cevada, apresentou bandas mais visíveis quando comparados com isolados brasileiros oriundos de trigo. Esse resultado pode indicar uma maior expressão da enzima para aquisição de nutrientes e com isso haver uma maior penetração na célula hospedeira. Pathuri *et al.* (2011) estudando susceptibilidade de cevada ao parasita *Blumeria graminis* f.sp. *hordei*, determinaram que ADH parece ser importante para manter o metabolismo de energia primária da célula hospedeira sob condições de aumento do fluxo metabólico e uma baixa pressão de oxigénio no local da infecção.

As isoenzimas glutamato desidrogenase, superóxido dismutase e peroxidase apresentaram apenas uma banda monomórfica sem variação de intensidade. Com os resultados dessas isoenzimas não foi possível agregar nenhuma informação a mais para que se pudesse estabelecer uma relação com a planta.

Os resultados obtidos com a enzima superóxido dismutase (SOD) não diferenciaram os isolados. Desta forma podemos inferir que esta enzima pode estar associada ao metabolismo basal do patógeno a sua importância biológica e de sobrevivência durante o processo de infecção. No trabalho realizado por Lambou *et. al.*(2010) com a análise funcional da família superóxido dismutase em *Aspergillus fumigatus* foi mostrado que a SOD pode ter funções metabólicas essenciais como controle de dormência e germinação de esporos.

A avaliação do dendrograma possibilitou observar um agrupamento bem definido entre isolados do Brasil e os oriundos de outros países.

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Tabela 1. Origem dos isolados de *Bipolaris sorokiniana.*

Código do Isolado	**Procedência**
98004 P, A, C	Cruz Alta, RS –Brasil
98007 P, A, C	Cruz Alta, RS -Brasil
98030 P, A, C	Cruz Alta, RS –Brasil
98032 P, A, C	Engenheiro Beltrão, PR Brasil
98011 P, A, C	Lagoa Vermelha, RS – Brasil
98012 P, B, C	Lagoa Vermelha, RS – Brasil
98031 P, A, C	Nova Estância, PR –Brasil
98034 P, A, C	Desconhecida
98025 P, A, C	Piratini, RS –Brasil
98026 P, B, C	Piratini, RS –Brasil
98042 P, A, C	Piratini, PR –Brasil
1992 P, B, C	Planaltina, GO – Brasil
98010 P, A, B	Santa Rosa, RS –Brasil
98023 P, A, B	União da Vitória, PR –Brasil
98013 P, B, C	União da Vitória, PR –Brasil
98041 P, B, C	União da Vitória, PR –Brasil
NRRL5851 P, B, C	África do Sul
CFO201 P, A, B	África do Sul
A20 P, B, C	Canadá
CEV48 P, A, C	Tapera, RS –Brasil
BS15M2 P, A, B	Delicias, Chihuahua – Mexico
BS16M1 P, A, C	Delícias, Chihuahua-México
BS18M2 P, B, C	Poza Rica,Vera Cruz-México
CMO105 P, B, C	México
BS52M1 P, B, C	Monterrey-Nuevo Leon
CS1004 P, A, B	Hanoi-Vietnam

P = Isolados polispóricos   A, B, C = Isolados monospóricos

Tabela 2. Sistemas e soluções utilizadas para análise das enzimas.

Enzima	Substrato/Sal	Tampão	Corante	Coenzima
Fosfatase ácida	$MgCl_2$ 1M, β-nafitilfosfato ácido de sódio1% em acetona 50%	Acetato 50mM, pH 5,5	Fast Black sal (50mg)	
Esterase	β- naftil acetato, α- Naftil acetato 1%, água destilada	Tris 0,5M pH 7,1	Fast Black sal (50mg)	
Álcool Desidrogenase	Etanol	Tris -HCl 0,1M pH 7,5	PMS(2mg), MTT(10mg)	NAD^+ (15mg)
Glicose desidrogenase	Glicose (9g)		PMS(5mg), MTT(5mg)	NAD^+ (20mg)
Glutamato desidrogenase	Glu,Na(400mg), $CaCl_2$ 10mM	Tris 0,1M pH 7,5	PMS(2mg), NBT(10mg)	NAD^+ (15mg)
Glicerol 3 fosfato desidrogenase	Piruvato de sódio (200mg)	Tris 0,06M pH 8,0 , D-L glicerol-3 fosfato Na_2 5 H_2O_2 (650mg), ágar 2%	PMS(1mg), MTT(1mg)	NAD^+ (20mg)
Superóxido Dismutase	Ribflavina (1mg), TEMED	Fosfato 50mM pH 7,5	MTT (20mg)	
Peroxidase	Metanol, H_2O_2	Acetato 0,1M pH 4,5, metanol	TMBZ(50mg)	
Malato Desidrogenase	DL Malato1M pH 7,5	Tris 0,1M pH 7,5	PMS(2mg), NBT(10mg)	NAD^+ (15mg)

Figura 1. Dendrograma com isolados de *Bipolaris sorokiniana* utilizando todos os sistemas enzimáticos. A matriz de dados foi gerada atribuindo 1 para presença e 0 para ausência de fragmento. A partir desses dados, uma matriz de similaridade foi construída com PAST (Paleontológico de Análise de Dados) por meio do coeficiente de similaridade de Jaccard. O dendrograma foi produzido usando o método UPGMA (Unweighted Pair Group Method with Arithmetic Mean). BR- Brasil, INTER- Internacional e CEV- Cevada

CAPÍTULO 4. ARTIGO CIENTÍFICO 4

Polymorphism in the internal transcribed spacers (ITS) and 5.8S of the ribosomal DNA of *Bipolaris sorokiniana* by PCR -RFLP

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Polymorphism of the internal transcribed spacers (ITS) and 5.8S of the ribosomal DNA of *Bipolaris sorokiniana* by PCR -RFLP

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Abstract

Spot blotch is one of the main wheat diseases and causes significant economic losses Worldwide. Its causal agent is *Bipolaris sorokiniana*, a phytopathogen with consistent morphological, physiological and genetic diversities. This study characterizes the molecular diversity of monospore and polyspore *B. sorokiniana* isolates from Brazil and other countries. Twenty-three polyconidial and 46 monoconidial isolates had the region ITS1-5.8S-ITS2 of rDNA amplified and digested with restriction enzymes. Profiles were analyzed by the UPGMA method. PCR – RFLP revealed important information on the genetic profiles of monospore and polyspore cultures, showing the distinctions between the three

obtaind isolates from the same polyspore strain. The dendrograms generated with the fragment obtained in the RFLP assay using the enzymes *Hae*III, *Hinf*I and *Hha*I cluster the isolates in to 24, 40 and 42 groups respectively with a 70% cutoff. The restriction pattern shared by the enzymes used caused a variation in the number of fragments and molecular weight. The results suggest that PCR-RFLP of the regions ITS1, 5.8S and ITS2 using restriction enzymes is able to assess the diversity of this pathogenic fungi.

Keywords Restriction enzymes – Polymorphism - Spot blotch

Introduction

Bipolaris sorokiniana is a well-known filamentous fungus that is pathogenic to wheat and other grasses, surviving in infected seeds, plant litter or in secondary hosts for several years (Nakajima et al. 1998). Harmfulness of *B. sorokiniana* might be explained by its ability to infect different plant parts, such as roots and leaves during all plant growth stages (Kumar et al. 2002). Symptoms mainly develop on sub-crown internodes, stem, leaves and seeds. The main disease caused by the pathogen is spot blotch. The early lesions on leaves are 1-2mm long, small and dark brown in color and there is no sign of chlorotic edge at the initial stage of infection. In general, in typically tropical regions, spot blotch is the most severe disease to affect wheat cultures. Tropical regions with temperature between 20 and 30 ^{o}C offer the best conditions for germination, infection, growth lesions and sporulation of *B. sorokiniana* (Mehta 1978).

This phytopathogen shows quite high morphological and genetic diversity, making its identification more difficult and laborious and, therefore, its

pathogenic evaluation becomes more complex. In recent years, molecular biology has been used to study *B. sorokiniana*, in an effort to discover fast identification and characterization methods of this fungus. RAPD has been used to analyze Brazilian *Bipolaris sorokiniana* isolates to determine intraspecific genetic variability between isolates (Oliveira et al. 2002, Muller et al. 2005). Mann et al. (2014) used URP-PCR to study *B. sorokiniana* isolates from Brazil and other countries, assessing the genetic variability of monospore cultures. Zhong and Steffenson (2001) used AFLP to study the virulence and molecular diversity of *B. sorokiniana* isolates, which infected barley in different barley-producing regions. Nascimento and Van Der Sand (2008) studied the ITS regions of rDNA to characterize pathogen of wheat plants. The aim of this work was to detect intraspecific polymorphisms among *B. sorokiniana* isolates using the ITS regions and 5.8S rDNA and to evaluate the profile of polyconidial and monoconidial isolates.

Materials and methods

Fungal cultures

The fungal isolates from different regions in Brazil were provided by Empresa Brasileira de Pesquisa Agropecuária - Trigo (EMBRAPA- Trigo, Passo Fundo), while the other isolates used in this study were kindly provided the International Maize and Wheat Improvement Center (CIMMYT – México). All isolates used were obtained from seeds and tissues of wheat plants. The biological material was deposited in the collection of the Environmental Mycology Laboratory, DMIP, ICBS, UFRGS.

Seventy-one *B. sorokiniana* isolates were used in this study, 23 were polyconidial and 46 were monoconidial *B. sorokiniana* isolates, one isolate of *Bipolaris oryzae* (BO) and one of *Bipolaris curvispora* (BC) (Table 1).

Monoconidial cultures

The monoconidial cultures were obtained from the aerial mycelia of the polyconidial cultures grown on plates with potato dextrose agar (PDA). A 0.85% saline solution was poured over the plated colonies, and the conidia were transferred to microcentrifuge tubes. The tubes were homogenized thoroughly to achieve complete conidia release. The suspension was transferred to a Petri dish with PDA and incubated at room temperature for 2h. Using a stereomicroscope with optical magnification of ×40, the conidia were transferred using plates with PDA media. The plates were maintained at 24 ±2ºC until the complete colonies had developed, and then were stored at 4ºC. Each spore culture was labeled with a letter (A, B, and C). The criteria used to select the spores for monoconidial cultures was random, since the spores were distant from one another.

Genomic DNA extraction

Genomic DNA was extracted following the protocol developed by Ashktorab and Cohen (1992), with modifications by Muller et al (2005). The isolates were grown in Erlenmeyer flasks containing 300 mL potato-dextrose broth (PD). After inoculation, incubation took place at room temperature under orbital agitation of 120 rpm for 7–10 days in order to obtain 1 g of mycelia (wet weight). After incubation, the mycelium of each isolate was strained and washed three times with sterile distilled water, and excess water was removed using filter paper.

Weighed mycelium was ground to fine powder using liquid nitrogen. The 300 mg powder was transferred to a tube and 1 mL/g of mycelium of extraction-lysis buffer (200 mM Tris–HCl, pH 8.0; 250 mM NaCl; 25 mM EDTA, pH 8.0; 2% SDS) was added along with 10 μL/mL β-mercaptoethanol (Sigma, Deisenhofen, Germany) and 50 μg/mL proteinase K (Promega, Mannheim, Germany). The tubes were incubated in a water bath at 65 °C for 1 h, shaken every 15 min, and then centrifuged at 2,795 × g for 20 min. The supernatant was transferred to clean centrifuge tubes and DNA was purified twice with 1 volume of phenol (pH 8.0) and three times with 1 volume of phenol–chloroform (0.5:0.5 v/v). Finally, 1 volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the supernatant. Pancreatic RNAse I (50 μg/mL) was added to the aqueous phase resulting from the centrifugation, and the mixture was incubated at 37°C for 20 min. DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of isopropanol at –20°C. DNA was collected with a glass rod, transferred to a falcon tube, washed with 70% ethanol (v/v), and centrifuged at 2,795 × g for 10 min. DNA was allowed to dry at room temperature and was then resuspended in Milli-Q water and stored at –20 °C.

ITS –PCR amplification

The amplification of the ITS1-5.8S-ITS2 region (Figure 1) was carried out in a final volume of 25 μL, of which 2.5 μL was reaction buffer (10×), 1.0 μL 2 mM $MgCl_2$, 1 μL of 2.5 mM each dNTP (dATP, dCTP, dGTP and dTTP), 10 pmol each primer ITS1 5′-TCCGTAGGTGAACCTGCGG-3′, ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (White et al. 1990), 1.0 U of Taq DNA polymerase, 50 ng DNA template, and autoclaved deionized water to complete

the final volume. The amplification reactions were performed with 35 cycles in a thermal cycler (TX96- Amplitherm). The cycling program started with a cycle of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 45 s and elongation at 72°C for 1 min. The reactions ended with final extension at 72°C for 5 min, and the amplified product was cooled at 4°C. All amplifications were repeated at least twice for each isolate in separate experiments.

PCR-RFLP

The amplification products were digested with five different endonuclease enzymes (Promega): *Hae*III, *Hha*I, *Hind*III, *Hin*fI, *Eco*RI. The reactions were prepared following the manufacturer's instructions individually for each enzyme. The digestion reactions took place in a water-bath accordingly to the manufacturer's instruction for each enzyme. All reactions were repeated twice.

Electrophoresis

Amplification products were separated by electrophoresis in a 3% (w/v) agarose gel, stained with ethidium bromide, and photographed under UV light using a Vilber Lourmat Doc-Print II. Digital photos of the gels were taken using a Kodak DC 120 digital camera and Kodak 1D (version 3.5.2) software.

DNA Sequencing

Some of the amplification products generated by the amplification of the ITS1-5.8S-ITS2 rDNA region from *B. sorokiniana* were submitted to nucleotide sequence analysis. Sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, UK) in an ABI-PRISM

3100 Genetic Analyzer (ABI, Foster City, CA), according to the protocol of the manufacturer. The sequences obtained were compared with homologous nucleotide sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

Phylogenetic analysis

Alignment of the nucleotide sequences was performed using the Clustal W application, version 2.0. The tree was calculated with the computer program MEGA 5, using the Maximum Parsimony method based on the data specific model (Nei and Kumar 2000). Ten *B. sorokiniana* isolates which were sequenced were compared with other phylogenetically related strains accessed from GenBank (GU934504: *Bipolaris sorokiniana* isolate OTU730), JX129889: *Cochliobolus sativus* isolate NES1, KC311473: *Cochliobolus sativus* isolate D_D44, GU345084: *Bipolaris sorokiniana* strain 7, and EF187908*: Bipolaris sorokiniana* strain DSM 62608).

Data analysis

The amplifications of all the isolates were used to construct a binary matrix by scoring the presence and absence of fragments as 1 and 0, respectively. The matrix was analyzed using PAST (Paleontological statistics) using Jaccard's coefficient of similarity. The dendrogram was produced using the unweighted pair-group method with arithmetic averages (UPMGA). The data sets were considered clustered if they had a similarity coefficient above 70%. All amplifications were repeated at least twice for each isolate in separate experiments.

Results

The profiles of DNA fragments obtained from the amplification of the ITS and 5.8S regions differed among the isolates, with size fragments ranging from 670 to 543 bp. The amplification products of all isolates were submitted to endonuclease restriction assays for different enzymes: *Hae*III, *Hinf*I, *Hha*I, *Eco*RI, *Hind*III and *Xho*I.

Restriction fragments obtained with all the endonucleases tested were used to determine genetic distances between the genotypes and cluster them into specific groups. The amplified products submitted to the restriction enzymes *Eco*RI and *Hind*III produced two fragments with molecular weight between 363 and 165 bp when *Eco*RI was used, while for *Hind*III the fragments varied from 414 to 202 bp. However, when the restriction enzymes *Hae*III, *Hinf*I and *Hha*I were used, 2 to 4 fragments were generated, with varying molecular weights. The fragments produced by *Hae*III varied from 493 to 101 bp, those generated with enzyme *Hinf*I ranged from 354 to 64 bp, and those generated with *Hha*I varied from 355 to 55 bp. The digestion with enzyme *Hha*I exhibited a clearly distinct fragment profile between isolates 98025P (Piratini/BR) and NRRL5851C (South Africa), compared with the others (Figure 2). However, the enzyme *Eco*RI presented only one cleavage site, which did not distinguish isolates of the phytopathogen.

The dendrograms generated with the fragment obtained in the RFLP assay using the enzymes *Hae*III, *Hinf*I and *Hha*I clustered isolates in 24, 31, 40 and 42 groups, respectively, with a 70% cutoff. The RFLP patterns obtained revealed substantial variability among the isolates. The highest number of polymorphic

loci was obtained for the *EcoR*I, *Hae*III *Hinf*I and *Hha*I. The dendrogram obtained with the data produced with *Hha*I enzyme showed 40 clusters, out of which nine had 100% of similarity (Figure 3). It was possible to observe a similarity from 100 to 0.2%, which reveals the high genetic variability of isolates. The digestion with *Hha*I enzyme generated a fragment of 154 bp that was common to 32 of the isolates, while with enzyme EcoRI a 315-bp product was common to 21 of the isolates. The pattern obtained using the enzyme *Hind*III was less polymorphic: 18 groups were formed, 12 of which showed 100% of similarity.

All endonucleases tested using the all isolates showed clusters with different profiles. *Xho*I was the only enzyme that did not have any recognition site in the ITS1-5.8S- ITS2 amplified fragment.

Isolates obtained from different geographical areas showed a profile that did not allow grouping them. The results obtained using PCR- RFLP showed the isolate *B. curvispora* grouping with *B. sorokiniana* with a coefficient of similarity lower than 10% when the enzyme *Hha*I was used. The patterns obtained with the other enzymes were similar. For the *B. oryzae* isolate, the coefficient of similarity with *B. sorokiniana* was lower than 35%, for all enzymes used.

Phylogenetic analysis

The amplified products of the ITS1- 5.8S- ITS2 rDNA region of 19 *B. sorokiniana* isolates were sequenced and these sequences were compared with *B. sorokiniana* sequences available in GenBank. The results showed 99% of

identical sequences. Of the 19 samples sequenced, nine have been deposited in GenBank. The *B. sorokiniana* sequences were deposited in the GenBank under the accession numbers: KF765399 (*B. sorokiniana* 98012C), KF765400 (*B. sorokiniana* 98026B), KF765401 (*B. sorokiniana* BS52M2P), KF765402 (*B. sorokiniana* NRRL5851C), KF765403 (*B. sorokiniana* NRRL5851B), KF765404 (*B. sorokiniana* B515M2C), KF765405 (*B. sorokiniana* 98026P), KF765406 (*B. sorokiniana* 98023B), KF765407 (*B. sorokiniana* 98023P) and KF765408 (*B. sorokiniana* 98026C).

Discussion

The ITS region is known to contain a relatively high level of variability among species, and a lack of variation in the ITS sequences is indicative of a close relationship among the microorganisms studied. Many authors suggest that the ITS sequences and the sequence adjacent 5.8S and 28S rRNA genes could be optimal targets for the development of PCR assays for fungi detection and species identification (Kumar & Shukla, 2005, Abd-Elsalam et al.,2003, Kumeda & Asao, 1996). The ITS region of rDNA has been useful for taxonomic discrimination of fungi at the species and sub-species levels. Kim et al., (2013) study multiple sequence alignment of the fungal ITS1 and ITS2 sequences showed a high degree of variability. The ITS regions play an important role in rRNA processing, as it forms specific secondary structures that are required for rRNA maturation, even though there can be substantial nucleotide sequence variation in the ITS regions (Liu and Schardl 1994).

The results obtained in the present study using enzymes *Hae*III, *Hinf*I and *Hha*I showed band variability, with molecular weight variations between monospore and polyspore isolates. Nascimento and Van Der Sand (2008) studied polymorphism by restriction analysis of the amplified ribosomal DNA spacers ITS1 and ITS2 of *B. sorokiniana*, and found that enzymes *Hae*III and *Hha*I were able to digest the DNA in the ITS1 region of all isolates, and reported a number of different fragments. PCR-RFLP of the ITS1-5.8S-ITS2 region using restriction enzymes afforded to assess the diversity of phytopathogenic fungi.

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Figure legends

Table 1. Origin of isolates of *Bipolaris* from different regions in Brazil and other countries.

Isolate code	**City, State, Country**
98030 P, B, C	Cruz Alta, RS –Brasil
98032 P, A, C	Engenheiro Beltrão, PR – Brasil
98011 P, A, C	Lagoa Vermelha, RS –Brasil
98012 P, A, C	Lagoa Vermelha, RS –Brasil
98031 P, A, C	Nova Estância, PR –Brasil
98025 P, A, B	Piratini, RS –Brasil
98026 P, B, C	Piratini, RS –Brasil
98042 P, A, C	Piratini, PR –Brasil
98010 P, A, B	Santa Rosa, RS –Brasil
98041 P, B, C	União da Vitória, PR –Brasil
98023 P, A, B	União da Vitória, PR –Brasil
98013 P, A, C	União da Vitória, PR –Brasil
98017 P, A, B	Samambaia, PR –Brasil
98034 P, A, C	Desconhecida
NRRL5851 P, B, C	África do Sul
CFO201 P, A, B	África do Sul
A20 P, B, C	Canadá
BS15M2 P, B, C	Delicias, Chihuahua – Mexico
BS16M1 P, A, C	Delícias, Chihuahua- México
BS18M2 P, A, C	Poza Rica,Vera Cruz-México
CMO105 P, B, C	México
BS52M1 P, B, C	Monterrey-Nuevo Leon
CS1004 P, A, B	Hanoi-Vietnam
Bbipolaris curvispora (BC)	Pelotas, RS–Brasil
Bipolaris oryzae(BO)	Pelotas, RS–Brasil

P: Polysporic *B. sorokiniana* isolate; A, B, and C: Monosporic *B. sorokiniana* isolate originated from the respective polysporic isolate; BO: *Bipolaris oryzae* and BC: *Bipolaris curvispora*.

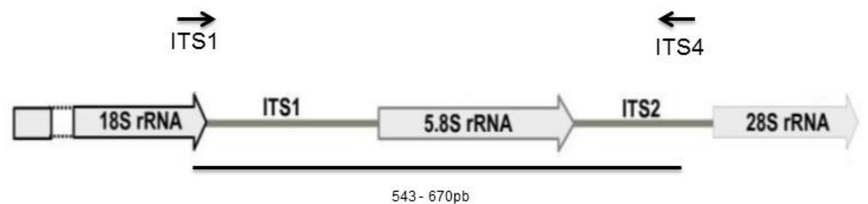

Fig. 1 Internal transcribed spacers (ITS) and 5.8S region should primers ITS1 and ITS4. A modified image from Embong et al. BMC Ophthalmology 2008 8:7.

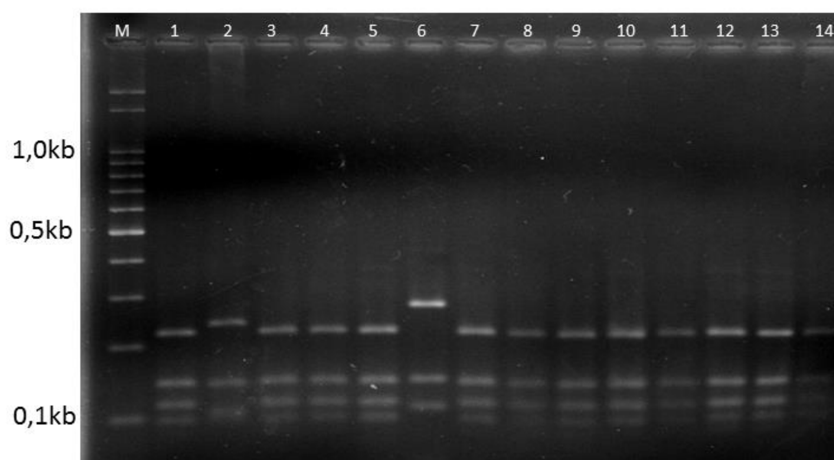

Fig. 2. Agarose gel 3.0 %, digestion of amplification products of *Bipolaris* sp. isolates using the restriction enzyme *Hha*I: (M) DNA marker ladder 100 bp; (1) 98013C; (2) 98025P; (3) 98025C; (4) 98031P; (5) 98041P; (6) NRRL5851C; (7) BS15M2C; (8) BS16M2A ; (9) BS18M2PP; (10) BS52M2A; (11) CF0201B; (12) CMO105B; (13) CS1004P and (14) CS1004B.

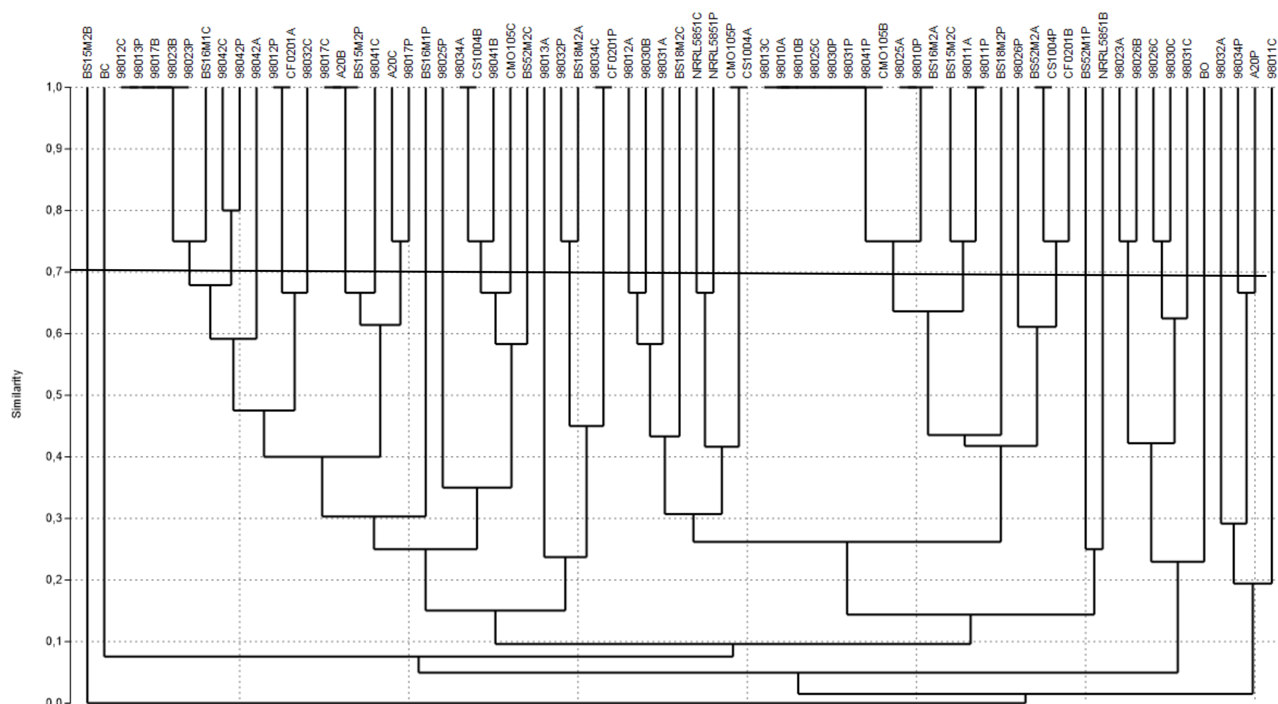

Fig. 3. Dendrogram of *Bipolaris sorokiniana* strains amplified with enzyme *Hha*I. A data matrix was generated by scoring the presence or absence of a fragment as 1 and 0 respectively. From these data, a similarity matrix was constructed with PAST (Paleontological Data Analysis) using the Jaccard's coefficient of similarity. The dendrogram was reproduced using UPMGA (unweighted pair-group method with arithmetic averages).

CAPÍTULO 5. ARTIGO CIENTÍFICO 5

Assessment of genetic diversity among isolates *Bipolaris sorokiniana* by REP, BOX and ERIC-PCR.

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Assessment of genetic diversity among isolates of *Bipolaris sorokiniana* by REP, BOX and ERIC-PCR

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Abstract

Spot blotch is caused by the pathogen *Bipolaris sorokiniana*, and is an important disease of wheat, with large economic losses worldwide. In this study, molecular variability in *B. sorokiniana* isolates collected from different regions of Brazil and other countries was investigated using REP-PCR, ERIC-PCR and BOX-PCR. Sixty-nine monosporic and polysporic *B. sorokiniana* isolates, one *Bipolaris curvipora* and one *Bipolaris oryzae* isolates were used. All primers used provided important data about the diversity of *Bipolaris*, and afforded to discriminate species. The amplification products obtained using the primers for REP and ERIC revealed higher diversity, while the products obtained using the

primer for BOX were more homogeneous. The isolates when amplified with the BOX primer generated a 700-bp monomorphic fragment which was sequenced. Based on this sequence, a par of primer was designed and the isolates were submitted to PCR. Amplified products in the 200-bp range were obtained for *Bipolaris*, but not the other microorganisms assessed (Gram-negative and Gram-positive bacteria, yeast and filamentous fungi). The results suggest that this pair of primers may be used as a molecular marker of the genus *Bipolaris*.

Keywords: molecular marker, diseases, phytopathogen.

Running title: REP, BOX and ERIC-PCR in *Bipolaris sorokiniana* genetic diversity

Introduction

Bipolaris sorokiniana is an economically important phytopathogen of wheat and other cereal species. Among the diseases that it causes, spot blotch is prevalent in warmer areas with high relative humidity, resulting in significant yield losses of wheat plantation (Mehta, 1998). Surveys on seeds traded in Brazil have demonstrated the constant presence of *B. sorokiniana* at levels that vary depending on year, place and cultivar, and the fungus may be present in 100% of seeds (Forcelini 1991). Seeds can harbor and transport microorganisms from all taxonomic groups, pathogenic or not. The detection and characterization of these organisms becomes an important tool in plant disease management. The advent of molecular biology has caused a significant shift on the approaches used to characterize and identify plant pathogens, and

to devise disease management strategies. Rep-PCR has been extensively used to identify pathogens, to differentiate strains, and to assess the genetic diversity of plant pathogens (Louws *et al.*1999; Yua *et al*. 2008) and can be used to determinate the genetic relationships within groups. Repetitive DNA PCR-Based Genomic Fingerprinting REP-PCR analysis was developed based on the observed occurrence of specific conserved repetitive sequences repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX elements distributed in the genomes of diverse bacteria (Versalovic *et al.* 1991; Versalovic *et al.* 1994). However, the term has been expanded to include the use of primers for PCR genomic fingerprinting that anneal to any repetitive DNA sequences (George 1997; George 1998). Three primer sets are commonly used for rep-PCR genomic fingerprinting analysis, corresponding to REP, ERIC and BOX.

A previous study examined the applicability of rep-PCR genomic fingerprinting to molecular typing of *Phaeoacremonium* and *Phaeomoniella* species Alves *et al.* (2004). The authors revealed that rep-PCR patterns clearly separated the species, and in some cases revealed intraspecific variability. The amplified DNA fragments, when separated by electrophoresis, constitute a genomic fingerprint that can be employed for subspecies discrimination and strain delineation of bacteria and fungi (Versalovic *et al.*, 2002).

The objective of this study was (i) to characterize polysporic and monosporic of *Bipolaris sorokiniana* isolates using analysis of the DNA fingerprints using primers for enterobacterial repetitive intergenic consensus (ERIC) sequences

and repetitive extragenic palindromic (REP) sequences, and BOX elements. (ii) to distinguish *Bipolaris sorokiana* from other species.

Materials and methods

Fungal cultures

The fungal isolates from different regions in Brazil were provided by Empresa Brasileira de Pesquisa Agropecuária - Trigo (EMBRAPA-Trigo, CNPT, Passo Fundo), while the other isolates used in this study were kindly provided the International Maize and Wheat Improvement Center (CIMMYT – México). All isolates used were obtained from seeds and tissues of wheat plants. The biological material was deposited in the collection of the Environmental Mycology Laboratory, DMIP, ICBS, UFRGS.

Seventy-one *B. sorokiniana* isolates were used in the assays, of which 23 were polyconidial and 46 were monoconidial cultures, one *B. oryzae* (BO) and one *B. curvispora* (BC). The isolate *Trichodema asperellum*, *Rhizopus*, Gram-positive bacteria, Gram-negative bacteria and yeasts isolates were employed only in the specificity assay (Table 1).

Genomic DNA extraction

Genomic DNA was extracted following a previously developed protocol (Ashktorab and Cohen, 1992), with modifications. The isolates were grown in Erlenmeyer flasks containing 300 mL potato-dextrose broth (PD). After inoculation, incubation took place at room temperature under orbital agitation of 120 rpm for 7–10 days in order to obtain 1 g of mycelia (wet weight). After

incubation, the mycelium of each isolate was strained and washed three times with sterile distilled water, and excess water was removed using filter paper. Weighed mycelium was ground to fine powder using liquid nitrogen. The 300 mg powder was transferred to a tube, and 1 mL/g of mycelium of extraction-lysis buffer (200 mM Tris–HCl, pH 8.0; 250 mM NaCl; 25 mM EDTA, pH 8.0; 2% SDS) was added along with 10 µL/mL β-mercaptoethanol (Sigma, Germany) and 50 µg/mL proteinase K (Promega, Mannheim, Germany). The tubes were incubated in a water bath at 65 °C for 1 h, shaken every 15 min, and then centrifuged at 2,795 ×*g* for 20 min. The supernatant was transferred to clean centrifuge tubes and DNA was purified twice with 1 volume of phenol (pH 8.0) and three times with 1 volume of phenol–chloroform (0.5:0.5 v/v). Finally, 1 volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the supernatant. Pancreatic RNAse I (50 µg/mL) was added to the aqueous phase resulting from the centrifugation, and the mixture was incubated at 37°C for 20 min. DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of isopropanol at –20°C. DNA was collected with a glass rod, transferred to a falcon tube, washed with 70% ethanol (v/v), and centrifuged at 2,795 × *g* for 10 min. DNA was allowed to dry at room temperature and was then resuspended in Milli-Q water and stored at –20 °C.

REP-PCR

The REP-PCR assays were carried out in a 25-µL reaction volume containing 300 ng of DNA, 1x PCR buffer, 4 mmol $MgCl_2$, 1.2 µM of primers REP 1R (5’-III ICG ICG ICA TCI GGC-3’) and REP 2R (5’- ICG ICT TAT CIG GCC TAC -3’)

(Louws *et al.*, 1999), 0.2 mmol of each dNTP, and 2U of Taq polymerase. The amplification conditions were an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 1 min 30 s with a final extension of 72°C for 5 min.

ERIC-PCR

The ERIC-PCR assays were performed in a 25-µL reaction volume containing 300 ng of DNA, 1x PCR buffer, 2 mmol $MgCl_2$, 0.6 µM of primers ERIC 1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC 2R (50-AAG TAA GTG ACT GGG GTG AGC G-3') (Hulton *et al.*, 1991), 0.2 mmol of each dNTP, BSA 1% (Bovine serum albumin) and 1U of Taq polymerase. Amplification conditions were an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 8 min with a final extension of 72°C for 5 min.

BOX-PCR

The BOX-PCR reactions were performed in a 25-µL reaction volume with 300 ng of purified DNA, 1x PCR buffer, 1.0 mmol $MgCl_2$, 2.4 mmol of primer BOX-PCR (5'-CTA CGG CAA GGC GAC GCT GAC G-3') (Louws *et al.*, 1999), 0.2 mmol of each dNTP, and 1U of Taq polymerase. The PCR conditions were an initial denaturation step of 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 44°C for 1 min and 65°C for 8 min with a final extension of 72°C for 5 min.

Electrophoresis

Amplification products were separated by electrophoresis in 1.5% (w/v) agarose gel, stained with ethidium bromide and photographed under UV light using a Vilber Lourmat Doc-Print II. Digital photos of the gels were taken using a Kodak DC 120 digital camera and Kodak 1D (version 3.5.2) software. All amplifications were repeated at least twice for each isolate in separate experiments.

Data analysis

The amplifications of all the isolates obtained were used to construct a binary matrix by scoring the presence and absence of fragments as 1 and 0, respectively. The matrix was analyzed using PAST using Jaccard's coefficient of similarity. The dendrogram was produced using the unweighted pair-group method with arithmetic averages (UPGMA). The data sets were considered clustered if they had a similarity coefficient above 70%.

Design of primers

Based on the result obtained in the amplification assay using BOX-PCR a pair of primers was designed using Software Oligo Explorer and preliminarily designated BIPO. Primer specificity was checked using nucleotide Basic Local Alignment Search Tool (nBLAST: http://blast.ncbi.nlm.nih.gov/).

Molecular marker assay

To assess the possibility to distinguish isolates of *B. sorokiniana* from other microorganisms, DNA amplification was performed using BIPO primers. The reactions were performed in a 25-µL final volume containing 100 ng of DNA, 1x PCR buffer, 2.0 mmol $MgCl_2$, 1 µL of 2.0 mM each dNTP (dATP, dCTP, dGTP and dTTP), 10 pmol of each primer BIPO (5´-TGCCATCTCCCTGTACGCCC-3´), BIPO (5´- CTCGGTATGGACACAGACACCC -3´), 200 ng/µL bovine serum albumin (BSA), 1U of Taq polymerase and sterile Milli-Q water. The PCR conditions were an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 64°C for 45 s and 72°C for 45 s with a final extension of 72°C for 5 min.

RESULTS

The primer pairs Rep1R/Rep2R (REP PCR), ERIC 1R/2R (ERIC-PCR) and BOX-PCR were tested for their abilities to differentiate a total of 69 *B. sorokiniana* isolates from Brazil and other countries, one *B. curvispora* isolate and one *B. oryzae* isolate from Brazil. The amplification products generated by PCR with the BOX, REP and ERIC primers yielded bands with sizes ranging from 95 to 3,400 bp.

REP-PCR revealed important information on isolate diversity (Figure 1). The different band profiles obtained among the isolates discriminated them from other *Bipolaris* species and even other isolates, of same conidial origin. The REP-PCR genomic fingerprint of different isolates revealed distinct patterns, produced bands that ranged from one to 16. The REP-PCR results formed

clusters with similarity under 50%, which indicates considerable diversity among isolates. Only isolates 98034P and 98034C showed 95% similarity.

The amplicons size for ERIC-PCR ranged between 144 - 3398 bp and showed multiple fragments. Fingerprints generated with ERIC-PCR contained from two to 10 bands. This primer also revealed considerable diversity between isolates, with similarity indices under 70%, only four isolates formed one cluster with 100% similarity (Figure 2).

The use of BOX-PCR amplified DNA fingerprints ranging from 1 to 13 bands. PCR amplification of multiple isolates of different localizations using primers BOX showed different intra-specific band patterns (Figure 3).

The dendrogram shows that the BOX primer formed clusters with different levels of similarity. However, some isolates formed clusters with 100% similarity (Figure 4). One 700-bp fragment was observed in all isolates of *Bipolaris* spp. when the amplification with the BOX primer was used. This fragment was removed from the gel, purified and sequenced. Sequence alignment was done and a 200-bp sequence was observed for all isolates. Based on this result, a pair of primers was designed for the amplification of this region and the comparison of that with other microorganisms (Table 1). The amplification product was observed for *B. sorokiniana, B. curvispora* and *B. oryzae* and none of the other microorganisms used in the assay showed any amplification (Figure 5).

DISCUSSION

Rep-PCR genomic fingerprinting with primers ERIC, BOX and REP had the capacity to discriminate *B. sorokiniana* isolates and revealed high level of genetic diversity among isolates. This is in accordance with results obtained in other studies (Carpenter *et al*., 1999; Mert-Türk *et al*., 2007. Non-degenerate oligonucleotides of either random, arbitrary sequences AP-PCR, or coding sequences of repeated genes (Gilson *et al*., 1990), have been used as primers to generate PCR-based fingerprints of bacterial genomes and fungi.

In the present study, rep-PCR revealed high variability between isolates, with similarity below 70%, which confirms the findings in a previous study (Berg *et al*., 2005). The authors reported that considerable intraspecific variability existed within *Penicillium* strains when BOX DNA fingerprints were used to distinguish strains. In *B. sorokiniana*, diversity may be the result of a series of factors, such as mutation and parasexual combination in this fungus population. PCR-ITS/RFLP was used to analyze polymorphism of ITS regions of *B. sorokiniana* rDNA, revealing great diversity among isolates from different regions and countries (Nascimento & Van Der Sand, 2008). A study that used 12 URP primers for the molecular characterization of 60 monosporic *B. sorokiniana* isolates from Brazil and other parts of the world observed a very high genetic variability among the monosporic isolates (Mann *et al.* 2014).

The design of the primer BIPO to identify the *Bipolaris* genus is considerably important in agriculture, since the phytopathogens has high morphological and genetic variability. Molecular markers are phenotypically neutral, and may be detected both in tissues and in seeds, are easily detectable and behave

predictably. Molecular markers allow selection and new crossings to be carried out in the same generation, which considerably increases the efficiency of improvement programs. They may be used even unmapped, that is, associated with a gene, a chromosome region or a phenotype, as long as they can be followed up in subsequent generations, proving their genetic nature (Ferreira & Grattapaglia, 1998; Milach, 1988).

With this preliminary result, we can say that the primer designed in this work is able to detect DNA from the genus *Bipolaris*, and that this pair of primers may be used as a marker of the *Bipolaris* genus, to differentiate pathogenic fungi accurately and quickly. More assays have to be carried out using different phytopathogenic fungi and bacteria especially to plants.

The results of the present study using BIPO-PCR as molecular marker of the *Bipolaris* genus affords to perform the genetic characterization of a large number of genotypes, based on a relatively simple and fast protocol. Current issues, like the assessment of cultures in efforts to develop quality control strategies in the field and in seed production in laboratory, may be more appropriately solved using accurate information on pathogens like *Bipolaris* spp.

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Acknowledgments

We thank de Bazilian government in the name of CNPq for the scholarship and to CAPES/PROAP for the financial support.

Table 1. Isolates used in the assays their codes and origins

Code isolate	Microorganism	Location, State, Country
98010 P, A, B	*Bipolaris sorokiniana*	Cruz Alta, RS, Brasil
98032 P, A, C	*Bipolaris sorokiniana*	Engenheiro Beltrão, PR, Brasil
98030 P, A, B,	*Bipolaris sorokiniana*	Cruz Alta, RS, Brazil
98011 P, A, C	*Bipolaris sorokiniana*	Lagoa Vermelha, RS, Brasil
98012 P, A, C	*Bipolaris sorokiniana*	Lagoa Vermelha, RS, Brasil
09,14,18,20,22*	*Trichoderma asperelum*	Goais, Brasil
BC	*Bipolaris curvispora*	Pelotas, RS,Brasil
BO	*Bipolaris oryzae*	Pelotas, RS,Brasil
Rh*	*Rhizopus* sp.	Pelotas, RS,Brasil
98025 P, A, C	*Bipolaris sorokiniana*	Piratini, RS, Brasil
98026 P, B, C	*Bipolaris sorokiniana*	Piratini, RS, Brasil
98042 P, A, C	*Bipolaris sorokiniana*	Piratini, PR, Brasil
1DE14*	*Escherichia coli*	Porto Alegre, RS, Brasil
3CC04*	*Citrobacter divresus*	Porto Alegre, RS, Brasil
2CC08*	Gram negative bacteria	Porto Alegre, RS, Brasil
DEC 26*	*Aureobasidium pullulans*	Rio Grande, RS, Brasil
DEC 45*	*Sporobolomyces ruberrimus*	Rio Grande, RS, Brasil
DEC 71*	*Yarrowia lipolytica*	Rio Grande, RS, Brasil
20E*	*Saccharomyces cerevisiae*	Santa Catarina, Brasil
98031 P, A, B	*Bipolaris sorokiniana*	Nova Estância, PR, Brasil
98023 P, A, B	*Bipolaris sorokiniana*	União da Vitória, PR, Brasil
98013 P, A, C	*Bipolaris sorokiniana*	União da Vitória, PR, Brasil
98041 P, B, C	*Bipolaris sorokiniana*	União da Vitória, PR, Brasil
98017 P, B, C	*Bipolaris sorokiniana*	Samambaia, PR, Brasil
98034 P, A, C	*Bipolaris sorokiniana*	unknown origin
NRRL5851 P, B, C	*Bipolaris sorokiniana*	África do Sul
CFO201 P, A, B	*Bipolaris sorokiniana*	África do Sul
A20 P, B, C	*Bipolaris sorokiniana*	Canadá
BS15M2 P, A, B	*Bipolaris sorokiniana*	Delicias, Chihuahua – Mexico
BS16M1 P, A, C	*Bipolaris sorokiniana*	Delícias, Chihuahua- México
BS18M2 P, A, C	*Bipolaris sorokiniana*	Poza Rica, Vera Cruz-México
CMO105 P, B, C	*Bipolaris sorokiniana*	México
BS52M1 P, A, C	*Bipolaris sorokiniana*	Monterrey, Nuevo Leon
CS1004 P, A, B	*Bipolaris sorokiniana*	Hanoi, Vietnam

P: Polysporic *B. sorokiniana* isolates; A, B, and C: Monosporic *B. sorokiniana* isolate originated from the respective polysporic isolates.* Isolates used only the specificity test

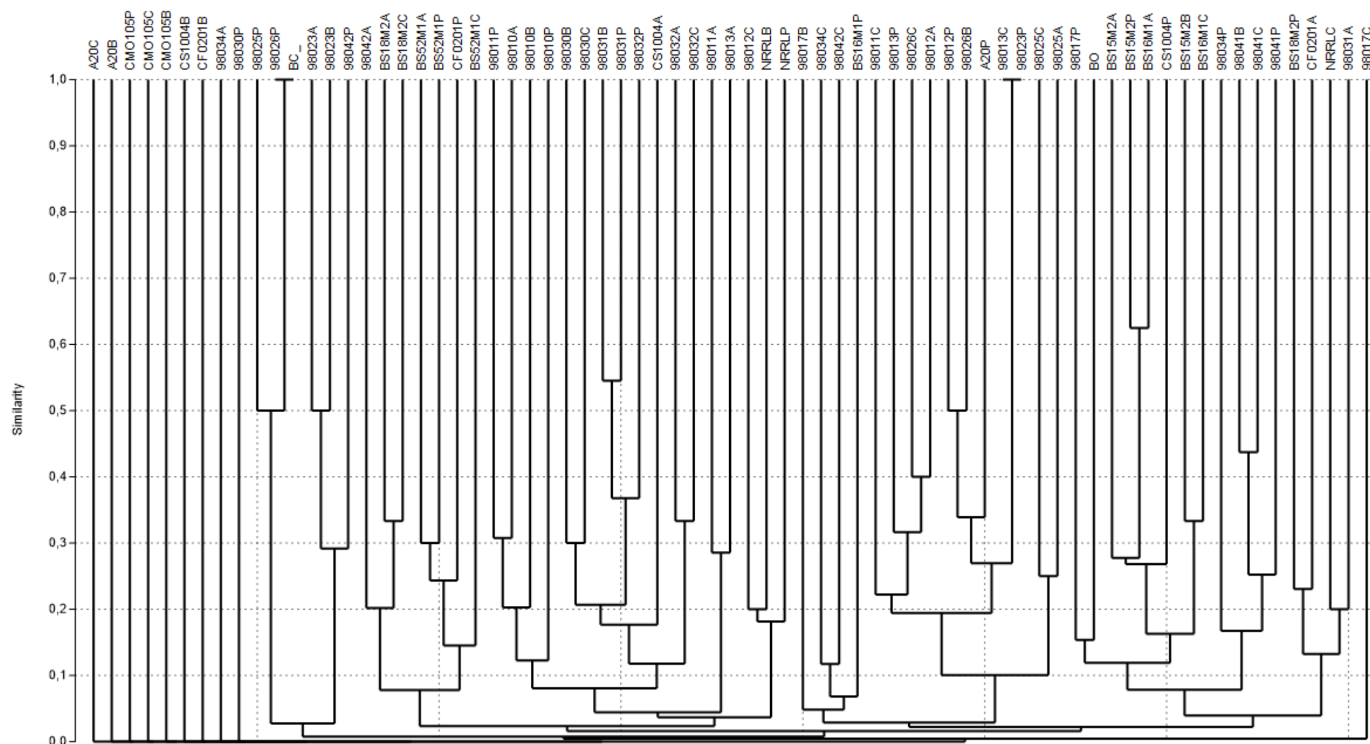

Fig 1. Dendrogram of *Bipolaris sorokiniana* strains amplified with primer ERIC. A data matrix was generated by scoring the presence or absence of a fragment as 1 and 0 respectively. From these data, a similarity matrix was constructed with PAST (Paleontological Data Analysis**)** using Jaccard's coefficient of similarity. The dendrogram was reproduced using UPMGA (unweighted pair-group method with arithmetic averages).

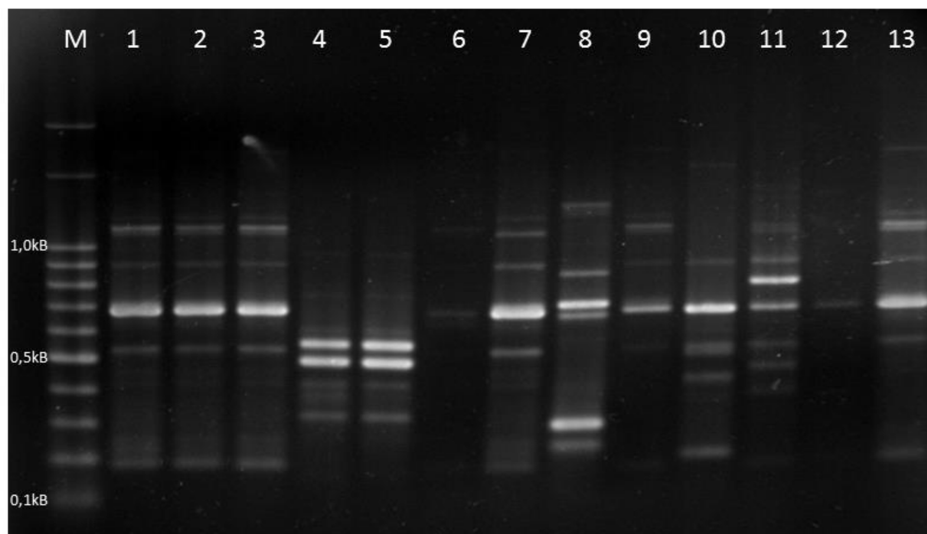

Fig 2. Agarose gel 1.5 %, genomic DNA amplification product isolates using primer BOX. (M) DNA marker ladder 100 bp; (1) 98032C; (2) 98034P; (3) 98041C; (4) BC; (5) BO; (6) BS15M2B; (7) BS18M2C; (8)NRRL5851B; (9) CF0201B; (10) 98017C; (11) 98034C; (12) BS15M2C (13) 98013C.

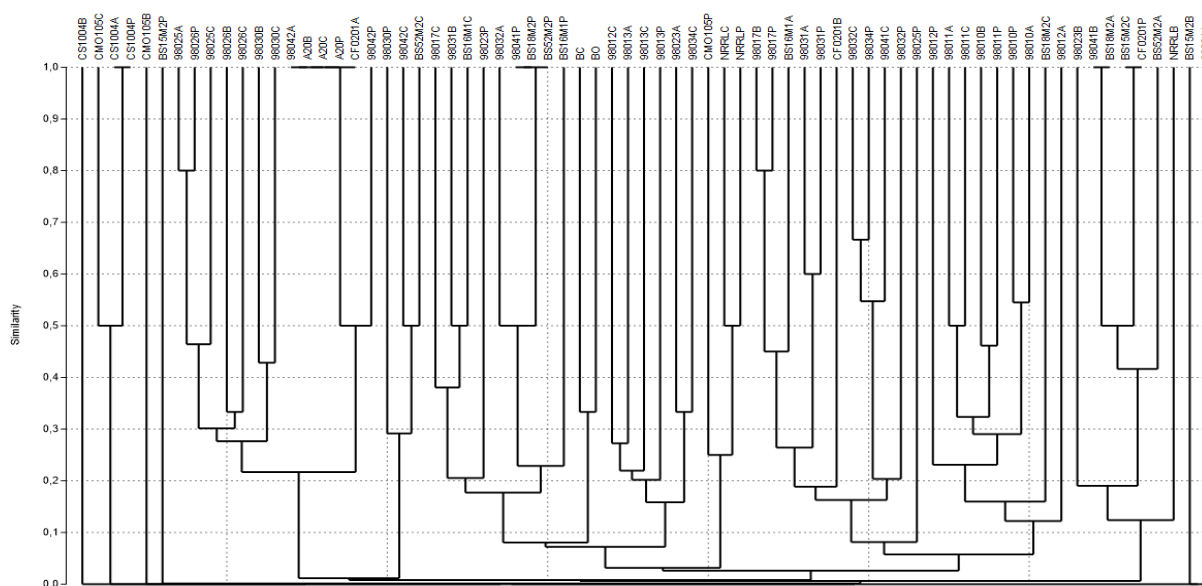

Fig.3. Dendrogram of *Bipolaris sorokiniana* strains amplified with primer BOX. A data matrix was generated by scoring the presence or absence of a fragment as 1 and 0 respectively. From these data, a similarity matrix was constructed with PAST (Paleontological Data Analysis**)** using Jaccard's coefficient of similarity. The dendrogram was reproduced using UPMGA (unweighted pair-group method with arithmetic averages).

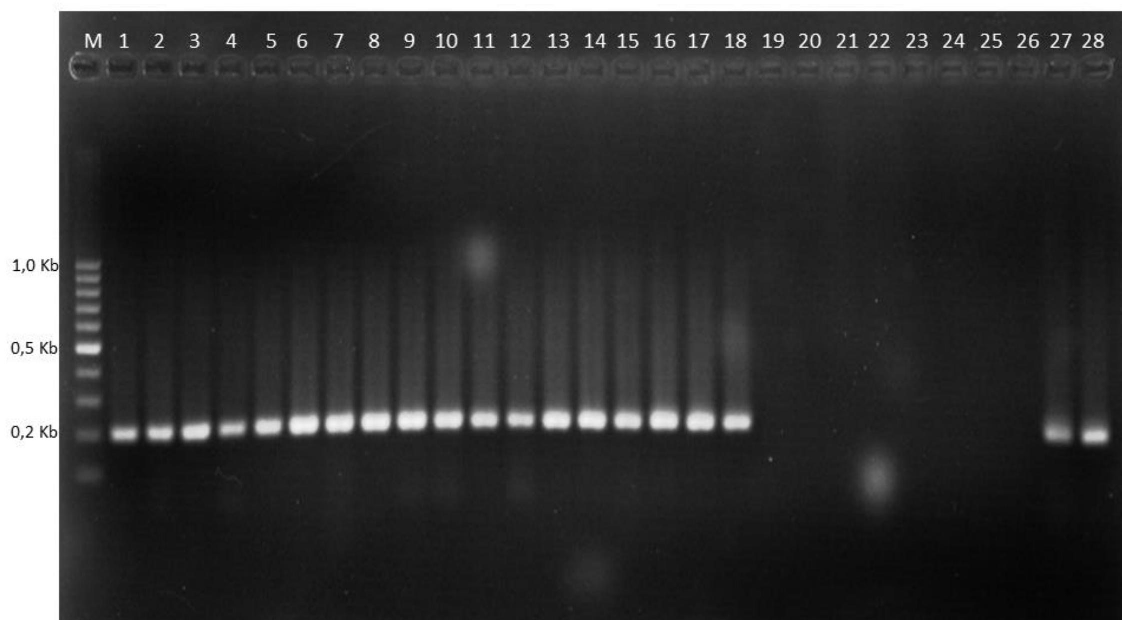

Fig.4. Genomic DNA amplification product isolates using primer BIPO. (M) DNA marker ladder 100 bp; (1) 98011P; (2) 98011A; (3) 98011C; (4) 98030P; (5) 98030B; (6) 98030C; (7) 98032P; (8)98032A; (9)98032C; (10)BS52M2P; (11) BS52M2A; (12) BS52M2C; (13)CS1004P; (14) CS1004A; (15) CS1004B; (16)NRRL5851P; (17) NRRL5851B; (18) NRRL5851C; (19) 3CC04; (20) 1DE14; (21) 20E; (22) 09; (23) 20; (24) DEC26; (25) DEC45; (26) RH; (27) BC; (28) BO.

CAPÍTULO 6. RESULTADOS E DISCUSSÃO GERAL

Neste capítulo optou-se por apresentar considerações que julgamos relevantes em complementação ao conteúdo já aceito para publicação no Artigo 2 e em relação aos artigos 1, 3, 4 e 5, submetidos. Serão levantados alguns pontos ainda não discutidos na versão aqui apresentada.

A cultura de trigo (*Triticum aestivum*) no sul do Brasil é afetada por um grande número de doenças causadas por fungos, bactérias e vírus. As doenças fúngicas são as mais numerosas, podendo atacar raízes, hastes, folhas e espigas de trigo. Dependendo da severidade do ataque, as doenças podem causar danos de magnitude econômica chegando, em alguns casos, a frustrar totalmente a produção de grãos.

O fitopatógeno *Bipolaris sorokiniana* é um fungo filamentoso com conídios fusóides e germinação bipolar que causa doenças em uma ampla variedade de gramíneas.

Problemas de ordem econômica são gerados pela incidência desses microrganismos na agricultura, acarretando queda na produtividade e desenvolvimento da planta. Atualmente medidas como melhoramento genético, rotação de culturas e fungicidas são utilizados para controle de fitopatógenos. No entanto, a aplicação de fungicida de forma indiscriminada e inadequada para conter a disseminação do fungo sobre a planta e sementes promovem reflexos negativos até mesmo em outras cadeias.

Portanto devido a estes problemas e a dificuldade de identificação resultante da variabilidade morfológica deste fitopatógeno este estudo buscou caracterizar, através de ferramentas enzimáticas e moleculares, o fungo *B. sorokiniana* buscando uma alternativa de diminuição nas perdas na produção. A proposta do trabalho foi aplicar métodos genotípicos e enzimáticos que fossem capazes de discriminar os isolados de *B.sorokiniana*. Neste trabalho foram utilizadas culturas monospóricas e polispóricas do fungo *Bipolaris sorokiniana* e que teve por finalidade conhecer a variabilidade molecular dos isolados.

A bibliografia mostra que URP-PCR (Universal Arroz Primers) têm sido utilizados para estudar a variabilidade genética inter e intra-específica de microrganismos, produzindo resultados rápidos e eficazes (Kang et al. 2001). Neste trabalho, com o uso da técnica de PCR-URP, foi observado a partir do dendrograma construído com todos os dados com culturas monospóricas e polispóricas de *B.sorokiniana,* que os isolados se diferenciaram formando agrupamentos muito distintos até mesmo entre os conídios de mesma origem polispórica. Aggarwal *et* al. (2010) utilizaram 12 *primers* de URP em um estudo com *B. sorokiniana* e obteveram sucesso, gerando perfis moleculares que diferenciaram isolados todos estes oriundos da Índia.

Outras técnicas de escolha foram REP-PCR, ERIC-PCR e BOX-PCR as duas primeiras revelaram maior diversidade, no entanto, as amplificações realizadas com o BOX-PCR apresentaram um perfil com maior similaridade entre os isolados. Um fragmento monomórfico foi observado e com base nesse resultado este fragmento foi isolado e sequenciado.

A análise dos resultados com o PCR-URP, BOX, ERIC, REP-PCR nos permite concluir que além da variabilidade morfológica, fisiológica que *B. sorokiniana* possui uma alta diversidade molecular.

Outro ensaio realizado neste trabalho foi de patogenicidade que teve por objetivo assegurar que as culturas, que vinham sendo mantidas em meio de cultura e passando por repiques, ainda encontra-se com seus genes de patogenicidade e de virulência ativos. É recomendado que não sejam realizadas mais que três transferências de microrganismos a partir da cultura original, evitando assim a perda das características originais do isolado. Dessa forma essas características devem ser constantemente avaliadas devido às peculiaridades de cada espécie. De acordo com Alfenas & Mafia (2007) a necessidade de se efetuar repicagens periódicas para garantir a viabilidade das culturas pode induzir o patógeno ao hábito saprofítico, a alteração de sua morfologia, a diminuição ou perda de sua capacidade de esporular e a diminuição de sua agressividade e, até mesmo, sua virulência.

Por esse motivo, foi avaliado o potencial patogênico dos isolados polispóricos e monospóricos, que revelou uma significativa diferença entre os grupos. Isolados polispóricos apresentaram maior severidade nas sementes quando comparados com a parte aérea. Reis & Forcelini (1993) relatam que esse sintoma nem sempre se manifesta, o que torna as sementes, muitas vezes, aparentemente sadias. A associação patógeno-semente é um mecanismo eficiente de sobrevivência, permitindo que o patógeno não se separe da principal fonte de nutrição, podendo esporular e se dispersar durante a germinação e emergência das plântulas em uma cultura recém -estabelecida

(Pretes et al, 1986). A comparação entre os grupos de tratamento (isolados monospóricos, polispóricos e controle) revelou uma diferença significativa entre os grupos.

Algumas considerações podem ser extraídas do trabalho de isoenzimas entre os perfis obtidos com a enzima esterase que apresentou um fenótipo monomórfico para isolados pertencentes ao Brasil e polimórfico para os isolados internacionais. De acordo com o estudo realizado por Bocchese *et al.*(2003), com caracterização enzimática com *Pyrenophora chaetomioides,* agente causal da mancha foliar da aveia, houve uma elevada associação da atividade enzimática de esterase com aqueles isolados que eram mais virulentos.

Variações quanto às exigências fisiológicas para crescimento micelial *in vitro*, decorrentes do isolamento geográfico, entre isolados do Brasil e de outros países, podem explicar as variações nos padrões isoenzimáticos. Os resultados mostraram que os todos isolados monospóricos, bem como, os polispóricos foram positivos para todos os sistemas enzimáticos testados, no entanto, se diferenciaram quanto à intensidade e número de bandas.

O PCR–RFLP também utilizado neste estudo, revelou informações importantes sobre os perfis genéticos de culturas polispóricas e monospóricas, mostrando as diferenças entre os três conídios isolados do mesma cepa polispórica. Os padrões de restrição apresentaram uma variação no número de fragmentos e de peso molecular. Outros trabalhos como de Nascimento e Van Der Sand (2008), verificaram uma alta diversidade genética entre isolados de *B. sorokiniana* e atribuíram essa diversidade à ocorrência de fluxo gênico entre

as populações do fungo geograficamente distantes através do comércio de sementes entre países.

Considerando que a variabilidade genética foi verificada em todas as técnicas utilizadas neste trabalho, cada uma com uma característica particular de agrupamento, pode-se concluir de uma forma geral, que o fitopatógeno exibiu alta diversidade. Uma possível explicação para essa variabilidade pode ser atribuída à condição multinucleada das células de *B. sorokiniana* como também à heterocariose, que pode levar à recombinação mitótica e polimorfismo.

Dentre todas as técnicas utilizadas neste trabalho, o primer BOX mostrou-se eficiente para avaliar diversidade e, ainda, a partir dos resultados obtidos neste ensaio, proporcionou a construção de um marcador molecular para identificar gênero *Bipolari*s sp..Questões atuais como a avaliação de culturas e esforços para desenvolver estratégias de controle de qualidade no campo e na produção de sementes em laboratório, podem utilizar esse marcador para obtenção de informações mais precisas sobre a presença deste fitopatógeno em culturas de trigo.

Os resultados mostram uma ampla diversidade genética e entre os isolados de diferentes origens geográficas, por este motivos se faz necessário testes quanto a resistência frente aos fungicidas. A resistência adquirida pela população do patógeno ao produto é diretamente proporcional às doses aplicadas, à frequência de aplicação, ao grau de cobertura obtido, à persistência na cultura ou no solo e ao tamanho da área tratada (Parreira et al., 2009). Por esses motivos se faz necessário conhecer a diversidade molecular

destes fungos para que medidas de controle e dosagens específicas sejam utilizadas.

CAPÍTULO 7 CONCLUSÕES

O primer BOX mostrou-se eficiente para avaliar diversidade e ainda proporcionou a construção de um marcador molecular que pode ser usado para identificar gênero o *Bipolari*s sp,

Um aspecto interessante no estudo da patogenicidade foi verificado em relação aos diferentes índices de virulência, a condição monospórica ou polispórica do patógeno e o órgão da planta afetado.

É possível concluir que os resultados obtidos com a URP-PCR no presente estudo permitiram uma análise da variabilidade rápida e eficiente para *B. sorokiniana*. Os isolados monospóricos e polispóricos analisados neste estudo mostraram uma alta diversidade genômica.

De maneira geral o estudo de isoenzimas forneceu informações relevantes sobre o perfil dos isolados do Brasil e de outros países, diferenciando os mesmos.

A técnica PCR-RFLP da região ITS1-5.8S-ITS2 utilizando enzimas de restrição foi capaz de acessar a diversidade de fungos fitopatogênicos e revelar a presença de polimorfismo intra-específico.

Todas as análises realizadas neste trabalho não permitiram agrupar os isolados de acordo com a região geográfica.

CAPÍTULO 8 PERSPECTIVAS

Para dar continuidade a esse trabalho sugerimos que:

Seja realizado sequenciamento dos diferentes fragmentos da região ITS, levando- se em conta a diversidade encontrada neste trabalho.

Realizar outros ensaios com um número maior de isolados fitopatogênicos com primer BIPO.

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